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Skin Microbiome in Small- and Large-plaque Parapsoriasis

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Staphylococcal enterotoxins have been shown to promote lymphoma-associated immune dysregulation. This study examined changes in the skin microbiome of parapsoriasis compared with intact skin. Swab microbiome specimens were taken of the parapsoriasis lesions of 13 patients. Control samples were taken from contralateral healthy sides of the body. Microbiotas were characterized by sequencing the V1–V3 region of the 16S ribosomal RNA bacterial genes on the Illumina MiSeq platform. The most common genera in the microbiome data were *Propionibacterium* (27.13%), *Corynebacterium* (21.20%) and *Staphylococcus* (4.63%). Out of the *Staphylococcus* sequences, 39.6% represented *S. epidermidis*, with the rest including *S. hominis*, *S. capitis* and unidentified species. No significant differences were observed between the patients' parapsoriasis and contralateral healthy skin or between large- and small-plaque parapsoriasis. Notable interpersonal variation was demonstrated. These results suggest that parapsoriasis is not associated with significant alterations in the cutaneous bacterial microbiome.

Novel molecular techniques have greatly improved our knowledge of the skin microbiome (1). Genomic studies with targeted sequencing of parts of the gene coding for ribosomal 16S RNA have shown that cutaneous microbial colonization is more complex than previously thought (2). Studies characterizing the microbiota of different body sites in humans have revealed that the spectrum of micro-organisms varies depending on numerous intrinsic and extrinsic factors (3–5). Common skin diseases, such as atopic dermatitis, have been linked to specific changes in the microbiome (6, 7). However, it remains unclear whether these changes are caused by microbes, or are secondary to factors such as changes in the skin barrier or immunological factors (8).

Parapsoriasis refers to a group of cutaneous lymphoproliferative disorders, ranging from a chronic dermatitis-like picture at one end to a picture mimicking patch-stage cutaneous T-cell lymphoma (CTCL) at the other (9). Clinical findings are traditionally used to classify the disease into small-plaque parapsoriasis (SPP), i.e. classical digitate dermatitis, and large-plaque parapsoriasis (LPP). Both subtypes may remain indolent for many years, but LPP progresses to CTCL, primarily mycosis fungoides, in up to 30% of cases (10), and parapsoriasis is difficult to differentiate from early CTCL by clinical features, histopathological characteristics or immunophenotype (11). There is no marker to identify cases prone to progression.

Various studies have confirmed the intimate interaction between skin microbiota and the host's immune system (11, 12). It has become apparent that, in addition to its physical characteristics, the skin's innate immune system, together with the resident commensal microbes, protects the skin by providing a functional immunological barrier. It was recently shown that Staphylococcal enterotoxin A (SEA) from the affected skin of patients with CTCL induces *in vitro* interleukin (IL)-17 production in primary malignant T cells of patients with Sézary syndrome when co-cultured with autologous non-malignant T cells (but not in monocultures of malignant T cells) (13). Since parapsoriasis often precedes mycosis fungoides (10), which is the most common form of CTCL, the aims of this study were to investigate whether parapsoriasis lesions would have a different skin surface microbiome compared with the individual's healthy (non-lesional) skin sites, and to explore the association of any specific bacteria with the chronic T-cell proliferation underlying parapsoriasis. A further aim was to investigate whether the skin microbiome (swab sampling) could offer a cost-effective and non-invasive diagnostic method to study parapsoriasis in patients whose skin is otherwise repeatedly biopsied.

METHODS

Patients and skin sampling

The clinical part of the study was carried out in January–May and September of 2014 at the Department of Dermatology and Allergology, Helsinki University Hospital, Helsinki, Finland. A total of 13 study subjects (6 with SPP and 7 with LPP) were recruited from patients with histopathologically confirmed parapsoriasis and followed up at the university clinic. All patients gave their informed consent. The skin characteristics and medical history of all the study subjects are presented in **Table I**. None of the subjects had received antibiotics or ultraviolet (UV) phototherapy within the previous 12 months, and none were predisposed to bacterial

Table I. Patient characteristics

Clinical type	Pat. No.	Sex/age, years	Diagnosis time (years prior sampling)	Sample site	Previous phototherapies (years prior to sampling)	Concomitant diseases
LPP	1	F/37	2012 (2)	Flank	None	Asthma, allergic rhinitis
LPP	3	M/86	2011 (3)	Upper arm	None	Hypothyroidism, hypertension, hypercholesterolaemia
LPP	4	M/81	2009 (5)	Upper arm	NB-UVB 2011 (3)	Coronary artery disease, hypertension, hypercholesterolaemia, prostatic hyperplasia, arthrosis
LPP	6	F/69	2010 (4)	Upper arm	None	Recurrent deep vein thrombosis, nephrolithiasis, colonic diverticulitis
LPP	10	M/60	2008 (6)	Flank	None	Hypertension, gout, prostatic hyperplasia
LPP	11	M/57	2001 (13)	Upper arm	NB-UVB 2013 (1), bath PUVA 2012 (2), bath PUVA 2011 (3)	None
LPP	12	F/59	2002 (12)	Thigh	Bath PUVA 2010 (4), NB-UVB 2009 (5), NB-UVB 2008 (6), NB-UVB 2006 (8), oral PUVA 2003 (11)	None
SPP	2	M/73	2002 (12)	Flank	2012 NB-UVB (2), 2011 NB-UVB (3), 2005 SUP (9), 2003 SUP (11), 2001 SUP (12)	Type 2 diabetes, hypertension, hypercholesterolaemia
SPP	5	M/47	2003 (11)	Flank	2013 NB-UVB (1), NB-UVB 2008 (6), SUP 2008 (6), SUP 2004 (10)	None
SPP	7	F/66	2008 (6)	Abdomen	NB-UVB 2013 (1)	Hypertension, hypercholesterolaemia
SPP	8	M/63	2012 (2)	Abdomen	None	Hypertension, migraine, arthrosis
SPP	9	M/71	2010 (4)	Thigh	NB-UVB 2011 (3), NB-UVB 2010 (4)	Hypertension, hypercholesterolaemia, prostatic hyperplasia, arthrosis
SPP	13	F/52	2013 (1)	Upper arm	None	None

LPP: large-plaque parapsoriasis; SPP: small-plaque parapsoriasis; NB-UVB: narrow-band ultraviolet B phototherapy; SUP: selective ultraviolet phototherapy; PUVA: psoralen ultraviolet A photochemotherapy.

or fungal skin infections. All patients were examined by the same experienced dermatologist. The clinical picture was documented using close-up photographs prior to the incisional biopsy. The ethics committee of the University of Helsinki approved the study protocol (approval number 12/13/03/01/2012).

Skin microbiome samples were collected under sterile conditions with a sterile swab (Copan Flocked Swab®, Copan Diagnostics Inc., Murrieta, CA, USA). The swab was first dipped in a buffer solution (sterile 0.15 mol/l NaCl with 0.1% Tween 20), then rubbed approximately 20 times in both directions over the target skin area. Lesional specimens were obtained directly from the parapsoriasis patches. The borders of the individual parapsoriasis lesion were not traversed and the skin was not manipulated or disinfected before sampling. Control samples were collected in a similar manner from healthy skin on the contralateral side of the same patient's body. No control group of healthy individuals was analysed. The approach of using the patient's own healthy skin as control was chosen to avoid inter-individual variation in the microbiome (4, 14) and because the patients were already diagnosed with parapsoriasis, we wanted to investigate whether skin swab specimens could provide additional information to traditional biopsies. In order to reduce the risk of sample cross-contamination, a new pair of sterile gloves was used for each separate sampling procedure. Both the lesion and the healthy skin were sampled twice. Samples were deposited immediately in sterile 1.5-ml Eppendorf® tubes (Eppendorf AG, Hamburg, Germany), frozen with liquid nitrogen, and stored at –80ºC.

DNA extraction, PCR and sequencing

Sample DNA was extracted with the FastDNA Spin Kit for Soil (MP Biomedicals, LLC, Santa Ana, CA, USA) according to the manufacturer's instructions. Each extraction batch included a kit blank with no template DNA. The PCR protocol consisted of 2 steps. The first step was run with 2×25-µl technical replicates of each sample, with the amount of template DNA ranging from 23 to 58 ng. The primers for the first step consisted of universal bacterial primers targeting the V1–V3 regions of the 16S rRNA gene, pA (AGAGTTTGATCMTGGCTCAG) (15) and pD' (GTATTACCG-CGGCTGCTG) (16) and partial Illumina TruSeq adapter (Illumina Inc., San Diego, CA, USA) sequences (ATCTACACTCTTTCC-CTACACGACGCTCTTCCGATCT and GTGACTGGAGTT-CAGACGTGTGCTCTTCCGATCT, respectively) added to the 5' ends of the primers. The PCR program was as follows: initial DNA denaturation at 98°C, followed by 15 cycles at 98°C for 10 s, 65°C for 30 s, and 72°C for 10 s, and final extension for 5 min at 72°C. Each PCR run included a PCR blank with no template DNA. Before the second step, the PCR products were purified with Exonuclease I (Thermo Scientific) and Thermosensitive Alkaline Phosphatase (FastAP; Thermo Scientific), Thermo Fisher Scientific, Waltham, CA, USA. Five ml per sample of the first PCR step's products were used for the second PCR step, which was run with full-length TruSeq P5 and Index-containing P7 adapters, and a PCR program identical to the first, except with 18 cycles. The final PCR products were purified with Agencourt AMPure XP magnetic

Table II. Overall abundances of the 10 most common genera in the microbiome data (% out of total sequence reads)

Phylum	Class	Order	Family	Genus	% of sequences
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	*Propionibacterium*	27.13
Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	*Corynebacterium*	21.20
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	4.63
Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Micrococcus*	3.58
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Ralstonia*	3.30
Actinobacteria	Actinobacteria	Actinomycetales	Dietziaceae	*Dietzia*	3.02
Firmicutes	Clostridia	Clostridiales	Clostridiales Incertae Sedis XI	*Anaerococcus*	2.72
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*	2.21
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Curvibacter*	2.05
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	1.87
Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	10.19
				Other	18.10

beads (Beckman Coulter Finland Oy, Vantaa, Finland) and pooled. All samples were sequenced in a single run on a MiSeq Sequencer (Illumina) using v2 600 cycle kit paired-end (325 bp + 285 bp). Raw data from sequencing have been uploaded to the European Nucleotide Archive (accession no PRJEB15287).

Sequence data analysis and statistics

Cutadapt (17) was used to trim primers and low-quality ends of sequences from the data, with the parameters -q 30 for both reads and -m 200 for the forward, -m 180 for the reverse read. Pairing the reads, further sequence quality control and taxonomic classification were done with mothur (18) following the recommended procedure for MiSeq-sequenced 16S rRNA data (19). All singleton Operational Taxonomic Units (OTUs) were trimmed before further analysis. Because of recent studies about a possible link between Staphylococcus enterotoxins and cutaneous lymphoma-associated immunological dysregulation, we further explored the sequences classified as *Staphylococcus* using oligotyping (20).

Visualization and statistics were performed in R (21). The final taxonomy and OTU tables from mothur were exported to the phyloseq (22) R package. Based on their high abundance in blanks, all bacteria of the genera *Halomonas* and *Shewanella* were discarded as likely contaminants. Since there were duplicate samples (each patient location was sampled twice), these were merged before further analyses. For beta diversity comparisons with Bray-Curtis dissimilarity, the data was subsampled with phyloseq (23) to the lowest amount of reads per sample, which was 30 285. Bray-Curtis dissimilarities were calculated and compared with vegan (24) (commands vegdist and adonis). Phyloseq was used for non-metric multidimensional scaling (NMDS) ordination based on the Bray-Curtis dissimilarities, as well as calculating the Shannon and inverse Simpson alpha diversity indices (commands plot_ordination and estimate_richness). Statistical significances of alpha diversity and pairwise beta diversity were assessed using the Kruskal–Wallis rank sum test and the Wilcoxon rank sum test with Holm-Bonferroni multiple comparison correction, with p-values ≤ 0.05 considered significant. Differential abundance of taxa was tested with the package DESeq2 (25).

RESULTS

General characteristics of the microbiome findings

The skin microbiome data for our patients with parapsoriasis represented a total of 410 genera, 39 classes and 21 phyla. Eighty-nine percent of the sequences represented 4 phyla: Actinobacteria (class Actinobacteria, 59.37%), Firmicutes (predominantly the classes Clostridia and Bacilli, 15.02%), Proteobacteria (mostly Alpha-, Gamma-, and Betaproteobacteria, 12.46%) and Bacteroidetes (mainly Bacteroidia, 2.16%). Of these sequences, 10.19% remained unclassified at the phylum level. The most common genera in the microbiome data were *Propionibacterium* (27.13%), *Corynebacterium* (21.20%) and *Staphylococcus* (4.63%). The relative abundances of the most abundant bacterial genera are shown in **Table II** and **Fig. 1**.

Oligotyping was used to further characterize the potential *Staphylococcus* species in our data. The optimal result, based on 2 nucleotide positions, suggested that the sequences can be split into 5 oligotypes. Based on
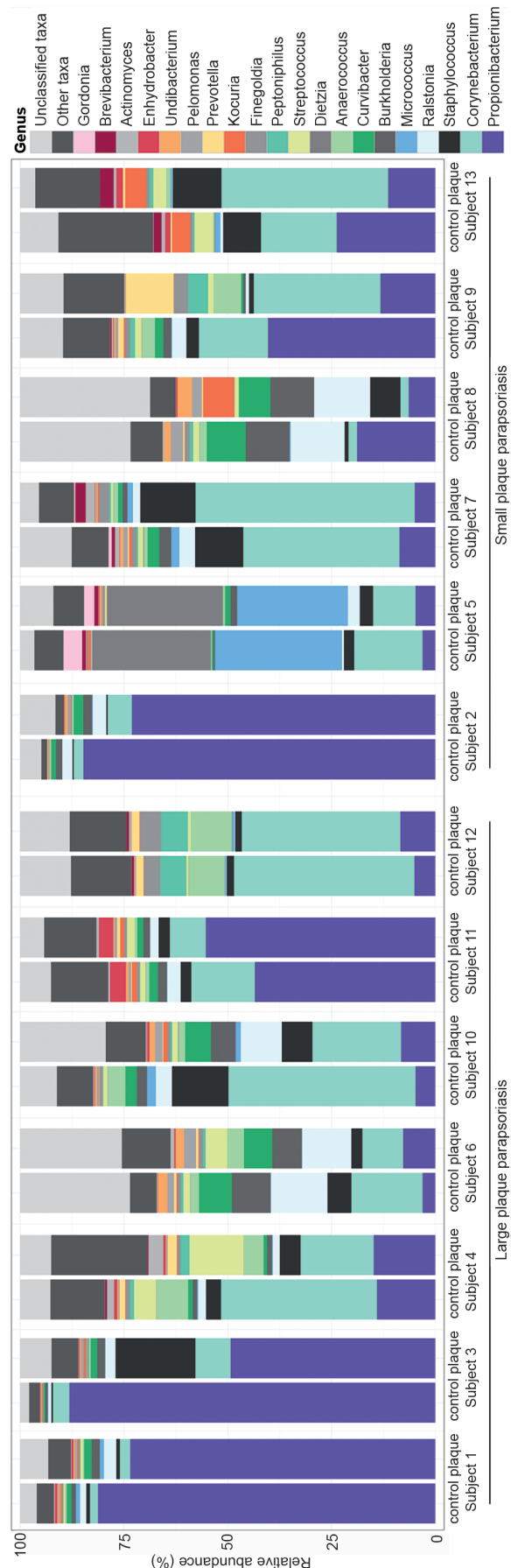

Fig. 1. Relative abundances of the 20 most common genera in the microbiome data, grouped by subject and sample type.
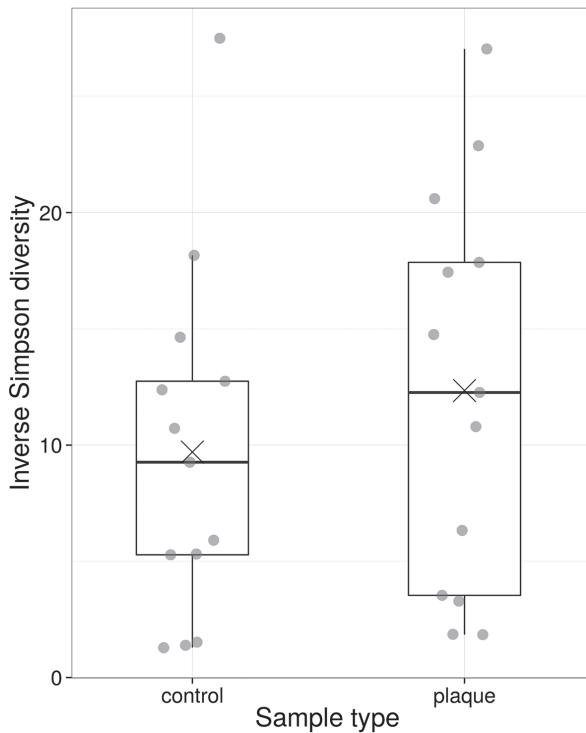
Fig. 2. Box-plot of the inverse Simpson diversity of the samples, grouped by sample type. *Lower and upper hinge* of the box: 1st and 3rd quartiles; *whiskers*: 1.5 * IQR; *line*: median; *cross*: mean; *grey points*: actual values for each sample.

comparisons with known sequences using BLAST, the 3 most common oligotypes represented *S. epidermidis* (39.63%), *S. hominis* (33.34%) and *S. capitis* (21.50%). The remaining 2 oligotypes (4.61% and 0.92%) could not be identified.
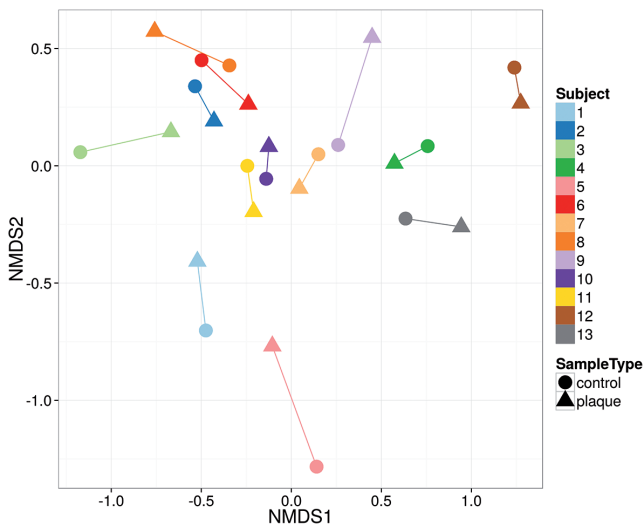

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarity. Colours correspond to subjects and shapes to sample types. The closer together 2 samples are on the plot, the more similar they are to each other.
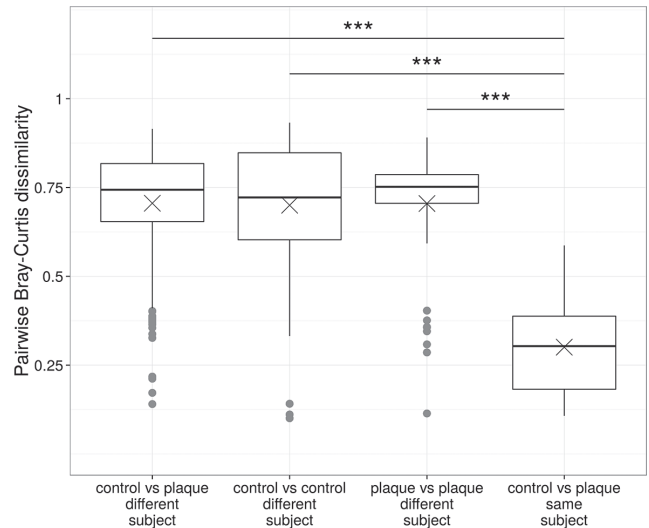

Fig. 4. Box plot of Bray-Curtis dissimilarities of all possible pairs of samples, in which each point represents the dissimilarity of 2 samples (the lower the value, the more similar the samples), grouped by type and subject of the compared samples. *Lower and upper hinge of the box*: 1st and 3rd quartiles; *whiskers*: 1.5 * IQR; *line*: median; *cross*: mean; *grey points*: outliers.

Microbial diversity

Alpha diversity indices (Shannon and inverse Simpson) were calculated for each microbiome sample, and these were compared statistically (**Fig. 2**). There was no statistically significant difference either between healthy (control) and lesional parapsoriasis skin (Kruskal–Wallis rank sum test; $p > 0.34$ for both indices), or between the small and large-plaque parapsoriasis groups ($p > 0.57$ for both indices).

In all patients, the microbial communities on lesional parapsoriasis skin and healthy control skin were very similar. A non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarity values, with samples coloured by subject, illustrates the similarity of each patient's samples (**Fig. 3**). The clustering by patient is highly statistically significant (adonis: $p = 0.00001$ and $R^2 = 0.88858$). On the other hand, neither ordination nor statistical testing with adonis suggested any kind of a community-wide effect for sample type or parapsoriasis subtype (data not shown). Grouped comparisons of pairs of Bray-Curtis dissimilarity values (**Fig. 4**) also demonstrate that pairs of samples from the same subject are highly similar, whereas pairs of samples from different patients, be they control vs. control, control vs. parapsoriasis plaque, or plaque vs. plaque, are equally dissimilar from one another. In other words, the microbiome was more alike between the same patient's samples, even though one was from lesional and the other from healthy skin, than compared with the samples of other patients, regardless of sample/lesion status or large-/small-plaque parapsoriasis type.

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Advances in dermatology and venereology

Comparisons of specific taxa

DESeq2 was used to search for differentially abundant taxa between the sample types (healthy skin vs. parapsoriasis lesion). A model that was corrected for the subject-specific variation produced a handful of significant taxa, but these were particularly abundant in one outlier sample, and were no longer significant when this outlier was left out of the analysis (data not shown). Unfortunately, the low number of samples per group did not allow for reliable comparisons between the small and large parapsoriasis subtypes. Statistical comparisons of the relative abundances of the 5 *Staphylococcus* oligotypes did not reveal any differences between plaques and healthy skin, or parapsoriasis subtypes (data not shown).

DISCUSSION

To our knowledge, this is the first publication on the skin microbiome in parapsoriasis. The abundances of bacterial taxa (based on the 16S rRNA gene) seen in our study were consistent with the results of preceding skin microbiome studies (14, 26). Also in line with previous studies, we observed significant interpersonal variation between subjects (27) and, thus, the use of an autologous healthy skin site as reference was appropriate. We did not find any differences between the lesional parapsoriasis skin and healthy control skin in our patients. Considering that the overall skin microbiota in our subjects appear to be similar to those found in subjects with healthy skin in many previous studies (28), the results of the current study suggest that overgrowth of any specific bacterial genus is not driving parapsoriasis, nor does parapsoriasis alter human skin bacterial communities.

While parapsoriasis belongs to the spectrum of lymphoproliferative diseases of the skin, the evolution of parapsoriasis is strongly influenced by the host's immune response. Considering the previously known association of the skin microbiome and cutaneous immune defence (29, 30) it could have been expected that lesional parapsoriasis would have an effect on the microbiome, or vice versa, local or systemic immunological factors of the patients would have had an effect on the cutaneous microbiome (31).

In recent studies, it has been demonstrated clearly that the composition of the skin microbiome is influenced by the host's native and adaptive immune system due to a constant interaction (32). Systemically acting or locally effective factors of the immune system have been shown to impact the cutaneous microbiological diversity (32–34). In addition, specific environmental factors, such as the patient's occupation, skin type, exposure to ultraviolet (UV)-light and the use of antibiotics, have been shown to influence the micro-organisms colonizing the skin (35, 36).

Some recent studies have investigated the role of the microbiome in skin cancer, but this field of research has only just begun to explore how the skin microbiome might influence the development of premalignant and malignant skin changes (37, 38). Some parallels have been drawn with the gut microbiome, which has been shown to directly impact the risk of cancer by promoting inflammation (39). The skin microbiome is almost as diverse as the gut microbiome, and might affect the risk of several diseases, including cancer (40).

Multiple reasons may explain why the skin microbiome in the patients' lesional parapsoriasis skin in our patient cohort showed no variation compared with their healthy control skin. One possibility would be the fact that the T-cell infiltration in parapsoriasis mainly occurs in the dermis. An epidermis of normal thickness contains only isolated, single atypical lymphoid cells, and therefore the cutaneous microbiome remains unaffected even in lesional skin. Another possibility would be that the stratum corneum is more intact in parapsoriasis than in other inflammatory skin diseases, e.g. atopic dermatitis, in which differences are seen.

Recent studies have shown a possible link between Staphylococcus enterotoxins and cutaneous lymphoma-associated immunological dysregulation (e.g. STAT3 activation and IL-17 expression in Sézary syndrome peripheral blood cell co-cultures). We further explored the sequences classified as *Staphylococcus* using oligotyping, but could not identify *S. aureus*. The role of Staphylococci has not been investigated in parapsoriasis earlier, but because of the fact that parapsoriasis belongs to the spectrum of cutaneous lymphoproliferative disorders and often precedes mycosis fungoides, the most prevalent type of CTCL (10), we expected to see changes in the skin microbiome. Specific differences between CTCL and parapsoriasis (T-cell infiltrate, localized and systemic disease), the metabolomics properties of the microbiome and unknown confounders may explain our observations. Based on these results, the role of *S. aureus per se* and SEA seem not to be relevant in parapsoriasis.

The presented observations were made in a small patient cohort, which should be considered as a major limitation of the study. In addition, we chose not to use a control group with healthy individuals, and such controls might reveal differences not seen here. The significant interpersonal variation and the small number of subjects may have masked minor differences between healthy and lesional skin, and elucidating them might only be possible in a larger cohort. In common skin diseases, such as atopic dermatitis, the demonstrated changes in the skin microbiome during disease progression and flares have been relatively characteristic, and this fact could be a potential target for future studies related to parapsoriasis (41, 42). Modern molecular tools for characterizing the skin microbiome have proved to be sensitive and less biased than older methods, and

could offer new insight into parapsoriasis and cutaneous lymphomas (43).

The role of the skin microbiome in parapsoriasis remains uncertain, but it would be important to further define how the microbiome changes during disease progression and to undertake metabolomics studies. Thus, as a further study we propose to compare the skin microbiome of large-plaque parapsoriasis with that of manifest cutaneous T-cell lymphoma. If changes in the microbiome in patients with common types of T-cell lymphoma can be reproduced, this would encourage further studies of large-plaque parapsoriasis in order to use disease-associated changes as a diagnostic tool. During disease progression of parapsoriasis a change in the cutaneous microbiome may be expected, as seen in inflammatory skin disorders such as atopic dermatitis. Investigation of the microbiome might therefore solve several aspects of the pathogenesis of parapsoriasis, leading to new diagnostic possibilities.

ACKNOWLEDGEMENTS

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