

The microbial environment and its influence on asthma prevention in early life

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There is accumulating evidence to suggest that the environmental microbiome plays a significant role in asthma development. The very low prevalence of asthma in populations highly exposed to microbial environments (farm children and Amish populations) highlights its preventive potential. This microbial diversity might be necessary to instruct a well-adapted immune response and regulated inflammatory responses to other inhaled and ingested environmental elements, such as allergens, particles, and viruses. Like the internal gut microbiome, which is increasingly recognized as an important instructor of immune maturation, the external environmental microbiome might shape immune responses on the skin, airway mucosal surfaces, and potentially also the gut early in life. The diversity of the external microbial world will ensure that of the many maladapted pathways leading to asthma development, most, if not all, will be counterbalanced. Likewise, important contributors to asthma, such as allergen sensitization and allergic manifestations early in life, are being suppressed. Thus the facets of innate immunity targeted by microbes and their compounds and metabolites might be the master switch to asthma and allergy protection, which has been found in environments rich in microbial exposures. (*J Allergy Clin Immunol* 2016;■■■:■■■-■■■.)

Key words: Microbiome, asthma, environment, children, epidemiology

The microbial world is a vast universe with innumerable species. Microbes have been found in the deep sea, caverns, hot springs, and other places not inhabitable by human subjects. However, microbes also populate our daily indoor and outdoor environments in urban and rural areas in great abundance.¹ This abundance had been underestimated as long as culture methods were the only possible means of investigation. Yet isolation and

Abbreviations used

ALEX: Protection Against the Development of Atopy: Relevant Factors from Farming Environments
aOR: Adjusted odds ratio
EPS: Extracellular polysaccharide
PAMP: Pathogen-associated molecular pattern
PARSIFAL: Prevention of Allergy–Risk Factors for Sensitisation in Children Related to Farming and Anthroposophic Lifestyle

cultivation have strong limitations because for many microbes, it is unknown under which conditions they grow.² For certain environments, only about 1% of all microbes can be cultured. Furthermore, cultivation methods are tedious and personnel intensive, which hampers their application to large population-based surveys.

Alternatively, exposure to microbes can indirectly be assessed by detecting substances derived from their cell walls, such as LPS and peptidoglycan. Although LPS or endotoxin is found on the cell walls of gram-negative bacteria, peptidoglycan is a component of the cell wall of gram-positive and also, to a smaller extent, gram-negative bacteria. The most commonly used test (the limulus amoebocyte lysate test) to detect endotoxin in dust samples has been largely applied in epidemiologic surveys. Others have measured levels of 3-hydroxy fatty acids from LPS, but levels correlate poorly with limulus amoebocyte lysate assay measurements. The same problem applies to different methods detecting muramic acid, a component of peptidoglycan.³ Other compounds reflecting exposure to fungi, such as extracellular polysaccharides (EPSs) and β -glucans, have also been measured.⁴ However, the specificity of these individual markers is limited.

The advent of DNA-based high-throughput analyses has revolutionized the field. These novel study instruments are comparable with the invention of the microscope, which also allowed making the invisible world seen. In 1977, Woese and colleagues⁵ discovered that the nucleotide sequence of 16S ribosomal RNA (16S rRNA) is present in every bacterial and archaeal cell but not in human or animal cells. 16S rRNA contains highly conserved regions among all bacteria. Thus detection of 16S rRNA signals the presence of bacteria and archaea but not protozoa, virus, vertebrate, and other animals or human subjects. These conserved regions flank sequence regions that are variable among different bacterial species and thus allow phylogenetic classification. Several techniques combining high-throughput methods, such as microarrays or next-generation sequencing, with 16S rRNA gene analysis are being increasingly applied to microbial investigations of environmental and human samples.⁶ The taxonomic classification of the bacteria is obtained

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by comparing the sequences against databases, such as the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/nucleotide>), the Ribosomal Database Project (<http://rdp.cme.msu.edu>), Greengenes (<http://greengenes.lbl.gov/cgi-bin/nph-index.cgi>), or SILVA (<http://www.arb-silva.de>). These techniques are advancing fast, and it is now possible to study all genetic material (human, animal, plant, microbes, and protozoa) recovered from environmental samples by using metagenomic shotgun sequencing. Given the exponential decrease in associated costs, more studies with larger sample sizes will embrace these techniques and provide novel insights into the interplay of these numerous actors.

It seems important to also discuss the limitations of these fantastic techniques. First, it is impossible to know whether the recovered material represents viable microbes, debris, or (most likely) both. The detection of viable bacteria and fungi might not be reflective of important metabolites produced by these microbes. In fact, phylogenetic diversity is probably not translated into functional diversity (ie, diversity of bacterial and fungal metabolites).⁷

These technological advances have allowed the investigation of the microbial world around us but also in and on us in a depth never seen before. The Human Microbiome Project and other studies have shown that the human body harbors 10-fold as many microbial than human cells and orders of magnitude more microbial genes than the human genome.^{8,9} The relation between the environmental microbiome, the human microbiome, and the development of childhood asthma and allergies is the theme of this review.

THE ENVIRONMENTAL MICROBIAL WORLD

The fungal and bacterial composition inside and outside of more than 1100 homes across the United States was studied by using novel DNA fingerprinting techniques.¹ Distinct microbial communities were found in indoor and outdoor dust samples, but these differences were larger for bacteria than for fungi.

It has been known that almost all indoor fungi originate from outdoors, except those associated with molds and water damage, wood degrading, and foods.¹ Therefore it is not surprising that an important portion of the variation among homes in fungal community composition was associated with geographic location and regional climatic and land use variables, such as temperature, mean annual precipitation, soil pH, and the diversity of vascular plants.¹⁰ Interestingly, 74% of these fungal taxa were unknown (ie, not represented in the reference sequence databases), highlighting our enormous gap in knowledge about the fungal world.

For bacteria, indoor communities were also found to be distinct from those found outdoors. Only a small proportion (1.6%) of all bacterial phylotypes was significantly more abundant inside homes, but many of these taxa were quite frequent and associated with skin and feces from human occupants.¹ Furthermore, the nonhuman occupants, such as insects, cats, and dogs, determined indoor bacterial composition. More than 50 genera were significantly more abundant in homes with dogs or cats, including members of the *Prevotella*, *Porphyromonas*, *Moraxella*, and *Bacteroides* genera, which occur in the mouths or feces of dogs and cats.¹ These bacterial phylotypes were so specific that they allowed prediction of dog and cat ownership with 92% and 83% accuracy, respectively. The influence of pets on indoor bacterial community structure has been shown repeatedly,^{11,12} but day

care attendance might also play a role.¹¹ Thus the presence, identity, and activities of home occupants are significant determinants of indoor bacterial composition in addition to a relevant input from outdoor sources, which relate to soil and climatic variables. Interestingly, the microbiome of outdoor sources was not affected by urbanization.⁷

Studies using endotoxin as a proxy for bacterial exposures have come to similar conclusions. For example, the Effects of Outdoor and Indoor Air Pollution on the Development of Allergic Disease in Children (AIRALLERG) study in 3 European countries concluded that keeping pets and having more than 4 persons living in the home were consistently associated with higher indoor endotoxin concentrations.¹³ Also, day care centers have high levels of endotoxin,¹⁴ and indoor storage of organic household waste increases endotoxin and fungal markers.¹⁵ The input from outdoor environments rich in bacterial exposures, such as farms, has been documented by using conventional culture methods and endotoxin measurements.¹⁶ Bacteria and fungi were more often detected and grown in higher quantities from farm children's bedrooms than bedrooms of children not exposed to farm activities. The microbial counts in farm children's bedrooms correlated with the counts in animal sheds. Similar findings have been reported for endotoxin,¹⁷ suggesting a significant transfer of bacteria from outdoor to indoor environments.

THE LUNG MICROBIOME

The new DNA-sequencing methods have shown not only that the indoor and outdoor air that we breathe daily contains thousands of microorganisms but also that these microbes actually enter and potentially colonize the lower airways. Hilty et al¹⁸ were among the first to demonstrate that in healthy children and adults bacteria are found in bronchoalveolar lavage fluid and airway brushings, respectively. The bacterial composition differed between healthy subjects and asthmatic patients in whom more Proteobacteria, in particular *Haemophilus influenzae*, were found. Lower airway samples can be contaminated by passage of a bronchoscope through the oropharynx. More recent studies suggest that the lower airway microbiome resembles a dilution of the upper airways and oral cavity microbiome. However, specific bacteria were detected in significantly higher abundance in the lungs (Enterobacteriaceae and *Haemophilus* species among other), suggesting also a unique airway microbiome.^{19,20} Other investigators have supported the notion of a greater representation of Proteobacteria, including *H influenzae* and *Pseudomonas*, *Neisseria*, and *Burkholderia* species, as well as Enterobacteriaceae, among asthmatic patients.²¹ These associations were independent of age,²² steroid treatment, and asthma severity.^{23,24}

Although these data all suggest a role for Proteobacteria in asthmatic patients, the hen-egg problem has not been solved. Inflammation in the airways of asthmatic patients might create a niche promoting the growth of these bacteria very early in life. In turn, colonization with certain bacteria can promote the development of asthma. In the COPSAC birth cohort the detection of *H influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* by means of conventional culture methods in the hypopharynx of 1-month-old infants preceded the development of persistent wheeze and asthma, as defined prospectively by the age of 6 years.²⁵ However, in the same cohort this asymptomatic colonization was also associated with increased risk of

pneumonia and bronchiolitis in the first 3 years of life independently of a future diagnosis of asthma,²⁶ questioning the specificity of this association. Moreover, airway inflammation assessed by levels of exhaled nitric oxide has been reported to precede asthma development,²⁷ thereby potentially creating a suitable niche for Proteobacteria.

THE GUT MICROBIOME

Bacterial colonization in the lower airways is more strongly constricted and regulated than in the gut, where microbes are found in much greater abundance. The microbial diversity is highest in the oral cavity and lowest in the stomach and increases again from the small intestine to the colon.²⁸ Dominated by anaerobes, commensal bacteria are involved in the conversion of indigestible food components, production of essential vitamins and cofactors, regulation of epithelial development, and instruction of immune maturation.⁶

Most findings about the potential role of the gut microbiome in asthmatic patients stem from experimental studies in mice, and little is known from human studies. The existence of a gut-lung axis has been proposed whereby factors affecting the gut microbiome result in changes in allergic and inflammatory airway responses. For example, oral administration of *Helicobacter pylori* has been shown to significantly reduce airway hyperresponsiveness, tissue inflammation, and goblet cell metaplasia.²⁹ Not just microbes but also their products and metabolites can play a significant role. Recent work by Trompette et al³⁰ has elegantly demonstrated that intake of fibers affected the gut microbiome, which resulted in increased levels of short-chain fatty acids. The increased short-chain fatty acid levels in the circulation influenced dendritic cell hematopoiesis and function and thereby affected allergic lung inflammation. Zwitterionic polysaccharides from *Bacteriodes fragilis* and tryptophan metabolites might play an equally important role in gut-lung communication.^{31,32} Another recent elegant study showed that gavage of mice with house dust from homes with dogs protected against ovalbumin- or cockroach allergen-mediated airway pathology.³³

THE MICROBIAL ENVIRONMENT AND ASTHMA DEVELOPMENT

The indoor bacterial microbiome is determined by its home occupants, their presence, and their activities, as outlined above. A number of studies have suggested an association between home occupants (ie, crowding, family size, day care, and pet keeping) on asthma development (Fig 1). Although it is suggestive to speculate that indoor bacterial composition accounts for these effects, few studies have investigated this question.

Home occupants: Crowding, family size, day care, and pets

There is strong evidence that crowding and particularly sibship size contribute to the expression of atopy.³⁴ Many authors have shown that the number of siblings is inversely related to the prevalence of atopic eczema, skin test reactivity, and specific serum IgE antibody levels in children, adolescents, and adults. Although the “sibling effect” is less clear for asthma, the consistency and strength of associations with atopy over all age groups and different populations is intriguing and suggests a major

underlying mechanism that has not been clarified. Interestingly, the sibling effect is not explained by farm exposures and *vice versa*.³⁵

Child day care can be seen as an indicator of early-life exposure to numerous other children and high indoor human occupancy. Day care attendance early in life was characteristic of the former East German lifestyle. In a large cross-sectional survey of East German children aged 5 to 14 years, Krämer et al³⁶ showed that only children entering day care in the first year of life were at significantly lower risk of hay fever and a positive skin prick test response than children attending day care after their second birthday. In 2 US birth cohorts day care attendance early in life was associated with decreased asthma rates at school age and adolescence, respectively.^{37,38} Finally, a number of studies have shown that the presence of pets, in particular dogs, early in life is associated with protection from allergic sensitization and partly also protection from asthma.^{39,40}

Farm studies

Farm studies have been performed in many countries worldwide since the turn of the century. The Swiss Cross Sectional Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution, Climate and Pollen (SCARPOL) enrolled schoolchildren of 3 rural communities.⁴¹ In this first landmark publication farming as a parental occupation was significantly associated with lower rates of sneezing attacks during the pollen season (adjusted odds ratio [aOR], 0.34; 95% CI, 0.12-0.89) and atopic sensitization (aOR, 0.31; 95% CI, 0.13-0.73), whereas the association with wheeze and itchy skin rash did not reach statistical significance. Interestingly, the risk of atopic sensitization was lower in children of full-time farmers (aOR, 0.24; 95% CI, 0.09-0.66) than those of part-time farmers (aOR, 0.54; 95% CI, 0.15-1.96), suggesting a dose-response effect. Two concomitant studies in Austria and southern Germany confirmed these associations but also added evidence of a protective farm effect on asthma and wheezing.^{42,43} Moreover, in both studies a role for contact with farm animals, such as livestock and poultry, was proposed. These 3 working groups subsequently conducted the cross-sectional Protection Against the Development of Atopy: Relevant Factors from Farming Environments (ALEX) study in Switzerland, Austria, and southern Germany, confirming their previous findings in other rural populations.⁴⁴ The ALEX study suggested that the timing of exposures might matter because exposure to animal sheds and consumption of milk produced on the farm in the first year of life showed significantly stronger effects than later exposures.

The farm effect on childhood asthma has been reproduced in numerous studies around the world.⁴⁵ A formal meta-analysis concluded that there was much heterogeneity between studies but that an overall 25% reduction in asthma risk can occur.³⁵ In one multicenter study country heterogeneity was attributable to differences in environmental exposure.⁴⁶ Interestingly, the protective farm effect was seen not only for atopic asthma but also for wheeze among nonatopic subjects and transient wheeze.⁴⁷ Most farm studies reported stronger effects on hay fever and atopy, with about half the prevalence among farm rather than nonfarm children.

The strongest protection has been reported for children in Amish populations, which retain very traditional farming practices, using horses for field work rather than tractors and other

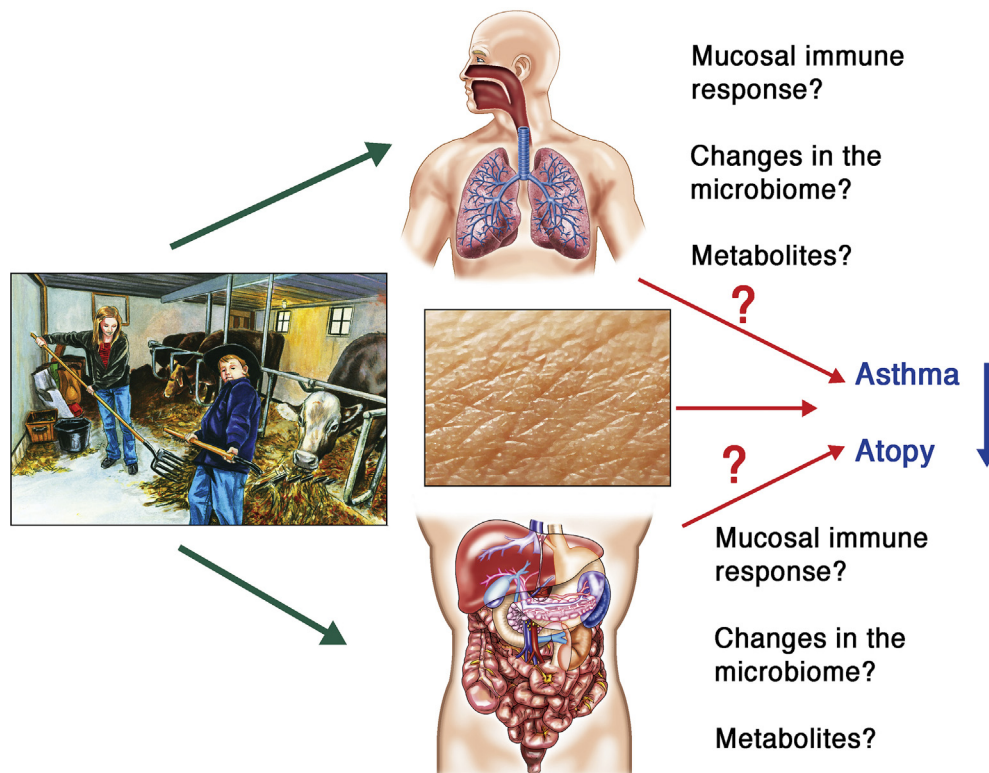


FIG 1. Environmental microbial exposures through siblings, day care, dogs, or farming environments can affect 3 compartments of the host (ie, the respiratory system, the gut, and/or the skin). Protective effects can be mediated through changes of the resident microbiome, which might result in the production of protective metabolites, such as short-chain fatty acids, tryptophan, or zwitterionic polysaccharides. Alternatively or additionally, pathogen-associated molecular patterns (PAMPs) or damage-associated-molecular patterns (DAMPs) from microbes might stimulate innate immune responses and thereby protect against asthma and allergies.

mechanized equipment.⁴⁸ Among these children, hay fever was almost nonexistent, and the rate of allergic sensitization to common aeroallergens amounted to 7.2%. These children had very little asthma, which was mostly triggered by viral infections and did not require regular treatment with anti-inflammatory medication. In contrast, Hutterites with otherwise very similar lifestyles and genetic backgrounds as the Amish embrace modern farming technologies, which is associated with a high prevalence of asthma and allergic sensitization (>15% and >40%, respectively).⁴⁹

The protection associated with growing up on a farm is sustained into adulthood.⁵⁰ Farm animal contact in childhood was associated with a decreased risk of allergic sensitization with and without accompanying nasal symptoms. Continued exposure to farm animals in adulthood further decreased the odds ratio of nasal allergies (aOR, 0.2; 95% CI, 0.1-0.4) but not of asymptomatic atopic sensitization. Von Mutius and Radon⁵¹ also reviewed the evidence from other studies of adult farmers. All reported an inverse association between being raised on a farm as a child and the prevalence of allergic sensitization to common allergens and allergic rhinitis in adulthood. Across these surveys, the largest protective effect was found when farm contact continued from childhood to adulthood.⁵² Some of these studies indicated that more than additive effects can occur. Interestingly, the loss of farm-related exposures in a Polish population after their country's accession to the European Union resulted in a doubling of atopy rates among adults and children in a remarkably

short period of only 9 years.⁵³ These data suggest that environmental microbial exposures might matter at all ages to suppress atopic immune responses.

Farm environments can differ from very traditional farming practices, as seen among the Amish, to farms raising only few animals, as seen in Poland before accession to the European Union⁵⁴ or in rural Mongolia,⁵⁵ to highly industrialized productions. Therefore the relevant exposures within farm environments merit attention. The importance of animal husbandry, in particular dairy farming, has been identified in a number of studies,^{46,52,56-59} whereas exposure to other animals, such as goats, sheep, horses, and rabbits, as the only source of animal contact might not be associated with protection.^{46,60} It seems noteworthy that the diversity of animal species encountered on a farm, particularly during pregnancy, was identified as a significant protective signal in a number of studies.⁶¹⁻⁶⁴

The consumption of unprocessed cow's milk is the other relevant exposure characteristic of a farm upbringing.^{57,65-68} Surprisingly, bacterial contamination of raw milk might not explain the beneficial effect of its consumption.^{67,69} Rather, heat-labile ingredients, such as whey proteins or microRNA, and fat composition of nonhomogenized and unpasteurized cow's milk might contribute to asthma protection.^{67,70,71} Other exposures, such as a family history of asthma and allergies, family size, maternal smoking, diet, a child's physical activity level, stress levels, and positive serology to *Toxoplasma gondii*, differ between farm

and nonfarm populations but do not explain the protection seen in these environments.^{35,44,50,72-74} Importantly, genetic background accounts for neither the farm effect in European studies nor for difference in asthma and allergy prevalence between Amish and Hutterite populations, as assessed in genome-wide studies.^{49,75}

Most farm studies enrolled school-aged children using a cross-sectional design but hinted at early-life exposures as being particularly relevant. In the ALEX study exposure to animal sheds and unprocessed cow's milk in the first year of life but not thereafter was associated with asthma and allergy protection.⁴⁴ In the Prevention of Allergy–Risk Factors for Sensitisation In Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) survey the prevalence of atopic sensitization was strongly determined by maternal exposure to stables during pregnancy, whereas current exposures had much weaker or no effects.⁴⁶ In New Zealand maternal exposure during pregnancy to animals, grain and hay, or both reduced the risk of asthma symptoms, hay fever, and eczema. However, continued exposure from pregnancy up to age 5 to 17 years showed the strongest effects.¹¹ Likewise, unprocessed cow's milk was inversely associated with allergic rhinitis and atopic dermatitis when consumed at less than 2 years of age in another New Zealand study.⁵⁷ However, these findings might be biased because of the retrospective assessment at school age and beyond.

There are few prospective farm studies. In an Austrian survey 1150 schoolchildren were followed for 3 years. Farm children had a higher likelihood to convert from a positive to a negative skin prick test response to common aeroallergens over this period.⁷⁶ In a Finnish birth cohort children born into a farm family had better lung function (FEV₁ and forced vital capacity) at age 31 years.⁷⁷ One large multicenter birth cohort, the Protection Against Allergy: Study in Rural Environments (PASTURE)/Mechanisms of Early Protective Exposures on Allergy Development (EFRAIM) survey in Europe, addressed timing prospectively and focused on the role of prenatal and early postnatal exposures for the development of atopic dermatitis, wheeze, and immune responses. In this birth cohort maternal exposures to animal sheds and in particular to diverse animal species during pregnancy has been shown to affect neonatal immune responses. Neonates born to exposed mothers had higher numbers of regulatory T cells with higher suppressive capacity in cord blood compared with newborns of nonexposed mothers.^{78,79} Moreover, cord blood mononuclear cells produced more IFN- γ and TNF- α when stimulated with phorbol 12-myristate 13-acetate plus ionomycin in neonates whose mothers had been exposed to diverse farm animal species and barns and had consumed unprocessed cow's milk.⁶² Finally, the presence of specific IgE antibodies to grass pollen, as assessed at a low detection level of 0.2 kU/L, in cord serum was inversely related to maternal activity in the barn in conjunction with handling of hay.⁶³ The allergen-specific IgE antibodies detected in cord blood correlated with the decreased IFN- γ production.⁸⁰

Not only cord blood immune responses were affected by maternal exposures in pregnancy. The risk of atopic dermatitis was reduced by more than half among offspring of mothers having contact with 3 or more farm animal species during pregnancy compared with children of nonexposed mothers.⁶¹ These findings clearly suggest an important prenatal window shaping a newborn's immune response and propensity to have atopic dermatitis very early in life.

Postnatal early-life exposures to microbial environments, such as those encountered in animal sheds, also play a significant role. Independent of maternal exposure, an infant's stay in animal sheds in the first year of life significantly reduced the risk of wheezing. This reduction in risk by more than half was found among infants with a susceptible genetic background at the chromosome 17q21 locus, which comprised about 75% of the study population.⁸¹ Wheezing symptoms in the first year of life are triggered by viral infections and are mostly unrelated to allergic mechanisms. Therefore it seems unsurprising that the infant's exposure to stables also significantly reduced the risk of rhinitis and fever episodes in the first year of life. In turn, the consumption of unprocessed milk in the first year of life was associated with less rhinitis and otitis media and a reduction in C-reactive protein levels compared with those in infants consuming pasteurized and ultra-heat-treated cow's milk.⁶⁸

Therefore upbringing in a farm environment not only confers protection from asthma and atopy but also from symptoms of viral infections and potentially inflammatory responses. Furthermore, farm upbringing has been inversely associated with the prevalence of inflammatory bowel disease and systemic lupus erythematosus in children and adults.⁸²⁻⁸⁴ In turn, no relation was found with juvenile type 1 diabetes and oligoarticular juvenile idiopathic arthritis.^{85,86}

Environmental microbial exposures in farm studies

It has long been known that air in animal sheds is highly contaminated with numerous species of molds and bacteria, which are transported indoors, as described above.¹⁶ Therefore endotoxin levels were measured in mattresses of children participating in the ALEX study.⁸⁷ A significant inverse association was seen with hay fever and atopic sensitization, suggesting that endotoxin exposure was an important player in the production of allergen-specific IgE antibodies and mediated allergic manifestations. The same relation was seen among nonfarm children in the lower range of endotoxin levels. In turn, no effect of endotoxin exposure was seen for patients with nonatopic wheeze, suggesting that the protective farm effect on childhood asthma might be attributable to endotoxin only in small part. In turn, children with higher mattress dust muramic acid concentrations as a reflection of exposure to gram-positive bacteria had a significantly lower prevalence of wheezing, regardless of farming status and endotoxin exposure.³ Furthermore, markers of fungal exposure, such as EPSs derived from *Penicillium* and *Aspergillus* species, reduced the risk of asthma in the PARSIFAL population,⁴⁶ whereas no association with $\beta(1,3)$ -glucans was seen.⁴

These microbial compounds merely reflect microbial exposures but do identify distinct bacterial or fungal species potentially underlying the reported effects. As described above, the new DNA-based methods allow a much deeper understanding of potentially relevant exposures. Therefore the bacterial microbiome was assessed by using the DNA-based single-strand conformation polymorphism method amplifying the 16S rRNA gene. Not a single bacterium, but the diversity of bacterial exposure was inversely related to asthma development.⁸⁸ Interestingly, no association between diversity and atopy was seen. Within that diversity, a "microbial cocktail" of various bacterial exposures was associated with asthma protection, suggesting that this diversity might not be interpreted as a dose effect

(ie, the more the better) but rather as a unique mixture of exposures that harbors a limited number of necessary ingredients in sufficient quantity.⁸⁹

This finding contrasts with analyses for atopic sensitization. Only *Acinetobacter lwoffii* and *Lactobacillus iners* were identified as protective exposures.⁸⁹ *A lwoffii* is an interesting bacterium because it has been recognized as a skin colonizer conferring protection from atopy in a Finnish study.^{89,90} Because *A lwoffii* is a gram-negative bacterium with a high LPS content, these findings are evocative of the studies relating high endotoxin levels to reduced risk of atopic sensitization.^{87,88}

The fungal mycobiome was assessed in the GABRIELA study by using the single-strand conformation polymorphism method, amplifying the internal transcribed spacer region characteristic for fungi. *Penicillium*, *Aspergillus*, and *Eurotium* species were identified as asthma-protective exposures,⁹¹ bringing to mind similar findings from previous surveys using either conventional culture methods⁸⁸ or EPSs (ETS) derived from *Penicillium* and *Aspergillus* species. Moreover, *Exophiala* species and *Glyphium elatum* were found in the GABRIELA study to be inversely associated with asthma.⁹¹ Other markers of fungal exposure, such as $\beta(1,3)$ -glucans, have not been related to asthma risk or protection.⁴

Experimental studies in mice support the epidemiologic evidence. Nasal application of cowshed bacteria identified in farm studies, such as *Lactococcus lactis*, *A lwoffii*, and *Staphylococcus sciuri*, result in prevention of experimental allergic asthma.⁹²⁻⁹⁴ Furthermore, maternal intranasal exposure to *A lwoffii* resulted in prevention of allergic asthma in offspring in a prenatal mouse model,⁹³ corroborating the population-based observations of protection through maternal exposure to animal sheds. Plant material, which is abundant in animal sheds because of hay and straw for feeding and bedding the animals, might also play a role. Peters et al⁹⁵ demonstrated that the grass-derived arabinogalactan can contribute to protection against allergic airway inflammation, airway hyperresponsiveness, and atopic sensitization.

Environmental microbial exposures in urban studies

Environmental microbes are not only found in farms but also in urban indoor environments, as discussed above. In the Boston metropolitan area endotoxin exposure has been related to a reduced risk of childhood atopy but an increased risk of wheeze.³⁸ A comprehensive meta-analysis confirmed this Janus-like feature of endotoxin exposure.⁹⁶ Among school-aged children, in whom asthma is more strongly associated with atopy, endotoxin exposure resulted in reduced risk, whereas among infants and toddlers with virus-triggered wheeze, endotoxin increased the risk.⁹⁶ Similar findings were reported from the National Health and Nutrition Examination Survey study enrolling more than 6000 subjects across the United States: endotoxin was a risk for wheeze but not asthma.⁹⁷

Less is known about other bacterial and fungal exposures in indoor urban homes. In the Boston cohort increased levels of muramic acid as a marker for gram-positive bacteria were inversely associated with current asthma but not with allergic sensitization. In the large Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort in The Netherlands EPS levels were inversely associated with asthma and persistent wheeze until the age of 4 years, whereas (1→3)- β -D-glucan levels were unrelated.⁹⁸ This association was no longer seen for

asthma at up to 6 to 10 years of age or found in the German Influence of Life-style Factors on the Development of the Immune System and Allergies in East and West Germany Plus the Influence of Traffic Emissions and Genetics (LISAPlus) and Spanish Infancia y Medio Ambiente (INMA) cohorts.⁹⁹

Thus the indoor microbiome might more reliably reflect relevant microbial exposures than more unspecific markers of microbial exposures, such as endotoxin and muramic acid. In a recent case-control study nested within a large high-risk birth cohort in inner-city areas of the United States, the bacterial content of house dust collected in the first year of life was assessed.¹⁰⁰ Furthermore, cat, dog, house dust mite, cockroach, and mouse allergen levels were measured. Differences in house dust bacterial content, especially reduced exposure to specific Firmicutes and Bacteroidetes, was associated with atopy and atopic wheeze. An important interaction between allergen levels and the bacterial microbiome was seen. Exposure to high levels of cockroach, mouse, and cat allergen and this subset of bacteria in the first year of life was most common among children without atopy or wheeze, suggesting that concomitant exposure to high levels of allergens and bacteria in early life might confer protection from asthma and allergy development.

Water damage in homes

Not all environmental microbial exposure has been associated with protection against childhood asthma. There is very consistent evidence that water damage and dampness indoors are strong risk factors. Findings from epidemiologic studies and meta-analyses have clearly shown that indoor dampness or mold was consistently associated with increased asthma development.^{101,102} Objective and replicable assessments of dampness have found exposure-response relations. The recent report of the Institute of Medicine in the United States concluded that a causal relationship exists between indoor dampness or dampness-related agents and asthma exacerbation in children.¹⁰³ In spite of all this epidemiologic evidence, the causative agents mediating risk for asthma are still unknown but might be related to chemical exposures, such as volatile organic compounds emitted by fungi in moldy environments.¹⁰¹ A causative role is further supported by findings from an intervention study.¹⁰⁴ Construction remediation aimed at the root cause of moisture sources and combined with a medical/behavioral intervention significantly reduced symptom days and health care use for asthmatic children living in homes with a documented mold problem.

Interplay between the environment and the human microbiome

As of today, very little is known about how the environmental microbiome might shape the human microbiome. Very recent studies have addressed this question in the farm environment. The diversity of the bacterial microbiome on farms resulted in increased diversity of the microbiome in the mattresses, noses, and throats of exposed children. However, the throat microbiome was unrelated to asthma, suggesting that the throat harbors a restricted and well-controlled microbial community. In contrast, the nasal microbiome was related to asthma: a reduction in risk was seen with higher diversity, whereas *Moraxella* species increased risk, suggesting that the nasal mucosa might affect asthma development, as proposed in the "united airways"

concept. The facultative pathogen *M catarrhalis* might be counterbalanced by a greater diversity of bacterial colonization, thereby preventing its outgrowth and subsequent harmful effects. The role of environmental microbial exposure for the gut microbiome is unknown.

Potential underlying mechanisms

Protection from environments rich in microbial exposures might be mediated not only by differential colonization of the mucosal surfaces. Substances produced by microbes, such as short-chain fatty acids or fungal metabolites, might mediate important immune effects, but these have not been assessed in human studies. Pathogen-associated molecular patterns (PAMPs), which are evolutionarily highly conserved structural components of the microbial cell wall, are recognized by similarly conserved receptors of the hosts' innate immune system, the pattern recognition receptors.¹⁰⁵ Examples for PAMPs are the bacterial compounds from bacteria and fungi that are used for exposure assessment, such as LPS (endotoxin), muramic acid, and EPS, as described above. PAMPs engage the pattern recognition receptors and thereby initiate innate host defense mechanisms but also provide signals required for initiating and modulating the adaptive immune response.¹⁰⁶ Remarkably, structural changes in the LPS molecule can result in opposite (ie, inflammatory and anti-inflammatory) effects.¹⁰⁷

There is much evidence to suggest that among farm children, innate immune responses are activated because increased gene expression of Toll-like receptors has been found repeatedly with different farm exposures.^{46,61,108} In the PARSIFAL survey gene expression of *IRAK1*, *IRAK2*, and *RIPK1*, all molecules involved in the Toll-like receptor downstream signaling cascade, was increased by farm exposure.¹⁰⁸ These pathways contributed to the observed asthma protection seen in the PARSIFAL study. Experimental studies corroborate the epidemiologic findings because maternal transmission of asthma protection to offspring was dependent on intact Toll-like receptor signaling.⁹³ Schuijs et al¹⁰⁹ recently reported that farm dust protects mice from mite allergic experimental asthma in ways similar to LPS exposure. This protection was mediated by A20, an inhibitor of the transcription factor nuclear factor κ B. Mice lacking A20 in lung epithelial cells were no longer able to abolish the allergic and asthmatic response to house dust mites after exposure to LPS or farm dust, suggesting a critical role for the first innate line of defense (ie, the epithelium) and the A20 enzyme.

Because all atopic asthma, nonatopic wheeze, transient wheeze, rhinitis, and fever episodes are less frequent among farm children, immune responses to allergens and viral infections can be dampened through common or additive mechanisms. Farm dust exposure might increase the epithelial barrier function, which might in turn contribute to viral defense and impaired allergen sensitization. Alternatively, protection from both airway allergen challenge and viral infection can be mediated through the gut microbiome.³³ Gavage of house dust from a home with a dog induced restructuring of the cecal microbiome with enrichment of, among others, *Lactobacillus johnsonii*, which induced protection from both airway allergen challenge and viral infection in a preventive mouse model.

However, more than 1 mechanism is likely to contribute to asthma protection in such diverse microbial environments. Experimental studies based on Amish and Hutterite populations

with strikingly different asthma prevalences, as described above, are telling.^{49,110,111} As in previous farm exposure studies, administration of Amish house dust protected animals from airway hyperresponsiveness and lung inflammation in an ovalbumin model of experimental asthma. In contrast, Hutterite house dust collected from indoor environments of Hutterite children with low microbial exposure exacerbated airway hyperresponsiveness in this model. Lung gene expression profiling revealed a multipronged suppression of ovalbumin-dependent pathways related to epithelial, dendritic, and T_H2 cell activation; alternative macrophage activation; and airway remodeling,¹¹¹ suggesting that many pathways of innate and adaptive immunity are involved in the protective effects. These findings are not surprising because the diversity of the microbial environmental exposure, which most likely contains a broad cocktail of numerous live microbes and compounds stemming from microbial and plant debris, exerts the asthma protective effect.

Future research

There are not many studies assessing environmental microbial exposure with DNA-based methods, but the costs of sequencing are still decreasing substantially. Given the complexity of the microbial exposures, large sample sizes will be needed to dissect the relevant exposures. Moreover, phylogenetic diversity might not correspond to functional diversity. Therefore the next step will be application of metagenomic shotgun sequencing to environmental samples, which amplifies DNA from every potential source (eg, human, animal, plant, bacteria, fungi, and archaea). This surge of big data will not only allow incorporating a variety of exposures (eg, microbial, plants, and animals) in one run but will also allow us to read the whole genome of relevant microbes, thereby identifying important genes and their products (ie, the microbial metabolites). Because microbes live in communities, interactions between them will also result in production of relevant metabolites.

Thus metagenome sequencing of single microbes might not be sufficient to capture the relevant environmental exposures. Alternatively, metabolomic studies of environmental samples might be needed. Ideally, the most comprehensive approach combining different genomics, transcriptomics, and metabolomics technologies should be applied to environmental samples. The environmental microbiome will be at least as complex, challenging, and interesting as the human microbiome and most relevant because environmental exposures are strong determinants of asthma. Moreover, there is much to learn about interactions between the microbiome at mucosal and skin surfaces and the environmental microbiome. To this aim, we need better computational resources. Although biostatistical methods can beautifully display differences between groups, much is to be learned about how to integrate such complex data into multivariate and other epidemiologic analyses.

Clinical applications

This exciting field of research presents possible future clinical applications. We know that environmental exposures can substantially suppress the manifestation of asthma and allergies, as demonstrated in Amish versus Hutterite populations with highly similar genetic ancestries but striking differences in asthma prevalence. If we can identify the relevant protective exposures

and mechanisms involved, in the future, we might be able to design novel prevention strategies to combat the ongoing asthma epidemic.

SUMMARY

There is accumulating evidence to suggest that the environmental microbiome plays a significant role in asthma pathogenesis. The very low prevalence of asthma in Amish and highly exposed European farm children, which cannot be explained by genetic factors, highlights its preventive potential. The microbial diversity around us might be necessary to instruct a well-adapted immune response and regulated inflammatory responses to other inhaled and ingested environmental elements, such as allergens, particles, and viruses. Like the internal gut microbiome, which is increasingly recognized as an important instructor of immune maturation, the external environmental microbiome might shape immune responses on the skin, airway mucosal surfaces, and potentially also the gut early in life in comparable ways. Such balanced immune responses to the external world will thereby counteract an asthmatic patient's propensity to mount inflammatory responses to respiratory viruses and allergens through a multitude of partly redundant pathways. The diversity of the external microbial world will ensure that of the many maladapted pathways leading to asthma development, most, if not all, will be counterbalanced. The facets of innate immunity targeted by microbes and their compounds and metabolites might thus be the master switch to asthma protection, which has been found in environments rich in microbial exposures.

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