Associations between early-life exposures and the infant skin microbiome

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Data availability: De-identified 16S sequencing data are available at Sequence Read Archive (SRA) under accession number 15024417. R code has been deposited on github (url pending).

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What is already known about this topic?

- The skin microbiome varies by body-site, and changes with age, particularly during the first year of life, and at puberty.
- Delivery method impacts the skin microbiome immediately after birth, but factors shaping the evolving infant skin microbiome are less-well defined.
- Atopic dermatitis (AD) is associated with alterations in the skin microbiome, with increased *Staphylococcus aureus* abundance, but the relationship between the infant skin microbiome and the development of AD remains uncertain.

What does this study add?

- During the infancy, the skin microbiome composition was more strongly associated with body-site and age, than with heritable and hygiene factors, or urban-vs-rural living.
- The microbiome composition was associated with skin barrier impairment, and with AD, but specific taxonomic associations were inconsistent, and *Staphylococcus aureus* was infrequently identified.
- More associations were identified with the microbiome at the elbow crease, compared to the volar forearm, which will inform future research.

Abstract

Background: Factors influencing the early-life skin microbiome, and the association with atopic dermatitis (AD), are relatively unexplored.

Objective: To evaluate associations with the infant skin microbiome during the first year of life.

Methods: 3-month-old infants from the Enquiring About Tolerance (EAT) birth cohort were examined for AD at enrolment, 1 and 3 years of age. Parent-completed questionnaires, trans-epidermal water loss (TEWL), and *filaggrin* mutation status were evaluated. Bacterial swabs were collected from the elbow crease and volar forearm in 148 infants at 3 months and 1 year of age, and the microbiome composition was characterized using 16S rRNA gene sequencing (V3-V4 region).

Results: Shannon diversity was significantly higher at the forearm compared to the elbow. *Staphylococcus, Acinetobacter*, and *Streptococcus* were the most abundant genera across time and body-site. Microbiome community composition was primarily associated with body-site and age ($p \leq 0.001$, both). Other significant associations were found with ethnicity ($p=0.009$), *filaggrin* status ($p \leq 0.001$), urban-vs-rural residence ($p=0.005$), older siblings ($p=0.041$), bath product usage at 3 months ($p=0.011$), but not with pets ($p=0.159$), systemic antibiotics ($p=0.27$) nor with bathing frequency ($p=0.109$). The microbiome was associated with elevated TEWL (3-months $p=0.004$, 1-year $p \leq 0.001$) and with concurrent AD (3-months $p=0.027$, 1-year $p \leq 0.001$). *Streptococcus parasanguinis* was significantly less abundant in non-lesional skin of infants with AD at 3 months.

Conclusion: In addition to age and body-site, the infant skin microbiome is associated with heritable factors, the home environment, hygiene practices, and with the presence of AD.

Introduction

The skin microbiome consists of the communities of bacteria, fungi and viruses residing on our skin. Body-site is the primary determinant of the bacterial skin microbiome because specific bacteria thrive in moist, dry, and sebaceous body-sites.[1,2] Once established, our individualized bacterial microbiome remains relatively stable over time, despite continuous interactions with environmental factors and external microbes.[2-4] Our understanding of factors shaping the development of the early-life skin microbiome is limited. Delivery method has been shown to influence the skin microbiome immediately after birth.[5-7] Additionally, age, pubertal status, contact with other family members, and with soil, and the wider living environment all contribute to the composition of the skin microbiome.[8-11]

Patients with atopic dermatitis (AD) are frequently colonized with *Staphylococcus aureus* and have a propensity to skin infections.[12-14] Beyond *S. aureus*, culture-independent microbiome studies provide evidence of wider bacterial dysbiosis amongst AD patients, as well as associations between the microbiome and AD severity and flares.[15,16] Perturbations of the early-life skin microbiome may predate the development of AD.[17-19] However, whether the early-life microbiome plays an active or bystander role in AD pathogenesis remains to be elucidated.

This study aimed to characterize the infantile skin microbiome, and to evaluate for associations with environmental and hygiene-related exposures, as well as associations with AD.

Patients and methods

Enquiring About Tolerance (EAT) was a population-based randomized trial of a dietary intervention aiming to prevent food allergy in 1303 term-born, three-month-old infants.[20] Questionnaires gathered information on the infants' general health, home environment, and family history. A numeric "hygiene score" was generated based on parent-reported bathing and hygiene factors (Table S1).[21,22] Infants' home locations were categorized as

urban or rural, using satellite-derived land-use data of the environment surrounding the home postcode (Figure S1).[23] Infants were examined for AD at enrolment (3 months), and at 1 and 3 years of age. AD was defined according to the photographic protocol of the International Study of Asthma and Allergies in Childhood (ISAAC) Phase II, based upon the UK working-party diagnostic criteria.[24] AD severity was recorded using the SCORing Atopic Dermatitis (SCORAD) index at each clinical assessment.[25] AD severity was dichotomized as mild versus moderate-severe (SCORAD ≥15), as previously described.[26] Ethics was granted by the Guy's & St Thomas' Hospital Research Ethics Committee (Reference 08/H0802/93) and the study was registered with the International Standard Randomized Controlled Trial Number Register (https://doi.org/10.1186/ISRCTN14254740). Further background and study methodology are available in Appendix S1.

Sample processing and 16S rRNA gene amplicon sequencing

EAT enrolled participants from November 2009. Beginning December 2011, skin swabs were collected from consecutive infants at enrolment and at the scheduled 1-year visits, according to a standardized procedure. Infants with longitudinal samples at both 3 months and 1 year were selected for this analysis (n=148). Table S2 compares infants participating in the EAT microbiome study to the overall EAT cohort. Microbiome samples were collected from the left volar forearm ("forearm") and antecubital fossa ("elbow") using a sterile, moistened Isohelix buccal DNA collection swab (Cell Projects, Kent, UK). An environmental control swab was collected for each visit. Further details of sample collection and laboratory and bioinformatic workflows are provided in Appendix S1, Table S3, and Figures S2-S6.

Statistical analysis

All analyses were performed using R version 4.3.1, as described in full in the Appendix S1. Shannon diversity and $\log_{10}$-transformed relative abundance were evaluated using linear mixed-effects models to account for the longitudinal and paired skin samples. Genus relative abundance were compared using Wilcoxon signed-rank tests, corrected for multiple hypotheses, using rabuplot[27]. Species differential abundance testing, subset to specific body-site and age combinations, was performed with function da.ds2 (an implementation of DESeq2, package: DAtest[28]) comparing species abundances by age, urban-vs-rural status and AD diagnosis, using a Wald test with sequencing run as a covariate. Bray-Curtis dissimilarity metrics were calculated using Hellinger-transformed, and total-sum-scaled counts, and associations with the microbiome community composition were evaluated with PERMANOVA (adonis2 function, package: vegan[29]), using a blocked-design, to account for body-site and age.

Results

Description of the cohort

Table 1 presents the demographic details of 148 EAT participants that contributed longitudinal skin swabs. Most infants were White (86.5%) and were born by vaginal delivery (78.4%). Fifty-four infants (36.5%) resided in highly urban environments. While 81.8% of infants had at least one atopic parent, only 8.1% carried a filaggrin (*FLG*) loss of function mutation (LOF).

Forty-five infants (30.4%) had AD at enrolment at 3 months, and 48.0% had active AD at least once before 3 years of age. AD was more prevalent in boys than girls at 3 months of age (38.2% vs 22.2%, P-value=0.049), and there was a trend towards a higher rate of AD in non-White infants (White: 27.3% vs non-White 50.0%, P-value=0.064). *FLG* LOF was associated with AD (15.6% vs 4.9%), but statistical significance was borderline (P-value=0.051). AD rates at enrolment were balanced amongst other demographic characteristics.

At enrolment, most infants were bathed two to four times weekly (n=79, 53.4%). Infants with AD at 3 months were bathed more frequently (more than once weekly) than those without AD, although the statistical significance was borderline (91.1% vs 78.6%, P-value=0.059). Bath product use (soap, shampoo, bubble bath) was equivalent among infants with and without AD at enrolment (P-value=0.58). There was no association between the 3-month hygiene score quintile and AD at 3 months of age (P-value=0.353).

The infant skin microbiome varies with body-site and age

Six samples with fewer than 2,000 reads were removed during quality control, resulting in a total of 586 clinical samples. Following contamination removal, 25,517 ASVs were observed. 87.9% of ASVs were annotated at genus-level and 69.9% at species-level. Body-site and age were the main determinants of alpha and beta diversity (Figure 1A&B). Shannon diversity was higher at the forearm, compared to the elbow, and at 1 year compared to 3 months (Figure 1A). The microbiome community composition was determined more by differences between the two body-sites, relative to differences by age (Figure 1B). Sequencing run and depth were also strongly associated with the community composition (Table S4), but these associations were attenuated following the removal of contaminant ASVs (Figure S4), and were accounted for in subsequent analyses.

Staphylococcus was the most abundant genus (mean relative abundance 25.2%) (Figure 1C, Figure S7, Table S5), followed by *Acinetobacter, Streptococcus, Corynebacterium, Moraxella, Pseudomonas* and *Bifidobacterium*. Linear mixed models demonstrated that body-site and age influenced the relative abundance of these seven genera (Figure 1D). S*taphylococcus* was significantly more abundant at the elbow compared to the forearm (P-value (site)<0.001). *Staphylococcus* relative abundance decreased between 3 months and 1 year of age (P-value (time)<0.001), more notably at the elbow (P-value (interaction)=0.041) (Figure 1C&D). *Streptococcus* relative abundance was significantly higher at the forearm (P-value (site)<0.001) and increased over time at both sites (P-value (time)<0.001, P-value (interaction)=0.997). Additional model results are presented in Table S6.

We observed similar findings at species level. *Staphylococcus epidermidis* was most abundant overall and predominated at the elbow (Figure S8). Notably, *S. aureus* was infrequently identified at 3 months (n=27 infants), and at 1 year (n=23 infants), and with a mean relative abundance of 0.07% (range: 0.002% - 2.27%).

Differential abundance testing identified numerous species that varied with time, at each body-site, including various *Streptococcus* species (Figure S9-S10, Table S7).

Associations between the early-life skin microbiome and intrinsic and extrinsic factors, including hygiene practices

Figure 2 presents PERMANOVA results evaluating associations between the species-level community composition and intrinsic and heritable factors, and extrinsic factors encompassing both the household and living environment and personal hygiene practices (Tables S8-S10). We observed significant variations in the infant skin microbiome according to infant's sex (P-value=0.046), ethnicity (White vs non-White: P-value=0.009), *FLG* LOF status (P-value≤0.001), paternal history of AD (P-value=0.03), paternal atopy (P-value=0.037), and having a sibling with AD (P-value=0.004). Delivery method, maternal history of AD and maternal atopy were not significantly associated with the microbiome composition (P-values>0.05).

The overall skin microbiome composition was associated with an objective urban-vs-rural classification of the infant's home environment at 3 months (P-value=0.005), and the association with the elbow microbiome at 1-year was highly significant (P-value≤0.001). *Corynebacterium* was more abundant in rural infants at the forearm at 3 months (P-value=0.025, Figure S11), however this finding was not significant following adjustment for multiple hypotheses (q-value 0.203). Rural living was associated with the abundance of four taxa in the 3-month forearm samples (Figure S12B). Meanwhile, at the elbow at 1 year,

we found a significant association between an urban-vs-rural environment and the abundance of three commensal *Staphylococcus* species, amongst others (Figure S12C). There were no differentially abundant species at other time-site combinations (Figures S12A&D).

A significant association was identified with the number of older siblings (P-value=0.041). Cigarette smoke exposure was only associated with the microbiome composition at the elbow in 1-year olds (P-value=0.025). There was no association with pet ownership at 3 months. There was also no association between the microbiome composition and systemic antibiotic use.

Bathing and personal hygiene factors were associated with significant variations in the infant skin microbiome (Table S10), particularly bath product use (P-value=0.011), frequency of hand wiping/washing (P-value=0.005) and using baby-wipes for cleaning infants' hands (P-value=0.005). There was no significant association between community composition and domestic water hardness (P-value=0.142). Bathing frequency at 3 months was not associated with the microbiome (P-value=0.109). However, we identified a borderline significant association when bathing frequency was dichotomized ('once per week or less' vs more frequent bathing, P-value=0.050), a variable previously associated with AD and barrier dysfunction in this cohort.[30] There was also a significant association with the hygiene score (numeric hygiene score: P-value=0.039, quintile of the hygiene score: P-value=0.001).

Many of the significant associations with the overall microbiome composition were not identified when PERMANOVAs were performed using only the swabs from each age and body-site combination (Tables S8-S10).

The presence of AD in infancy is associated with the overall composition of the skin microbiome

Table 2 summarizes AD prevalence and associated characteristics, including distribution, age of onset and severity. AD was more frequent in 3-month-old and 1-year-old infants, compared to 3-year olds (30.4% and 28.4%, vs 14.7%, respectively). AD severity remained mostly mild-moderate during the study; only 9 infants had SCORAD index ≥15. The median SCORAD index was highest at 3 years (median 11.50, IQR 6.30-39.20).

Shannon diversity was significantly lower in those with AD at 3 months (P-value=0.026, Figure S13), and specifically at the elbow (P-value=0.004) but not the forearm (P-value=0.588). There was no association between raised TEWL and Shannon diversity at 3 months (Figure S14). At 1 year, there was no association between Shannon diversity and AD

(Figure S15), however Shannon diversity was significantly lower in infants with raised TEWL at 1 year (P-value=0.008, Figure S16), specifically at the elbow (P-value=0.018).

Given the fluctuating nature of AD over time, we assessed associations between AD and the skin microbiome composition at corresponding time points: AD at 3 months with 3-month samples ("Overall 3m") and AD at 1 year with 1-year samples ("Overall 1y") (Figure 3, Tables S11 and S12). At 3 months, AD was associated with the overall microbiome composition (P-value=0.027). AD at the elbow at 3 months was associated with the overall microbiome composition (P-value=0.008), and specifically with the microbiome at the elbow (P-value=0.017)." Similar patterns emerged at 1 year (Figure 3B). AD was associated with the overall microbiome composition (P-value≤0.001), and with the microbiome composition at the elbow (P-value=0.022), but not the forearm (P-value=0.224). AD at the elbow at 1 year was associated with the overall microbiome composition (P-value=0.041), but not with the elbow microbiome (P-value=0.148) as was seen at 3 months. AD on the forearm was only associated with the overall microbiome at 1 year (P-value=0.006). We found a borderline association between AD severity and the overall microbiome composition at 1 year (P-value=0.063). Despite small numbers, AD severity was associated with the elbow microbiome at 1 year (P-value=0.038). In longitudinal analysis (Figure 3C), the microbiome during the first year of life was not associated with the presence of AD at 3 years of age (P-value=0.109).

Skin barrier impairment, at both 3 months and 1 year, was associated with the microbiome composition (P-value (3m)=0.004, P-value (1y)≤0.001). Moisturizer frequency was not associated with the skin microbiome composition.

Taxonomic differences associated with AD in infancy

Staphylococcus was more abundant at the elbow in infants with AD (P-value 0.035), but this finding lost significance after adjustment for multiple testing (q-value=0.212) (Figure S17). Unaffected 1-year-old infants had significantly higher *Pseudomonas* relative abundance at the elbow, surviving adjustment (P-value=0.003, q-value=0.025). Other associations with AD at 1 year were not significant after adjustment.

At species-level, 3-month-old infants with AD had lower *Streptococcus parasanguinis* abundance, at the elbow and the forearm (Figures S18 and S19, Table S13). AD was associated with lower *Corynebacterium vitaeruminis* abundance at the elbow at 3 months (Figure S18), and at the forearm at 1 year (Figure S20). Other taxa were differentially abundant only at single time-site combinations (Figures S18-S21). *S. aureus* was not frequently identified, and thus was excluded from the differential abundance analysis following species filtering. Other *Staphylococcus* species were not significantly associated with the presence of AD (Table S13).

Discussion

We studied the evolution of the skin microbiome of term-born infants during the first year of life by sequencing longitudinal skin swabs from both a moist and a dry ecological niche. Body-site and age were the most significant determinants of the skin microbiome in this early-life cohort. Sex, ethnicity, *FLG* LOF status, paternal AD, urban-rural living and the hygiene practice score demonstrated significant associations in both the overall microbiome composition analysis, and in certain body-site and age-specific analyses. Other associations were only significant with the overall microbiome composition, including the number of older siblings, having a sibling with AD, bathing frequency, and using bath products. Delivery method, the presence of pets and hard domestic water were not associated with the microbiome composition. The association between the skin microbiome and AD was stronger at 1 year, than at 3 months. There was no association between the skin microbiome during the first year of life and the presence of AD at 3 years.

Strengths and limitations

Our study contributes to the limited body of evidence relating to the early-life skin microbiome. Low-biomass sites, such as the skin, require rigorous contamination control measures throughout sampling and sequencing procedures. Environmental control samples collected during skin sampling were particularly important during quality control.

Taxonomic identification beyond genus using 16S rRNA gene sequencing methods is often restricted by shared sequence homology.[31,32] To improve taxonomic resolution, we utilized AnnotIEM to leverage multiple annotation databases.[33-35] A similar approach has been used by an independent research group, supporting this methodology.[36] Shotgun metagenomic sequencing may allow more definite species- and strain-level analysis, as well as interrogation of the functional importance of the skin microbiome.

30% of infants already had active AD at the first microbiome sampling at 3 months. In order for future studies to answer whether the microbiome plays a direct role in AD development,[37] samples need to be collected prior to disease initiation, as well as longitudinally thereafter to monitor changes throughout the disease trajectory.

Finally, the associations with intrinsic, environmental, and hygiene-related factors were systematically evaluated using the marginal effects of each variable in separate permutational models. PERMANOVA results were adjusted for sequencing run and

sequencing depth, with permutations constrained by body-site and the infants' age, to address the significant impact of these variables on the skin microbiome.

Placing our results in wider context

A small number of studies have evaluated for associations between AD and the infantile skin microbiome.[7,15,17-19,38-44] Prior to our study, only Rapin *et al*. comprehensively evaluated associations with AD-associated epidemiological factors and skin barrier changes.[7] PreventADALL was an allergy-focused population-based randomized trial with longitudinal skin sampling, allowing direct comparison with our results. However, there were important methodological differences, including sampling body-site (outer upper arm in PreventADALL), and technical factors, including 16S rRNA gene sequencing primer choices (V4 in PreventADALL).

Rapin *et al*. demonstrated an association with delivery method on day 1 of life, which was no longer significant by 3 months of age, in keeping with our results, as well as existing literature.[5,6,17] While we found no association with pets in the home at 3 months, Rapin *et al*. reported that exposure to dogs during pregnancy was associated with the microbiome composition.[7]

We objectively classified the home environment using satellite data, and demonstrated significant associations between urban-rural living and the composition of the microbiome, similar to Rapin *et al*. who found an association with a highly urban birth location. Rural infants were more likely to be White (93.6% vs 74.1%, P-value=0.002), and to live with pets (56.4% vs 38.9%, P-value=0.06), but otherwise the groups were similar. Urban-rural living was most significantly associated with the 1-year elbow microbiome, which we attribute to infants' developmental milestones, where older infants increasingly interact with their surroundings, and hence more environmental microbes. Previous studies indicated an association between an objective urban-rural classification and the skin microbiome in older children,[11] as well as the infantile gut and airway microbiome.[23] Future work should investigate how demographic, socioeconomic and environmental factors (e.g. air pollution) shape microbial differences in urban-vs-rural areas, as this may provide insights into higher rates of AD in urban populations.

FLG mutation status and skin barrier dysfunction were significantly associated with the skin microbiome in EAT. PreventADALL found no significant association with *FLG* mutations,[7] but we characterized the *FLG* gene more comprehensively, and the PreventADALL analysis may have been underpowered.

We found a significant association between the microbiome composition and the presence of concurrent AD, at both 3 months and 1 year. However, we did not identify strong signals

for any AD-associated genera or species. The low prevalence and abundance of *S. aureus* in this study was noteworthy, despite the high prevalence of AD, as there is a vast literature linking AD and *S. aureus* in older children and adult populations.[12,15,16,45] Scarcity of *S. aureus* in our cohort may reflect the by-and-large mild AD in the EAT cohort, and that we did not purposely swab lesional skin.[45] Our findings are, however, consistent with a growing body of literature which does not implicate *S. aureus* in infantile AD.[7,17-19,42] Sampling from active dermatitis (lesional skin) may be informative regarding any potential link between *S. aureus* and infantile AD.

In summary, the infant skin microbiome was primarily influenced by body-site and age and was associated with a variety of epidemiological factors relevant to AD, including urban-rural living, siblings, family history of atopy, and infant bathing and hygiene practices. We identified significant associations between the skin microbiome and AD. The differential abundance of bacterial species in infants with AD, and in infants from urban compared to rural environments, warrants further study. This research contributes to our understanding of the "biodiversity hypothesis", which suggests that the reduction in personal and environmental biodiversity is responsible for the increased prevalence of chronic inflammatory diseases such as AD and allergies.

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Figure legends

Figure 1. The infant skin microbiome is highly influenced by body-site and age.

[A] Violin plots of Shannon diversity according to the infants' age ("time") and body-site sampled. Paired samples from each time point are joined by dashed lines. A linear mixed-effects model (text box) demonstrated the significant effect of the body-site and the infants' age on Shannon diversity, adjusting for sequencing depth, natural logarithm-transformed sequencing depth and sequencing run as fixed-effects, and the individual infants as random-effects. The interaction (between age and body-site) was not statistically significant. Shannon diversity was significantly higher at 1 year compared to 3 months at both the elbow and the forearm. P-values within the plot were generated using Wilcoxon signed-rank tests, adjusted for multiple hypothesis testing (Holm method). [B] Principal Coordinate Analysis (PCoA) ordination demonstrating that genus-level community composition (Bray-Curtis dissimilarity index) was more strongly associated with body-site than with infants' age. R2 and P-values (inset) were generated using PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) based upon 999 permutations constrained by age and body-site, and adjusted for sequencing run and sequencing depth. Count data were Hellinger transformed and total sum scaled prior to PERMANOVA. Stacked barplots and line plots [C], and violin plots [D] depict the relationship between the relative abundance of the seven most abundant genera, the infant's age and body-site sampled. Linear mixed models [D] were generated using transformed relative abundances, to facilitate visualization of right-skewed data (log_{10}-transformation following the addition of a pseudocount of 0.01). Linear mixed models were generated with sequencing depth, natural logarithm-transformed sequencing depth and sequencing run as fixed-effects, and the individual infants as random-effects. *Staphylococcus* was the most abundant genus overall, particularly at the elbow at 3 months. *Staphylococcus* abundance was significantly associated with age and with body-site, and abundance decreased between 3 months and 1 year at the elbow while abundance remained relatively stable at the forearm, as evidenced by the significant interaction between body-site and age from the linear mixed model. Conversely, while *Streptococcus* abundance was significantly associated with age and with body-site, and was higher at the forearm compared to the elbow in both 3-month and 1-year samples, the interaction between age and body-site was not statistically significant, which signified a consistent relationship between *Streptococcus* abundance and body-site and age.

Figure 2. The early-life skin microbiome is associated with a variety of intrinsic and heritable, extrinsic and hygiene-related factors.

Bar plots of pseudo-F statistics, and P-values, evaluating the association between the species-level composition of the infant skin microbiome and [A] intrinsic and heritable factors, [B] extrinsic factors, including the home environment and systemic antibiotic use

and [C] personal hygiene factors. PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) was performed using the Bray-Curtis dissimilarity index, assessing the marginal effect of each variable, with 999 permutations (constrained by age and body-site), and adjusted for sequencing run and sequencing depth. Bar plots were annotated with P-values ≤ 0.1, with significant P-values (≤ 0.05) being highlighted with an asterisk (*). Data were Hellinger transformed and total-sum scaled (TSS) prior to PERMANOVA. Overall PERMANOVA results (calculated using all samples) are presented as blue bars, while associations with specific age and body-site specific results are presented with purple and green bars. In panel B, overall PERMANOVA regarding antibiotic use in the month preceding the 1-year visit utilized 1-year samples only. In panel C, infrequent bathing refers to bathing once weekly or less. Exposure to bath products involved parent-reported use of soap, shampoo and/or bubble bath. The EAT hygiene score was calculated according to the responses regarding weekly bathing frequency, daily hand and face cleaning frequency, and the use of baby-wipes for cleaning the infant's hands, and was evaluated as a linear descriptor of hygiene practice, and categorized into quintiles. Full PERMANOVA results are presented in Tables S8-S10.

Figure 3. Atopic dermatitis (AD), AD severity and skin barrier impairment are associated with the composition of the infant skin microbiome.

Bar plots of pseudo-F statistics, and P-values, evaluating the association between the species-level composition of the infant skin microbiome and AD and related factors at [A] 3 months, [B] at 1 year, and [C] and longitudinally up to 3 years of age. PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) was performed using the Bray-Curtis dissimilarity index, assessing the marginal effect of each variable, with 999 permutations (constrained by age and body-site), and adjusted for sequencing run and sequencing depth. Bar plots were annotated with P-values ≤ 0.1, with significant P-values (≤ 0.05) being highlighted with an asterisk (*). Data were Hellinger transformed and total-sum scaled (TSS) prior to PERMANOVA. Overall PERMANOVA results (calculated using all samples) are presented as blue bars, while associations with specific age and body-site specific results are presented with purple and green bars. Panels A and B report time-congruous analyses evaluating associations between clinical outcomes and the microbiome at the same time point (either 3-month or 1-year outcomes and respective 3-month and 1-year samples only). In panel C, 'atopic dermatitis (3y)' refers to the presence of AD on examination at 3 years of age, while 'atopic dermatitis (ever)' refers to the diagnosis of AD at any point between enrolment and 3 years of age. Age of onset categorizes infants as early-onset AD (present at 3 months), late-onset (present at 1 year or later) or unaffected (by 3 years of age). SCORAD: SCORing of Atopic Dermatitis index, TEWL: Trans-Epidermal Water Loss. Full PERMANOVA results are presented in Tables S11-S12.

Table 1. Baseline demographics of Enquiring About Tolerance (EAT) infants included in microbiome analysis, according to atopic dermatitis (AD) status at enrolment. Microbiome samples were collected from sequential EAT infants, however only infants with swabs at both 3 months and 1 year were analyzed. P-values calculated using Fisher's exact test.

	No AD at enrolment (3 months)	AD at enrolment (3 months)	P-value
	n=103 (69.6%)	n=45 (30.4%)	
Demographics and birth history			
Sex (male)	47 (45.6%)	29 (64.4%)	0.049
Ethnicity			0.064
White	93 (90.3%)	35 (77.8%)	
Non-White	10 (50%)	10 (50%)	
Filaggrin gene status			0.051
Wild type	81 (78.6%)	33 (73.3%)	
Any loss of function mutation	5 (4.9%)	7 (15.6%)	
Missing	17 (16.5%)	5 (11.1%)	
Caesarean delivery	21 (20.4%)	11 (24.4%)	0.665
Family history (self-reported)			
Maternal AD (%)	34 (33.0%)	17 (37.8%)	0.578
Maternal atopy (asthma, AD, hayfever, food allergy) (%)	62 (60.2%)	25 (55.6%)	0.717
Paternal AD (%)	20 (19.4%)	14 (31.1%)	0.139
Paternal atopy (asthma, AD, hayfever, food allergy) (%)	57 (55.3%)	27 (60.0%)	0.719
Older sibling(s) (%)	51 (49.5%)	27 (60.0%)	0.284
Number of siblings			0.474
None (%)	52 (50.5%)	18 (40.0%)	
One (%)	38 (36.9%)	19 (42.2%)	
Two or more (%)	13 (12.6%)	8 (17.8%)	
Sibling(s) with AD (%)	24 (23.3%)	16 (35.6%)	0.159
Home environment			
Urban-Rural classification (Urban) (%)	36 (35.0%)	18 (40.0%)	0.582
Pets in the home (%)	56 (54.4%)	18 (40.0%)	0.152
Cat(s) (% of those with a cat)	33 (80.5%)	8 (19.5%)	
Dog(s) (% of those with a dog)	18 (60.0%)	12 (40.0%)	
Cigarette smoke exposure (%)	12 (11.7%)	7 (15.6%)	0.595
"Hard" domestic water [$CaCO_3$ concentration ≥257mg/L] (%)	52 (50.5%)	22 (48.9%)	1
Systemic antibiotic use*			
Antibiotics prior to enrolment	13 (12.6%)	5 (11.1%)	1

Missing	5 (4.9%)	2 (4.4%)	
Antibiotics in the 30 days preceding the 1-year visit	23 (22.3%)	4 (8.9%)	0.065
Missing	4 (3.9%)	3 (6.7%)	
Bathing and hygiene variables (3-month questionnaire)			
Bathing frequency (%)			0.102
Once per week or less	17 (16.5%)	2 (4.4%)	
2-4 times per week	49 (47.6%)	30 (66.7%)	
5-6 times per week	8 (7.8%)	3 (6.7%)	
Daily or more than daily	24 (23.3%)	8 (17.8%)	
Missing	5 (4.9%)	2 (4.4%)	
Infrequent (weekly or less) bathing (%)	17 (16.5%)	2 (4.4%)	0.059
Missing	5 (4.9%)	2 (4.4%)	
Bath product use (soap, shampoo, bubble bath) (%)	60 (58.3%)	24 (53.3%)	0.58
Missing	5 (4.9%)	2 (4.4%)	
Frequency of hand washing/wiping (%)			0.295
Not at all	11 (10.7%)	9 (20.0%)	
1-2 times per day	72 (69.9%)	27 (60.0%)	
3-4 times per day	15 (14.6%)	7 (15.6%)	
≥ 5 times per day	0	0	
Missing	5 (4.9%)	2 (4.4%)	
Wet wipes used to clean hands (%)	23 (22.3%)	7 (15.6%)	0.641
Missing	16 (15.5%)	11 (24.4%)	

Systemic antibiotic use was based on parental recall, and did not specify the which antibiotic was used, the indication, nor the duration of treatment.

1

Table 2 The diagnosis and severity of atopic dermatitis (AD), and skin barrier impairment in infants up to 3 years of age

	Yes (%)	No (%)	Missing
3-month variables			
AD at 3 months	45 (30.4%)	103 (69.6%)	0
Active AD on the forearms (% of those with AD)	5 (11.1%)	40 (88.9%)	0
Active AD at the antecubital fossae (% of those with AD)	6 (13.3%)	39 (86.7%)	0
Median SCORAD [IQR]	3.70 [3.60, 7.10]	NA	
Moderate to severe AD (SCORAD ≥ 15)	3 (6.7%)	42 (93.3%)	103
Median TEWL [IQR]	12.72 [10.77, 15.26]		
Raised TEWL (≥ 15.0g/m²/h)	43 (29.1%)	105 (70.9%)	
1-year variables			
AD at 1 year	42 (28.4%)	106 (71.6%)	0
Active AD on the forearms (% of those with AD)	8 (19.0%)	34 (81.0%)	0
Active AD at the antecubital fossae (% of those with AD)	10 (23.8%)	32 (76.2%)	0
Median SCORAD [IQR]	7.70 [3.73, 14.37]	NA	
Moderate to severe AD (SCORAD ≥ 15)	9 (21.4%)	33 (78.6%)	106
Median TEWL [IQR]	14.06 [11.81, 17.07]		16
Raised TEWL (≥ 16.1g/m²/h)	44 (33.3%)	88 (66.7%)	16
3-year and longitudinal variables			
AD at 3 years	21 (14.7%)	122 (85.3%)	5
Active AD on the forearms (% of those with AD)	6 (28.6%)	15 (71.4%)	5
Active AD at the antecubital fossae (% of those with AD)	9 (42.9%)	12 (57.1%)	5
Median SCORAD [IQR]	11.50 [6.30, 39.20]	NA	0
Moderate to severe AD (SCORAD ≥ 15)	9 (47.4%)	10 (52.6%)	129
AD at least once by 3 years (cumulative AD ever)	71 (48.6%)	75 (51.4%)	2
Age of onset of AD			2
Early-onset AD (present at 3 months) **Late-onset AD (developed after 3 months)** **Unaffected by AD by 3 years of age**	45 (30.8%) 26 (17.8%) 75 (51.4%)		

SCORAD: the total SCORing of Atopic Dermatitis index was recorded at each visit in infants with AD (range 0-103). A SCORAD ≥ 15 was used to define moderate to severe AD, as per our previous publications.[26]

TEWL: trans-epidermal water loss was measured on clinically unaffected skin on the left volar forearm in triplicate, and the mean value was calculated for each infant, at each visit. Results were dichotomized ("Raised TEWL") if the value was above the upper quartile of TEWL measurements in infants without visible AD at that age (median TEWL 3 months 12.4g m^{-2} h^{-1} (IQR 10.4-15.0), median TEWL 1 year 13.7g m^{-2} h^{-1} (IQR 11.4-16.1).[26,46] Further information is provided in Appendix S1.

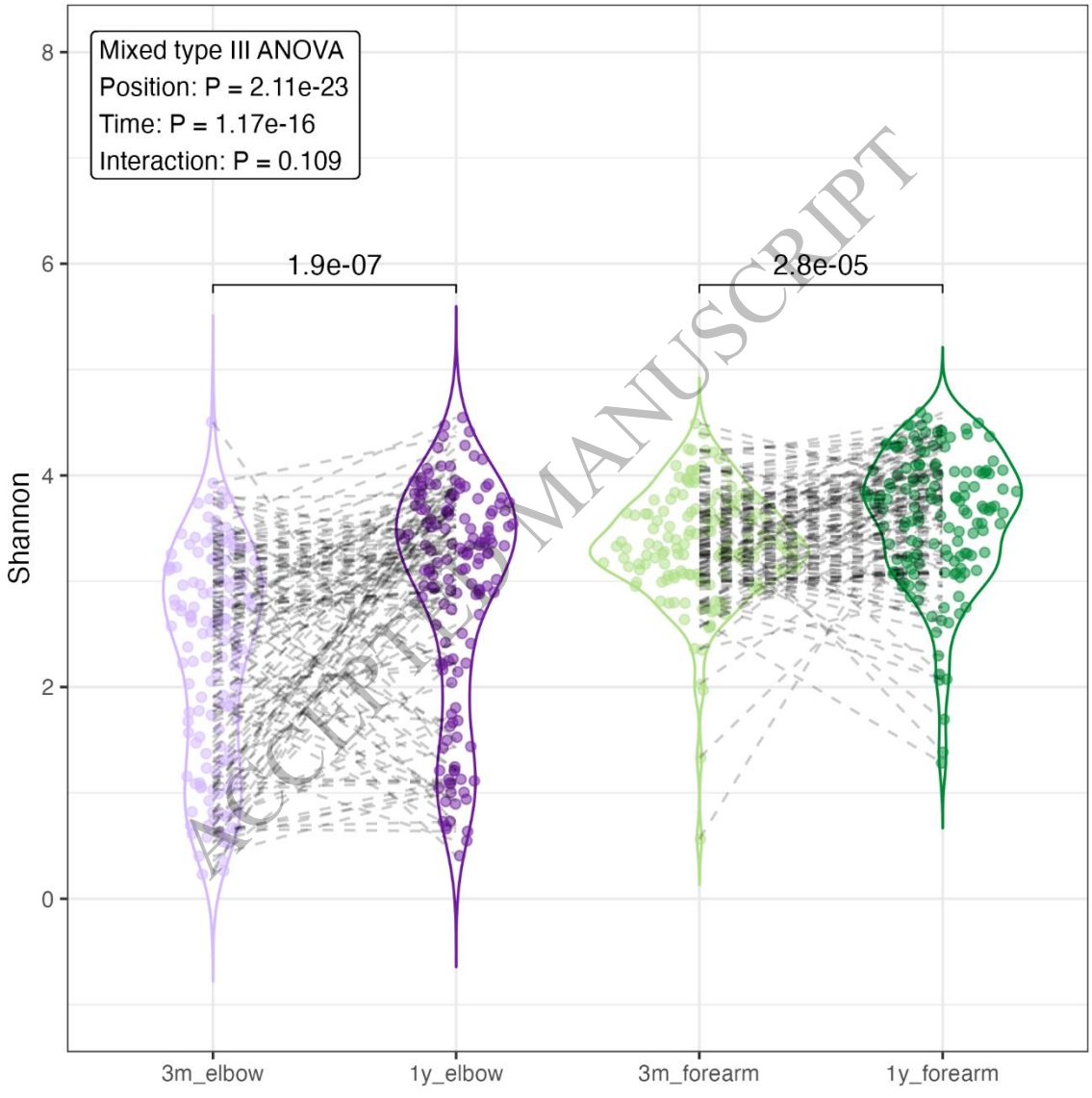

Figure 1A
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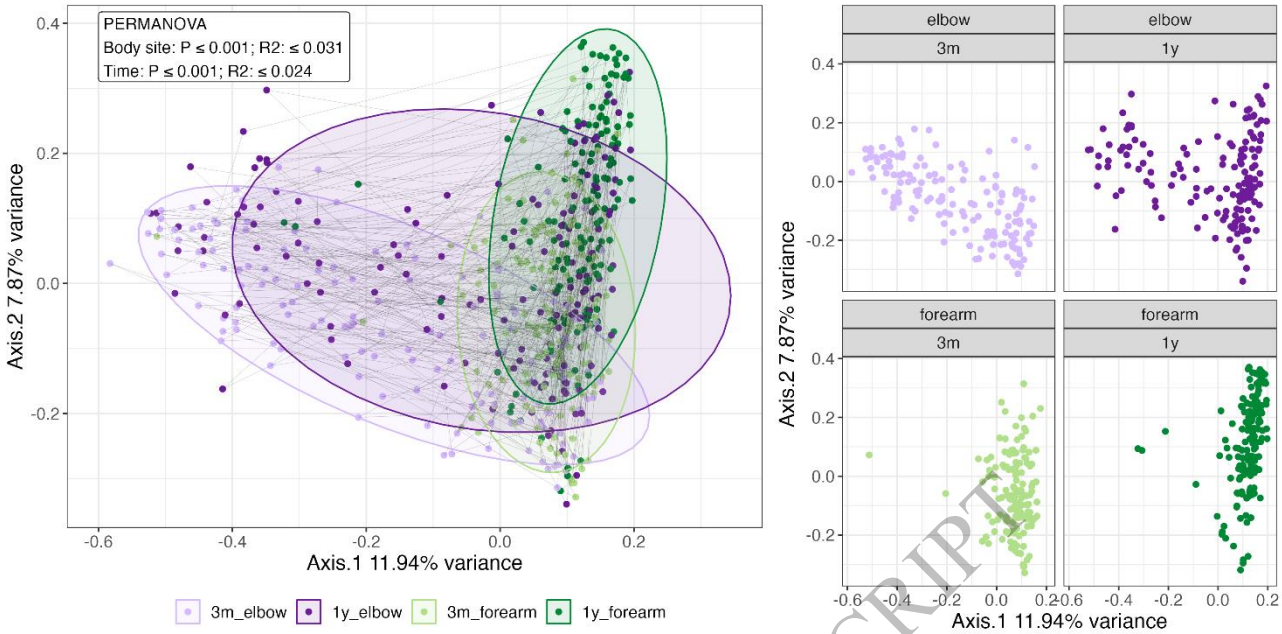

Figure 1B
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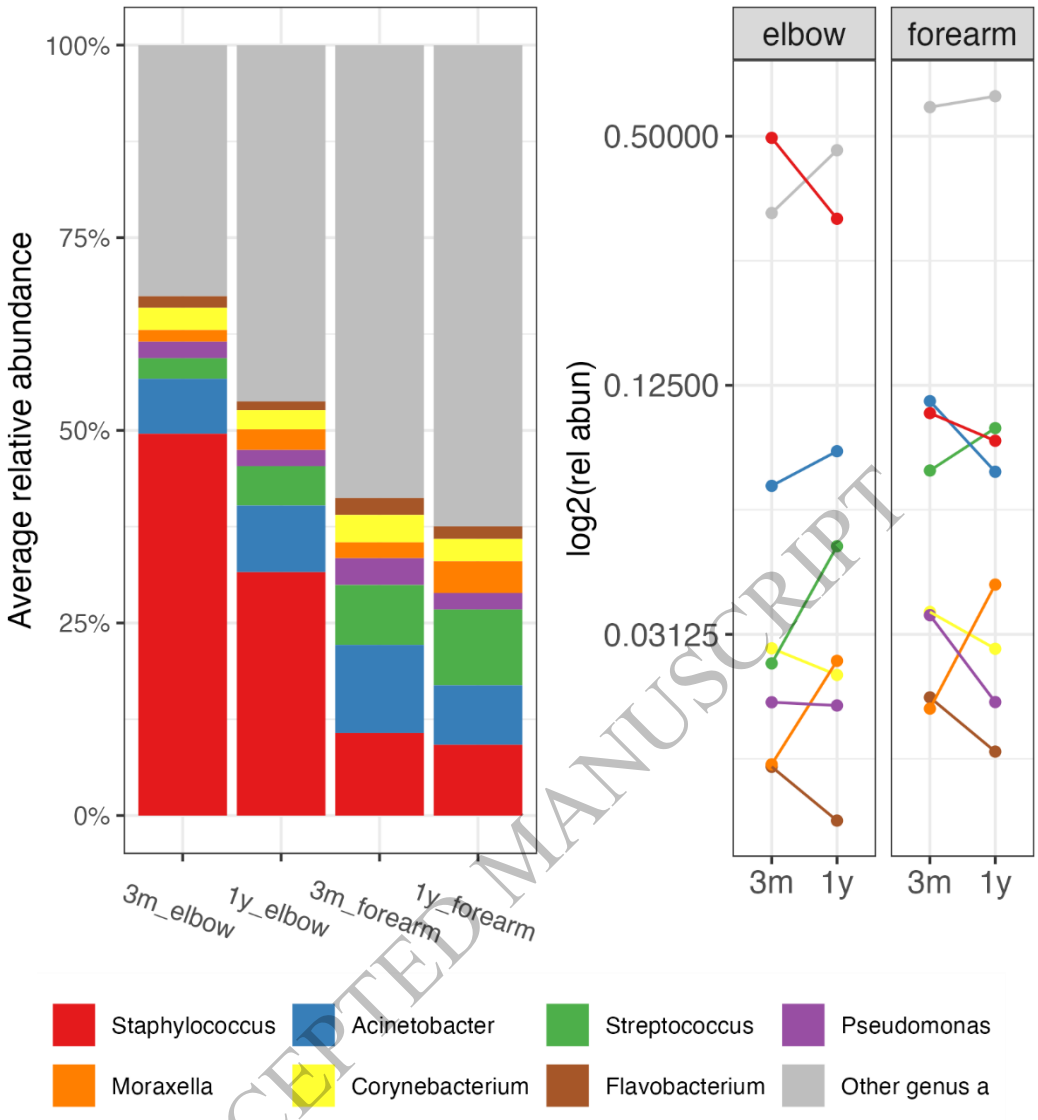

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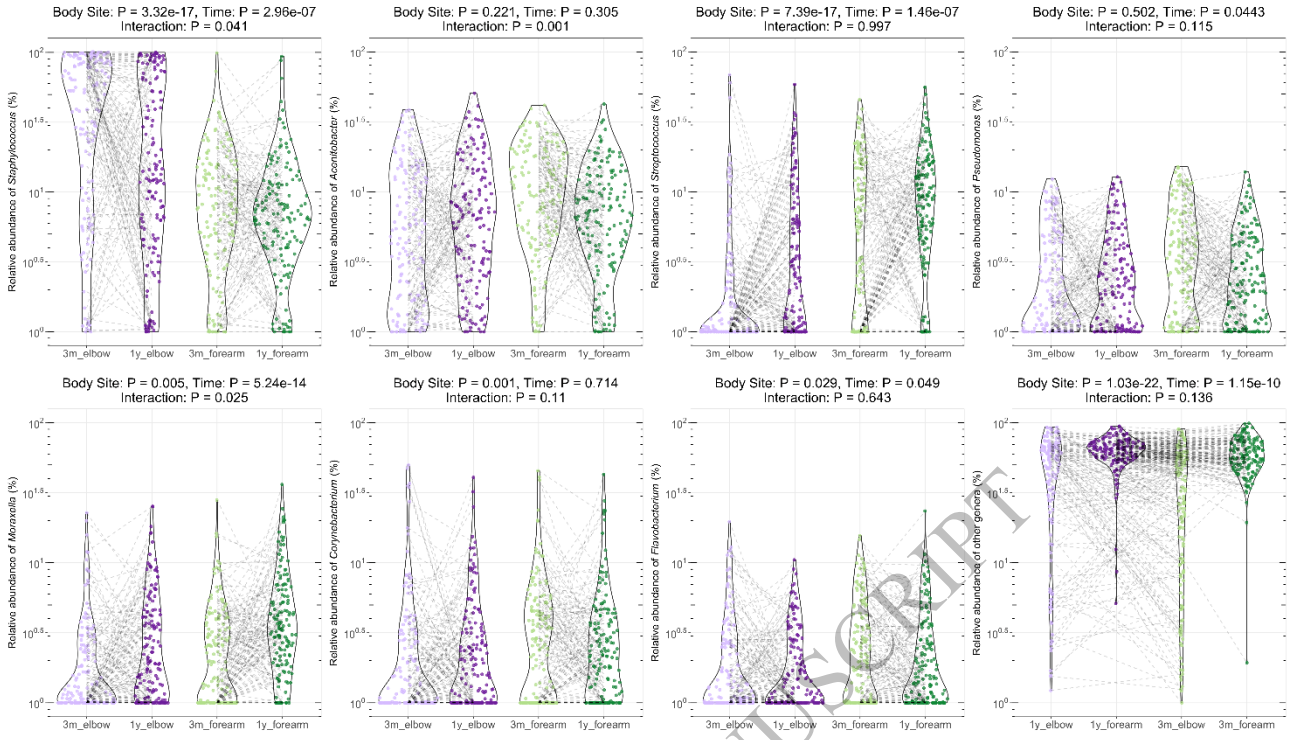

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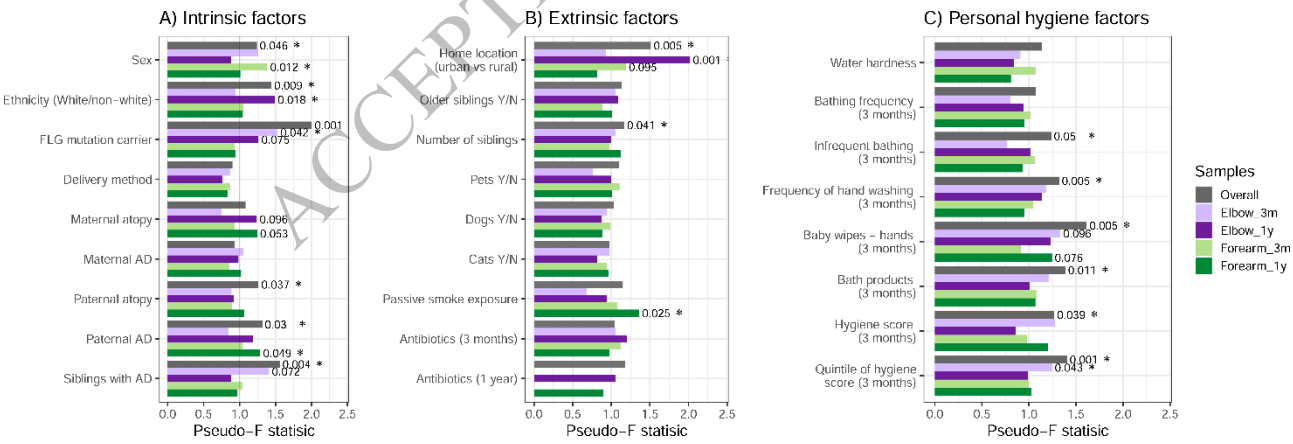

Figure 2
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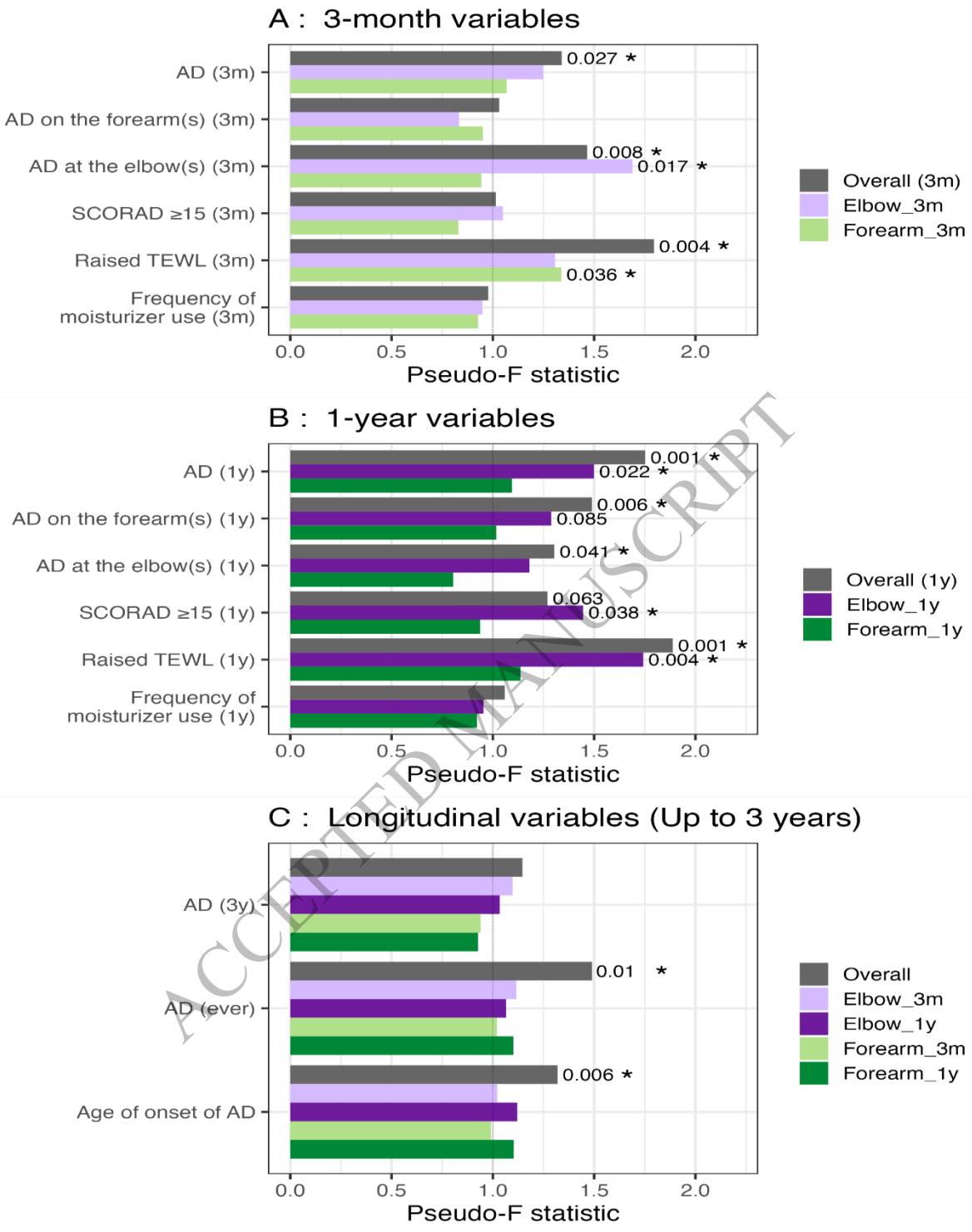

Figure 3
164x246 mm (DPI)