# Protective and risk factors for childhood asthma in observational birth cohorts: the role of vitamin D and allergic rhinitis

**Inauguraldissertation**

zur Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

**Mascha Rochat**
aus Le Lieu, Vaud, Schweiz

Basel, 2015

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von Prof Dr. med. Charlotte Braun-Fahrländer und Prof. Dr. Christian Lengeler

Basel, den 09. Dezember 2014

Prof. Dr. Jörg Schibler
Dekan

**Table of Contents**

## Summary

**Background:** Asthma is an inflammatory disease of the airways of the lung. There has been an increase in the prevalence of asthma and other allergic diseases over the past decades and, due to its high prevalence, it is of major public health concern. The reasons for this increase are still largely unknown, but interactions between various types of environmental exposures in populations with different genetic backgrounds have been proposed. Complicating matters, childhood asthma is not one disease but rather a syndrome characterised by several distinct wheezing phenotypes, rendering research even more challenging. The best way to research risk and protective factors is with birth cohorts that follow children from birth over the first years of their life, allowing temporal sequence of exposure and the development of asthma or allergic diseases to be analysed.

**Aim:** The aim of the present thesis was to achieve an overview of birth cohorts on asthma and allergic diseases; to investigate potential risk and potential protective factors for the development of asthma in childhood; and to determine whether a tool to help monitor children with respiratory diseases throughout their life would be necessary and helpful.

**Methods:** The overview of birth cohorts was achieved with a systematic PubMed search of all existing birth cohorts on asthma and allergic diseases. Vitamin D was investigated as a potential protective factor within the Protection against Allergy-Study in Rural Environments (PASTURE) birth cohort, which is an ongoing observational birth cohort on the development of allergic diseases in 5 European countries. Over 1,100 pregnant women were enrolled and their offspring is being followed up to the age of 6 years.

Allergic rhinitis was investigated as a potential risk factor within the German Multicentre Atopy (MAS) study which is another observational birth cohort on the development of atopic diseases in early childhood in five German cities. Over 1,300 children were followed from birth to the age of 13 years.

Lung function reference equations were developed using data from the LuftiBus project, (Germany, Switzerland, Austria, Finland and France)which is a mobile bus equipped with flow-sensing spirometers that tours the greater Zurich (Switzerland) area and offers spirometry measurements to the general population. Spirometry data are recorded electronically along with data on basic health and lifestyle information.

**Results:** An overview of birth cohorts on asthma and allergic diseases with special emphasis on risk and protective factors is presented.

Within the PASTURE cohort, vitamin D supplementation during pregnancy was shown to increase cord blood mRNA levels of Immunoglobulin-Like Transcripts (ILT)3 and ILT4, which are two inhibitory receptors on tolerogenic dendritic cells. This finding may point towards an early induction of tolerogenic immune responses by maternal vitamin D intake, potentially influencing the prenatal immune system and possibly the development of atopic diseases.

Within the MAS cohort, allergic rhinitis in preschool children was shown to be a predictor for subsequent wheezing onset. Preschool children with rhinitis might thus benefit from early assessment of allergic sensitization to identify the children at high risk of wheezing.

Within the LuftiBus project, the lung function data allowed the development of spirometry reference equations for a central European population between 8 and 90 years of age that can be implemented in a wide range of clinical settings.

**Conclusion:** During the last decades many risk and protective factors for the development of asthma were identified but only a few have enough evidence to issue population based recommendations. As protective factor, vitamin D supplementation during pregnancy may influence the prenatal immune system and play a role in the development of atopic diseases. As risk factor, allergic rhinitis was found to be a precursor for subsequent wheezing onset. Both factors can be influenced and prospective studies are needed to determine whether they may change the prevalence of asthma and other allergic diseases. The spirometry reference equations developed are a useful tool for clinicians working in central Europe and following patients over years. As they spans across all ages they are practical to track disease progression from childhood to adulthood and assess effectiveness of therapy over time.

The PhD thesis allowed a few more insights to be added to the complex entity of respiratory diseases in childhood, helping to understand risk and protective factors. The spirometry tool developed may help clinicians monitor children with respiratory diseases throughout their life.

In the years to come, more research is needed to determine whether the protective and risk factors studied can be influenced and whether their alteration has any effect on the incidence of asthma.

# Chapter I: General introduction and background

Asthma is a chronic inflammatory disease of the airways of the lung that causes recurrent episodes of breathlessness, cough, wheezing and chest tightness in susceptible individuals. [1]. It is a heterogeneous condition with variable signs and symptoms in patient groups, as well as variability within each individual patient over time [2]. Small children begin with wheezing symptoms, and, based on the time of onset, the different risk factors and determinants, the wheezing either resolves or can progress into asthma and may persist into adulthood [2]. Taking into account these different phenotypes, it can be postulated that asthma may not be one disease but rather a syndrome and that distinct mechanisms underlie the apparently equivalent clinical manifestations. Current asthma therapies are effective in cases of mild asthma, but severe asthma remains very difficult to treat. Around 80% of the cost arises from the 20% of individuals with severe disease. According to the Asthma and Allergy Foundation of America, asthma is among the most common chronic condition of children, producing the greatest number of school absences, and is the third ranking cause of childhood hospitalization [3]. In Europe around 30 million adults and children suffer from asthma [4]. In 2007, the total incremental cost of asthma to society in the USA was estimated at $56 billion, with productivity losses caused by morbidity accounting for $3.8 billion and productivity losses caused by mortality accounting for $2.1 billion [5].

## Epidemiology of childhood asthma

The fact that the prevalence of asthma and allergic diseases increased at a higher than expected rate over the past 40-50 years [6-8] resulted in a great amount of research, mainly due to the public health concerns of this intriguing phenomenon.

In 1976 Gerrard et al. proposed that it was ñthe price to be paid by the white community é for their relative freedom from diseases due to viruses, bacteria and helminthsò [9] and in 1989 Strachan introduced the idea of the nowadays well known hygiene hypothesis, after observing a decreased risk of hay fever with increasing birth order [10]. The initial definition of the hygiene hypothesis has since been broadened, because of the observation that the ñindustrialized way of lifeò at large, with its reduced microbial exposure, may be responsible for the increased prevalence of atopic diseases. As a result, an increasing number of studies were performed, focusing not only on risk but also on protective factors for the development of atopic diseases and asthma with the aim of finding the cause of the diseases and the reason for their increase. Research groups have focused on understanding their onset, their risk and

protective factors, their pathogenesis and finally on finding treatments [11]. However, after decades, the reasons for the increase are still largely unknown, but interactions between various types of environmental exposures in populations with distinct genetic backgrounds have been proposed [7].

Research has focused on a number of environmental contributors such as stress [12], nutrition, [13] or second-hand smoke [14] to name a few, but also on the lung [15] or the immune system [16] development or on genetic factors [17]. Achieving an overview of the potential causes for the development of asthma is challenging but was one of the aims of the present dissertation.

## Types of studies

The study of any disease begins with its description, the identification of the population at risk and the diseaseôs natural history. Epidemiology then aims at uncovering the intrinsic and extrinsic determinants of the disease [11].

Research often starts with cross sectional studies which allow the identification of risk and protective factors. Cross sectional studies measure simultaneously the prevalence of exposures and diseases in a given population. They are useful to generate hypotheses but cannot definitively give answers on a time sequence between a potential risk or protective factor and a disease. The advantage of cross sectional studies is that they can be done in a short period of time with, potentially, very large populations.

Prospective studies have the advantage of documenting temporal sequences, meaning that the cause precedes the disease begin. Conclusions on causal relations are thus facilitated. Typically, a group of individuals is recruited before the onset of a disease and they are followed commonly for years. The incidence of the disease is then documented. These groups of individuals are commonly called cohorts and the studies, cohort studies. A cohort can be drawn from a general population or a population at high risk of developing the disease. Recruiting high risk populations reduces the number of individuals needed, increases the participation rate of the probands thus reducing the cost of the study.

Cohort studies can be either observational or interventional. An observational cohort study is an epidemiologic study of subjects who are exposed in different degrees to a risk or protective factor hypothesized to influence the occurrence of a given disease or outcome. The probands are followed over a period of time and the natural evolution of the disease is observed. The study can either observe one population or different populations in parallel (farmers vs. reference children [18]). In interventional cohort studies, the probands are allocated into groups to evaluate the efficacy and safety of a preventive or therapeutic regimen. At least one group is

left to its natural course and is used as a control. The probands are then followed for a given period of time [19]. The term birth cohort study is generally used to describe a cohort in which children are either recruited pre- or post-nataly and observed over the next years to examine associations between early-life exposures and childhood outcomes.

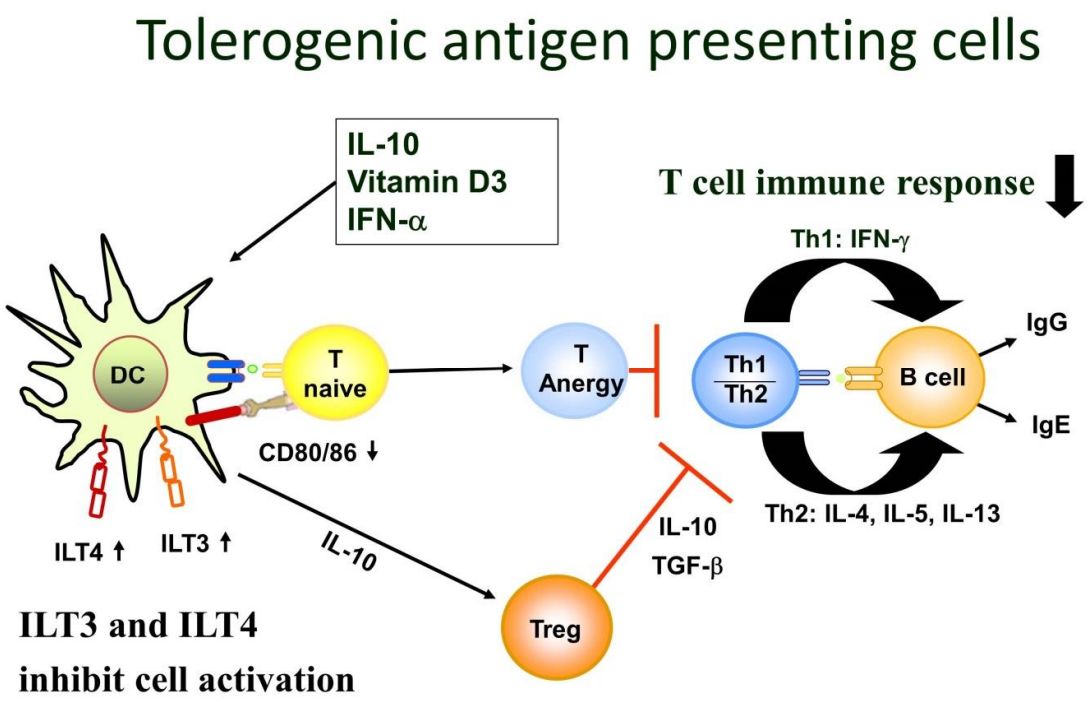
The advantage of cohort studies is the discovery of risk and protective factors, the disadvantages are the cost of the follow-up and the years necessary for the study completion both on the probands and on the researcherôs side. Additionally a proper randomization and double blinding is not always possible, often for ethical reasons (randomized studies on parachute use to prevent death and major trauma is not possible [20])

## Vitamin D and the immune system

The idea of dietary manipulation to prevent a disease is interesting as it would be a simple and cheap manner to influence health. Additionally, many nutriments ingested by mothers cross the placenta and may, therefore, influence the child prenatally. Vitamin D has gained interest in asthma research as it has been associated with disease control [21, 22] and disease prevention when taken during pregnancy [23].

Vitamin D is also known to have strong immunomodulator properties inducing tolerogenic dendritic cells. Dendritic cells are specialized antigen-presenting cells of the immune system that process antigen material and present it on the cell surface to the T helper (Th) cells [24]. In response to the antigens, the T helper cells communicate with B cells to mediate appropriate immune responses. Immunoglobulin-Like Transcripts (ILT)3 and ILT4 are two inhibitory receptors that can be up-regulated on tolerogenic dendritic cells in response to external stimulation [25] (Fig. 1). After up-regulation of ILT3 and ILT4, a possible pathway in which dendritic cells influence T cells is by the enhanced secretion of Interleukin (IL)-10 [26, 27].

**Figure 1: Proposed action of ILT3 and ILT4 on the immune system**




*CD: Cluster of Differentiation; DC: dendritic cells; ILT: Immunoglobulin-Like Transcripts; IFN-Ŭ: Interferon alpha; IL: Interleukin; T: T cell; Th: T helper cell; TGF-ß: Transforming growth factor; Treg: T regulatory cell*

Whether the effect of vitamin D on the development of allergic diseases is mediated by tolerogenic dendritic cells or through the enhanced secretion of IL-10 is unknown.

## Rhinitis and the united airway concept

Allergic rhinitis is clinically defined as a symptomatic disorder of the nose induced after allergen exposure and mediated by an immunoglobulin E (IgE) inflammation of the membranes lining the nose [28]. It was defined in 1929 by Hansel as ñThe three cardinal symptoms in nasal reactions occurring in allergy are sneezing, nasal obstruction and mucous dischargeò [29]. The link between upper and lower respiratory tracts has been observed in the past decades but only studied during the last years [30]. Particular attention has been paid to the relationship between rhinitis and asthma, i.e. between the upper and lower inflammation of the airways. The increasingly detailed knowledge of the mechanisms sustaining allergic inflammation in the respiratory tract (i.e. antigen presentation, cytokines, chemokines and adhesion molecules) have

partly clarified the functional relationships between nose and bronchi. Therefore, it is logical to expect that allergy is not a disease of a specific target organ, but rather a disorder of the whole respiratory tract with a broad spectrum of clinical manifestations [30]. These observations lead to the ñunited airways conceptò which suggests that the respiratory system functions as an integrated unit. Mostly adult studies have suggested that there may be a causal effect between rhinitis and asthma [31], with the former being a risk factor the later. Studies in children on a link between rhinitis and asthma are sparse and in small children lacking. Complicating matter, in early childhood, there are several wheezing phenotypes, with some resolving after a few years but some persisting into adulthood [32]. However, by the age of six years patterns of wheezing prevalence and levels of lung function appear to be established and seem to track to adulthood [33, 34]. The idea of a risk factor, such as rhinitis, in early childhood that could possibly alter these patters is tempting and warrants further research.

## Lung functions to track disease progression

Establishing a diagnosis of asthma involves a careful process of history taking, physical examination and diagnostics especially in the pediatric population. Once a diagnosis is confirmed, disease progression needs to be monitored, often over years. Lung functions are used for the diagnosis and the monitoring of patients with asthma. Their interpretation relies on the comparison of data from the patient with reference values based on healthy subjects from a general population. Reference values should be obtained from studies of ñnormalò or ñhealthyò subjects with the same anthropometric (e.g. sex, age and height) and, where relevant, ethnic characteristics of the patient being tested and should not be extrapolated beyond the published age-range [35]. Such reference equations are hard to obtain as most reference equations are either outdated, derived from a different population or do not span across all ages [36, 37]. Additionally, a major challenge in reference equations are the complex statistical models needed to develop them, as there is a steep increase of lung function throughout childhood, followed by a regular decline until old age [38]. Therefore, most studies have published reference equations either for children or for adults. However, in every day practice, reference equations spanning across all ages, including the transition point from childhood to adulthood would be of great use.

## Aims of the present PhD

The overall aim of the thesis was to achieve an overview of risk and protective factors in birth cohorts on asthma; and, among the abundance of them, to analyse two different ones in separate ongoing observational birth cohorts. An additional aim was to determine whether a tool could be developed to help children with asthma.

In particular, the following research questions were addressed:

1. What are the known risk and protective factors for the development of childhood asthma.
2. What questions still remain to be investigated?

To answer those questions, an extensive research was performed in PubMed with words such as ñbirth cohortsò and ñasthmaò, ñchildhood asthmaò, ñasthma developmentò, ñrisk factors for asthma developmentò or ñprotective factors for asthmaò. The results are presented in **chapter III**.

Among other health benefits, maternal vitamin D intake during pregnancy has been associated with a reduced risk of wheeze in early childhood. One next research step is to determine which pathway is used, as this may lead to a better understanding of the protective mechanism and bring us one step closer to a treatment possibility. Therefore, in **chapter IV,** the following questions were addressed:

3. Does vitamin D supplementation during pregnancy influence the immune system of the new born child, specifically the mRNA levels of ILT3 and ILT4?
4. Is the effect of vitamin D mediated by IL-10?

Rhinitis, which is a disease of the upper airways, has been shown to be a predictor for adult-onset asthma, but whether this is true for childhood wheezing is not known. Rhinitis would therefore be a risk factor and its treatment could potentially lead to a diminution of the number of children developing asthma. In **chapter V,** three specific questions were addressed:

5. What are the temporal sequences of the development of rhinitis and wheezing?
6. Does sensitization play a role in the development of rhinitis and wheezing?
7. Is rhinitis a risk factor for wheezing onset in children?

Once children develop asthma, they need regular clinical follow-up over years. Lung function measurements help clinicians manage the asthma. Their interpretation relies on reference equations that need to be as similar to the patient tested as possible.

8. Are there such reference equations for Central European children and adults?
9. If not, is it possible to develop practical ones that can be used by clinicians in their daily practice?

These questions were addressed in **chapter VI**.

# Chapter II: Methods

## Overview of birth cohort studies

For the overview of birth cohorts studies on asthma a PubMed search was performed. From the reference section of the papers identified additional cohorts were found, which finally gave an extensive overview of the existing cohorts. After studying the papers selected, an overview was attempted and is presented in chapter III.

## The Protection against Allergy-Study in Rural Environments (PASTURE)

The PASTURE study is funded through a European Commission Framework 5 grant and is composed of a multidisciplinary team of clinicians, epidemiologists, immunologists, experts in genetics and microbiologists from 6 European countries. Itôs main aim is to investigate the temporal sequence of immunological and genetic mechanisms involved in the determination of individual responses to environmental influences leading to the development of allergies [18].
It is a longitudinal birth cohort study in rural areas of 5 European countries (Germany, Switzerland, Austria, Finland and France). Over 1ô100 pregnant women were enrolled and their offspring are currently being followed up to the age of 6 years.
Exposure assessment: The main exposure categories are identified through comprehensive questionnaires and interviews. Sources of exposures such as stables, stable animals, barns, fodder, farm milk, harvesting etc. were assessed in great detail through interviews. Potential confounding factors such as socio-economic status, family size, maternal smoking, and the family history of allergic illnesses were also asked. Blood sampling was performed for genetic polymorphism and immunological studies. Dust, milk and stool samples were collected. Measures of microbial exposures such as levels of endotoxin from gram negative bacteria and extracellular polysaccharides from fungi were performed and will also serve as reference for the questionnaire/ interview assessed exposures. Immunologic pathways are being investigated.
Outcome assessment: The incidence of asthma, hay fever and atopic eczema are assessed via standardized interviews to the parents. The cumulative incidence of symptoms of the respective diseases is the main outcome variable. In addition, a physical examination of the children at age 1, 4.5 and 6 years is available as objective outcome measures. Allergic sensitisation was defined as the presence of specific IgE antibodies towards inhalant and food allergens in the serum at ages 1 and 4.5 years.

## The German Multicentre Atopy (MAS-90) study

The MAS study, an observational birth cohort, followed children from birth until 13 years old. Its main aims were to evaluate the predictive value of various clinical and immunological parameters as well as the significance of early environmental exposures to allergens and trigger factors for the development of atopic diseases in early childhood [39].

A questionnaire on atopic diseases, symptoms, as well as sociodemographic variables was distributed in the delivery wards to the parents of infants born in 5 German cities (Berlin Düsseldorf, Freiburg, Mainz, München) between the 1.1. and the 31.12.1990. For the parents who accepted the study cord blood IgE was determined. A total of 1ó314 children were recruited into the study, 499 new-borns with risk factors for atopy and 815 newborns with no atopy risk factors. Regular follow-up visits were organized when the children were 1, 3, 6, 12, 18, 24 months and then yearly until the age of 13 years.

Exposure assessment: At the controls questionnaires were completed on a number of subjects such as health symptoms, nutrition, development, environmental factors, housing conditions, psychological problems and demographic features. Additionally parents kept a diary in which details of the infantôs diet, diseases and important events were recorded. The infants had a standardized physical examination by a physician with special emphasis on skin conditions. Carpet dust samples were analysed for allergens at 6, 18 and 36 months of age. Blood samples were taken at 12 and 24 months for the determination of total IgE against 9 common food and inhalant allergens. Spot urine samples for cotinine determinations were collected at 12 and 24 months of age.

Outcome assessment: Clinical manifestations of atopic diseases and atopic sensitisation were treated separately. The diagnosis of the family physician, of the examining doctor and, additionally, a statistical analysis of the relevant symptoms or signs were used to define an obvious or probable atopic manifestation in the first 24 months of life. Consistent vomiting, diarrhoea and colic without infection after consumption of allergenic foods, were regarded as symptoms and signs for food intolerance. The child was considered sensitized, when the IgE antibody concentration against at least 1 of 9 common allergens was higher than 0.35 kU/1.

## The LuftiBus project

In an effort to improve health care in the Zurich metropolitan area, the ongoing ĂLuftiBusñ project was initiated in 1993 as a joint venture between the Zurich Lung Association and the Medical Society of Zurich [40] (ĂLuftiBusñ may be translated into ñAirBusò). The bus tours

the Zurich area allowing the population to have their lung function tested. It can either be encountered in random public places in the greater Zurich area or be leased by schools, sport teams/events or any other given group. In the first case, passers-by pay a fee of 10 Swiss Francs for adults and 5 Swiss Francs for children (half the cost of a light meal) to get their lung function tested. If the bus is leased, the test and the counselling are free of charge for all participants. The bus is equipped with two computer assisted spirometers that allow accurate lung function measurements to be performed. Specially trained lung function technicians perform the spirometry, instruct and supervise the people being tested, and subsequently counsel them. After the spirometry tests have been performed, the participants receive a comprehensive report of their lung function results, are advised to get professional medical advice when the results are pathological (i.e. FEV1%pred <80%) and are shortly counselled on general health as well as specific pulmonary issues. Additionally, the ÄLuftiBusñ-team collects epidemiological and basic health data from each person tested. Subsequently, all results are rendered anonymous and entered into a general database from where they can be extracted for statistical analysis. As of 1998, the ÄLuftiBusñ team has been active in children and adolescents health care prevention and has therefore actively gone to schools and youth events.

# Chapter III

## Birth Cohort Studies for Prevention of Allergy, New Perspectives, where do we go from now?

This book chapter has been published:

**Rochat MK,** von Mutius E. Birth Cohort Studies for Prevention of Allergy, New Perspectives, where do we go from now?
In: Springer, editor. Allergy Frontiers: Therapy and Prevention. Japan: Springer; **2010**.

# Birth Cohort Studies for the Prevention of Allergy: New Perspectives—Where Do We Go from Now?

**Mascha Rochat and Erika von Mutius**

## Introduction

There has been an increase in the prevalence of asthma and other allergic diseases in both industrialized and developing countries over the past decades [1]. In population based studies, prevalence estimates of asthma, atopic dermatitis, and allergic rhinitis vary from 7–10%, 15–20%, and 15–20%, respectively [2, 3], and different increase rates of each of these diseases have been reported in countries around the world [1, 4]. The reasons for this increase are still largely unknown, but interactions between various types of environmental exposures in populations with distinct genetic backgrounds have been proposed [1]. Allergic diseases have therefore become a major public health problem as well as a burden to health care resources, and they not only adversely affect the quality of life of millions of children and adults, but most importantly can be life-threatening in their most severe form. An urgent need to formulate strategies, leading to a reduction of their morbidity and mortality is thus required. This reduction could be achieved through primary or secondary prevention, and much research in both areas has been undertaken.

## Study Design

A necessary prerequisite for effective primary prevention measures is to know the different determinants of allergic diseases. Many determinants have been studied, discovered and defined through different study types during the last decades; but as described below, each of these study designs has distinct advantages and disadvantages, and should be adopted after due consideration.

M. Rochat and E. von Mutius (✉)
University Children's Hospital, Lindwurmstr. 4, 80337, Munich, Germany
e-mail: Erika.Von.Mutius@med.uni-muenchen.de

R. Pawankar et al. (eds.), *Allergy Frontiers: Therapy and Prevention*,
DOI 10.1007/978-4-431-99362-9_24, © Springer 2010

## *Cross-Sectional Studies*

Most of the risk and protective factors such as housing conditions (dampness), allergen exposure, active and passive smoking, pet keeping, breastfeeding or viral infections were primarily identified through cross-sectional or retrospective studies. The term cross sectional study is used to describe a study design that measures the prevalence of exposures and diseases in a given population simultaneously. The potential of cross-sectional studies is to generate hypotheses and, in case of consistent findings across numerous studies, suggest causal relations, particularly, if dose-response patterns can be observed. They are, however, limited in their capacity to describe a time sequence between potential risk factors and health effects. The fact that they can be done in a relatively short period of time and are less expensive than cohort studies confers advantages.

## *Cohort Studies*

To assess whether a time sequence, meaning the necessity for the cause to precede the outcome, is present, the study design should be prospective and include accepted well-defined diagnostic criteria and outcome measures, a sufficient duration of follow-up, and a proper sample size for adequate statistical evaluation [5]. In Ancient Rome, a cohort was one of ten divisions of a Roman military legion and was constituted with young men of similar age coming from one region. Members were often injured or killed in service but were not replaced. The cohort was then disbanded when the term of enlistment was over [6]. Historically, the term "cohort" was introduced into epidemiological studies in 1935 by Dr. Wade Frost, the leading US-American epidemiologist of the time [7]. He used the term to describe what would now be called generation studies. Over the years, its current meaning slowly emerged and a cohort study is now a well defined study design. A cohort study tracks outcome(s) forward in time. At the beginning of the observation period, the probands can be divided into two or more groups based on exposure, treatment, and/or risk factors. The groups are then followed for a period of time and the incidence of the outcome(s) is documented. The cohort can be drawn from the general population or a population at high risk for developing the respective disease. A birth cohort is a cohort of individuals born within a given period of time. It is often used to define a cohort of children followed since birth (or since pregnancy). Unfortunately cohort studies are very expensive and time consuming, and absence of follow-up may cause major problems.

## *Observational Birth Cohort Studies*

Observational cohort studies are considered "natural" experiments where the natural course is observed. Several observational birth cohort studies investigating allergies were initiated during the last decades. The aims were to identify risk factors that

could be modified to prevent or limit the development of allergic diseases; and predictors to target potential prevention strategies in the susceptible population.

### *Interventional Birth Cohort Studies*

For conclusive proof of evidence for causal relationships, well-designed, double-blind randomised controlled trials or interventional cohort studies including a control for confounders, as well as proper registration of compliance and follow-up of dropouts are required [5]. In interventional studies, two or more groups are recruited at the beginning of the trial and one or more interventions are initiated. At least one group is left to its natural course and is used as a control. All groups are then followed for a given period of time. Unfortunately, interventional studies with proper randomisation and double blinding are not always possible. The effect of breast-feeding or maternal smoking, for example, cannot be tested by means of randomised controlled trials for obvious ethical reasons.

Even though interventional studies can address either the general population or children at high risk of developing allergic diseases, most of them have been performed on high risk children to maximize the benefits, reduce the expense of recruiting a large population, and obtain maximum co-operation from the parents. The definition of high risk is, however, very heterogeneous. Some studies used a questionnaire based approach asking about allergy or asthma in first degree relatives (mother, biological father, siblings). Others confirmed the diagnosis with laboratory tests (specific IgE, skin-prick test or elevated cord blood IgE). Similarly, hereditary was defined either as single or multiple.

In general, interventions aiming at the prevention of allergies can be categorized into two aspects: “avoidance” and “immune deviation”. The “avoidance” concept is based on the expectation that by avoiding allergens during the critical period of sensitization, the immune system will not react to these allergens later on and allergic diseases will not occur. The “immune deviation” concept postulates that the immune system can be “educated” to develop tolerance to environmental allergens. During the last decades, there have been an increasing number of prospective interventional birth cohorts, testing either one or multiple of the known risk or protective factors. Most of them have been based on the avoidance concept.

## Defining Allergies

As mentioned above, the determinants of allergic diseases are a prerequisite for preventive measures, but a precise definition of the disease is equally mandatory. Even though such a statement seems obvious, it is in fact a challenge to define an allergic “outcome”, as the term allergy describes various diseases among which asthma, allergic rhinitis and atopic dermatitis are the most common. But other diseases such as urticaria or food allergy can also be found under this umbrella term.

Therefore, different research groups reporting on allergies may actually be reporting on very different disease entities. Atopic diseases were originally grouped under one term because they are associated with an elevation of either total or specific immunoglobulin E (IgE) (also termed "atopy"). This association is, however, loose and many patients with "atopic" illnesses have normal IgE levels and lack specific IgE antibodies in their serum. The relevance of allergic sensitization as an outcome is furthermore to be debated since not many children with a positive allergy test either as prick or RAST test are asymptomatic [8]. Given this ambiguity, the EAACI recently revised the nomenclature and proposed standard definitions in 2001 and subsequently in 2004 [9]. But, as these have not always been used, comparisons between the different studies are often difficult, if not impossible. This may partly explain why controversial results emerge from different studies.

To complicate matters even further, some diseases have been divided into different phenotypes. Asthma, for example, which is a chronic inflammatory disorder of the airways that can cause recurrent episodes of wheezing, breathlessness, chest tightness, and cough in susceptible individuals has been shown to begin in early life with wheezing symptoms. Based on the time of onset, the natural course and the different risk factors and determinants, wheezing children can be grouped into at least three different phenotypes: transient wheezers (present only in the first 3 years of life); persistent wheezers (beginning in the first 3 years and persisting beyond 3 years of age); and late-onset wheezers (beginning between 3 and 6 years of age) [10]. Taking into account these different phenotypes, it can be postulated that asthma may not be one disease but rather a syndrome and that distinct mechanisms underlie the apparently equivalent clinical manifestations.

## Natural Course of Allergic Illnesses

It is very important to take into consideration the natural course of the disease when evaluating potential preventive measures. Even though no clinical symptoms are detectable at birth, recent studies show evidence that food and inhalant allergens can reach the fetal circulation [11]. However, the role of this exposure in determining subsequent patterns of immunologic memory and disease is conflicting [12, 13]. In infancy, the main allergic outcomes are atopic dermatitis, gastrointestinal symptoms and recurrent wheezing, whereas asthma and allergic rhinoconjunctivitis are the main diseases later in childhood [14]. Adverse reactions to foods, mainly cow's milk, hen's egg, peanut, wheat and soy protein are most common in the first years of life, whereas allergy to inhalant allergens such as house dust mites, animal dander and pollen mostly occurs later [15]. Recent findings from the German Multicentre Allergy Study (MAS) birth cohort suggest that in most cases asthma does not follow a first manifestation of atopic dermatitis claimed as "atopic march". Rather, in most cases both conditions are either manifest in an individual child or the infant is only affected by one illness. The combination of conditions results in more severe asthma as well as more severe eczema, and lung function parameters are significantly reduced in these children at school age [16].

## Risk Factors and Protective Exposures

Prospective birth cohorts have helped us greatly to understand the natural history of allergic diseases by following a cohort of either high risk children or children representative of the general population for various periods of time. Additionally, most of them have studied, and reported specific risk or protective factors. These factors are briefly detailed below, with an emphasis on the ones that have been studied in interventional studies.

### *Nutrition*

#### Maternal Diet

There is strong evidence that maternal dietary allergens can cross the placental barrier and pass into the breast milk [17, 18]. The hypothesis, that reduced maternal allergen intake may reduce fetal allergen exposure and thus avoid early sensitization was therefore tested in a number of studies. Mothers were asked to refrain from consuming a varying number of allergenic products (cow's milk, eggs, peanuts, fish, citrus fruits) [19–21] either during pregnancy, lactation or both [19–22] for a period of time ranging from four weeks to a few months. Despite the variety of study approaches, no conclusive evidence of a preventive effect of maternal dietary restrictions during pregnancy or lactation on the development of allergic diseases was observed [19–23]. On the contrary, potential adverse effects on maternal nutritional status and on gestational weight gain, fetal growth and preterm birth have been observed [23].

As maternal dietary interventions did not have an effect on the development of allergic diseases, other dietary habits were investigated. The substantial shift in dietary fatty acid intake in many populations around the world favouring n-6 polyunsaturated fatty acid (PUFA) (margarine, vegetable oil) over n-3 PUFA (oily fish) resulted in the hypothesis that the intake of certain fatty acids may increase the risk of developing allergies. Observational studies assessing maternal dietary intake during the last 4 weeks of pregnancy [24] or the PUFA content of breast milk [25] concluded that low levels of n-3 PUFA intake correlated with the development of allergic diseases or atopic eczema during the first two years of life. These findings were, however, not confirmed when using skin test reactivity as an outcome [26]. Interventional studies are needed before any firm recommendation can be issued.

#### Breastfeeding

A recent review of the literature [27] reported a protective effect of exclusive breastfeeding on the development of allergic diseases especially among high risk children. Even though one long-term prospective birth cohort confirmed these findings [28],

several recent long-term observational birth cohort studies not included in the previously published meta-analyses have shown breastfeeding to be a risk factor for the development of allergic diseases mainly in high risk children [29]. Matheson et al [30] followed a cohort from childhood to middle-age and concluded that "in high risk children, breast-feeding, although important for the protection of the infant against early wheezing illness (asthma), eczema, and food allergies, does not appear to protect against the development of asthma, allergic rhinitis, or food and inhalant allergies in the long term". Truly randomised interventional birth cohorts on exclusive breastfeeding cannot be performed for ethical reasons, but Kramer et al [31] used a similar approach and performed a cluster randomised trial by selecting at random study areas in Bellarussia where breastfeeding was promoted. The investigators followed 13,889 children up to the age of 6 years. Children in the interventional sites were significantly more likely to be breastfed and by the age of 6 years no protective effect of prolonged and exclusive breastfeeding on asthma or allergy was found.

## Hypoallergenic Infant Formula

If breastfeeding is not possible, products with highly reduced allergenicity based on hydrolysed protein or amino acid mixtures can be used as a substitute. A number of interventional cohort studies on different formulas have been undertaken. Most of the studies were performed in high risk populations, only a few were in the general population. The study designs and formulas under investigation differed considerably between the studies, which rendered comparisons difficult [32]. Cow's milk was compared to hydrolyzed or extensively hydrolyzed casein formula or to partially hydrolyzed or extensively hydrolyzed whey formula in different combinations. Taken together, the results indicate that a hydrolysed formula is not superior to exclusive breastfeeding for the prevention of allergy. Among high risk infants who cannot be exclusively breastfed, there is some evidence to suggest that prolonged feeding with a hydrolysed formula as compared to a cow's milk formula reduces transiently the incidence of infant and childhood atopic dermatitis, but not asthma in the first years of life [33]. Longitudinal studies following children up to school age are needed to see whether there is any prolonged benefit from such interventions.

## Infantile Diet

The evidence that introduction of solid foods to infants before 4 months of age increases the risk of allergic diseases is conflicting. In general, there is insufficient evidence to suggest that on its own, the early introduction of solids to infants is associated with an increased incidence of asthma, food allergy and allergic rhinitis. Unfortunately, many studies lack a rigorous design and are thus susceptible to multiple biases. No evidence was found to support a delayed introduction

of solid foods beyond the sixth month of life for the prevention of atopic dermatitis or atopic sensitization [34]. However, there is a consistent association between the persistence of eczema and the introduction of solid foods before age 4 months that is supported by long term follow-up studies and the dose-dependent nature of the association. Furthermore, it is also noteworthy that the preventive effect of breastfeeding and hydrolyzed formulas was only observed in studies including avoidance of complementary foods during at least the first 4 months of life [35]. Clearly, additional evidence from well designed randomised longitudinal studies is needed to endorse recommendations on delayed solid food introduction in infancy.

Prospective studies investigating the potential effects of other dietary factors such as fish oil, omega-3 fatty acids, antioxidants (vitamin C, vitamin E and selenium), magnesium or sodium which have been found to be either protective or risk factors in cross-sectional studies are still awaited.

## Probiotics

Observational studies have found a correlation between the colonization of the gastrointestinal tract with lactobacillus as well as eubacteria and a decrease in allergic diseases [34]. During the last years, interventional birth cohorts have therefore examined the effect of different doses of probiotics (live microbial food ingredients) on the development of allergic diseases. All studies were conducted on high risk infants and each studied a different probiotic bacterial strain.

After administering either the probiotics Lactobacillus GG, Lactobacillus reuteri or a mixture of four probiotics and one prebiotic to pregnant women and subsequently to their offspring for the first 6 [36, 37] and 12 [38] months, three studies saw a protective effect on the incidence of eczema [36] and atopic eczema [37, 38] up to the age of 2 [37, 38] and 7 years [36]. One survey administered Lactobacillus acidophilus to infants during the first 6 months, but not to their mothers during pregnancy [39]. The authors found an increase in atopic eczema, but did not see an effect on the cumulative incidence of eczema at the age of 12 months. Whether these contrasting results are attributable to different bacterial strains or to the postnatal administration remains unclear. Moro et al. showed a beneficial effect of a mixture of prebiotic oligosaccharides (GOS/FOS) added to bottle fed infants in reducing the incidence of atopic eczema during the first six months of age [40]. Taken together, the current evidence suggests that probiotics might have a preventive effect on the development of eczema. However, in our opinion, a number of issues remain unanswered which preclude firm recommendations on the use of probiotics for the prevention of allergies: which is the best probiotic strain? What is the optimal dose? Is there any effect among children from the general population without a family history of atopic diseases in first degree relatives? Is this preventive effect prolonged into childhood years? Are other allergic illnesses such as asthma and hay fever also amenable to prevention by the administration of probiotics?

## *Environmental Factors*

As mentioned above, two concepts prevail with respect to potential preventive strategies. One concept is based on the rationale of risk factor avoidance, particularly of allergen avoidance. The other complementing concept is based on the identification of protective factors, in particular the hygiene hypothesis postulating that "allergies may be the result of a misdirected immune response in the absence of infection" [41]. Many, mostly cross-sectional studies aiming at the identification of harmful and protective environmental exposures for the development of allergies have been performed. In the following sections we will focus on cohort studies investigating either avoidance or immune modulation pathways.

### Environmental Tobacco Smoke

Environmental tobacco smoke (ETS) exposure either during pregnancy or early life is one of the most consistent risk factors for the development of respiratory symptoms, wheezing and asthma [1]. Active smoking has been associated with the onset of asthma in adolescents and adults in a number of studies [1]. Exposure to ETS is therefore the most important preventable inducer and trigger of asthma. Because of the consistence of findings across numerous populations worldwide, the avoidance of ETS is warranted for the prevention of asthma and other respiratory diseases. There is less evidence, however, to suggest that ETS exposure also affects the incidence of atopic sensitisation.

### Traffic Related Pollution

While it is well accepted that air pollution in general can trigger symptoms in children with established asthma [42], its influence on the development of allergies is not clear. Cross sectional studies have shown strong evidence to support an association between air pollutants associated with traffic exposure and respiratory health, however, the evidence is conflicting regarding the development of allergic diseases and atopic sensitization. To our knowledge there are only two observational cohort studies among children investigating traffic pollution and health outcome at the ages of two and four years, respectively. As acknowledged by the authors, the short duration of follow-up, limits the interpretation of the results, and the positive relation with wheezing, asthma, and sensitization to food allergens must therefore be interpreted with caution [43, 44].

### Inhaled Allergens

The level of aeroallergen exposure varies according to the geo-economic situation. House dust mite (HDM) is the most important allergen in humid climates; pet allergens might be more relevant in colder countries; Alternaria species prevail in dry

climates and cockroach is one of the dominant allergens in inner-city areas in the US. These allergens have been investigated in numerous studies over the past decades, because their exposure affects a persons risk of developing IgE antibodies against them [45]. Without allergens in the environment, specific sensitization towards that allergen cannot occur. In a German birth cohort, the levels of HDM and cat allergen concentrations in domestic carpet dust were strongly related to the development of atopic sensitization towards these allergens in the first 3–7 years of life [46]. A clear dose-response relationship was found as well as a strong effect modification by the familial background, for atopic diseases. In the group of children with a positive family history of atopy, mite allergen concentration below 750 ng/gm dust resulted in a 3% sensitization rate, whereas in the group of children without a positive family exposure up to 25,000 ng/mg dust was associated with a sensitization rate of 3%. These findings indicate that no general exposure threshold for allergic sensitization can be proposed. As might be expected HDM exposure was not related to the propensity of mounting IgE responses towards other environmental antigens. Likewise, the propensity to develop asthma may not be affected by levels of environmental allergen exposure. This notion is supported by findings from prospective cohort studies where no relation between the level of exposure to HDM and the incidence of asthma and persistent wheeze was found [45]. However, the interaction of higher indoor allergen exposure and the development of atopic sensitization towards these allergens in the first 3 years of life resulted in more severe asthma [47].

Recent findings from a number of interventional studies have further corroborated these notions. In the interventional studies, HDM reduction measures were initiated either during pregnancy or at birth, and the children were followed until the ages of 7–8 years old. Although a reduction of HDM could be documented after radical allergen reduction measures were undertaken, the results of one study showed an increased risk in mite sensitization at the age of 3 years, an increase in atopic dermatitis and no significant differences in respiratory symptoms between the interventional and the control groups by the age of 8 [48]. Other studies, using more practical interventional measures such as mattress covers, showed no protective effect on the prevalence of asthma and atopic sensitization up to the age of 2 years [49, 50]. In inner-city areas in the US comprehensive environmental interventions to decrease indoor allergen levels, including cockroach and dust-mite allergens, resulted in reduced asthma-associated morbidity among 5 to 11 year old children [51].

## Pets

As with other allergens, exposure to pet allergen increases the risk of subsequent specific sensitization and thus potentially increases the incidence of allergic diseases. This notion was challenged in 1999 by studies showing that early life exposure to pets was associated with a lower prevalence of allergic rhinitis and asthma at school age and reduced cat sensitization as well as atopy in adulthood. A review of the associations between pet exposure, asthma and asthma like

symptoms was therefore undertaken by Apelberg in 2001 who concluded that in children over 6 years of age there was a significant increase in the risk of asthma or wheezing, but in children younger than 6 years, exposure to pets had a protective effect [52]. Since then, there have been a number of prospective studies showing a reduced risk of allergic diseases or allergic sensitization in children exposed to cats or dogs in infancy, but consistency among these studies is lacking. Parental, mostly maternal history of asthma or atopy seems to exert significant effect modification with pet exposure being a risk factor in high risk children and a protective factor in low risk children. Additionally, there seems to be marked differences between different animal species, with exposure to dogs showing a more consistent pattern of protection than exposure to cats [53]. An interesting question in analyzing the relationship between pet exposure and allergic sensitization or allergic disease is whether it is exposure to high levels of allergens given off by the pet or exposure to an undefined environmental factor(s) related to the pet that contribute(s) protectively to the determination of the outcome, presumably by effects on the maturation of the immune system [54]. Furthermore, these results must be regarded with caution because of the possibility of reverse causation due to already implemented avoidance strategies in families with atopic heredity. As of today, the available literature does not provide conclusive guidance regarding the effects of pet exposure early in life.

## Family Size/Day Care

Among the most consistent associations is Strachan's original observation that exposure to siblings reduces the risk of developing allergic diseases such as hay fever and eczema [55]. Whether this effect is attributable to an increase of the number of infections transmitted by "unhygienic" contact with older siblings or due to other unknown factors associated with an increased number of pregnancies is unknown. In the absence of a large family, exposure to children in early child care seems to have a similar protective effect [56]. Several observational studies have confirmed these findings in recent years [57, 58].

One cross-sectional study from Kramer et al. found an association between day-care attendance in the first year of life and the development of allergic diseases in single child families [59]. Positive associations were reported in two prospective birth cohorts. Celedon et al. found an inverse association between day care attendance in the first year of life and total serum IgE levels at the age of two [60], and eczema, as well as asthma and recurrent wheezing in the first 6 years of life among high risk children. This effect was not seen among offsprings of mothers with a history of asthma, where day care attendance was associated with an increased risk of wheezing [61]. In the Tucson birth cohort, young children's exposure to other children in or out of the home was found to result in more frequent wheezing in the first few years of life and subsequently to decreased levels of serum IgE concentrations, skin test reactivity and to protection against the development of asthma and frequent wheezing later in childhood [62].

## Infections

Few prospective birth cohorts have investigated the potential effect of early infections on the development of atopy [63–66]. Three studies showed a protective effect, while no effect was seen in one survey. With respect to wheeze and asthma, studies investigating the effect of early life respiratory tract infections must be interpreted with caution. Bias by reverse causation is likely to occur since viral infections are the most potent trigger of asthma exacerbations. Respiratory syncitial virus (RSV) may, however, be particularly associated with asthma. A number of studies have consistently shown that children with a history of RSV bronchiolitis have an increased risk of developing repeated wheezing and asthma up to school age. Whether the RSV infection is the culprit or whether the determining factor is an asthmatic's susceptibility to develop (severe) bronchiolitis after RSV infection remains unresolved. A recent cohort study provided some evidence that both arguments may be justified [67].

Findings from cross-sectional studies, mainly based on serological results, have suggested that other infectious agents such as hepatitis A, herpes, EBV, salmonella, helicobacter pylori, mycobacteria and lactobacilli [68] may be linked to the development of allergies. However, little is known from prospective studies.

## Microbial Exposures in the Environment

There is increasing evidence from cross-sectional studies to suggest that an environment rich in microbial compounds may protect from the development of allergic diseases. So far, few prospective studies have investigated the role of such exposures and these studies have focussed on assessing endotoxin, the cell wall component of gram negative bacteria. Two prospective birth cohorts found that endotoxin exposure in infancy among high risk children was associated with an increased incidence of wheeze and inconsistently with an increased incidence of atopic dermatitis, but not rhinitis or atopy in the first years of life [69, 70]. In contrast, another study showed that prenatal exposure to endotoxin was associated with a reduction in cord blood immunoglobulin E production suggesting that maternal cytokine production modulates the foetal immune cells [71]. But the infant's exposure to endotoxin had no protective effect on the development of atopy until the age of 2 years [72]. These discrepancies between studies might be explained by recent data from a birth cohort showing that exposure to endotoxin exerts a protective effect only in individuals with a particular genetic background (CC genotype for CD14/_159) [73].

## Antibiotics/Vaccinations/Paracetamol

Antibiotics and vaccinations have been postulated to interfere with the immune response in the developing immune system and thus to enhance the development of allergic diseases. But here again, results from observational cohort studies are conflicting.

While no association has been found between antibiotics and the subsequent development of asthma in a number of prospective birth cohorts and recently summarised in a meta-analysis [74], some prospective birth cohorts have shown a positive association between antibiotics administered during pregnancy or lactation and the development of allergic diseases in the offspring [75, 76].

According to the hygiene hypothesis, infections might confer protection from the development of allergic illnesses. Following this argument, it was proposed that vaccinations may play a role in the incidence of allergic disorders. However, two recent reviews of the literature came to the conclusion, that there is no epidemiological evidence to suggest an association between infant vaccinations and the development of allergic diseases [77, 78].

Since 2000, cross sectional, case control and ecologic studies have analysed and consistently reported a dose response association between paracetamol (acetaminophen) use and the development of wheeze and asthma. One possible mechanism might be mediated by an imbalance in the oxidant/antioxidant equilibrium leading to oxidant damage in the lung [79]. Reverse causation may, however, significantly bias these associations. Since paracetamol is given for fever and infectious diseases, the reported associations may merely reflect the strong relation between viral infections and asthma. In this context it is interesting to note that a prospective birth cohort found an association between paracetamol administered during late pregnancy and the development of asthma, wheeze and elevated total IgE by the age of 6–7 years [80].

## *Multifaceted Interventional Studies*

The studies described above used a monofaceted approach, studying one protective exposure or one preventive measure. Overall, the results of theses studies are rather disappointing. One explanation might be that the development of complex diseases, such as allergic illnesses, is attributable to a number of environmental influences. Accordingly, the avoidance of one single factor may not be sufficient. Some studies have therefore used a multifaceted approach, asking the parents to avoid or eliminate various known risk factors. All were performed among high risk children.

The Isle of Wight study started the intervention postnatally. The investigators combined dietary restrictions in the lactating mothers and their children during the first year of their life with HDM reduction measures. The results at the age of 8 years suggest that such a combined approach is mostly effective for reducing atopy and may prevent some cases of childhood asthma depending on the phenotypic characteristic of the child [81]. The Canadian Childhood Asthma Primary Prevention Study used a combination of dietary restrictions in the mothers during pregnancy and lactation and encouraged them to breast-feed and delay the introduction of solid foods during the first year of life of their children. Additionally, intervention measures introduced before birth and during the first year of life included avoidance of HDM, pets, environmental tobacco smoke and day care facilities. The results showed a significant reduction in the prevalence of asthma and asthma symptoms, but not allergic rhinitis, atopic dermatitis, atopy or bronchial hyperresponsiveness at 7 years

of age. Since there was no difference between the groups for the objective parameters and since the controls could not be blinded towards the intervention, the preventive effect may at least in part be attributable to some underreporting in the active arm [82]. The Prevention of Asthma in Children study (PREVASC) combined prenatal HDM and pet allergen avoidance strategies with dietary interventions and restrictions in passive smoking. The results showed no effective reduction in asthma-like symptoms at the age of two years [83]. The Australian Childhood Asthma Prevention Study (CAPS) combined a reduction in HDM allergen measures with dietary fatty acid supplementation. The results at 5 years showed that the measures did not prevent the onset of asthma, eczema or atopy. As in other studies the reduction of HDM increased the risk of eczema [84].

## Secondary and Tertiary Prevention in Cohort Studies

There have been a number of studies investigating the potential of secondary and tertiary preventive measures through medications aiming at either preventing the onset of disease among predisposed subjects such as the Early Treatment of the Atopic Child (ETAC) study or at the prevention of a chronic course of asthma. The ETAC study, a multi-centre European double-blind, randomised, placebo-controlled study, investigated the potential of cetirizine (antihistamine) to prevent the development of asthma in infants with atopic dermatitis. However, this trial failed since no effect on asthma prevention was seen in the active arm [85]. The hypothesis that the treatment with inhaled corticosteroids at the onset of infant asthma might improve long-term asthma outcome is built on the observation that in many asthmatic children an ongoing chronic inflammatory process is associated with a loss of lung function by early childhood which extends into adult life. Within a birth cohort, a subsample of infants was randomised after either one prolonged (>1 month) or two medically confirmed wheezy episodes to a treatment with Fluticasone propionate twice daily or to a control group. At the age of 5 years, the results showed that the early use of inhaled corticosteroids had no effect on the natural history of asthma or wheeze later in childhood and did not prevent lung function decline or reduce airway reactivity [86]. Likewise, a nested interventional study of another cohort showed that early intermittent inhaled budesonide therapy had no effect on the progression from episodic to persistent wheezing and no short-term benefit during episodes of wheezing in the first three years of life [87].

## Conclusion

Birth cohort studies investigating determinants of allergic illnesses have substantially contributed to our understanding of the natural course of allergic diseases and associated features. They have identified a number of potential harmful and protective determinants, but consistency across populations is often lacking. Recommendations on a

population level, based on these findings, can in our opinion, only be issued for tobacco smoke avoidance for the prevention of asthma and other respiratory conditions. With respect to eczema and food allergy, the administration of hypoallergenic milk formula can be recommended for high risk children if prolonged breastfeeding is not possible. All other strategies have to be scrutizined in view of the results obtained so far.

## Where do we go from now?

The lack of understanding of the significant determinants of allergic illnesses may be attributable to a number of reasons [1]. A basic requirement for the demonstration of a causal relation between an exposure and a disease is the temporal sequence of events. Only exposures occurring before the first symptoms of an illness can influence its inception. Birth cohorts have the advantage of documenting temporal sequences and thus facilitating conclusions on causal relations. But the disparity and heterogeneity of findings in the literature are remarkable, and reflect the complexity of allergic diseases.

Environmental exposures potentially causing the new onset of allergies are likely to occur during fetal life and early childhood. Any potential risk factor is likely to interact with an underlying, genetically determined pathway resulting in the manifestation of disease. But the mechanisms underlying allergic illnesses are not fully understood. This lack of understanding may in part be attributable to the fact that allergic illnesses are a syndrome of many diseases rather than one entity.

The context in which exposures occur and interact with a subject's individual pathophysiological pathways is another important factor in the causation of allergies. Variation in these pathways is determined by genes. Environmental exposures interact with these pathways. However, these interactions do not occur in isolation. Pathways have more than one component and the function of each component is determined by the gene or genes contributing to and regulating the processes. If in fact more than one pathway leads to the development of allergies, then not only many genes with small individual contributions, but also many environmental factors and gene–environment interactions will eventually be found to contribute to disease manifestation. Hence, different contexts in which a number of different exposures interact with various genetic backgrounds in a range of racial or ethnic groups will eventually result in changes in the incidence of allergies.

The challenge in the years to come will be to integrate complex interactions between multiple exposures and numerous genetic variants to achieve an understanding of the causation of asthma.

## References

1. Eder W, Ege MJ, von Mutius E (2006) The asthma epidemic. N Engl J Med 355(21): 2226–35
2. Sly RM (1999) Changing prevalence of allergic rhinitis and asthma. Ann Allergy Asthma Immunol 82(3):233–48; quiz 248–52

3. Schultz Larsen F (1996) Atopic dermatitis: an increasing problem. Pediatr Allergy Immunol 7(9 Suppl):51–3
4. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, Williams H (2006) Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 368(9537):733–43
5. Host A, Halken S (2002) Can we apply clinical studies to real life? Evidence-based recommendations from studies on development of allergic diseases and allergy prevention. Allergy 57(5):389–97
6. Webb P, Bain C, Pirozzo S (2005) Essential Epidemiology, an introduction for Students and Health Professionals. Second ed, Cambridge, Cambridge University Press, pp
7. Frost WH (1995) The age selection of mortality from tuberculosis in successive decades. 1939. Am J Epidemiol 141(1):4–9; discussion 3
8. Stevenson MD, Sellins S, Grube E, Schroer K, Gupta J, Wang N, Khurana Hershey GK (2007) Aeroallergen sensitization in healthy children: racial and socioeconomic correlates. J Pediatr 151(2):187–91
9. Johansson SG, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, Motala C, Ortega Martell JA, Platts-Mills TA, Ring J, Thien F, Van Cauwenberge P, Williams HC (2004) Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol 113(5):832–6
10. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ (1995) Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med 332(3):133–8
11. Prescott SL (2006) Maternal allergen exposure as a risk factor for childhood asthma. Curr Allergy Asthma Rep 6(1):75–80
12. Rowe J, Kusel M, Holt BJ, Suriyaarachchi D, Serralha M, Hollams E, Yerkovich ST, Subrata LS, Ladyman C, Sadowska A, Gillett J, Fisher E, Loh R, Soderstrom L, Ahlstedt S, Sly PD, Holt PG (2007) Prenatal versus postnatal sensitization to environmental allergens in a high-risk birth cohort. J Allergy Clin Immunol 119(5):1164–73
13. Hagendorens MM, Ebo DG, Bridts CH, Van de Water L, De Clerck LS, Stevens WJ (2004) Prenatal exposure to house dust mite allergen (Der p 1), cord blood T cell phenotype and cytokine production and atopic dermatitis during the first year of life. Pediatr Allergy Immunol 15(4):308–15
14. Wahn U (2000) What drives the allergic march? Allergy 55(7):591–9
15. Nickel R, Lau S, Niggemann B, Gruber C, von Mutius E, Illi S, Kulig M, Wahn U (2002) Messages from the German Multicentre Allergy Study. Pediatr Allergy Immunol 13(Suppl 15):7–10
16. Illi S, von Mutius E, Lau S, Nickel R, Gruber C, Niggemann B, Wahn U (2004) The natural course of atopic dermatitis from birth to age 7 years and the association with asthma. J Allergy Clin Immunol 113(5):925–31
17. Vadas P, Wai Y, Burks W, Perelman B (2001) Detection of peanut allergens in breast milk of lactating women. Jama 285(13):1746–8
18. Edelbauer M, Loibichler C, Nentwich I, Gerstmayr M, Urbanek R, Szepfalusi Z (2004) Maternally delivered nutritive allergens in cord blood and in placental tissue of term and preterm neonates. Clin Exp Allergy 34(2):189–93
19. Falth-Magnusson K, Kjellman NI (1992) Allergy prevention by maternal elimination diet during late pregnancy--a 5-year follow-up of a randomized study. J Allergy Clin Immunol 89(3):709–13
20. Lilja G, Dannaeus A, Foucard T, Graff-Lonnevig V, Johansson SG, Oman H (1989) Effects of maternal diet during late pregnancy and lactation on the development of atopic diseases in infants up to 18 months of age--in-vivo results. Clin Exp Allergy 19(4):473–9
21. Hattevig G, Sigurs N, Kjellman B (1999) Effects of maternal dietary avoidance during lactation on allergy in children at 10 years of age. Acta Paediatr 88(1):7–12
22. Lovegrove JA, Hampton SM, Morgan JB (1994) The immunological and long-term atopic outcome of infants born to women following a milk-free diet during late pregnancy and lactation: a pilot study. Br J Nutr 71(2):223–38

23. Kramer MS, Kakuma R (2006) Maternal dietary antigen avoidance during pregnancy or lactation, or both, for preventing or treating atopic disease in the child. Cochrane Database Syst Rev 3:CD000133
24. Sausenthaler S, Koletzko S, Schaaf B, Lehmann I, Borte M, Herbarth O, von Berg A, Wichmann HE, Heinrich J (2007) Maternal diet during pregnancy in relation to eczema and allergic sensitization in the offspring at 2 y of age. Am J Clin Nutr 85(2):530–7
25. Hoppu U, Rinne M, Lampi AM, Isolauri E (2005) Breast milk fatty acid composition is associated with development of atopic dermatitis in the infant. J Pediatr Gastroenterol Nutr 41(3):335–8
26. Stoney RM, Woods RK, Hosking CS, Hill DJ, Abramson MJ, Thien FC (2004) Maternal breast milk long-chain n-3 fatty acids are associated with increased risk of atopy in breastfed infants. Clin Exp Allergy 34(2):194–200
27. van Odijk J, Kull I, Borres MP, Brandtzaeg P, Edberg U, Hanson LA, Host A, Kuitunen M, Olsen SF, Skerfving S, Sundell J, Wille S (2003) Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966–2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. Allergy 58(9):833–43
28. Saarinen UM, Kajosaari M (1995) Breastfeeding as prophylaxis against atopic disease: prospective follow-up study until 17 years old. Lancet 346(8982):1065–9
29. Bergmann RL, Diepgen TL, Kuss O, Bergmann KE, Kujat J, Dudenhausen JW, Wahn U (2002) Breastfeeding duration is a risk factor for atopic eczema. Clin Exp Allergy 32(2):205–9
30. Matheson MC, Erbas B, Balasuriya A, Jenkins MA, Wharton CL, Lai-Kuan Tang M, Abramson MJ, Walters EH, Hopper JL, Dharmage SC (2007) Breast-feeding and atopic disease: A cohort study from childhood to middle age. J Allergy Clin Immunol
31. Kramer MS, Matush L, Vanilovich I, Platt R, Bogdanovich N, Sevkovskaya Z, Dzikovich I, Shishko G, Mazer B (2007) Effect of prolonged and exclusive breast feeding on risk of allergy and asthma: cluster randomised trial. Bmj
32. Osborn DA, Sinn J (2006) Formulas containing hydrolysed protein for prevention of allergy and food intolerance in infants. Cochrane Database Syst Rev(4):CD003664
33. von Berg A, Koletzko S, Filipiak-Pittroff B, Laubereau B, Grubl A, Wichmann HE, Bauer CP, Reinhardt D, Berdel D (2007) Certain hydrolyzed formulas reduce the incidence of atopic dermatitis but not that of asthma: three-year results of the German Infant Nutritional Intervention Study. J Allergy Clin Immunol 119(3):718–25
34. Bjorksten B, Naaber P, Sepp E, Mikelsaar M (1999) The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy 29(3):342–6
35. Halken S (2004) Prevention of allergic disease in childhood: clinical and epidemiological aspects of primary and secondary allergy prevention. Pediatr Allergy Immunol 15 Suppl 16:4–5, 9–32
36. Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E (2001) Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet 357(9262):1076–9
37. Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M (2007) Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. J Allergy Clin Immunol 119(1):192–8
38. Abrahamsson TR, Jakobsson T, Bottcher MF, Fredrikson M, Jenmalm MC, Bjorksten B, Oldaeus G (2007) Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol 119(5):1174–80
39. Taylor AL, Dunstan JA, Prescott SL (2007) Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. J Allergy Clin Immunol 119(1):184–91
40. Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G (2006) A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child 91(10):814–9

41. Strachan DP (1989) Hay fever, hygiene, and household size. Bmj 299(6710):1259–60
42. Tatum AJ, Shapiro GG (2005) The effects of outdoor air pollution and tobacco smoke on asthma. Immunol Allergy Clin North Am 25(1):15–30
43. Morgenstern V, Zutavern A, Cyrys J, Brockow I, Gehring U, Koletzko S, Bauer CP, Reinhardt D, Wichmann HE, Heinrich J (2007) Respiratory health and individual estimated exposure to traffic-related air pollutants in a cohort of young children. Occup Environ Med 64(1):8–16
44. Brauer M, Hoek G, Smit HA, de Jongste JC, Gerritsen J, Postma DS, Kerkhof M, Brunekreef B (2007) Air pollution and development of asthma, allergy and infections in a birth cohort. Eur Respir J 29(5):879–88
45. Lau S, Illi S, Sommerfeld C, Niggemann B, Bergmann R, von Mutius E, Wahn U (2000) Early exposure to house-dust mite and cat allergens and development of childhood asthma: a cohort study. Multicentre Allergy Study Group. Lancet 356(9239):1392–7
46. Wahn U, Lau S, Bergmann R, Kulig M, Forster J, Bergmann K, Bauer CP, Guggenmoos-Holzmann I (1997) Indoor allergen exposure is a risk factor for sensitization during the first three years of life. J Allergy Clin Immunol 99(6 Pt 1):763–9
47. Illi S, von Mutius E, Lau S, Niggemann B, Gruber C, Wahn U (2006) Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. Lancet 368(9537):763–70
48. Woodcock A, Lowe LA, Murray CS, Simpson BM, Pipis SD, Kissen P, Simpson A, Custovic A (2004) Early life environmental control: effect on symptoms, sensitization, and lung function at age 3 years. Am J Respir Crit Care Med 170(4):433–9
49. Horak F, Jr., Matthews S, Ihorst G, Arshad SH, Frischer T, Kuehr J, Schwieger A, Forster J (2004) Effect of mite-impermeable mattress encasings and an educational package on the development of allergies in a multinational randomized, controlled birth-cohort study – 24 months results of the Study of Prevention of Allergy in Children in Europe. Clin Exp Allergy 34(8):1220–5
50. Brunekreef B, Smit J, de Jongste J, Neijens H, Gerritsen J, Postma D, Aalberse R, Koopman L, Kerkhof M, Wilga A, van Strien R (2002) The prevention and incidence of asthma and mite allergy (PIAMA) birth cohort study: design and first results. Pediatr Allergy Immunol 13 Suppl 15:55–60
51. Morgan WJ, Crain EF, Gruchalla RS, O'Connor GT, Kattan M, Evans R, 3rd, Stout J, Malindzak G, Smartt E, Plaut M, Walter M, Vaughn B, Mitchell H (2004) Results of a home-based environmental intervention among urban children with asthma. N Engl J Med 351(11):1068–80
52. Apelberg BJ, Aoki Y, Jaakkola JJ (2001) Systematic review: Exposure to pets and risk of asthma and asthma-like symptoms. J Allergy Clin Immunol 107(3):455–60
53. Gern JE, Reardon CL, Hoffjan S, Nicolae D, Li Z, Roberg KA, Neaville WA, Carlson-Dakes K, Adler K, Hamilton R, Anderson E, Gilbertson-White S, Tisler C, Dasilva D, Anklam K, Mikus LD, Rosenthal LA, Ober C, Gangnon R, Lemanske RF, Jr. (2004) Effects of dog ownership and genotype on immune development and atopy in infancy. J Allergy Clin Immunol 113(2):307–14
54. Ownby DR, Johnson CC (2003) Does exposure to dogs and cats in the first year of life influence the development of allergic sensitization? Curr Opin Allergy Clin Immunol 3(6):517–22
55. Karmaus W, Botezan C (2002) Does a higher number of siblings protect against the development of allergy and asthma? A review. J Epidemiol Community Health 56(3):209–17
56. Marks GB (2006) Environmental factors and gene-environment interactions in the aetiology of asthma. Clin Exp Pharmacol Physiol 33(3):285–9
57. Biagini JM, LeMasters GK, Ryan PH, Levin L, Reponen T, Bernstein DI, Villareal M, Khurana Hershey GK, Burkle J, Lockey J (2006) Environmental risk factors of rhinitis in early infancy. Pediatr Allergy Immunol 17(4):278–84
58. Infante-Rivard C, Amre D, Gautrin D, Malo JL (2001) Family size, day-care attendance, and breastfeeding in relation to the incidence of childhood asthma. Am J Epidemiol 153(7):653–8

59. Kramer U, Heinrich J, Wjst M, Wichmann HE (1999) Age of entry to day nursery and allergy in later childhood. Lancet 353(9151):450–4
60. Celedon JC, Litonjua AA, Ryan L, Weiss ST, Gold DR (2002) Day care attendance, respiratory tract illnesses, wheezing, asthma, and total serum IgE level in early childhood. Arch Pediatr Adolesc Med 156(3):241–5
61. Celedon JC, Wright RJ, Litonjua AA, Sredl D, Ryan L, Weiss ST, Gold DR (2003) Day care attendance in early life, maternal history of asthma, and asthma at the age of 6 years. Am J Respir Crit Care Med 167(9):1239–43
62. Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL (2000) Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. N Engl J Med 343(8):538–43
63. Illi S, von Mutius E, Lau S, Bergmann R, Niggemann B, Sommerfeld C, Wahn U (2001) Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. Bmj 322(7283):390–5
64. Zutavern A, von Klot S, Gehring U, Krauss-Etschmann S, Heinrich J (2006) Pre-natal and post-natal exposure to respiratory infection and atopic diseases development: a historical cohort study. Respir Res 7:81
65. Nafstad P, Brunekreef B, Skrondal A, Nystad W (2005) Early respiratory infections, asthma, and allergy: 10-year follow-up of the Oslo Birth Cohort. Pediatrics 116(2):e255–62
66. Ramsey CD, Gold DR, Litonjua AA, Sredl DL, Ryan L, Celedon JC (2007) Respiratory illnesses in early life and asthma and atopy in childhood. J Allergy Clin Immunol 119(1):150–6
67. Gern JE, Brooks GD, Meyer P, Chang A, Shen K, Evans MD, Tisler C, Dasilva D, Roberg KA, Mikus LD, Rosenthal LA, Kirk CJ, Shult PA, Bhattacharya A, Li Z, Gangnon R, Lemanske RF, Jr. (2006) Bidirectional interactions between viral respiratory illnesses and cytokine responses in the first year of life. J Allergy Clin Immunol 117(1):72–8
68. Schaub B, Lauener R, von Mutius E (2006) The many faces of the hygiene hypothesis. J Allergy Clin Immunol 117(5):969–77; quiz 978
69. Park JH, Gold DR, Spiegelman DL, Burge HA, Milton DK (2001) House dust endotoxin and wheeze in the first year of life. Am J Respir Crit Care Med 163(2):322–8
70. Gillespie J, Wickens K, Siebers R, Howden-Chapman P, Town I, Epton M, Fitzharris P, Fishwick D, Crane J (2006) Endotoxin exposure, wheezing, and rash in infancy in a New Zealand birth cohort. J Allergy Clin Immunol 118(6):1265–70
71. Heinrich J, Bolte G, Holscher B, Douwes J, Lehmann I, Fahlbusch B, Bischof W, Weiss M, Borte M, Wichmann HE (2002) Allergens and endotoxin on mothers' mattresses and total immunoglobulin E in cord blood of neonates. Eur Respir J 20(3):617–23
72. Bolte G, Bischof W, Borte M, Lehmann I, Wichmann HE, Heinrich J (2003) Early endotoxin exposure and atopy development in infants: results of a birth cohort study. Clin Exp Allergy 33(6):770–6
73. Simpson A, John SL, Jury F, Niven R, Woodcock A, Ollier WE, Custovic A (2006) Endotoxin exposure, CD14, and allergic disease: an interaction between genes and the environment. Am J Respir Crit Care Med 174(4):386–92
74. Marra F, Lynd L, Coombes M, Richardson K, Legal M, Fitzgerald JM, Marra CA (2006) Does antibiotic exposure during infancy lead to development of asthma?: a systematic review and metaanalysis. Chest 129(3):610–8
75. Kummeling I, Stelma FF, Dagnelie PC, Snijders BE, Penders J, Huber M, van Ree R, van den Brandt PA, Thijs C (2007) Early life exposure to antibiotics and the subsequent development of eczema, wheeze, and allergic sensitization in the first 2 years of life: the KOALA Birth Cohort Study. Pediatrics 119(1):e225–31
76. Jedrychowski W, Galas A, Whyatt R, Perera F (2006) The prenatal use of antibiotics and the development of allergic disease in one year old infants. A preliminary study. Int J Occup Med Environ Health 19(1):70–6
77. Gruber C, Nilsson L, Bjorksten B (2001) Do early childhood immunizations influence the development of atopy and do they cause allergic reactions? Pediatr Allergy Immunol 12(6):296–311

78. Koppen S, de Groot R, Neijens HJ, Nagelkerke N, van Eden W, Rumke HC (2004) No epidemiological evidence for infant vaccinations to cause allergic disease. Vaccine 22(25–26): 3375–85
79. Allmers H (2005) Frequent acetaminophen use and allergic diseases: is the association clear? J Allergy Clin Immunol 116(4):859–62
80. Shaheen SO, Newson RB, Henderson AJ, Headley JE, Stratton FD, Jones RW, Strachan DP (2005) Prenatal paracetamol exposure and risk of asthma and elevated immunoglobulin E in childhood. Clin Exp Allergy 35(1):18–25
81. Arshad SH, Bateman B, Matthews SM (2003) Primary prevention of asthma and atopy during childhood by allergen avoidance in infancy: a randomised controlled study. Thorax 58(6):489–93
82. Chan-Yeung M, Ferguson A, Watson W, Dimich-Ward H, Rousseau R, Lilley M, Dybuncio A, Becker A (2005) The Canadian Childhood Asthma Primary Prevention Study: outcomes at 7 years of age. J Allergy Clin Immunol 116(1):49–55
83. Schonberger HJ, Dompeling E, Knottnerus JA, Maas T, Muris JW, van Weel C, van Schayck CP (2005) The PREVASC study: the clinical effect of a multifaceted educational intervention to prevent childhood asthma. Eur Respir J 25(4):660–70
84. Marks GB, Mihrshahi S, Kemp AS, Tovey ER, Webb K, Almqvist C, Ampon RD, Crisafulli D, Belousova EG, Mellis CM, Peat JK, Leeder SR (2006) Prevention of asthma during the first 5 years of life: a randomized controlled trial. J Allergy Clin Immunol 118(1):53–61
85. Wahn U (1998) Allergic factors associated with the development of asthma and the influence of cetirizine in a double-blind, randomised, placebo-controlled trial: first results of ETAC. Early Treatment of the Atopic Child. Pediatr Allergy Immunol 9(3):116–24
86. Murray CS, Woodcock A, Langley SJ, Morris J, Custovic A (2006) Secondary prevention of asthma by the use of Inhaled Fluticasone propionate in Wheezy INfants (IFWIN): double-blind, randomised, controlled study. Lancet 368(9537):754–62
87. Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F (2006) Intermittent inhaled corticosteroids in infants with episodic wheezing. N Engl J Med 354(19):1998–2005

# Chapter IV

## Maternal vitamin D intake during pregnancy increases gene expression of ILT3 and ILT4 in cord blood

This article has been published:

**Rochat MK**, Ege MJ, Plabst D, Steinle J, Bitter S, Braun-Fahrlander C, et al. Maternal vitamin D intake during pregnancy increases gene expression of ILT3 and ILT4 in cord blood. ClinExp Allergy **2010** May;40(5):786-94.

doi: 10.1111/j.1365-2222.2009.03428.x

*Clinical & Experimental Allergy*, **40**, 786–794



ORIGINAL ARTICLE **Epidemiology of Allergic Disease**

# Maternal vitamin D intake during pregnancy increases gene expression of ILT3 and ILT4 in cord blood

**M. K. Rochat[1]*, M. J. Ege[1]*, D. Plabst[1], J. Steinle[2], S. Bitter[3], C. Braun-Fahrländer[3], J-C. Dalphin[4], J. Riedler[5], M. Roponen[6], M-R. Hirvonen[6,7], G. Büchele[8], H. Renz[9], R. Lauener[2,10], S. Krauss-Etschmann[1,11]*, E. von Mutius[1]* and the PASTURE Study group**

*[1]Children's Hospital, University of Munich, Munich, Germany, [2]Children's Hospital, University of Zurich, Zurich, Switzerland, [3]Institute of Social and Preventive Medicine at Swiss Tropical Institute. Associated Institute of the University of Basel, Basel, Switzerland, [4]Department of Respiratory Disease, University Hospital of Besançon, Besançon, France, [5]Children's Hospital, Schwarzach, Austria, [6]Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland, [7]Department of Environmental Sciences, University of Kuopio, Kuopio, Finland, [8]Institute of Epidemiology, University of Ulm, Ulm, Germany, [9]Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Germany, [10]Hochgebirgsklinik/Children's Allergy and Asthma Hospital, Davos, Switzerland and Christine Kühne Center for Allergy Research and Education, Davos, Switzerland and [11]Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany*

Clinical & Experimental Allergy

*Correspondence:*
M. Rochat, Dr von Haunersche Kinderklinik, Lindwurmstrasse 4, 80337 Munich, Germany.
E-mail: mascha.rochat@med.uni-muenchen.de

*Cite this as*: M. K. Rochat, M. J. Ege, D. Plabst, J. Steinle, S. Bitter, C. Braun-Fahrländer, J-C. Dalphin, J. Riedler, M. Roponen, M-R. Hirvonen, G. Büchele, H. Renz, R. Lauener, S. Krauss-Etschmann, E. von Mutius and the PASTURE Study group, *Clinical & Experimental Allergy*, 2010 (40) 786–794.

## Summary

*Background* Recent studies indicate that prenatal vitamin D intake may protect against the development of atopic diseases in young children. Vitamin D has been shown to induce tolerogenic antigen-presenting cells such as dendritic cells. Whether the allergy-protective potential of prenatal vitamin D is mediated through such mechanisms is, however, unknown.
*Objective* To evaluate the association between prenatal vitamin D supplementation and tolerogenic antigen-presenting cells in cord blood (CB) as determined by mRNA measurement of immunoglobulin-like transcripts (ILT)3 and ILT4.
*Methods* A prospective multi-centre birth cohort was established in rural areas of five European countries. Information on maternal exposures including vitamin D intake was collected by questionnaires during pregnancy. The gene expression of ILT3 and ILT4 was analysed by real-time PCR in the CB of 927 children. Maternal vitamin D supplementation was assessed in Finland and France ($n = 349$).
*Results* Maternal vitamin D supplementation during pregnancy was associated with an increase in the gene expression of ILT3 ($P = 0.012$) and ILT4 ($P < 0.001$). This association remained significant for ILT4 ($P = 0.020$) and showed a positive trend for the gene expression of ILT3 ($P = 0.059$) after multivariate analysis controlling for various confounders.
*Conclusions* Vitamin D supplementation during pregnancy may increase the mRNA levels of ILT3 and ILT4 in CB. This finding may point towards an early induction of tolerogenic immune responses by maternal vitamin D intake.

**Keywords** atopic disease, birth cohort, farming environment, ILT3 and ILT4, vitamin D
*Submitted 22 July 2009; revised 29 October 2009; accepted 3 November 2009*

The PASTURE study group: Gertraud Weiß, Ellen Üblagger, Claudia Humer, Manuela Rußegger (Austria); Juha Pekkanen, Raija Juntunen, Reetta Tiihonen, Pekka Tiittanen, Kati Huttunen, Suvi Virtanen, Timo Kauppila, Aino Nevalainen, Sami Remes, Tomi-Pekka Tuomainen, Anne Karvonen, Mikko Lappalainen (Finland); Marie-Laure Dalphin, Dominique Vuitton, Renaud Piarroux, Gabriel Reboux, Sandrine Roussel, Bertrand Sudre (France); Michael Kabesch, Susanne Schmid, Sabina Illi, Nicola Korherr, Jon Genuneit, Richard Peter, Serdar Sel, Nicole Blümer, Petra Pfefferle (Germany); Bert Brunekreef, Ulrike Gehring, Gert Dockes (The Netherlands); Felix H. Sennhauser, Susanne Loeliger, Remo Frei, Caroline Roduit (Switzerland).
*Contributed equally to this work.

## Introduction

Increasing evidence suggests that gestational exposures interacting with distinct genetic backgrounds influence the development of atopic diseases. Several mechanisms have been proposed, suggesting that a variety of immune cell types and maturational processes may be involved [1, 2].

Recent studies indicate that prenatal, but not postnatal [3], vitamin D intake protects from the development of

wheeze and atopic diseases in 3- [4] and 5- [5, 6] year-old children. These data may point towards different effects of vitamin D on the prenatal and postnatal immune system and possibly the development of atopic diseases. In turn, others have suggested that vitamin D supplementation may be a cause for atopic diseases [7, 8].

Yet, vitamin D, a known immunomodulator, has been shown to induce tolerogenic dendritic cells (DC) [9]. A general feature of tolerogenic DCs is the up-regulation of the two inhibitory receptors immunoglobulin-like transcripts (ILT)3 and ILT4 [10]. The expression of these two receptors results in the inhibition of nuclear factor (NF)-κB activation, a main transcription factor for inflammatory responses [11, 12]. Beyond their ability to induce regulatory T cells (Treg) [13], tolerogenic DCs render naïve T cells anergic, inhibiting their capacity to respond [11]. Tolerogenic DCs are therefore interesting candidates in the development of atopic diseases.

We hypothesized that prenatal vitamin D supplementation could induce tolerogenic DC at birth. To evaluate this hypothesis in an epidemiological setting, we quantified the gene expression levels of ILT3 and ILT4 in cord blood (CB) samples of a population-based birth cohort of farm and reference children.

## Materials and methods

### *Study population*

The Protection against Allergy-Study in Rural Environments (PASTURE) is an ongoing multi-centre birth cohort in rural areas of five European countries (Austria, Finland, France, Germany and Switzerland) designed to assess the role of exposure to various microbial products for the development of childhood asthma and allergies. The aims of the study as well as the study design have been described in detail elsewhere [14]. Briefly, women were contacted in the third trimester of pregnancy. Women who lived or worked on family-run farms where any kind of livestock was kept were assigned to the farm group. The reference group was composed of women from the same rural areas not living or working on a farm. Exclusion criteria were living on farms without livestock, maternal age below 18 years, twin pregnancies, home births, premature deliveries, genetic disease in the offspring, absent telephone connection, insufficient knowledge of the country's language, intention to move away from the study area and commuting to a metropolitan area. The main exposure categories were identified through comprehensive questionnaires and interviews derived from internationally validated questionnaires. Maternal and paternal variables were assessed during the third trimester of pregnancy. Supplementation with vitamin D during pregnancy was assessed by the following question: 'Do you currently take any vitamin, minerals or other dietary supplements? Vitamin D yes/no,' followed by a list of other vitamins and multivitamin preparations. Vitamin D supplementation was only analysed in the Finnish and the French populations as they were the only two populations included in the PASTURE study with recommendations for vitamin D supplementation during pregnancy (Finland: 10 μg of vitamin D supplementation per day between November and the end of March. France: a single parenteral dose of 2500 μg of vitamin D is recommended in the seventh month of pregnancy). Birth and early childhood variables were assessed when the child was 2 months of age. The study was approved by the national ethical boards of the five study centres and informed consent was obtained from the children's parents for questionnaires and blood samples.

### *Blood sampling*

Blood samples were collected from the umbilical cord at birth. For the assessment of mRNA, the blood was collected in a PAXgene® Blood RNA tube containing an RNA-stabilizing solution (PreAnalytiX/Qiagen, Hilden, Germany) and then frozen to −80 °C within 24 h. In a central laboratory (Zurich, Switzerland), the RNA was isolated using the PAXgene 96 Blood RNA Kit (PreAnalytiX/Qiagen) supplemented with RNase-free DNase (Qiagen). The RNA was then quantitatively measured using the NanoDrop ND-1000 UV/VIS-Spectralphotometer (PeqLab Biotechnologie GmbH, Erlangen, Germany). Next, the mRNA was retro-transcribed into cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Weiterstadt, Germany). A pre-developed assay with quantitative real-time PCR (TaqMan, Applied Biosystems, Rotkreuz, Switzerland) was used to measure gene expression levels for ILT3 and ILT4 (Applied Biosystems; Hs00275975_m1, Hs00429000_m1), as well as for two housekeeping genes (18S-ribosomal RNA; Applied Biosystems; Hs99999901_s1 and β-2-microglobulin; Applied Biosystems; B2M-Hs99999907_m1) as endogenous controls. From the values of the respective target genes, the geometric mean threshold cycle ($C_t$) value of the two housekeeping genes was subtracted, resulting in delta $C_t$ values. Calibrating the delta $C_t$ values on the first reference child with detectable $C_t$ values for all genes produced delta $C_t$ values, which were used for statistical analysis [15, 16].

Allergen-specific IgE against 20 common inhalant and food allergens was measured using the Allergy Screen test panel for atopy (Mediwiss Analytic, Moers, Germany) in a central laboratory (Marburg, Germany). This test panel has previously been validated against the *in vitro* IgE CAP system (Pharmacia, Freiburg, Germany) [17].

After stimulation of whole CB cells at the local study centres with a standardized protocol that has been described elsewhere [18], cytokines were measured in the

supernatants in one central laboratory (Marburg, Germany). Briefly, after stimulation with phorbol 12-myristate 13-acetate (5 ng/mL) and ionomycin (1 mg/mL) (P/I) (both from Sigma, Deisenhofen, Germany) for 24 and 48 h at 37 °C, cell-free supernatants were obtained by centrifugation. Cytokine concentrations were measured using ELISA techniques (Opteia, BD, Heidelberg, Germany) according to the manufacturer's instructions. The French study group did not contribute to the cytokine data at birth.

### *Statistical analyses*

Statistical analysis was performed using SAS 9.2 (The SAS Institute, Cary, NC, USA).

The frequencies of the population characteristics were compared between farm and reference children using the $\chi^2$-test for nominal or categorical variables and the *t*-test for continuous variables. β estimates from linear regression models are given with 95% confidence intervals (CI). Reported *P*-values are two-sided. *P*-values $< 0.05$ were considered significant. Homogeneity of effects was tested by adding multiplicative interaction terms to the regression models following a pre-specified protocol.

In epidemiological studies, adjustment of *P*-values for multiple testing is not performed usually 'because it will lead to fewer errors of interpretation when the data under evaluation are not random numbers but actual observations on nature.' [19]

mRNA data were analysed as continuous values after log transformation of the delta $C_t$ values. This resulted in a satisfactory approximation to a normal distribution.

Vitamin D, farming status, gender as well as the other variables analysed were selected due to their known association with atopic diseases as well as their potential immunomodulatory function. As $< 3\%$ of the total mRNA values were below the detection limit, the analyses were performed with multiple linear regressions models including all observations according to a pre-specified analysis protocol. Statistical methods for censored data such as Tobit regression models were explored in sensitivity analyses, but did not reveal major differences.

Specific CB IgE levels were dichotomized at their detection limit of 0.2 IU/mL. Levels of cytokine production after P/I stimulation were standardized on the number of leucocytes. Log transformation of the detectable values gave satisfactory approximation to a normal distribution. The analysis was performed using a multivariable Tobit regression model where left censored cytokine values were taken into account.

## Results

Of the 2871 pregnant women contacted for the PASTURE study, 62% were eligible, and of these 1133 agreed to participate in the study [14]. CB samples were available for the gene expression analysis in 927 subjects (Fig. 1). Vitamin D supplementation during pregnancy differed significantly between the countries [Finland: 53 (28.2%), France: 26 (16.2%), Germany: 11 (5.9%), Switzerland: 2 (1.0%), and Austria: 1 (0.6%), *P*-value = $<0.001$], but did not differ between farm and reference mothers (*P*-value = 0.346). In Germany, Switzerland and Austria, further analyses on vitamin D supplementation were not possible because of low exposure frequencies. Therefore, all vitamin D supplementation analyses were performed in the Finnish and the French populations. Table 1 shows the general characteristics of mothers taking vitamin D supplementation in these two populations. In the Finnish and the French populations, children with and without CB mRNA measurements did not differ, with the exception of a lower proportion of caesarean section in the children with mRNA measurements (10% vs. 29%, $P = 0.025$).

When analysing the associations between vitamin D supplementation during pregnancy and the mRNA levels of ILT3 and ILT4 in Finland and France, higher levels were seen for ILT3 ($\beta = 0.18$, $P = 0.059$) and ILT4 ($\beta = 0.21$, $P = 0.020$) in neonates whose mothers had supplemented vitamin D during pregnancy (Fig. 2). The association was independent of farming status, gender and centre as well as all potential confounders with a *P*-value below 0.15 in Table 1 (gestational age, birth weight, primigravida, maternal age, season of birth and current smoking of mothers). Interestingly, vitamin D was also significantly associated with gestational age and birth weight in a bivariate analysis (Table 1). After adjustment for potential confounders, however, the association between vitamin D and gestational age was no longer significant ($\beta = 0.06$, $P = 0.721$), whereas the relation with birth weight remained highly significant ($\beta = 161.64$, $P = 0.005$).

In the Finnish population, vitamin D supplementation occurred more frequently during the winter months as

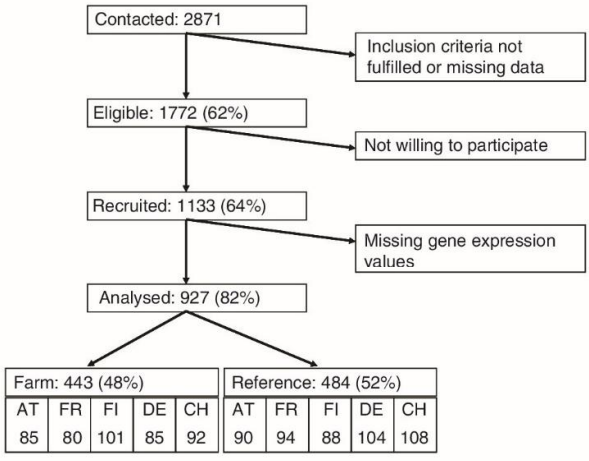



Fig. 1. Recruitment of the study population. AT, Austria, FR, France, FI, Finland, DE, Germany, CH, Switzerland.

Table 1. Population characteristics

| | Maternal intake of vitamin D during pregnancy in the Finnish and French population | | |
|---|---|---|---|
| | Yes ($n = 79$) No. percentage | No ($n = 270$) No. percentage | *P*-value |
| Female sex | 43 [54.00%] | 134 [49.63%] | 0.453 |
| Gestational age (weeks)* | 40 [1.2] | 40 [1.09] | **0.117** |
| Birth weight (g)* | 3720 [523] | 3543 [449] | **0.002** |
| Mode of birth | | | |
| Spontaneous | 64 [82.05%] | 227 [84.70%] | 0.290 |
| Vaginal with complications | 7 [8.97%] | 12 [4.48%] | |
| Caesarean section | 7 [8.97%] | 29 [10.82%] | |
| Pregnancy complications | 7 [9.00%] | 35 [12.96%] | 0.324 |
| Season of birth | | | |
| Dec/Jan/Feb | 30 [37.97%] | 65 [24.07%] | **<0.001** |
| Mar/Apr/May | 30 [37.97%] | 72 [26.67%] | |
| Jun/Jul/Aug | 12 [15.19%] | 47 [17.41%] | |
| Sept/Oct/Nov | 7 [8.86%] | 86 [31.86%] | |
| Primigravida | 23 [29.00%] | 47 [17.41%] | **0.022** |
| Child with more than two siblings | 25 [32.00%] | 107 [39.63%] | 0.198 |
| Maternal age | | | |
| < 30 years old | 37 [46.84%] | 102 [37.78%] | **0.009** |
| Between 30 and 35 years old | 34 [43.04%] | 96 [35.56%] | |
| Older than 35 | 8 [10.13%] | 72 [26.67%] | |
| Mother smoking currently | 3 [4.00%] | 31 [11.48%] | **0.043** |
| Parental atopy | 58 [81.00%] | 207 [84.84%] | 0.386 |
| Mother living or working on a farm | 34 [43.00%] | 138 [51.11%] | 0.207 |
| Exposure to pets during pregnancy | 55 [70.00%] | 171 [63.33%] | 0.304 |
| Exposure to a stable during pregnancy | 37 [47.00%] | 130 [49.62%] | 0.735 |
| Farm milk consumption during pregnancy | 27 [34.00%] | 101 [37.41%] | 0.600 |
| Mother lived on a farm during childhood | 38 [48.00%] | 113 [41.85%] | 0.324 |

*Mean (standard deviation). Significant *P*-values (<0.05) are in bold.
The frequency is compared between mothers with and without vitamin D intake using Pearson's $\chi^2$-test for categorical variables and the *t*-test for continuous variables.

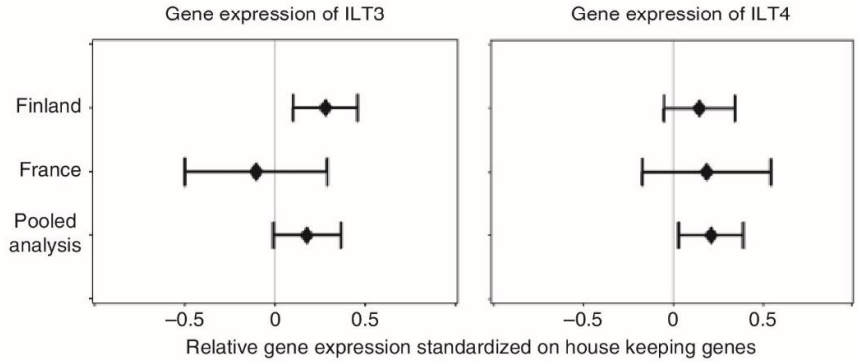



Fig. 2. Association between vitamin D intake during pregnancy and gene expression of ILT3 and ILT4 in Finland and France ($n = 349$). β-estimates and 95% confidence intervals (CI) are shown. A β-estimate greater than 0 indicates a higher gene expression of ILT3 and ILT4, whereas a β-estimate smaller than 0 indicates a lower gene expression of ILT3 and ILT4 in children whose mother supplemented vitamin D during pregnancy. An association with a CI including 0 is not statistically significance at a 0.05 significant level. The models were adjusted for gender, farming status, gestational age, birth weight, primigravida, maternal age, season of birth, current smoking of mothers, and in the pooled analysis, additionally for centre. Reported *P*-values are two-sided.

compared with the summer months (winter months 37.4%, summer months 17.9%; $P = 0.003$). Because of a lower frequency of vitamin D supplementation in the French population, the seasonal distribution could not be assessed. As vitamin D effects strongly depend on sunlight exposure, we additionally analysed the direct association

between season of birth (Winter = December–February; Spring = March–May; Summer: June–August; Autumn: September–November) and ILT3 or ILT4. For ILT3, no association was found ($P = 0.2023$), and for ILT4 the association was significant ($P = 0.001$). However, the association between farming or vitamin D supplementation on ILT4 was not substantially modified by season of birth (change in estimate < 10%).

The mRNA levels of ILT3 and ILT4 differed between farm and reference children, with considerable heterogeneity across the countries as determined by a *P*-value for homogeneity of effects of 0.01 for ILT3 and 0.009 for ILT4. In Austria, Germany and France, gene expression was increased among farm children, whereas in Switzerland, farm children had lower levels than reference children. In Finland, mRNA levels did not vary between farm and reference children. Heterogeneity of associations of ILT3 and ILT4 with farming was formally shown between Switzerland and the other countries (ILT3: *P*-value 0.002, ILT4: *P*-value 0.004), but not between the remaining countries (data not shown). In Switzerland, an inverse association was seen between mRNA levels of ILT3 ($\beta = -0.28$, $P = 0.066$) and ILT4 ($\beta = -0.14$, $P = 0.069$) in the CB of farm children as compared with reference children. In turn, in all countries other than Switzerland, significant increases in the mRNA levels of ILT3 (ILT3: $\beta = 0.14$, $P = 0.014$) and ILT4 ($\beta = 0.15$, $P = 0.003$) were seen in the CB of farm children as compared with reference children. These analyses were adjusted for gender, vitamin D supplementation during pregnancy and, in the pooled analysis with all countries except Switzerland, for centre. In all countries other than Switzerland, farm and reference children differed substantially in many aspects. However, none of the prenatal exposures assessed explained the farming effect on the mRNA levels of ILT3 and ILT4.

In boys, mRNA levels were lower for ILT4 ($\beta = 0.09$, $P = 0.032$) and for ILT3 ($\beta$: 0.10, $P = 0.059$) than in girls across all countries and independently of farming, centre or supplementation with vitamin D during pregnancy.

No association was found between specific IgE to inhalant or food allergens in CB and the mRNA levels of ILT3 or ILT4 either in all the countries together (Fig. 3) or in Finland and France (data not shown). Furthermore, no association was found between the mRNA levels of ILT3 or ILT4 and the production of the cytokines IL-12, IL-5, TNF$\alpha$ and IFN$\gamma$ (data not shown). IL-10 production, however, was inversely associated with mRNA levels of ILT3 and ILT4 after 24 and 48 h of stimulation (Fig. 4). When IL-10 production was dichotomized at the detection limit of 11.4 pg/mL, similar results were obtained for mRNA levels of ILT3 and ILT4 (data not shown).

## Discussion

The present analysis revealed a positive association between maternal vitamin D supplementation during pregnancy and mRNA levels of ILT3 and ILT4 in CB. Additionally, prenatal exposure to a farming environment in some countries and female gender in all countries were found to be positively associated with mRNA levels of ILT3 and ILT4. The ILT3 and ILT4 mRNA levels were inversely associated with IL-10 production after stimulation. No relation was found either with IL-5, IL-12, TNF-$\alpha$, IFN-$\gamma$ or with CB IgE.

ILT3 and ILT4 transcripts were quantified in whole CB. Therefore, the cellular origin of the transcripts is unknown. However, it has been shown that ILT3 and ILT4 are expressed on DCs, macrophages, monocytes and endothelial cells [10, 20]. As macrophages and endothelial cells are not present in peripheral blood, the mRNA of the

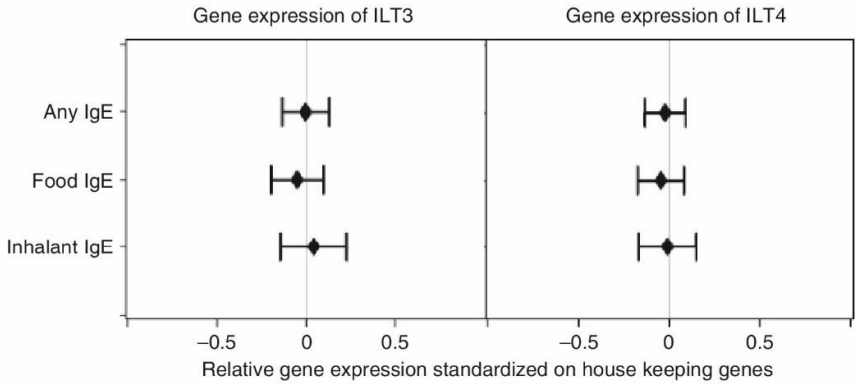



**Fig. 3.** Association between IgE levels and gene expression of ILT3 and ILT4 ($n = 843$). $\beta$-estimates and 95% confidence intervals (CI) are shown. A $\beta$-estimate greater than 0 indicates a higher gene expression of ILT3 and ILT4, whereas a $\beta$-estimate smaller than 0 indicates a lower gene expression of ILT3 and ILT4. An association with a CI including 0 is not statistically significant at a 0.05 significant level. The models were adjusted for farming status, gender, vitamin D intake during pregnancy and centre. Reported *P*-values are two-sided.

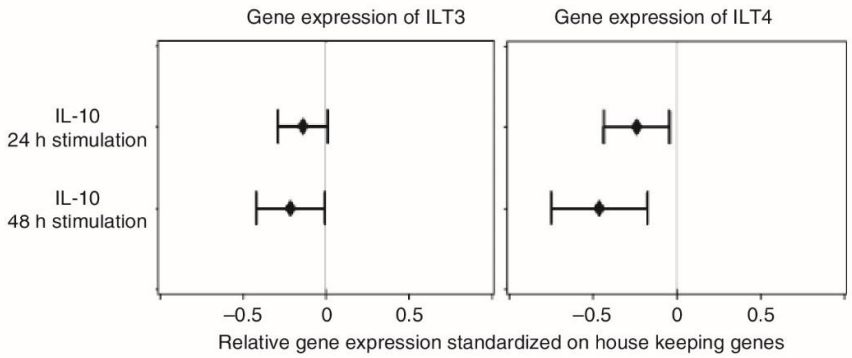



Fig. 4. Association between IL-10 cytokine levels and gene expression of ILT3 and ILT4 ($n = 522$). Continuous IL-10 cytokine values are shown after 24 and 48 h, respectively, after stimulation with P/I. β-estimates and 95% confidence intervals (CI) are shown. A β-estimate greater than 0 indicates a higher gene expression of ILT3 and ILT4, whereas a β-estimate smaller than 0 indicates a lower gene expression of ILT3 and ILT4. An association with a CI including 0 is not statistically significant at a 0.05 significant level. The models were adjusted for farming status, gender, vitamin D intake during pregnancy and centre. Reported *P*-values are two-sided.

two inhibitory receptors is most likely derived from monocytes and DCs.

The association of vitamin D supplementation with markers of tolerogenic DCs is interesting because the activated form of vitamin D, $1,25(OH)_2D3$, has pronounced immunoregulatory properties [7–9]. The biologic effects of $1,25(OH)_2D3$ are mediated by the vitamin D receptor, which is expressed on antigen-presenting cells such as DCs [21]. A number of studies have shown that *in vitro* treatment with vitamin D induces tolerogenic DC [22], up-regulates ILT3 [10] and – in combination with dexamethasone – up-regulates ILT4 [23]. ILT3 has been suggested to act as a master switch in the regulation of antigen-specific responses mediated by CD8+and CD4+T-cells in transplantation as well as cancer immunology, autoimmunity and allergy [24]. The tolerogenic effects of $1,25(OH)_2D3$ and its analogues have been observed on murine DCs *in vitro* and *in vivo* [25].

Despite an established link between maternal vitamin D intake during pregnancy and a reduced risk of wheeze in early childhood [4–6] as well as emerging data on an association between vitamin D intake and food allergy [26], only a few studies have investigated the effects of prenatal vitamin D on the human fetal immune system. One study reported a suppression of Th1 as well as Th2-associated cytokine production after *in vitro* treatment of human CB with 1α,25(OH)2D3 [27], thereby supporting the idea of an immunomodulatory effect of vitamin D on the human developing immune system. Another study found a positive association between human CB IL-10 and vitamin D levels [28]. This finding is contrary to what may be expected from our data. Both studies, however, are not comparable at many levels. One major difference is that we did not measure IL-10 levels directly after exposure to vitamin D as in the study by Zittermann et al. [28] but measured the cellular capacity to produce IL-10 after stimulation with P/I in neonates whose mother had or had not been exposed to vitamin D. The inverse association between ILT3 and ILT4 mRNA levels and IL-10 found in our study should, nevertheless, be interpreted with caution because it is not in line with other studies. For example, the study by Pedersen et al. [23] showed a positive association between ILT3 and ILT4 gene expression and IL-10 production by vitamin D-stimulated DCs.

As a potential predictor for the development of atopic diseases, we assessed whether the detection of specific IgE antibodies was associated with ILT3 and ILT4 mRNA levels. No association was found. Although high total IgE levels in CB have been shown to be risk factors for the development of allergic diseases [29], $<5\%$ of the neonates in the PASTURE study had IgE levels above 0.35 kU/L [30]. As the predictive value of specific IgE in CB for the incidence of allergic illnesses later in life is currently unknown, subsequent analyses of the PASTURE cohort are needed to determine the significance of this lack of association.

Findings from a recently published Finnish birth cohort showed an inverse association between maternal vitamin D intake during pregnancy and the risk of asthma and allergic rhinitis in 5-year-old children [6]. In this more urban population, enriched for susceptibility to type 1 diabetes, the effects were linked to common dietary intake of vitamin D and not to supplementary intake. Therefore, it remains to be seen whether ILT3 or ILT4 mRNA levels will be predictive of atopic disease.

A number of epidemiological studies have shown that prenatal and early childhood exposure to a farming environment protect from the development of atopic diseases [31, 32]. Different pathways by which farm-related environmental exposures might affect immune responses have been suggested [1, 33–35] but the mechanisms remain unclear. We assessed whether ILT3 and ILT4 mRNA levels were associated with prenatal

exposure to a farming environment and found a positive association in most study areas.

Although previous studies have shown that the protective farming effect is mediated by specific farm characteristics [36], in the present analysis, none of the assessed individual farm-related exposures accounted for the different gene expression levels between farm and reference children in all countries except for Switzerland. Unknown exposures not covered by the questionnaires may therefore be relevant for ILT3 and ILT4 gene expression. Sunlight exposure, which was not assessed by the questionnaire, might be such an exposure. Farmers are regularly exposed to sunlight during various outdoor activities and sunlight is known to induce the production of vitamin D in the skin [28, 37]. However, if sunlight would account for the farming effect, the levels of ILT3 and ILT4 expression should show seasonal fluctuations and confound the association between farming and ILT3 or ILT4 gene expression. Even though the child's season of birth was significantly associated with ILT4 expression, it did not change the estimate of the associations between farming or vitamin D with ILT4 expression. We therefore suggest that the observed farm effect cannot merely be attributed to increased sun exposure of pregnant farming women.

As in many multi-centre studies, heterogeneity was observed between study centres. Contrary to the other countries, Swiss farm children had lower levels of ILT3 and ILT4 compared with their reference children. Although the protective effect of living on a farm has clearly been shown in Switzerland for specific IgE and clinical symptoms of allergic rhinitis [38], no effect was seen for asthma [36]. Furthermore, maternal exposure during pregnancy was only weakly associated with protection from atopic sensitization [35]. Therefore, farm exposures in Switzerland may differ from other countries. It remains to be seen whether the centre heterogeneity in the CB gene expression of ILT3 and ILT4 will lead to differences in disease manifestation in the different countries.

Boys had lower levels of ILT3 and ILT4 mRNA than girls at birth. This underlines the potential biological relevance of ILT3 and ILT4 expression. However, whether the higher incidence of atopic sensitization and atopic disease observed in boys during the first years of life [39, 40] is related to this finding remains to be elucidated.

The main strengths of the PASTURE study are a well-defined prospective birth cohort study population and well-specified laboratory outcomes. However, some potential limitations exist. The gene expression association with vitamin D could only be assessed in Finland and with some limitations in France. The association with vitamin D remained after controlling for different factors including gestational age, birth weight, primigravida, maternal age, season of birth and current smoking of mothers. Therefore, major confounding by these factors is unlikely. In Finland, vitamin D supplementation during pregnancy is officially recommended. The current recommendations for pregnant women are 10 μg (400 IU) of vitamin D supplementation per day between November and the end of March. Every pregnant woman should therefore supplement vitamin D during 2–5 of her pregnancy months. The questionnaires, which were administered at recruitment at the end of pregnancy, assessed current oral vitamin D supplementation. This partly explains the low (28%) overall point prevalence of vitamin D supplementation in Finland. Nevertheless, the recommendation is reflected in our data, with more women supplementing vitamin D during the winter months than during the summer months. Moreover, the percentage of mothers taking vitamin D supplementation is similar to that of another Finnish birth cohort [6], which strengthens the plausibility of the questionnaire-based answers. Quantification of 25(OH)Vitamin D3 would have reduced non-differential misclassification, which would have strengthened the association and minimized potential bias towards the null. However, in our analysis, the association was still detectable with a relevant and significant estimate. Furthermore, the mothers answered the questionnaire blindly because at the time of data collection (before birth) they were unaware of the ILT3 or ILT4 levels.

In France, a single intramuscular dose of 2500 μg (100 000 IU once) of vitamin D is recommended in the seventh month of pregnancy. Thus, the question on oral supplementation may have led to underreporting of vitamin D supplementation in the French population. In the other countries, there are no recommendations for vitamin D supplementation during pregnancy. Hence, specific vitamin D supplementation was only reported sporadically in the other centres rendering the assessment of a potential association of vitamin D with gene expression of ILT3 and ITL4 in Germany, Austria and Switzerland impossible. In Austria and Switzerland, multivitamin supplementation was reported more frequently. However, multivitamin supplementation was not associated with either ILT3 or ILT4 (data not shown). Additionally, as we did not assess the specific formulations of multivitamins in the different countries, we do not consider multivitamin supplementation an adequate substitute for vitamin D intake. Apart from cod-liver oil preparations, no other dietary sources of vitamin D were assessed in the questionnaire. Cod-liver oil intake was reported scarcely, rendering association analyses for gene expression impossible.

In summary, the present analysis of the PASTURE birth cohort showed vitamin D supplementation during pregnancy to be associated with an increased level of CB gene expression of ILT3 and ILT4, two hallmarks of tolerogenic DCs. Exposure to a farming environment and female gender were independent determinants of increased CB gene expression of ILT3 and ILT4. These factors may

therefore be involved in the induction of tolerogenic DCs and consequently of Tregs, ultimately impacting on the Th1/Th2 balance. Whether this hypothesis can be extended to disease manifestation, and possibly to its prevention, will be left to the follow-up of the PASTURE birth cohort, when, at school age, atopic disease will have become manifest.

## Acknowledgements

We thank the participating families, the fieldworkers and other team workers of the PASTURE study.

Supported by the European Union (Research Grant QLK4-CT-2001-00250).

M.K.R. was supported by a Marie Curie Actions research grant MEST-CT-2005-020524-GALTRAIN.

*Conflict of interest:* Prof. Dr Erika von Mutius is a consultant to UCB, GSK and ProtectImmun. She receives research grants from Airsonnet AB for conducting a clinical trial. The other authors declare no conflict of interest.

## References

1 Schaub B, Liu J, Hoppler S *et al.* Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. *J Allergy Clin Immunol* 2009; **123**:774–82, e5.

2 Dietert RR, Zelikoff JT. Early-life environment, developmental immunotoxicology, and the risk of pediatric allergic disease including asthma. *Birth Defects Res* 2008; **83**:547–60.

3 Back O, Blomquist HK, Hernell O, Stenberg B. Does vitamin D intake during infancy promote the development of atopic allergy? *Acta Dermatol Venereol* 2009; **89**:28–32.

4 Camargo CA Jr, Rifas-Shiman SL, Litonjua AA *et al.* Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age. *Am J Clin Nutr* 2007; **85**:788–95.

5 Devereux G, Litonjua AA, Turner SW *et al.* Maternal vitamin D intake during pregnancy and early childhood wheezing. *Am J Clin Nutr* 2007; **85**:853–9.

6 Erkkola M, Kaila M, Nwaru BI *et al.* Maternal vitamin D intake during pregnancy is inversely associated with asthma and allergic rhinitis in 5-year-old children. *Clin Exp Allergy* 2009; **39**:875–82.

7 Zittermann A, Tenderich G, Koerfer R. Vitamin D and the adaptive immune system with special emphasis to allergic reactions and allograft rejection. *Inflam Allergy Drug Target* 2009; **8**:161–8.

8 Wjst M. The vitamin D slant on allergy. *Pediatr Allergy Immunol* 2006; **17**:477–83.

9 Adorini L, Penna G. Dendritic cell tolerogenicity: a key mechanism in immunomodulation by vitamin D receptor agonists. *Human Immunol* 2009; **70**:345–52.

10 Manavalan JS, Rossi PC, Vlad G *et al.* High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. *Transplant Immunol* 2003; **11**:245–58a.

11 Chang CC, Ciubotariu R, Manavalan JS *et al.* Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nat Immunol* 2002; **3**:237–43.

12 Liang S, Ristich V, Arase H, Dausset J, Carosella ED, Horuzsko A. Modulation of dendritic cell differentiation by HLA-G and ILT4 requires the IL-6–STAT3 signaling pathway. *Proc Natl Acad Sci USA* 2008; **105**:8357–62.

13 Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 2003; **18**:605–17.

14 von Mutius E, Schmid S. The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe. *Allergy* 2006; **61**:407–13.

15 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, CA)* 2001; **25**:402–8.

16 Bieli C, Frei R, Schickinger V *et al.* Gene expression measurements in the context of epidemiological studies. *Allergy* 2008; **63**:1633–6.

17 Herzum I, Blumer N, Kersten W, Renz H. Diagnostic and analytical performance of a screening panel for allergy. *Clin Chem Lab Med* 2005; **43**:963–6.

18 Pfefferle PI, Sel S, Ege MJ *et al.* Cord blood allergen-specific IgE is associated with reduced IFN-gamma production by cord blood cells: the Protection against Allergy-Study in Rural Environments (PASTURE) Study. *J Allergy Clin Immunol* 2008; **122**:711–6.

19 Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology (Cambridge, MA)* 1990; **1**:43–6.

20 Suciu-Foca N, Manavalan JS, Scotto L *et al.* Molecular characterization of allospecific T suppressor and tolerogenic dendritic cells: review. *Int Immunopharmacol* 2005; **5**:7–11.

21 Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat Rev* 2004; **4**:24–34.

22 Penna G, Roncari A, Amuchastegui S *et al.* Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+Foxp3+regulatory T cells by 1,25-dihydroxyvitamin D3. *Blood* 2005; **106**:3490–7.

23 Pedersen AE, Gad M, Walter MR, Claesson MH. Induction of regulatory dendritic cells by dexamethasone and 1alpha,25-Dihydroxyvitamin D(3). *Immunol Lett* 2004; **91**:63–9.

24 Vlad G, Cortesini R, Suciu-Foca N. CD8+T suppressor cells and the ILT3 master switch. *Human Immunol* 2008; **69**:681–6.

25 van Etten E, Dardenne O, Gysemans C, Overbergh L, Mathieu C. 1,25-Dihydroxyvitamin D3 alters the profile of bone marrow-derived dendritic cells of NOD mice. *Ann NY Acad Sci* 2004; **1037**:186–92.

26 Camargo CA Jr, Clark S, Kaplan MS, Lieberman P, Wood RA. Regional differences in EpiPen prescriptions in the United States: the potential role of vitamin D. *J Allergy Clin Immunol* 2007; **120**:131–6.

27 Pichler J, Gerstmayr M, Szepfalusi Z, Urbanek R, Peterlik M, Willheim M. 1 alpha,25(OH)2D3 inhibits not only Th1 but also Th2 differentiation in human cord blood T cells. *Pediatr Res* 2002; **52**:12–8.

28 Zittermann A, Dembinski J, Stehle P. Low vitamin D status is associated with low cord blood levels of the immunosuppressive

cytokine interleukin-10. *Pediatr Allergy Immunol* 2004; **15**:242–6.
29 Wahn U, Bergmann RL, Nickel R. Early life markers of atopy and asthma. *Clin Exp Allergy* 1998; **28** (Suppl. 1):20–1; discussion 32–6.
30 Ege MJ, Herzum I, Buchele G *et al.* Prenatal exposure to a farm environment modifies atopic sensitization at birth. *J Allergy Clin Immunol* 2008; **122**:407–12, 12 e1–4.
31 Douwes J, Cheng S, Travier N *et al.* Farm exposure in utero may protect against asthma, hay fever and eczema. *Eur Respir J* 2008; **32**:603–11.
32 Riedler J, Braun-Fahrlander C, Eder W *et al.* Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001; **358**:1129–33.
33 Debarry J, Garn H, Hanuszkiewicz A *et al. Acinetobacter lwoffii* and *Lactococcus lactis* strains isolated from farm cowsheds possess strong allergy-protective properties. *J Allergy Clin Immunol* 2007; **119**:1514–21.
34 Braun-Fahrlander C, Riedler J, Herz U *et al.* Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002; **347**:869–77.
35 Ege MJ, Bieli C, Frei R *et al.* Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol* 2006; **117**:817–23.
36 Ege MJ, Frei R, Bieli C *et al.* Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* 2007; **119**:1140–7.
37 Weiss ST, Litonjua AA. Maternal diet vs lack of exposure to sunlight as the cause of the epidemic of asthma, allergies and other autoimmune diseases. *Thorax* 2007; **62**:746–8.
38 Braun-Fahrlander C, Gassner M, Grize L *et al.* Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. *Clin Exp Allergy* 1999; **29**: 28–34.
39 Lau S, Nickel R, Niggemann B *et al.* The development of childhood asthma: lessons from the German Multicentre Allergy Study (MAS). *Paediatr Respir Rev* 2002; **3**:265–72.
40 Wright AL. Epidemiology of asthma and recurrent wheeze in childhood. *Clin Rev Allergy Immunol* 2002; **22**:33–44.

# Chapter V

## Allergic rhinitis as a predictor for wheezing onset in school-aged children

This article has been published:

**Mascha K. Rochat**, Sabina Illi, Markus J. Ege, Susanne Lau, Thomas Keil, Ulrich Wahn, Erika von Mutius, and the Multicentre Allergy Study (MAS) group, Allergic rhinitis as a predictor for wheezing onset in school-aged children, Journal of Allergy and Clinical Immunology, **2010** Dec;126(6):1170-5.e2

# Allergic rhinitis as a predictor for wheezing onset in school-aged children

**Mascha K. Rochat, MD,[a] Sabina Illi, PhD,[a] Markus J. Ege, MD,[a] Susanne Lau, MD,[b] Thomas Keil, MD, MSc,[c] Ulrich Wahn, MD,[b] Erika von Mutius, MD,[a] and the Multicentre Allergy Study (MAS) group*** *Munich and Berlin, Germany*

**Background: Rhinitis in older children and adults has been shown to be a predictor for adolescent- and adult-onset asthma. These findings suggest an interaction between the upper and lower airways. Whether rhinitis is a predictor for childhood-onset asthma is unknown.**

**Objective: We sought to investigate whether rhinitis in early childhood is an independent predictor for wheezing between the ages of 5 and 13 years in the German Multicentre Allergy Study birth cohort.**

**Methods: The German Multicentre Allergy Study cohort initially included 1314 healthy children. They were followed from birth to the age of 13 years with regular questionnaires and interviews. Specific IgE levels were measured at yearly intervals. Airway hyperresponsiveness was assessed at 7 years.**

**Results: Allergic rhinitis until the age of 5 years was found to be a predictor for developing wheezing between the ages of 5 and 13 years, with an adjusted relative risk of 3.82 ($P < .001$). This association was not attributable to the type of sensitization, the severity of sensitization, or atopic dermatitis during the first 2 years of life. In this group of children, 41.5% of all new cases of wheezing occurred among children with preceding allergic rhinitis.**

**Conclusions: The first manifestation of allergic rhinitis occurs in preschool children in whom it is a predictor for subsequent wheezing onset. Preschool children with rhinitis might thus benefit from early assessment of allergic sensitization to identify the children at high risk of wheezing. (J Allergy Clin Immunol 2010;126:1170-5.)**

***Key words:*** *Allergic rhinitis, birth cohort study, wheezing, allergic sensitization, risk factors*

*Abbreviations used*
AHR: Airway hyperresponsiveness
MAS: German Multicentre Allergy Study
PARF: Population-attributable risk fraction
RR: Relative risk

Rhinitis, even in the absence of atopy, has been shown to be a strong predictor for adult-onset asthma,[1,2] suggesting an interaction between the upper and lower airways. This link has been explored in a number of studies,[3] leading to the "united airways concept,"[4] which suggests that the respiratory system functions as an integrated unit. Support for this concept can be found in studies showing that pathophysiological processes involving the upper airways generally occur in conjunction with lower airway diseases and that diffuse inflammation often affects the respiratory mucosa at different sites simultaneously.[5]

Rhinitis until the age of 7 years has also been shown to be a strong predictor for adolescent- and adult-onset asthma.[6] It is, however, unknown whether rhinitis is associated with childhood-onset asthma. Childhood asthma is not one disease but rather a syndrome characterized by several distinct wheezing phenotypes.[7-9] The new onset of most wheezing phenotypes occurs in the first and second years of life,[10] too early for other symptoms to precede them. Yet other wheezing phenotypes begin after the age of 2 years or even later.[10]

These phenotypes are strongly associated with atopy and airway hyperresponsiveness (AHR) later in life.[10,11] Furthermore, pulmonary function abnormalities associated with persistent wheezing become established during early childhood and track to adult life.[12,13] The identification of a potential predictor for wheezing onset in early childhood, an age at which wheezing phenotypes might still be altered or progression to asthma halted,[10,12] might thus lead to new therapeutic strategies and possibly influence asthma morbidity.[12]

Therefore we investigated whether rhinitis in early childhood was an independent risk factor for childhood-onset wheezing in the German Multicentre Allergy Study (MAS) population-based birth cohort.

## METHODS

### Study population

The MAS, a birth cohort, recruited 1314 healthy mature infants born in 1990 in 5 German cities. A detailed description of the sampling method and the study participants has been published elsewhere.[14] Briefly, 499 newborns with risk factors for atopy (increased cord blood IgE levels [≥0.9 kU/L], ≥2 atopic family members, or both) and 815 newborns without these risk factors were included in the cohort and the presented analyses (see the Methods section in this article's Online Repository at www.jacionline.org).

From [a]Children's Hospital, University of Munich, and [b]the Department of Pediatric Pneumology and Immunology and [c]the Institute for Social Medicine and Epidemiology, Charité-Universitätsmedizin Berlin.

*The MAS study group: Volker Wahn, Marketa Groeger, Antje Schuster (Dusseldorf, Germany); Fred Zepp, Imke Bieber, Wolfgang Kamin (Mainz, Germany); Johannes Forster, Uta Tacke (Freiburg, Germany); Carl-Peter Bauer (Gaisach, Germany); Renate Bergmann, Young-Ae Lee, Renate Nickel (Berlin, Germany); Ute Hoffmann (Munich, Germany).

Supported by the German Ministry of Education and Research (BMBF), grant number 01 EE9406, and cofunded by an unrestricted educational grant from AstraZeneca Germany. M. K. R. was supported by a Marie Curie Actions research grant (MEST-CT-2005-020524-GALTRAIN).

Disclosure of potential conflict of interest: M. J. Ege has received research grants from the European Union, the German Research Foundation (DFG), and the Behring-Rontgen Foundation. The rest of the authors have declared that they have no conflict of interest.

Received for publication January 31, 2010; revised September 7, 2010; accepted for publication September 8, 2010.

Available online November 9, 2010.

Reprint requests: Mascha K. Rochat, MD, Dr. von Haunersche Kinderklinik, Lindwurmstrasse 4, 80337 Munich, Germany. E-mail: wehntaler@gmail.com.

0091-6749/$36.00



doi:10.1016/j.jaci.2010.09.008

## Blood sampling

Serum samples were obtained from the children at 1, 2, 3, 5, 6, 7, and 10 years of age. Specific IgE antibodies to food allergens (cow's milk, egg white, soy bean, and wheat) and inhalant allergens (house dust mites *Dermatophagoides pteronyssinus*, cat dander, mixed grass, birch pollen, and dog dander from age 3 years on) were determined with ImmunoCAP (Phadia, Freiburg, Germany).

## Definition of rhinitis, sensitization, and wheezing

Current rhinitis was defined as parents answering yes to the question "Has your child had a congested or runny nose since our last follow up?" and no to the question "Were these symptoms during a cold?" until the age of 2 years. After this time, it was defined as answering yes to the following question: "Did your child have sneezing attacks (at least 5 consecutively) or a congested or runny nose without a cold or an infection during the last 12 months?" To avoid underreporting of rhinitis and to increase the probability of not only assessing children with severe rhinitis,[15] we included parent-reported rhinitis and not a physician's diagnosis of rhinitis. Sensitization was defined as specific IgE levels of 0.35 kU/L or greater to at least 1 allergen.

In accordance with international guidelines,[16] children were stratified into 4 rhinitis phenotypes at each follow-up depending on the presence of rhinitis or sensitization until the age at the respective follow-up. First, children were stratified into the group of allergic rhinitis if they had rhinitis and were sensitized. Second, children with rhinitis but without sensitization were stratified into the group of nonallergic rhinitis. Third, children sensitized but without rhinitis were stratified into the group of atopy without rhinitis. Finally, the children with neither rhinitis nor sensitization formed the control group.

Current wheeze was defined as answering yes to the following question: "Has your child wheezed during the last 12 months or since our last visit?"

## AHR

At the age of 7 years, an airway challenge was performed in 647 of the 800 children whose lung function had been measured. According to a standard procedure, the airway challenge was performed with a body plethysmograph (Master-Lab; E Jaeger, Würzburg, Germany), with doses of histamine ranging from 0.5 to 8.0 mg/mL.[17] A histamine concentration of 0.85 mg/mL or less caused a 20% decrease in $FEV_1$ ($PC_{20}$ FEV1) in 10% of the population. Therefore AHR was defined as a $PC_{20}$ $FEV_1$ value of less than this. A dichotomous variable was used for the analysis. A sensitivity analysis has been performed and described elsewhere.[17]

## Statistical analyses

Cumulative incidence was defined as the number of new cases within the age range divided by all children at risk (without the symptom) at the beginning of the age range. Period prevalence refers to the time period from birth to the respective age. It was defined as the sum of cases in the respective time period divided by the number of children at the end of the time period (ie, the respective age). The occurrence of rhinitis, wheezing, or sensitization was assessed when the children were 2 and 5 years old. We distinguished 2 time periods.

First, rhinitis, wheezing, or sensitization until a given age (ie, 2 or 5 years) was defined as having rhinitis, wheezing, or being sensitized at least once until the given age. For example, a child beginning to wheeze at the age of 3 years was coded as negative for wheezing until the age of 2 years but positive for wheezing until the age of 5 years.

Second, for the purpose of the study, we defined incidence of rhinitis or wheezing as having rhinitis or wheezing at least once after the given age but not before. For example, a child beginning to wheeze at the age of 3 years was coded as positive for incidence of wheezing after the age of 2 years but negative for incidence of wheezing after the age of 5 years. Each reference group was composed of children present at more than 50% of all follow-up visits until the given age.

Cox proportional-hazard regression models were used to study the relation between rhinitis, wheezing, or sensitization and the new onset of wheezing or rhinitis. The association between rhinitis and sensitization with AHR at the age of 7 years was performed by using multivariate logistic regression analysis. For both approaches, study center, parental atopy, sex, parental education, maternal smoking during pregnancy, and 1 or more older siblings were forced into the model as potential confounders.

The population-attributable risk fraction (PARF) was calculated by using the population prevalence of the different rhinitis phenotypes (p) and the adjusted relative risk (RR) associated with the exposure as follows: $p(RR - 1)/(1 + p[RR - 1])$. The 95% CI for the PARF was estimated by using a substitution method described by Daly.[18]

**TABLE I.** Temporal relation between rhinitis, wheeze, and atopic sensitization

| Relation | Age | Adjusted RR | 95% CI | *P* value |
|---|---|---|---|---|
| Rhinitis before wheezing onset | At 2 y | 0.95 | 0.56-1.63 | .862 |
| | At 5 y | **1.83** | **1.06-3.17** | **.030** |
| Wheezing before rhinitis onset | At 2 y | 1.22 | 0.96-1.56 | .104 |
| | At 5 y | 1.12 | 0.78-1.60 | .550 |
| Sensitization before wheezing onset | At 2 y | **1.62** | **1.12-2.33** | **.009** |
| | At 5 y | **2.24** | **1.26-4.01** | **.006** |
| Sensitization before rhinitis onset | At 2 y | **1.89** | **1.49-2.39** | **<.001** |
| | At 5 y | **1.92** | **1.36->2.71** | **<.001** |

Cox regression models are mutually adjusted within the model, as well as adjusted for at least 2 first-degree family members with atopic diseases, parental education, maternal smoking during pregnancy, at least 1 older sibling, sex, and center. Significant *P* values (<.05) are shown in boldface.

# RESULTS

## Study population

Of the 1314 children recruited at birth, 1092 (83.1%) were followed to the age of 2 years, 1004 (76.4%) to the age of 5 years, 939 (71.5%) to the age of 7 years, and 766 (58.3%) to the age of 13 years.

## Prevalence and incidence of rhinitis, wheezing, sensitization, and AHR

Rhinitis point prevalence was highest at 3 years at 9.2% and ranged between 2.5% and 7.7% throughout the other years. By the age of 13 years, the cumulative incidence of rhinitis was 47.8%. Wheezing point prevalence was highest at the end of the second year, with a value of 19.8% decreasing to less than 7% throughout the following years. The cumulative incidence of wheezing was 40.5% by the age of 13 years. Proportions of 89.1% and 93.7% of all children with wheezing beginning after the ages of 2 and 5 years, respectively, were sensitized to at least 1 allergen during the whole follow-up period. The cumulative incidence of sensitization was 41.2% by the age of 10 years. In the high-risk stratum it was 46.3%, and in the low-risk stratum it was 38.0%. At the age of 7 years, the prevalence of AHR was 16.1%.

## Predictors for wheezing and rhinitis onset

To explore the temporal sequence of wheezing and rhinitis onset, we analyzed whether these 2 symptoms predicted each other at the ages of 2 and 5 years (Table I). Rhinitis significantly predicted the incidence of wheezing only at the age of 5 years, whereas wheezing was not a predictor for the incidence of rhinitis at either age considered. Independent of the age considered, sensitization was a predictor for the incidence of wheezing and rhinitis onset (Table I).

To better characterize the association between sensitization and rhinitis on the incidence of wheezing, we defined the following 4

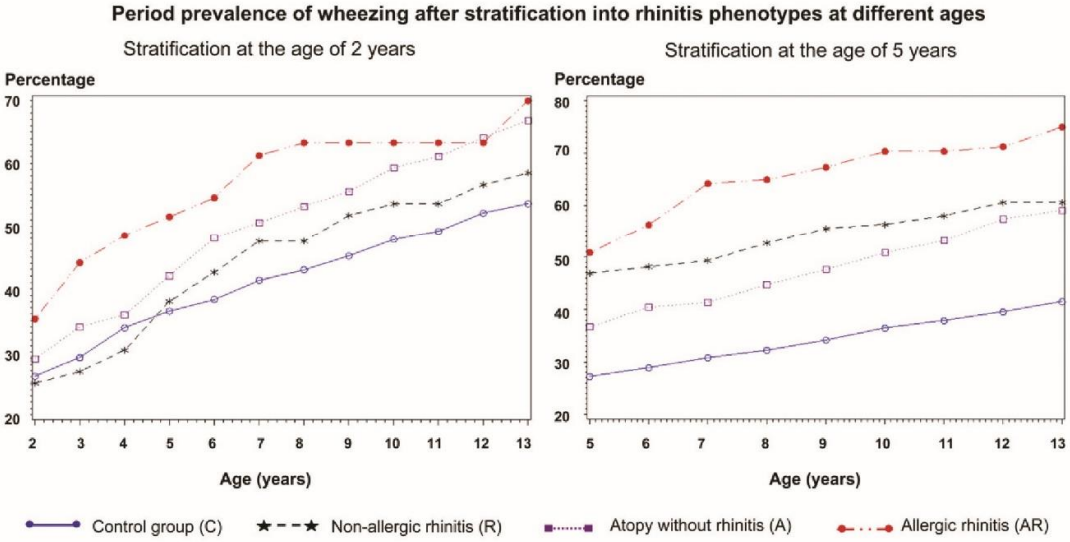



**FIG 1.** The period prevalence of wheezing (sum of the incidences until each age divided by the number of children in the follow-up at that age) after stratification in the 4 rhinitis phenotypes at different ages is shown. The 4 rhinitis phenotypes are allergic rhinitis, nonallergic rhinitis, atopy without rhinitis, and the control group.

rhinitis phenotypes: allergic rhinitis, nonallergic rhinitis, atopy without rhinitis, and control groups.

## Period prevalence of wheezing stratified by rhinitis phenotypes at different ages

The period prevalence of wheezing varied depending on the rhinitis phenotypes and the age of stratification (Fig 1). When defining the stratum at the age of 2 years, the period prevalence of wheezing was lower both in the control group and in the group with nonallergic rhinitis compared with that seen in the other 2 groups, suggesting a difference between children sensitized and nonsensitized to any allergen. This difference reached statistical significance at the age of 6 years (Fig 1).

When defining the stratum at the age of 5 years, the 4 rhinitis phenotypes tracked proportionally along the initial values, with significant differences throughout the follow-up period. Contrary to the definition at 2 years, nonallergic rhinitis showed significantly higher period prevalences than atopy without rhinitis (Fig 1).

## Baseline characteristics and selected outcomes according to rhinitis phenotype

At the age of 2 years, there were 604 children in the control group, 197 with atopy without rhinitis, 68 with nonallergic rhinitis, and 34 with allergic rhinitis. A comparison of baseline characteristics and selected outcomes did not differ among the rhinitis phenotype strata when these were defined at 2 years of age (data not shown). At the age of 5 years, there were significantly more boys, more children with a family history of atopy, and more children with asthma or AHR at the age of 7 years in the group with allergic rhinitis compared with the control group. Additionally, the children with allergic rhinitis were sensitized to more outdoor and indoor inhalant allergens compared with the group with atopy without rhinitis (Table II).

## Stratified analysis of new-onset wheeze

Fig 2 shows the probability of remaining free of wheezing for each rhinitis phenotype at the ages of 2 and 5 years, respectively. The probability of remaining free of wheezing is the inverse of the probability of wheezing onset. When assessing the probability of wheezing onset after the ages of 2 and 5 years, respectively, distinct patterns emerged. At the age of 2 years and relative to the control group, a significant increase in the incidence of wheeze was seen for children with atopy without rhinitis (adjusted RR, 1.70; $P = .007$) but not for the other strata. Even though an increased risk was seen for allergic rhinitis (adjusted RR, 1.37; $P = .496$), the association was not significant, possibly because of small numbers in this group.

When analyzing the probability of wheezing onset after the age of 5 years, however, only the children with allergic rhinitis had a significantly increased probability (adjusted RR, 3.82; $P < .001$). This association was not attributable to specific allergens because the risk of subsequent wheeze was increased consistently for all allergens tested (see Table E1 in this article's Online Repository at www.jacionline.org). Additionally, the association was not attributable to the severity of sensitization (additionally adjusting for total IgE: RR, 3.23; $P = .002$) or atopic dermatitis during the first 2 years of life (additionally adjusting for atopic dermatitis during the first 2 years of life: RR, 3.44; $P < .001$; additionally adjusting for both total IgE and atopic dermatitis: RR, 2.86; $P = .008$). As can be seen in Fig 2, at the age of 5 years, the group with allergic rhinitis varied substantially from the other 3 groups. Therefore we analyzed the effect of allergic rhinitis compared with a pooled group of the 3 other phenotypes on the risk for subsequent wheezing onset and found a significant increase (adjusted RR, 3.37; $P < .001$).

## PARF

The PARF of rhinitis phenotypes on the incidence of wheezing was highest for allergic rhinitis until the age of 5 years (41.5%; 95% CI, 20.0% to 61.3%), followed by sensitization to any allergen until the age of 5 years (39.0%; 95% CI, 10.2% to

**TABLE II.** Characteristics of children at the age of 5 years according to rhinitis phenotype

| | Total (n= 699) | Control group (n= 235) | Nonallergic rhinitis (n = 79) | Atopy without rhinitis (n = 207) | Allergic rhinitis (n = 178) |
|---|---|---|---|---|---|
| Girls | 331/699 (47.4%) | 121/235 (51.5%) | 42/79 (53.2%) | 95/207 (45.9%) | 73/178 (41.0%)* |
| High parental education | 367/662 (55.4%) | 126/222 (56.8%) | 34/76 (44.7%) | 110/195 (56.4%) | 97/169 (57.4%) |
| Maternal smoking during pregnancy | 166/697 (23.8%) | 51/235 (21.7%) | 15/79 (19.0%) | 52/205 (25.4%) | 48/178 (27.0%) |
| One or more older siblings | 326/699 (46.6%) | 113/235 (48.1%) | 36/79 (45.6%) | 102/207 (49.3%) | 75/178 (42.1%) |
| Early-life exposure to cats (≤ 3 y) | 74/683 (11.6%) | 32/219 (14.6%) | 5/72 (6.9%) | 20/188 (10.6%) | 17/159 (10.7%) |
| Early-life exposure to dogs (≤ 3 y) | 43/639 (6.7%) | 15/221 (6.8%) | 7/71 (9.9%) | 17/189 (9.0%) | 4/158 (2.5%) |
| Family history of atopy | 150/697 (21.5%) | 40/234 (17.1%) | 22/79 (27.8%)* | 38/207 (18.4%) | 50/177 (28.2%)† |
| Outdoor sensitization until 5 y | 204/326 (62.6%) | | | 94/172 (54.7%) | 110/154 (71.4%)§ |
| Food sensitization until 5 y | 271/353 (76.8%) | | | 149/196 (76.0%) | 122/157 (77.7%) |
| Indoor sensitization until 5 y | 140/319 (43.9%) | | | 66/173 (38.2%) | 74/146 (50.7%)‡ |
| Inhalant sensitization until 5 y | 267/349 (76.5%) | | | 131/186 (70.4%) | 136/163 (83.4%)§ |
| Asthma at 7 y | 50/649 (7.7%) | 7/222 (3.2%) | 1/74 (1.4%) | 9/191 (4.7%) | 33/162 (20.4%)† |
| AHR at 7 y | 83/496 (16.7%) | 17/169 (10.1%) | 7/60 (11.7%) | 23/146 (15.8%) | 36/121 (29.8%)† |

The frequency between the 4 rhinitis phenotypes is compared by using the Pearson $\chi^2$ test.
*$P < .05$ compared with the control group.
†$P < .01$ compared with the control group.
‡$P < .05$ compared with the atopy without rhinitis group.
§$P < .01$ compared with the atopy without rhinitis group.

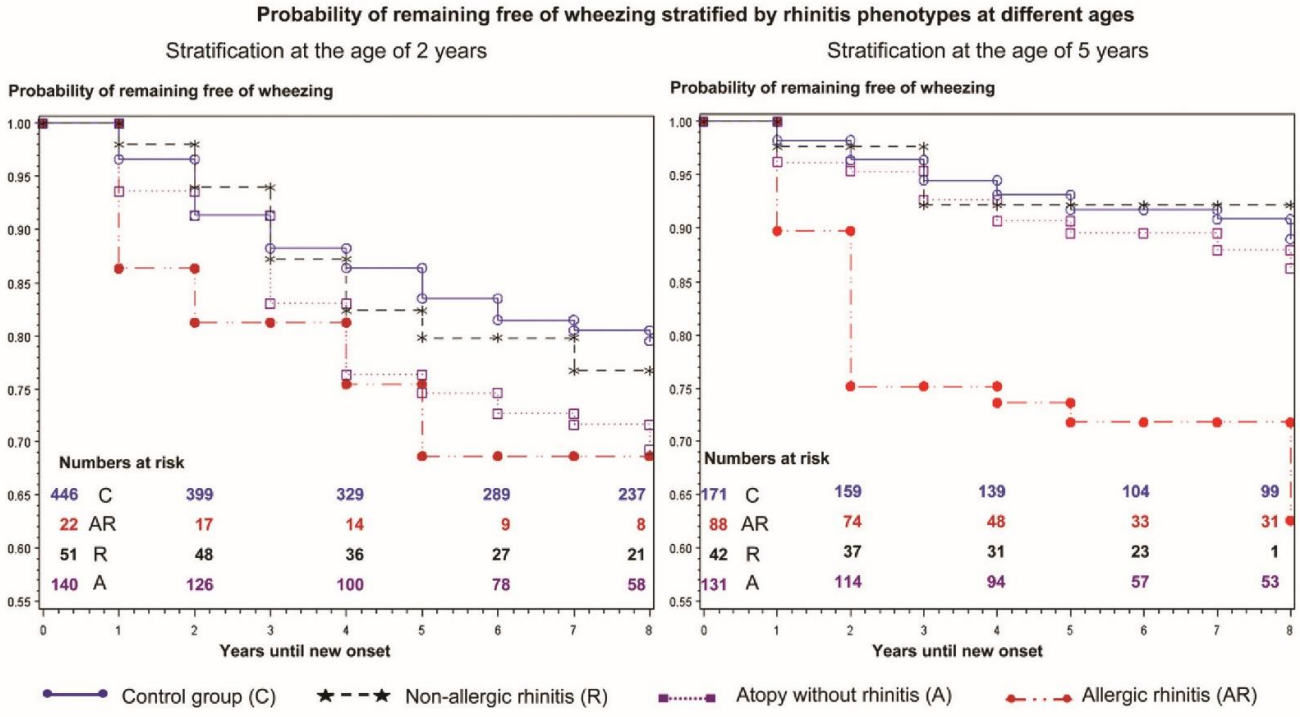



**FIG 2.** Kaplan-Meier plots of the probability of remaining free of wheezing stratified by the 4 rhinitis phenotypes at the ages of 2 and 5 years. The 4 rhinitis phenotypes were allergic rhinitis, nonallergic rhinitis, atopy without rhinitis, and the control group. The probability of remaining free of wheezing decreases over time. In the figure the probability ranges from 0.55 to 1. One represents a 100% probability of remaining free of wheezing in the corresponding year. A probability of 0.75, for example, represents a 75% probability of remaining free of wheezing, meaning a 25% probability of wheezing onset.

61.1%). The PARF for the other rhinitis phenotypes ranged between 0.7% and 24.1% depending on age and the phenotype analyzed.

## AHR

When analyzing the prevalence of AHR at the age of 7 years, a similar pattern as for wheezing emerged. Children with allergic rhinitis until the age of 5 years had the highest risk for AHR at the age of 7 years (Table III).

## DISCUSSION

The findings of this study suggest that the first manifestation of allergic rhinitis occurs in preschool children. In this age group allergic rhinitis is a risk factor for subsequent wheezing onset. In

**TABLE III.** Multivariate analysis of the effect of rhinitis and sensitization on AHR at the age of 7 years

| | OR | 95% CI | *P* value |
|---|---|---|---|
| Any sensitization until age 2 y | **2.61** | **1.58-4.33** | **<.001** |
| Ever rhinitis until age 2 y | 0.85 | 0.39-1.88 | .69 |
| Stratified analysis | | | |
| Control until age 2 y | 1 | | |
| Atopy without rhinitis until age 2 y | **2.32** | **1.35-3.97** | **.002** |
| Nonallergic rhinitis until age 2 y | 0.50 | 0.15-1.73 | .276 |
| Allergic rhinitis until age 2 y | **3.37** | **1.17-9.73** | **.025** |
| Any sensitization until age 5 y | **2.09** | **1.22-3.60** | **.008** |
| Ever rhinitis until age 5 y | **1.68** | **1.01-2.79** | **.047** |
| Stratified analysis | | | |
| Control until age 5 y | 1 | | |
| Atopy without rhinitis until age 5 y | 1.53 | 0.77-3.10 | .221 |
| Nonallergic rhinitis until age 5 y | 0.99 | 0.38-2.56 | .977 |
| Allergic rhinitis until age 5 y | **3.30** | **1.71-6.38** | **<.001** |

Multivariate logistic regressions are mutually adjusted for at least 2 first-degree family members with atopic diseases, parental education, maternal smoking during pregnancy, at least 1 older sibling, sex, and center.
Significant *P* values (<.05) are shown in boldface.
*OR*, Odds ratio.

contrast, neither the rare manifestation of allergic rhinitis in the first 2 years of life nor nonallergic rhinitis up to the age of 5 years was associated with the onset of wheezing in childhood. These findings suggest that some childhood wheezing phenotypes are associated with rhinitis in children.

Rhinitis in the first 2 years of life was not associated with wheezing thereafter. As reported in a previous analysis of the MAS cohort,[19] as well as in other studies,[7,20,21] sensitization is a driving risk factor for the development of wheezing in this age group. This finding contrasts with the concept of united airways,[4] which implicates an association between rhinitis and wheezing. Additionally, the discrepancy between the cumulative incidence of rhinitis (47.8%) and its peak period prevalence (9.2%) suggests that rhinitis is a transient phenomenon in early childhood and manifests years before wheezing begins. However, the effect of rhinitis on the incidence of wheezing might require a certain amount of time to become clinically apparent and might therefore not be detectable before school age. This notion is supported by the fact that although allergic rhinitis until the age of 2 years was not associated with subsequent wheeze, it was associated with AHR at the age of 7 years, which might precede the clinical manifestation of wheeze. Indeed, AHR has been suggested as an intermediate factor between allergic rhinitis and asthma in adults.[1] In children allergic rhinitis has also been shown to increase the prevalence of AHR,[22] and the presence of AHR with concomitant atopic manifestation increases the risk of asthma.[23,24]

Rhinitis in preschool children is a risk factor for wheezing onset after the age of 5 years, but only when associated with atopic sensitization. Although this finding goes in line with the concept of united airways,[4] nonallergic rhinitis would also be expected to be associated with wheezing onset in this concept. As reported previously in the MAS cohort, only the atopic children wheeze beyond the ages of 5 to 7 years. The nonatopic children stop wheezing around that age.[19] Therefore this cohort might not have the power to detect the effect of nonallergic rhinitis on the incidence of nonallergic wheezing.

Together, these findings suggest that in young children, as in adults, a progression from rhinitis to wheezing can be found. This progression could possibly be mediated by common features or by an interaction between the upper and lower airways. Several potential mechanisms might explain the observed association.

One potential mechanism implies impaired mucosal function. An impaired mucosal barrier leads to an increased allergen uptake and a mounting of IgE responses, which eventually results in allergic airway inflammation, AHR, and asthma.[25] Conversely, impaired mucosa of the upper and lower airways might be more susceptible to viral infections, which could interact with the process of allergic sensitization and thereby increase the risk of asthma.[26] This hypothesis is supported by studies showing that mutations in the gene encoding filaggrin, a protein known to have an important role in the integrity of the epithelial barrier,[27] were also associated with the prevalence of allergic rhinitis.[28]

Additionally, recent studies have shown that asthma is also associated with polymorphisms in genes expressed in the airway mucosa, indicating that events at epithelial cell surfaces might be driving disease processes.[29]

Another hypothesis linking the upper and lower airways implies a shared airway inflammation throughout the respiratory system. The mucosa of the different parts of the respiratory system being structurally and physiologically uniform, disorders of the entire respiratory system present with similar inflammatory responses.[30] Additionally, stimulation of one part of the respiratory mucosa with antigens has been shown to result in system-wide inflammatory changes within hours.[31] These results suggest that inflammatory crosstalk or immune communication throughout the respiratory tract is responsible for system-wide changes, resulting in an upregulation of airway inflammation.[31,32] Therefore continuous antigenic stimulation of the respiratory tract could lead to a cumulative increase of airway inflammation, resulting in the observed progression of airway inflammation from allergic rhinitis to asthma.[5] Our results support this hypothesis by showing a clear progression from rhinitis to wheezing but not from wheezing to rhinitis.

Independent of the underlying mechanism, the PARF of wheezing incidence from allergic rhinitis in preschool children in our study was substantial. Similar PARFs can be found in adult populations.[1] In adults, however, the second-highest PARF is seen for nonallergic rhinitis, whereas in children it is seen for atopy without rhinitis. This, once more, illustrates the difference in wheezing phenotypes between school-aged children and adults.

A considerable proportion of children with persistent asthma are not identified until their disease has already consolidated, and thus any potential benefits of early intervention are not achieved.[8] If the association we found is truly causal, the question arises whether the natural history of wheezing illnesses could be altered or the progression to asthma could be halted by reducing childhood allergic rhinitis. Efforts at primary prevention of asthma through rhinitis have concentrated on immunomodulation in children already sensitized or immunoprophylaxis in children not yet sensitized.[33] Allergen immunotherapy has been well established as an effective treatment of allergic rhinitis,[34,35] yet few of these studies have included children younger than 5 years.[36] Results from studies with subcutaneous and sublingual immunotherapy for secondary prevention of asthma have had mixed results.[36,37]

Data are either lacking[33] or inconclusive[38] on other therapeutic approaches, such as antihistamines, intranasal corticosteroids, leukotriene receptor antagonists, or anti–IL-5.

Therefore more studies are required to further assess whether early intervention can result in disease modification, especially in preschool children.

The main strengths of the MAS are a well-defined prospective birth cohort study population, the use of standardized questionnaires, and objective sensitization measurements. As in all epidemiologic studies, misclassification because of the outcome relying on parental recall might have biased the results. However, similar results were seen when analyzing the association between rhinitis and AHR, which is an objective measure of lower airway disorders.

The diagnosis of rhinitis in early childhood presents several diagnostic challenges because the symptoms might not be distinguishable from those of the common cold. Although we used a stepwise assessment of allergic rhinitis in 1- and 2-year-olds, we cannot exclude that a considerable part of the symptoms in the first years of life might be related to viral infections. However, viral infections interacting with atopy in infancy have been reported to promote asthma onset.[26] Because we did not find any association between rhinitis in infancy and the subsequent incidence of wheezing, misclassification of rhinitis is improbable.

In conclusion, the present analysis suggests that the first manifestation of allergic rhinitis occurs in preschool children, in whom it is a risk factor for subsequent wheezing onset. Preschool children with rhinitis might thus benefit from early assessment of allergic sensitization to identify children at high risk of wheezing. Identification of these children might help targeted selection for clinical trials on secondary prevention of asthma at school age and provide individual benefit by ensuring they are actively monitored. However, because the outcome cannot be predicted with certainty for an individual patient, parents should be counseled cautiously.

We thank the study participants and their parents, as well as the data manager, Andreas Reich (Berlin, Germany).

**Clinical implications: Allergic rhinitis in preschool children is a risk factor for subsequent wheezing onset. Preschool children with rhinitis might benefit from early assessment of allergic sensitization.**

## REFERENCES

1. Shaaban R, Zureik M, Soussan D, Neukirch C, Heinrich J, Sunyer J, et al. Rhinitis and onset of asthma: a longitudinal population-based study. Lancet 2008;372:1049-57.
2. Dixon AE. Rhinosinusitis and asthma: the missing link. Curr Opin Pulm Med 2009;15:19-24.
3. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). Allergy 2008;63 (suppl 86):8-160.
4. Passalacqua G, Ciprandi G, Canonica GW. The nose-lung interaction in allergic rhinitis and asthma: united airways disease. Curr Opin Allergy Clin Immunol 2001;1:7-13.
5. Krouse JH. The unified airway—conceptual framework. Otolaryngol Clin North Am 2008;41:257-66, v.
6. Burgess JA, Walters EH, Byrnes GB, Matheson MC, Jenkins MA, Wharton CL, et al. Childhood allergic rhinitis predicts asthma incidence and persistence to middle age: a longitudinal study. J Allergy Clin Immunol 2007;120:863-9.
7. Stein RT, Martinez FD. Asthma phenotypes in childhood: lessons from an epidemiological approach. Paediatr Respir Rev 2004;5:155-61.
8. Sly PD, Boner AL, Bjorksten B, Bush A, Custovic A, Eigenmann PA, et al. Early identification of atopy in the prediction of persistent asthma in children. Lancet 2008;372:1100-6.
9. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med 1995;332:133-8.
10. Henderson J, Granell R, Heron J, Sherriff A, Simpson A, Woodcock A, et al. Associations of wheezing phenotypes in the first 6 years of life with atopy, lung function and airway responsiveness in mid-childhood. Thorax 2008;63:974-80.
11. Kurukulaaratchy RJ, Fenn MH, Waterhouse LM, Matthews SM, Holgate ST, Arshad SH. Characterization of wheezing phenotypes in the first 10 years of life. Clin Exp Allergy 2003;33:573-8.
12. Morgan WJ, Stern DA, Sherrill DL, Guerra S, Holberg CJ, Guilbert TW, et al. Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence. Am J Respir Crit Care Med 2005;172:1253-8.
13. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med 2003;349:1414-22.
14. Bergmann RL, Bergmann KE, Lau-Schadensdorf S, Luck W, Dannemann A, Bauer CP, et al. Atopic diseases in infancy. The German multicenter atopy study (MAS-90). Pediatr Allergy Immunol 1994;5:19-25.
15. Marinho S, Simpson A, Lowe L, Kissen P, Murray C, Custovic A. Rhinoconjunctivitis in 5-year-old children: a population-based birth cohort study. Allergy 2007; 62:385-93.
16. Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. J Allergy Clin Immunol 2001;108(suppl):S147-334.
17. Niggemann B, Illi S, Madloch C, Volkel K, Lau S, Bergmann R, et al. Histamine challenges discriminate between symptomatic and asymptomatic children. MAS-Study Group. Multicentre Allergy Study. Eur Respir J 2001;17:246-53.
18. Daly LE. Confidence limits made easy: interval estimation using a substitution method. Am J Epidemiol 1998;147:783-90.
19. Illi S, von Mutius E, Lau S, Niggemann B, Gruber C, Wahn U. Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. Lancet 2006;368:763-70.
20. Lowe L, Murray CS, Custovic A, Simpson BM, Kissen PM, Woodcock A. Specific airway resistance in 3-year-old children: a prospective cohort study. Lancet 2002; 359:1904-8.
21. Joseph-Bowen J, de Klerk N, Holt PG, Sly PD. Relationship of asthma, atopy, and bronchial responsiveness to serum eosinophil cationic proteins in early childhood. J Allergy Clin Immunol 2004;114:1040-5.
22. Choi SH, Yoo Y, Yu J, Rhee CS, Min YG, Koh YY. Bronchial hyperresponsiveness in young children with allergic rhinitis and its risk factors. Allergy 2007;62:1051-6.
23. Porsbjerg C, von Linstow ML, Ulrik CS, Nepper-Christensen S, Backer V. Risk factors for onset of asthma: a 12-year prospective follow-up study. Chest 2006; 129:309-16.
24. Ferdousi HA, Zetterstrom O, Dreborg S. Bronchial hyper-responsiveness predicts the development of mild clinical asthma within 2 yr in school children with hayfever. Pediatr Allergy Immunol 2005;16:478-86.
25. Holgate ST. Epithelium dysfunction in asthma. J Allergy Clin Immunol 2007;120: 1233-46.
26. Kusel MM, de Klerk NH, Kebadze T, Vohma V, Holt PG, Johnston SL, et al. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. J Allergy Clin Immunol 2007;119:1105-10.
27. Bonness S, Bieber T. Molecular basis of atopic dermatitis. Curr Opin Allergy Clin Immunol 2007;7:382-6.
28. Weidinger S, O'Sullivan M, Illig T, Baurecht H, Depner M, Rodriguez E, et al. Filaggrin mutations, atopic eczema, hay fever, and asthma in children. J Allergy Clin Immunol 2008;121:1203-9, e1.
29. Cookson W. The immunogenetics of asthma and eczema: a new focus on the epithelium. Nat Rev Immunol 2004;4:978-88.
30. Bachert C, Vignola AM, Gevaert P, Leynaert B, Van Cauwenberge P, Bousquet J. Allergic rhinitis, rhinosinusitis, and asthma: one airway disease. Immunol Allergy Clin North Am 2004;24:19-43.
31. Braunstahl GJ, Kleinjan A, Overbeek SE, Prins JB, Hoogsteden HC, Fokkens WJ. Segmental bronchial provocation induces nasal inflammation in allergic rhinitis patients. Am J Respir Crit Care Med 2000;161:2051-7.
32. Braunstahl GJ. The unified immune system: respiratory tract-nasobronchial interaction mechanisms in allergic airway disease. J Allergy Clin Immunol 2005;115:142-8.
33. Holt PG, Sly PD. Prevention of allergic respiratory disease in infants: current aspects and future perspectives. Curr Opin Allergy Clin Immunol 2007;7:547-55.
34. Compalati E, Penagos M, Tarantini F, Passalacqua G, Canonica GW. Specific immunotherapy for respiratory allergy: state of the art according to current meta-analyses. Ann Allergy Asthma Immunol 2009;102:22-8.
35. Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. Cochrane Database Syst Rev 2007;(1):CD001936.
36. Passalacqua G, Durham SR. Allergic rhinitis and its impact on asthma update: allergen immunotherapy. J Allergy Clin Immunol 2007;119:881-91.
37. Panettieri RA Jr, Covar R, Grant E, Hillyer EV, Bacharier L. Natural history of asthma: persistence versus progression-does the beginning predict the end? J Allergy Clin Immunol 2008;121:607-13.
38. Corren J. The connection between allergic rhinitis and bronchial asthma. Curr Opin Pulm Med 2007;13:13-8.

# METHODS

## Study population

The MAS, a birth cohort, recruited 1314 healthy mature infants born in 1990 in 5 German cities. A detailed description of the sampling method and the study participants has been published elsewhere.[E1] Briefly, 499 newborns with risk factors for atopy (increased cord blood IgE level [≥0.9 kU/L], ≥2 atopic family members, or both) and 815 newborns without these risk factors were included in the cohort and the presented analyses. All children were followed up at ages 1, 3, 6, 12, 18, and 24 months and from then on yearly until the age of 13 years. The study was approved by local ethics committees. The parents of each child provided written informed consent at the time of enrollment. At each follow-up visit, parents either gave structured interviews to a study physician or completed postal questionnaires (at ages 8, 9, 11, and 12 years). The main topics of these interviews were asthmatic and atopic symptoms and disease. From the age of 5 years onward, questions relating to wheeze corresponded to the International Study of Asthma and Allergies in Childhood core questions.[E2]

To assess potential participation bias, we analyzed the number of attended follow-up visits per child, testing whether some children participated in more follow-up visits than other children based on data collected at birth. Of the 1314 children included in the study at birth, 52 attended only 1 follow-up visit, and 454 attended the maximum number of 17 follow-up visits. Children who have an atopic parent ($P < .001$ for trend), whose parents have a high level of education ($P < .001$ for trend), or whose mothers did not smoke during pregnancy ($P < .001$ for trend) were significantly more likely to attend regular follow-up visits than children without these characteristics. All multivariate models were therefore adjusted for these variables, as stated in the statistics section.

## Statistical analyses

Statistical analysis was performed with SAS 9.2 software (SAS Institute, Inc, Cary, NC).

Reported $P$ values are 2-sided. $P$ values of less than .05 were considered significant. $\chi^2$ Tests were used to compare the prevalence between groups.

Cumulative incidence was defined as the number of new cases within the age range divided by all children at risk (without the symptom) at the beginning of the age range. Period prevalence refers to the time period from birth to the respective age. It was defined as the sum of cases in the respective time period divided by the number of children at the end of the time period (ie, the respective age). The occurrence of rhinitis, wheezing, or sensitization was assessed when the children were 2 and 5 years old. We distinguished 2 time periods.

First, rhinitis, wheezing, or sensitization until a given age (ie, 2 or 5 years) was defined as having rhinitis, wheezing, or being sensitized at least once until the given age. For example, a child beginning to wheeze at the age of 3 years was coded as negative for wheezing until the age of 2 years but positive for wheezing until the age of 5 years.

Second, for the purpose of the study, we defined incidence of rhinitis or wheezing as having rhinitis or wheezing at least once after the given age but not before. For example, the child beginning to wheeze at the age of 3 years was coded as positive for the incidence of wheezing after the age of 2 years but negative for the incidence of wheezing after the age of 5 years. Each reference group was composed of children present at more than 50% of all follow-up visits until the given age.

Cox proportional-hazard regression models were used to study the relation between rhinitis, wheezing, or sensitization and the new onset of wheezing or rhinitis. The timescale was the number of years from the given age until the end of the follow-up period (age 13 years). All possible interactions between potential confounders and rhinitis, sensitization, and the different rhinitis phenotypes were tested and found to be nonsignificant. The proportional-hazard assumption was checked by testing the time dependence of all variables in the regression model. In the stratified analysis we only considered results with a cell frequency of 10 or more children.

The association between rhinitis and sensitization with AHR at the age of 7 years was performed by using multivariate logistic regression analysis. For both approaches, study center, parental atopy, sex, parental education, maternal smoking during pregnancy, and 1 or more older siblings were forced into the model as potential confounders.

The maximum number of subjects with available data was used for all analyses, resulting in varying sample sizes.

### REFERENCES

E1. Bergmann RL, Bergmann KE, Lau-Schadensdorf S, Luck W, Dannemann A, Bauer CP, et al. Atopic diseases in infancy. The German multicenter atopy study (MAS-90). Pediatr Allergy Immunol 1994;5:19-25.

E2. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J 1995;8:483-91.

**TABLE E1.** Specific sensitization pattern until the ages of 2 and 5 years

**Risk for new onset of wheezing after age 2 y**

| | Atopy without rhinitis | | | Allergic rhinitis | | |
|---|---|---|---|---|---|---|
| | **RR** | **95% CI** | ***P* value** | **RR** | **95% CI** | ***P* value** |
| Birch | 2.02 | 0.87-4.67 | .102 | * | | |
| Cat | **2.28** | **1.09-4.77** | **.028** | * | | |
| Egg | **1.67** | **1.03-2.70** | **.038** | 1.49 | 0.54-4.09 | .441 |
| Grass | 1.47 | 0.76-2.83 | .252 | * | | |
| Milk | **1.93** | **1.18-3.17** | **.009** | 0.49 | 0.07-3.54 | .477 |
| Mite | 2.08 | 0.90-4.81 | 0.089 | * | | |
| Soy | **5.14** | **2.39-11.03** | **<.001** | * | | |
| Wheat | 1.71 | 0.79-3.72 | .173 | * | | |

**Risk for new onset of wheezing after age 5 y**

| | Atopy without rhinitis | | | Allergic rhinitis | | |
|---|---|---|---|---|---|---|
| | **RR** | **95% CI** | ***P* value** | **RR** | **95% CI** | ***P* value** |
| Birch | 2.57 | 0.95-6.99 | .064 | **5.51** | **2.50-12.11** | **<.001** |
| Cat | 1.88 | 0.42-8.36 | .405 | **3.04** | **1.11-8.36** | **.031** |
| Egg | 0.96 | 0.32-2.86 | .944 | **3.68** | **1.74-7.80** | **.001** |
| Grass | **3.26** | **1.42-7.48** | **.005** | **6.57** | **3.29-13.10** | **<.001** |
| Milk | 1.22 | 0.49-3.06 | .665 | **3.70** | **1.73-7.89** | **.001** |
| Mite | 2.38 | 0.89-6.36 | .084 | **3.52** | **1.30-9.55** | **.013** |
| Soy | 1.27 | 0.17-9.80 | .816 | **9.15** | **4.00-20.92** | **<.001** |
| Wheat | **2.95** | **1.08-8.06** | **.035** | **7.26** | **3.13-16.84** | **<.001** |

Cox regression models are mutually adjusted within the model, as well as adjusted for at least 2 first-degree family members with atopic diseases, parental education, maternal smoking during pregnancy, at least 1 older sibling, sex, and center. Significant *P* values (<.05) are shown in boldface.
*Estimation was not possible because of low cell numbers ($n \leq 10$).

# Chapter VI

## Spirometry Reference Equations for Central European Populations from School Age to Old Age

This article has been published:

**Rochat MK**, Laubender RP, Kuster D, Braendli O, Moeller A, Mansmann U, von Mutius E, Wildhaber J. Spirometry reference equations for central European populations from school age to old age. PLoS One. **2013**;8(1):e52619.

OPEN ACCESS Freely available online

PLOS ONE

# Spirometry Reference Equations for Central European Populations from School Age to Old Age

**Mascha K. Rochat[1,6*☯], Ruediger P. Laubender[2☯], Daniela Kuster[3], Otto Braendli[4], Alexander Moeller[3], Ulrich Mansmann[2], Erika von Mutius[1], Johannes Wildhaber[5]**

**1** Children's Hospital, University of Munich, Munich, Germany, **2** Institute for Medical Informatics, Biometry und Epidemiology, University of Munich, Munich, Germany, **3** Division of Respiratory Medicine, Children's Hospital, University of Zurich, Zurich, Switzerland, **4** Zurich Lung Association, Zurich, Switzerland, **5** Department of Paediatrics, Hospital of Fribourg and Faculty of Medicine, University of Fribourg, Fribourg, Switzerland, **6** Department of Pediatrics, Lausanne University Hospital, Lausanne, Switzerland

## Abstract

*Background:* Spirometry reference values are important for the interpretation of spirometry results. Reference values should be updated regularly, derived from a population as similar to the population for which they are to be used and span across all ages. Such spirometry reference equations are currently lacking for central European populations.

*Objective:* To develop spirometry reference equations for central European populations between 8 and 90 years of age.

*Materials:* We used data collected between January 1993 and December 2010 from a central European population. The data was modelled using "Generalized Additive Models for Location, Scale and Shape" (GAMLSS).

*Results:* The spirometry reference equations were derived from 118'891 individuals consisting of 60'624 (51%) females and 58'267 (49%) males. Altogether, there were 18'211 (15.3%) children under the age of 18 years.

*Conclusion:* We developed spirometry reference equations for a central European population between 8 and 90 years of age that can be implemented in a wide range of clinical settings.

**Citation:** Rochat MK, Laubender RP, Kuster D, Braendli O, Moeller A, et al. (2013) Spirometry Reference Equations for Central European Populations from School Age to Old Age. PLoS ONE 8(1): e52619. doi:10.1371/journal.pone.0052619

**Editor:** Mehrdad Arjomandi, University of California San Francisco, United States of America

**Received** August 1, 2012; **Accepted** November 20, 2012; **Published** January 8, 2013



**Funding:** The work of R.P. Laubender was partially supported by the LMUinnovativ project Munich Center of Health Sciences (McHealth). No additional external funding was received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: wehntaler@gmail.com

☯ These authors contributed equally to this work.

## Introduction

"Spirometry measurements are important in diagnosis and follow-up of patients with respiratory diseases and their interpretation relies on the availability and use of appropriate reference equations [1]. In Europe, the most commonly used reference equations are outdated [2] and the continued publication of new reference equations [3] reflects the widespread recognition of the limitations of the existing ones. Most reference equations are indeed inappropriate for central European populations as they have either been derived from a small or non-European population [3] or used statistical methods that cannot adequately model the complexity of age-dependent lung function [2]". Additionally, published reference values are mostly derived from healthy never-smoking populations of restricted age ranges [3] and should not be extrapolated beyond the published range [1,4,5]. Practically, however, clinicians often track disease progression over long periods or assess effectiveness of therapy over time in patients who are not "healthy never-smokers". There is, therefore, an important need for practical reference values spanning across all ages derived from a population most similar to that for which the equations are to be used.

Such reference equations are statistically challenging as on the one hand individual spirometry measurements are determined by age, sex, height, health status, ethnicity, equipment and general population characteristics (so called "cohort effect") [1,4,6] and the European Respiratory Society (ERS)/American Thoracic Society (ATS) recommend taking these characteristics into account when developing and updating reference equations [1]. On the other hand, the lung volume changes according to height and age with a skewed distribution [7,8]. Statistical methods taking multiple variables as well as this complex distribution into account have been developed and compared [9] in recent years. A possible approach that has been applied to spirometry data are Generalized Additive Models for Location, Scale and Shape (GAMLSS) methods. GAMLSS allows modelling of data with skewed and kurtotic distribution and is therefore ideal for spirometry reference equations including transition from childhood to adulthood [10,11].

The aim of this study was to develop reference equations for a central European Population between 8 and 90 year olds.

## Materials and Methods

In this study we used data collected by the "LuftiBus" which is a project that has been described in detail previously [12,13]. Briefly, the "LuftiBus" is a mobile bus equipped with two flow-sensing spirometers that tours the greater Zurich (Switzerland) area and offers spirometry measurements to the general population. Spirometry data were recorded electronically along with data from a standardised interviewer-administered questionnaire collecting basic information on health and lifestyle of the subjects. Lung function tests were charged 10 CHF for adults and 5 CHF for children if the bus was not leased by an organisation or a community in which case the test was free of charge. When the bus was leased by schools, entire classrooms were tested. In children, weight (kg) and standing height (cm) were measured according to WHO recommendations [14], in adults they were either asked or measured.

### Study Population

For this analysis we used the data collected from volunteers between January 1993 and December 2010. In the course of the years the "LuftiBus" visited each village of the Zurich County. In each village a similar proportion of the population was tested. This proportion ranged from 0.66% in Andelfingen to 2.05% in Dielsdorf. Additionally, the age distribution of the "LuftiBus" dataset is similar to the age distribution of the Swiss population with the exception for an over-representation of teenagers [15]. Although the population tested was mainly of Western European descent, ethnicity was recorded as of 2004 (33.7% of the whole population). Non-Western European descent participants accounted for 375 (2.04%) men and 355 (1.98%) women and were excluded from the analysis. They were the only individuals excluded from the dataset. The Zurich population is representative of Central and Western European populations [16], or North-West/Central European populations [17].

### Spirometry

The "LuftiBus" is equipped with two computerised pneumotachographs (SensorMedics1 Vmax Legacy 20c spirometer run by Vision 7-2b softwarc; VIASYS, Yorba Linda, CA, USA). Thc volume signal of the equipment was calibrated at least once daily with a 3-L syringe. Tests were performed in a sitting position according to American Thoracic Society (ATS) guidelines until end of 2005 and ATS/European Respiratory Society (ERS) guidelines as of 2006 without nose-clips and after oral instruction by the technician [18,19]. Participants were assisted by trained spirometry technicians who performed immediate on-screen evaluation of major acceptability criteria (including start, duration and end of test) in addition to the automated review performed by the computer software. As recommended by the ATS/ERS task force [19] subjects were asked to perform up to a maximum of eight manoeuvres in an attempt to obtain reproducible results. The largest forced vital capacity (FVC) and forced expiratory volume in one second (FEV1) were selected. All other parameters [FEV1/FVC ratio, peak expiratory flow (PEF), mean expiratory flow at 75%, 50%, 25% of expired volume ($MEF_{75,\ 50,\ 25}$)] were taken from the trial with the largest sum of FVC and FEV1.

### Definition of variables

For the analysis we defined the two exploratory variables "smoking" and "sick". Smoking was defined as a cumulative self-reported smoking history of more than one pack-year. A pack-year being defined as years of smoking times the number of cigarettes smoked per day divided by 20. For the exploratory variable "smoking" passive smokers were considered non-smokers. Sick volunteers were defined as meeting one of the following criteria: i) common cold at the time of the measurement or ii) lung disease at the time of the measurement, which included acute bronchitis or respiratory symptoms (cough, wheezing, phlegm, shortness of breath during rest or exertion); asthma medication at the time of the measurement; history of asthma; history of chronic obstructive pulmonary disease; chronic bronchitis or a history of other lung diseases (e.g. lung surgery, pulmonary embolism). Volunteers with non-respiratory diseases such as diabetes or heart diseases were included in the healthy group. For the analysis we defined 4 health groups: healthy/non-smoker, healthy/smoker, sick/non-smoker and sick/smoker.

### Statistical analysis

Statistical analysis was performed with the statistical software "R" version 2.13.1 (R Development Core Team 2011) with the packages 'gamlss' (version 4.0-8) and 'gamlss.tr' (version 4.0-4) for the GAMLSS models [10,11,20] and with the package 'quantreg' (version 4.71) for the quantile regression models [21]. Within the GAMLSS framework we used the four-parametric Box-Cox power exponential density distribution function (BCPE($\boldsymbol{\mu}$, $\boldsymbol{\sigma}$, $\boldsymbol{\nu}$, $\boldsymbol{\tau}$)) as this distribution allows modelling of the expectation ($\boldsymbol{\mu}$), the variance ($\boldsymbol{\sigma}$), the skewness ($\boldsymbol{\nu}$) as well as the kurtosis ($\boldsymbol{\tau}$) [10] and a truncated BCPE distribution for FEV1/FVC as that endpoint cannot exceed 100%. Due to the non-linear relation between the spirometry parameters and age we used a bent hyperbola model for the $\boldsymbol{\mu}$ link with two change points and two transition smoothness parameters. Further, the non-linear relation between the spirometry parameters and age for the $\boldsymbol{\sigma}$ link was modelled by fractional polynomials of the 2$^{nd}$ degree. The change points and the transition smoothness parameters were estimated using the L-BFGS-B algorithm and within the GAMLSS models framework using the generalized Akaike's information criteria (GAIC) with a penalty of 3 and Bayesian Information Criterion (BIC). Continuous variables are presented as median and inter-quartile range. We modelled the relation between the spirometry parameters and the covariates age, height, sex, smoking status and disease status. Besides, several models with interaction terms formed of the variables age, sex and height were fitted and selected using GAIC with a penalty 3 and BIC.

## Results

### Study population

From a total of 128'568 measurements 9'677 were excluded due to age (<8 years, >90 years) incomplete data or non-Western-European origin. The spirometry reference equations were derived from 118'891 individuals consisting of 60'624 (51%) females and 58'267 (49%) males. In total there were 18'211 (15.3%) children under the age of 18 years. The age distribution of the study population is shown in Figure 1. The main characteristics of the study population can be taken from Table 1. In adults 58.9% of the women and 43.8% of the men were never smokers. All together 34.9% of the individuals under the age of 18 were either active (19.9%) or passive smokers (14.9%). Of all individuals, 66.3% where healthy, 6.8% had a common cold at the time of the measurement, 17.0% had a lung disease and 9.8% a non lung-related disease such as diabetes or heart disease.

### Reference equation modelled with GAMLSS

The lung function parameters FEV1, FVC, PEF, MEF25, MEF50, MEF75 were modelled with the Box-Cox power exponential density distribution function (BCPE($\boldsymbol{\mu}$, $\boldsymbol{\sigma}$, $\boldsymbol{\nu}$, $\boldsymbol{\tau}$)). A

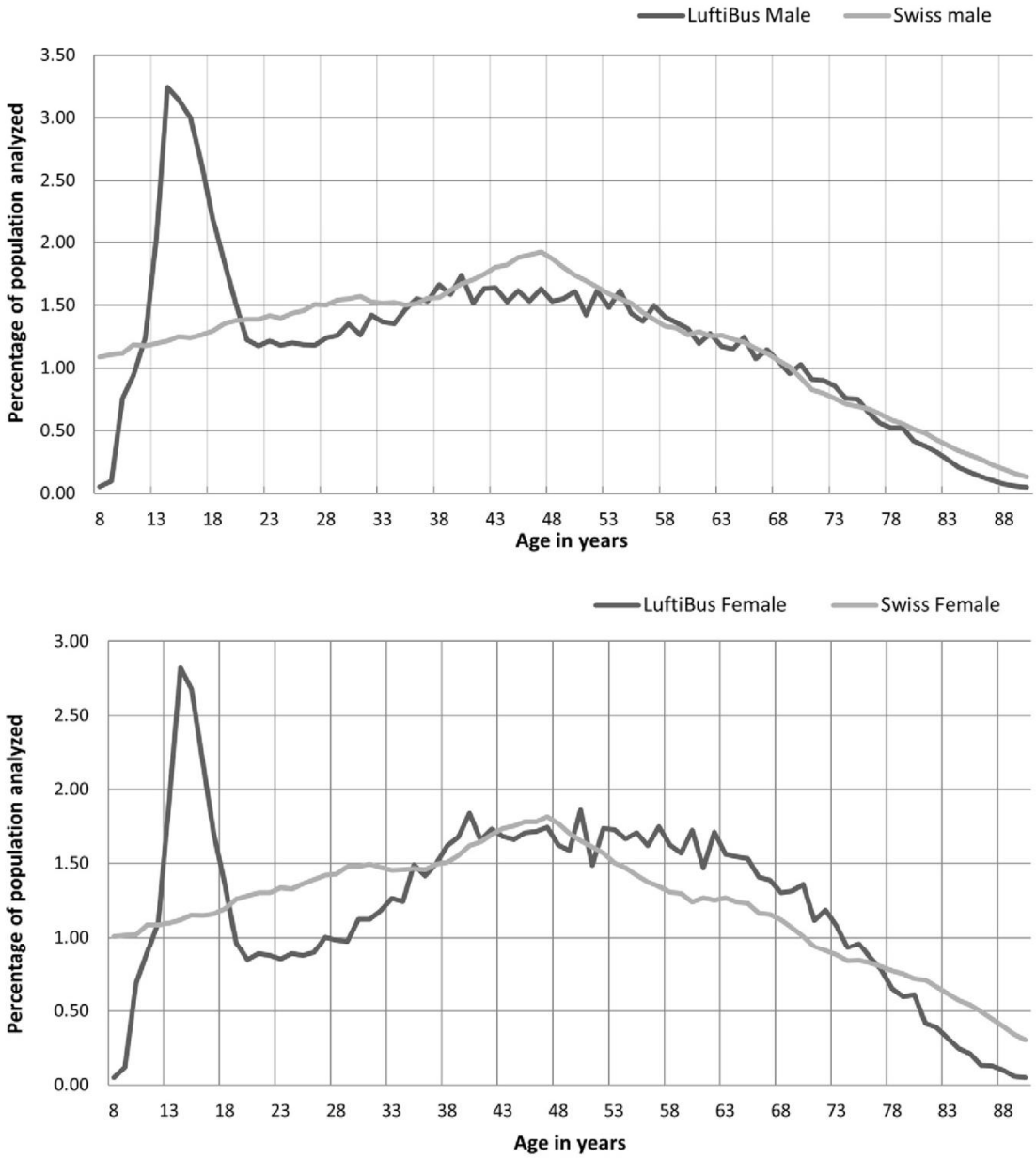



**Figure 1. Age distribution of the reference population.** A comparison with the age distribution of the Swiss population in 2011 is made.
doi:10.1371/journal.pone.0052619.g001

truncated BCPE($\mu$, $\sigma$, $\nu$, $\tau$) function was used to model the lung function parameter FEV1/FVC. The BCPE distribution was necessary as it was not possible to renounce modelling the kurtosis (when using e.g. the BCCG distribution) as this would worsen the model fit and increases the BIC (GAIC) in the models for all endpoints. Residual analyses based on worm plot were done in order to identify model inadequacies and were performed graphically for all models (Figures S1 and S2). A good model fit was achieved as only about 1484 (1.21%) individuals were not on the QQ-line. The BCPE($\mu$, $\sigma$, $\nu$, $\tau$) function gives a distribution from which the 5th quantile can be predicted. This is the quantile generally recommended for the lower limit of the normal range. The reference values (5th quantile), according to the GAMLSS model, can be calculated by the four functions in Table 2 and transformed to z-scores as described by the formula 1 of reference [10] (Figure S3).

## Comparison between the four health groups

Our reference equations not only include information on age, sex and height but also on health and smoking status. This allows us to model the entire population and produce adaptable reference equations, where smokers can be compared to a smoking

**Table 1.** Characteristics of the study population.

| N = 118'891 | Female n = 60'624 | | Male n = 58'267 | |
|---|---|---|---|---|
| | **Adults** | **Children (<18)** | **Adults** | **Children (<18)** |
| | 52'245 (100%) | 8'379 (100%) | 48'435 (100%) | 9'832 (100%) |
| **Age** | 50 (25) | 14 (3) | 47 (27) | 15 (3) |
| **Smoking:** | | | | |
| **Never-Smokers** | 30'753 (58.9%) | 5'664 (67.6%) | 21'207 (43.8%) | 6'169 (62.7%) |
| **Smokers/Ex- Smokers** | 18'419 (35.3%) | 1'436 (17.1%) | 25'769 (53.2%) | 2'208 (22.5%) |
| **Passive smokers** | 3'073 (5.9%) | 1'279 (15.3%) | 1'459 (3.0%) | 1'455 (14.8%) |
| **Health status:** | | | | |
| **Healthy** | 33'256 (63.7%) | 5'628 (67.2%) | 33'211 (68,6%) | 6'770 (68.9%) |
| **Common cold** | 2'610 (5.0%) | 1'134 (13.5%) | 3'040 (6.3%) | 1'263 (12.8%) |
| **Lung diseases** | 9'745 (18.7%) | 1'479 (17.7%) | 7'378 (15.2%) | 1'652 (16.8%) |
| **Other diseases** | 6'634 (12.7%) | 138 (1.6%) | 4'806 (9.9%) | 147 (1.5%) |

For age we reported medians and inter-quartile range (in brackets) since the distribution was skewed.
Other diseases include all non-lung diseases such as diabetes, heart diseases, etc.
doi:10.1371/journal.pone.0052619.t001

population. To illustrate this concept, Figure 2 shows a graphical representation of four different populations: "healthy/non-smoker" (54'488, 45.5%), "healthy/smoker" (36'760, 30.7%), "sick/non-smoker" (17'127, 14.3%) and "sick/smoker" (11'391, 9.5%). The biggest difference between these populations can be seen for the 5$^{th}$ quantile which is generally used as the lower limit of normal. Not surprisingly, the individuals with the highest prediction values are the "healthy/non-smokers". The "sick" individuals have the lowest values. A mean difference of 0.33 litres in men and 0.27 litres in women is seen between healthy/non-smokers and sick/smokers.

### Quantile Regression reference equation and comparison with GAMLSS

As equations modelled with GAMLSS are complex and cannot be implemented in every spirometer we developed reference equations with quantile regression to increase the implementation possibilities. However, residual analyses revealed a worse fit than for the GAMLSS models for all endpoints (additional information can be found in the supporting information online).

### Sensitivity analysis

A sensitivity analysis was performed for the following variables:

**Compulsory measurement in children.** In 66.5% of all children and adolescents spirometry was done in a compulsory setting. No significant difference was seen when excluding children measured in a volunteer setting.

**Years of data collection.** As the data was collected over a period of 17 years we analysed a linear time trend but did not find any significant difference over time.

**Body Mass Index (BMI).** Only marginal differences were found when comparing the reference equations for BMI cut-of values of <25, 25–30 and >30 for adults and their equivalents for children [22].

**Common cold.** Reference values for common cold alone were only marginally different than reference values for healthy individuals.

As only marginal differences were found in all sensitivity analysis (data not shown) all individuals and years were included in the final population.

## Discussion

We developed spirometry reference equations for 8–90 year olds from a very large, cross-sectional sample of a Central European population.

"Spirometry reference values are important for the interpretation of individual spirometry measurements and may influence clinical decision making. Most published reference equations use statistical methods that cannot adequately model the complexity of age-dependent lung function [2] and very few span across all ages [3] introducing discontinuities at the transition points with potential clinical implications for individuals with chronic lung diseases."

One exception are the recently published spirometry reference equations by Stanojevic et al., developed with complex statistical methods for individuals aged 4–80 years of age [8]. As their reference equations were derived from 4 pooled datasets collected in 4 different countries (USA, Canada, UK and Belgium) their reference values can be generalized to other mixed populations with similar ethnic backgrounds. The reference equations we developed are complementary to theirs as they also span from school age to old age and use similar statistical methods. However, they are derived from a single Central European population with homogenous local environmental factors and genetic background and the data was collected using the same instruments and testing procedures throughout the years. Nevertheless, both equations result in similar values [8] (Figure 2: healthy non-smoker). In boys, the peak lung function is reached at the age of 20 years with almost 4.5 l followed by an age-dependent decline to just under 2.75 l at the age of 80 years. In girls, the peak lung function is reached at 19 years with 3.4 l followed by an age-dependent decline to 2 l at the age of 80 years. The decline is initially less steep in the LuftiBus population with 3.2 l at the age of 40 years compared to 3 l in the Stanojevic reference equations.

Spirometry reference equations should be derived from a population as similar to the population from which the patient originates as possible [23]. However, most spirometry reference equations are derived from healthy non-smoking individuals [3] who are generally a small subsample and have higher reference values than the general population [24]. Some authors have therefore included smokers in their reference population when the

**Table 2.** GAMLSS reference equations.

**Reference equation for the mean (μ):**

$g_1(\mu) = \beta_0+\beta_1*age+\beta_2*\sqrt{((age-\alpha_1)^2+\gamma_1^2)}+\beta_3*\sqrt{((age-\alpha_2)^2+\gamma_2^2)}+\beta_4*sex+\beta_5*height+\beta_6*smoker+\beta_7*sick+\beta_8*age*sex+\beta_9*\sqrt{((age-\alpha_1)^2+\gamma_1^2)}*sex+\beta_{10}*\sqrt{((age-\alpha_2)^2+\gamma_2^2)}*sex+\beta_{11}*height*sex$

| | FEV1 | FVC | FEV1/FVC | PEF | MEF25 | MEF50 | MEF75 |
|---|---|---|---|---|---|---|---|
| Regression parameters (β) | | | | | | | |
| $\beta_0$ | −3.708 | −5.593 | 105.386 | −0.841 | 0.956 | −0.627 | −3.745 |
| $\beta_1$ | 0.044 | 0.025 | 0.516 | 0.154 | 0.098 | 0.097 | 0.071 |
| $\beta_2$ | −0.068 | −0.047 | −0.919 | −0.203 | −0.148 | −0.146 | −0.118 |
| $\beta_3$ | −0.013 | −0.017 | 0.254 | −0.088 | −0.063 | −0.017 | 0.046 |
| $\beta_4$ | 1.586 | 2.140 | 3.520 | 0.550 | 0.284 | 1.596 | 0.551 |
| $\beta_5$ | 0.043 | 0.061 | −0.153 | 0.073 | 0.048 | 0.029 | 0.010 |
| $\beta_6$ | −0.033 | 0.024 | −1.443 | −0.135 | −0.048 | −0.149 | −0.126 |
| $\beta_7$ | −0.086 | −0.061 | −1.028 | −0.162 | −0.322 | −0.277 | −0.091 |
| $\beta_8$ | −0.024 | −0.018 | −0.083 | −0.110 | −0.074 | −0.054 | −0.028 |
| $\beta_9$ | 0.027 | 0.024 | −0.088 | 0.119 | 0.088 | 0.063 | 0.029 |
| $\beta_{10}$ | 0.003 | 0.000 | 0.172 | 0.035 | 0.029 | 0.005 | 0.004 |
| $\beta_{11}$ | −0.011 | −0.015 | −0.005 | −0.013 | −0.008 | −0.009 | −0.003 |
| Changepoints (α) | | | | | | | |
| $\alpha_1$ | 18.789 | 22.080 | 15.964 | 19.688 | 19.436 | 17.194 | 16.505 |
| $\alpha_2$ | 37.006 | 38.368 | 23.828 | 50.902 | 57.602 | 56.979 | 20.000 |
| Transition smoothness (γ) | | | | | | | |
| $\gamma_1$ | 0.789 | 0.587 | 0.285 | 1.067 | 1.494 | 0.965 | 0.100 |
| $\gamma_2$ | 12.143 | 9.122 | 6.771 | 27.527 | 17.050 | 3.257 | 74.096 |

**Reference equation for the variance (σ):**

$g_2(\sigma) = \exp[\beta_0+\beta_1*(age^{p1})+\beta_2*(age^{p2})+\beta_3*height+\beta_4*sex+\beta_5*smoker+\beta_6*sick]$

| | FEV1 | FVC | FEV1/FVC | PEF | MEF25 | MEF50 | MEF75 |
|---|---|---|---|---|---|---|---|
| Regression parameters (β) | | | | | | | |
| $\beta_0$ | −1.543 | −1.483 | −2.719 | −0.690 | −1.410 | −0.991 | −2.041 |
| $\beta_1$ | 0.269 | 0.276 | −0.174 | −0.162 | 0.056 | 0.014 | −1.020 |
| $\beta_2$ | 0.012 | 0.011 | 0.025 | 0.011 | 0.001 | −0.001 | 1.465 |
| $\beta_3$ | −0.004 | −0.004 | 0.002 | −0.005 | −0.001 | −0.002 | −0.003 |
| $\beta_4$ | −0.095 | −0.048 | −0.009 | −0.063 | −0.148 | −0.113 | −0.026 |
| $\beta_5$ | 0.052 | 0.022 | 0.062 | 0.050 | 0.061 | 0.078 | 0.069 |
| $\beta_6$ | 0.132 | 0.069 | 0.094 | 0.069 | 0.128 | 0.118 | 0.078 |
| Power for the fractional polynomials # | | | | | | | |
| p1 | −1 | −1 | 1 | 0.5 | 0 | 2 | 0 |
| p2 | 2 | 2 | 2 | 2 | 3 | 3 | 0.5 |

**Table 2.** Cont.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Reference equation for the variance (σ):** | | | | | | | |
| **$g_2(\sigma) = \exp[\beta_0+\beta_1*(age^{P1})+\beta_2*(age^{P2})+\beta_3*height+\beta_4*sex+\beta_5*smoker+\beta_6*sick]$** | | | | | | | |
| | **FEV1** | **FVC** | **FEV1/FVC** | **PEF** | **MEF25** | **MEF50** | **MEF75** |
| # if p1≠p2 then β1*(age $^{P1}$)+β2*(age $^{P2}$); if p1 = p2 = p then β1*(age $^{P}$)+β2*(age $^{P}$)* ln(age); If p1 = 0 then use the natural logarithm (ln) | | | | | | | |
| **Reference equation for the skewness (ν):** | | | | | | | |
| **$g_3(\nu) = \beta_0+\beta_1 * age+\beta_2 * height+\beta_3 * sex+\beta_4 * smoker+\beta_5 * sick$** | | | | | | | |
| | **FEV1** | **FVC** | **FEV1/FVC** | **PEF** | **MEF25** | **MEF50** | **MEF75** |
| Regression parameters (β) | | | | | | | |
| $\beta_0$ | −0.130 | −3.053 | 2.762 | −0.854 | 0.291 | 0.946 | 0.618 |
| $\beta_1$ | 0.007 | 0.005 | 0.006 | 0.005 | 0.010 | 0.001 | −0.005 |
| $\beta_2$ | 0.005 | 0.021 | −0.007 | 0.009 | 0.001 | −0.003 | −0.001 |
| $\beta_3$ | 0.021 | −0.093 | −0.347 | −0.206 | −0.044 | 0.074 | −0.022 |
| $\beta_4$ | 0.245 | 0.120 | 0.431 | 0.148 | 0.112 | 0.103 | −0.003 |
| $\beta_5$ | 0.326 | 0.173 | 0.907 | 0.019 | 0.197 | 0.201 | 0.074 |
| **Reference equation for the kurtosis (τ):** | | | | | | | |
| **$g_4(\tau) = \exp[\beta_0+\beta_1 * age+\beta_2 * height+\beta_3 * sex+\beta_4 * smoker+\beta_5 * sick]$** | | | | | | | |
| | **FEV1** | **FVC** | **FEV1/FVC** | **PEF** | **MEF25** | **MEF50** | **MEF75** |
| Regression parameters (β) | | | | | | | |
| $\beta_0$ | 0.166 | −0.121 | −0.046 | 0.528 | 0.106 | 0.280 | 0.603 |
| $\beta_1$ | 0.001 | 0.001 | −0.001 | 0.001 | 0.001 | 0.001 | 0.002 |
| $\beta_2$ | 0.002 | 0.003 | 0.003 | 0.001 | 0.003 | 0.002 | −0.001 |
| $\beta_3$ | −0.014 | −0.014 | 0.014 | −0.066 | −0.090 | 0.060 | 0.023 |
| $\beta_4$ | 0.015 | 0.007 | 0.015 | 0.022 | 0.038 | 0.003 | 0.018 |
| $\beta_5$ | −0.026 | −0.047 | 0.049 | −0.039 | −0.006 | 0.002 | 0.040 |

The variables are coded as followed:
Age: years; height: cm, sex: male = 0, female = 1; smoker: non-smoker = 0; smoker = 1*; sick: healthy = 0; sick = 1*.
*definition of smoker and sick can be found in the methods section of the paper.
**Note:** to compare a patient with a "healthy-non-smoker" population, "smoker" and "sick" must be set to "0" even if the patient is a smoker and has a pulmonary pathology.
doi:10.1371/journal.pone.0052619.t002

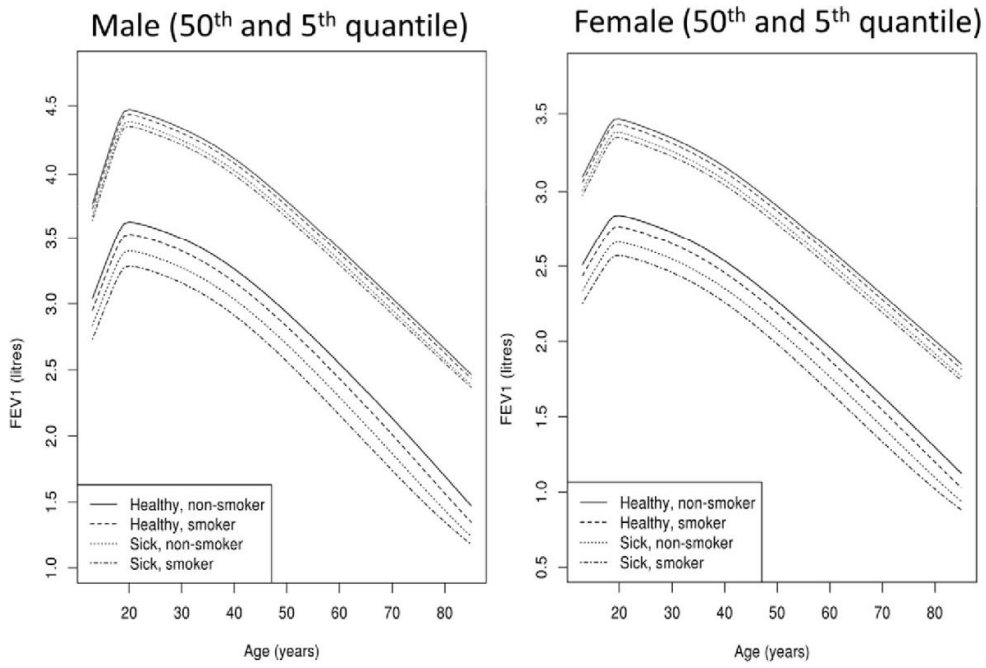



**Figure 2. Comparison between the four health groups.** The lung function parameter FEV1 is compared between the four health groups at ages between 8–90 years old. For this comparison only men of 175 cm and women of 165 cm were included. The 5th quantile indicates the lower limit of normal for each group. FEV1: forced expiratory volume in one second. The four health groups are: healthy/non-smoker, healthy/smoker, sick/non-smoker and sick/smoker.
doi:10.1371/journal.pone.0052619.g002

smoking prevalence was high [25]. The statistical methods we used permitted us to model the entire population while including information on smoking and health status. This allows clinicians to choose which reference values are most appropriate for a given individual. Indeed, although in most situations reference values for healthy-never-smokers will be used, reference values for healthy-smokers might be more appropriate for certain patients when tracking disease progression or assessing effectiveness of therapy over time. By including information on disease the reference equations allow a comparison between healthy and sick individuals. As can be seen in Figure 2 our data confirm that individuals with lung diseases have lower spirometry values than healthy individuals. Even though individuals with common cold where included in the "sick" group, they did not have significantly different reference values than healthy individuals, suggesting as recently published [24] that not all respiratory symptoms need to be accounted for when performing spirometry in patients.

Practically, the reference values according to the GAMLSS model can be calculated by the four functions in Table 2 and the formula found in Figure S3 [10]. To begin with, the values age, sex, and height of a person have to be known. Smoker and sick are for the clinician to decide. If the clinician would like to compare a person to a "healthy-non-smoker" population then "smoker" and "sick" should be set to zero. The values calculated with the Table 2 must then be inserted in the function found in Figure S3 from where the quantiles can be calculated. However, since these are complex algebraic equations, the reference values are best obtained by using the statistical software package R where the function 'qBCPE' implemented in the package 'gamlss' can be used. R is a free language and environment for statistical computing and graphics that can be downloaded from the following internet site (http://www.r-project.org/). Additionally, upon request, the authors will gladly provide the source code in R, thus facilitating its implementation in spirometry devices.

To allow the reference equations to be implemented in a wide range of spirometers we additionally developed reference equations with quantile regression [12,26,27] using the same endpoints (Table S1). A comparison between GAMLSS and quantile regression models was done in Figure S5. However, compared to the GAMLSS models, residual analyses revealed a worse model fit for all endpoints (Figure S1, S4). Therefore, while the GAMLSS reference equations should be used whenever possible as they give the most accurate reference values the quantile regression equations can be implemented as an alternative.

The reference equations for 18–80 years old recently published by Kuster et al [12] are derived from the same data set. However, the two reference equations are not directly comparable. Indeed, we included data spanning from school age to old age thus modelling the growth spurt of puberty and the transition from childhood to adulthood. The equations presented herein therefore expand and complement the reference equations from Kuster at al.

The ATS/ERS task force recommends that reference values be derived from a "representative sample of healthy subjects in a general population"; but, alternatively, can also be derived from a "large group of volunteers, provided that criteria for normal selection and proper distribution of anthropometric characteristics are satisfied" [1]. Although the population visiting the "LuftiBus" consisted mostly of volunteers and was thus possibly motivated by personal health concerns we believe that the "LuftiBus" population can be considered a "large group of volunteers" representative of the Zurich population. First, in the course of the 18 years

the "LuftiBus" visited each village of the Zurich County and a similar proportion of the population of each village is represented in the dataset. Second, the age distribution of the "LuftiBus" dataset is similar to the age distribution of the Swiss population apart from an over representation of teenagers [15]. Third, when the "LuftiBus" was leased by schools whole classrooms were tested which allowed us to perform a sensitivity analysis between the children being tested in a compulsory or a voluntary setting. No significant difference was found. Lastly, we excluded all "sick" and "smoking" individuals from our "healthy/non-smoking" reference values, thus reducing possible biases caused by health concerns.

Lung function has been shown to be influenced by various factors such as cohort effect [1], ethnicity [28] or BMI [29]. As only marginal differences were found when performing sensitivity analysis we did not exclude individuals or years tested but rather considered them as part of our "general representative" population.

We developed spirometry reference equations spanning from school age to old age for a Central European population. The equations were derived from a large general population and are intended for every day clinical use as they can be implemented in most clinical settings. Additionally they allow clinicians to choose reference values depending on a given clinical situation.

## Supporting Information

**Supporting Information S1 Results S1; Quantile Regression reference equation and comparison with GAMLSS.**
(DOC)

**Table S1 Quantile regression reference equation.**
(DOCX)

**Figure S1 Residual plots for FEV1 from the GAMLSS model.** Residuals of FEV1 from the GAMLSS model using BCPT are shown: (a) against fitted values of μ (b) against each person (c) kernel density estimate (d) normal QQ plot. The Figures show that the model is adequately fitted as the plots are homogenous, compact, well centred around the zero in the density estimate plot and only about 1484 individuals are not on the QQ-line. GAMLSS: Generalized Additive Models for Location, Scale and Shape. BCPE: Box-Cox power exponential density distribution function. FEV1: forced expiratory volume in one second. μ: mean.
(TIF)

**Figure S2 Worm plot of the residuals of the GAMLSS reference equation for FEV1.** The worm plot shows that the model is well fitted at every age. The top bar shows the 20 age ranges tested (displayed in steps from 6 to 99 years). The 20 corresponding 20 QQ plots (quantile-quantile plots) are probability plots, which is a graphical method for comparing the residuals of the GAMLSS model. They read from bottom left to top right and correspond to the 20 age ranges. GAMLSS: Generalized Additive Models for Location, Scale and Shape. FEV1: forced expiratory volume in one second.
(TIF)

**Figure S3 Formula for calculating quantiles.** Formule taken from Rigby RA, Stasinopoulos DM (2004) Smooth centile curves for skew and kurtotic data modelled using the Box-Cox power exponential distribution. Stat Med 23: 3053–3076.
(TIF)

**Figure S4 Residual plots for FEV1 for quantile regression.** Residuals from the quantile regression model for the 50th and the 5th quantile are shown. (a) against fitted values of μ (b) against each person (c) kernel density estimate (d) normal QQ plot. The residuals show a slight skewed distribution which is accentuated in the 5th quantile. This can be seen by the plots being less centred and less compact, having individuals at −4 but non at +4 in the density estimate plot and having less individuals on the QQ-line. FEV1: forced expiratory volume in one second. μ: mean.
(TIF)

**Figure S5 Comparison between the GAMLSS and Quantile Regression reference equations.** The lung function parameter FEV1 is compared between the GAMLSS and the Quantile Regression model between the ages of 8–90 years old. For this comparison only healthy non-smoking men of 175 cm and women of 165 cm were included. The $5^{th}$ quantile indicates the lower limit of normal for each group. GAMLSS: Generalized Additive Models for Location, Scale and Shape. Quantreg: quantile regression.
(TIF)

## Acknowledgments

The authors acknowledge and thank the Lung Association Zurich (Switzerland) for providing the funding for the spirometry data.

## Author Contributions

Conceived and designed the experiments: MKR DK OB AM JW. Analyzed the data: MKR RPL UM EvM. Contributed reagents/materials/analysis tools: OB UM EvM. Wrote the paper: MKR RPL EvM.

## References

1. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, et al. (2005) Interpretative strategies for lung function tests. Eur Respir J 26: 948–968.
2. Stanojevic S, Wade A, Stocks J (2010) Reference values for lung function: past, present and future. Eur Respir J 36: 12–19.
3. Liou TG, Kanner RE (2009) Spirometry. Clin Rev Allergy Immunol.
4. Stocks J, Quanjer PH (1995) Reference values for residual volume, functional residual capacity and total lung capacity. ATS Workshop on Lung Volume Measurements. Official Statement of The European Respiratory Society. Eur Respir J 8: 492–506.
5. Subbarao P, Lebecque P, Corey M, Coates AL (2004) Comparison of spirometric reference values. Pediatr Pulmonol 37: 515–522.
6. Kunzli N, Kuna-Dibbert B, Keidel D, Keller R, Brandli O, et al. (2005) Longitudinal validity of spirometers–a challenge in longitudinal studies. Swiss Med Wkly 135: 503–508.
7. Golshan M, Amra B, Soltani F, Crapo RO (2009) Reference values for lung volumes in an Iranian population: introducing a new equation model. Arch Iran Med 12: 256–261.
8. Stanojevic S, Wade A, Stocks J, Hankinson J, Coates AL, et al. (2008) Reference ranges for spirometry across all ages: a new approach. Am J Respir Crit Care Med 177: 253–260.
9. Borghi E, de Onis M, Garza C, Van den Broeck J, Frongillo EA, et al. (2006) Construction of the World Health Organization child growth standards: selection of methods for attained growth curves. Stat Med 25: 247–265.
10. Rigby RA, Stasinopoulos DM (2004) Smooth centile curves for skew and kurtotic data modelled using the Box-Cox power exponential distribution. Stat Med 23: 3053–3076.
11. Cole TJ, Stanojevic S, Stocks J, Coates AL, Hankinson JL, et al. (2009) Age- and size-related reference ranges: a case study of spirometry through childhood and adulthood. Stat Med 28: 880–898.
12. Kuster SP, Kuster D, Schindler C, Rochat MK, Braun J, et al. (2008) Reference equations for lung function screening of healthy never-smoking adults aged 18–80 years. Eur Respir J 31: 860–868.
13. Egger S, Wieland R, Ludin M, Brandli O, Vetter W, et al. (2001) [Overweight and obesity in the Zurich canton. A LuftiBus study]. Praxis (Bern 1994) 90: 531–538.

14. (1995) Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser 854: 1–452.
15. Official website of Switzerland ps (Accessed 27 november 2012) http://www.bfs.admin.ch/bfs/portal/de/index/themen/01/02/blank/key/alter/gesamt.html.
16. Nelis M, Esko T, Magi R, Zimprich F, Zimprich A, et al. (2009) Genetic structure of Europeans: a view from the North-East. PLoS One 4: e5472.
17. Lao O, Lu TT, Nothnagel M, Junge O, Freitag-Wolf S, et al. (2008) Correlation between genetic and geographic structure in Europe. Curr Biol 18: 1241–1248.
18. (1991) Lung function testing: selection of reference values and interpretative strategies. American Thoracic Society. Am Rev Respir Dis 144: 1202–1218.
19. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, et al. (2005) Standardisation of spirometry. Eur Respir J 26: 319–338.
20. Stasinopoulos RARDM (2007) Generalized Additive Models for Location Scale and Shape (GAMLSS) in R. Journal of Statistical Software 23: 1–46.
21. Koenker R (2005) Quantile Regreesion. Cambridge University Press Cambridge.
22. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH (2000) Establishing a standard definition for child overweight and obesity worldwide: international survey. Bmj 320: 1240–1243.
23. Pistelli F, Bottai M, Carrozzi L, Baldacci S, Simoni M, et al. (2007) Reference equations for spirometry from a general population sample in central Italy. Respir Med 101: 814–825.
24. Johannessen A, Omenaas ER, Eide GE, Bakke PS, Gulsvik A (2007) Feasible and simple exclusion criteria for pulmonary reference populations. Thorax 62: 792–798.
25. Thompson JE, Sleigh AC, Passey ME, Barnes A, Streatfield RW (1992) Ventilatory standards for clinically well aboriginal adults. Med J Aust 156: 566–569.
26. Brandli O, Schindler C, Kunzli N, Keller R, Perruchoud AP (1996) Lung function in healthy never smoking adults: reference values and lower limits of normal of a Swiss population. Thorax 51: 277–283.
27. Brandli O, Schindler C, Leuenberger PH, Baur X, Degens P, et al. (2000) Re-estimated equations for 5th percentiles of lung function variables. Thorax 55: 173–174.
28. Oscherwitz M, Edlavitch SA, Baker TR, Jarboe T (1972) Differences in pulmonary functions in various racial groups. Am J Epidemiol 96: 319–327.
29. Jones RL, Nzekwu MM (2006) The effects of body mass index on lung volumes. Chest 130: 827–833.

# RESULTS S1

### Quantile Regression reference equation and comparison with GAMLSS

As equations modelled with GAMLSS are complex and cannot be implemented in every spirometer we developed reference equations with quantile regression to increase the implementation possibilities. Within the framework of the quantile regression we estimated the 5% and 50% quantiles for all spirometry values. The reference values (5th quantile) can be calculated by the function in Table S1. Graphically based residual analyses were done in order to identify model inadequacies (Figure S4). However, residual analyses revealed a worse fit than for the GAMLSS models for all endpoints. A comparison between GAMLSS and quantile regression models was done by comparing regression coefficients of the mean of the GAMLSS model with the regression coefficients of the quantile regression models. In Figure S5 the estimated values for the 50th and 5th quantiles for each age can be seen. As the other covariates were fixed, only healthy non-smoking men of 175 cm and women of 165 cm were included. Although the GAMLSS model shows a much better fit (Figure S1, S2) than the quantile regression fit (Figure S4) the estimated values obtained by both methods were similar (Figure S5).

## Table S1: Quantile regression reference equation

### Reference equation for the 50th quantile

$$g_{QR} = \beta_0 + \beta_1 * age + \beta_2 * \sqrt{((age - \alpha_1)^2 + \gamma_1^2)} + \beta_3 * \sqrt{((age - \alpha_2)^2 + \gamma_2^2)} + \beta_4 * sex + \beta_5 * height + \beta_6 * smoker + \beta_7 * sick + \beta_8 * age * sex + \beta_9 * \sqrt{((age - \alpha_1)^2 + \gamma_1^2)} * sex + \beta_{10} * \sqrt{((age - \alpha_2)^2 + \gamma_2^2)} * sex + \beta_{11} * height * sex$$

| | FEV1 | FVC | FEV1/FVC | PEF | MEF25 | MEF50 | MEF75 |
|---|---|---|---|---|---|---|---|
| **Regression parameters (β):** | | | | | | | |
| $\beta_0$ | -3.757 | -5.705 | 105.338 | -0.113 | 0.799 | -0.493 | -2.671 |
| $\beta_1$ | 0.049 | 0.029 | 0.453 | 0.152 | 0.102 | 0.110 | 0.064 |
| $\beta_2$ | -0.074 | -0.047 | -0.778 | -0.211 | -0.154 | -0.159 | -0.120 |
| $\beta_3$ | -0.013 | -0.022 | 0.162 | -0.096 | -0.065 | -0.016 | 0.046 |
| $\beta_4$ | 1.738 | 2.384 | 3.346 | 0.750 | 0.803 | 1.694 | 0.783 |
| $\beta_5$ | 0.043 | 0.061 | -0.146 | 0.073 | 0.049 | 0.028 | 0.012 |
| $\beta_6$ | -0.046 | 0.013 | -1.450 | -0.147 | -0.062 | -0.172 | -0.122 |
| $\beta_7$ | -0.092 | -0.065 | -0.942 | -0.188 | -0.360 | -0.297 | -0.086 |
| $\beta_8$ | -0.029 | -0.020 | -0.104 | -0.109 | -0.077 | -0.063 | -0.033 |
| $\beta_9$ | 0.033 | 0.026 | 0.010 | 0.123 | 0.094 | 0.074 | 0.034 |
| $\beta_{10}$ | 0.002 | -0.001 | 0.097 | 0.041 | 0.033 | 0.005 | 0.003 |
| $\beta_{11}$ | -0.011 | -0.016 | -0.004 | -0.017 | -0.013 | -0.008 | -0.003 |
| **Changepoints (α)** | | | | | | | |
| α1 | 18.735 | 21.359 | 15.989 | 19.838 | 19.373 | 17.176 | 16.573 |
| α2 | 37.027 | 35.958 | 25.750 | 53.841 | 57.885 | 57.004 | 12.000 |
| **Transition smoothness (γ)** | | | | | | | |
| γ1 | 0.864 | 0.230 | 0.329 | 1.010 | 1.257 | 0.821 | 0.206 |
| γ2 | 13.578 | 12.185 | 5.537 | 29.938 | 17.258 | 1.012 | 49.328 |

### Reference equation for the 5th quantile

$$g_{QR} = \beta_0 + \beta_1 * age + \beta_2 * \sqrt{((age - \alpha_1)^2 + \gamma_1^2)} + \beta_3 * \sqrt{((age - \alpha_2)^2 + \gamma_2^2)} + \beta_4 * sex + \beta_5 * height + \beta_6 * smoker + \beta_7 * sick + \beta_8 * age * sex + \beta_9 * \sqrt{((age - \alpha_1)^2 + \gamma_1^2)} * sex + \beta_{10} * \sqrt{((age - \alpha_2)^2 + \gamma_2^2)} * sex + \beta_{11} * height * sex$$

| | FEV1 | FVC | FEV1/FVC | PEF | MEF25 | MEF50 | MEF75 |
|---|---|---|---|---|---|---|---|
| **Regression parameters (β)** | | | | | | | |
| $\beta_0$ | -3.335 | -4.901 | 102.764 | -3.120 | 0.041 | -0.929 | -12.466 |
| $\beta_1$ | 0.032 | 0.024 | 0.338 | 0.102 | 0.080 | 0.064 | 0.058 |
| $\beta_2$ | -0.065 | -0.052 | -0.722 | -0.145 | -0.142 | -0.104 | -0.075 |
| $\beta_3$ | -0.012 | -0.017 | -0.156 | -0.076 | -0.045 | -0.009 | 0.075 |
| $\beta_4$ | 1.310 | 2.129 | 2.612 | 2.056 | 0.866 | 1.359 | 1.972 |
| $\beta_5$ | 0.038 | 0.052 | -0.139 | 0.067 | 0.035 | 0.021 | 0.008 |
| $\beta_6$ | -0.094 | -0.012 | -2.734 | -0.283 | -0.281 | -0.257 | -0.085 |
| $\beta_7$ | -0.224 | -0.131 | -4.056 | -0.338 | -0.764 | -0.429 | -0.095 |
| $\beta_8$ | -0.019 | -0.022 | -0.115 | -0.077 | -0.046 | -0.027 | -0.022 |
| $\beta_9$ | 0.028 | 0.032 | 0.200 | 0.090 | 0.067 | 0.033 | 0.023 |
| $\beta_{10}$ | 0.004 | 0.001 | 0.095 | 0.025 | 0.021 | 0.004 | -0.008 |
| $\beta_{11}$ | -0.009 | -0.014 | -0.020 | -0.017 | -0.008 | -0.007 | -0.002 |
| **Changepoints (α)** | | | | | | | |
| α1 | 19.240 | 21.023 | 15.000 | 19.224 | 17.777 | 17.006 | 17.071 |
| α2 | 43.283 | 41.375 | 57.648 | 42.908 | 50.031 | 43.955 | 57.324 |
| **Transition smoothness (γ)** | | | | | | | |
| γ1 | 0.532 | 0.000 | 0.000 | 0.000 | 1.139 | 0.000 | 0.116 |
| γ2 | 5.778 | 8.201 | 0.100 | 19.986 | 6.869 | 2.077 | 150.000 |

The variables are coded as followed:

Age: years; height: cm, sex: male = 0, female = 1; smoker: non-smoker = 0; smoker = 1*; sick: healthy = 0; sick = 1*

*definition of smoker and sick can be found in the methods section of the paper.

**Note:** to compare a patient with a "healthy-non-smoker" population, "smoker" and "sick" must be set to "0" even if the patient is a smoker and has a pulmonary pathology.

**Figure S1 Residual plots for FEV1 from the GAMLSS model**

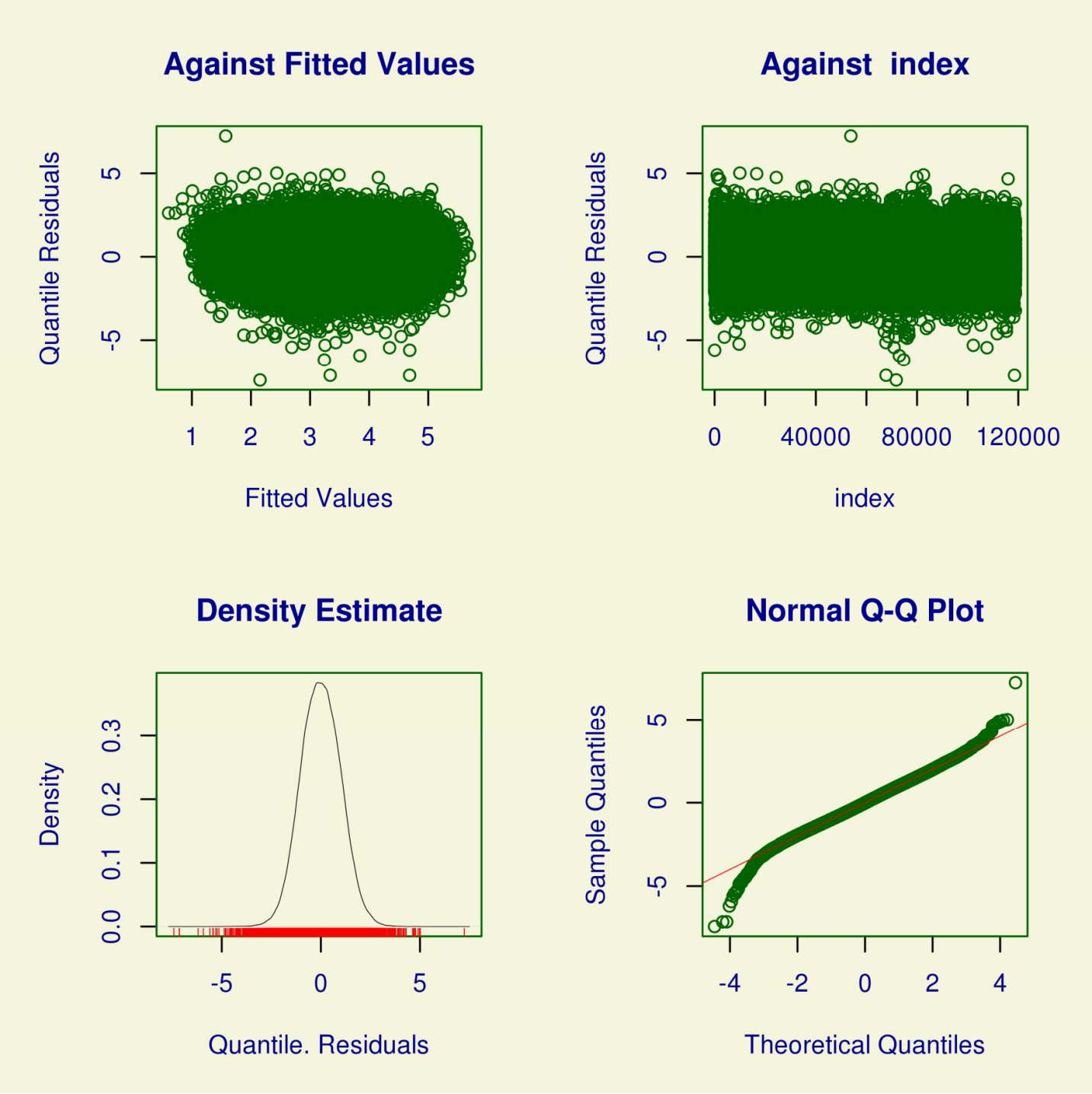


**Figure S2 Worm plot of the residuals of the GAMLSS reference equation for FEV1**

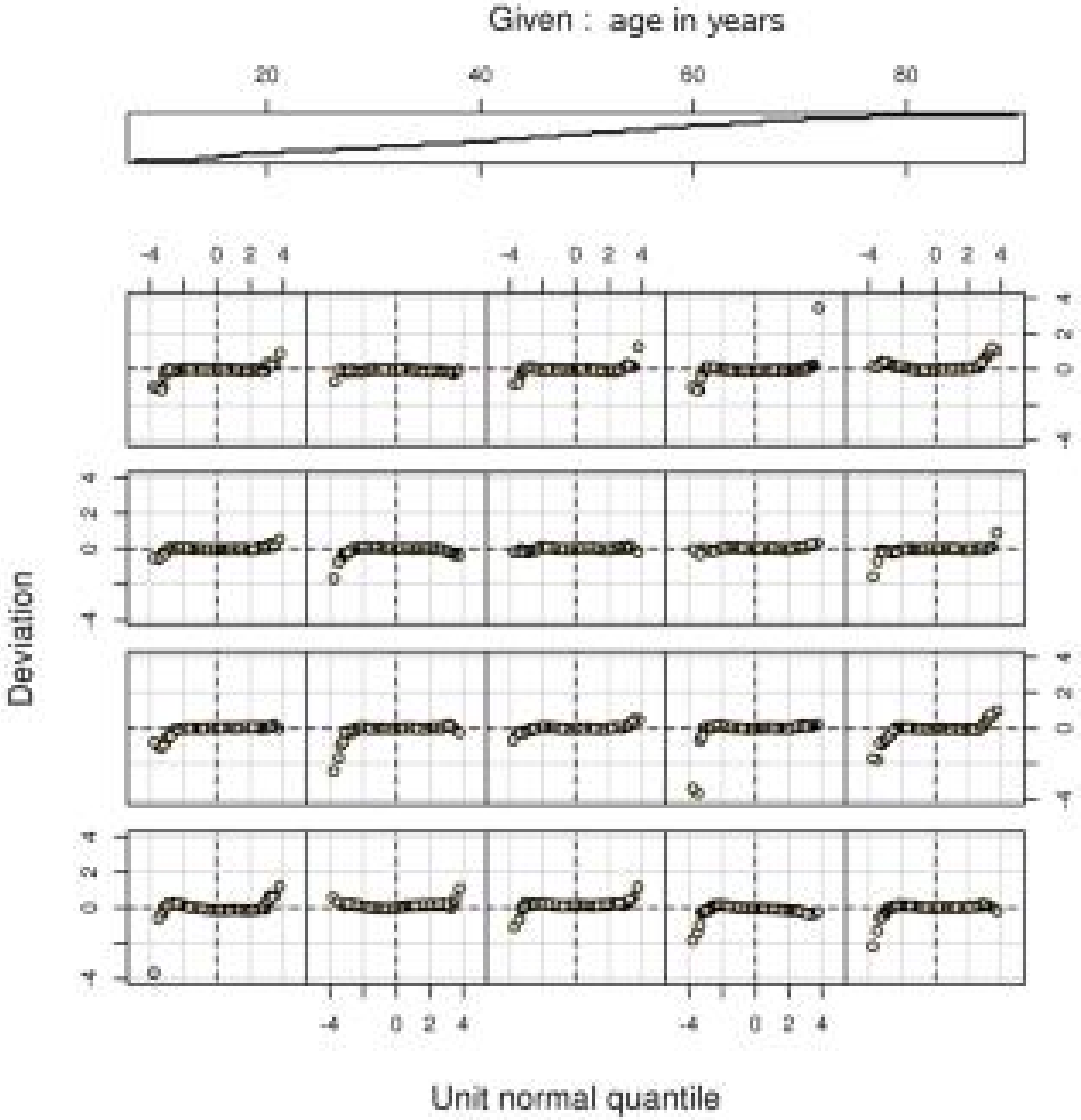


**Figure S3 Formula for calculating quantiles**

$$y_{\propto} = \begin{cases} \mu[1 + \sigma v z_{\alpha}]^{1/v} & \text{If } v \neq 0 \\ \mu \exp[\sigma z_{\alpha}] & \text{If } v = 0 \end{cases}$$

**Figure S4 Residual plots for FEV1 for quantile regression**

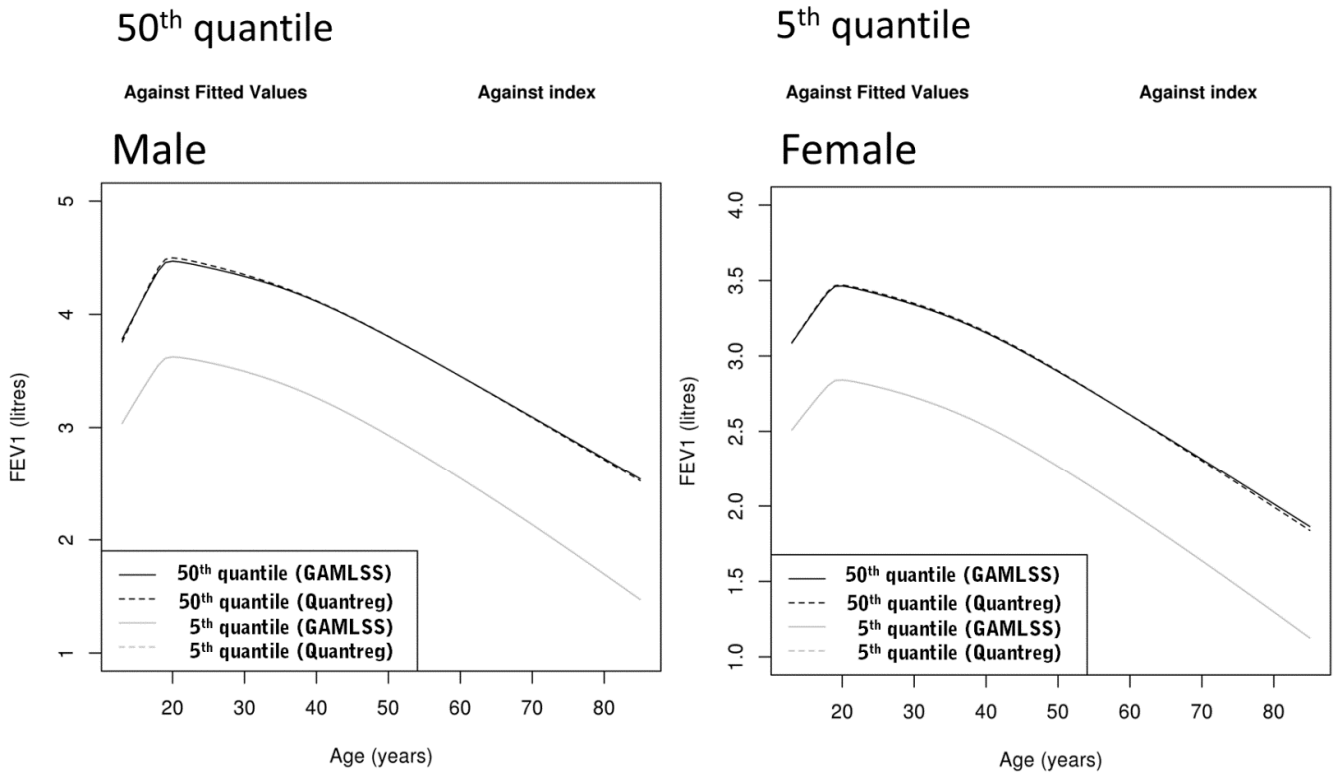



**Figure S5 Comparison between the GAMLSS and Quantile Regression reference equations**

# Chapter VII: General discussion and outlook

## General aspects of the results from the birth cohort chapter

To begin, a summary of the main findings of the book chapter is presented which answers the first question of the thesis, namely:

1. What are the known risk and protective factors for the development of childhood asthma?

In observational as well as interventional birth cohorts on asthma or allergies two major topics have been investigated in the last years: nutrition and environmental factors.
Nutritional factor studies have concentrated on maternal diet, breastfeeding, infant formula and the introduction of solid foods. Most studies were done in high risk children, many of these studies were observational and some interventional studies could not be done in a proper randomised way for obvious ethical reasons (breastfeeding). Taken together, no effect could be seen on the development of allergic diseases except for the introduction of solid food after the $4^{th}$ month and probiotics given to pregnant mothers and their children for the first few months after birth. Therefore, to be able to make recommendations, interventional studies with proper randomization, following children into childhood or even adolescence would be needed.
Research on environmental factors have focussed on a number of subjects such as environmental tobacco smoke, traffic pollution, house dust mites, pet exposure, family size or early day care, infections, microbial exposures or medication. From all these studies only environmental tobacco smoke can be conclusively seen as a risk factor for the development of asthma.
Even multi-faceted studies, which have tried a combination of avoidance of known risk factors and promotion of protective factors, have not been able to pin-point a combination of factors that show conclusive evidence on the development of asthma. Additionally, studies on secondary or tertiary prevention were not able to influence the disease outcome.

This brings us to the second question of the thesis, namely:

2. What questions still remain to be investigated?

Although the huge body of research on risk and protective factors seems rather disappointing as only few recommendations can be made and disease incidence cannot yet be influenced, the birth cohort studies have contributed substantially to the understanding of the natural course of allergic diseases. And, even though consistency across populations is often lacking, they have

identified a number of potential harmful and protective determinants, allowing the research community to identify targets for treatment and preventive strategies. Nevertheless, these hypothesis-generating observations must be validated with carefully designed interventional birth cohorts before population based recommendations can be made.

### Window of opportunity

Although different birth cohorts often have contradictory results an increasing amount of research points towards a sensitive window of opportunity during pregnancy and early childhood [3]. While genetic background, allergic status of parents, and predisposition for atopy and inflammation play an important role, prenatal and early childhood environmental conditions seem to be able to alter the course of the immune and respiratory system development and thus influence the incidence of asthma [3]. The respiratory and immune systems are functionally immature at birth and undergo prolonged periods of pre- and postnatal development and, as such, are vulnerable to a variety of environmental exposures [41]. The environmental influence may be mediated by alterations that maintain the Th2 bias seen during gestation, blocking the maturation of innate immune cells and creating inflammatory dysfunction in the infant that may provide the foundation for childhood asthma [3]. A potential way to achieve conclusive evidence would thus be to concentrate future research on this sensible time period, following the children subsequently until adulthood.

### Gene-environment interaction

The observational and interventional birth cohort studies reported in the book chapter did not investigate gene-environment interactions. As mentioned in the introduction, asthma is a complex disease, and its incidence is probably determined by an interplay of genetic and environmental factors [42]. There are possibly many genes with small effects that interact with a personôs environment and determine the incidence of asthma [42]. Many studies have linked genetics to characteristics of asthma helping to identify risk factors for specific phenotypes of asthma [43], but gene-environment interactions introduce complications into these studies [44]. Complicating matters even more, recent studies have not only found gene-environment interactions, but also environment-environment interactions (Table 2 in [3]). To be able to, not only take gene-environment, but also environment-environment interactions into consideration, future research would need to include an interdisciplinary group of researchers and a big research population. This challenging task, however, may give an insight into the development of asthma.

### Conclusion

Because asthma is connected to the risk of several comorbid chronic conditions, the benefit of asthma risk reduction and prevention is greater than may be initially apparent.

No single risk factor can fully explain the increased prevalence of asthma in recent decades but it is assumed that the rapid increase is due to environmental and/or epigenetic changes [3]. Research, therefore, should not focus on individual factors, genes or pathways but on the combination of host, social and environmental factors in both disease inception and control [11]. To achieve this, large, interventional, longitudinal studies following children into adulthood, involving many research areas would be necessary.

## General aspects of the results from the vitamin D study

To begin, a summary of the main findings of the paper is presented which answers the two following thesis questions:

3. Does vitamin D supplementation during pregnancy influence the immune system of the new born child, specifically the mRNA levels of ILT3 and ILT4?

Maternal vitamin D supplementation during pregnancy was found to increase significantly gene expression of ILT3 and ILT4 in cord blood. The immune system of the new born can therefore be influenced by maternal food intake. Their increase may point towards an early induction of tolerogenic immune response. However, whether cord blood ILT3 and ILT4 mRNA levels are of relevance for the allergy risk later in life is unclear.

4. Is the effect of vitamin D mediated by IL-10?

ILT3 and ILT4 mRNA levels were found to be inversely associated with IL-10 production after stimulation. This finding is contrary to what could be expected and should be interpreted with caution as it contradicts other studies.

The potential effect of maternal supplementation of vitamin D on the new born immune system is an interesting hypothesis and the results of the paper do seem to point towards an association. However, there are several aspects that need to be taken into consideration:

### Maternal vitamin D

In the PASTURE study, maternal vitamin D supplementation was assessed via a questionnaire completed by pregnant mothers in their third trimester, as the serum (blood component)

concentrations were not available. The question inquired about ***current*** vitamin D intake: ôDo you currently take any vitamin, minerals or other dietary supplements? Vitamin D yes/no.ô

Very little is currently known about the effect of vitamin D on the immune system of an unborn child. The results of the paper raise a series of research questions, namely:

- At what stage does vitamin D affect the immune system?
- Is there a window of opportunity where the immune system is particularly sensitive to vitamin D influence (eg. in early or late pregnancy [45])?
- Is vitamin D deficiency a risk factor, or are high doses protective against allergic diseases?
- Is there a dose-response effect of maternal vitamin D supplementation on cord blood dendritic cells?

Answers to these questions would necessitate serial measurements of maternal serum vitamin D throughout pregnancy and up until the time of birth. Such measurements were not done in our study and could be suggested for future research.

In our study, only two populations recommend vitamin D supplementation during pregnancy (France and Finland), and those countries do not recommend a daily dose during the whole year. Due to the type of question asked (see above), the number of women supplementing vitamin D during pregnancy was potentially underestimated. Additionally, there are three sources of vitamin D: diet, supplements and sunlight [46]. As it was not possible to assess all sources of vitamin D in the PASTURE study, different questions might have given different prevalenceôs and thus different results.

### The role of IL-10

In our study the potential effect of vitamin D on the new born immune system was not mediated by the cytokine IL-10 which is an astonishing result as it has been shown that 1,25(OH)2D-treated human dendritic cells have the capacity to convert CD4 T cells into IL-10ï secreting Treg cells and suppress the proliferation of T cells [47]. The PASTURE study is not specifically designed to evaluate the effect of vitamin D on the new born immune system [18] which is the reason why IL-10 levels were not measured directly after exposure to vitamin D as in other studies [48]. Rather, the cellular capacity to produce IL-10 was measured after stimulation with phorbol 12-myristate 13-acetate and ionomycin (P/I). To answer the question of whether the vitamin D effect is mediated by IL-10, future research on the subject, should be made with direct blood measurements of both vitamin D and IL-10.

### The effect of tolerogenic dendritic cells on the development of asthma

Beyond a central role in calcium and bone physiology, vitamin D metabolism (specifically the conversion of 25(OH)D to the active form vitamin D (1,25[OH]2D)) has effects on epithelial cell, T-cell, B-cell, and dendritic cell functions that are important to innate and adaptive immunity [49]. Although tolerogenic dendritic cells are interesting candidates for the pathway in which vitamin D could influence the development of asthma, there are other mechanisms that are just as plausible [46]. Therefore, whether the results correlate with asthma incidence needs to await data from the cohort in a few years, when the children will, or will not, have developed asthma.

### Conclusion

The hypothesis generated by the results in our paper are interesting but do not allow for definitive conclusions to be made. Future research is necessary to determine whether maternal serum vitamin D levels during pregnancy are associated with tolerance induction of the neonatal immune system and whether this association can be extended to disease manifestation in school-age children. Due to the complexity of the pathogenesis of asthma, the questions would require collaborative work among clinicians, epidemiologists, immunologists, and geneticists and would have to be done in an interventional, randomized, double blind birth cohort study. The results could help understanding the immunological mechanism of vitamin D on the maturing human immune system. The implications could open new avenues into the development of preventive, diagnostic and therapeutic strategies to reduce the burden of asthma and allergies.

## General aspects of the results from the rhinitis study

To begin, a summary of the main findings of the paper is presented which answers the three following thesis questions:

5. What are the temporal sequences of the development of rhinitis and wheezing?

Rhinitis point prevalence was highest at 3 years with 9.2% and ranged between 2.5% and 7.7% throughout the other years. By the age of 13 years, the cumulative incidence of rhinitis was 47.8%. Wheezing point prevalence was highest at the end of the second year with 19.8%, decreasing to less than 7% throughout the following years. The cumulative incidence of wheezing was 40.5% by the age of 13 years. Although both clinical manifestations appear during the first years of life, rhinitis was not associated with wheezing during the first two years

of life, but significantly predicted the incidence of wheezing by the age of 5 years, whereas wheezing was not a predictor for the incidence of rhinitis at either 2 or 5 years.

6. Does sensitization play a role in the development of rhinitis and wheezing?

Independent of the age considered, sensitization was a predictor for the incidence of wheezing and rhinitis. At the age of two years, sensitization to any allergen influenced the period prevalence of wheezing. By the age of 5 years, allergic rhinitis was associated with the highest period prevalence of wheezing, and non-allergic rhinitis showed significantly higher period prevalenceôs than atopy without rhinitis.

7. Is rhinitis a risk factor for wheezing onset in children?

The findings of the paper suggest that allergic rhinitis in preschool children is a risk factor for subsequent wheezing onset. In contrast, neither the rare manifestation of allergic rhinitis in the first two years of life nor non-allergic rhinitis up to the age of five years was associated with the onset of wheezing in childhood. Therefore, some childhood wheezing phenotypes are associated with rhinitis. Additionally, the discrepancy between the cumulative incidence of rhinitis (47.8%) and its peak period prevalence at the age of 3 years (9.2%) suggests that rhinitis is a transient phenomenon in early childhood and manifests years before wheezing begins. Thus, the findings of the paper strongly suggest that a progression from rhinitis to wheezing can be found.

Rhinitis in preschool children was found to increase the risk of wheezing in preschool children. Wheezing is often a precursor to asthma, and asthma is a major risk factor for chronic obstructive pulmonary diseases (COPD) [50]. Given the importance of the finding of a potential risk factor, a few questions should be considered:

### Mechanism for the progression from rhinitis to wheezing

As discussed in the paper, there are several potential mechanisms that might explain the association found between rhinitis and wheezing. Elucidating the exact mechanism could help understand the disease progression, pin-point windows of opportunity and aid in targeting specific therapies to either slow down or halt the progression. Therefore, major areas of future research might concentrate on similarities in histological structures, inflammatory mechanisms and anatomical proximity [29].

## Secondary prevention of allergic rhinitis

The fact that allergic rhinitis in preschool children was found to be a risk factor for wheezing onset in childhood is, of course, appealing as it opens the possibility for secondary prevention. Secondary prevention has been tried in older children as allergic rhinitis is a known risk factor for the development of asthma in school children and adults. One of the concepts tried is immunological intervention [51]. The concept is to downgrade the overall propensity of the immune system to produce Th2-skewed responses to environmental and innocuous antigens [52]. This interesting prevention strategy is not allergen-specific as it influences the overall innate immune response and, as a consequence, the polarization of the adaptive immune responses. But research in this area has not yet been conclusive [53].

By contrast, allergen-specific approaches have been successful in tertiary prevention, showing that allergic rhinitis treatment influences asthma control [54]; nasal inhaled steroid can modify asthma symptoms as well as airways hyperreactivity; and antihistaminic drugs can reduce the most relevant asthma triggers [55]. Allergen-specific immunotherapy is the only treatment found that can modify allergic rhinitis progress and reduce the risk of asthma in patients with allergic rhinitis [56]. Although these results are promising, most of the trials were open studies, with the control groups receiving symptomatic medication alone and with limited follow-up periods to assess any long-term effects [57], so clearly, more research is needed.

Another option could be an ñimmunoprophylaxis of atopyò targeted at children who are either not sensitized or only sensitized to a few allergens but still healthy. It could be done at a very early age when the immune response is more susceptible to be corrected or re-addressed. The prophylactic intervention could even be tailored on the childôs molecular sensitization profile to enhance efficacy [53, 58]. The target of the intervention could be, for examples, to prevent the onset of seasonal allergic rhinoconjunctivitis, thus keeping the IgE response to grass pollen at a subclinical level. Even if rhinitis were not prevented, its onset could be delayed and its symptoms diminished and perhaps its progression to asthma halted. [53].

## Conclusion

Allergic rhinitis was found to be a risk factor for the development of wheezing and asthma, and treating the underlying allergy may represent an attractive method for asthma prevention. This should be done in double-blind, placebo-controlled randomized trials to assess the preventive effect of the different therapies on asthma development.

## General aspects of the results from the lung function study

To begin, a summary of the main findings of the paper is presented which answers the two following thesis questions:

8. Are there such reference equations for Central European children and adults?

A PubMed research with words such as ñlung functionò and ñspirometryò reference equations revealed that there are many spirometry reference equations for different populations of different age ranges, but none that span across all ages for Central European populations.

9. If not, is it possible to develop practical ones that can be used by clinicians in their daily practice?

In the paper we present spirometry reference equations for 8ï 90 year olds that we derived from a very large, cross-sectional sample of a Central European population.

Once asthma has been diagnosed, the follow-up of patients is extremely important, especially in childhood, as any deterioration should lead to the adaptation of therapy to prevent long term effects as much as possible. Lung functions are used as objective measures of control and their interpretation relies on reference equations. The reference equations presented in the paper have certain advantages and certain disadvantages that merit a short discussion.

### Complicated statistics

While the GAMLSS method is a very good statistical method that allows modelling of data with skewed and kurtotic distributions, it is not easily integrated in every lung function device. The aim, when developing reference equations, is naturally that they be used. Although the paper points out how they could be applied, the authors fear that the complicated statistics will deter busy clinicians from integrating the reference equations in their devices. To broaden the spectrum of potential users, the quantile regression equations were developed. They do not model the data as precisely as the GAMLSS equations, but might be adopted by more clinicians. Regardless of the equation selected, little is known about the real life application of the spirometry reference equations presented in the paper. In future papers about reference equations, thought might be given to practical dissemination of the equations.

### "Ideal" Reference equations

The ñLuftiBusò collected an impressive amount of spirometry data that has been used to create the reference equations. Nevertheless, the aim of the ñLuftiBusò is not to create reference equations but to offer a service to the population. In that regard, the population is not selected to be representative, or healthy, but is probably skewed towards people who have health questions. Future reference equations might be derived from a healthy, representative population selected in view of creating reference equations.

## Conclusions and outlook

In this thesis four chapters are presented and discussed. First, an overview of birth cohorts on asthma was done, second and third a protective and a risk factor for the development of asthma were studied and last spirometry reference equations were developed.

Thus, the thesis added a few more insights into the complex entity of respiratory diseases in childhood, helping to understand risk and protective factors. The spirometry tool developed may help clinicians monitor children with respiratory diseases throughout their life. The challenge in the years to come will be to integrate all the knowledge acquired into complex analysis taking, not only host, social and environmental factors into consideration but also their interactions, to achieve a better understanding of the causes of asthma and possibly develop treatments and implement prevention strategies.

## References


1. Martinez, F.D., et al., *Asthma and wheezing in the first six years of life. The Group Health Medical Associates.* N Engl J Med, 1995. **332**(3): p. 133-8.
2. Martinez, F.D., *Development of wheezing disorders and asthma in preschool children.* Pediatrics, 2002. **109**(2 Suppl): p. 362-7.
3. Dietert, R.R., *Maternal and childhood asthma: risk factors, interactions, and ramifications.* Reprod Toxicol, 2011. **32**(2): p. 198-204.
4. Samolinski, B., et al., *Prevention and control of childhood asthma and allergy in the EU from the public health point of view: Polish Presidency of the European Union.* Allergy, 2012. **67**(6): p. 726-31.
5. Barnett, S.B. and T.A. Nurmagambetov, *Costs of asthma in the United States: 2002-2007.* J Allergy Clin Immunol, 2011. **127**(1): p. 145-52.
6. Burr, M.L., et al., *Changes in asthma prevalence: two surveys 15 years apart.* Arch Dis Child, 1989. **64**(10): p. 1452-6.
7. Eder, W., M.J. Ege, and E. von Mutius, *The asthma epidemic.* N Engl J Med, 2006. **355**(21): p. 2226-35.
8. Asher, M.I., et al., *Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys.* Lancet, 2006. **368**(9537): p. 733-43.
9. Gerrard, J.W., et al., *Serum IgE levels in white and metis communities in Saskatchewan.* Ann Allergy, 1976. **37**(2): p. 91-100.
10. Strachan, D.P., *Hay fever, hygiene, and household size.* BMJ, 1989. **299**(6710): p. 1259-60.
11. von Mutius, E. and T. Hartert, *Update in asthma 2012.* Am J Respir Crit Care Med, 2013. **188**(2): p. 150-6.
12. Mathilda Chiu, Y.H., et al., *Prenatal and postnatal maternal stress and wheeze in urban children: effect of maternal sensitization.* Am J Respir Crit Care Med, 2012. **186**(2): p. 147-54.
13. Muraro, A., et al., *Dietary prevention of allergic diseases in infants and small children. Part III: Critical review of published peer-reviewed observational and interventional studies and final recommendations.* Pediatr Allergy Immunol, 2004. **15**(4): p. 291-307.
14. Neuman, A., et al., *Maternal smoking in pregnancy and asthma in preschool children: a pooled analysis of eight birth cohorts.* Am J Respir Crit Care Med, 2012. **186**(10): p. 1037-43.
15. Bisgaard, H., S.M. Jensen, and K. Bonnelykke, *Interaction between asthma and lung function growth in early life.* Am J Respir Crit Care Med, 2012. **185**(11): p. 1183-9.
16. Folsgaard, N.V., et al., *Neonatal cytokine profile in the airway mucosal lining fluid is skewed by maternal atopy.* Am J Respir Crit Care Med, 2012. **185**(3): p. 275-80.
17. Moffatt, M.F., et al., *A large-scale, consortium-based genomewide association study of asthma.* N Engl J Med, 2010. **363**(13): p. 1211-21.

18. von Mutius, E., S. Schmid, and P.S. Group, *The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe.* Allergy, 2006. **61**(4): p. 407-13.

19. Host, A. and S. Halken, *Can we apply clinical studies to real life? Evidence-based recommendations from studies on development of allergic diseases and allergy prevention.* Allergy, 2002. **57**(5): p. 389-97.

20. Smith, G.C. and J.P. Pell, *Parachute use to prevent death and major trauma related to gravitational challenge: systematic review of randomised controlled trials.* BMJ, 2003. **327**(7429): p. 1459-61.

21. Brehm, J.M., et al., *Vitamin D insufficiency and severe asthma exacerbations in Puerto Rican children.* Am J Respir Crit Care Med, 2012. **186**(2): p. 140-6.

22. Wu, A.C., et al., *Effect of vitamin D and inhaled corticosteroid treatment on lung function in children*. Am J Respir Crit Care Med, 2012. **186**(6): p. 508-13.

23. Erkkola, M., et al., *Maternal vitamin D intake during pregnancy is inversely associated with asthma and allergic rhinitis in 5-year-old children.* Clin Exp Allergy, 2009. **39**(6): p. 875-82.

24. Zittermann, A., G. Tenderich, and R. Koerfer, *Vitamin D and the adaptive immune system with special emphasis to allergic reactions and allograft rejection.* Inflamm Allergy Drug Targets, 2009. **8**(2): p. 161-8.

25. Manavalan, J.S., et al., *High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells.* Transpl Immunol, 2003. **11**(3-4): p. 245-58.

26. Pedersen, A.E., et al., *Induction of regulatory dendritic cells by dexamethasone and 1alpha,25-Dihydroxyvitamin D(3).* Immunol Lett, 2004. **91**(1): p. 63-9.

27. Hackstein, H. and A.W. Thomson, *Dendritic cells: emerging pharmacological targets of immunosuppressive drugs.* Nat Rev Immunol, 2004. **4**(1): p. 24-34.

28. Bousquet, J., et al., *Allergic rhinitis and its impact on asthma.* J Allergy Clin Immunol, 2001. **108**(5 Suppl): p. S147-334.

29. Bousquet, J., et al., *Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen).* Allergy, 2008. **63 Suppl 86**: p. 8-160.

30. Passalacqua, G., G. Ciprandi, and G.W. Canonica, *The nose-lung interaction in allergic rhinitis and asthma: united airways disease.* Curr Opin Allergy Clin Immunol, 2001. **1**(1): p. 7-13.

31. Shaaban, R., et al., *Rhinitis and onset of asthma: a longitudinal population-based study.* Lancet, 2008. **372**(9643): p. 1049-57.

32. Stein, R.T. and F.D. Martinez, *Asthma phenotypes in childhood: lessons from an epidemiological approach*. Paediatr Respir Rev, 2004. **5**(2): p. 155-61.

33. Henderson, J., et al., *Associations of wheezing phenotypes in the first 6 years of life with atopy, lung function and airway responsiveness in mid-childhood.* Thorax, 2008. **63**(11): p. 974-80.

34. Morgan, W.J., et al., *Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence.* Am J Respir Crit Care Med, 2005. **172**(10): p. 1253-8.

35. Pellegrino, R., et al., *Interpretative strategies for lung function tests.* Eur Respir J, 2005. **26**(5): p. 948-68.

36. Stanojevic, S., A. Wade, and J. Stocks, *Reference values for lung function: past, present and future.* Eur Respir J, 2010. **36**(1): p. 12-9.

37. Liou, T.G. and R.E. Kanner, *Spirometry.* Clin Rev Allergy Immunol, 2009. **37**(3): p. 137-52.

38. Stanojevic, S., et al., *Reference ranges for spirometry across all ages: a new approach*. Am J Respir Crit Care Med, 2008. **177**(3): p. 253-60.

39. Bergmann, R.L., et al., *Atopic diseases in infancy. The German multicenter atopy study (MAS-90).* Pediatr Allergy Immunol, 1994. **5**(6 Suppl): p. 19-25.

40. Egger, S., et al., *[Overweight and obesity in the Zurich canton. A LuftiBus study]*. Praxis (Bern 1994), 2001. **90**(13): p. 531-8.

41. Sly, P.D. and P.G. Holt, *Role of innate immunity in the development of allergy and asthma*. Curr Opin Allergy Clin Immunol, 2011. **11**(2): p. 127-31.

42. von Mutius, E., *Gene-environment interactions in asthma.* J Allergy Clin Immunol, 2009. **123**(1): p. 3-11; quiz 12-3.

43. Szefler, S.J., *Advances in pediatric asthma in 2011: moving forward.* J Allergy Clin Immunol, 2012. **129**(1): p. 60-8.

44. Vercelli, D., *Gene-environment interactions: the road less traveled by in asthma genetics.* J Allergy Clin Immunol, 2009. **123**(1): p. 26-7.

45. Holt, P.G. and C.A. Jones, *The development of the immune system during pregnancy and early life.* Allergy, 2000. **55**(8): p. 688-97.

46. Cook-Mills, J.M. and P.C. Avila, *Vitamin E and D regulation of allergic asthma immunopathogenesis.* Int Immunopharmacol, 2014.

47. Unger, W.W., et al., *Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1.* Eur J Immunol, 2009. **39**(11): p. 3147-59.

48. Zittermann, A., J. Dembinski, and P. Stehle, *Low vitamin D status is associated with low cord blood levels of the immunosuppressive cytokine interleukin-10.* Pediatr Allergy Immunol, 2004. **15**(3): p. 242-6.

49. Vassallo, M.F. and C.A. Camargo, Jr., *Potential mechanisms for the hypothesized link between sunshine, vitamin D, and food allergy in children.* J Allergy Clin Immunol, 2010. **126**(2): p. 217-22.

50. Svanes, C., et al., *Early life origins of chronic obstructive pulmonary disease.* Thorax, 2010. **65**(1): p. 14-20.

51. Hamelmann, E., et al., *Primary prevention of allergy: avoiding risk or providing protection?* Clin Exp Allergy, 2008. **38**(2): p. 233-45.

52. Holt, P.G., *Prevention--what is the most promising approach?* Pediatr Allergy Immunol, 2014. **25**(1): p. 12-4.

53. Matricardi, P.M., *Allergen-specific immunoprophylaxis: toward secondary prevention of allergic rhinitis?* Pediatr Allergy Immunol, 2014. **25**(1): p. 15-8.

54. Durham, S.R., *Effect of intranasal corticosteroid treatment on asthma in children and adults.* Allergy, 1999. **54 Suppl 57**: p. 124-31.

55. Braido, F., et al., *Allergic rhinitis: current options and future perspectives.* Curr Opin Allergy Clin Immunol, 2014. **14**(2): p. 168-76.

56. Barr, J.G., et al., *Allergic rhinitis in children.* BMJ, 2014. **349**: p. g4153.

57. Valovirta, E., et al., *Design and recruitment for the GAP trial, investigating the preventive effect on asthma development of an SQ-standardized grass allergy immunotherapy tablet in children with grass pollen-induced allergic rhinoconjunctivitis.* Clin Ther, 2011. **33**(10): p. 1537-46.

58. Holt, P.G., et al., *Prophylactic use of sublingual allergen immunotherapy in high-risk children: a pilot study.* J Allergy Clin Immunol, 2013. **132**(4): p. 991-3 e1.

## Acknowledgements

My research fellowship and my thesis were supported by a Marie-Curie Grant within the GALTRAIN project for which I am very grateful. It allowed me to spend three fascinating years learning about research in a fast moving field, with an international and interdisciplinary group of highly qualified researchers. The yearly GALTRAIN meetings in different European cities were always highly instructive, interesting and supportive.

I am very grateful to Prof. Erika von Mutius for letting me be part of her research team; she supported and challenged me constantly. After receiving a phone call saying: ñWould you like to leave to München immediately to do three years of research?ò I arrived in München three months later not knowing what to expect. I ended up spending three very intensive years, jet-setting across Europe and learning so much more than I had expected. But above all I acquired a life experience that would not have been possible under any other circumstances.

All my colleagues in München gave me a pleasant and enjoyable working and studying environment, full of interesting discussions. I would, however, like to thank my three ñroom-collegesò specially. Markus Ege, who, with a never ending patience, taught me SAS and statistics; Martin Depner for always being there and listening to me, and Sabina Illi with whom I spend countless hours discussing about life and family.

I also want to thank specifically two of the GALTRAIN colleagues. Anna, with whom I spend hours talking and many evenings roaming together in München and Elena with whom I visited 9 countries in 4 continents during our three years of research!

And of course my fellow ñmaster in public health studentsò for spending two years with me, sharing entertaining discussions on the extravagant studies we had to come up with for our studies. Specially, I would like to thank Anja for being there and listening to me and Rebecca for the support and time we spend together.

Big thanks go to Prof. Charlotte Braun-Fahrländer for supporting the idea of the thesis, making it possible to study at the University of Basel and being my Doktor-Mutter throughout all these years; and to Prof. Christian Lengeler for accepting to be the Korreferat and being present and supporting whenever I was ready to take up the work.

Finally, I am very grateful to my husband, Lukas, who accepts and supports me whenever I decide to move to another city or country, and decide to do yet another course, exam or project; and who, during the last months, made it possible for me to write this thesis. I would also like to thank my mother for coming all the way to Vucherens to take care of our daughter while I had to work or write, and to Amiel, who in spite of the ñmamamamaôsò, spend many days playing with a lot of different people while I was writing.