**Microbes and asthma: opportunities for intervention**

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Abbreviations: allergic bronchopulmonary mycosis (ABPM), dendritic cells (DC), G-protein coupled receptor 41 (GPR41), γ-glutamyl transpeptidase (GGT), immunoglobulin E (IgE), interferon-gamma (IFN-γ), long-acting β-agonists (LABA), natural killer T cells (NKT), regulatory T (Treg) and B (Breg) cells, respiratory syncytial virus (RSV), Toll-like receptor (TLR), short-chain fatty acids (SCFAs), Thelper 2 cell (Th2 cell), Trichuris suis eggs (TSO), vacuolating cytotoxin (VacA).

**ABSTRACT**

The worldwide incidence and prevalence of asthma continues to increase. Asthma is now understood as an umbrella term for different phenotypes or endotypes which arise through different pathophysiological pathways. Understanding the many factors contributing to the development of the disease is important for the identification of novel therapeutic targets for treatment of certain asthma phenotypes.

The hygiene hypothesis has been formulated to explain the increasing prevalence of allergic disease, including asthma. This hypothesis postulates that decreased exposure to certain infectious agents, as a result of improved hygiene, increased antibiotic use and vaccination, as well as changes in lifestyle and dietary habits, is associated with changes in the immune system, which predispose individuals to allergy. Many microbes, during their co-evolution with humans, developed mechanisms to manipulate the human immune system and to increase their chances of survival.

Improving models of asthma as well as choosing adequate endpoints in clinical trials will lead to a more complete understanding of the underlying mechanisms, thus providing an opportunity to devise primary and secondary interventions at the same time as identifying new molecular targets for treatment. This article reports the discussion and conclusion of a workshop under the auspices of the Netherlands Lung Foundation, to extend our understanding of how modulation of the immune system by bacterial, parasitic and viral infections may impact on the development of asthma, and to map out future lines of investigation.

Keywords: Hygiene hypothesis, asthma, sensitization, microbes, microbiome; helminths, viruses, immune regulation

In recent decades, there has been a marked increase in the incidence of many non-communicable diseases, including asthma, which is now estimated to affect 300 million individuals worldwide.(1) Patients with asthma suffer from a variable degree of airflow obstruction, breathlessness, and bronchial hyperresponsiveness, associated with chronic airway inflammation and excessive mucus production. Various specific and unspecific triggers have been identified which can lead to an increase in inflammation, obstruction and symptoms. Traditionally, asthma, and especially allergic asthma, has been considered as an inflammatory disease associated with T-helper (Th) 2 cells, production of IgE antibodies, accumulation of eosinophils in the lungs and goblet cell hyperplasia. It is now recognized that asthma is a complex syndrome in which many different phenotypes exist, including early onset allergic asthma, late onset eosinophilic asthma, exercise-induced, obesity related and non-eosinophilic asthma.(2) Recently, the definition of asthma has shifted further with the introduction of endotypes, which distinguish asthma variants by their underlying molecular mechanisms. Probably the best described endotype is the Type 2-induced form of disease.(3) Other endotypes are less well defined and include patients without Type 2 –induced airway inflammation (probably driven by Th1 or Th17 cells) and allergic bronchopulmonary mycosis (ABPM) as an asthma endotype.(4)

Most asthma patients have a mild form of the disease, which can be managed with inhaled corticosteroids and long-acting β-agonists (LABA). However, patients with more severe disease and particularly those with a non-Th2 endotype might not respond well to currently available therapies. Particularly in patients with asthma, personalized medicine may open novel approaches to accommodate the heterogeneity of the disease. asthma better understanding of mechanisms and endotypes will provide opportunities both for prevention and for causal treatment.

In the last years interactions of microbes, including worm parasites, with their host have been identified: Exposure to microorganisms not only triggers but also effectively suppresses immune responses, and beneficial effects of microorganisms are increasingly recognized and mechanistically understood. Strategies are emerging to potentially implement these effects in novel interventions to prevent or treat allergic diseases such as allergic asthma (figure 1). To endorse such strategies and to improve and refine interventions, a better understanding of the disease in its many guises at a basic level is needed. In this context, a group of clinicians and basic scientists with wide-ranging fields of expertise convened in Amersfoort, Netherlands, under the auspices of the Netherlands Lung Foundation for a workshop to assess our current understanding of the disease and identify challenges and opportunities for the prevention and treatment of asthma with microbial intervention, as the guiding theme for the workshop.

**Hygiene Hypothesis and ‘Old Friends’ Hypothesis**

The so-called hygiene hypothesis is frequently invoked to help explain the increasing prevalence of asthma. The hypothesis has its origins in observations published in 1989 by Strachan(5), who noted that declining family size was associated with hay fever in developed countries and suggested that this might be related to a lower degree of sibling-related childhood infections and microbial exposure. In extension of the hygiene hypothesis, Rook has postulated the ‘Old friends’ hypothesis(6), in which many infectious agents and microbes in their co-evolution with humans, have developed mechanisms to modulate and evade the host immune system (Figure 1). Immunomodulatory microorganisms have been described to activate various cells of the regulatory network, such regulatory T (Treg) and B (Breg) cells and to modulate or even re-program certain antigen-presenting cells, leading to tolerogenic dendritic cells (DCs) and/or alternatively-activated macrophages. A more detailed understanding of how these infectious agents accomplish this, can provide indicators for primary prevention strategies and may help to identify new molecular targets for novel treatments. This is especially relevant since in various non-communicable inflammatory diseases, such as asthma, these regulatory networks seemed to be underrepresented and poorly developed.

***Rural exposure and ‘archaic’ microbiome***

As discussed above, microbes (‘old friends’) form a central part of the (extended) hygiene hypothesis.(6;7) Interestingly, this has not so much to do with ‘personal hygiene’ (as often interpreted from the hygiene hypothesis), as a recent study showed that personal or home cleanliness was not associated with a risk to develop asthma or allergy.(8) The ‘old friends’ mostly accounts for a group of microbes that the human race has co-evolved with and that in the past 50 years was rapidly lost due to changes in life style, living conditions or occupations. Prime candidates are microbes associated with rural living, like farming, and various members of an ‘archaic’ microbiome, responsible for a richer composition of our personal microbial hemisphere, which includes compartments such as gut, lung and skin. In this perspective, helminths are regarded as a natural and ancient (evolutionary conserved) partner of the microbial community, which is still the case in many parts of the world but no longer in Westernized countries. The likelihood that archaic microbes play an essential role in protection against asthma and allergic diseases is framed by several landmark studies.

Several studies noted that living on farms offers a protective effect against atopy, hay fever, and asthma, especially in children.(9) Further analysis suggested a link with increased exposure to a variety of bacteria and fungi related to farming and protection from asthma.(10) Interestingly, several gene environment interactions were found for early farm exposure. A number of single nucleotide polymorphisms in children living in rural Europe were linked to farming, e.g. in the genes transcribing CD14 or Toll-like receptors.(11) Remarkably, a recent farm-study also reports on associations with the ‘asthma risk’ alleles on chromosome 17q21, suggesting two faces: the same genotype constitutes a genetic risk to asthma, but at the same time is susceptible to environmental influences.(12) This would imply options for future preventive strategies. In addition, in particular farm exposure during pregnancy seems to influence gene expression patterns by DNA methylation in specific asthma- and allergy-related genes further contributing to its protective effect.(13) In big contrast, a higher prevalence of asthma and a higher morbidity can be found in children living in inner cities. Various environmental risk factors have been recognized, including high indoor allergen, pollutant and endotoxin exposure(14), although part of this effect might be explained by differences in ethnicity and demographics.(15)

Also the composition of gut microbiota influences the development of allergic diseases such as asthma. Indeed, intestinal gene expression and ultimately immune system development is different in “germ-free” mice, lacking a gut microbiome, compared to conventional mice.(16) In addition, germ-free mice exhibit increased susceptibility to allergen-induced airway disease and this has been linked to different mechanisms such as elevated IgE production and basophil numbers at mucosal sites as well as the induction of natural killer T cells (NKT).(17-19) Also in humans the composition of gut microbiota has been linked to airway disease, as stool samples from babies that later on develop allergies and asthma have a different composition and contain less Lactobacilli, Bacteroidetes and Bifidobacteria.(20)

Finally, early and chronic parasitic worm infections of, or linked to, the gastrointestinal tract protected against autoimmune diseases such as multiple sclerosis(21) and inflammatory bowel disease.(22) This effect has also been observed for respiratory allergies and, in the case of hookworm infections also for asthma.(23) Nevertheless, not all parasitic worm infections are protective: in particular early and chronic infections tend to be protective, while in some studies low-burden and sporadic infection were associated with enhanced allergic reactions.(24) -Interestingly, some studies have suggested a possible interaction between gut parasites and microbiota, as they both inhabit the same organ. For example, helminth infection in humans is linked to an increased diversity of the microbiota(25;26). Furthermore, murine *H. polygyrus* promotes the colonization of Lactobacillus spp. Interestingly, helminth-modified microbiota mediated protection against experimental allergic asthma.(27;28) Knowledge on the interaction between worm parasites and the microbiota is still in its infancy and what factors are crucially involved is subject of future research.

**Respiratory Viruses**

Unlike parasitic and bacterial infections, respiratory viral infections are not associated with protection against atopy or asthma. In contrast, bronchiolitis induced by respiratory syncytial virus (RSV) or rhinovirus have been consistently associated with increased risk of later asthma in numerous studies.(29;30) For example, in a prospective cohort study of children hospitalized for respiratory syncytial virus (RSV) bronchiolitis in the first year of life Sigurs et al(31) found that viral hospitalized bronchiolitis was significantly associated with asthma (defined as three episodes of bronchial obstruction) at 3 years of age, which persisted into early adulthood.(32) The COAST study(33) found that in children selected for a high risk of asthma development, rhinovirus wheeze was the strongest predictor of wheezing at the age of 3 years(34) and this effect was carried over to 6 years of age.(35) Importantly, allergic sensitization (as indicated by measurement of allergen-specific IgE) preceded the onset of recurrent viral wheeze. These findings, also supported by others(36;37), also suggest that rhinovirus wheezing can be used as a marker for children at risk of developing asthma.

Interestingly, some recent studies have suggested a putative interplay between viruses and pathogenic bacteria in the nose and upper respiratory tract of young children, based positive associations between the bacteria *H. Influenza, S. pneumonia, S. Aureus, M. catarrhalis* and/or rhinoviruses and RSV.(38;39) Importantly, the presence of these pathogenic bacteria was associated with increased respiratory symptoms and asthma exacerbations.(40) Though the question remains whether the colonization of those pathogenic bacteria precedes the respiratory viral infections and asthma symptoms or whether this is the consequence of persistent viral infections in the upper airways. New preventive strategies could be designed based on disrupting those reinforcing interactions between viruses and pathogenic bacteria.

**Opportunities for Intervention**

Several new therapies in the pipelines of the pharmaceutical industry will enter the market in the coming years. These therapies cover a number of different molecular targets, thereby enabling the tailoring of therapy according to a particular endotype. However, many of these new therapies focus on controlling Th2 induction and so are unlikely to cover all patient needs.(41) Prevention of the development of asthma may therefore be a more efficient and sustainable approach to reducing the disease burden in the long term.

***Primary Prevention***

As discussed above, exposure to microorganisms may afford a potential protective effect and a number of mechanisms have been proposed to explain the effect. An important question is whether these mechanisms can be harnessed to develop interventions to reduce the burden of asthma. One of the most promising approaches seems to be primary prevention. However, timing is crucial: after birth, the window of opportunity for primary prevention rapidly closes as the immune system matures. It is important to identify individuals who are at risk of developing asthma at an early stage in order to target such interventions effectively. To increase the effectivity of such interventions some studies suggest that preventive strategies should already be started before birth. Prenatal priming may induce or reset long-term epigenetic check-points allowing the immature immune system to respond sufficiently to microbial stimuli and quickly develop a strong and sustained regulatory network.

*Farm bacteria*

A possible source of the bacterial diversity encountered in farm living is stable dust, a rich source of a highly diverse bacterial ecosystem. From this large pool of farm-related micro-organisms, two species in particular, *Acinetobacter lwoffii* F78 and *Lactococcus lactis* G121, have been tested and potently inhibited allergic reactions in mice.(42) Interestingly, a recent study shows that farm dust reduced the production of innate type 2 cytokines by epithelial cells, which was attributed to the ubiquitin-modifying enzyme A20 in lung epithelium.(43)Not only neonate – but also maternal exposure to microorganisms can reduce the risk of offspring of developing allergic diseases such as asthma. Epigenetic changes following farm exposure, may be responsible for increasing the number and function of cord blood Treg cells.(44) This may then lead to lower Th2 cytokine secretion and lymphocyte proliferation upon innate exposure. In the specific case of exposure to *Acinetobacter lwoffii* F78, a murine model suggested that maternal bacterial exposures was directly related to functional maternal TLR signalling, resulting in asthma protection in the progeny.(45;46) The mechanism was IFN-γ dependent, possibly through protection from loss of IFN-γ promotor-associated histone 4 (H4) acetylation.(46)

*Bacteria in the Lungs*

Also the lungs support a complex microbiota originating from inhaled microbes and flora from the digestive system.(47) Low microbial diversity in the lung has been found in diseases such as asthma (with an increased proportion of *Proteobacteria* such as *Haemophilus,* *Neisseria* or *Streptococcus*). Absence of a lung microbiota in germ-free mice is associated with increased Th2 responses and increased allergic airway disease suggesting a strong inhibitory effect of the lung microbiota on Th2 development.(48) Furthermore, new studies have linked the composition of the lung microbiome to therapy responses to corticosteroids by uncovering a difference in microbiota composition in steroid responsive and steroid resistant patients.(49) New therapies targeting the lung microbiome would be an interesting approach in the prevention or early treatment of asthma. However, in this stage more information is needed on the ideal composition of a ‘healthy’ lung microbiome or alternatively which bacteria species should be avoided or removed before new therapeutic strategies can be designed.

*Gut Bacterial Biodiversity and Bacterial Intestinal Infections*

Recently published work on a murine model suggests that the gut microbiota metabolizes dietary fibres resulting in increased circulating short-chain fatty acids (SCFAs).(50) Importantly, the authors demonstrated that SCFAs, via ligation of G-protein coupled receptor 41 (GPR41), ultimately induced seeding of the lungs with DCs with an impaired ability to promote Th2 cell effector function (but high phagocytic capacity). These results therefore suggest a mechanism whereby diet, especially its fibre content, in association with the intestinal microbiota could have a direct influence on the development of asthma.

In addition to gut microbial biodiversity and diet, individual gut bacteria such as *Helicobacter pylori* (*H. pylori*) have received considerable attention recently. *H. pylori* infection is better known for its pathogenic properties, bening linked to conditions such as peptic ulcer and gastric cancer.(51) However, infection by *H. pylori*, especially in early childhood, may confer benefits as protective effects were described of *H. pylori* infection against the development of asthma and allergies.(52;53) Also when mice were infected during the neonatal period, they were subsequently protected against the development of allergic airway disease.(54) The mechanisms by which this protection is enforced involve the induction of Treg cells and reprogramming of DCs towards a tolerogenic phenotype.(55) Various persistence determinants of *H. pylori*, the γ-glutamyl transpeptidase (GGT), vacuolating cytotoxin (VacA) and urease, have been shown to be critically important to the protective effects and are currently evaluated for further therapeutic applications.(56;57)

The evidence from clinical trials for the effectiveness of primary prevention by the use of probiotic strains is at present, patchy. Trials are heterogeneous with respect to e.g. timing of the intervention, bacterial strain, and use of mono- or combination therapy.(58) Endpoints are also somewhat varied, with few studies focussing on asthma itself. Findings can vary according to the population included, as evidenced by the different results with *Lactobacillus GG* reported with the same treatment protocol and endpoints. In these studies an effect was seen for mothers who had at least one first-degree relative [or partner] with atopic eczema, allergic rhinitis, or asthma (59) but not when at least one member [mother, father, or child] had atopic disease (60). Taken together, the body of evidence to date suggests that a combined antenatal and postnatal approach is the most promising, while monotherapy with *Lactobacilli* seems to be the most promising agent, although there is plenty of room for improvement in the quality of the clinical trials. In particular, there is a need for validated surrogate endpoints for the development of asthma to provide a read-out more quickly than would be possible using asthma itself. Finally, the ethics and safety concerns of prenatal exposure need to be taken into account.

*Worm Parasites*

It is not surprising that organisms that co-evolved with humans have developed mechanisms for modulating human inflammatory responses to promote their own survival. In general, worm parasites seem to be able to manipulate both innate immunity (by affecting toll-like receptor [TLR]-induced responses and triggering the inflammasome (61)) and adaptive immunity.(62) In the case of adaptive immunity, unlike bacteria, helminths strongly induce Th2 responses and elevated IgE.(63) Interestingly though, this Th2 response is not associated with an increased predisposition to asthma. In fact, the IgE detected is primarily cross-reactive to carbohydrate epitopes present in parasites and not to the protein component of allergens, and does not lead to mast cell degranulation. Currently, it is hypothesized that cross-reactive IgE may help to prevent atopic sensitization and the development of allergic diseases in children with helminth infections. Knowledge on the processes that lead to IgE immunoglobulins against cross-reactive carbohydrates may help to implement these strategies in children at risk and prevent the development of high-affinity IgE molecules against proteins of allergens and thereby clinical symptoms (64)

Helminths are also master inducers of immunoregulatory processes.(65) Detailed studies in schistosome-infected mice or humans have shown increased numbers of Breg and Treg cells with an enhanced regulatory capacity, which at least in mice, were crucial for protection against allergic airway disease (66-69) Also infections with *H. polygyrus* or exposure to its excretory/secretory antigens (HES - collected from cultures of live adults) could prevent experimentally induced airway allergy(70;71) and this has been linked to suppression of IL-33 release.(72) Ultimately, the goal of future work in this line of research is to identify the immunomodulators within the HES fraction and translate these molecules into novel preventive therapies.

Interestingly, prenatal parasite exposure of the mother may also confer protection against allergic diseases, as illustrated by the higher incidence of eczema among Ugandan children born to schistosome-infected Ugandan mothers who had been treated with the deworming agent praziquantel during pregnancy.(73) Furthermore, mouse studies of maternal schistosome infection have pointed in the same direction and showed a crucial role for the maternal cytokine milieu within the placenta ultimately determining whether allergic responses are promoted or suppressed in the offspring.(74;75)

***Secondary and tertiary prevention***

Given the effects of certain microbes in the context of primary interventions, the possibilities for secondary and tertiary prevention are less well investigated. Secondary prevention aims to detect and treat disease that has not yet become symptomatic, whereas tertiary prevention is directed at those who already have symptomatic disease, to prevent further deterioration. These approaches include anti-inflammatory treatment, treatment with anti-viral agents, and administration of non-viable lysates from the bacterial causative agents of acute respiratory tract illness. Recent trials suggest that systemic corticosteroid treatment is beneficial in first-time wheezing children susceptible to rhinovirus infection, especially in those with high virus loads at presentation.(76;77) The marked reduction of relapses and asthma during long-term follow-up supports the role for high rhinovirus load as an important marker of those children with early pulmonary inflammation that may benefit from early intervention with anti-inflammatory treatment. The importance of airway inflammatory or allergen exposure control is also supported by trial with the anti-IgE omalizumab which nearly abolished autumn peaks in exacerbations typically caused by viral infections.(78)

Prevention of repeated RSV-associated wheezing, which has also been associated with asthma, is another potential area for intervention. Monthly injections of palivizumab, a humanized anti-RSV monoclonal antibody, has been shown to effectively reduced recurrent wheeze during the first year of life (79). Since children with recurrent infant wheeze are at a high risk of asthma development, it could be speculated that the intervention also reduces the risk of developing asthma. While this trial is undoubtedly valuable for understanding the mechanisms by which viral infections can lead to asthma, such treatments for RSV are expensive. Furthermore, in the case of rhinovirus, the variability in the virus (> 100 serotypes) is a barrier to developing effective monoclonal antibodies against infection.

Finally, evidence from mouse models suggests that bacterial lysates can reduce airway inflammation and lead to selective recruitment of Treg cells in the tracheal compartment (80), while evidence from clinical studies suggests that treatment increases secretory IgA levels at mucosal surfaces. Analysis of the clinical trials in this setting provide at best only weak support for a beneficial effect of the intervention. The most promising agent seems to be the bacterial lysate (OM-85 BV), which has been shown to prevent wheezing attacks provoked by acute respiratory tract illnesses in children.(81) Similarly, studies have been conducted to treat allergic rhinitis patients with Trichuris suis eggs (TSO)(82) or asthma patients with hookworm larvae(83), but did not show major clinical improvement of the disease symptoms. It is unclear whether the lack of effect can be explained by the type of worm, the dosing, the timing, the duration or the disease state.(24;84) In general though, the studies conducted to date often are inadequately designed or lack statistical power.

***Treatment of asthma***

Increasing knowledge of the immunomodulatory effects of microbial-host interaction might also offer a chance to develop novel therapeutic treatments for patients with asthma. Indeed, treatment of the disease is mainly based a one-size-fits-all approaches, mostly relying on inhaled corticosteroids as anti-inflammatory treatment. One example of transferring the understanding of the pathophysiological mechanism into therapeutic approaches has been the development and use of monoclonal antibodies for the treatment of severe asthma. Indeed, several novel opportunities are now available to specifically target Type 2 driven inflammation(41) . The increasing knowledge of the impact of microbial-host interaction on allergy and asthma may also result in novel therapeutic approaches. Whether microbial-based interventions in patients are useful first needs to be assessed in therapeutic mouse models. Indeed, one promising study showed that microbial interventions also work as a treatment for experimental allergic airway inflammation.(50) Treatment using the short chain fatty acid propionate modulates the intestinal microbiome and effectively treats allergic airway disease in adult mice, suggesting that microbial interventions might not only be used for preventive approaches.(50) However, so far only little data is available from human patients. Bacterial derived lysates have been used in infancy to prevent the development of atopy(85), but only few studies have investigated these compounds in patients with already established disease. Compounds targeting certain TLRs have been used in patients with asthma with somewhat conflicting results. Administration of a TLR9 agonist resulted in improved asthma control during steroid reduction in patients on moderate or high-dose inhaled steroids (86) whereas a different TLR9 agonist showed no additional benefit in patients with insufficiently controlled moderate-to-severe allergic asthma.(87) Whether microbial compounds will be a useful addition to the current therapeutic options needs to be determined in further studies.

**Conclusions**

For the foreseeable future, asthma will remain a disease difficult to prevent or to cure. Microbial infections and certain microbial metabolites and secretions seem to point at protection against asthma and offer numerous interesting opportunities. To reduce the overall burden of disease, primary or secondary prevention seems the most promising approach. Identification of populations at risk is of utmost importance to ensure that primary prevention is delivered where it is most needed. Indeed, there is some evidence to support the effectiveness of interventions in pregnant women, although ethical and safety issues would need to be carefully addressed and the risk-balance equation would need to be accurately assessed. Beyond the primary prevention setting, there are opportunities for secondary or tertiary intervention but earlier interventions are more likely to be successful. Finally, in the treatment setting, the goal of new asthma treatments should be disease modification. In this sense, a focus on underlying immune mechanisms would seem the approach most likely to deliver promising results but the effects of alterations of the structural compartment should not be ignored. A shift to animal models that better represent the complex conditions and phenotypes of human asthma would not only enhance our understanding of the disease, but also help identify better therapeutic candidates. Given that asthma develops over a long period of time, robust clinical trials can be challenging, but are needed to assess the effectiveness novel therapeutic approaches. In addition, it is necessary to elucidate if these novel approaches only offer prevention of airway disease or if they also offer a therapeutic benefit in already established disease.

Acknowledgements: We thank the Lung Foundation Netherlands for hosting and organising this 2-day workshop in Amersfoort, The Netherlands.

**Figure 1. Schematic overview of the hygiene/’old friends’ hypothesis**. Several microbial signals, such as environmental exposure (rural/farm environment) and an archaic microbiota (containing more diverse and abundant bacteria and typical helminth parasites) provide strong signals to develop regulatory responses (tolerance), whereas a urbanized environment and/or a westernised microbiota does not, tipping the immune balance more towards inflammation. Tolerance leads to good health while in its absence an inflammatory profile leads to airway diseases, such as respiratory allergies and asthma. Composition and diversity of the microbiota is affected by diet. Viral infections, sinusitis and air pollution trigger the immune system to move towards inflammation while a diverse microbiota may reduce these inflammatory signals.

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