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Abstract

Background: Farm exposures in early life reduce the risks for childhood allergic diseases and asthma. There is less information about how farm exposures relate to respiratory illnesses and mucosal immune development.

Objective: We hypothesized that children raised in farm environments have a lower incidence of respiratory illnesses over the first two years of life than non-farm children. We also analyzed whether farm exposures or respiratory illnesses were related to patterns of nasal cell gene expression.

Methods: The Wisconsin Infant Study Cohort included farm (n=156) and non-farm (n=155) families with children followed to age 2 years. Parents reported prenatal farm and other environmental exposures. Illness frequency and severity were assessed using illness diaries and periodic surveys. Nasopharyngeal cell gene expression in a subset of 64 children at age two years was compared to farm exposure and respiratory illness history.

Results: Farm vs. non-farm children had nominally lower rates of respiratory illnesses (rate ratio 0.82 [0.69,0.97]) with a stepwise reduction in illness rates in children exposed to 0, 1, or ≥2 animal species, but these trends were non-significant in a multivariable model. Farm exposures and preceding respiratory illnesses were positively related to nasal cell gene signatures for mononuclear cells and innate and antimicrobial responses.

Conclusions: Maternal and infant exposure to farms and farm animals was associated with nonsignificant trends for reduced respiratory illnesses. Nasal cell gene expression in a subset of children suggests that farm exposures and respiratory illnesses in early life are associated with distinct patterns of mucosal immune expression.

Key Messages

- Prenatal and early life farm animal exposures were related to trends for a reduced incidence of respiratory illnesses.
- Both farm exposures and respiratory illnesses were related to increased innate and antiviral gene expression in the nasal mucosa.

Capsule summary

Prenatal and early postnatal exposure to farm animals was associated with trends for reduced respiratory illnesses and enhanced nasal cell expression of gene networks related to innate responses.

Key words

Farm, respiratory illness, virus, gene expression, nasal epithelial cells, children

Abbreviations

WISC, Wisconsin Infant Study Cohort

Introduction

Environmental exposures in early life greatly influence the risk of developing allergic diseases and asthma. For example, children growing up on dairy farms have a reduced risk of developing atopic dermatitis, allergic sensitization, and childhood asthma.[1-5] Further, allergic diseases are uncommon among children in families with traditional agrarian cultures such as the Amish or Old Order Mennonites.[6, 7] There is some evidence that farm exposures are associated with reduced respiratory illness frequency and severity.[8] In Central European traditional farms, consumption of raw farm milk has been associated with reduced respiratory illnesses during infancy.[9] A cross-sectional survey in Wisconsin also linked early-life farm exposures to reduced rates of severe respiratory illnesses in early childhood.[10]

The protective effects of farming are related to specific exposures, such as diverse environmental microbes, the interaction of mothers or children with multiple animal species, time spent in animal sheds or barns, and consumption of unprocessed cow milk.[8, 11, 12] These exposures are associated with changes in immune development measured in peripheral blood cells, including enhanced responses to LPS and maturation of tolerance mechanisms, which may protect against allergic diseases.[13, 14] The development of RNA sequencing technologies has enabled analysis of upper airway cellular responses to identify patterns of gene expression that relate to respiratory outcomes, including allergy, asthma, and respiratory illnesses.[15, 16]

The Wisconsin Infant Study Cohort (WISC) is a case-control birth cohort study comparing children with dairy farm exposure to children from small towns or rural areas in the same region of Central Wisconsin.[17] We prospectively monitored respiratory illnesses through age 2 years and obtained nasal epithelial samples in a subset of children to evaluate relationships between farm exposure, respiratory illnesses, and patterns of nasal cell gene expression. We hypothesized that young children with farm exposures in early life have lower rates of respiratory illnesses and wheezing. In addition, we tested whether farm exposures are

associated with increased expression of nasal epithelial cell genes that prime antipathogen responses and whether gene expression patterns relate to respiratory illness frequency and severity.

Methods

Population and cohort design

All families provided written informed consent before study enrollment, and the Marshfield Clinic Health System Human Subjects Institutional Review Board approved the study protocol. WISC recruited two groups of children in rural Wisconsin. A farm family was defined as those who either reside within 1/8th mile of a farm or work on a farm or have a household member who works on a farm. On the other hand, non-farm mothers are those who do not live within 1/8th mile of a farm or have any personal or household connection to farm work.[17] Pregnant mothers were recruited during their prenatal care within the Marshfield Clinic Health System, which serves a population in central Wisconsin living in rural areas or small towns. Study visits were conducted prenatally and every three months following birth with periodic questionnaire administration to assess farming demographics and medical history (Supplemental Table 1). Biospecimen collection included nasal mucus swabs at surveillance visits conducted every three months and during family-reported illnesses and nasopharyngeal swabs at two years of age for transcriptomics.

Farm Exposures

At the prenatal, 2-month, and 9-month visits, families completed questionnaires to assess the mother's and child's exposure to farm animals and forage. The amounts of time spent in various activities such as milking and cleaning were asked for specific animals, with separate questions for child and maternal exposures. In a previous study of farm exposures and atopic dermatitis

(AD), we performed a cluster analysis and found that AD incidence was inversely related to exposure to more animals.[5] The current analysis used that idea to model the exposure classification. For this analysis, we calculated a farm exposure score representing the number of animal species the family (either maternal or child) were exposed to "regularly" (at least weekly). Some farm families had minimal farm animal exposure for mother and child and thus had an exposure score of 0, as did nearly all non-farm children. Farm families in our study with a score of 1 were exposed to cows only due to the prevalence of dairy farms in central Wisconsin. The families with a score of 2+ also have frequent exposure to at least one other farm animal (goats, pigs, chickens, horses, sheep).

Illness assessment

At each quarterly visit, parents were asked to report the number of colds, illnesses with a cough, and illnesses with wheezing since their last visit. We used the maximum report of the number of colds or the number of illnesses with a cough to represent the number of respiratory illnesses their child experienced since the last visit. Nasal swabs were collected at each quarterly visit.

We asked parents to keep illness diaries with each respiratory illness and record the severity of individual symptoms ("cold," "cough," and "wheeze") on a scale from 0-3 for each day the child was ill.[18, 19] We summed the scores for each symptom for the day, adding an additional point for fever. Illness burden was defined as the sum of daily scores over the entire course of the illness (area under the curve). Families were asked to provide a nasal swab for viral PCR on day 2 of each acute illness, with swabs repeated every two weeks for the duration of the illness. In addition, coordinators collected nasal swabs during scheduled visits (ages 2, 6, 9, 12, 18, and 24 mo) to estimate the frequency of viral infections

Viral diagnostics. Nasal mucus samples were tested for all common respiratory viruses using multiplex PCR (NxTAG® Respiratory Pathogen Panel [Luminex, Austin TX], SARS-CoV-2

added after the pandemic). Pan-rhinovirus RT-PCR and partial sequencing identified rhinovirus species and types.[20]

Nasal transcriptomics. Coordinators obtained nasal cells and secretions by inserting a swab (#516CS01, Copan Inc., Murrieta, CA) into the nasopharynx, rotating it three times, and then immediately immersing it in a cell lysis buffer (RLT buffer, Qiagen, Ann Arbor MI) to preserve the RNA. RNA processing and sequencing is described in the online supplement.

Of the 100 samples submitted for sequencing, we removed samples that failed QC checks or did not have corresponding respiratory illness data (see Online Data Supplement). Sixty-four samples (22 farm, 42 non-farm) were included in the analyses of differential gene expression.

Statistical analysis (see online supplement for additional details)

The main outcome for illness analyses was the self-reported number of illnesses reported at each study visit. Secondary outcomes included illness burden from illness diaries measured over the first two years of age and virus detection. SAS (SAS Institute Inc., Cary, NC), version 9.4, was used for all illness analyses.

The number of illnesses reported from the questionnaires (roughly every 3 months) was analyzed as longitudinal data (multiple responses from each child) using mixed effects models, and subject was included as a random effect. The mixed model appropriately accounts for missing data, allowing us to include children that had yet to reach the age of a two-year visit. The main predictor(s) were farm status and the number of animal exposures, with adjustment for age. The potential interaction between farm status (or farm exposure) and time was evaluated and not significant, so a common effects model was applied. In additional models, we evaluated the impact of other covariates including sex, day care, older sibs, smoke exposure, breastfeeding, delivery mode, season of report, and timing relative to the pandemic (defined as

information collected beginning April 2020). In models examining the impact of other covariates, each covariate was considered alone as added to the baseline model.

Illness burden (secondary outcome) was obtained by summing daily diary scores over all illnesses within a period of time of interest to have a single data point for each child and analyzed using standard regression/ANOVA approaches. We looked at birth through age 1 as well as birth through age 2. Since not all children have information through the first two years, we only included diary data for children who have completed their the age 1- or 2-year visit.

Virus detection (secondary outcome) was analyzed for all samples obtained for individuals up through their age 2 visit. Viral detection frequency rates was analyzed by generalized mixed models with a logit link and subject as the random effect.

To identify genes that varied with farm status or the number of respiratory illnesses in the first two years of life, we performed differential expression analysis using the R package DESeq2.[21] Next, we applied Gene Set Enrichment Analysis[22] to compare the gene expression trends for farm status and high respiratory illness count. The number of respiratory illnesses was totaled over each visit within a specific age period (through age 1, through age 2) in order to have a single summary measure for each child. Children with incomplete visits (typically because have not yet reached that age) within these time frames were dropped from the respective analyses. We obtained two signed, ranked lists of genes from the DESeq2 results: one list ranking genes for farm status (farm vs. non-farm) and one for high respiratory illness count (highest tertile vs. all others).

We employed WGCNA (Weighted Gene Correlation Network Analysis)[23] to identify co-expressed gene modules in the transcriptomics data. To determine whether any modules were associated with farm status or respiratory illness frequency, we quantified the eigengenes for each module and then analyzed for differential expression. To interpret the gene modules, we

used tested for significant overlap with gene sets from three databases: nasal cell type signatures,[24] Hallmark gene sets,[25] and gene networks from nasal cells of children with and without asthma.[26]

Results

Study population. The WISC study enrolled a total of 311 patients from farm (n=156) and non-farm (n=155) families (Table 1). There was a higher proportion of males in the farm group than in the non-farm group. Farm children had higher rates of prenatal exposure to raw milk. Non-farm children had higher daycare attendance in the first year of life and had higher rates of prenatal maternal smoking exposure.

Respiratory illnesses. The farm and non-farm children had similar age-related patterns of respiratory diseases, with lower rates in the first few months and a peak at around nine months of age. Through two years of age, the farm group participants reported lower respiratory illness rates than the non-farm group (rate ratio 0.82 [0.69,0.97], p=0.020, Fig 1A). There was no significant interaction with age. On average, the 3-month illness rate was 0.65 in farm children and 0.79 in non-farm children. The two groups had similar rates of reported wheezing illnesses through the first two years of life (rate ratio 0.84 [0.43,1.65], Figure 2).

While adjusting for individual covariates had relatively minor effects on the results, the illness rates were no longer significant after adjusting for daycare (RR 0.86 [0.73, 1.01], p=0.07, Fig 1B). Illness rates were significantly lower during the peak of the SARS-CoV-2 pandemic (April 2020 - April 2021, rate ratio 0.52 [0.45, 0.59], p<.001, Supplemental Figure 1). Most of the children had completed the age-two visit before the pandemic, so among the data used for these analyses, only 10% of the visits were during the pandemic period. Adjusting for illnesses reported during the pandemic also attenuated the association with any respiratory illness (RR 0.86 [0.73,1.01], p=0.07, Fig 1B).

We next analyzed relationships between farm status and respiratory illness rates in a multivariable model that included sex and variables that were associated with illness rates (Table 2). In model 1, the main effect persisted after adjusting for seasonal effects and the pandemic. In model 2, the relationship between farm status and respiratory illness rates was no longer significant (RR = 0.89, p = 0.157) after adjustment for these variables plus daycare, breastfeeding, and sex.

Farm exposures and respiratory illnesses. Based on a previous analysis of atopic dermatitis,[5] we hypothesized that regular farm animal exposure would be a marker for the degree of farm exposure in the first two years of life. We grouped all mothers and children into having 0, 1, or 2+ animal species exposures at least weekly. The number of unique animal exposures was inversely related to illness frequency in the first two years of life. Exposure to at least two unique species (cattle plus at least 1 other) was associated with a significantly reduced illness frequency compared to no farm animal exposure (Table 2). In the multivariate model, this association persisted after adjustment for season and pandemic (p = 0.027) but not in the fully adjusted model (p = 0.101).

Farm status and illness burden. We next tested whether the illnesses reported by the farm vs. non-farm children differed in severity, as assessed from symptom diaries taken by participants during acute illnesses. The families submitted diaries for a subset (33% of illnesses in each group) of the illnesses reported on the quarterly calls. The illness burdens across illnesses were similar for the first year (RR 0.81 [0.38, 1.73]) and the first two years of life (RR 0.97 [0.48, 1.95]) for the farm and non-farm groups (Table 3)

Viral detection. Virus detection was higher during symptomatic illnesses compared to the scheduled periods (90% vs. 38%). The farm and non-farm groups had a similar number of positive tests and distribution of respiratory virus types detected during routine study visits and also during symptomatic illnesses (Supplemental Figure 2).

Nasal epithelial gene expression. We conducted an exploratory analysis of differential gene expression in nasal epithelial cells from a subset of children in the farm and non-farm groups at the age two scheduled visits. Of the 100 samples that we collected, 64 (22 farm, 42 non-farm) that passed quality assessments (Supplemental Figures 3-5) and had associated respiratory illness data were selected for further analysis (Supplemental Figure 6). While no individual genes were differentially expressed in the farm vs. non-farm groups (adjusted $p<0.05$), several gene sets were (Figure 3). The farm group had increased gene signatures for monocytes and epithelial cell subsets including secretory and cycling basal cells. The farm group had increased expression of a module ("magenta") related to several immunoregulatory gene sets, including the p53 pathway, Th2/ILC2 cells, lysosomal proteins, and antiviral/innate responses. The farm group also had increased expression of a "purple" module associated with injury response, oxidative phosphorylation, DNA repair, and mitochondrial/ribosomal function. One module ("cyan") downregulated in the farm group was associated with mRNA processing, and intracellular vesicle and protein transport.

We next tested whether the number of respiratory illnesses in the first two years was associated with changes in nasal cell gene expression at age two. Frequent respiratory illnesses were related to the upregulation of 1100 genes and reduced expression of 193 genes (Supplemental Figure 7). Respiratory illnesses were associated with differential expression of cell-associated gene sets, including positive associations with secretory epithelial cell, monocyte, and T cell/NK cell-related genes and negative associations with cycling basal, deuterosomal, and multiciliated airway epithelial cell genes (Figure 4). Frequent illnesses were also associated with functional pathways, including those related to innate, antiviral responses, antimicrobial responses, and genes regulating T cell activation, Th2/ILC2, and epithelial integrity/leukocyte migration. There were negative relationships with gene sets associated with leukotriene and lipid metabolism and epithelial cell tight junctions and cilium.

Leading edge genes for each of the functional and cell-specific modules and their expression levels are listed in the Online Supplement (Supplemental Table 2).

Discussion

Studies worldwide have linked prenatal and early-life farming exposures to health benefits, including a lower risk of allergic diseases and asthma. Based on a prospective study in Central Europe[9] and a survey of children on Wisconsin dairy farms,[10] we hypothesized that children of dairy farm families would have lower rates of respiratory illnesses in the first two years of life. In the WISC birth cohort study comparing farming and nonfarming rural and small-town families from Central Wisconsin, we found that farm children had lower rates of respiratory illnesses over the first two years of life, although these relationships were not significant in a multivariable model after adjusting for covariates including daycare and the pandemic. When exposure to animals was considered, regular exposure of mother and children to farm animal species (cattle plus at least one other) was associated with a trend for lower rates of respiratory illnesses. This association suggests a dose-dependent relationship between the degree of farm exposure and the risk of early-life respiratory illnesses. Finally, we evaluated nasal cell gene expression in a subset of the farm and non-farm children at two years of age. Both farm status and the number of respiratory illnesses were positively associated with differential expression of genes related to cell composition and function. In general, the results of our gene expression studies suggest that both farm exposures and a history of previous infections have immunostimulatory effects on nasal mucosal immune responses.

Farm exposure was not significantly related to wheezing illnesses in early life. While the rate ratios for wheezing illnesses and total respiratory illnesses were similar, the number of reported wheezing illnesses was lower than expected. This study was underpowered to identify a significant relationship. Part of the study was conducted during the pandemic, which was associated with reduced respiratory and wheezing illnesses.[27, 28] Additionally, surveillance nasal

swabs were obtained at set intervals to identify whether nasal viral microbiome is associated with farm status and illness frequency. Viral populations were similar between the two groups, suggesting that farm status is related to rates of illnesses but not infections during infancy. The severity of illnesses was also similar between the two groups.

We found trends for lower rates of respiratory illnesses in farm children with greater exposure to animals and barns. We previously reported similar dose-related relationships between animal exposure and reduced atopic dermatitis in the WISC study.[5] Biodiverse animal and farm exposures are consistently associated with better allergic outcomes in European children.[29, 30] Additional supportive data from cross-sectional and longitudinal studies of in-home pets (especially dogs) exist.[31, 32] Furthermore, in disadvantaged urban settings in the USA, exposure to pets and even pests (e.g., mice and cockroaches) in early life are associated with reduced rates of wheezing and asthma.[33, 34] Part of the benefits of animal exposure could be due to animal-associated microbes. These microbes could stimulate innate immune sensors within the epithelium or mucosal surfaces, and some may colonize the children's skin, gut, and respiratory tract and secrete immunomodulating metabolites.

Notably, animal exposure could also have beneficial effects independent of the microbiome. In an urban birth cohort study, house dust levels of cockroach, mouse, and cat proteins were associated with reduced preschool wheeze, while exposure to a rich microbiome was associated with reduced allergic sensitization.[33] Other immunostimulatory substances of animal origin include carbohydrates and proteins. For example, N-glycolulneuraminic acid (Neu5AC) is a sialic acid derivative in animal but not human cells. Neu5AC exposure is increased in farm children and associated with reduced non-atopic wheeze.[35] Beta-lactoglobulin is a bovine protein in high concentrations in barn dust that can complex with zinc and other molecules and has immunomodulatory properties, including stimulation of IL-6 and IFN-γ responses.[36] Interestingly, the 17q12-21 gene locus, closely related to childhood asthma risk, interacts with

dog, cat, and farm exposures concerning wheezing or asthma.[13, 37, 38] Polymorphisms in 17q12-21 associated with asthma risk regulate expression of *GSDMB* on airway epithelial cells,[39] suggesting that these cells, which are exposed to both viruses and airborne farm exposures, might mediate farm effects on respiratory outcomes.

The nasal cell gene expression results provide new information linking farm exposure to differences in mucosal immunity and epithelial gene expression in toddlers. Samples from farm children had increased signals effectors of innate immune processes including monocytes and secretory epithelial cells, which are both active in antimicrobial responses.[40] Gene networks associated with innate responses such as apoptosis, TNF and NFkB signalling, and Th2/ILC2 responses also had increased expression. While isolated enhancement of T2 responses is associated with allergy and increased susceptibility to infection,[41, 42] the patterns of nasal gene expression suggest that farm exposures may generally promote immune responsiveness. Previous studies of blood cells have linked farm exposures to enhanced expression of innate sensors such as TLR and CD14 and increased LPS-induced cytokine responses.[13, 43-45] Similar positive relationships between animal exposure and blood cell immune responses have been noted in suburban children exposed to dogs[46] or urban children exposed to cockroaches, mice, and cats.[47] These findings suggest that biodiverse exposures could promote immune development in early childhood.

The frequency of respiratory illnesses in the first two years was also associated with gene expression patterns that overlapped with those associated with farm exposures. Respiratory illnesses were positively associated with the cellular signatures for monocytes and secretory cells. There were also positive associations with functional pathways, including innate immune responses, antibacterial responses, and Th2/ILC2 cells. Respiratory illnesses were inversely related to the expression of genes associated with leukotriene metabolism, which in other studies have been linked to allergic asthma and acute wheezing illnesses.[15, 26] Illness-

associated changes in epithelial cell gene expression could alter differentiation away from ciliated cells, which are the target of infection with many common viruses, and towards secretory cells, which are essential contributors to early innate response to viral infection.[40] These findings indicate that early viral infections could promote responsiveness to viral and bacterial pathogens, perhaps enhancing defenses against future infections.[40] The reduced barrier function and changes in lipid metabolism could also influence responses to subsequent infections, and additional studies are needed to identify specific consequences of these changes.

Our study provides new information about the relationships between farm exposures, respiratory illnesses, and mucosal immune responses. The major strength of this birth cohort is its prospective design and frequent study visits, allowing for accurate estimates of illness frequency. We also identified associations between farm status, respiratory illnesses, and patterns of nasal mucosal gene expression. While significant relationships were identified, the studies were conducted on a subset of 64 individuals, which limited the power to identify associations with individual genes. Our study also has limitations to consider. While adjusting for individual covariates had only small effects on the rate ratio for respiratory illnesses, differences in illness rates were no longer significant after adjusting for daycare and the pandemic. Our analysis was limited by a modest sample size, and replication in another population is needed to determine whether farm exposures influence illness rates during infancy. We assessed wheeze by parental report, which is more sensitive but less specific than wheezing diagnosed by a health care provider. Our study was underpowered to identify associations between farm status and wheezing outcomes and analyze how specific farm exposures influence respiratory illnesses. The WISC population is predominantly White and resides in rural areas or small towns, so findings may not be generalizable to other social and ethnic groups.

In conclusion, farm exposures were associated with a reduced rate of respiratory illnesses in preschool children. While these relationships were attenuated in a multivariable model, the association was strongest in children and mothers who were exposed to multiple animals. Both farm exposures and respiratory illnesses were related to increased expression of gene networks related to innate and antipathogen responses. These findings are consistent with a growing literature suggesting that broad exposures during the prenatal period and early life stimulate immune development to prime innate and adaptive responses to respond to subsequent pathogens. Respiratory illnesses are particularly problematic for young children and those with asthma. Identifying mechanisms linking animal exposures to reduce illnesses could lead to new therapeutic approaches for at-risk children.

References

1. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrlander C, et al. Exposure to environmental microorganisms and childhood asthma. N Engl J Med 2011; 364:701-9.
2. Deckers J, Marsland BJ, von Mutius E. Protection against allergies: Microbes, immunity, and the farming effect. Eur J Immunol 2021; 51:2387-98.
3. Kirjavainen PV, Karvonen AM, Adams RI, Taubel M, Roponen M, Tuoresmaki P, et al. Farm-like indoor microbiota in non-farm homes protects children from asthma development. Nat Med 2019; 25:1089-95.
4. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001; 358:1129-33.
5. Steiman CA, Evans MD, Lee KE, Lasarev MR, Gangnon RE, Olson BF, et al. Patterns of farm exposure are associated with reduced incidence of atopic dermatitis in early life. J Allergy Clin Immunol 2020; 146:1379-86 e6.
6. Stein MM, Hrusch CL, Gozdz J, Igartua C, Pivniouk V, Murray SE, et al. Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children. N Engl J Med 2016; 375:411-21.
7. Seppo AE, Bu K, Jumabaeva M, Thakar J, Choudhury RA, Yonemitsu C, et al. Infant gut microbiome is enriched with Bifidobacterium longum ssp. infantis in Old Order Mennonites with traditional farming lifestyle. Allergy 2021; 76:3489-503.
8. Loss GJ, Depner M, Hose AJ, Genuneit J, Karvonen AM, Hyvarinen A, et al. The Early Development of Wheeze. Environmental Determinants and Genetic Susceptibility at 17q21. Am J Respir Crit Care Med 2016; 193:889-97.
9. Loss G, Depner M, Ulfman LH, van Neerven RJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. J Allergy Clin Immunol 2015; 135:56-62.
10. Ludka-Gaulke T, Ghera P, Waring SC, Keifer M, Seroogy C, Gern JE, et al. Farm exposure in early childhood is associated with a lower risk of severe respiratory illnesses. J Allergy Clin Immunol 2018; 141:454-6 e4.
11. Loss GJ, Depner M, Hose AJ, Genuneit J, Karvonen AM, Hyvarinen A, et al. The Early Development of Wheeze: Environmental Determinants and Genetic Susceptibility at 17q21. Am J Respir Crit Care Med 2015.
12. Zanobetti A, Ryan PH, Coull B, Brokamp C, Datta S, Blossom J, et al. Childhood Asthma Incidence, Early and Persistent Wheeze, and Neighborhood Socioeconomic Factors in the ECHO/CREW Consortium. JAMA Pediatr 2022; 176:759-67.
13. Illi S, Depner M, Pfefferle PI, Renz H, Roduit C, Taft DH, et al. Immune Responsiveness to LPS Determines Risk of Childhood Wheeze and Asthma in 17q21 Risk Allele Carriers. Am J Respir Crit Care Med 2022; 205:641-50.
14. Lluis A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D, et al. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. J Allergy Clin Immunol 2014; 133:551-9.
15. Altman MC, Gill MA, Whalen E, Babineau DC, Shao B, Liu AH, et al. Transcriptome networks identify mechanisms of viral and nonviral asthma exacerbations in children. Nat Immunol 2019; 20:637-51.
16. Altman MC, Kattan M, O'Connor GT, Murphy RC, Whalen E, LeBeau P, et al. Associations between outdoor air pollutants and non-viral asthma exacerbations and airway inflammatory responses in children and adolescents living in urban areas in the USA: a retrospective secondary analysis. Lancet Planet Health 2023; 7:e33-e44.

17. Seroogy CM, VanWormer JJ, Olson BF, Evans MD, Johnson T, Cole D, et al. Respiratory health, allergies, and the farm environment: design, methods and enrollment in the observational Wisconsin Infant Study Cohort (WISC). BMC Research Notes 2019.
18. Kloepfer KM, Lee WM, Pappas TE, Kang TJ, Vrtis RF, Evans MD, et al. Detection of pathogenic bacteria during rhinovirus infection is associated with increased respiratory symptoms and asthma exacerbations. J Allergy Clin Immunol 2014; 133:1301-7 e3.
19. Kloepfer KM, Olenec JP, Lee WM, Liu G, Vrtis RF, Roberg KA, et al. Increased H1N1 infection rate in children with asthma. Am J Respir Crit Care Med 2012; 185:1275-9.
20. Bochkov YA, Grindle K, Vang F, Evans MD, Gern JE. Improved molecular typing assay for rhinovirus species A, B, and C. J Clin Microbiol 2014; 52:2461-71.
21. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014; 15:550.
22. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102:15545-50.
23. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 2008; 9:559.
24. Deprez M, Zaragosi LE, Truchi M, Becavin C, Ruiz Garcia S, Arguel MJ, et al. A Single-Cell Atlas of the Human Healthy Airways. Am J Respir Crit Care Med 2020; 202:1636-45.
25. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst 2015; 1:417-25.
26. Altman MC, Calatroni A, Ramratnam S, Jackson DJ, Presnell S, Rosasco MG, et al. Endotype of allergic asthma with airway obstruction in urban children. J Allergy Clin Immunol 2021; 148:1198-209.
27. Poole S, Brendish NJ, Tanner AR, Clark TW. Physical distancing in schools for SARS-CoV-2 and the resurgence of rhinovirus. Lancet Respir Med 2020; 8:e92-e3.
28. Perez A, Lively JY, Curns A, Weinberg GA, Halasa NB, Staat MA, et al. Respiratory Virus Surveillance Among Children with Acute Respiratory Illnesses - New Vaccine Surveillance Network, United States, 2016-2021. MMWR Morb Mortal Wkly Rep 2022; 71:1253-9.
29. Roduit C, Wohlgensinger J, Frei R, Bitter S, Bieli C, Loeliger S, et al. Prenatal animal contact and gene expression of innate immunity receptors at birth are associated with atopic dermatitis. J Allergy Clin Immunol 2011; 127:179-85, 85 e1.
30. Illi S, Depner M, Genuneit J, Horak E, Loss G, Strunz-Lehner C, et al. Protection from childhood asthma and allergy in Alpine farm environments-the GABRIEL Advanced Studies. J Allergy Clin Immunol 2012; 129:1470-7 e6.
31. Gern JE, Reardon CL, Hoffjan S, Nicolae D, Li Z, Roberg KA, et al. Effects of dog ownership and genotype on immune development and atopy in infancy. J Allergy Clin Immunol 2004; 113:307-14.
32. Ownby DR, Johnson CC, Peterson EL. Exposure to dogs and cats in the first year of life and risk of allergic sensitization at 6 to 7 years of age. JAMA 2002; 288:963-72.
33. Lynch SV, Wood RA, Boushey H, Bacharier LB, Bloomberg GR, Kattan M, et al. Effects of early-life exposure to allergens and bacteria on recurrent wheeze and atopy in urban children. J Allergy Clin Immunol 2014; 134:593-601 e12.
34. O'Connor GT, Lynch SV, Bloomberg GR, Kattan M, Wood RA, Gergen PJ, et al. Early-life home environment and risk of asthma among inner-city children. J Allergy Clin Immunol 2018; 141:1468-75.

35. Frei R, Ferstl R, Roduit C, Ziegler M, Schiavi E, Barcik W, et al. Exposure to nonmicrobial N-glycolylneuraminic acid protects farmers' children against airway inflammation and colitis. J Allergy Clin Immunol 2018; 141:382-90 e7.
36. Pali-Scholl I, Bianchini R, Afify SM, Hofstetter G, Winkler S, Ahlers S, et al. Secretory protein beta-lactoglobulin in cattle stable dust may contribute to the allergy-protective farm effect. Clin Transl Allergy 2022; 12:e12125.
37. Schoettler N, Dissanayake E, Craven MW, Yee JS, Eliason J, Schauberger EM, et al. New Insights Relating Gasdermin B to the Onset of Childhood Asthma. Am J Respir Cell Mol Biol 2022.
38. Tutino M, Granell R, Curtin JA, Haider S, Fontanella S, Murray CS, et al. Dog ownership in infancy is protective for persistent wheeze in 17q21 asthma-risk carriers. J Allergy Clin Immunol 2022.
39. Ober C, McKennan CG, Magnaye KM, Altman MC, Washington C, 3rd, Stanhope C, et al. Expression quantitative trait locus fine mapping of the 17q12-21 asthma locus in African American children: a genetic association and gene expression study. Lancet Respir Med 2020; 8:482-92.
40. Basnet S, Mohanty C, Bochkov YA, Brockman-Schneider RA, Kendziorski C, Gern JE. Rhinovirus C causes heterogeneous infection and gene expression in airway epithelial cell subsets. Mucosal Immunol 2023; 16:386-98.
41. Contoli M, Ito K, Padovani A, Poletti D, Marku B, Edwards MR, et al. Th2 cytokines impair innate immune responses to rhinovirus in respiratory epithelial cells. Allergy 2015; 70:910-20.
42. Esquivel A, Busse WW, Calatroni A, Togias AG, Grindle KG, Bochkov YA, et al. Effects of Omalizumab on Rhinovirus Infections, Illnesses, and Exacerbations of Asthma. Am J Respir Crit Care Med 2017; 196:985-92.
43. Lauener R, Birchler T, Adamski J, Braun-Fahrlander C, Bufe A, Herz U, et al. Expression of CD14 and Toll-like receptor 2 in farmers' and non- farmers' children. Lancet 2002; 360:465.
44. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002; 347:869-77.
45. Pfefferle PI, Buchele G, Blumer N, Roponen M, Ege MJ, Krauss-Etschmann S, et al. Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy: The PASTURE Study. J Allergy Clin Immunol 2009.
46. Bufford JD, Reardon CL, Li Z, Roberg KA, DaSilva D, Eggleston PA, et al. Effects of dog ownership in early childhood on immune development and atopic diseases. Clin.Exp.Allergy 2008; 38:1635-43.
47. Gern JE, Calatroni A, Jaffee KF, Lynn H, Dresen A, Cruikshank WW, et al. Patterns of immune development in urban preschoolers with recurrent wheeze and/or atopy. J Allergy Clin Immunol 2017; 140:836-44 e7.

507 **Table 1.** Characteristics of the Study Population

	Farm (N=156)	Non-farm (N=155)	P value
Maternal Characteristics			
Age (yrs)	30.5 (IQR 6.1)	30.5 (IQR 5.6)	0.78
Education			0.81
High school or less	6%	7%	
Associate degree or some college	31%	28%	
Bachelor's degree	46%	43%	
Graduate degree	14%	19%	
Unknown	3%	3%	
Employed outside of the home	67%	81%	**0.008**
Smoking			**0.003**
Never	79%	59%	
Past history	10%	23%	
In year before pregnancy	6%	11%	
During pregnancy	1%	3%	
Unknown	3%	3%	
History of allergic rhinitis (ever)	28%	31%	0.72
History of asthma (ever)	18%	19%	0.95
Consumption of raw farm milk during pregnancy	14%	1%	**<0.001**
Child Characteristics			
Sex (% male)	55%	43%	**0.03**
Race/ethnicity			0.35
White	98%	95%	
Other	2%	5%	
C-section	21%	19%	0.59
Daycare attendance in first year	42%	57%	**0.007**
Ever breastfed	89%	94%	0.32
Visits during the pandemic*	9.3%	4.2%	**0.001**
Household Characteristics			
Any other children in the household	69%	78%	0.09
Indoor dog	51%	52%	0.82
Indoor cat	38%	35%	0.56
Farm exposure score (postnatal)			**<0.001**
No farm animal exposure	12%	99%	
Cattle only	41%	1%	
Cattle plus other species exposure	47%	1%	

508 *Refers to the percent of visits that occurred during the SARS-CoV-2 pandemic (after April 2020).

509 **Table 2.** Exposure to Animals in the First Year of Life and Rates of Respiratory Illnesses
510 Through Age Two Years
511

Adjustment	Comparison	Rate Ratio	95% CI	P value
Age	Farm vs Non-farm	0.82	0.69 - 0.97	0.020
	Number of farm animal species	.		0.006
	x 0 vs 1	0.86	0.69 - 1.07	.
	x 1 vs 2+	0.82	0.63 - 1.08	.
	x 0 vs 2+	0.71	0.57 - 0.88	.
Model 1 + season and pandemic	Farm vs Non-farm	0.85	0.72 - 0.99	0.043
	Number of farm animal species	.		0.027
	x 0 vs 1	0.93	0.76 - 1.13	.
	x 1 vs 2+	0.81	0.63 - 1.04	.
	x 0 vs 2+	0.75	0.61 - 0.93	.
Model 2 + daycare, breastfeeding, and sex	Farm vs Non-farm	0.89	0.76 - 1.04	0.157
	Number of farm animal species	.		0.101
	x 0 vs 1	0.98	0.81 - 1.18	.
	x 1 vs 2+	0.82	0.64 - 1.04	.
	x 0 vs 2+	0.80	0.64 - 0.98	.

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514 **Table 3.** Cumulative illness burden between farm and non-farm children at 1 and 2 years of life.

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Age Interval	Farm (N)	Non-farm (N)	Rate Ratio (95% CI)	p-value
Birth – 1 Year	31.3 (134)	38.9 (146)	0.81 (0.38, 1.73)	0.58
Birth - 2 Year	81.5 (103)	84.1 (124)	0.97 (0.48, 1.95)	0.93

516

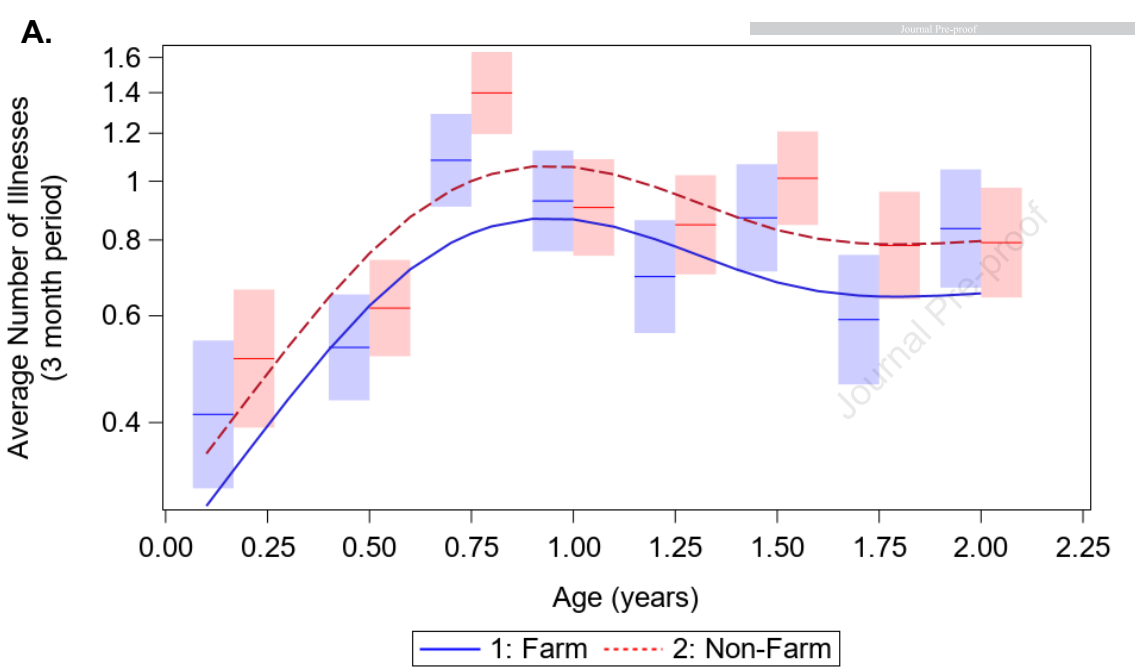
Figure Legends

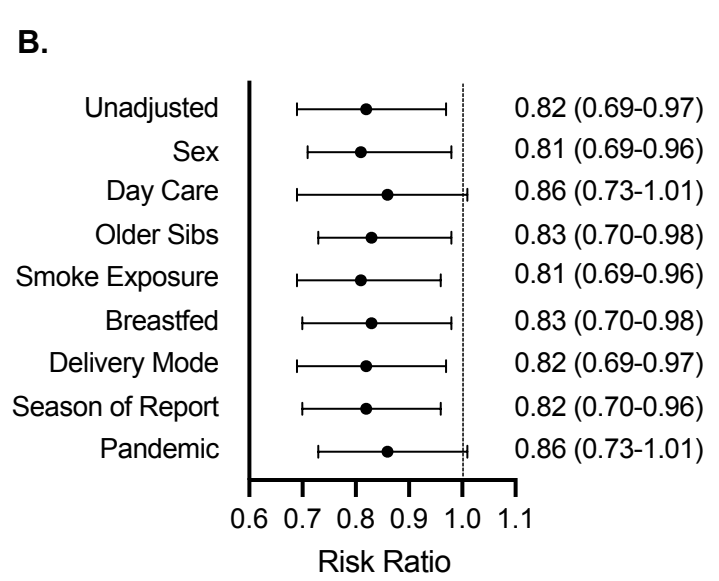
FIG 1. Farm status and rates of respiratory illnesses for the first two years of life. Boxes (A) represent the average reported illnesses at each visit with 95% confidence intervals. Lines represent age-specific predicted rates (with a common effect and natural cubic spline for age). The overall rate ratio is 0.82 (0.69-0.97, p=0.02) for the farm vs. non-farm groups; covariate adjustment had little effect on this ratio (B). Bars are 95% confidence intervals. Each bar represents a separate model fit with the addition of that covariate.

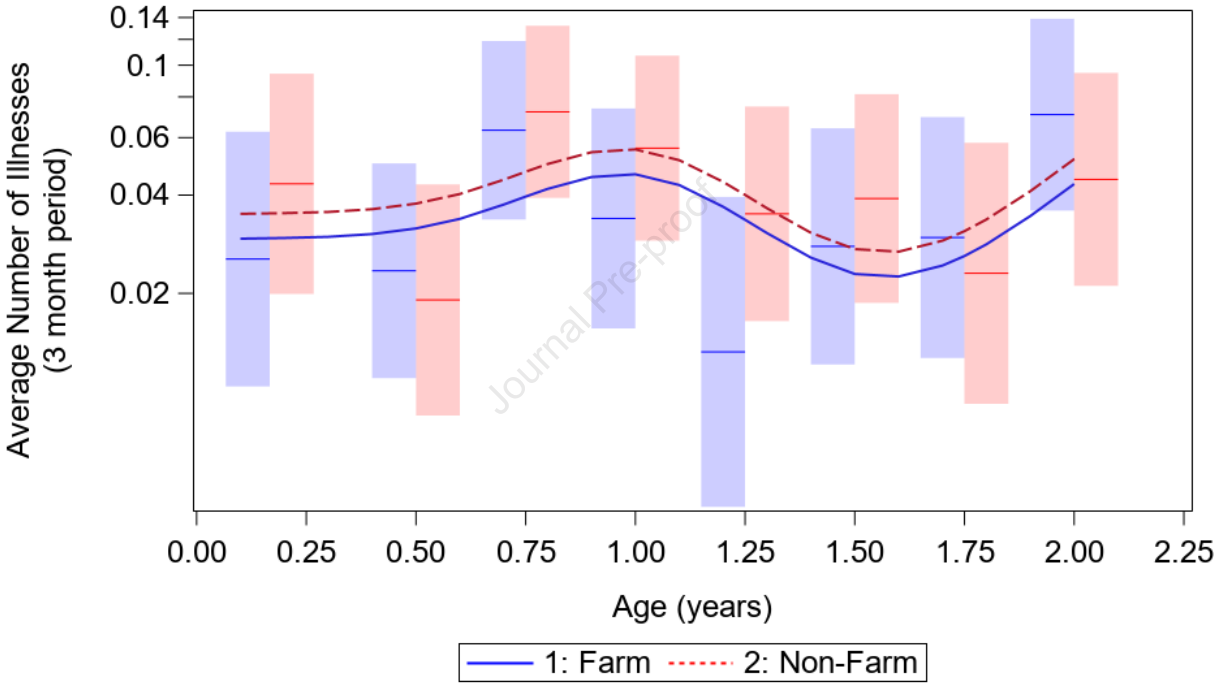
FIG 2. Farm status and rates of wheezing illnesses for the first two years of life. Boxes represent average reported illnesses at each visit with 95% confidence intervals. Lines represent age-specific predicted rates (with a common effect and natural cubic spline for the period). The rate ratio for the farm vs. non-farm groups is 0.84 (0.43-1.65, p=0.61).

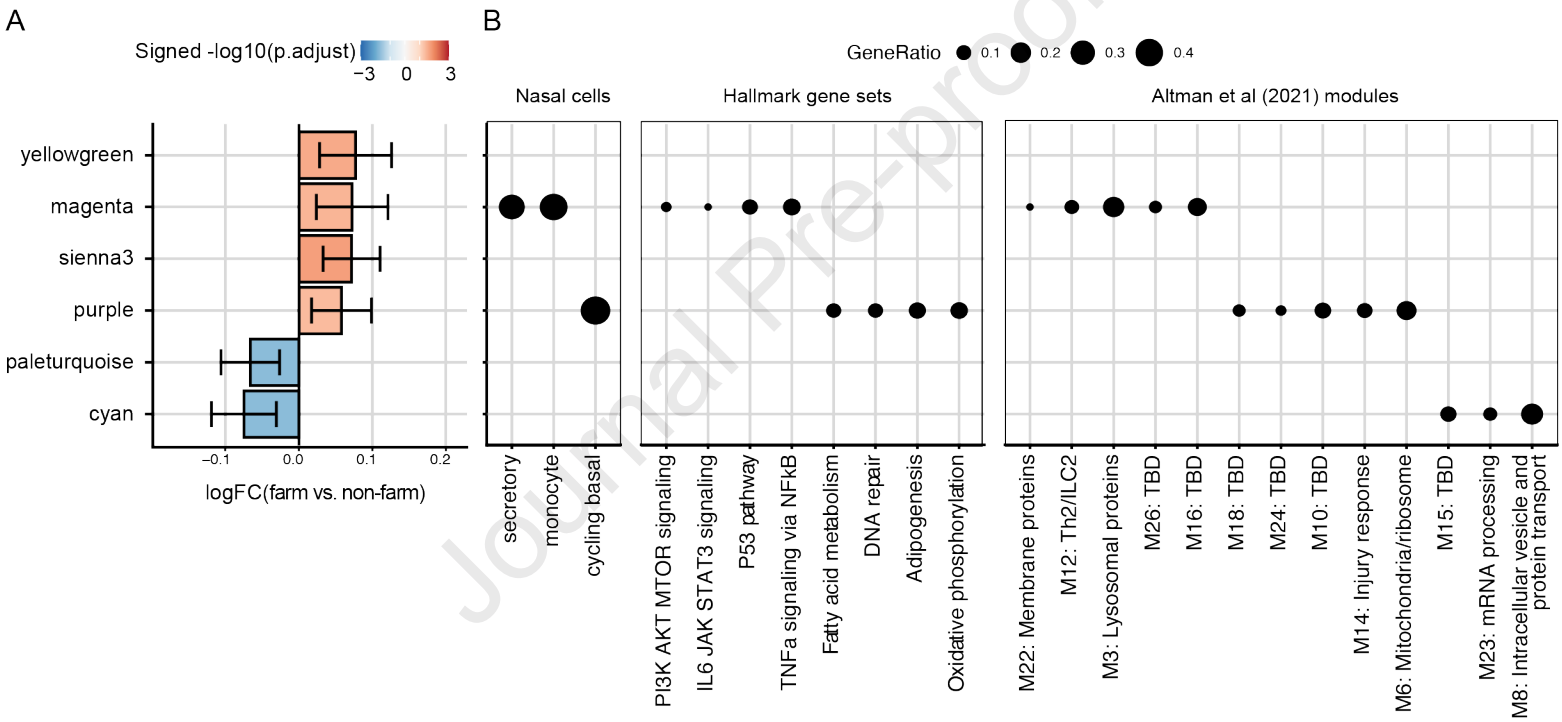
FIG 3. WGCNA expression modules associated with farm differences (adjusted $p < 0.05$). A: Bars show log fold change of module summary expression value for the comparison between farm and non-farm, with 95% confidence intervals. B: Significantly overlapping nasal cell type signatures from Deprez et al,[24] Hallmark gene sets,[25] and gene sets from Altman et al 2021.[26]

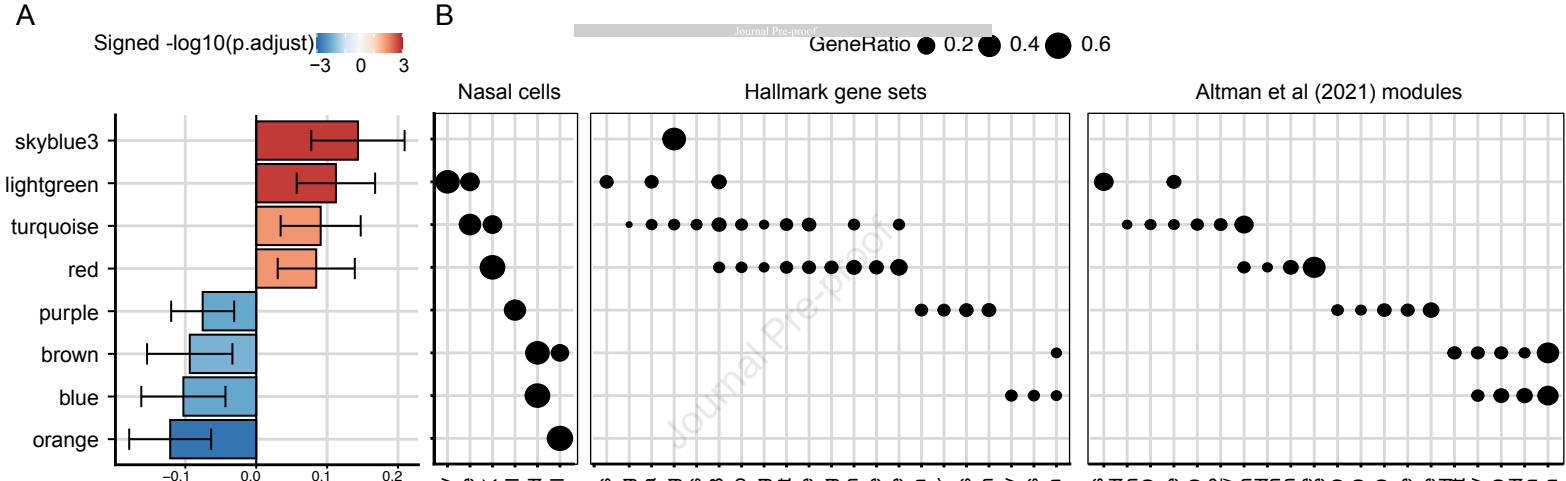
FIG 4. WGCNA expression modules associated with 10+ respiratory illnesses in the first two years of life compared to fewer than 10 (adjusted $p < 0.05$). A: Bars show log fold change of module summary expression value for the comparison, with 95% confidence intervals. B: Significantly overlapping nasal cell type signatures from Deprez et al,[24] Hallmark gene sets,[25] and gene sets from Altman et al 2021.[26]










A
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blue
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logFC(10+ illnesses vs. fewer)
B
GeneRatio 0.2 0.4 0.6
Nasal cells
secretory
monocyte
LT/NK
cycling basal
multiciliated
deuterosomal
Hallmark gene sets
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TGFbeta signaling
Hypoxia
MTORC1 signaling
Apoptosis
TNFa signaling via NFkB
KRAS signaling up
IL6 JAK STAT3 signaling
Complement
Inflammatory response
IL2 STAT5 signaling
Allograft rejection
Interferon alpha response
Interferon gamma response
Fatty acid metabolism
DNA repair
Adipogenesis
Oxidative phosphorylation
Estrogen response early
Myogenesis
Bile acid metabolism
Altman et al (2021) modules
M3: Lysosomal proteins
M20: Epithelial integrity and leukocyte migration
M16: TBD
M19: Antibacterial response
M15: TBD
M12: Th2/ILC2
M5: Innate immune resolnse/ rmyeloid activation
M25: Antigen processing and presentation
M17: T cell activation
M1 : Antiviral/innate immune response
M18: TBD
M24: TBD
M10: TBD
M14: Injury response
M6: Mitochondria/ribosome
M8: Intracellular vesicle and protein transport
M9: Antioxidant activity
M13: TBD
M11: Leukotriene and lipid metabolism
M2: Tight junction/cilium