

# Does diversity of environmental microbial exposure matter for the occurrence of allergy and asthma?

Dick Heederik, PhD,<sup>a</sup> and Erika von Mutius, MD, PhD<sup>b</sup> *Utrecht, The Netherlands, and Munich, Germany*

This review describes the recent literature on microbial exposures and protective effects for asthma and atopy. Certain microorganism-associated molecular patterns have been identified as agents that might influence the development of the immune system, which in turn leads to protective effects for asthma and atopy. Endotoxins from gram-negative bacteria were the first agents associated with a reduced risk for asthma and atopy. In later studies,  $\beta(1 \rightarrow 3)$ glucans, extracellular polysaccharides, and muramic acid from, respectively, molds and gram-positive bacteria were associated with a reduced risk of allergy and asthma separately in rural and urban populations. These results already suggested that not just one but several independent microbial signals from gram-negative and gram-positive bacteria, as well as molds, might play a role in explaining the protective effects. Recently, the diversity of microbial exposure has been associated with such a reduced risk in farmers' children. Surprisingly, the diversity of both fungal and bacterial exposure seemed to have protective effects. These results open new areas of research and create complex challenges. Methodological issues, such as environmental exposure characterization and assessment and elucidation of potential underlying mechanisms, are discussed because these aspects have a major influence on how microbial diversity can be studied in future studies in relation to protective effects for asthma and atopy. (*J Allergy Clin Immunol* 2012;■■■■:■■■■-■■■■.)

**Key words:** *Environmental exposure, farming exposure, microbial exposure, allergy, asthma, microbiome, protective effects*

Asthma prevalence has increased in recent decades and seems to have stabilized in affluent countries.<sup>1-3</sup> Established risk factors for asthma, such as allergen exposure, could not explain the increase in prevalence, and the role for atopic asthma seems smaller than earlier thought.<sup>4</sup> Many population studies indicate that a change in lifestyle ("westernization") seems to play a role in

## Abbreviations used

EPS: Extracellular polysaccharide  
MAMP: Microorganism-associated molecular pattern  
PAMP: Pathogen-associated molecular pattern  
TLR: Toll-like receptor

the change of occurrence in asthma. For instance, studies comparing Eastern and Western Germany shortly after unification showed a higher asthma prevalence in Western Germany than in Eastern Germany, although house dust mite allergen levels did not clearly differ.<sup>5</sup> Studies in the Finnish and Russian Karelia region indicate a difference in allergic asthma occurrence between populations at either side of the border, despite the fact that ethnic background and geoclimatic environments are similar.<sup>6,7</sup> Asthma is more clearly attributable to IgE sensitization in Finnish than in Russian children.<sup>6</sup> Studies that explored the contents of pooled house dust samples from Finnish and Russian homes showed high levels of muramic acid in Russian homes, a marker of gram-positive bacteria.<sup>8</sup> The gram-positive bacteria were predominantly animal-associated species, and it was hypothesized that exposure to these species might have skewed development of the immune system, which led to a protective effect with regard to development of sensitization in Russian children. However, the study had an ecological design, and no direct association was observed between the presence of microorganisms and asthma.

Results of the Karelian studies seem in agreement with those of more recent studies in farming communities. Increasing numbers of studies report a reduced risk of atopy, hay fever, and asthma in farmer's children, adolescents, and adult farmers, most likely as a result of early-life microbial exposure.<sup>9</sup> Endotoxins from the cell walls of gram-negative bacteria were the first specific group of microbial agents, which were considered to play an important role in protective effects for allergy and possibly asthma.<sup>10</sup> More recently, protective associations have been observed for muramic acid, a marker for gram-negative and gram-positive bacteria, and extracellular polysaccharides (EPSs) and glucans, markers indicative of fungal exposure not limited to farmers' children.<sup>11-16</sup>

There are numerous studies that have now associated exposure to microbial markers in the home environment, even in inner-city populations. For instance, endotoxin appeared to be associated with protective effects for eczema<sup>17</sup> and asthma, with the latter seen in cross-sectional<sup>18-21</sup> and longitudinal<sup>13,22</sup> studies among rural, urban, and inner-city children from across the world. In some studies these signals are present after mutual adjustment indicating that several independent microbial signals from bacteria and molds can exert protective effects.<sup>13,16</sup> Exposures early in

From <sup>a</sup>IRAS, Division of Environmental Epidemiology, Utrecht University, and <sup>b</sup>Dr von Hauner Children's Hospital, University of Munich.

Disclosure of potential conflict of interest: D. Heederik receives research support from the European Commission. E. von Mutius has consultant arrangements with Novartis, GlaxoSmithKline, ALK-Abelló, and Protectimmune; receives speaker's fees from InfectoPharm; receives research support from Airsonett AB; is a member of the expert panel for the UK Research Excellence Framework; and is an Associate Editor of the *Journal of Allergy and Clinical Immunology*.

Received for publication September 13, 2011; revised January 19, 2012; accepted for publication January 23, 2012.

Corresponding author: Dick Heederik, PhD, Division of Environmental Epidemiology, IRAS Utrecht University, PO Box 80198, 3508 TD Utrecht, The Netherlands.  
E-mail: d.heederik@uu.nl.

0091-6749/\$36.00

© 2012 American Academy of Allergy, Asthma & Immunology  
doi:10.1016/j.jaci.2012.01.067

life, including the prenatal period, appear particularly protective, but it has been suggested that continued exposure might be required to maintain optimal protection.<sup>23</sup> Studies among adults with high occupational exposures and without early childhood exposures because they were not raised on a farm have also shown clear protective effects associated with endotoxin exposure. However, these associations were observed at endotoxin exposure levels that are orders of magnitude higher than usually encountered in the home environment.<sup>24</sup>

Molecules, such as endotoxins, EPSs, glucans, and muramic acid, which originate from the cell walls of microorganisms, are recognized by the innate and humoral immune system and referred to as pathogen-associated molecular patterns (PAMPs) or microorganism-associated molecular patterns (MAMPs). PAMPs are recognized by cellular receptors, such as the so-called Toll-like receptors (TLRs), of the innate immune system and are known potent proinflammatory agents.<sup>25-27</sup> TLRs are now seen as key receptors that alert the immune system to the presence of microbial infection. Thirteen TLRs (TLR1-TLR13) have been identified in human subjects and recognize ligands from different microorganisms ranging from endotoxin from gram-negative bacteria, heat shock proteins, lipoproteins, lipopeptides or flagellin from bacteria, bacterial DNA, or viral RNA. Molecules that mediate TLR signaling have also been identified, and TLR signaling pathways have been unraveled to a large extent. The effect of exposure to complex mixtures containing multiple MAMPs on TLR signaling has not been studied.

## MAMP EXPOSURE AND EXPOSURE ROUTES

How are subjects exposed to microorganisms and MAMPs, and where do they come from? Early studies have already shown that animal husbandry is associated with high exposures to gram-positive and gram-negative bacteria from animal gut flora, animal fur, or plant-derived animal fodder.<sup>28,29</sup> Microbial exposure on the farm might also involve different zoonotic species (*Coxiella burnetii* [Q-fever], *Mycobacterium* species, and *Campylobacter* species, *Clostridium perfringens*, *Enterococcus* species, *Escherichia coli*, and *Salmonella* species), which originate from farm animals and can cause infections in human subjects.<sup>29,30</sup> When parents with young children visit a stable or barn, they are directly exposed because they inhale stable air, which contains high levels of microorganisms up to 1000-fold higher levels than in dwellings (see Fig 1).<sup>28,31</sup> Subjects will bring in microorganisms from the stables into the farmhouse through their skin, hair, clothing, and shoes. These microorganisms can become airborne again and can be inhaled after resuspension. Direct transport through the air from farm to living areas has been observed in different studies.<sup>31,32</sup> As a result, outdoor microorganisms or microorganisms that can be found in farmhouses are found in air, floor dust, and even in mattress dust in homes.<sup>31-33</sup> At higher age, the child will play in the farmyard and might enter stables and have direct contact with animals that will lead to oral uptake and respiratory exposure.

Particulates in the air of stables are relatively large and generally have aerodynamic diameters of greater than 5  $\mu\text{m}$ .<sup>34,35</sup> They will predominantly deposit in the higher airways and will be cleared through the gastrointestinal tract. Thus inhalation of large particulates leads to both oral and respiratory uptake of microorganisms. Direct skin contact and mouthing behavior of the child will also contribute to oral uptake.

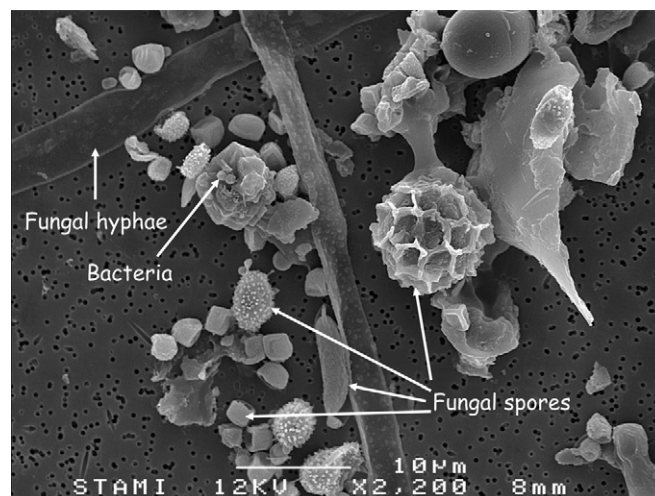


**FIG 1.** An air sample taken at the farm during grain handling, which clearly illustrates the heterogeneous exposure to various bacteria and mold fragments (hyphae) and spores. Gram-negative bacteria will contribute to endotoxin exposure, and gram-positive bacteria will contribute to muramic acid exposure. Mold fragments and spores contribute to glucan and EPS exposure, with the latter depending on the species. With courtesy of Dr Anne S. Halstensen and Dr Wijnand Eduard, National Institute for Occupational Health, Oslo, Norway.

In (sub)urban environments the main sources of microbial exposure are the presence of pets in the house, shedding by human occupants, and microbial growth resulting from specific climatic and housing conditions (home dampness). Animal contact of children as part of leisure activities, such as horseback riding, can be expected to be sources of short but high microbial exposure.<sup>36</sup> Human subjects shed especially gram-negative and gram-positive bacteria; for instance, endotoxin levels in dwellings have been shown to be correlated to occupancy.<sup>37,38</sup> Companion animals bring in soil particulates, which contain microorganisms, and the animals themselves are a source of gram-positive and gram-negative bacteria through shedding of hair, dander, saliva, and fecal particulates. Factors such as the presence of carpeted floors and cleaning practices by the occupants of houses are known to modify the exposure.<sup>37,38</sup>

## MICROBIAL ENVIRONMENTAL DIVERSITY AND ASTHMA AND ATOPY

The association between microbial exposures and the protective effect for asthma and allergy has been put in a completely new perspective by results from 2 independent population surveys.<sup>39</sup> In one study microbial exposure to bacteria was characterized by analyzing mattress dust samples from the child by using molecular techniques. For this study, a fragment of the variable regions 4 and 5 of the 16S ribosomal DNA was amplified and analyzed by means of single-strand conformational polymorphism gel electrophoresis. This led to a total of 76 detectable bands, with each band representing most likely 1 bacterial species. The association between bacterial diversity and asthma was strong, with an odds ratio of 0.62 for each additional 10-band increase in microbial diversity. The contribution of each band was further analyzed by using factor analysis. Bands that mapped to significant factors were excised, and DNA in each

band was cloned, sequenced, and compared with a database for phylogenetic identification. Two clusters containing various clustered bacterial species were found, and these 2 clusters appeared also associated with a clear protective effect on asthma, although less so for atopy. One factor consisted of the species *Listeria monocytogenes*, *Bacillus licheniformis* and other *Bacillus* species, *Corynebacterium* species, *Methylobacterium* species, *Xanthomonas* species, *Enterobacter* species, *Pantoea* species, and others. The other factor consisted of bands coding for *Staphylococcus* species, *Salinicoccus* species, *Macrococcus* species, *Bacillus* species, *Jeotgalicoccus* species, and others.

In the second survey microbial exposure was characterized by measuring viable bacteria and fungi in settled dust samples from the bedroom air of the child, and results were described for 9 fungal taxa (including yeasts). Of the viable fungi, *Eurotium* and *Penicillium* species were associated inversely with asthma but not after adjustment for multiple comparisons. The surveys were complementary because in one the emphasis was on bacterial species while in the other also mold diversity was established. In both studies there was a striking association between an increasing diversity of microbial exposure and a gradually decreasing prevalence of asthma.

## WHAT DO WE KNOW ABOUT MICROBIAL DIVERSITY IN THE ENVIRONMENT?

The abovementioned studies are the first that describe such an association. Molecular technology is rapidly advancing, and new higher-resolution techniques have become available that measure microbial diversity in a high-throughput fashion. It is timely to consider the results of studies on measurement of environmental microbial diversity and some methodological issues to optimize a new generation of epidemiologic surveys in the field of asthma and allergy. Most molecular diversity studies conducted thus far have been exploratory. A typical metagenomic study includes a limited number of samples. Variability between environments or over time is yet poorly understood. However, the first results are exciting and open a completely new field, especially because these techniques open up a world that was invisible until recently. Surveys of outdoor environments (soil and water samples) reveal the presence of thousands and sometimes even millions of different species.<sup>40</sup> A significant fraction of the clone sequences appear to be novel, although quality control issues with sample treatment and DNA amplification might complicate interpretation.<sup>41</sup> An intriguing example are organisms from the domain Archaea, formerly classified as Archaea bacteria. Some Archaea species live in the gut of ruminants. They are extreme anaerobes, sensitive to oxygen, and difficult to culture. Levels in stable air appeared extremely high up to  $10^8$  per cubic meter on the basis of quantitative PCR measurements, and some species have been suggested to have protective properties.<sup>42</sup> Bacterial diversity in outdoor air samples seems highly variable over time and dependent on meteorologic conditions<sup>43</sup> and higher in areas with agriculture than in suburban areas and forests.<sup>44</sup> House dust from the low-allergy Russian Karelia contained more gram-positive bacteria (Firmicutes and Actinobacteria), predominantly Staphylococcaceae and Corynebacteriaceae, probably with an animal source as the likely origin.<sup>8</sup> Finnish Karelian dust contained more gram-negative bacteria (mainly Proteobacteria) and clones of plant-associated bacterial species.

In general, the biodiversity of indoor air samples is lower than that of samples from outdoor environments.<sup>45</sup> Moreover, indoor

samples contain fewer novel sequences than outdoor environmental samples. Bacteria found indoors are more often human related, although libraries with sequences are likely to be biased in the direction of human pathogens.<sup>45,46</sup> Taubel et al<sup>46</sup> studied bacterial diversity on skin samples and mattress and floor dust samples. Analysis of samples from 4 houses showed that mattress dust samples are dominated by gram-positive bacteria. The specter resembles the specter of human skin, and most microorganisms thus seem to have been shed by human subjects. Floor dust samples had a higher microbial diversity, with more species of likely environmental origin. Therefore probably many microorganisms on the floor are brought in from outside by human subjects or pets (walk-in exposures) or are deposited after air exchange between the outdoor and indoor environments (ventilation). More gram-negative bacteria were found in the floor dust samples, and as expected, LPS concentrations in floor dust samples were increased as well. Households with cats or dogs and with children who attend day care centers have distinctly different bacterial communities.<sup>47</sup> Day care centers exhibit a high bacterial diversity, and it appears that diaper changing contributes significantly to bacterial load.<sup>48</sup> Korthals et al<sup>33</sup> detected several outdoor related species, such as *Acinetobacter* species, *Brevicompectum* species, and *Arthrobacter* species, in mattresses of children living on farms. Bacterial diversity appeared strongly dependent on location; exposure in close proximity of the source differs from the pattern at greater distance.<sup>49</sup> Considerably less information is available for fungal diversity in the air. In a recent large-scale ecologic study, indoor fungal composition appeared to have a clear geographic pattern and, surprisingly, appeared more diverse in temperate zones than in tropical areas,<sup>50</sup> pointing to strong environmental selection.

Thus farm environments seem to have a higher diversity, with species that originate from animals, plants, and fodder, but the evidence is still limited. Bacterial diversity is lower in indoor environments and more strongly dominated by species that are shed by human subjects. Variability over time and between different environments needs to be studied in greater detail.

## FUTURE CHALLENGES

The study by Ege et al,<sup>39</sup> highlighting the potential protective role of microbial diversity, raises many questions and methodologic issues relevant for future studies and does raise potential new hypotheses that have to be explored in the near future:

- *Environmental exposures as a cause of asthma and allergy.* The association between microbial diversity of the environment and allergy re-emphasizes the environment as the cause of asthma and allergy. The genetic revolution of the past few years led to the identification of a number of novel asthma and allergy genes but was somewhat disappointing in the sense that associations were relatively weak and cannot explain solely the occurrence of asthma and allergy in a satisfactory way. The same genetic technologies applied to environmental samples will hopefully be the starting point for a revolution in microbial exposure assessment and a more thorough exploration of the role of the microbial environment in asthma and allergy. Eventually, gene-environment interactions based on well-designed exposure studies will have to be further explored.
- *Is there a relation between environmental exposures and the human microbiome?* Until recently, the lung was

believed to be a sterile environment; this now appears not to be the case.<sup>51-53</sup> It is not clear whether the lung has a stable microbial community or whether transient colonization occurs more regularly and whether these changes are associated with environmental exposures. Repeated skin and fecal measurements in 2 subjects have shown that only a small fraction of all taxa found within a single body site are present across all time points.<sup>54</sup> This suggests that the human core microbiome is small, whereas many taxa were present at some but not each occasion. It seems plausible that external influences, such as inhalation or ingestion of microorganisms, can temporarily change the composition of the microbiome and that farm or animal-associated microorganisms might compete with microorganisms promoting asthma. Whether this leads to changed colonization patterns, for instance in which pathogens are outcompeted, needs to be established. *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* colonization at neonate age have been associated with the occurrence of asthma in childhood<sup>55</sup>; however, their causal role has been debated,<sup>56</sup> and it is not clear whether these organisms occurred because of absence of competition with other species in particular environments. Several studies have shown that diversity of the gut flora is related, although not consistently, to allergic sensitization.<sup>57-61</sup> Children living on farms consume raw milk, and raw milk consumption has also been associated with protective effects against the development of asthma and allergy.<sup>62</sup> Inhalation of microorganisms also leads to oral exposure. Whether inhalatory exposure to microorganisms or oral intake of raw milk can influence the human gut microbiome is not clear yet but is a relevant question to explore in future studies. Changes in the microbiome need not be permanent but might occur transiently and repeatedly over time. Alternate disease models taking the potential benefit of the variability of exposures into account might have to be explored to understand relations between microbial exposures and occurrence of disease.<sup>63</sup>

- **Underlying mechanisms.** What are the underlying mechanisms that explain the protective effects of exposure to microorganisms? Different hypotheses exist:

- A plausible hypothesis is that the protective effects of microorganisms are the result of signaling through the innate immune system by PAMPs. The fact that gene-environment interactions have been found for innate immune system-related genes involved in TLR signaling illustrates the potential relevance of this mechanism.<sup>64</sup> Although correlations between endotoxin, muramic acid, EPS, and glucan exposure on the one hand and microbial diversity on the other hand have not been studied, it can be expected that when more species are present, levels of these microbial markers are higher. Which marker correlates best with microbial diversity depends on the microbial community present in a certain environment. This raises the question of whether the diversity or composition of the microbial community itself plays a causal role or whether it is merely an issue of the dose of proinflammatory markers that triggers protective immunologic responses. Strong indications exist that microbes or microbial components stimulate

innate immune receptors and thereby favor the generation of regulatory T cells that promote immunologic tolerance through complex signaling pathways of the immune system.<sup>25</sup> Differences seem to exist among the few species studied; some species elicit stronger neutrophilic inflammatory responses, whereas other species seem to better promote tolerance, in particular in intergenerational asthma models (*Acinetobacter lwoffii*; *Bacillus licheniformis*; *Lactococcus* species, such as *Lactococcus lactis*; *Listeria monocytogenes*; *Lactobacillus rhamnosus*; *Mycoplasma pneumoniae*; and *Mycobacterium tuberculosis*).<sup>65-73</sup> Thus different species might differentially contribute to the immunologic properties of complex environmental mixtures. Given that the innate immune defense is a wide-meshed filter that recognizes MAMPs rather than individual microbial species, redundancy of exposures or reinforcement of signals might play a role. If the dose of protective microbial signals is the driving factor, it will depend on the composition of the underlying microbial community.

- Other signaling molecules than the MAMPs identified thus far might also play a role. Microorganisms are known to have communication systems, such as quorum sensing, which make use of signaling molecules, enabling a cooperative response, potentially at the expense of some species, at high cell densities though activation of gene transcription.<sup>74</sup> Quorum-sensing molecules have broad biological activities beyond their role in bacterial communication. They are known to have antimicrobial, pharmacologic, and immunomodulatory activities.<sup>75,76</sup> The profile of these molecules at any given time might reflect the status of a microbial community. This opens new areas of research in environmental exposure to microorganisms. All the above examples indicate that the hypothesis influences how diversity should be assessed.
- An alternate hypothesis might be that a more complex community of microorganisms protects more optimally against colonization or even infection by certain pathogens? If so, viability of the different microorganisms might play a role. Molecular techniques measure a combined viable and nonviable signal. Colonization and infection both depend on viability. Viability seems less relevant for mechanisms based on TLR signaling by MAMPs. In case of TLR signaling, other issues might play a role: Should microorganism be intact, and should there be several parallel microbial signals from different species?
- **Optimal exposure assessment.** Optimal exposure assessment by measuring a subject's environmental exposure with similar accuracy and precision as a subject's genome has been mentioned as a requirement for future studies.<sup>77</sup> This can most likely not be achieved for the following reasons. First, it is still questionable whether reservoir measurements are a good proxy for inhalatory exposure of inhabitants of houses.<sup>78,79</sup> Second, environmental exposure is highly variable,<sup>79</sup> as observed for many exposures, including endotoxin in floor dust<sup>80,81</sup> and viable microbial counts.<sup>82</sup> The variability in the composition of the environmental microbiome raises additional problems, and together, these issues lead to different types of errors.<sup>83</sup>

These errors in the exposure assessment might lead to underestimation of exposure-response relations but can also create other difficulties in the interpretation of studies and complicate requirements for replication.<sup>63</sup>

- Type I errors refer to false-positive results from the large number of comparisons. When microbial diversity is measured with high-resolution techniques, an enormous stream of information becomes available, and this immediately introduces multiple comparison issues. The studies conducted thus far give a snapshot of the present microbial exposure. The dynamics of microbial populations in the environment are poorly understood. Potential analytic errors will be superimposed on top of the large environmental variability over time and space. Repeated measurement studies are required in more powerful future studies to be able to distinguish between microorganisms, which are transiently and permanently present.
- Type II errors refer to the lack of sensitivity of measurement techniques resulting in false-negative results. Levels for some of the individual species in environmental samples are expected to be low, and the available material for analysis is often limited. Moreover, analytic techniques, such as single-strand conformational polymorphism and denaturing gradient gel electrophoresis, have relatively high detection limits.<sup>33</sup> Thus the likelihood that these errors might arise is high. Alternative techniques, such as deep sequencing, are expected to be considerably more sensitive.
- Type III errors refer to the lack of biological relevance of some microorganisms detected in statistical analyses. This is a crucial issue because the underlying mechanisms are only partially unraveled, and the role of individual microorganisms or microbial communities in the cause of asthma and allergies is not yet understood.
- *Replication of associations.* Replication of associations with environmental exposures is more difficult because of the inherently high variability in exposure over time.<sup>63,83</sup> This raises the following question: When can we speak of replication? To a large extent, this depends on our hypothesis and explanatory models. Do we expect to reproduce the concept of diversity in another study, or do we expect also the same microbial pattern associated with the protective effect for asthma and atopy?<sup>84</sup> Because indications exist that both the environmental and human microbiomes are to some extent variable, this might not be an easy task. What do we expect from future microbial diversity studies? In any case replication requires accurate exposure assessment, and thus repeated measurement studies are needed, embedded in large cohort studies of asthma and allergy.

In conclusion, the recent development of molecular techniques to study the microbiome has opened new approaches that will help in understanding the contribution of environmental exposure to microorganisms and their diversity to human health. The microbiome does not only relate to human body habitats but also to a human subject's exposure to diverse microbial communities in the exterior environment and its potential effect on human health. These developments might require application of new concepts, such as the investigation of microbial ecologic systems rather than the identification of individual pathogens. Many

questions relating to the best methodologies to assess exposures and their effect on human health are still open and need to be explored in a new generation of large interdisciplinary studies.

## REFERENCES

- James AL, Knuiman MW, Divitini ML, Hui J, Hunter M, Palmer LJ, et al. Changes in the prevalence of asthma in adults since 1966: the Busselton health study. *Eur Respir J* 2010;35:273-8.
- Anandan C, Nurmatov U, van Schayck OC, Sheikh A. Is the prevalence of asthma declining? Systematic review of epidemiological studies. *Allergy* 2010;65:152-67.
- Pearce N, Douwes J. Commentary: asthma time trends—mission accomplished? *Int J Epidemiol* 2005;34:1018-9.
- Pearce N, Douwes J, Beasley R. Is allergen exposure the major primary cause of asthma? *Thorax* 2000;55:424-31.
- Hirsch T, Range U, Walther KU, Hederer B, Lassig S, Frey G, et al. Prevalence and determinants of house dust mite allergen in East German homes. *Clin Exp Allergy* 1998;28:956-64.
- Hugg T, Ruotsalainen R, Jaakkola MS, Pushkarev V, Jaakkola JJ. Comparison of allergic diseases, symptoms and respiratory infections between Finnish and Russian school children. *Eur J Epidemiol* 2008;23:123-33.
- Hugg TT, Jaakkola MS, Ruotsalainen R, Pushkarev V, Jaakkola JJ. Exposure to animals and the risk of allergic asthma: a population-based cross-sectional study in Finnish and Russian children. *Environ Health* 2008;7:28.
- Pakarinen J, Hyvarinen A, Salkinoja-Salonen M, Laitinen S, Nevalainen A, Makela MJ, et al. Predominance of Gram-positive bacteria in house dust in the low-allergy risk Russian Karelia. *Environ Microbiol* 2008;10:3317-25.
- von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* 2010;10:861-8.
- 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.
- van Strien RT, Engel R, Holst O, Bufe A, Eder W, Waser M, et al. Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health. *J Allergy Clin Immunol* 2004;113:860-7.
- Schram-Bijkerk D, Doekes G, Douwes J, Boeve M, Riedler J, Ublagger E, et al. Bacterial and fungal agents in house dust and wheeze in children: the PARSIFAL study. *Clin Exp Allergy* 2005;35:1272-8.
- Douwes J, van Strien R, Doekes G, Smit J, Kerkhof M, Gerritsen J, et al. Does early indoor microbial exposure reduce the risk of asthma? The Prevention and Incidence of Asthma and Mite Allergy birth cohort study. *J Allergy Clin Immunol* 2006;117:1067-73.
- Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, et al. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* 2007;119:1140-7.
- Sordillo JE, Alwis UK, Hoffman E, Gold DR, Milton DK. Home characteristics as predictors of bacterial and fungal microbial biomarkers in house dust. *Environ Health Perspect* 2011;119:189-95.
- Sordillo JE, Hoffman EB, Celedon JC, Litonjua AA, Milton DK, Gold DR. Multiple microbial exposures in the home may protect against asthma or allergy in childhood. *Clin Exp Allergy* 2010;40:902-10.
- Perzanowski MS, Miller RL, Thorne PS, Barr RG, Divjan A, Sheares BJ, et al. Endotoxin in inner-city homes: associations with wheeze and eczema in early childhood. *J Allergy Clin Immunol* 2006;117:1082-9.
- Gehring U, Strikwold M, Schram-Bijkerk D, Weinmayr G, Genuneit J, Nagel G, et al. Asthma and allergic symptoms in relation to house dust endotoxin: Phase Two of the International Study on Asthma and Allergies in Childhood (ISAAC II). *Clin Exp Allergy* 2008;38:1911-20.
- Gehring U, Heinrich J, Hoek G, Giovannangelo M, Nordling E, Bellander T, et al. Bacteria and mould components in house dust and children's allergic sensitisation. *Eur Respir J* 2007;29:1144-53.
- Gereda JE, Leung DY, Liu AH. Levels of environmental endotoxin and prevalence of atopic disease. *JAMA* 2000;284:1652-3.
- Gereda JE, Leung DY, Thatayatikom A, Streib JE, Price MR, Klinnert MD, et al. Relation between house-dust endotoxin exposure, type I T-cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet* 2000;355:1680-3.
- Tischer C, Gehring U, Chen CM, Kerkhof M, Koppelman G, Sausenthaler S, et al. Respiratory health in children, and indoor exposure to (1,3)-beta-D-glucan, EPS mould components and endotoxin. *Eur Respir J* 2011;37:1050-9.
- Douwes J, Cheng S, Travier N, Cohet C, Niesink A, McKenzie J, et al. Farm exposure in utero may protect against asthma, hay fever and eczema. *Eur Respir J* 2008;32:603-11.

24. Smit LA, Heederik D, Doekes G, Lammers JW, Wouters IM. Occupational endotoxin exposure reduces the risk of atopic sensitization but increases the risk of bronchial hyperresponsiveness. *Int Arch Allergy Immunol* 2010;152: 151-8.
25. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* 2009;22:240-73.
26. Taylor PR, Tsoni SV, Willment JA, Dennehy KM, Rosas M, Findon H, et al. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol* 2007;8:31-8.
27. Palma AS, Feizi T, Zhang Y, Stoll MS, Lawson AM, Diaz-Rodriguez E, et al. Ligands for the beta-glucan receptor, Dectin-1, assigned using "designer" microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides. *J Biol Chem* 2006;281:5771-9.
28. Omland O. Exposure and respiratory health in farming in temperate zones—a review of the literature. *Ann Agric Environ Med* 2002;9:119-36.
29. Schenker M, Ferguson T, Gamsky T. Respiratory risks associated with agriculture. *Occup Med* 1991;6:415-28.
30. Letourneau V, Duchaine C, Cote C, Letellier A, Topp E, Masse D. Presence of zoonotic pathogens in physico-chemically characterized manures from hog finishing houses using different production systems. *Bioresour Technol* 2010;101:4048-55.
31. Normand AC, Vacheyrou M, Sudre B, Heederik DJ, Piarroux R. Assessment of dust sampling methods for the study of cultivable-microorganism exposure in stables. *Appl Environ Microbiol* 2009;75:7617-23.
32. Roussel S, Sudre B, Reboux G, Waser M, Buchele G, Vacheyrou M, et al. Exposure to moulds and actinomycetes in Alpine farms: a nested environmental study of the PASTURE cohort. *Environ Res* 2011;111:744-50.
33. Korthals M, Ege MJ, Tebbe CC, von Mutius E, Bauer J. Application of PCR-SSCP for molecular epidemiological studies on the exposure of farm children to bacteria in environmental dust. *J Microbiol Methods* 2008;73:49-56.
34. Attwood P, Brouwer R, Ruigewaard P, Versloot P, de Wit R, Heederik D, et al. A study of the relationship between airborne contaminants and environmental factors in Dutch swine confinement buildings. *Am Ind Hyg Assoc J* 1987;48:745-51.
35. Attwood P, Versloot P, Heederik D, de Wit R, Boleij JS. Assessment of dust and endotoxin levels in the working environment of Dutch pig farmers: a preliminary study. *Ann Occup Hyg* 1986;30:201-8.
36. Samadi S, Wouters IM, Houben R, Jamshidifard AR, Van Eerdenburg F, Heederik DJ. Exposure to inhalable dust, endotoxins, beta(1-3)-glucans, and airborne microorganisms in horse stables. *Ann Occup Hyg* 2009;53:595-603.
37. Tavernier G, Fletcher G, Gee I, Watson A, Blacklock G, Francis H, et al. IPEADAM study: indoor endotoxin exposure, family status, and some housing characteristics in English children. *J Allergy Clin Immunol* 2006;117:656-62.
38. Giovannangelo M, Gehring U, Nordling E, Oldenwening M, Terpstra G, Bellander T, et al. Determinants of house dust endotoxin in three European countries—the AIRALLERG study. *Indoor Air* 2007;17:70-9.
39. 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.
40. Tringe SG, Hugenholtz P. A renaissance for the pioneering 16S rRNA gene. *Curr Opin Microbiol* 2008;11:442-6.
41. von Wintzingerode F, Gobel UB, Stackebrandt E. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* 1997;21:213-29.
42. Nehme B, Gilbert Y, Letourneau V, Forster RJ, Veillette M, Villemur R, et al. Culture-independent characterization of archaeal biodiversity in swine confinement building bioaerosols. *Appl Environ Microbiol* 2009;75:5445-50.
43. Brodie EL, DeSantis TZ, Parker JP, Zubietta IX, Piceno YM, Andersen GL. Urban aerosols harbor diverse and dynamic bacterial populations. *Proc Natl Acad Sci U S A* 2007;104:299-304.
44. Bowers RM, McLetchie S, Knight R, Fierer N. Spatial variability in airborne bacterial communities across land-use types and their relationship to the bacterial communities of potential source environments. *ISME J* 2011;5:601-12.
45. Tringe SG, Zhang T, Liu X, Yu Y, Lee WH, Yap J, et al. The airborne metagenome in an indoor urban environment. *PLoS one* 2008;3:e1862.
46. Taubel M, Rintala H, Pitkaranta M, Paulin L, Laitinen S, Pekkanen J, et al. The occupant as a source of house dust bacteria. *J Allergy Clin Immunol* 2009;124: 834-40, e47.
47. Maier RM, Palmer MW, Andersen GL, Halonen MJ, Josephson KC, Maier RS, et al. Environmental determinants of and impact on childhood asthma by the bacterial community in household dust. *Appl Environ Microbiol* 2010;76:2663-7.
48. Lee L, Tin S, Kelley ST. Culture-independent analysis of bacterial diversity in a child-care facility.  *BMC Microbiol* 2007;7:27.
49. Just N, Kirychuk S, Gilbert Y, Letourneau V, Veillette M, Singh B, et al. Bacterial diversity characterization of bioaerosols from cage-housed and floor-housed poultry operations. *Environ Res* 2011;111:492-8.
50. Amend AS, Seifert KA, Samson R, Bruns TD. Indoor fungal composition is geographically patterned and more diverse in temperate zones than in the tropics. *Proc Natl Acad Sci U S A* 2010;107:13748-53.
51. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. *PLoS one* 2010;5:e8578.
52. Huang YJ, Kim E, Cox MJ, Brodie EL, Brown R, Wiener-Kronish JP, et al. A persistent and diverse airway microbiota present during chronic obstructive pulmonary disease exacerbations. *OMICS* 2010;14:9-59.
53. Huang YJ, Nelson CE, Brodie EL, Desantis TZ, Baek MS, Liu J, et al. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J Allergy Clin Immunol* 2011;127:372-81, e1-3.
54. Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, et al. Moving pictures of the human microbiome. *Genome Biol* 2011;12:R50.
55. Bisgaard H, Hermansen MN, Buchvald F, Lohland L, Halkjaer LB, Bonnelykke K, et al. Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007;357:1487-95.
56. von Mutius E. Of attraction and rejection—asthma and the microbial world. *N Engl J Med* 2007;357:1545-7.
57. Adlerberth I, Strachan DP, Matricardi PM, Ahrne S, Orfei L, Aberg N, et al. Gut microbiota and development of atopic eczema in 3 European birth cohorts. *J Allergy Clin Immunol* 2007;120:343-50.
58. Bisgaard H, Li N, Bonnelykke K, Chaves BL, Skov T, Paludan-Muller G, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol* 2011;128: 646-52, e1-5.
59. Sjogren YM, Jenmalm MC, Bottcher MF, Bjorksten B, Sverremark-Ekstrom E. Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clin Exp Allergy* 2009;39:518-26.
60. Stsepitova J, Sepp E, Julge K, Vaughan E, Mikelsaar M, de Vos WM. Molecularly assessed shifts of *Bifidobacterium* spp. and less diverse microbial communities are characteristic of 5-year-old allergic children. *FEMS Immunol Med Microbiol* 2007;51:260-9.
61. Wang M, Karlsson C, Olsson C, Adlerberth I, Wold AE, Strachan DP, et al. Reduced diversity in the early fecal microbiota of infants with atopic eczema. *J Allergy Clin Immunol* 2008;121:129-34.
62. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, et al. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* 2011;128:766-73, e4.
63. Vineis P, Khan AE, Vlaanderen J, Vermeulen R. The impact of new research technologies on our understanding of environmental causes of disease: the concept of clinical vulnerability. *Environ Health* 2009;8:54.
64. Ege MJ, Strachan DP, Cookson WO, Moffatt MF, Gut I, Lathrop M, et al. Gene-environment interaction for childhood asthma and exposure to farming in Central Europe. *J Allergy Clin Immunol* 2011;127:138-44, e1-4.
65. Conrad ML, Ferstl R, Teich R, Brand S, Blumer N, Yildirim AO, et al. Maternal TLR signaling is required for prenatal asthma protection by the nonpathogenic microbe *Acinetobacter lwoffii* F78. *J Exp Med* 2009;206:2869-77.
66. Debarry J, Garn H, Hanuszkiewicz A, Dickgreber N, Blumer N, von Mutius E, et al. *Acinetobacter lwoffii* and *Lactococcus lactis* strains isolated from farm cowsheds possess strong allergy-protective properties. *The J Allergy Clin Immunol* 2007;119:1514-21.
67. Debarry J, Hanuszkiewicz A, Stein K, Holst O, Heine H. The allergy-protective properties of *Acinetobacter lwoffii* F78 are imparted by its lipopolysaccharide. *Allergy* 2010;65:690-7.
68. Hansen LT, Gill TA. Solubility and antimicrobial efficacy of protamine on *Listeria monocytogenes* and *Escherichia coli* as influenced by pH. *J Appl Microbiol* 2000; 88:1049-55.
69. Blumer N, Sel S, Virna S, Patrascan CC, Zimmermann S, Herz U, et al. Perinatal maternal application of *Lactobacillus rhamnosus* GG suppresses allergic airway inflammation in mouse offspring. *Clin Exp Allergy* 2007;37: 348-57.
70. Vogel K, Blumer N, Korthals M, Mittelstadt J, Garn H, Ege M, et al. Animal shed *Bacillus licheniformis* spores possess allergy-protective as well as inflammatory properties. *J Allergy Clin Immunol* 2008;122:307-12, e1-8.
71. Chu HW, Honour JM, Rawlinson CA, Harbeck RJ, Martin RJ. Hygiene hypothesis of asthma: a murine asthma model with *Mycoplasma pneumoniae* infection. *Chest* 2003;123(suppl):390S.
72. Chu HW, Honour JM, Rawlinson CA, Harbeck RJ, Martin RJ. Effects of respiratory *Mycoplasma pneumoniae* infection on allergen-induced bronchial hyperresponsiveness and lung inflammation in mice. *Infect Immun* 2003;71: 1520-6.
73. Korf J, Stoltz A, Verschoor J, De Baetselier P, Grooten J. The *Mycobacterium tuberculosis* cell wall component mycolic acid elicits pathogen-associated host innate immune responses. *Eur J Immunol* 2005;35:890-900.

74. Williams P, Winzer K, Chan WC, Camara M. Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond B Biol Sci* 2007;362:1119-34.
75. Khajanchi BK, Kirtley ML, Brackman SM, Chopra AK. Immunomodulatory and protective roles of quorum-sensing signaling molecules N-acyl homoserine lactones during infection of mice with *Aeromonas hydrophila*. *Infect Immun* 2011;79:2646-57.
76. Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, et al. The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. *Chem Biol* 2007;14:87-96.
77. Wild CP. Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. *Cancer Epidemiol Biomarkers Prev* 2005;14:1847-50.
78. Noss I, Wouters IM, Visser M, Heederik DJ, Thorne PS, Brunekreef B, et al. Evaluation of a low-cost electrostatic dust fall collector for indoor air endotoxin exposure assessment. *Appl Environ Microbiol* 2008;74:5621-7.
79. Horick N, Weller E, Milton DK, Gold DR, Li R, Spiegelman D. Home endotoxin exposure and wheeze in infants: correction for bias due to exposure measurement error. *Environ Health Perspect* 2006;114:135-40.
80. Topp R, Wimmer K, Fahlbusch B, Bischof W, Richter K, Wichmann HE, et al. Repeated measurements of allergens and endotoxin in settled house dust over a time period of 6 years. *Clin Exp Allergy* 2003;33:1659-66.
81. Giovannangelo M, Nordling E, Gehring U, Oldenwening M, Bellander T, Heinrich J, et al. Variation of biocontaminant levels within and between homes—the AIR-ALLERG study. *J Expo Sci Environ Epidemiol* 2007;17:134-40.
82. Verhoeff AP, van Wijnen JH, Brunekreef B, Fischer P, van Reenen-Hoekstra ES, Samson RA. Presence of viable mould propagules in indoor air in relation to house damp and outdoor air. *Allergy* 1992;47:83-91.
83. Foxman B, Goldberg D. Why the human microbiome project should motivate epidemiologists to learn ecology. *Epidemiology* 2010;21:757-9.
84. Foxman B, Goldberg D, Murdock C, Xi C, Gilsdorf JR. Conceptualizing human microbiota: from multicelled organ to ecological community. *Interdiscip Perspect Infect Dis* 2008;2008:613979.