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URBANIZED MICROBIOTA IN INFANTS, IMMUNE CONSTITUTION AND LATER RISK OF ATOPIC DISEASES

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ABSTRACT

Background Urbanization is linked with an increased burden of asthma and atopic traits. A putative mechanism is insufficient exposure to beneficial microbes early in life leading to immune dysregulation as previously shown for indoor microbial exposures.

Objective To investigate whether urbanization is associated with the microbiota composition in the infants' body and early immune function, and whether these contribute to the later risk of asthma and atopic traits.

Methods We studied the prospective COPSAC₂₀₁₀ mother-child cohort of 700 children growing up in areas with different degrees of urbanization. During their first year of life, airway and gut microbiota as well as immune marker concentrations were defined. At six years of age, asthma and atopic traits were diagnosed by pediatricians.

Results In adjusted analyses, the risk of asthma and aeroallergen sensitization were increased in urban infants. The composition of especially airway, but also gut microbiota differed between urban and rural infants. The living environment related structure of the airway microbiota associated with immune mediator concentrations already at one month of age. An urbanized structure of airway and gut microbiota associated with an increased risk of asthma coherently during multiple time points, and also with the risks of eczema and sensitization.

Conclusion Our findings suggest that urbanization related changes in the infant microbiota may elevate the risk of asthma and atopic traits, probably via crosstalk with the developing immune system. The airways may facilitate this effect as they are open for colonization by environmental, airborne microbes and serve as immune interface.

Key messages

- Airway and gut microbiota as well as immune marker concentrations differ between rural and urban infants.
- Urbanized composition of microbiota in infancy increases the risk of asthma, eczema and allergic sensitization at six years of age.
- Urban environments may predispose to the development of a disease-promoting composition of the infant microbiota.

Capsule summary In a prospective cohort study, an urban living environment and urbanized infant microbiota associated with increased risks of asthma, eczema and aeroallergen sensitization at six years of age probably due to early immune dysregulation.

Keywords microbiome; residential environment; non-communicable diseases; urbanization; childhood

Abbreviations

aOR = adjusted odds ratio

ANOVA = Analysis of variance

CCL = (C-C motif) chemokine ligand

CRP = C-reactive protein

COPSAC = Copenhagen Prospective Studies on Asthma in Childhood

CXCL = (C-X-C motif) chemokine ligand

IL = Interleukin

INF = Interferon

TGF = Transforming growth factor

- 90 TNF = Tumor necrosis factor
- 91 OR = Odds ratio
- 92 PAM = Partitioning Around Medoids
- 93 PCA = Principal Component Analysis
- 94 PCoA = Principal Coordinates Analysis
- 95 PERMANOVA = Permutational Multivariate Analysis of variance
- 96 RMSE = Root Mean Squared Error
- 97 sIgE = specific Immunoglobulin E
- 98 SPT = skin prick test
- 99 sPLS = Sparse Partial Least Squares

INTRODUCTION

Urban children tend to have a higher risk of developing asthma^{1,2} and atopic traits such as allergic sensitization³, rhinitis⁴ and eczema⁵ than rural children. Several factors differ between these populations including larger family size and increased exposure to animals in rural areas, which may contribute to the difference in disease risk^{6–8}. Also, the microbial exposures are different between rural and urban areas, which has been shown for the composition of outdoor, indoor and human microbiota^{9–12}. These microbial differences may mediate the protective effect of a rural environment, as demonstrated for farming-related indoor microbiota, which has been repeatedly associated with asthma protection^{13–15}, also in experimental settings^{14,16,17}.

Microbial exposures are especially important during infancy and early childhood, when the constitution of the immune system is primed by extensive immune-microbiota crosstalk^{18,19}. Indeed, accumulating evidence shows that infant microbiota is altered prior to the development of allergic sensitization, rhinitis²⁰, eczema²¹ and/or asthma^{22–24}, potentially influencing this early crosstalk. A recent report suggested that growing up in farms can support the maturation of infants' gut microbiota and thus reduce their risk of asthma when compared to other rural children²⁵. However, it remains unclear how constantly increasing urbanization of our living environments influence on early immune-microbiota crosstalk and later risk of disease.

To address the hypothesis that dissimilar microbiota between urban and rural infants contribute to the development of their immune system and the later risk of disease, we utilized longitudinal data from the Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC₂₀₁₀) mother-child cohort. We defined the degree of urbanization in their living environment during infancy and studied its associations with asthma and atopic traits at age six years. We further investigated whether the early microbiota composition of airways and

gut as well as the local airway and systemic immune function may convey associations between living environment and disease.

METHODS

Cohort

Children in the COPSAC₂₀₁₀ mother-child cohort (n=700) have been followed prospectively since birth with numerous scheduled visits at a dedicated research unit. Comprehensive descriptions of the cohort and the research approach are published^{26–28}. The COPSAC pediatricians were solely responsible for diagnosis and treatment of all respiratory, allergy and skin-related symptoms.

Definition of asthma and atopic traits

Asthma by age 6 years was diagnosed prospectively based on a previously detailed quantitative symptom algorithm^{26,29}(**Online repository methods**).

Eczema by age 6 years was diagnosed prospectively based on the criteria of Hanifin and Rajka³⁰ requiring the presence of 3 of 4 major criteria and at least 3 of 23 minor criteria.

Allergic rhinitis at age 6 years was based on sensitization and clinical interviews of the parents on history of significant nasal congestion, sneezing and/or runny nose outside periods with common cold and in the relevant period of the sensitized aeroallergen.

Allergic sensitization was determined at age 6 years, defined as any positive skin prick test (SPT) ≥ 3 mm (ALK-Abello, Horsholm, Denmark) or specific Immunoglobulin E (sIgE) in serum ≥ 0.35 kUa/L against common inhalant and/or food allergens (ImmunoCAP; Thermo Fisher Scientific, Allerod, Denmark). “Not sensitized” were both SPT and sIgE negative (**Online repository methods**).

Definition of urbanization gradient

We utilized the land cover database CORINE (<https://land.copernicus.eu/>) from the year 2012 for an unbiased definition of the living environment. Addresses at birth ($n=686$) were translated to coordinates using Google API services³¹. Land cover types around each coordinate point were extracted from the CORINE2012 raster map (100 m resolution) with a three-kilometer buffer (**Table E1**). The correlation matrix of proportions of five major land cover types within the buffer region were analyzed with Principal Component Analysis (PCA)³². The first axis explained 44% of the total variation and made clear separation between artificial and natural (i.e. agricultural and forested) land cover types (**Fig E1a**). The first axis was extracted for downstream analyses to represent a gradient of urbanization running from the most rural to the most urban environments in the study region. Land cover information was also dichotomized into rural and urban groups ($n_{\text{rural}}=314$, $n_{\text{urban}}=372$; **Fig E1b**) with Partitioning Around Medoids (PAM) clustering on Euclidean distances³³. Most children ($n=577$) did not move during their first year of life or moved later than 50 weeks after birth ($n=5$). From the 109 children who did move, 77% did not change between rural and urban groups (**Online repository methods**).

Covariates

Differences between rural and urban living environments were tested for common disease-associated factors including pet ownership, daycare attendance during the first year of life, length of breastfeeding period, passive smoking exposure, income of family, parental education, number of older siblings, home type, mode of delivery, parental diagnosis of asthma, eczema and rhinitis, and use of antibiotics during the first year of life (**Fig E2**).

Microbiota samples

Airway microbiota samples were collected at one week (n=549), one (n=647) and three months (n=657) of age³⁴, gut microbiota samples were collected at one week (n=544), one month (n=615) and one year (n=625) of age²² (**Online repository methods**).

DNA was extracted from airway and gut samples using the PowerMag® Soil DNA Isolation Kit optimized for epMotion® (MO-BIO Laboratories, Inc., Carlsberg, CA, USA) using the epMotion® robotic platform model 5075 (Eppendorf, Hamburg, Germany). The manufacturer's protocol was mildly modified (described earlier³⁴). Extracted DNA was stored at -20 °C degrees prior to amplification of the 16S rRNA gene variable region 4 (V4) and sequencing with the Illumina MiSeq System (Illumina Inc., CA, USA) as previously described³⁴. The bioinformatics analysis pipeline is detailed in the **Online repository methods**.

Immune mediators

Airway immune mediator samples (upper airway mucosal lining fluid) were collected from one month old infants (n=620) with a synthetic absorptive matrix placed in both nostrils for 2 minutes³⁵. Matrixes were stored immediately at -80 °C degrees. Blood for systemic immune mediator analysis was drawn in an EDTA tube from a cubital vein at the age of 6 months (n=568), centrifuged to separate plasma and cells, and plasma was immediately stored at -80°C until analysis³⁶.

The levels of the *a priori* selected cyto- and chemokines^{37,38} were determined by using high-sensitivity immunoassays based on electrochemiluminescence in a multiplex setting. Samples were read in duplicates by using the Sector Imager 6000 (Meso Scale Discovery, Gaithersburg, MD).

Statistics

Odds ratios for atopic traits in response to urban and rural (baseline) classes as well as urbanization gradient were calculated with logistic regression models adjusted for lifestyle variables. Adjustment included other covariates associated with urbanization (**Fig E2**), while house type was excluded due to collinearity with the explanatory variable of interest (urban/rural groups).

The R-package phyloseq³⁹ was used for handling the microbiota data. Observed richness and Shannon diversity were used as the alpha diversity metrics. The community compositions of microbiotas (beta diversity) were visualized with Principal Coordinates Analysis (PCoA) for Bray-Curtis⁴⁰, Weighted UniFrac and Unweighted UniFrac⁴¹ distances and tested with Permutational Multivariate Analysis of variance (PERMANOVA)³² with 1000 permutations. The dissimilarities within rural and urban individuals were defined with the distance of each sample from group mean⁴².

Immune mediators were log transformed and centered log ratio scaled in order to remove biological variation due to differing amount of protein per volume of fluid from the dataset as well as to increase the comparability between immune mediators following methodology developed previously³⁸. The normalized concentrations were tested between rural and urban groups in adjusted linear models.

Sparse Partial Least Squares (sPLS) models with relative abundances of taxa agglomerated to genus level, log-transformed, and cleaned to include those present at least in 5% of samples were used to predict the urbanization gradient^{38,43}. The best model was selected based on the Root Mean Squared Error (RMSE) statistics, from repeated 10-fold cross-validation, in order to avoid overfitting. The predicted values from each model were extracted to represent a bacterial score for the urbanization gradient. All analyses were conducted in R version 3.5.2⁴⁴.

215 ***Ethics statement***

216 This study was approved by the local Ethics Committee (H-B-2008-093), and the Danish Data
217 Protection Agency (2015-41-3696). Both parents gave verbal and written informed consent
218 before enrolment.

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RESULTS

Asthma and atopic traits in rural and urban infants

All subjects spent their first year of life in a westernized, densely populated, and geographically concise area in Zealand (7,031 km²), Denmark (**Fig 1a**). Few children lived on the farms.

Several lifestyle variables such as pet ownership, number of older siblings, income in the family and education of the parents differed between rural and urban infants (**Fig E2**). Other screened variables, including mode of delivery, antibiotics during the first year of life, vitamin D or fish oil intervention groups, and atopic diseases in the parents, all previously associated with atopic diseases^{27,45,46}, did not differ between rural and urban infants.

The prevalence of asthma at six years of age or earlier was 22.3% (n=146), allergic rhinitis 6.8% (n=45), and eczema 31.7% (n=210). At six years of age, 31.7% (n=157) of children were sensitized, of which 23.7% (n=117) had aeroallergen and 14.7% (n=73) food sensitization. The prevalence of asthma, allergic rhinitis and aeroallergen sensitization at six years of age were higher in children spending their first year of life in an urban compared to a rural environment (**Figs 1b and 1d**). Allergic sensitization and eczema showed similar trends. Urban infants developed more specific sensitization to peanut, house dust mite, timothy, birch, and mugwort allergens (**Table E2**). After adjustment for lifestyle features, the odds ratios for asthma (aOR 2.31, 95% CI 1.47-3.68, P=0.0003) and aeroallergen sensitization (aOR 1.77, 95% CI 1.05-3.02, P=0.0348) were increased in urban infants (**Figs 1c and 1e**). The adjusted, predicted prevalence of asthma and aeroallergen sensitization showed a large increase along the urbanization gradient while only food sensitization remained completely unrelated (**Fig E3**).

Microbiota and immune mediator concentrations in rural and urban infants

The airway microbiota at all three time points showed significant differences between rural and urban infants regardless of the metric used to define the similarity of microbiota between subjects (**Fig E4, Table E3**). These differences remained significant after adjustment for each lifestyle variable as well as in a fully adjusted model (PERMANOVA, **Table E4**). A part of the observed rural-urban differences was explained by the larger family size and more common pet ownership in rural environments. Shannon diversity and richness in the airway microbiota were higher in urban infants at one month of age (ANOVA, $P=0.004$ and 0.005 respectively). Remarkably, urban infants had more homogenous airway microbiota than rural infants at all time points (i.e. urban children had more similar microbiota to each other than rural children; **Fig E5**). Among the fifteen most abundant genera in the airways, several showed dissimilar relative abundances between rural and urban infants (**Fig E6**). *Veillonella* showed a consistent association with an urban living environment through all time points. *Haemophilus* and *Rothia* associated with urban environment while *Dolosigranulum* and *Moraxella* associated with rural environment in more than one time point (**Fig. E6**).

The composition of the gut microbiota associated with the living environment only at one year of age regardless of distance metric (**Fig E4, Table E3**). Nevertheless, in fully adjusted model, this association attenuated (**Table E5**), mainly due to the influence of older siblings. The fifteen most abundant genera in the gut microbiota at different time points did not show differences between rural and urban environments after adjustment for multiple testing (**Fig E7**).

However, the *Firmicutes/Bacteroidetes*-ratio was lower in rural infants at one year of age (mean in rural: 3.4, urban: 4.1; ANOVA, $P=0.0004$). A closer look at this ratio revealed that the richness within *Bacteroidetes* was higher in rural than urban infants at the same time point (mean in rural: 13.4, urban: 11.5; ANOVA, $P=0.0003$).

Relative concentrations of both airway (1 month) and systemic (6 months) immune mediators differed between urban and rural infants in adjusted analyses for both sample types (**Fig 2; Table E6**). In airways (**Fig 2a**), Type 2-related cytokine and chemokine concentrations showed a large disparity between urban and rural infants. For example, urban infants had higher relative concentrations of CCL11, CCL13 and CCL17, while CCL22 and CCL26 were higher in rural infants. Furthermore, IL-12p70 (Type 1) was found in higher relative concentrations in rural infants as well as IL-10 (Regulatory), which has anti-inflammatory properties. A different set of immune mediators was measured in the systemic samples (**Fig 2b**). CXCL8 (Type 17) was measured in both airway and systemic samples and showed higher relative concentration in urban infants in both sample types. However, other immune mediators measured from both sample types, IL-1 β and TNF- α , did not show similar responses.

Urbanized infant microbiota

We defined the bacterial community profiles most indicative of urbanization gradient separately for each time point and sampling site. We utilized cross-validated sPLS models to find a minimal (sparse) set of jointly contributing taxa that discriminated between urban and rural samples. The models predicted better during later time points in both airway and gut samples, indicating an accumulated influence of living environment with age (**Fig 3**). Higher relative abundances of the genera *Veillonella*, *Rothia*, *Gemella*, *Bergyella* and *Streptococcus* in airway microbial profiles were indicative for urban living environments, while *Moraxella*, *Paracoccus* and *Dolosigranulum* were characteristic of a rural environment (**Fig 3a**) at all time points corresponding to univariate analyses (**Figs E6**). Some airway bacteria such as *Bacillus* and *Listeria* associated with a rural environment at the first week of life but the direction shifted later to association with urban environments.

Moraxella was also indicative for rural environment and *Veillonella* for urban environment in the gut microbial profiles (**Fig 3b**), but only at a single time point each. *Bifidobacterium* in gut was indicative for rural environment, already in the first week of life. In the gut profiles, only *Bacteroides* switched direction from urban to rural environment at the last time point. Overall, living environment-related members of gut communities did not match much between time points primarily due to the low predictability at one week and one month as well as the strong shifts in community composition during the first year of life (as described previously²²).

Urbanized microbial profiles, risk of disease and concentrations of immune mediators

A bacterial score was extracted for each sample (how much did the composition of the sample resemble the sPLS trained bacterial signature), with higher values indicating more urbanized bacterial profiles in infants.

Having an urbanized bacterial profile in the samples was associated with higher risk of asthma for all but one week gut and three months airway bacterial scores (*Airway 1 week*: OR 1.25, 95% CI 1.01-1.55, $P=0.0473$; *Airway 1 month*: OR 1.22, 95% CI 1.00-1.48, $P=0.0498$; *Gut 1 month*: OR 1.29, 95% CI 1.05-1.59, $P=0.0175$; *Gut 1 year*: OR 1.24, 95% CI 1.02-1.53, $P=0.0368$; **Fig 4**). The risk of eczema increased only in response to an urbanized gut bacterial profile at one week of age (OR 1.24, 95% CI 1.04-1.48, $P=0.0161$). Similarly, the risk of sensitization was restricted to one time point and compartment. An urbanized bacterial profile in gut at one year of age was associated with the risk of any sensitization (OR 1.28, 95% CI 1.03-1.59, $P=0.0281$) and aeroallergen sensitization (OR 1.24, 95% CI 0.98-1.57, $P=0.0812$) though borderline significant (**Fig 4**).

The airway bacterial score at one month of age was correlated with immune mediator concentrations at the same time point (**Fig 5; Table E6**) and the direction of these associations

were similar to our comparison between urban and rural groups (**Fig 2**), but with important distinctions. The systemic CXCL8 concentration was higher with an urbanized bacterial profile (**Fig 5**) corresponding to results for living environment (**Fig 2**), but these associations were borderline after correction for multiple testing. In contrast to the living environment associations, airway IL-2 and IL-13 showed lower concentrations in infants with urbanized bacterial profile. Bacterial scores for the gut microbiota did not significantly associate with immune mediator profiles (**Fig E8; Table E6**).

DISCUSSION

Primary Findings

The higher risk of asthma and atopic traits in urban than in rural children is a well-established phenomenon¹³. Our prospective, observational cohort study of 700 children supports the hypothesis that the early development of the human microbiota, and its influential crosstalk with the immune system, shapes the predisposition to these diseases in childhood. We found that an urban living environment in infancy was associated with a higher risk of asthma and aeroallergen sensitization in childhood. Urbanized microbiota both in the airways and the gut in infancy was positively associated with the risk of having asthma. The urbanized gut microbiota profile at one week of age also related with the risk of eczema and at one year of age with allergic sensitization. Furthermore, several immune markers showed significant associations with living environment related airway bacteria suggesting an influence of these bacteria in immune development.

Strengths and Limitations

A major strength of the study was the extensive and prospective clinical assessment of outcomes and environmental exposures. All children were uniformly diagnosed and repeatedly monitored for development of symptoms of asthma and atopic traits at the COPSAC clinic, in

which also microbial and immunological samples utilized in the study were collected. Our definition of the living environment was based on land cover data providing unbiased characterization of rural and urban individuals. However, our observational study is unable to confirm causal relationships and depends on future studies with interventional or experimental approaches to validate the mechanisms behind our findings. Further, methodology we used for microbiota analysis can hide some details such as importance of bacterial strains.

Even though our findings support the role of urbanized microbiota in disease development, other explanatory variables can shape the risk. The examined diseases are believed to originate from multiple triggers acting via a myriad of gene-environment interactions⁴⁷. These can include exposures other than bacteria such as indoor fungi, which has been associated with childhood asthma⁴⁸. Additionally, exposure to other molecules e.g. allergens may be crucial as both allergens and bacterial exposure can be needed for normal immune development⁴⁹. Further, our analysis lacks information on (air) pollution exposure that has previously been associated with the development of asthma⁵⁰ and is known to differ between rural and urban Denmark^{51,52}. Therefore, potential differences in the exposure to non-bacterial items could partly explain the identified differences between living in urban or rural environment. Finally, we did not have information about mothers' behavior, environment and health during pregnancy, which can be important. Moreover, our cohort has limited ethnic diversity, which reduces the generality of our findings.

Interpretation

Exposure to farming-associated indoor microbes, i.e. the "external" microbiota early in life is associated with asthma protection^{13-16,53}. Here, we focused on the "internal" microbiota, i.e. the infant's own microbiota, in rural and urban children and showed corresponding findings, especially for airway microbiota. Recently, living in farm has been shown to support the

development of gut microbiota in infants, which can decrease risk of asthma²⁵. These associations are also suggested by *Biodiversity* and *Old Friends* hypotheses^{54,55}. External and internal microbiotas are likely to have intimate relations. The airway microbiota is more open for colonization by air-borne microbes than the gut (because inhaled microbes continuously travel to the lungs) and thus reflects the external microbial exposures. Active mucosal layers in the airways and lungs can respond to microbial exposures and shape the function of immune system. However, as we did not sample from the gut mucosa, we cannot exclude its potential importance.

Bacteria associated with predisposing and protective effects vary between studies⁵⁶. Members of urbanized bacterial communities probably act together, but *Veillonella* was an interesting genus associated with urbanization both in the airways and the gut. *Veillonella* in the gut at one year of age was previously associated with an increased risk of asthma in this cohort, whereas another study found a protective association^{22,23}. In contrast to our findings, airway colonization with *Moraxella*, an opportunistic pathogen⁵⁷, was previously associated with increased risk of asthma⁵⁷⁻⁵⁹, also in the previous COPSAC₂₀₀₀ cohort, in which culture-based methodology was applied⁵⁹. We suspect that the opportunistic (pathogenic) nature of some urban and rural indicator bacteria, such as rural-associated *Moraxella*, *Acinetobacter*^{60,61} and *Mycobacterium*⁶², may explain their potential importance in atopic diseases as well as their cohort specific roles in diseases.

The hypothesized window of susceptibility, i.e. the period when extrinsic microbial stimuli can permanently influence immune responsiveness, is suggested to be during the first 100 days after birth²³ or during the entire infancy including the prenatal period¹⁸. In this study, the window associated with asthma development was the first year of life while much narrower for eczema and allergic sensitization. Our study was not designed for defining such a window of susceptibility, however. Previously, the window associated with asthma development has

been restricted to shorter periods after birth (less than 100 days and less than 12 months)^{23,24} and longer period for eczema (until 12 month of age)²¹, leaving it unclear when the exposure is most important.

We found large disparities in the immune mediator concentrations between urban and rural children as well as between urbanized and rural bacterial profiles. Therefore, we conclude that the living environment as well as associated bacteria may influence immune function during infancy. Further, our findings showed interesting similarities to our previous study discovering that asthma-associated bacteria at one-month of age were positively associated with CCL2 and CCL17 while negatively associated to TNF- α and IL-1 β ³⁸. These same associations were found in response to urbanized airway bacteria at one-month of age. However, *in vivo* cyto- and chemokine concentrations do not reflect easily delimited individual immune pathways. For example, both Type-2 and Type-17 related cytokines differed between rural and urban infants in a non-orchestrated manner. Some individual mediators showed expected patterns such as CXCL8, a neutrophil chemotactic Type-17 mediator that is enriched in moderate to severe asthma⁶³, which was increased in urban children as well as in response to urbanized airway microbiota. Deficiency in regulatory responses is suspected to be important for the trajectory towards hyperreactivity against allergens and development of manifest clinical allergy⁶⁴.

Accordingly, urban infants had lower concentrations of the anti-inflammatory, regulatory type mediator IL-10. Having an urbanized airway microbiota associated with decreased IL-2 in the airways, which is interesting as suppressed IL-2 expression in infants has been associated with food allergy⁶⁵.

There are several potential explanations for some immune mediator concentrations differing from the expected. First, the influence of an individual mediator depends on other mediators. For example, a higher concentration of IL-13 (Type-2) in response to rural airway bacteria can indicate that when combined with IL-12p70, TNF- α , IL-1 β , IL-10 and IL-2, it does not provide

increased susceptibility to Type-2-based symptoms. Second, pro-inflammatory immune mediators can be beneficial during certain developmental stages such as higher IL-4, IL-5 and IL-13 in one-month old rural infants. Our results underscore that the current understanding about an adequately stimulated immune system during different developmental stages is not complete.

Conclusions

We found that an urbanized living environment, an urbanized microbiota in infants, *in vivo* immune mediators and the risk of childhood asthma and atopic traits were associated, in a geographically concise cohort of 700 unselected children. Our findings support an important role of the infant microbiota in the association between urbanization and disease, probably via crosstalk with the developing immune system.

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447 **Governance:** We are aware of and comply with recognized codes of good research practice,
448 including the Danish Code of Conduct for Research Integrity. We comply with national and
449 international rules on the safety and rights of patients and healthy subjects, including Good
450 Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the
451 International Conference on Harmonisation's (ICH) good clinical practice guidelines and the
452 Helsinki Declaration. Privacy is important to us which is why we follow national and
453 international legislation on General Data Protection Regulation (GDPR), the Danish Act on
454 Processing of Personal Data and the practice of the Danish Data Inspectorate.

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Figure legends

Figure 1. Prevalence of asthma and atopic diseases in rural and urban infants. The proportions of main land cover types in a radius of 3km around the home at birth are condensed to a gradient from mostly rural to highly urbanized living environments by Principal Components Analysis (PCA). Figure **a** shows geographical distribution of children, colored by the urbanization gradient. Urban children are mainly clustered around the capital, Copenhagen, as expected. Coordinate information is slightly jittered on the map to preserve anonymity. Figures **b** and **d** show the crude prevalence of outcomes at six years of age in children living in urban and rural environment during infancy. Differences are tested with Fisher's exact tests. Figures **c** and **e** show the adjusted odds ratios from logistic regression models for the outcomes in urban vs. rural (baseline) living environment. Urban infants have increased risk for asthma and aeroallergen sensitization. Analyses are adjusted for socio-economics, pet ownership, older siblings, passive smoking exposure and total length of breastfeeding.

Figure 2. Relative concentration of airway and systemic immune mediators differ between urban and rural infants. The airway immune profile at one month of age ($n = 586$, **a**) and systemic immune profile at six months of age ($n = 665$, **b**) in response to urban and rural classes defined by linear models adjusted for living environment associated lifestyle features. Positive estimates indicate higher relative concentration in urban infants while negative estimates indicate higher relative concentration in rural infants. Error bars represent the 95% CI. False positive rate corrected p-values are presented in Table E6.

Figure 3. The composition of living environment-related microbial profiles for each time point. sparse Partial Least Squares (sPLS) models were run for (sub-)genera present in at least 5% of samples. Selection of the best model for each time point was done by comparing Root Mean Square Error (RMSE) values between models with differing numbers of taxa and components. Each selected model for airway (**a**) and gut (**b**) microbiota had one component. Included taxa were ordered in the heatmap by their summed loadings across all time points. Taxa with positive loadings are more common in urban (gold) living environments, while taxa with negative loadings are more common in rural (green) living environments. Taxa colored white are not included in the present model.

Figure 4. Airway and gut bacterial scores indicative of living environment and the risk of asthma and atopic traits at 6 years of age. Bacterial scores were scaled to a standard deviation of one in order to ease interpretation. For airways (**a-c**), the risk of developing asthma increases with higher values of bacterial score, i.e. urban-like bacterial profiles at one week and one month of age. For gut (**d-f**), eczema associates with the bacterial score from one week (**d**), while the one-month and one-year time points are important for asthma development (**e**). The risk of sensitization increases with higher values of the gut bacterial score at one year of age (**f**). Error bars represent the 95% CI.

Figure 5. Airway bacterial score versus the relative concentration of airway and systemic immune mediators. The figure shows normalized and scaled relative concentrations of immune mediators in relation to airway bacterial score per standard deviation. Higher values indicate association with an urban-like bacterial community composition. Panel A shows the topical immune mediators at one month of age, and panel B the systemic immune mediators at six months of age in response to bacterial scores. Linear models were adjusted for living environment associated lifestyle features. Error bars represent 95% CI. False positive rate corrected p-values are presented in Table E6.

<i>Buffer</i>	<i>Rural</i>		<i>Urban</i>		Silhouette width
	n	%	n	%	
3 km	314	45.8	372	54.2	0.61
1 km	204	29.7	482	70.3	0.70
500 m	134	19.5	552	80.5	0.78

		Rural (n = 238)					Urban (n = 289)					p-value (>0.35)
specific IgE		% >0.35	n >0.35	median	mean	range	% >0.35	n >0.35	median	mean	range	
Food	Egg	6.7	16	0	0.06	0;2.01	5.9	17	0	0.11	0;16.96	0.72
	Milk	9.7	23	0	0.09	0;2.56	7.6	22	0	0.09	0;3.46	0.53
	Wheat	5.0	12	0	0.05	0;1.70	5.5	16	0	0.12	0;4.85	1
	Peanut	0.8	2	0	0.01	0;0.54	3.8	11	0	0.13	0;15.54	0.045
	Seafood	0.8	2	0	0.04	0;7.49	2.4	7	0	0.19	0;37.45	0.19
Aeroallergen	House dust mite	6.7	16	0	3.07	0;100	12.1	35	0	2.50	0;100	0.039
	Cat	2.9	7	0	0.51	0;100	3.5	10	0	1.47	0;100	0.81
	Dog	2.1	5	0	0.17	0;36.08	3.5	10	0	0.28	0;49.91	0.44
	Horse	0.4	1	0	0.08	0;18.10	0.7	2	0	0.02	0;2.58	1
	Timothy	6.7	16	0	0.12	0;6.08	14.2	41	0	3.2	0;100	0.007
	Birch	3.8	9	0	0.9	0;100	9.7	28	0	2.29	0;100	0.006
	Mugworth	1.7	4	0	0.02	0;0.81	6.6	19	0	0.19	0;18.94	0.005
Molds	C. Herbarum	0.4	1	0	0.02	0;5.28	1.0	3	0	0.02	0;1.57	0.63
	A. Fumigatus	0.4	1	0	0	0;0.98	0	0	0	0	0;0.2	0.45
	A. Tenuis	0.4	1	0	0.14	0;33.96	1.4	4	0	0.03	0;4.65	0.38

Body site	Age	n	Distance Metric					
			Bray-Curtis		Weighted UniFrac		Unweighted Unifrac	
			R2	p	R2	p	R2	p
Gut	1 week	547	0.002	0.3	0.003	0.2	0.001	0.8
	1 month	600	0.001	0.9	0.001	0.5	0.002	0.2
	1 year	612	0.003	0.005	0.004	0.02	0.003	0.005
Airways	1 week	538	0.006	0.001	0.008	0.001	0.003	0.038
	1 month	629	0.006	0.001	0.007	0.001	0.004	0.002
	3 months	633	0.006	0.001	0.007	0.001	0.004	0.002

Airway microbiota

<i>Living environment gradient +</i>	1 week			1 month			3 months		
	<i>n</i>	<i>R2</i>	<i>p</i>	<i>n</i>	<i>R2</i>	<i>p</i>	<i>n</i>	<i>R2</i>	<i>p</i>
<i>Unadjusted model</i>	538	0.005	0.001	629	0.006	0.001	633	0.006	0.001
<i>Furry pet at home</i>	530	0.004	0.001	622	0.003	0.002	624	0.005	0.001
<i>Daycare attendance before age 1</i>	538	0.006	0.001	629	0.006	0.001	633	0.006	0.001
<i>Total breastfeeding duration</i>	533	0.005	0.001	626	0.007	0.001	628	0.006	0.001
<i>Passive smoking exposure</i>	530	0.005	0.001	622	0.007	0.001	624	0.006	0.001
<i>Socio-economics</i>	516	0.005	0.001	604	0.007	0.001	609	0.006	0.001
<i>Older siblings</i>	531	0.005	0.001	622	0.005	0.001	626	0.004	0.001
<i>Adjusted for all</i>	514	0.004	0.002	602	0.005	0.001	605	0.004	0.002

Gut microbiota

<i>Living environment gradient +</i>	1 week			1 month			1year		
	<i>n</i>	<i>R2</i>	<i>p</i>	<i>n</i>	<i>R2</i>	<i>p</i>	<i>n</i>	<i>R2</i>	<i>p</i>
<i>Unadjusted model</i>	547	0.002	0.3	600	0.002	0.6	612	0.004	0.002
<i>Furry pet at home</i>	540	0.002	0.2	591	0.002	0.6	609	0.003	0.003
<i>Daycare attendance before age 1</i>	547	0.002	0.4	600	0.001	0.7	612	0.004	0.002
<i>Total breastfeeding duration</i>	543	0.002	0.4	595	0.002	0.5	610	0.004	0.002
<i>Passive smoking exposure</i>	540	0.002	0.3	591	0.002	0.7	609	0.004	0.001
<i>Socio-economics</i>	527	0.002	0.3	574	0.002	0.5	592	0.004	0.001
<i>Older siblings</i>	541	0.002	0.3	592	0.002	0.6	611	0.002	0.04
<i>Adjusted for all</i>	525	0.002	0.2	572	0.001	0.8	589	0.002	0.2

Explanatory variable	Time	Immune marker	Immune response type	Estimate	Std.error	Conf.low	Conf.high	p-value	q-value
Living environment (Rural/urban)	One month	CCL2	Type 1	1.346	0.091	1.125	1.610	0.001	0.006
		CCL4	Type 1	1.176	0.09	0.985	1.404	0.074	0.134
		CXCL10	Type 1	1.116	0.093	0.931	1.339	0.236	0.363
		IFNg	Type 1	1.027	0.095	0.854	1.236	0.776	0.786
		IL12p70	Type 1	0.778	0.092	0.649	0.931	0.006	0.018
		TNFa	Type 1	1.104	0.093	0.92	1.325	0.287	0.383
		CCL11	Type 2	1.344	0.091	1.125	1.607	0.001	0.006
		CCL13	Type 2	1.360	0.09	1.139	1.624	0.001	0.006
		CCL17	Type 2	1.301	0.087	1.097	1.544	0.003	0.009
		CCL22	Type 2	0.724	0.09	0.607	0.863	0	0.006
		CCL26	Type 2	0.792	0.092	0.662	0.949	0.012	0.026
		IL13	Type 2	0.842	0.094	0.701	1.011	0.066	0.132
		IL4	Type 2	0.903	0.093	0.753	1.084	0.275	0.383
		IL5	Type 2	0.875	0.094	0.728	1.051	0.154	0.257
		CXCL8	Type 17	1.267	0.091	1.061	1.513	0.009	0.023
		IL17	Type 17	1.097	0.093	0.915	1.317	0.318	0.398
		IL1b	Type 17	0.942	0.092	0.786	1.128	0.514	0.604
		IL10	Regulatory	0.749	0.092	0.625	0.898	0.002	0.008
		IL2	Regulatory	0.975	0.093	0.812	1.171	0.786	0.786
		TGFb1	Regulatory	1.035	0.092	0.864	1.241	0.708	0.786
	Six months	TNFa	Type 1	0.867	0.09	0.727	1.034	0.113	0.188
		CXCL8	Type 17	1.450	0.087	1.223	1.720	0	0
		IL1b	Type 17	0.704	0.089	0.591	0.837	0	0
		IL6	Type 17	0.918	0.092	0.766	1.101	0.357	0.357
		CRP	Generic	1.129	0.09	0.947	1.345	0.177	0.221
Bacterial score (airways 1 month)	One month	CCL2	Type 1	1.107	0.044	1.015	1.207	0.022	0.063
		CCL4	Type 1	0.96	0.043	0.882	1.044	0.34	0.433
		CXCL10	Type 1	1.043	0.045	0.956	1.138	0.346	0.433
		IFNg	Type 1	1.043	0.044	0.958	1.136	0.334	0.433
		IL12p70	Type 1	0.909	0.044	0.833	0.992	0.033	0.077
		TNFa	Type 1	0.9	0.043	0.826	0.98	0.015	0.056
		CCL11	Type 2	1.048	0.044	0.962	1.142	0.28	0.431
		CCL13	Type 2	1.086	0.043	0.998	1.182	0.056	0.101
		CCL17	Type 2	1.125	0.041	1.038	1.219	0.004	0.021
		CCL22	Type 2	1.031	0.043	0.948	1.121	0.479	0.563
		CCL26	Type 2	0.977	0.044	0.896	1.065	0.599	0.666
		IL13	Type 2	0.877	0.045	0.804	0.958	0.004	0.021
		IL4	Type 2	0.981	0.045	0.899	1.070	0.661	0.696
		IL5	Type 2	1.066	0.043	0.979	1.159	0.141	0.235
		CXCL8	Type 17	0.919	0.04	0.849	0.995	0.037	0.077
		IL17	Type 17	0.913	0.044	0.837	0.995	0.039	0.077
		IL1b	Type 17	0.863	0.043	0.793	0.94	0.001	0.011
		IL10	Regulatory	0.902	0.043	0.83	0.981	0.017	0.056
		IL2	Regulatory	0.867	0.044	0.796	0.944	0.001	0.011
		TGFb1	Regulatory	1.007	0.044	0.924	1.098	0.866	0.866
Bacterial score (airways 3 months)	Six months	TNFa	Type 1	0.946	0.042	0.871	1.027	0.183	0.458
		CXCL8	Type 17	1.102	0.041	1.017	1.193	0.017	0.087
		IL1b	Type 17	1.009	0.041	0.931	1.094	0.823	0.956

Bacterial score (gut 1 month)	Time	Cytokine	Type	Bacterial score (gut 1 month)							
				IL6	Type 17	1.002	0.043	0.922	1.089	0.956	0.956
Bacterial score (gut 1 month)	Time	Cytokine	Type	CRP	Generic	0.977	0.042	0.9	1.060	0.573	0.956
				CCL2	Type 1	1.038	0.043	0.954	1.129	0.391	0.784
Bacterial score (gut 1 month)	Time	Cytokine	Type	CCL4	Type 1	1.001	0.042	0.922	1.087	0.982	0.982
				CXCL10	Type 1	1.025	0.043	0.942	1.115	0.57	0.784
Bacterial score (gut 1 month)	Time	Cytokine	Type	IFNg	Type 1	0.987	0.045	0.904	1.078	0.772	0.908
				IL12p70	Type 1	0.978	0.044	0.897	1.066	0.617	0.784
Bacterial score (gut 1 month)	Time	Cytokine	Type	TNFa	Type 1	0.968	0.043	0.888	1.054	0.448	0.784
				CCL11	Type 2	1.043	0.043	0.959	1.134	0.328	0.784
Bacterial score (gut 1 month)	Time	Cytokine	Type	CCL13	Type 2	1.054	0.041	0.972	1.142	0.204	0.784
				CCL17	Type 2	1.034	0.041	0.953	1.121	0.419	0.784
Bacterial score (gut 1 month)	Time	Cytokine	Type	CCL22	Type 2	0.975	0.041	0.899	1.057	0.539	0.784
				CCL26	Type 2	1.001	0.043	0.92	1.089	0.979	0.982
Bacterial score (gut 1 month)	Time	Cytokine	Type	IL13	Type 2	0.913	0.044	0.837	0.995	0.04	0.784
				IL4	Type 2	0.963	0.044	0.884	1.049	0.386	0.784
Bacterial score (gut 1 month)	Time	Cytokine	Type	IL5	Type 2	1.024	0.043	0.942	1.114	0.579	0.784
				CXCL8	Type 17	1.023	0.043	0.941	1.112	0.589	0.784
Bacterial score (gut 1 month)	Time	Cytokine	Type	IL17	Type 17	1.067	0.043	0.981	1.162	0.132	0.784
				IL1b	Type 17	0.992	0.043	0.912	1.079	0.85	0.944
Bacterial score (gut 1 month)	Time	Cytokine	Type	IL10	Regulatory	1.036	0.044	0.951	1.128	0.417	0.784
				IL2	Regulatory	0.974	0.044	0.894	1.062	0.553	0.784
Bacterial score (gut 1 month)	Time	Cytokine	Type	TGFb1	Regulatory	1.021	0.043	0.938	1.112	0.627	0.784
				TNFa	Type 1	1.002	0.043	0.922	1.089	0.969	0.969
Bacterial score (gut 1 month)	Time	Cytokine	Type	CXCL8	Type 17	1.046	0.041	0.966	1.132	0.272	0.676
				IL1b	Type 17	0.966	0.041	0.892	1.047	0.405	0.676
Bacterial score (gut 1 month)	Time	Cytokine	Type	IL6	Type 17	1.010	0.043	0.928	1.100	0.812	0.969
				CRP	Generic	1.060	0.042	0.976	1.152	0.165	0.676

a**Airway. 1 month**

Journal Pre-proof

b**Systemic. 6 months**

Airway bacterial scores

Gut bacterial scores

a**Airway immune profile (1 month) +
airway bacterial score (1 month)** **b****Systemic immune profile (6 months) +
Airway bacterial score (3 months)**