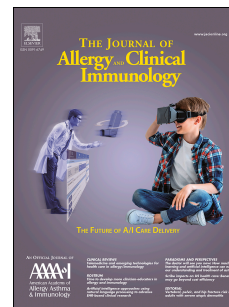


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Atopic dermatitis microbiomes stratify into ecological dermatotypes enabling microbial virulence and disease severity

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CONFLICT OF INTEREST DECLARATION

The authors have no conflicts of interest to disclose.

KEY WORDS

Atopic dermatitis, skin, microbiome, nonlesional, stratification, biomarkers

ABBREVIATIONS

Ac (antecubital fossae), AD (atopic dermatitis), AMP (antimicrobial peptide), BCAA (branched chain amino acid), CCL (chemokine (C-C motif) ligand), CoNS (coagulase-negative Staphylococcus), FLG (filaggrin), IgE (immunoglobulin E), IL (interleukin), KLK (kallikrein), MCP (monocyte chemotactic protein), MDC (macrophage-derived chemokine), NMF (natural moisturizing factor), PCA (2-pyrrolidone-5-carboxylic acid), SC (stratum corneum), SCORAD (SCORing Atopic Dermatitis) TARC (thymus and activation regulated chemokine), TEWL (transepidermal water loss), Th2 (T helper type 2), UCA (urocanic acid (or 4-imidazoleacrylic acid))

ABSTRACT

Background: Atopic dermatitis (AD) is a common skin disease affecting up to 20% of the global population, with significant clinical heterogeneity and limited information about molecular subtypes and actionable biomarkers. While alterations in the skin microbiome have been described in AD subjects during progression to flare state, the prognostic value of baseline microbiome configurations has not been explored.

Objective: To identify microbial signatures on AD skin that are predictive of disease fate.

Methods: Nonlesional skin of AD patients and healthy control subjects were sampled at 2-timepoints separated by at least 4 weeks. Using whole metagenome analysis of skin microbiomes for AD and control subjects (n=49, 189 samples), we identified distinct microbiome configurations (dermotypes A and B). Blood was collected for immunophenotyping and skin surface sampling was analyzed for correlations with natural moisturizing factors and anti-microbial peptides.

Results: Dermotypes were robust and validated across two additional cohorts (63 individuals), with strong enrichment of AD subjects in dermotype B. Dermotype B was characterized by reduced microbial richness, depletion of *Cutibacterium acnes*, *Demacoccus* and *Methylobacterium* species, individual-specific outlier abundance of *Staphylococcus* species (e.g. *S. epidermidis*, *S. capitis*, *S. aureus*), and enrichment in metabolic pathways (e.g. BCAA and arginine biosynthesis) and virulence genes (e.g. β -toxin, δ -toxin)_that defined a pathogenic ecology. Skin surface and circulating host biomarkers exhibited a distinct microbial-associated signature, that was further reflected in more severe itching, frequent flares and increased disease severity in patients harboring the dermotype B microbiome.

Conclusion: We report distinct clusters of microbial profiles that delineate the role of microbiome configurations in AD heterogeneity, highlight a mechanism for ongoing inflammation, and provide prognostic utility towards microbiome-based disease stratification.

Key Messages

- Distinct skin microbiome signatures at baseline stratify AD patients and help identify a group with more severe itching, frequent flares and increased disease severity.
- Differences in host skin response and circulating immune profiles highlight a mechanism for ongoing systemic inflammation driven by altered microbial ecology in dermatotype B patients.
- Microbiome-associated disease exacerbations can be monitored and serve as a basis for stratified treatment.

Capsule Summary

Distinct microbiome dermatotype signatures on nonlesional AD skin provide unique insights into altered ecological conditions that facilitate microbial virulence and could be predictive of AD disease outcomes.

INTRODUCTION

Atopic dermatitis (AD; OMIM 603165) is a chronic inflammatory skin disease that is impacted by complex interactions between barrier function, the immune system and environmental factors¹. Affecting approximately 1 in 5 people globally, AD is usually a lifelong disease, with relapsing-remitting periods more prevalent in childhood but often persisting into adulthood². Clinical management with topical and systemic medications can alleviate acute flare symptoms to maintain long periods of latency^{3,4}. However, adequate disease control is often complicated by heterogeneity of the condition, owing to its multifactorial aetiology and currently unknown mechanisms that drive disease flares.

Molecular signatures associated with AD have been explored extensively from a genetic and immunological perspective, but their application to disease stratification and management has been limited⁵. To date, more than 30 genetic loci have been identified for susceptibility to AD, mostly encoding epidermal barrier and immune response genes⁶. Immunological studies based on epidemiological data and serum biomarker profiles have demonstrated that AD is highly heterogeneous, implicating several immune pathways and highlighting that molecular stratification is necessary to understand the complex underlying endotypes and phenotypes⁷⁻¹². Microbial markers and stratification studies have been limited, primarily investigating the association between *Staphylococcus aureus* and AD¹³, and establishing that profound microbiome alterations are seen during flares¹⁴ and more subtle changes in non-lesional skin^{15,16}.

Microbe-host interactions at the skin interface are known to influence skin immune responses, thus likely contributing to AD disease pathogenesis¹⁷. In particular, shifts in skin microbiome configurations could serve as a trigger for the initiation of flares^{14,18}, highlighting the potential value of the baseline, non-lesional skin microbiome for providing vital information about skin homeostasis^{15,19}. The analysis of microbial DNA from skin can characterize the metabolic and virulence potential of resident species, in addition to their identities, thus serving to capture information about microbial ecology, skin surface metabolites and microbe-host interplay by proxy. Skin microbiomes can thus serve as a readily sampled and assayed source of biomarkers with predictive utility to track skin health and disease progression, that currently remains underexploited.

A potential challenge for the use of skin microbiome biomarkers is the natural

variability in species compositions, that can result in part from ready exposure to environmental perturbations. To circumvent this limitation, we conducted a two-timepoint sampling study separated by >4 weeks of standard-of-care treatment to identify stable signatures. Based on analysis of 189 metagenomic profiles from 49 subjects we identified two distinct microbial configurations (dermotypes A and B) that were stable across time, validated across multiple cohorts, and with marked enrichment of AD subjects in dermotype B. The microbial ecology of dermotype B subjects was characterized by reduced richness and individual-specific outlier abundance of *Staphylococcus* species, which in conjunction with increased abundance of specific metabolic and virulence genes indicates greater pathogenic potential for the skin microbiome. These differences were further reflected in host responses on skin (antimicrobial peptide and protease expression), circulating immune profiles (cytokine, chemokine and IgE levels), and patient phenotypes (itch and frequency of flares), highlighting a mechanism for microbiome-driven disease exacerbation that can be readily monitored and serve as a basis for stratified treatment.

MATERIALS AND METHODS

Study subjects

We recruited 50 subjects from the National Skin Centre (NSC), Singapore between March 2015 and July 2017. This included 34 AD patients, 15 healthy controls (and 1 patient with ichthyosis vulgaris who was excluded from the analysis). The study protocol was approved by the National Healthcare Group Domain Specific Review Board (DSRB, Reference 2013/01212) and in compliance with the Helsinki declaration. All study subjects gave written informed consent before participation. The inclusion criteria for AD patients were an unequivocal diagnosis of AD based on the UK Working Party criteria²⁰, age above 18 years old, with mild-to-severe AD as defined by objective SCORing Atopic Dermatitis (SCORAD) scores of <15 for mild AD, 15–40 for moderate AD, >40 for severe AD²¹ and with stable disease as defined by usual and stable disease state and the ability to tolerate at least 7 days without topical AD treatment to the intended sampling sites and at least 4 weeks off both oral corticosteroids and systemic treatment prior to the first recruitment visit. Exclusion criteria were a recent disease flare in the past 2 weeks, the use of any ultraviolet light therapy, systemic therapy, including corticosteroids or immunosuppressants within the past 4 weeks, the use of systemic antibiotics within the past 2 weeks, or the use of any creams, washes, soaps and personal care products that contain antiseptics or antibiotics over the intended sampling sites for the preceding 7 days. Controls were recruited with no reported personal history of atopic diseases (AD, asthma or allergic rhinitis).

Study design

Recruited subjects were assessed and sampled at two study visits, namely “visit 1” and “visit 2” that were between 4 to 6 weeks apart dependent on recall availability and clinical status of the subjects. Subjects were instructed not to use any soap over the intended sampling sites for 12 hours prior to each study visit. At each visit, study subjects underwent a clinical examination, skin physiological measurements and completed a full questionnaire related to medical and medication history. In between visits 1 and 2, patients resumed standard AD treatments prescribed by their physician. This included maintenance topical therapy such as topical steroids, antimicrobials and emollients but not systemic AD therapy including oral corticosteroids or phototherapy. In the event of a disease flare between visits 1 and 2, as defined by an episode requiring escalated treatment at the site of sampling, patients were

recalled at least 4 weeks after the flare episode to ensure that this study was focused on non-flare skin.

Skin physiological measurements

At each study visit, measurements of transepidermal water loss (TEWL), pH and stratum corneum (SC) hydration were performed over a fixed point, defined as 10 cm distal to the antecubital crease on uninvolved flexor volar forearm skin. Transepidermal water loss was measured using the VapoMeter (Delfin Technologies), pH was measured using the Seven2Go™ pH/mV Meter (Mettler-Toledo) and SC hydration was measured using a MoistureMeterSC Compact (Delfin Technologies). Three consecutive readings were taken and averaged. Measurements were performed in the same room under controlled temperature of 25 °C and after 15 min acclimatization.

Skin prick tests and serum total IgE

At the first visit, subjects were assessed for house dust mite (HDM) allergen sensitization using skin prick tests (SPT) to *Dermatophagoides pteronyssinus* and *Blomia tropicalis*. A positive SPT response is defined as a wheal diameter ≥ 3 mm. A positive control with histamine and negative control with saline was included in the panel. Atopy is defined as a positive SPT response to either of the HDM allergens, the major allergens in Singapore²². Serum total IgE was determined using a solid-phase, chemiluminescence immunometric assay on an Immulite 2000 analyzer (Siemens Healthcare). In this study, we defined elevated total IgE as >501 IU/mL. No controls had elevated total IgE but some had sensitization to HDM due to the high rates in the general population in Singapore (Table E1)²².

Sample collection

At visits 1 and 2, skin tape stripping of the antecubital fossae (Ac) of subjects was done using adhesive D-Squame tape discs (CuDerm Corporation) by repeated sticking and peeling actions for a period of 2 minutes, until the tapes were saturated with skin surface material as previously described¹⁵. Four tape strips were collected from the Ac of each arm at both timepoints, giving a total of 196 sampling sites (49 subjects at two Ac sites, across two visits) and a collective total of 784 tape strips for analysis. All samples were immediately placed on ice and transferred to the laboratory on the same day where they were stored at -20 °C before further processing. Peripheral blood samples were collected in EDTA-treated

Vacutainer™ tubes (lavender top; Becton Dickinson), inverted to mix to prevent clotting and transferred to the laboratory where they were processed for DNA extraction and isolation of peripheral blood mononuclear cells (PBMCs) on the same day.

Quantification of natural moisturizing factors on D-Squame tapes

Natural moisturizing factors (NMF) on D-Squame tape discs (2 discs per subject; bilaterally sampled at visit 1) were extracted in 175 µL of acetonitrile containing 15 ng/ml internal standards, vortexed for 2 min and 200 µL of water was added. An aliquot of 50 µL was used for protein quantification using the Micro BCA™ Protein Assay Kit (Pierce, Thermo Scientific) according to the manufacturer's instructions. Analysis of NMFs was done using ultrahigh-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) by combining the ACQUITY UPLC I-Class system (Waters Corporation) with the Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation).

To separate the four amino acids (L-Arginine, L-Histidine, 4-imidazoleacrylic acid or urocanic acid, UCA), 2-pyrrolidone-5-carboxylic acid (PCA) and internal standard (imipramine 15 ng/ml), chromatography was performed with UPLC–Kinetex Evo C18 column (100 × 2.1 mm i.d.; 1.7µm) joined to an UPLC guard column (Phenomenex Inc). A gradient method was used for chromatographic separation with mobile phase A (10mM ammonium acetate with 0.1% formic acid) and mobile phase B (methanol with 0.1% formic acid). The following gradient was used to separate L-Arginine, L-Histidine, UCA, PCA and internal standard: 0–0.2 min (25% A), 0.2–4.85 min (70% A), 4.85–4.86 min (25% A), 4.86–5 min (25% A). The mobile phase flow rate was 0.4 mL/min till 0.2 mins and increased to 0.5 mL/min till 4.85 mins and reduced back to 0.4 mL/min from 4.86 mins till 5 mins. The total run time was 5 min and the injection volume was 3 µL. The column temperature was set to 40 °C. The UPLC flow was introduced into the Xevo TQ-S. Electro-spray ionization (ESI+) was used as ionization source, and detection was obtained by multiple reaction monitoring (MRM) with the following transitions for L-Arginine, L-Histidine, UCA, PCA and internal standard: m/z 175.2→70, 156.1→110, 139.12→120.96, 130→84 and 281.16→58.11 respectively. The ESI–MS/MS parameters settings were as follows: capillary voltage, 3.9 kV; cone voltage, 90V; source temperature, 150 °C; desolvation temperature, 250 °C; gas flow to cone, 150 L/hr; desolvation gas flow, 1250 L/h (nitrogen). Argon was introduced as the collision gas into the collision cell at a flow rate of 0.15 mL/min. Data acquisition was done by Masslynx 4.1

software and processed by TargetLynx (Waters Corporation). The levels of L-Arginine, L-Histidine, UCA and PCA were normalized for the protein value of each tape.

Serum biomarker measurements

With the peripheral blood collected at visits 1 and 2, the levels of 50 serum cytokines and chemokines were assayed using the MILLIPLEX[®] MAP Human Cytokine/Chemokine Magnetic Bead Panel 1, 41-plex (Merck) and a Human Customized Panel (Merck): sCD40L, EGF, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN- α 2, IFN- γ , IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, MCP-1, MCP-3 (CCL7), MDC (CCL22), MIP-1 α , MIP-1 β , PDGF-AB/BB, RANTES, TGF- α , TNF- α , TNF- β , VEGF, Eotaxin (CCL11), PDGF-AA, Eotaxin-3 (CCL26), TARC (CCL17), MCP-4 (CCL13), MIG (CXCL9), MIP-3 α (CCL20), S100A9, MMP-12, IL-19 and IL-4R α . Samples and standards were incubated with fluorescent-coded magnetic beads, which had been pre-coated with the respective capture antibodies. After an overnight incubation at 4°C, plates were washed twice. Biotinylated detection antibodies were incubated with the complex for an hour and subsequently Streptavidin-PE was added and incubated for another 30 mins. Plates were washed twice again, then beads were re-suspended with sheath fluid before imaging on the FLEXMAP[®] 3D (Luminex) using xPONENT[®] 4.0 (Luminex) acquisition software. Data analysis was done on Bio-Plex Manager[™] 6.1.1 (Bio-Rad). Standard curves were generated with a 5-PL (5-parameter logistic) algorithm, reporting values for both median fluorescence intensity (MFI) and concentration data. Hierarchical clustering (Euclidean distance, complete linkage) was done using 19 AD-associated biomarkers from published studies^{8, 9, 23-25} and ANOVA analysis (p -value<0.05).

Dot blot assay

Proteins collected on D-Squame tape discs (2 discs per subject; bilaterally sampled at visit 1) were extracted in 1 mL of 0.1× phosphate buffered saline (PBS) and frozen in a -80 °C freezer overnight and thawed in a 37 °C water bath for 3 min. The freeze-thaw cycle was repeated two more times to release keratinocytes from the tapes. Samples were then sonicated for 2 cycles of 15 min on ice to lyse the cells. Samples were centrifuged at 16,000 × g for 5 min and concentrated by lyophilization. Lyophilized protein samples from both arms of a subject were pooled in 100 μ L of Tris-HCl buffer and transferred into a 384 well plate. A standard curve was generated with two-fold serial dilutions of a protein sample

pooled from 12 random individuals. A 384 solid pin Multi-Blot™ replicator (V&P Scientific, Inc.) was used to blot 0.2 µL of samples onto a nitrocellulose membrane for 5 times and the membranes were completely dried.

The nitrocellulose membrane was blocked in 4% non-fat milk in 1×PBS-T (0.1% Tween-20) for 1 hr, followed by 4 washes in 1×PBS-T. Primary antibody incubation was performed at 4 °C for 16 to 22 hr, followed by 4 washes in 1×PBS-T and secondary antibody incubation for 2 hr and 4 more washes in 1×PBS-T. Detection was performed with enhanced chemiluminescence and visualized with GeneSys software. Relative concentrations of each sample were calculated using the pooled sample as relative standards, and results were expressed in percent arbitrary units (%AU).

DNA extraction from blood and filaggrin genotyping

DNA was extracted from blood samples using standard protocols (Nucleon Illustra™ BACC2, GE Healthcare). Filaggrin (*FLG*) genotyping was carried out on all study subjects using a PCR-based tiled amplicon assay using 48.48 Access Array microfluidic technology (Fluidigm) and next generation sequencing (MiSeq, Illumina), as previously described²⁶.

DNA extraction for shotgun metagenomics

D-Squame tape discs (CuDerm Corporation) were transferred into Lysing Matrix E tubes (MP Biomedicals). 500 µL of ATL Buffer (Qiagen) was added and samples were subjected to bead-beating in a FastPrep-24 Instrument (MP Biomedicals) at 6.0 m/s for 40 s. Samples were centrifuged at 16,000 ×g for 5 min. 200 µL of the supernatant was treated with Proteinase K (Qiagen) and incubated at 56 °C for 15 min. DNA was extracted using the EZ1 Advanced XL Instrument (Qiagen) and EZ1 DNA Tissue Kit (Qiagen), and quantified using the Qubit dsDNA HS Assay Kit (Life Technologies) and stored at –20 °C.

Library construction and read preprocessing for metagenomics

50 µL of extracted DNA was fragmented using a Covaris LE220 Focused-ultrasonicator (Covaris). DNA libraries were constructed using GeneRead DNA Library I Core Kit (Qiagen) according to the manufacturer's protocol with the use of barcode adaptors. Custom index primers with 14 PCR cycles were used for enrichment of DNA libraries and libraries were quantified using the Agilent DNA 1000 Kit (Agilent Technologies) on an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples were then normalized and pooled, followed by

paired-end sequencing (2×101 bp reads) on an Illumina HiSeq 2000 Instrument at the Next Generation Sequencing Platform (Genome Institute of Singapore). Sequenced bases with quality score <3 were trimmed, and reads <30 bp in length were removed from the analysis. Human reads were filtered out by aligning to the human reference genome (GRCh38) using BWA-MEM²⁷ (version 0.7.10-r789). An average of 18.6% of the reads were found to be usable after filtering for human contamination (**Table E2**).

Taxonomic and statistical analysis

To profile the relative abundance of bacteria, eukaryotes and viruses, we used MetaPhlAn²⁸ with default parameters. Data from all cohorts was normalized using Cumulative Sum Scaling (CSS; cumNormMat function in metagenomeSeq 1.18.0) and control samples were compared across cohorts to confirm that no differentially abundant taxa were detected (genus-level; Wilcoxon test p-value >0.5). Differentially abundant species (mean relative abundance $>0.1\%$) were detected using Wilcoxon test and False Discovery Rate (FDR) adjusted p-values were reported. Alpha diversity and beta diversity analysis was performed using the vegan package (version 2.4–3). Principal coordinate analysis was performed based on Bray–Curtis dissimilarity using the ‘phyloseq’ package and the “ordinate” function to visualize relationships between case and control samples. Hierarchical clustering was performed on the first three principal coordinates to identify stable clusters with pvclust²⁹ using Euclidean distance and the ‘average’ clustering method with 1,000 bootstrap replicates.

Ecological modelling

To learn generalized Lotka-Volterra models (gLVM) for the two dermatotypes³⁰, species with low relative abundances ($<0.1\%$) were treated as absent in a sample, and rare species (present in <100 samples) were excluded from model fitting (samples with $>50\%$ of their relative abundance in rare species were also ignored). The dataset for dermatotype A was subsampled to match the number of samples in dermatotype B. Models were learned separately for the two dermatotypes using the BEEM-Static R package (commit: 07fbc4e; $\alpha=0.5$).

***Staphylococcus* virulence gene and strains**

Metagenomic reads were mapped using BWA (version 0.7.10-r789) to a curated set of *Staphylococcus* virulence genes including cytolytic toxin genes (beta-hemolysin and delta-

toxin), enterotoxin genes (*seA*, *seB* and *seC*) and exfoliative toxin genes (*etA* and *etB*; GenBank accession numbers S72497.1, AF288215.1, M18970.1, M11118.1, X05815.1, M17347.1 and M17348.1). Mapped read counts were used to determine the presence or absence of a gene and test for enrichment between dermatypes in R. Strain analysis for *Staphylococcus aureus* was performed using StrainPhlAn (version 2.7.7) with the “--relaxed_parameters3” option.

Pathway and correlation analysis

Analysis of differential functional pathways between groups was performed with LEfSe³¹ using relative abundance tables from metaCyC pathways produced with HUMAnN2³² (version 0.9.2). Pearson correlation test was used to examine the correlation between pairs of continuous variables. For all box plots, centre lines represent the median and the whiskers represent 1.5× inter-quartile range. Differences between groups were tested using the Wilcoxon rank-sum test and FDR corrected *p*-values were reported where applicable. For functional experiments, statistical significance was first determined by analysis of variance (ANOVA) followed by pairwise t-test comparisons (*p*-value <0.05).

Data availability

All sequencing reads are available from the NCBI sequence read archive (SRA) under accession number SRP160100.

RESULTS

Skin microbiome configurations stratify AD patients into distinct dermatotypes

A clinically well-characterized cohort of adult AD patients (n=34) and controls (n=15) was recruited from the National Skin Centre in Singapore (NSC), and sampled during two study visits separated by at least 4 weeks of standard of care treatment (no treatment for controls), to capture stable skin microbiome signatures in a natural history setting (no flare in past two weeks; **Table 1, Table E1**). Tape strips were collected at each visit from clinically intact skin of the left and right antecubital fossae (Ac, 196 samples), a site of disease predilection, and extracted DNA was analyzed with Illumina whole metagenome shotgun sequencing (189 successful libraries; 2×101bp, >10 million reads on average; **Table E2; Methods**) to study the skin microbiome (**Fig. E1**)¹⁵. Disease severity was assessed quantitatively using SCORAD²¹, along with physiological clinical parameters, trans-epidermal water loss (TEWL), skin hydration and pH during both visits. Taxonomic analysis based on metagenomic data across timepoints showed that the skin microbiome in AD subjects did not change significantly compared to healthy controls in this cohort, despite being maintained on their baseline routine topical anti-inflammatory therapies and emollients (Wilcoxon test p -value=0.38; Bray-Curtis index; **Table E1, E3; Methods**). This was accompanied by median objective SCORAD being unchanged (23.0) for the patient group at both visits (**Table 1**). Correspondingly, principal coordinates analysis (PCoA, with Bray-Curtis index) revealed two distinct microbiome configurations represented by a high density of samples, that were bilaterally consistent (**Fig. 1A**), and temporally stable across study visits compared to differences between the configurations (Wilcoxon test p -value<0.001, **Fig. 1B**). These microbial community types have not been observed previously in microbiomes for specific skin sites and are hence referred to as “dermatotypes” here.

To assess the robustness of this analysis, we included metagenomic data (n=44) from a previously sequenced cohort¹⁵ and observed that hierarchical clustering consistently revealed the same two high confidence (>90%) clusters (**Fig. 1C; Methods**). While one of the community types was well-represented in both control and AD subjects (dermatotype A), the second community type was found to predominantly have AD subjects (dermatotype B; Fisher’s exact test p -value<0.05). A similar distribution of subjects in dermatotypes was also seen when metagenomic data for this body site was included from another cohort³³ (n=19;

Fig. E2). Furthermore, modelling of microbial interactions based on generalized Lotka-Volterra equations confirmed that the dermatotypes represent distinct community types in terms of carrying capacities and interaction hubs (**Fig. E3; Methods**). Together, these results highlight the presence of a distinct skin microbiome dermatotype in the clinically intact skin of a large fraction of AD patients (~30%) whose structure and function has not been previously explored.

Dermotype B represents a configuration with reduced microbial richness and a niche favoring specific *Staphylococcus* species

By comparing the taxonomic profiles at the species level between healthy controls (n=15; all dermatotype A) and AD patients from dermatotypes A (n=18) and B (n=16), we observed that while community richness was preserved in AD patients from dermatotype A compared to controls, there was a significant loss of species in dermatotype B subjects (**Fig. 2A**). This was accompanied and contributed to by reduced abundances of key commensal species such as *Cutibacterium acnes*, *Demacoccus* and *Methylobacterium* species, some of which have previously been associated with AD¹⁵ (FDR-corrected Wilcoxon *p*-value <0.05; abundances normalized to account for compositionality, **Methods; Table 2; Fig. 2B**). In addition, several species that have previously been reported to inhibit the growth of *Staphylococcus aureus* (e.g. *Staphylococcus hominis*³⁴, *Streptococcus mitis*¹⁵) were found to be specifically or more markedly depleted in dermatotype B, compared to controls and AD patients from dermatotype A (**Fig. 2C**). Among enriched genera in dermatotype B, *Staphylococcus*, *Micrococcus* and *Enhydrobacter* were notable, though not statistically significant (FDR-corrected Wilcoxon *p*-value >0.05). Specific *Staphylococcus* species including *S. epidermidis*, *S. capitis* and *S. aureus* were seen in high abundance in several dermatotype B patients (>3 standard deviations away from mean for controls) and enriched in frequency compared to dermatotype A AD patients (Fisher's exact test *p*-value <0.05), though the median abundance was not significantly different for any species between dermatotype A and B microbiomes (FDR-corrected Wilcoxon *p*-value >0.05; **Fig. 2D**). Overall, these findings point to an altered microbiome configuration in dermatotype B characterized by reduced richness, depletion of several commensals and sporadic individual-specific enrichment of *Staphylococcus* species.

Metabolic and virulence factors have distinct microbial pathogenicity across dermatotypes

Leveraging shotgun metagenomic data, we compared the genetic potential for various metabolic and virulence pathways between controls and AD patients belonging to dermatotypes A and B (**Table E4; Methods**). As expected, pathway abundance profiles of samples from the two dermatotypes exhibited broadly distinct patterns (**Fig. E4**). Overall, several pathways related to branched chain amino acids (BCAA) biosynthesis were over-represented in dermotype B subjects (**Fig. 3A**). BCAAs are known to enhance signalling pathways key to immune cell function^{35, 36} and over-supplementation may contribute to hypersensitive immune systems characterized by an increase in Th2 cytokines. Additionally, enrichment in arginine biosynthesis pathways was noted specifically in dermotype B, while genes for arginine conversion to ammonia were shown previously to be enriched across AD patients¹⁵ (**Fig. 3A**). The production of ammonia can raise skin pH and adversely affect antimicrobial acid stress³⁷, and furthermore, reduce the availability of arginine for urea production via the arginase pathway and thus contribute to dry skin³⁸. In agreement with its higher arginine conversion (but not microbial biosynthesis) potential, arginine levels on the skin of dermotype A AD patients were lower than controls (UPLC–MS/MS data; t-test p -value <0.008 ; **Fig. 3B**). In contrast, dermotype B patients with enriched microbial biosynthesis and conversion pathways maintain arginine levels closer to controls (**Fig. 3B**). These results underline the distinct metabolic functions enriched across dermatotypes and their potential to contribute to skin chemistry and physiological differences seen in AD.

We further investigated the abundance of virulence genes in the skin microbiomes of dermotype A and B patients compared to controls to study the pathogenic potential of these communities. Normalizing by species abundance, several *Staphylococcus* virulence genes¹³ including β -toxin, δ -toxin and enterotoxin genes (*SEA* and *SEB*) were found to be significantly enriched for detection in dermotype B compared to dermotype A AD patients and controls (FDR corrected χ^2 test p -value <0.1 ; **Fig. 3C**). This was despite the absence of any significant differences in *S. aureus* strain type distribution across dermatotypes (**Fig. E5**).

Host responses on skin have a pronounced microbial signature in Dermotype B patients

Skin physiology measurements (hydration, TEWL) were found to be significantly different between controls and AD patient groups, though differences between dermatotype A and B patients were less defined (**Fig. 4A**). As loss-of-function mutations in the *FLG* gene causing skin barrier defects are the strongest known genetic risk factor for AD and may impact the skin microbiome⁴⁰⁻⁴², we genotyped all patients by whole gene sequencing²⁶ and measured filaggrin specific natural moisturizing factors (NMF) (2-pyrrolidone-5-carboxylic acid - PCA, urocanic acid - UCA and histidine by UPLC–MS/MS). The frequency of *FLG* mutations did not differ significantly between dermatotype A and B patients (Fisher's exact test p -value=0.43), and while surface levels of corresponding NMF components were significantly lower in AD patients compared to controls, this did not differ between dermatotypes (**Fig. 4B**).

To investigate host response at the stratum corneum (SC), we extracted proteins from tape strips and performed dot blot assays for a subset of severity-matched dermatotype A and B patients (**Table E5; Methods**). While antimicrobial peptides (AMPs) such as defensins have been reported to be up-regulated in AD lesions compared to normal skin⁴³, we observed increased levels of human β -defensin-3 (hBD-3) and α -defensin-3 (HNP-3) in clinically intact skin of dermatotype B patients⁴⁴ compared to controls and dermatotype A patients (FDR-corrected t-test p -value <0.1; **Fig. 4C**). In addition, the kallikrein KLK13, a serine protease predominantly expressed in the upper granular layer and stratum corneum of the epidermis⁴⁵, was specifically elevated in dermatotype B patients (FDR-corrected t-test p -value <0.05; **Fig. 4D**), reflecting an anti-microbial response that can lead to barrier disruption and inflammation⁴⁶⁻⁴⁸. Finally, Cathepsin S, a cysteine protease whose overexpression has been implicated in inducing scratching behavior in mice⁴⁹, was also specifically higher in dermatotype B patients (FDR-corrected t-test p -value <0.05; **Fig. 4D**). Thus overall, microbial dermatotype B patients exhibit distinct stratum corneum biomarker expression, indicating neutrophil-activation (e.g. neutrophil specific defensin, HNP-3) and anti-microbial response.

Dermotype B subjects exhibit distinct immune profiles in blood and increased disease severity

Analysis of 50 biomarkers in blood serum (Luminex multiplex technology) was used to study if differences in dermatotype B microbial virulence and host responses were also reflected in

circulating immune profiles (**Table E6; Methods**). Clustering using AD-associated biomarkers^{8,9,23-25} grouped all but one dermatotype B patient separately from dermatotype A patients and controls, as a cluster enriched for Th2 and innate immunity biomarkers (**Fig. 5A; Methods**). Differential abundance analysis with all 50 serum biomarkers identified 7 analytes (FDR-corrected Wilcoxon p -value <0.1) that were significantly higher only in dermatotype B patients compared to controls (CCL17/TARC, CCL13, CCL22/MDC, CCL26, IL-13, TNF- β , S100A9; **Fig. 5B, E6**), with 4 analytes exhibiting significant Th2 (CCL17, CCL22) and innate (CCL13, CCL26) response differences between dermatotype A and B patients (**Fig. 5B**). Quantifying the number of circulating immune cells by FACS analysis showed no significant difference in dermatotype B patients compared to controls, though changes in monocytes, B cells and NK cells were seen in dermatotype A patients (**Fig. E7**). Together, this data confirms that the distinct microbial metabolism, virulence and skin surface responses seen in dermatotype B versus dermatotype A patients are also reflected in systemic Th2 and innate immune responses in blood (including higher IgE levels, Wilcoxon p -value <0.01). Overall, our data supports a model where dermatotype B represents a distinct microbiome configuration, with altered metabolism and virulence, that can influence susceptibility to microbial triggers. Correspondingly, we investigated medical records of the patients in our cohort and observed that dermatotype B patients were more prone to severe itching (Wilcoxon p -value <0.05 ; **Fig. 5C**) and recurrent flares that exacerbated their disease state (50% in dermatotype B; 17% in dermatotype A; χ^2 test p -value <0.04 ; **Fig. 5C**). With a higher frequency of flares, dermatotype B patients also exhibit more pronounced innate and adaptive immune response (**Fig. 4C; Fig. 5A, B**), and higher clinical severity scores (Wilcoxon p -value <0.02 ; **Fig. 5D**). Finally, dermatotype B AD subjects were found to have a higher propensity for other common atopic conditions in adults (allergic rhinitis or asthma; 81% in dermatotype B; 46% in dermatotype A; Fisher's exact test p -value <0.023), highlighting the clinical utility of dermatotypes for stratifying AD patients.

DISCUSSION

Atopic dermatitis has a high degree of clinical heterogeneity, and a stratified approach to defining patient groups will be beneficial for defining treatment strategies⁵. Several studies have focused on identifying host biomarkers of disease activity in skin and blood identifying various endotypes^{9, 16, 25, 50, 51}. By showing for the first time that intrinsic skin surface microbiome configurations alone can stratify AD patients with markedly different disease phenotypes and endotypes, we provide an alternative, conveniently accessible and biologically meaningful microbiome definition for AD subgroups. Our study establishes the functional features of stratification, by using integrated analysis to identify clear clinical associations between cutaneous microbiome, host biomarkers, and disease severity.

The defining characteristic in the microbiome configuration of dermatotype B subjects is an altered carrying capacity for various species that results in a distinct microbiome assemblage with reduced species richness (**Table 2**). Reduced abundance was observed in several species that can protect against *S. aureus* overgrowth, including *S. hominis*³⁴, *S. mitis*, *S. sanguinis*¹⁵ and *C. acnes*⁵². We also observed enrichment of *Micrococcus luteus*, which can augment proliferation and virulence of *S. aureus*⁵³. However, strikingly, no significant change in *S. aureus* abundance was observed on non-flare AD skin. Instead, several *Staphylococcus* species were sporadically enriched across dermatotype B subjects. We also observed increased virulence gene exposure, such as δ -toxin, in dermatotype B subjects that could contribute to inflammation by activating mast cells, inducing allergic skin disease and thus would be predicted to result in higher serum IgE³⁹. Dermatotype B thus defines a distinct microbiome state with reduced richness that favours *Staphylococcus* species on non-flare AD skin and an interactome that is less hostile to *S. aureus*.

Additionally, skin surface topography can profoundly shape the skin microbiome with physiologically comparable body sites sharing similar taxonomic profiles⁵⁴. However, we identified something different across the dermatotypes with stable, yet distinct, microbial configurations at the antecubital fossa site. The underlying skin surface and systemic biomarkers were also differentiating between dermatotype A and B, however, no clear differences were observed in skin physiological parameters (TEWL, skin hydration, NMFs). The two dermatotypes did have a clear differentiator in that IgE was significantly higher in dermatotype B subjects identifying a group of extrinsic/allergic AD subjects²³. It is also worth

noting that while dermatotype B was enriched in AD patients, we also identified healthy individuals with dermatotype B within published cohorts^{15, 33}. The biological implications for healthy subjects that harbour dermatotype B microbiomes requires further investigation as published studies did not provide in-depth analysis of host-microbe interactome that could have indicated subclinical skin barrier alterations or susceptibility to atopic disease. Currently, how these microbial signatures are established and could be modulated by different treatment strategies is unexplored.

Skin microbiomes have typically been described as associated with health or disease using broad measures of diversity from a small number of cohorts, however their metagenomic predictive value and functional utility have not been fully explored. The ecological description of skin microbiome dermatotypes that we provide here is analogous to enterotypes described for the gut microbiome and allows for stratification of groups of subjects⁵⁵. We have identified that these assemblages of microbial communities can provide valuable indications related to underlying host biology that is predictive of clinical phenotypes. Our observations provide unique insights into the distinct functional features at the taxonomic level and as mapped to genetic and metabolic pathways. This was highlighted by shifts in carrying capacity and altered arginine and branched-chain amino acid synthesis pathways between the identified dermatotypes. Additionally, skin surface and circulating biomarkers of host immunity were distinct between dermatotypes, suggestive of a host-microbe interactome with multiple nodes of homeostatic plasticity to maintain skin health. We envisage that monitoring the environmentally sensitive skin surface microbiome could act as a predictive beacon for atopic dermatitis disease outcomes.

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FIGURE LEGENDS

Figure 1. Identification of distinct skin microbiome dermatotypes. (A) Principal coordinates analysis (PCoA) plot (species-level Bray-Curtis index) highlighting that skin microbiome taxonomic profiles have two distinct configurations as indicated by regions of high density, with bilateral representation (red: left Ac, blue: right Ac). Contour lines represent regions that have the same sample density. The distribution of Pearson correlation values between left and right Ac profiles is shown above the PCoA plot to highlight the extent of bilateral concordance. (B) Boxplots showing the Bray-Curtis dissimilarity of taxonomic profiles between visits for the same site and across dermatotypes. Statistical significance was determined using the Wilcoxon test (***: p -value <0.01). (C) Clustering of microbiome profiles from two consistently analysed cohorts (this study and Chng et al¹⁵) using average-linkage clustering (values indicate bootstrap confidence). Among the two top-level clusters, the cluster on the left was observed to be enriched for AD subjects (dermatotype B) compared to the cluster on the right (dermatotype A; orange: AD, green: healthy). Numbers indicate the bootstrap probability as percentages.

Figure 2. Microbial diversity and taxonomic features of dermatotype B communities. (A) Species richness of skin microbiomes for dermatotype A controls ($n=15$) and dermatotype A ($n=14$) and B ($n=14$) patients. (B) Boxplots depicting the depletion of AD-associated commensal species¹⁵ in dermatotype B subjects. (C) Boxplots depicting the depletion of species antagonistic to *S. aureus* growth in dermatotype B subjects. (D) Stacked bar graphs depicting the relative abundance of *Staphylococcus* species present in the skin microbiomes of dermatotype A controls, and dermatotype A and B patients. Colored boxes at the bottom indicate samples with outlier values (>3 standard deviation from the mean) relative to the controls group. A χ^2 test was used to identify enrichment of outlier samples among dermatotype B versus dermatotype A patients. FDR corrected Wilcoxon p -values were computed for subfigures B and C. *: p -value <0.1 , **: p -value <0.05 , ***: p -value <0.01

Figure 3. Functional shifts and enrichment of virulence genes in the skin microbiome of dermatotype B patients. (A) Differentially abundant pathways (linear discriminant analysis or LDA score >3) in skin metagenomes of dermatotype A controls and dermatotype A and B patients (each group vs other two). (B) Boxplot showing that controls and dermatotype B patients have significantly higher arginine levels compared to dermatotype A patients

(Wilcoxon test). (C) Barplot showing the percentage of patients from the two dermatotypes with virulence genes detected in the skin microbiome. Multiple virulence factor genes were significantly enriched for detection in patients from dermatotype B (χ^2 test with FDR correction; SEA, SEB and SEC: Staphylococcal enterotoxin A, B and C, ETA: exfoliative toxin A). *: p -value <0.1; **: p -value <0.05; ***: p -value <0.01

Figure 4. Consistency of skin surface parameters in patients and variation of host response markers by dermatotype. (A) Boxplots showing data from transepidermal water loss (TEWL; left) and skin hydration (right) measurements across subject groups (Wilcoxon test). (B) Boxplot showing that natural moisturizing factors (PCA+UCA+HIS) are similarly depleted in patients (dermatotype A and B) versus controls (Wilcoxon test). Boxplots showing the significant enrichment of (C) defensins (HBD-3: human β -defensin 3; HNP-3: human neutrophil peptide 3 or α defensin 3) and (D) proteases (KLK13: kallikrein 13; cathepsin S) in the skin of dermatotype B patients compared to dermatotype A patients and control subjects. Statistical significance for host response markers was determined by pairwise t-test comparisons and FDR correction. *: p -value <0.1; **: p -value <0.05; ***: p -value <0.01

Figure 5. Systemic and phenotypic differences in dermatotype B patients. (A) Clustering based on AD-associated serum biomarker revealed a cluster of dermatotype B patients enriched for Th2 and innate immunity markers. (B) Boxplots of serum biomarker analytes exhibiting significant increase in dermatotype B compared to dermatotype A AD subjects. Statistical significance was determined by FDR-corrected Wilcoxon tests. (C) Graphs showing that dermatotype B patients were more prone compared to dermatotype A patients to severe itching (Wilcoxon test; left) and recurrent flares in the past one year (χ^2 test; right). (D) Boxplot showing the significantly higher disease severity in dermatotype B versus dermatotype A patients (Wilcoxon test; SCORAD: SCORing atopic dermatitis). *: p -value <0.1; **: p -value <0.05; ***: p -value <0.01

771 **Table 1.** Clinical characteristics of the study subjects

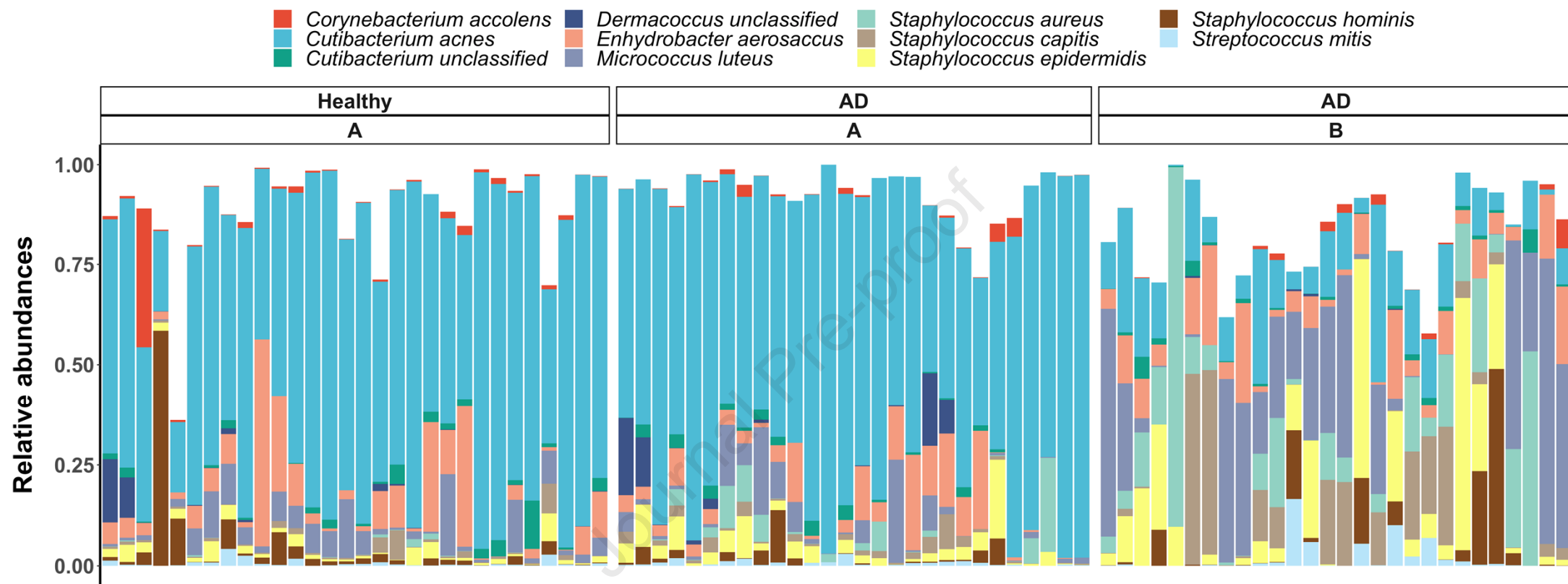
	Atopic dermatitis (AD)	Control
Subjects analyzed (n)	34	15
Age, median (range)	27 (18–48)	27 (22–45)
Male:female	30:4	5:10
Visit 1 objective SCORAD, median (range)	23.0 (7.2–46.2)	N/A
Visit 2 objective SCORAD, median (range)	23.0 (3.7–45.8)	N/A
IgE in IU/mL, median (range)	2965 (21–15000)	39 (3–501)
Age of onset, no. (%)		
< 2 years	(23.5)	0 (0)
2–4 years	(11.8)	0 (0)
5–14 years	(61.8)	0 (0)
Missing data	1 (2.9)	0 (0)
Family history of AD, no. (%)	20 (58.8)	0 (0)
Atopic diseases, no. (%)		
Asthma	20 (58.8)	0 (0)
Allergic conjunctivo-rhinitis	22 (64.7)	0 (0)
Positive skin-prick test, no. (%)		
<i>Blomia tropicalis</i>	29 (85.3)	7 (46.7)
<i>Dermatophagoides pteronyssinus</i>	30 (88.2)	7 (46.7)
Missing data for <i>Blomia tropicalis</i>	3 (8.8)	0 (0)
Missing data for <i>Dermatophagoides pteronyssinus</i>	2 (5.9)	0 (0)

772

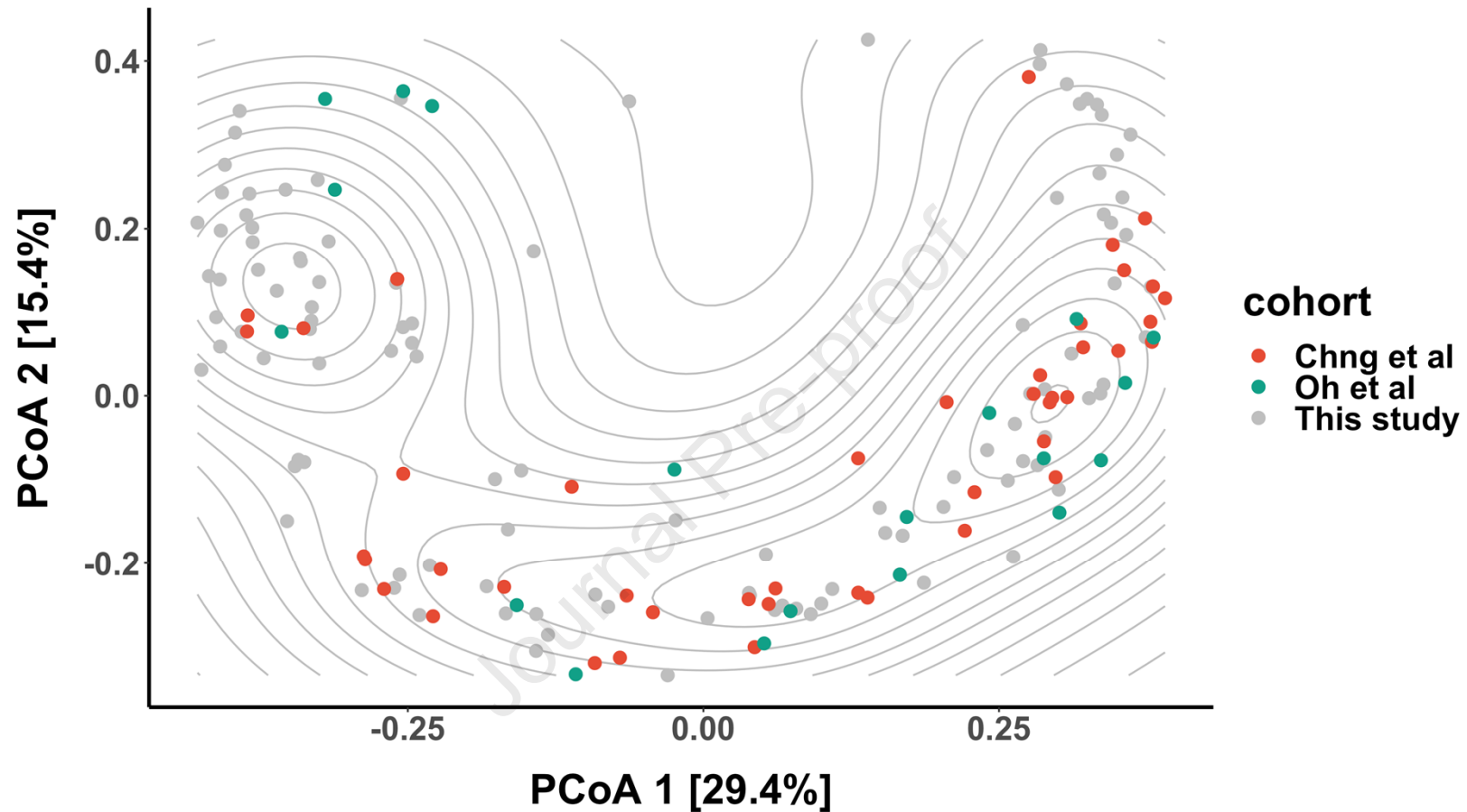
773

Table 2. Differentially abundant species in dermatotype B patients compared to dermatotype A patients and controls (FDR corrected p -values<0.1 for both comparisons).

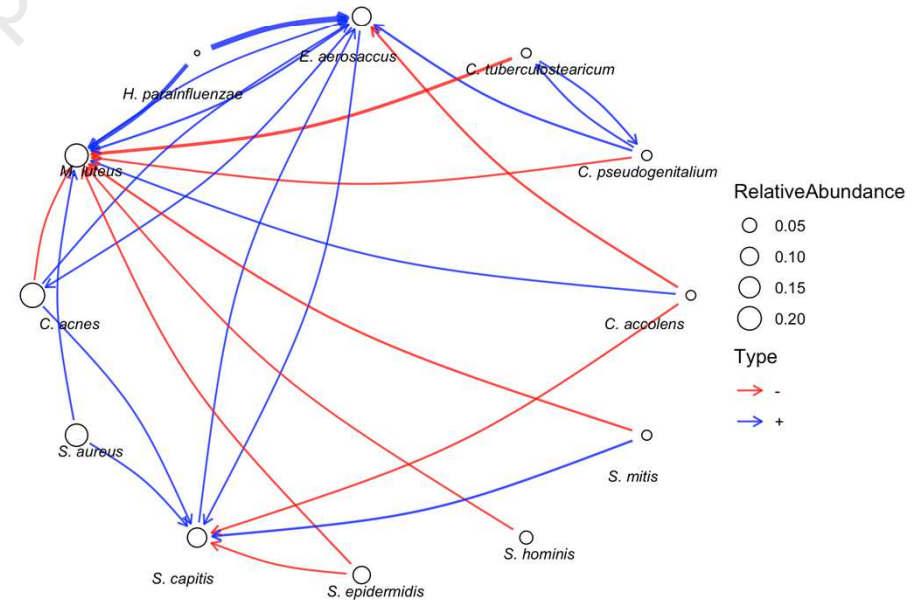
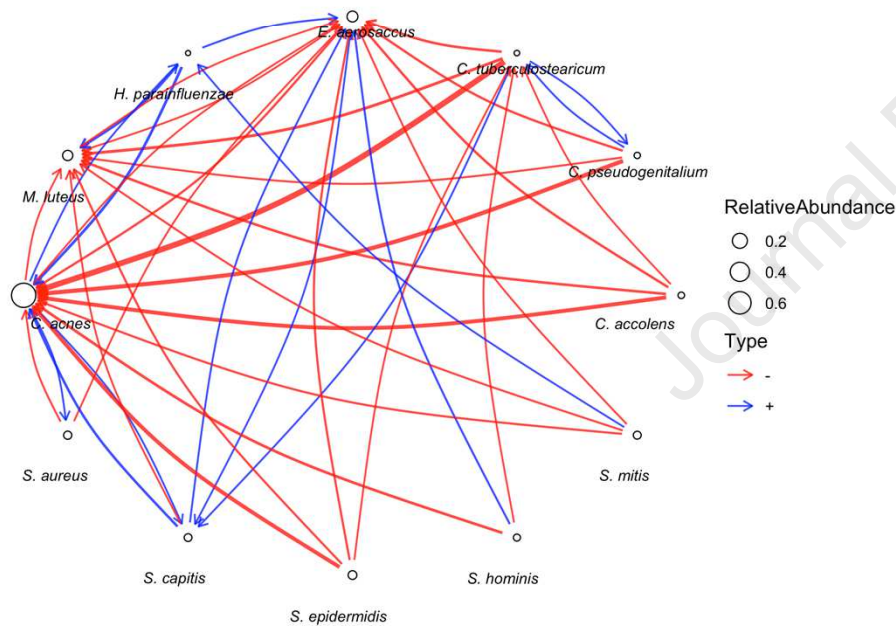
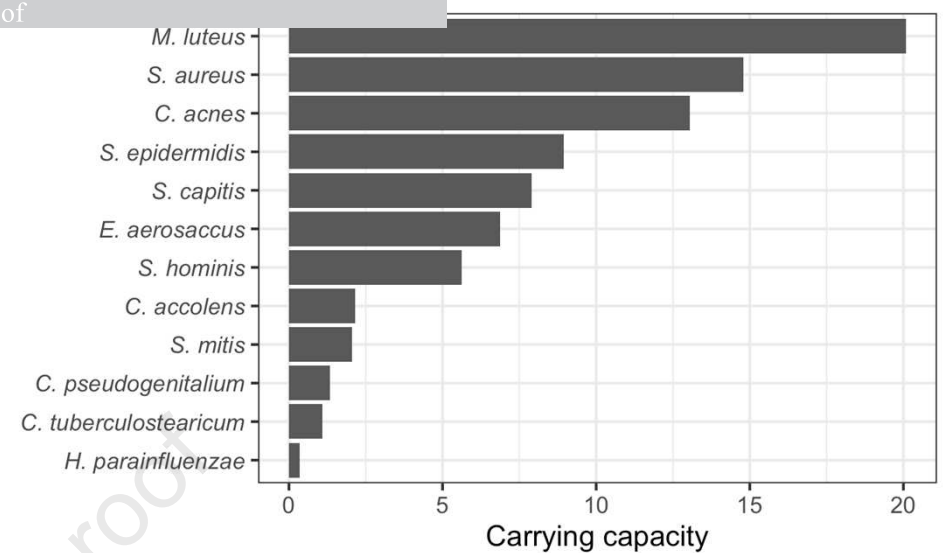
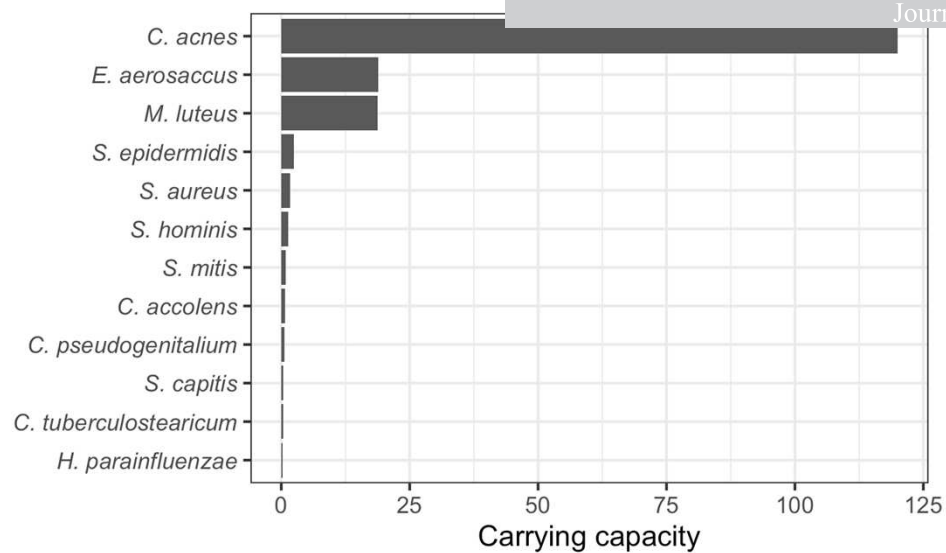
Species	Dermatotype B patients vs dermatotype A controls	Dermatotype B patients vs dermatotype A patients
<i>Acinetobacter junii</i>	0.0011	0.024
<i>Corynebacterium matruchotii</i>	0.0033	0.0017
<i>Cutibacterium acnes</i>	<0.0001	<0.0001
<i>Deinococcus unclassified</i>	0.034	0.038
<i>Dermaococcus unclassified</i>	0.0012	0.0092
<i>Haemophilus parainfluenzae</i>	0.000034	0.000059
<i>Methylobacterium unclassified</i>	0.00047	0.0016
<i>Pseudomonas unclassified</i>	0.025	0.0038
<i>Rothia mucilaginosa</i>	0.011	0.048
<i>Staphylococcus hominis</i>	0.00033	0.24
<i>Streptococcus mitis</i>	0.0039	0.006
<i>Streptococcus sanguinis</i>	0.013	0.0062
<i>Veillonella parvula</i>	0.0072	0.0066



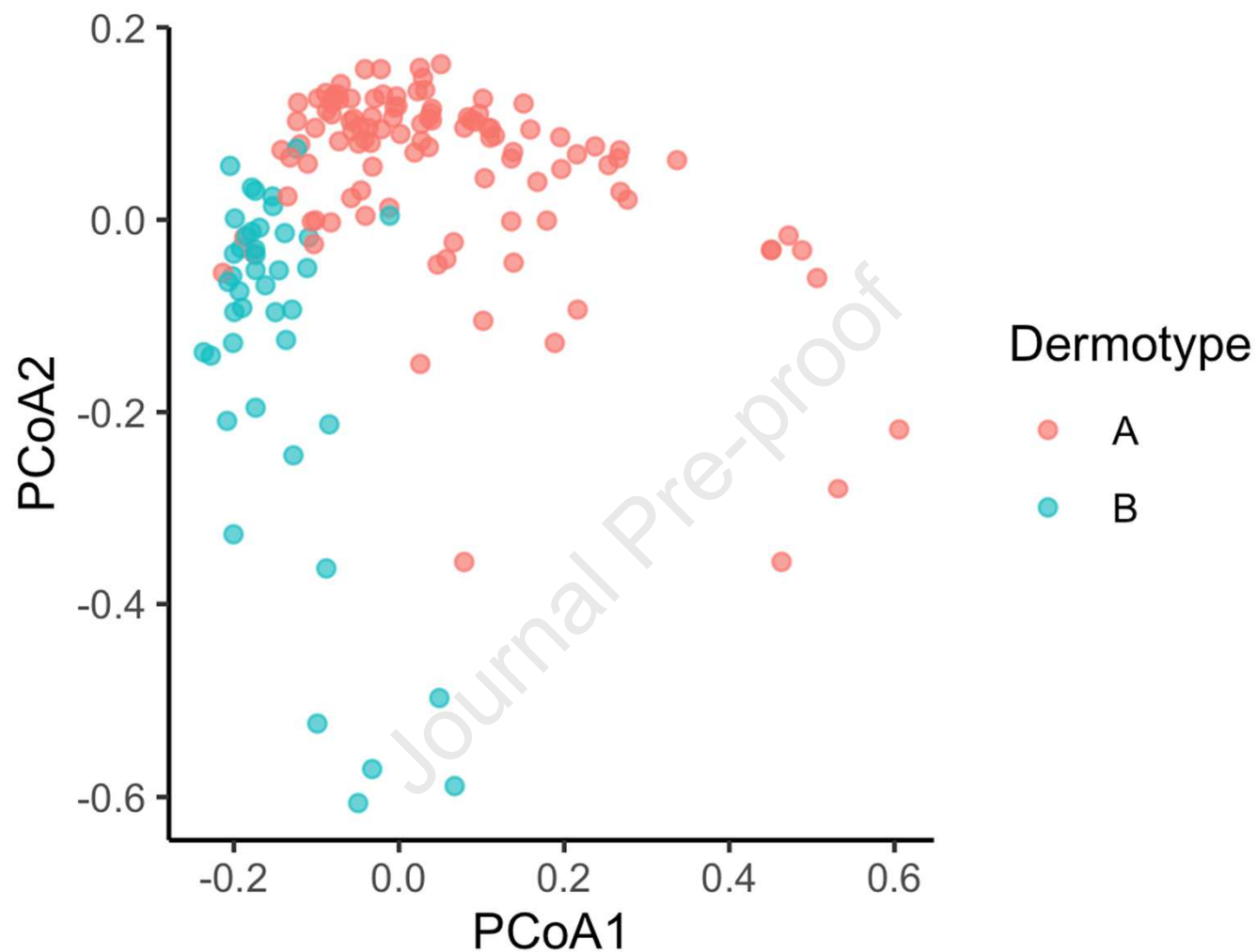
Supplemental Figure E1: Stacked bar graphs showing the relative abundances of major species (mean relative abundance >1%) in dermotype A controls, and dermotype A and B patients.



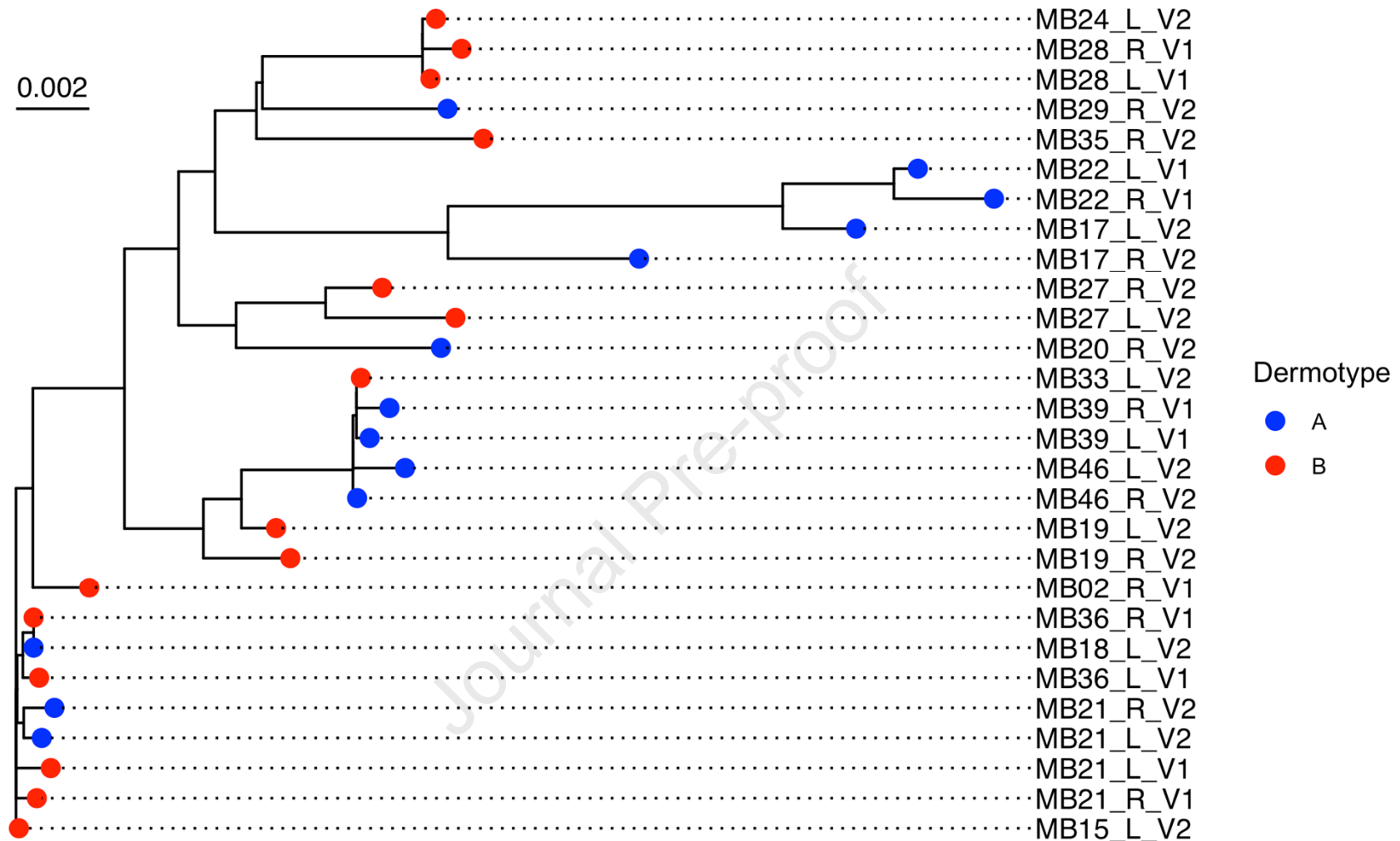
Supplemental Figure E2: Principle coordinate analysis plot (Bray-Curtis dissimilarity) based on skin microbiome data for multiple cohorts, including data from this study (grey), from Oh et al (n=19, green) and from Chng et al (n=44, red). All samples were from the antecubital fossa and visually undamaged skin.



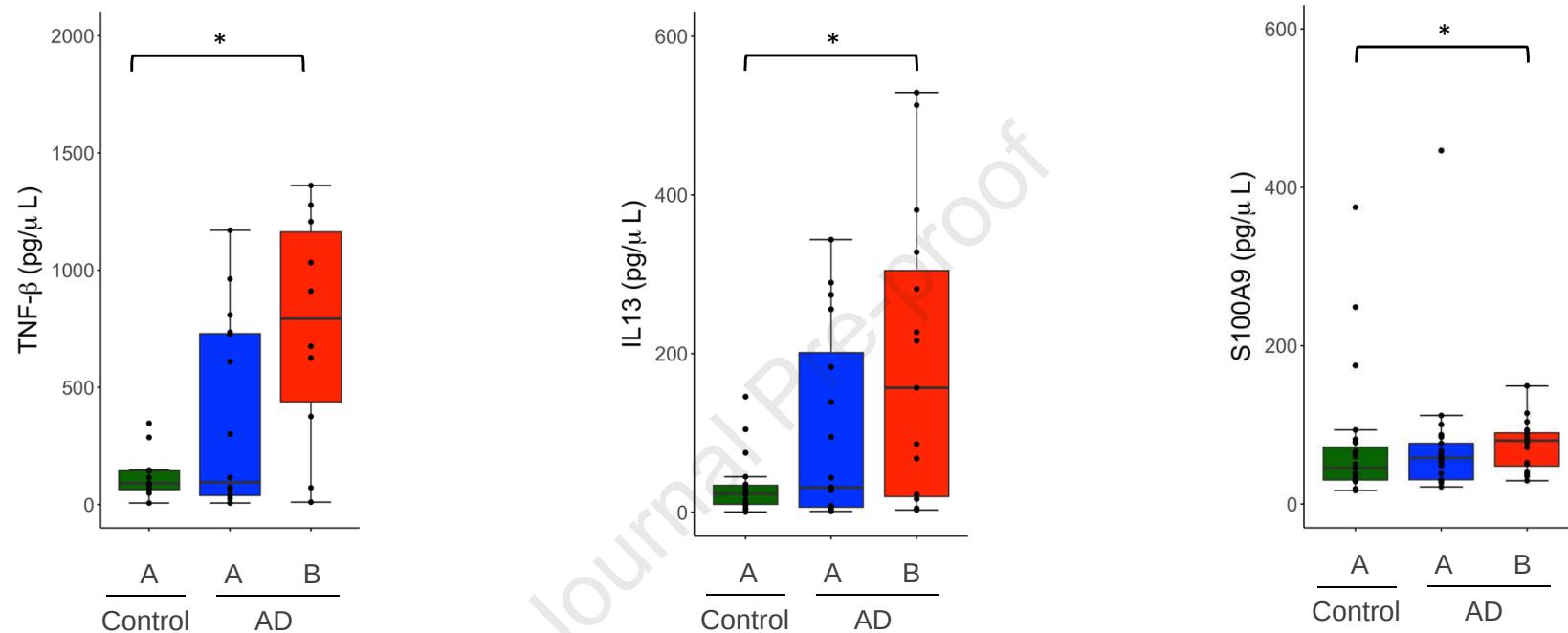
Supplemental Figure E3: Carrying capacities (relative) and interaction networks for dermotype A (left) and B (right) subjects based on generalized Lotka-Volterra modelling. Node sizes and edge widths are proportional to mean relative abundances and interaction strengths, respectively. Edges are colored by interaction types (positive: blue; negative: red). In general, dermotype A seems to have a favorable carrying capacity for *C. acnes* with many other species having inhibitory interactions with it that potentially contribute to community stability. Dermototype B was found to favor a wider range of species and with fewer inhibitory interactions that may indicate a more unstable equilibrium.



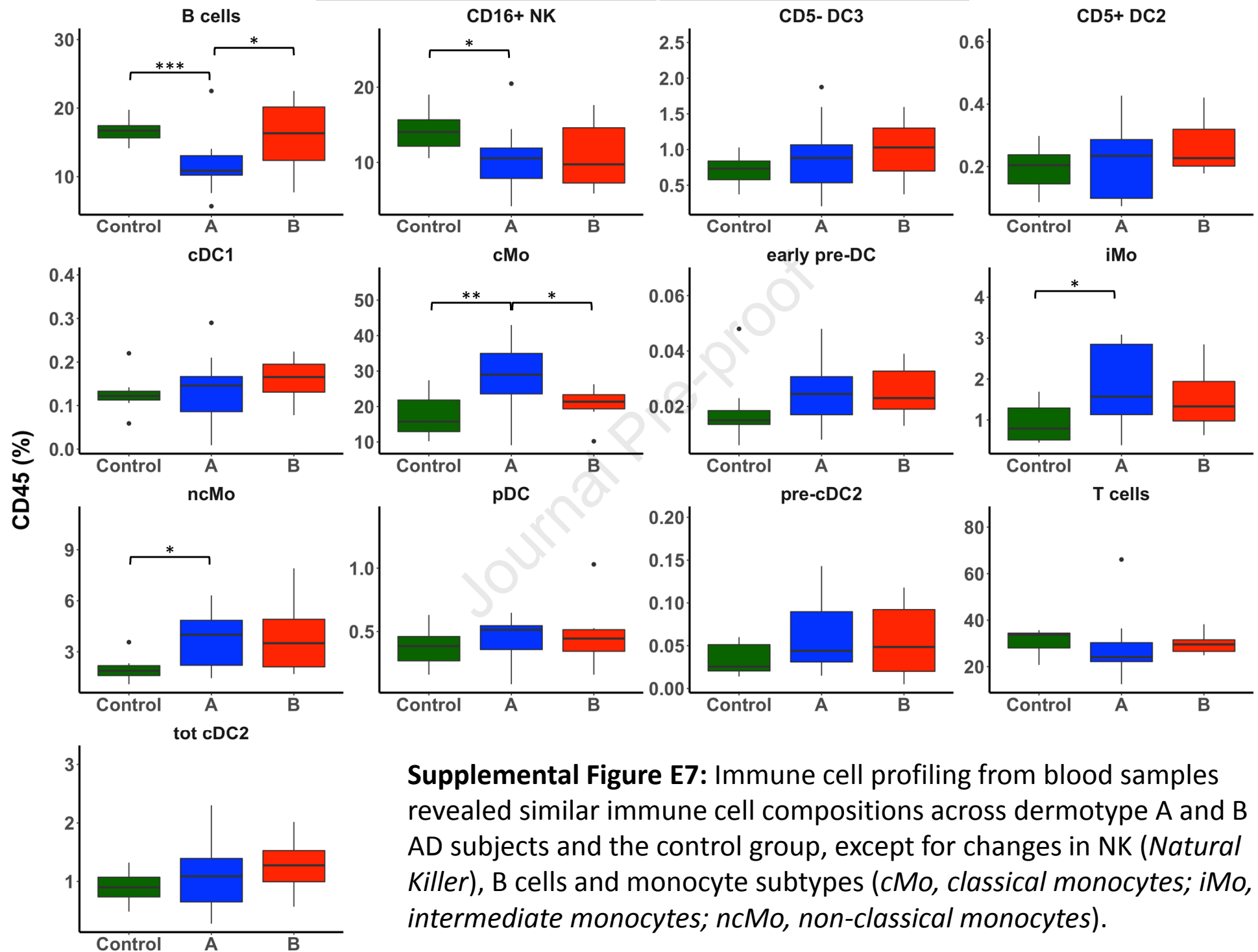
Supplemental Figure E4: Principal coordinates analysis (PCoA) plot showing the distinct functional profiles (using Bray-Curtis distance for pathway abundances) of Dermotype A and Dermotype B samples.

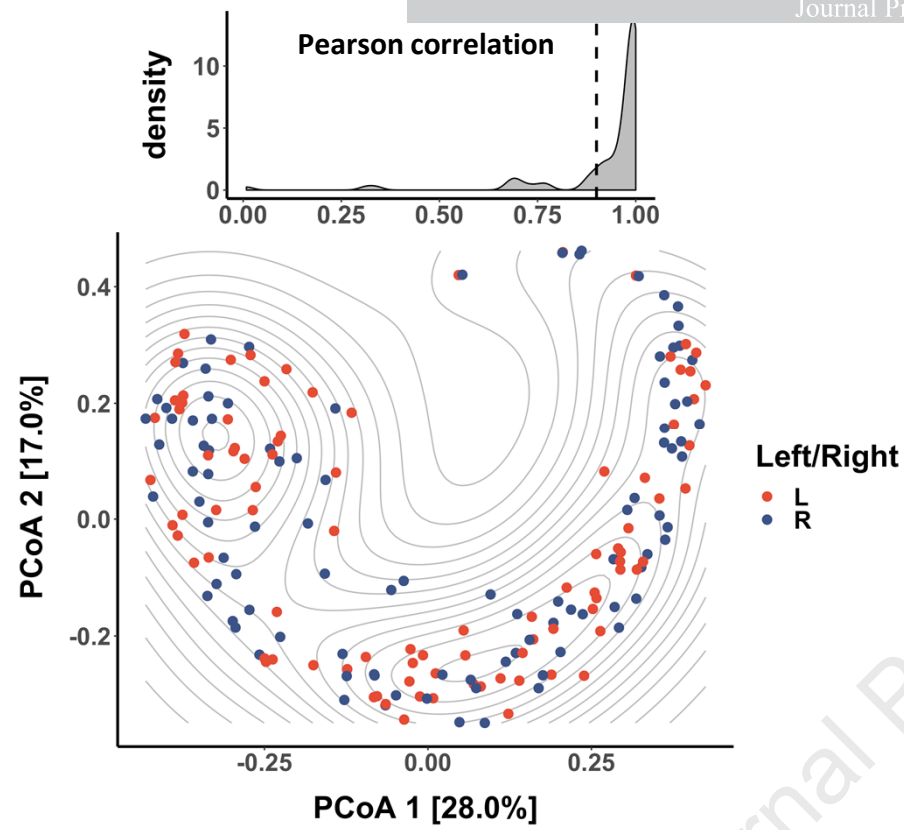
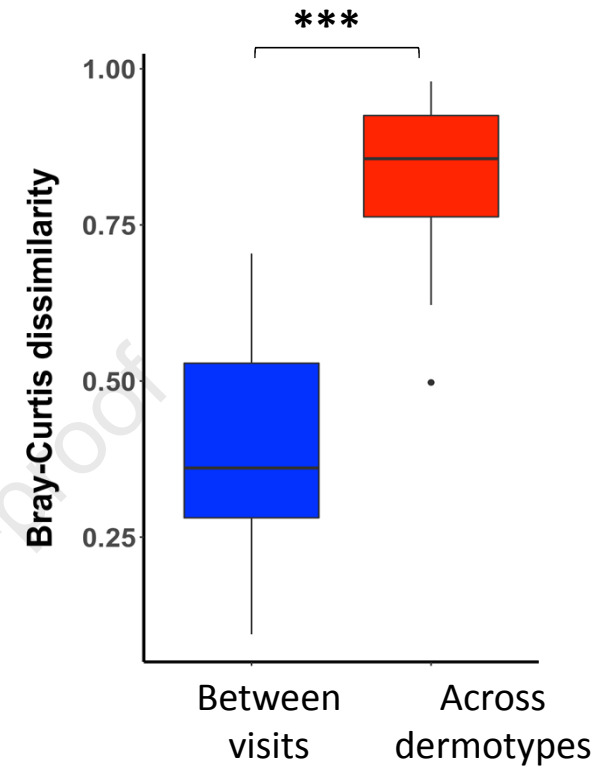
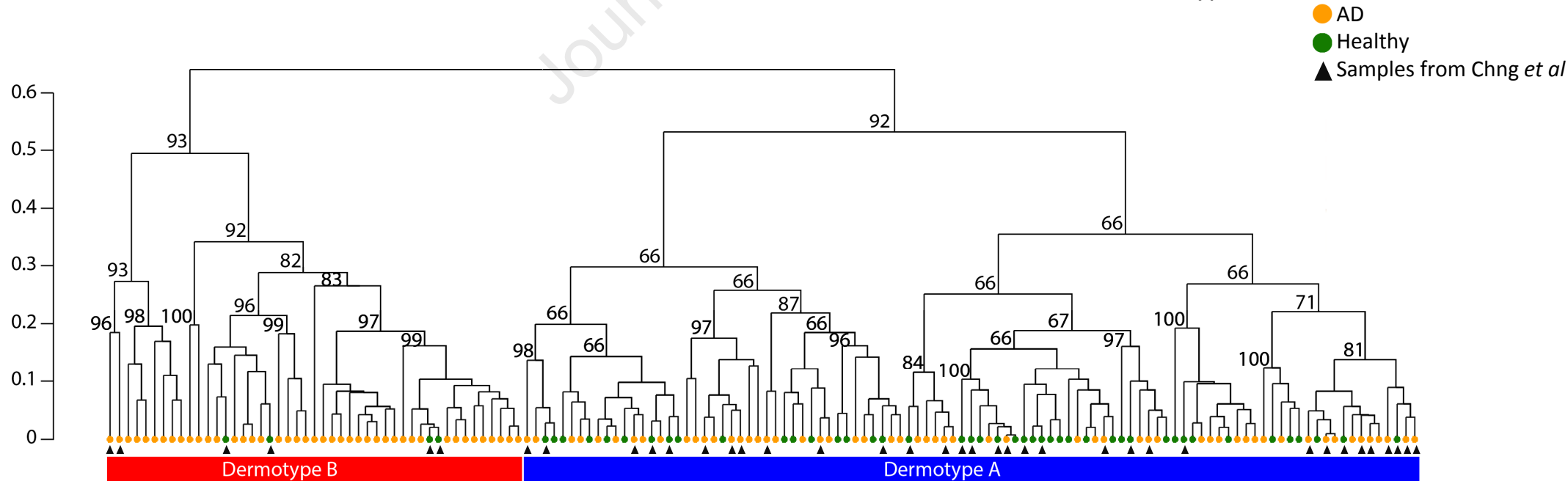


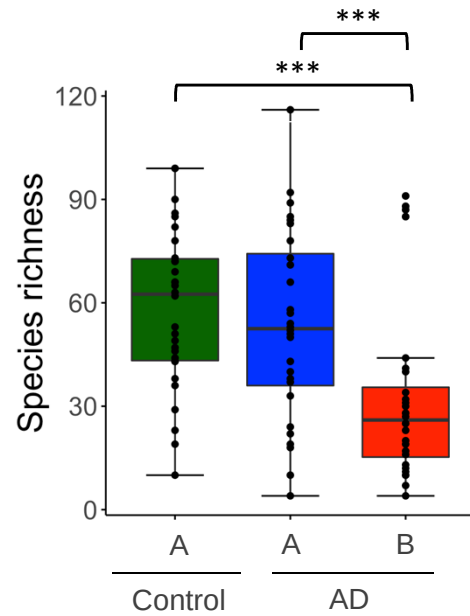
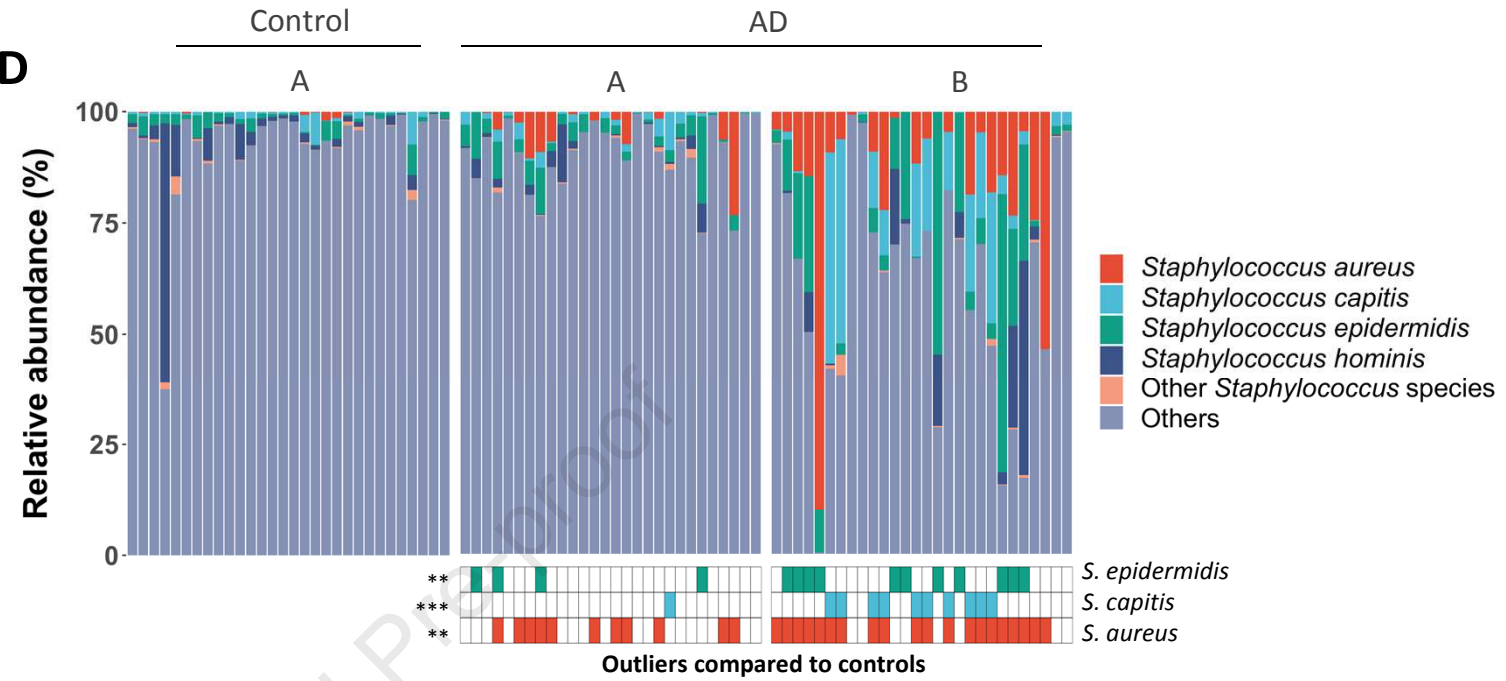
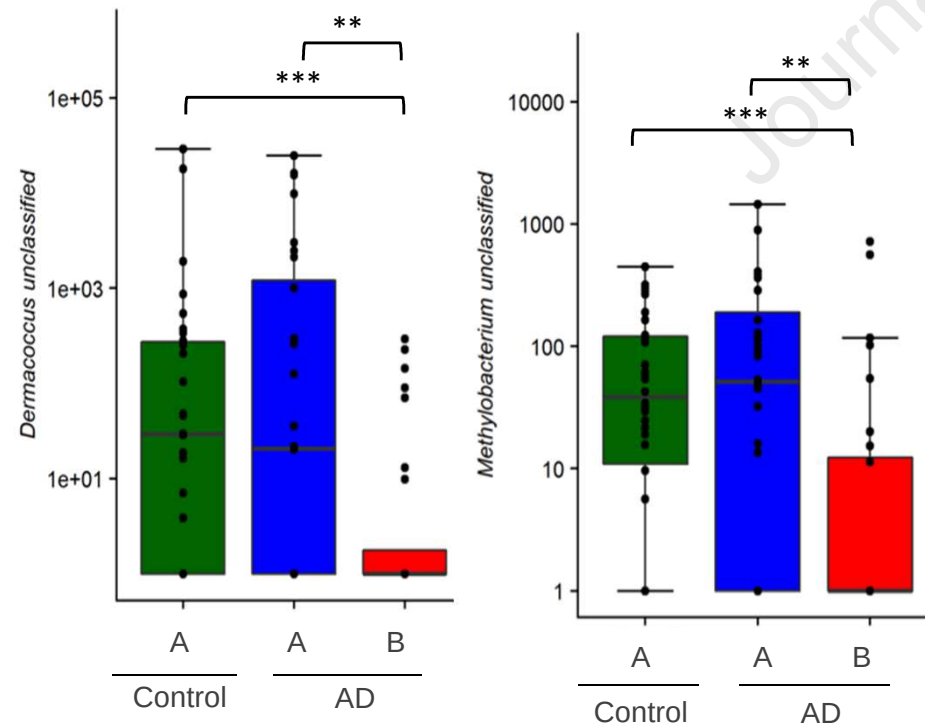
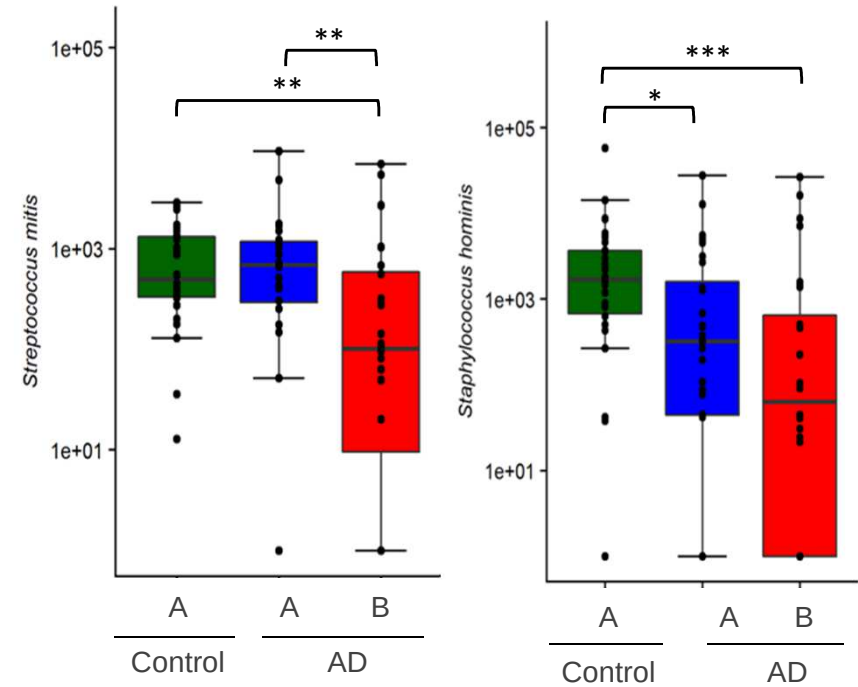
Supplemental Figure E5: Phylogenetic tree of *S. aureus* strains in the skin microbiomes of subjects in this study showing that the dermotypes do not segregate by strain type. Strain genotypes were obtained using StrainPhlAn analysis (with the "--relaxed_parameters3" option) and used to construct a maximum likelihood phylogeny with FastTree2.



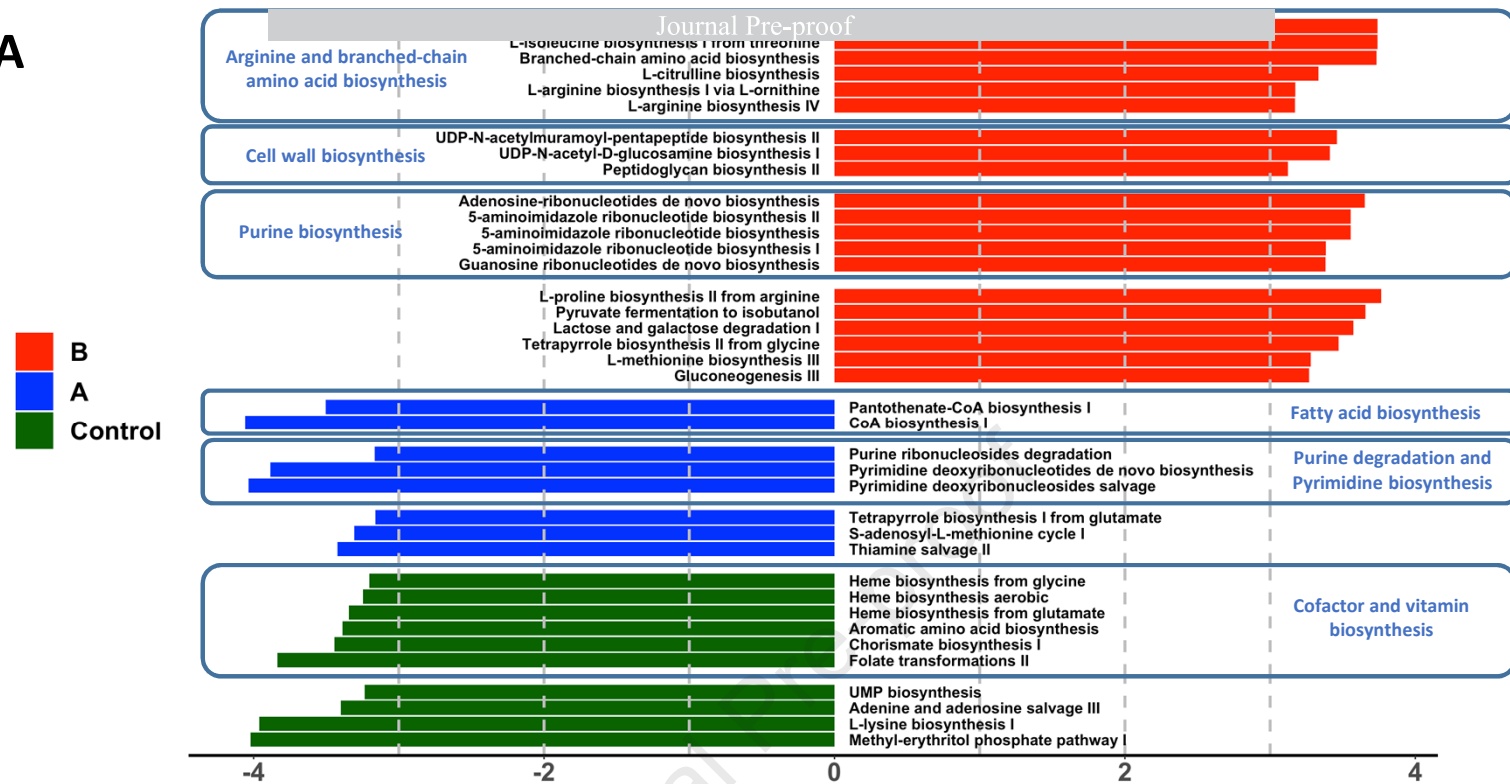
Supplemental Figure E6: Boxplots showing data for serum biomarkers that were significantly higher in dermotype B subjects compared to controls. Boxplot whiskers represent 1.5 $\times$ inter-quartile range.



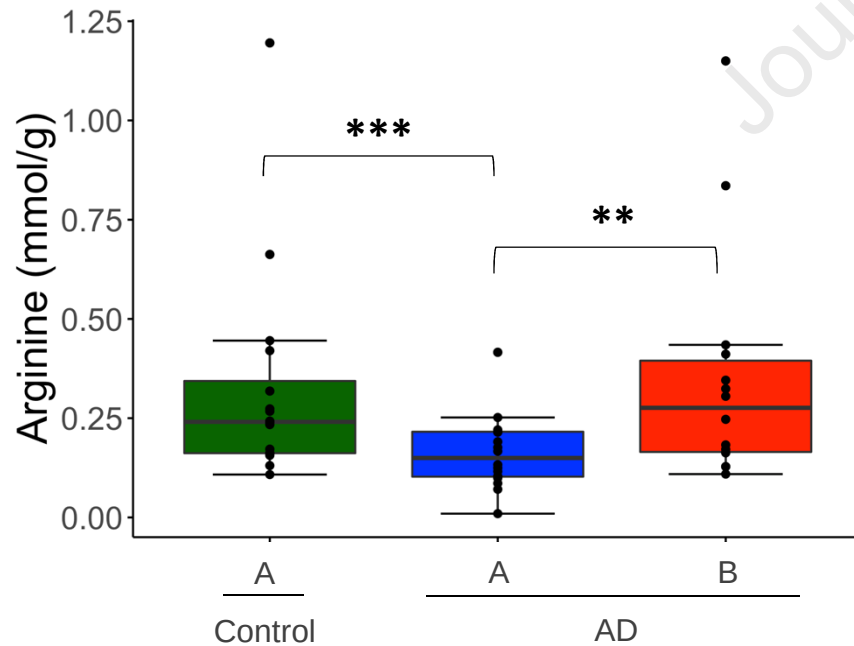
A**B****C**

A**D****B****C**

A



B



C

LDA score

