

Interplay between skin microbiota and immunity in atopic individuals

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To the Editor:

The prevalence of allergic diseases continues to increase worldwide along with industrialization and urbanization. Emerging evidence provides insights into the connection between the development of chronic inflammatory disorders, such as asthma and allergies, and the human microbiota through the immune-modulatory function of the commensal microbes^{1,2}. In addition to the well-established evidence on the role of the gut microbiota in human health and disease, other microbial communities, such as cutaneous microbiota, play an important role in establishing and tuning the host immune system towards a balanced response against commensal and pathogenic microorganisms³.

Additionally, the skin microbiota may promote immune tolerance towards environmental allergens both locally and systemically⁴. Interindividual differences and alterations in the skin microbiota composition, and its associations with human health and disease is evident, but a greater clarification of the factors that modify the composition of microbiota is needed^{1,2}. Factors that have been investigated for their potential interaction with the microbiota include lifestyle, use of medication, and the exposure to a changing environmental biodiversity^{1,5}.

Here, we explore the composition of skin microbiota in 62 healthy and atopic asymptomatic young adults, and associate that with the characteristics of their childhood and present living environments, allergen-specific serum IgE levels, and the immune function of their peripheral blood mononuclear cells (PBMCs) (see Fig. 1A and supplementals for detailed methodological description). We observed distinct associations between the skin microbiota and allergen-specific serum IgE levels and the expression of cytokines, and identified several taxa that may play an important role in augmenting or suppressing the systemic inflammatory immune responses.

Allergen-specific serum IgE measurements revealed that approximately half of the subjects were sensitized to airborne and food allergens (aIgE >0.35 kU_A/L, n=30), and of these 43% were sensitized to birch pollen (bIgE >0.35 kU_A/L, n=13), and 50% to timothy pollen (tIgE >0.35 kU_A/L, n=15) (Supplementary Fig. 1A–C). Principal component analysis (PCA) of the allergen-specific IgE levels revealed clustering of all subjects, except those with very high (>12.5 kU_A/L) aIgE, bIgE or tIgE (Fig. 1B), which are referred to as Highly Sensitized (HS; n=11) individuals in

subsequent analyses. Subjects with aIgE below 0.35 kU_A/L are referred to as Non-Sensitized (NS; n=32) individuals. A total of 19 subjects exhibited intermediate allergen-specific IgE levels (0.35 – 12.5 kU_A/L).

The relative levels of *IL10*, *IL4*, *IL5*, *IL13* and *IFNG* mRNA were measured in PBMCs after 6 hours of stimulation with either birch (Bet v 1) or timothy (Phl p 1) pollen allergen, and compared between the groups. HS individuals showed significantly higher expression of Th2-type cytokine *IL4* in Bet v 1 and Phl p 1 stimulated cells ($p < 0.05$) and Th1-type cytokine *IFNG* in Phl p 1 stimulated cells ($p < 0.05$) compared with NS. There was no difference in *IFNG* expression between HS and NS after Bet v 1 stimulation. Furthermore, the expression of anti-inflammatory cytokine *IL10* was significantly lower at the mRNA level in HS individuals compared with NS (in Phl p 1 stimulated PBMCs, $p < 0.05$; but not in Bet v 1 stimulated PBMCs, $p < 0.1$) (Fig. 1C, results from Bet v 1 stimulated PBMCs in Supplementary Fig. 2). The mRNA levels correlated closely with protein levels measured in cell supernatants (Supplementary Fig. 3). In conclusion, we show that high allergen-specific serum IgE levels in our study subjects are associated with potent pro-inflammatory, and attenuated anti-inflammatory responses to allergens.

The skin microbiotas, analyzed by 16S rRNA gene sequencing, mainly consisted of four phyla, including Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Fig. 1D), representing typical skin microbial communities consistent with previous observations². We further explored the microbial compositions using distance-based redundancy analysis (dbRDA), testing the possible influence of living environments and allergen-specific IgE levels. Our results suggest that childhood living environments (urban, agricultural or forest) may have impacted the skin microbiota (Fig. 2A) in a manner which is still discernible when constraining the analysis by the effect of current living environment (Supplementary Fig. 4). Notably, the study subjects had moved from their first childhood homes since several years before the current study (Supplementary Table 1). Finally, the constrained ordination analysis (dbRDA) suggested that high allergen-specific serum IgE levels may influence skin microbial compositions (Fig. 2B). However, the results did not reach statistical significance, likely due to the low number of HS individuals.

To further explore associations between the skin microbiota and host immunity, we analyzed links between serum IgE levels, cytokine expression in PBMCs and the relative abundance of microorganisms, using Spearman's rank correlation. Our exploratory analysis revealed several taxa, including *Burkholderia-Caballeronia-Paraburkholderia*, *Ralstonia*, *Pelomonas*, *Curvibacter*, *Sediminibacterium* and *Thermoactinomyces vulgaris*, which correlated

positively with aIgE levels and *IL5* mRNA expression, and negatively with the expression of *IL10* ($p < 0.05 - 0.1$) (Fig. 2C–E, Supplementary Table 2). *Burkholderia-Caballeronia-Paraburkholderia*, *Ralstonia*, *Pelomonas* and *Curvibacter* are members of the *Burkholderiaceae* family, which includes organisms recognized as pathogens in the lungs, and an increase in their abundance has been associated with decreased pulmonary function in cystic fibrosis patients⁵. Furthermore, *T. vulgaris* is known as one of the causative organisms in farmer's lung disease, which is a form of hypersensitivity pneumonitis⁶.

In contrast, *Acinetobacter lwoffii* correlated positively with Phl p 1 induced *IL10* ($p < 0.01$) and negatively with *IL5* ($p < 0.05$), (Fig. 2C, Supplementary Table 2). *Acinetobacter* species are known to stimulate anti-inflammatory immune signaling in the skin, induce immune tolerance and protect against allergies^{4,7}. Further, *Porphyromonas*, which negatively regulates airway allergic inflammation⁸, correlated negatively with aIgE levels ($p < 0.05$). Moreover, *Anaerococcus* and *Finegoldia* correlated negatively with *IL4* expression (Fig. 2F) ($p < 0.01$ and $p < 0.05$, respectively). These gram-positive anaerobic cocci have been found to be scarce in filaggrin deficient ichthyosis vulgaris patients, suggesting that they may have protective effects in the skin⁹. Furthermore, *Lactobacillus* and *Veillonella* correlated positively with *IFN* γ and *IL10* expression, respectively ($p < 0.05$). An increase in *Veillonella* abundance is associated with decreased inflammation in the lungs of cystic fibrosis patients⁵.

In conclusion, we have identified distinct associations between the composition of the skin microbiota and immune signaling by comparing atopic individuals with healthy individuals. Our results suggest that the skin of allergen sensitized asymptomatic individuals may be colonized by less favorable, pro-inflammatory microbial species, and is characterized by a general scarcity of beneficial anti-inflammatory microbes.

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Figure legends main figures

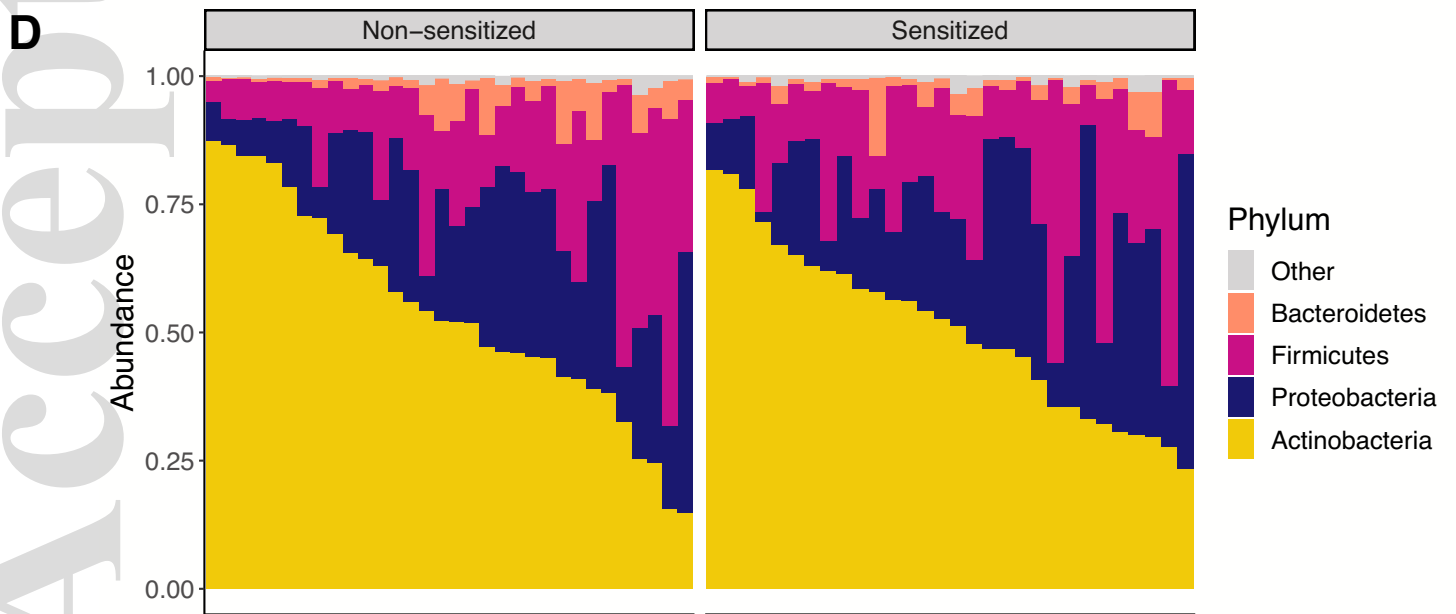
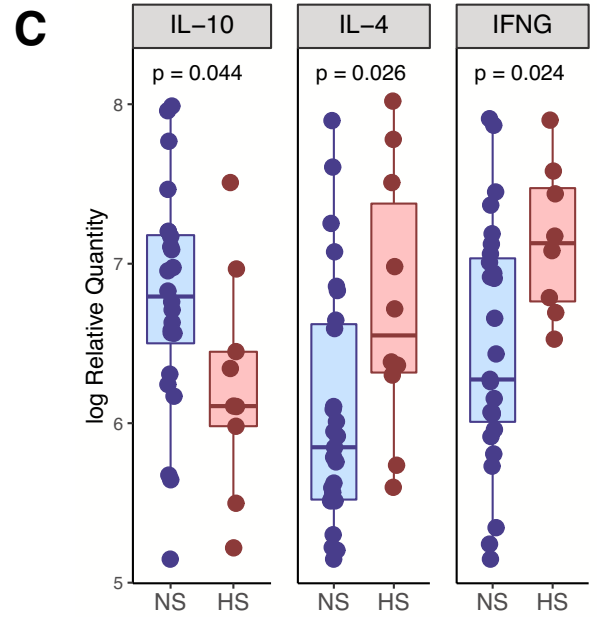
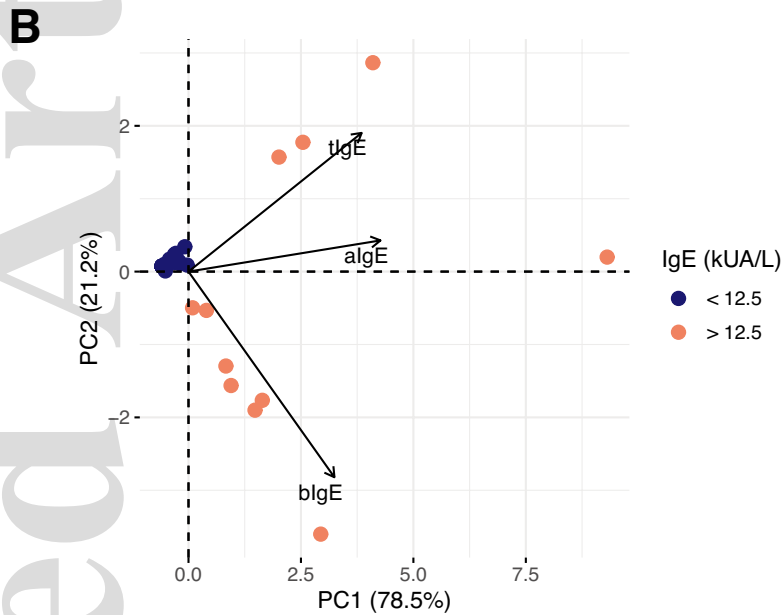
Figure 1. Serum IgE levels, immune function and skin microbiota composition. (A) Study population characteristics: group size (n), female ratio (F%), mean IgE levels specific for airborne and food (aIgE), birch (bIgE) and timothy (tIgE) allergens. (B) Principal component analysis of IgE levels (IgE <12.5 kU_A/L (blue); >12.5 kU_A/L (pink)). (C) *IL10*, *IL4* and *IFNG* mRNA levels in Phlp1 stimulated peripheral blood mononuclear cells. *P*-values calculated with Wilcoxon rank sum test. Log transformation using natural logarithm. (NS, non-sensitized; HS, highly sensitized individuals). (D) Taxa relative abundance at phylum level in non-sensitized (aIgE <0.35 kU_A/L) and sensitized (aIgE >0.35 kU_A/L) individuals.

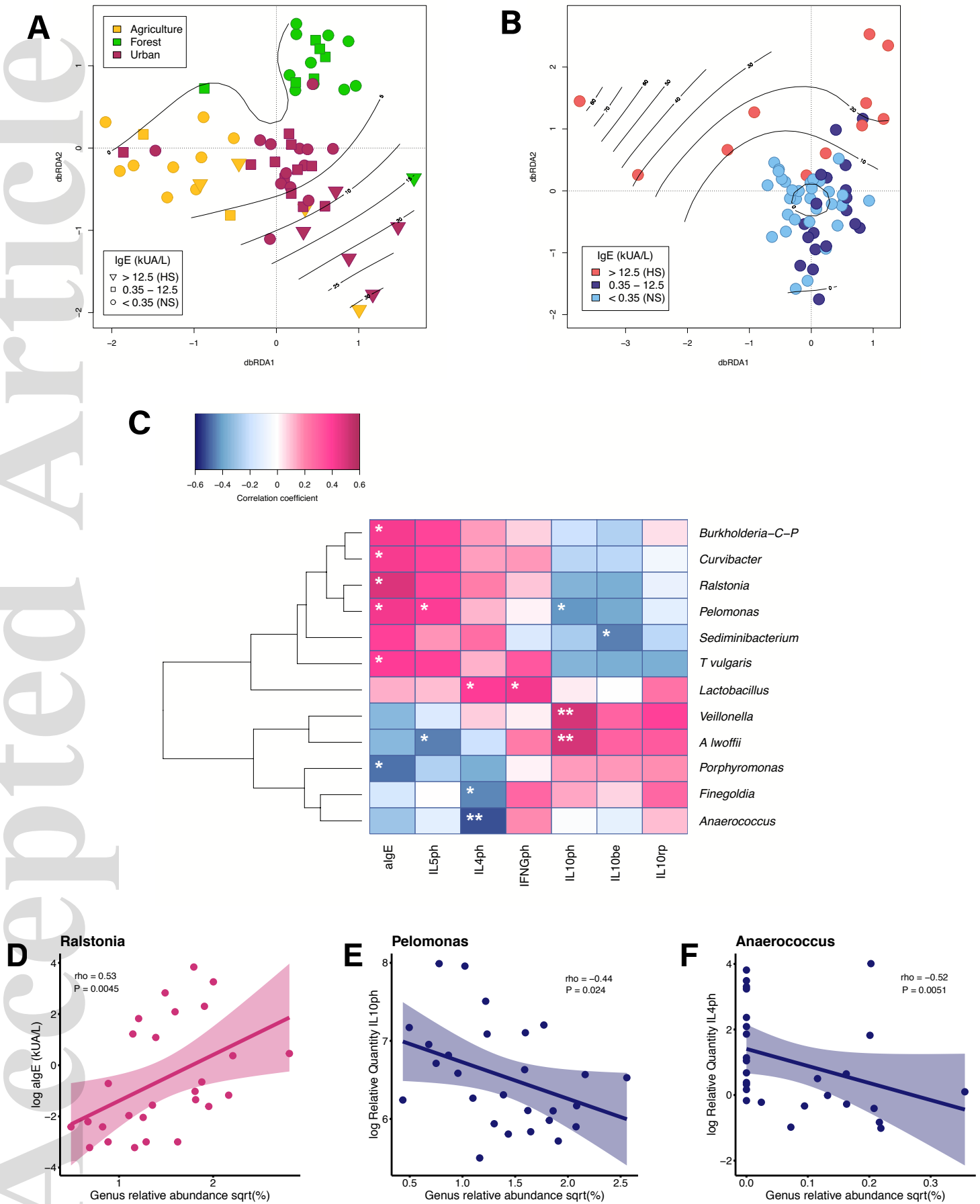
Figure 2. Association between skin microbiota and immune function. (A,B) Distance-based redundancy analysis (dbRDA) constrained by (A) aIgE and childhood environment (aIgE, black lines; agriculture, yellow; forest, green; urban, purple; NS, non-sensitized, circles; HS, highly sensitized, triangles; intermediate IgE levels, squares), and by (B) IgE levels (aIgE, black lines; birch; timothy; HS, pink symbols, NS, light-blue; intermediate, dark-blue). (C) Hierarchical clustering of taxa correlating with aIgE and cytokines (rp; non-stimulated; ph, Phlp1; be, Betv1 stimulated peripheral blood mononuclear cells (**p* < 0.05, ***p* < 0.01). (D,E,F) Spearman's rank correlation between the relative abundance of (D) *Ralstonia* and aIgE, (E) *Pelomonas* and *IL10*, (F) *Anaerococcus* and *IL4* levels. Log transformation using natural logarithm.

A

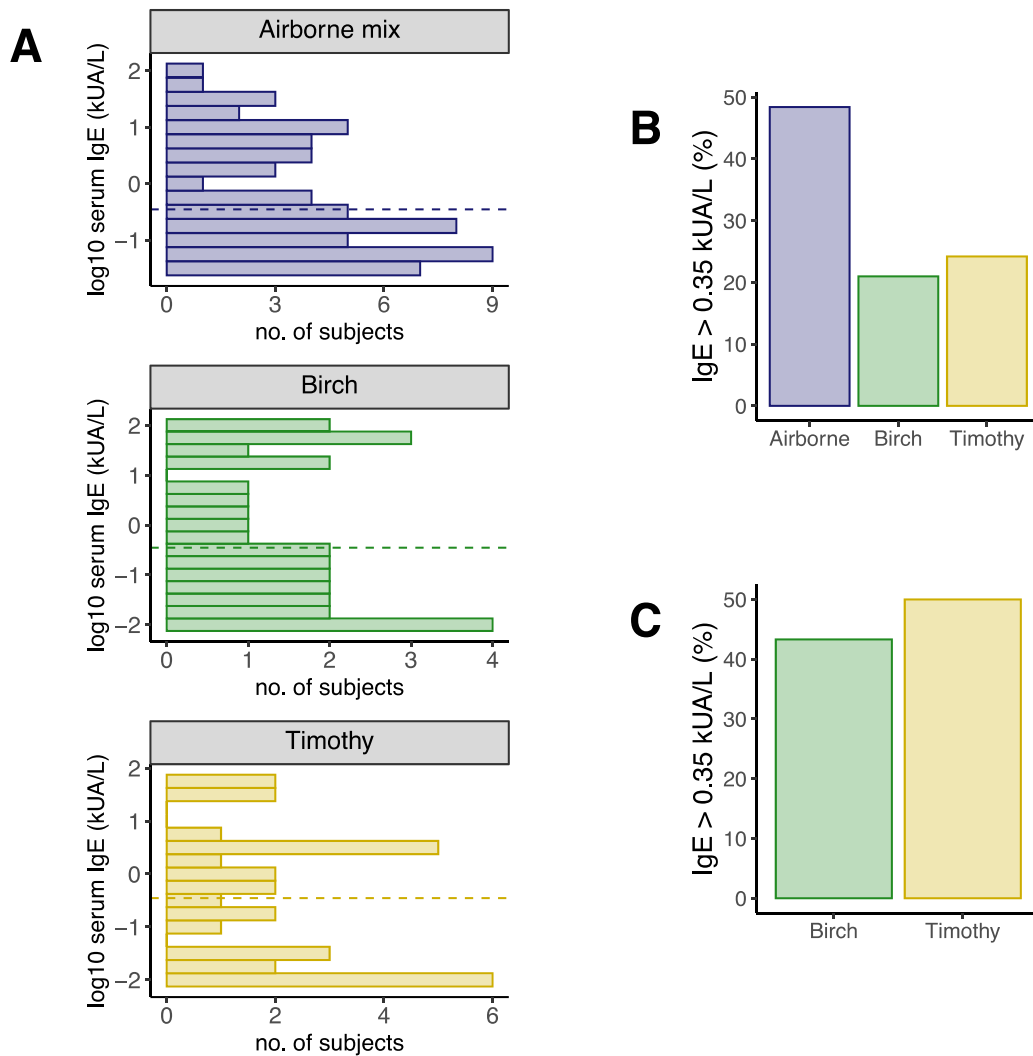
	Sensitization [†]		Childhood living environment		
	Non-sensitized	Sensitized	Urban	Agriculture	Forest
n	32	30	30	15	17
F%	65.5	46.7	44.8	80	58.8
algE mean	0.11	11.59	6.73	6.90	2.71
blgE mean	n/a	14.70	12.54	4.17	0.13
tlgE mean	n/a	6.92	2.65	5.93	2.32
Sensitized %			56.7	40.0	41.2

[†]Sensitization is defined according to the levels of serum IgE specific for airborne allergens.
Non-sensitized: algE <0.35 kUA/L; sensitized: algE ≥0.35 kUA/L.

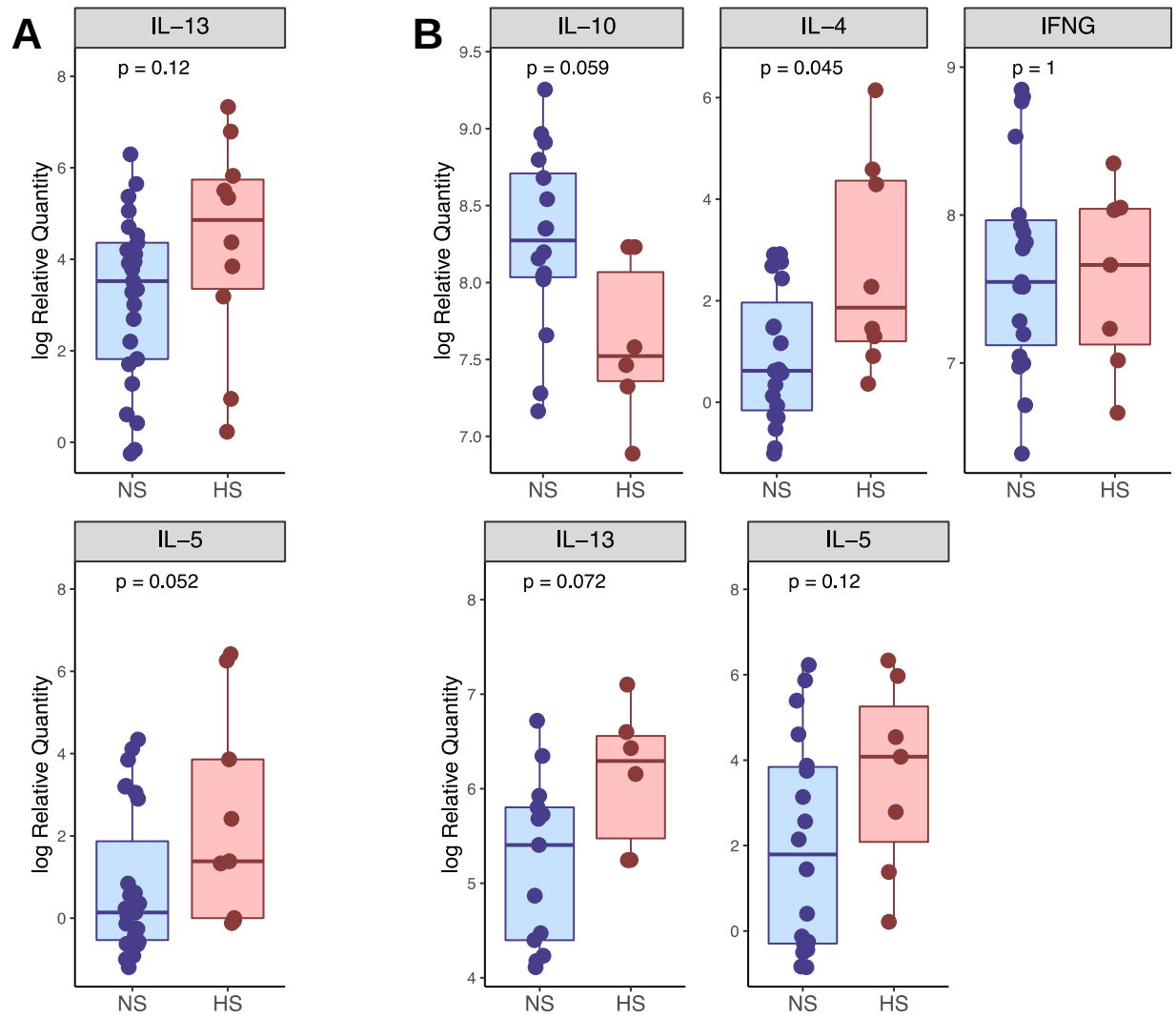




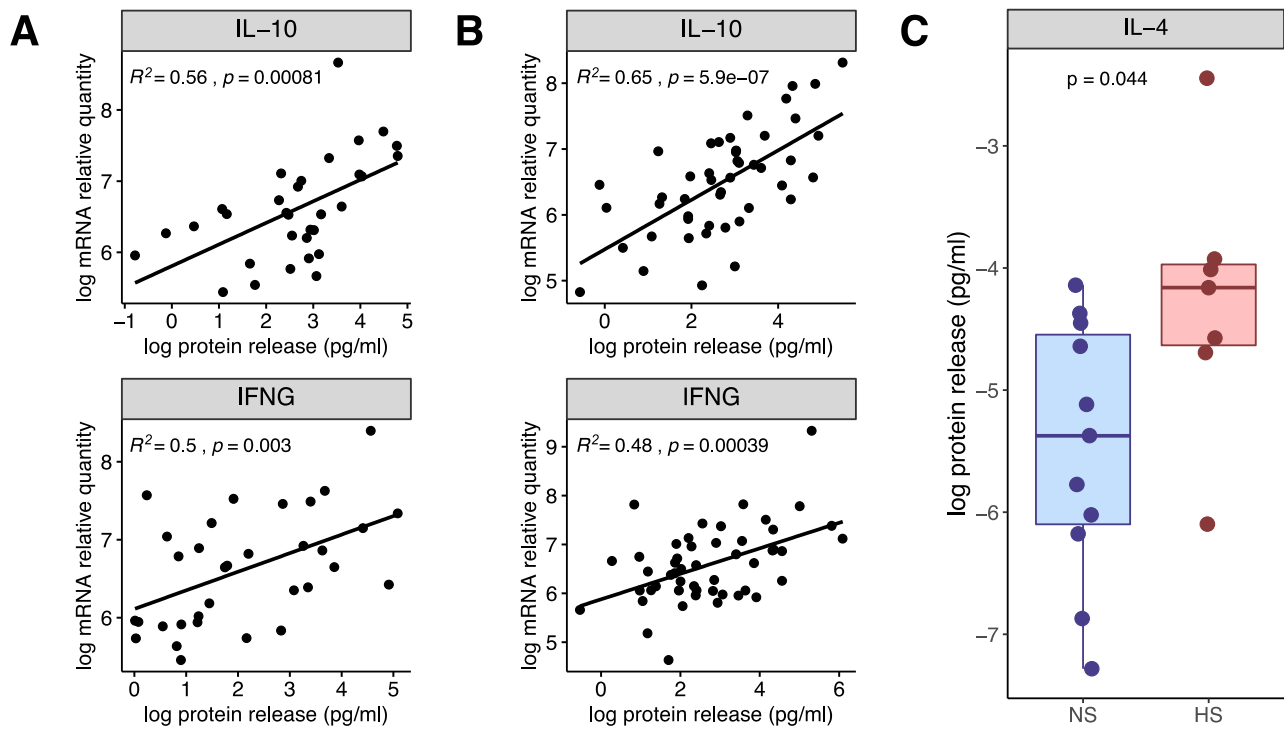
SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Distribution of allergen-specific serum IgE levels in the study population. (A) Distribution of IgE levels specific to a mix of airborne allergens and birch and timothy pollen allergens. The dashed line indicates the value 0.35 kUA/L. **(B)** Ratio of individuals sensitized (IgE >0.35 kUA/L) to a mix of airborne allergens, and birch and timothy pollen allergens in the study population. **(C)** Ratio of sensitized individuals (IgE specific for a mix of airborne allergens > 0.35 kUA/L, n=30) who are also sensitized to birch and timothy pollen allergens.



Supplementary Figure 2. The expression of pro- and anti-inflammatory cytokines in non-sensitized and highly sensitized subjects. Peripheral blood mononuclear cells were stimulated with (A) timothy pollen allergen (Phl p 1) and (B) birch pollen allergen (Bet v 1) for 6 hours. The expression levels for anti-inflammatory cytokine *IL10* and pro-inflammatory cytokines *IL13*, *IL4*, *IL5* and *IFNG* were measured after the stimulation and compared between non-sensitized (NS) and highly sensitized (HS) subjects. *P*-value was calculated with Wilcoxon rank sum test. The data was transformed using natural logarithm.



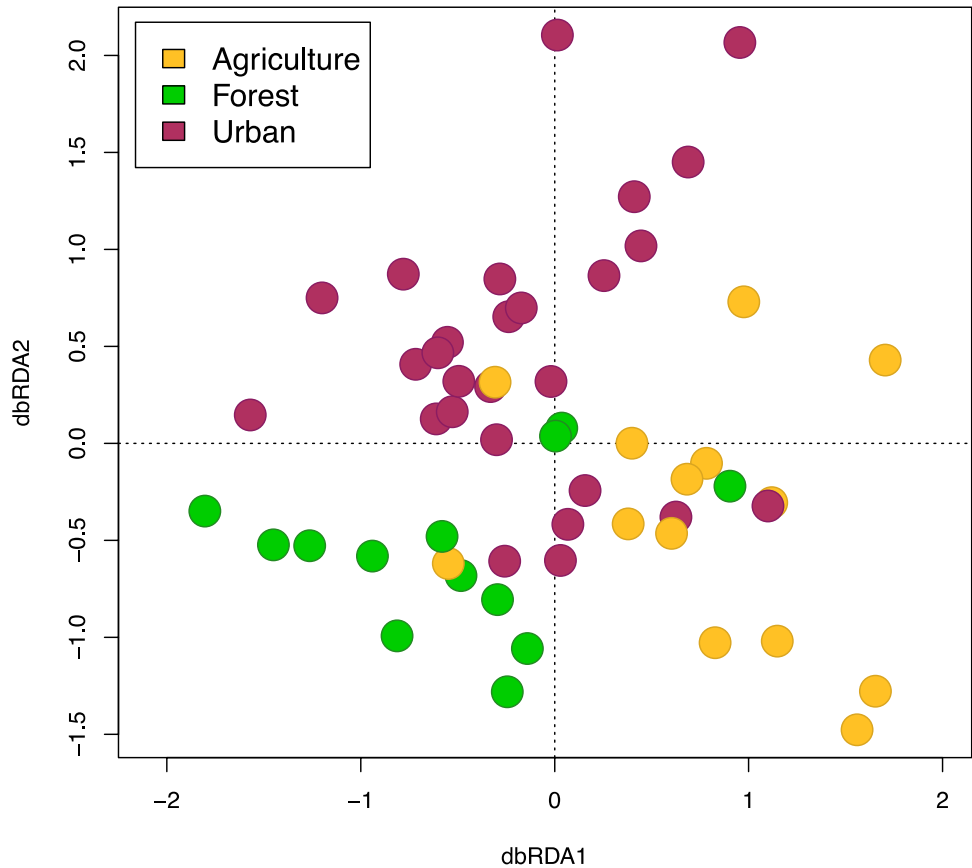
Supplementary Figure 3. mRNA expression levels correlate closely with protein secretion in Phl p 1 stimulated and non-stimulated peripheral blood mononuclear cells. Protein production and expression at the mRNA level of IL-10, IFN- γ and IL-4 measured in **(A)** non-stimulated and **(B)** Phl p 1 stimulated cells. **(C)** The secretion of IL-4 protein following Phl p 1 stimulation was significantly higher in highly sensitized (HS) individuals compared to non-sensitized (NS) individuals. *P*-value was calculated with Wilcoxon rank sum test. The data were log transformed using natural logarithm.

Supplementary Table 1. Information on the years spend in the childhood home.

	Mean	SD
Age at the time of the study	23	± 2.7
Age in year 2000 [†]	9	± 2.7
Years lived in 1 st childhood home	11.6	± 3.1
Years lived in 2 nd childhood home [‡]	5.5	± 2.3
Years lived away from childhood home	3.9	± 2.5

[†]The land use analysis regarding the first childhood home was extracted from the open access land cover data CORINE2000

[‡]The ratio of study subjects who lived in two different addresses before age 18 was 58,1%



Supplementary Figure 4. The influence of childhood and current living environment on the skin microbiota composition. Distance-based redundancy analysis using Bray-Curtis distance, constrained by dust-specific IgE serum levels, and childhood and current living environment type. The color of the points represents childhood living environment, that is, agriculture (yellow), forest (green) and urban (purple).

Supplementary Table 2. Spearman's rank correlation between several taxa and serum IgE levels and cytokine expression levels in PBMCs. Relative abundance of 12 taxa (columns) were correlated with serum IgE levels specific for airborne allergens (aIgE), and IL-4, IFN- γ and IL-10 expression in stimulated (ph, stimulated with Phl p 1; be, stimulated with Bet v 1) and non-stimulated (rp) PBMCs (rows). The correlation coefficient (Spearman's rank correlation; Corr. coeff.) is followed by its corresponding *p*-value and FDR corrected *p*-value (adj. *p*-value). Significant *p*-values are marked with asterisks (**p*<0.05, ***p*<0.01).

		<i>Burkholderia-C-P</i>	<i>Ralstonia</i>	<i>Pelomonas</i>	<i>Curvibacter</i>	<i>Sediminibacterium</i>	<i>T. vulgaris</i>	<i>Lactobacillus</i>	<i>Veillonella</i>	<i>A. Iwofffii</i>	<i>Finexgoldia</i>	<i>Anaerococcus</i>	<i>Porphyromonas</i>
aIgE	Corr. coeff.	0.42	0.5	0.43	0.42	0.38	0.4	0.13	-0.34	-0.34	-0.17	-0.3	-0.47
	<i>p</i> -value	0.033*	0.01*	0.029*	0.031*	0.054	0.042*	0.53	0.089	0.088	0.417	0.142	0.015*
	adj. <i>p</i> -value	0.079	0.079	0.079	0.079	0.093	0.084	0.53	0.119	0.119	0.455	0.17	0.079
IL5ph	Corr. coeff.	0.38	0.37	0.41	0.38	0.17	0.38	0.11	-0.14	-0.45	0	-0.11	-0.26
	<i>p</i> -value	0.057	0.062	0.04*	0.059	0.409	0.055	0.608	0.492	0.02*	0.985	0.585	0.207
	adj. <i>p</i> -value	0.124	0.124	0.124	0.124	0.613	0.124	0.663	0.656	0.124	0.985	0.663	0.355
IL4ph	Corr. coeff.	0.16	0.22	0.12	0.15	0.25	0.12	0.4	0.08	-0.19	-0.44	-0.52	-0.37
	<i>p</i> -value	0.436	0.291	0.568	0.452	0.21	0.55	0.044*	0.694	0.347	0.025*	0.007**	0.064
	adj. <i>p</i> -value	0.603	0.582	0.62	0.603	0.504	0.62	0.176	0.694	0.595	0.15	0.084	0.192
IFNGph	Corr. coeff.	0.07	0.09	0.02	0.17	-0.15	0.32	0.43	0.03	0.22	0.28	0.19	0.02
	<i>p</i> -value	0.719	0.65	0.925	0.416	0.466	0.108	0.029*	0.899	0.283	0.167	0.35	0.912
	adj. <i>p</i> -value	0.925	0.925	0.925	0.799	0.799	0.648	0.348	0.925	0.799	0.668	0.799	0.925
IL10ph	Corr. coeff.	-0.2	-0.36	-0.42	-0.23	-0.27	-0.35	0.03	0.51	0.51	0.14	-0.02	0.16
	<i>p</i> -value	0.332	0.073	0.034	0.25	0.175	0.078	0.877	0.008**	0.008**	0.486	0.908	0.443
	adj. <i>p</i> -value	0.498	0.187	0.136	0.429	0.35	0.187	0.908	0.048*	0.048*	0.583	0.908	0.583
IL10be	Corr. coeff.	-0.25	-0.37	-0.38	-0.23	-0.45	-0.37	-0.01	0.28	0.29	0.07	-0.1	0.17
	<i>p</i> -value	0.212	0.065	0.055	0.254	0.021*	0.064	0.963	0.159	0.148	0.732	0.633	0.413
	adj. <i>p</i> -value	0.363	0.195	0.195	0.381	0.195	0.195	0.963	0.318	0.318	0.799	0.76	0.551
IL10rp	Corr. coeff.	0.05	-0.09	-0.11	-0.06	-0.23	-0.37	0.24	0.38	0.32	0.27	0.1	0.19
	<i>p</i> -value	0.815	0.65	0.598	0.784	0.254	0.063	0.235	0.056	0.114	0.174	0.615	0.365
	adj. <i>p</i> -value	0.815	0.78	0.78	0.815	0.508	0.378	0.508	0.378	0.456	0.508	0.78	0.626

Materials and methods

Subject recruitment

Students and workers at the University of Helsinki, Finland were recruited to the study by utilizing email-lists of several faculties and departments. Voluntary participants who met the inclusion and exclusion criteria (aged 19 – 30, no chronic disease demanding medication, or antibiotic treatment in the past three months, no symptoms of allergic disease or atopic skin disease at the time of the study) were admitted to the study. The participants ($n = 62$; mean age 23, $sd \pm 2.7$) consisted of equal proportions of sensitized and non-sensitized individuals, as well as individuals with a rural or urban background regarding childhood and current living environments, as described in Fig. 1A.

The experimental protocol followed the Declaration of Helsinki principles and was approved by the Ethics Committee of Helsinki University Central Hospital (approval number 313/13/03/01/2013). All participants signed an informed consent form.

Sample collection

All samples were collected during October 2014. Venous blood samples were collected from the study subjects on their first visit to the clinic for the isolation of peripheral blood mononuclear cells (PBMC) and serum IgE measurements. The subjects were advised to use DOVE soap for 7 days for hygiene, and refrain from bathing for 24 hours prior to the second visit, which took place 8 days after the first visit. During the second visit, skin microbial samples were collected from the left volar forearm using sterile swabs (Copan Flocked Swab), pre-moistened in sterile sampling solution (0.15M NaCl with 0.1% Tween20). The swabs were rubbed against the skin altogether 20 times inside a 2cm x 2cm area.

Serum IgE level measurements

Sera were analyzed at The Skin and Allergy Hospital (Helsinki University Central Hospital) using the Phadiatop Combi® –test (ThermoFisher Scientific), which included allergens derived from birch, timothy grass, cat, dog dandruff, horse, *Dermatophagoides petronyssinus*, *Cladosporium*, egg white, milk, fish, wheat, peanut and soybean. If the serum IgE levels specific for this combination of airborne and food allergens were higher than the clinical reference 0.35 kU_A/L, further allergen-specific analyses were performed for birch and timothy pollens.

Land use analysis

Information regarding the childhood and current residential address was collected in a questionnaire. Addresses of the first childhood home were transformed into coordinates using the R package ggmap (v3.0.0)¹, which were then utilized to extract land use information from the publicly available land cover data CORINE2000 and CORINE2012 for childhood and current living environment, respectively. Level 1 land use classification was used in both cases, and land use was calculated from a 2 km buffer zone around the home location for childhood, and 1 km buffer zone for current living environment. The study subjects were categorized into three groups, that is, urban ($n = 29$), agriculture ($n = 15$) and forest ($n = 17$) according to the childhood home land use analysis results simplified in a

principal component analysis (PCA). The first principal component (PC1) separated urban from rural, while PC2 separated agricultural from forested areas.

PBMC stimulation and the cytokine expression rate

PBMCs were collected from blood samples and cultured in 24-well plates at a density of 2×10^6 cells/ml in complete RPMI-1640 medium supplemented with 10% inactivated FBS, 1% L-glutamine, 1% PEST, 1% sodium pyruvate, 1% MEM NEAA, 1% HEPES and 1% filtered 2-ME, at 37°C in a 5% CO₂ atmosphere. The cells were stimulated with 10 µg/ml birch pollen allergen (Bet v 1) or 10 µg/ml timothy pollen allergen (Phl p 1) (Indoor Biotechnologies, Charlottesville, Va) for 6 or 24 hours. After the stimulation, cell supernatant was collected and stored at -20 °C and total RNA was extracted from the cells using TRIsure reagent (Bioline, London, United Kingdom). A sample input of 300 ng RNA per sample was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Foster City, Calif). The reverse transcription was performed in 25 µl and at 25°C for 20 minutes, followed by 120 minutes at 37°C.

mRNA expression levels of *IL4*, *IL5*, *IL13*, *IL10* and *IFNG* were analyzed using quantitative RT-PCR that applied iTaq Universal Probes Supermix (Bio-Rad, Hercules, Calif), TaqMan chemistry, and the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, Calif). The run consisted of 30 seconds at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A housekeeping gene *GAPDH* was simultaneously amplified in each sample in order to normalize the expression rates of the target genes between the samples. The results were calculated from the comparative cycle threshold, resulting in relative quantity of each target gene (*IL4*, *IL5*, *IL13*, *IL10* and *IFNG*)². The release of cytokines IL-10, IFN-γ and IL-4 at the protein level from Phl p 1 stimulated and non-stimulated PBMCs after 24 h stimulation was measured from the cell supernatant using the V-PLEX cytokine assay kit for human Proinflammatory Panel 1 (Meso Scale Discovery, Rockville, Maryland).

Skin microbiota

After swabbing, the tip of the swab was placed in a sterile tube and immediately snap frozen, and stored in -20°C until further analysis. DNA was extracted from the skin swabs and negative control samples using the FastDNA spin kit for soil (MP Biomedicals, Solon, Ohio) following the manufacturer's instructions with few modifications: Tissue Lyser II (Qiagen) was used for bead beating, and afterwards the samples were centrifuged two times longer than proposed in the protocol. Next, the V1 to V3 region in the 16S rRNA gene was amplified using the primer pair 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-GTATTAACCGCGGCTGCTG-3'. The PCR reaction consisted of 30s initial denaturation at 98°C followed by 18-35 cycles of 10s denaturation at 98°C, 30s annealing at 65°C and 15s extension at 72°C, and finished with 5 min extension at 72°C. The PCR reaction was repeated using 18 – 35 cycles in order to yield adequate results three times. The three per-sample amplicons were pooled into one representative sample and sequenced at the Institute of Molecular Medicine Finland (FIMM, University of Helsinki) using paired-end sequencing in Illumina Miseq with v3 chemistry. These sequence data have been submitted to the GenBank database under accession number 'PRJNA655626'.

Sequencing data processing

Adapter sequences were cut off from the sequencing reads using Cutadapt (v 2.6)³. The trimmed reads were further analyzed using R software (v3.5.2, R core team 2019). DADA2 (v 1.10.1) pipeline⁴ was utilized for filtering out low quality and erroneous reads introduced by PCR amplification and sequencing. The forward and reverse reads were truncated at position 280 and 250, respectively, in order to cut off the tails with low quality scores ($Q < 30$) off. Filtered reads were inferred into amplicon sequence variants (ASVs) and the ASVs were taxonomically assigned by aligning them against the SILVA rRNA gene database (release 132)⁵. Contaminating ASVs were removed from the data with the help of the negative control samples from DNA extraction, thus ASVs that were present only in the negative controls were removed from the data. Since the ASV counts varied greatly between the samples, ranging from 4438 to 309385, the counts were normalized with Cumulative Sum Scaling (CSS) and log-transformed using the R package metagenomeSeq (v 1.24.1)⁶.

Microbiota composition analysis

The associations between the skin microbiota composition and allergen specific IgE levels (for airborne allergens, timothy pollen and birch pollen) was observed with distance-based redundancy analysis (dbRDA) in R package vegan (v 2.5.6)⁷, using Bray-Curtis distance. The analysis was supervised by the serum IgE levels specific for airborne, birch pollen and timothy pollen as well as IgE level -based characterization of the subjects into three groups. Moreover, sex was set as a co-variable in the analysis due to its significance in driving the differences in skin microbiota between the subjects. For this analysis, serum IgE levels specific for birch and timothy pollen in non-sensitized individuals were set to a pseudo-value of 0.00 kU_A/L. Variable-wise significance was calculated with anova.cca in vegan using 999 permutations.

To examine the influence of childhood living environment on the skin microbiota composition observed in adolescence, the study subjects were categorized into urban (n = 29), agriculture (n = 15) and forest (n = 17) according to the childhood home land use analysis. The differences in skin microbiota between individuals growing up in urban, agricultural or forested environment were investigated with dbRDA using Bray-Curtis distance. The analysis was supervised by the serum IgE levels specific for airborne allergens and the childhood environment type. The sex was set as co-variable. Variable-wise significance was calculated with anova.cca in vegan using 999 permutations.

Distinctly differing ASVs between the non-sensitized and highly sensitized individuals were inspected using the fitFeatureModel method for differential abundance testing in the R package metagenomeSeq⁶. Associations between the most abundant genera, that is, genera present in >80% of the samples, and the differentially abundant ASVs, and serum IgE levels specific for airborne allergens and mRNA levels of *IL10*, *IL4*, *IL5* and *IFNG* was explored using Spearman's rank correlation. Relative abundance of the taxa was used for the analysis and was calculated from the raw ASV counts. ASVs were agglomerated down to genus level to calculate the relative abundance of the most abundant genera. This exploratory correlation analysis was done using only samples with no missing values in any of the variables (n=27). The *p*-values for the correlations were adjusted using the false discovery rate (FDR)⁸.

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