Early-life Infections, Bacteria and Childhood Asthma Development
The Generation R Study

Evelien Rosanne van Meel

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Early-life Infections, Bacteria and Childhood Asthma Development
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Vroege infecties, bacteriën en de ontwikkeling van astma op kinderleeftijd
Het Generation R onderzoek

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Chapter 1

Introduction and design

BACKGROUND

Asthma is a common disease during childhood. The prevalence of asthma symptoms ranges from 20% in preschool children with wheezing, to 5% at school age with a doctor diagnosis of asthma[1 2]. In the Netherlands, around 3.5% of school-aged children visit the doctor for asthma[3].

Asthma is a heterogeneous disease, characterized by chronic airway inflammation. Symptoms include episodes of wheezing, shortness of breath and cough, which can be triggered by factors such as allergens or infections[4]. Diagnosis is based on history of respiratory symptoms, combined with variable expiratory airflow limitation. The most commonly used lung function test for the diagnosis of asthma is spirometry. Most typically, spirometry in asthmatic children would demonstrate airway obstruction, reflected by a lower Forced Expiratory Volume in the first second (FEV_1), or a lower FEV_1/Forced Vital Capacity (FVC). Additionally, the Forced Expiratory Flow after exhaling 75% of the FVC (FEF_{75}) could be decreased, which reflects airflow limitation of the smaller airways[5]. However, lung function as measured by spirometry is not necessarily abnormal in children with asthma, which is reflected by a high positive predicting value, but a low negative predicting value[6].

It has been proposed that asthma has its origins in early life[7 8]. Early-life exposures and infectious diseases could have an effect on the developing immune and respiratory systems. This might lead to developmental adaptations, resulting in a lower lung function and an increased risk of asthma in later childhood. Additionally, lower lung function and asthma in childhood might predispose to respiratory disease in adulthood, such as chronic obstructive pulmonary disease[9 10].

Various environmental exposures during fetal life and infancy could be related to childhood lung function and asthma, possibly through a mediating effect of lower respiratory tract infections. Both infections during fetal life or childhood, as well as carriage of bacteria, either specific or combined in the microbiome, might be associated with childhood lung function and asthma. Lastly, respiratory tract infections and airway bacteria might influence each other, further complicating the possible relation with lung function and asthma. The identification of these early-life exposures and infectious diseases contributing to adverse respiratory health helps to understand the development of disease, and is the first step for developing future prevention strategies.

Fetal and infant exposures

Previously, maternal psychological distress during pregnancy has been associated with an up to 2-fold increased risk of wheezing and asthma in the offspring at preschool age[11-13]. An underlying mechanism could be disturbances in the development of the fetal hypothalamic-pituitary-adrenal axis[14 15]. Unmeasured confounding of socio-economic and lifestyle factors might play a role as well. Maternal psychological distress reflects intra-uterine mechanisms, while paternal psychological distress reflects unmeasured confounding od socio-economic and lifestyle factors. Therefore, studying both maternal and paternal psychological distress during pregnancy might help in understanding the associations of exposure to psychological distress during pregnancy with childhood asthma[16]. Their long-term effects need to be elucidated. Additionally, psycho-

logical distress during pregnancy might be associated with lower respiratory tract infections, and these infections might mediate the association of psychological distress during pregnancy with childhood lung function and asthma[17].

During infancy, breastfeeding, and specifically exclusive or prolonged breastfeeding is protective against early-life respiratory tract infections, wheezing and asthma[18-20]. Whether there is an effect of breastfeeding on lung function or asthma at school-age remains inconclusive[21 22]. Possibly, associations of breastfeeding with later-life respiratory health might be mediated by early-life respiratory tract infections.

Fetal and childhood infections

The immune system plays a key role in the pathogenesis of asthma[23]. Early-life infections might lead to a disturbance of the still developing immune system, thereby affecting the risk of asthma. Upper respiratory tract infections, such as the non-specific common cold, are very frequent in the first years of life[24 25]. The prevalence of specific upper respiratory tract infections, such as otitis media, is 0.3-1.6 episodes per child year[24 25]. The prevalence of lower respiratory tract infections, such as pneumonia and bronchitis, has been estimated at 0.02-0.37 episodes per child year[24 26]. Early-life respiratory tract infections have been associated with an increased risk of asthma in early childhood, and chronic obstructive respiratory disease in adulthood[27-30]. One of the proposed mechanisms for these associations is allergic sensitization, leading to airway inflammation, obstruction and hyperreactivity[31-33]. Thus far, the associations of respiratory tract infections with respiratory health at school age, specifically lung function, are not well known.

Specifically, maternal *Chlamydia trachomatis* infection during pregnancy may predispose individuals to an increased risk of childhood asthma. Maternal *Chlamydia trachomatis* infection during pregnancy has been associated with chorioamnionitis, which might lead to fetal immune changes, such as increased production of Interleukins or IgE[34-37]. Mice studies have demonstrated that the murine biovar of *Chlamydia trachomatis*, is associated with specific IgE in bronchoalveolar lavage fluid and an increased production of interleukins when mice are infected in the neonatal period[38]. Additionally, infection in these mice in the neonatal period is associated with an increase in alveolar diameter, suggesting that the infection also has an effect on the respiratory system[39]. Lastly, *Chlamydia trachomatis* has been associated with an increased risk of prematurity, which could be a mediator in the association with asthma[40-42]. In humans, it is unknown if maternal *Chlamydia trachomatis* infection during pregnancy affects the risk of childhood asthma.

Viral infections during childhood might also affect the immune system and thereby the risk of asthma . Specifically, Epstein-Barr virus and Cytomegalovirus have been proposed to be associated with atopy[43-46]. Infection with either of these herpesviridae is common in childhood[47 48]. The immune response to these infections is however different, with predominantly a T-helper-2 type response for Epstein-Barr virus, and a T-helper-1 type response for Cytomegalovirus[49 50]. Therefore, infection, and specifically possible combinations of these infections, might lead to a T-helper cell mediated response including memory T-cell expansions, and subsequently an increased risk of asthma or atopy.

Childhood bacterial carriage and the microbiome

Next to infections, bacterial carriage or the microbiome may be important for development of childhood asthma.[51]. Both bacterial carriage and the microbiome reflect the community of micro-organisms in or on the human body. The most interesting locations of the microbiome related to respiratory health are the airway and the gut[52-55].

Airway bacterial carriage, specifically carriage of *Haemophilus influenzae, Moraxella catarrhalis* and *Streptococcus pneumonia* in infancy, has been associated with an increased risk of lower respiratory tract infections, wheezing and asthma[56 57]. Respiratory tract infections might also influence airway bacterial carriage[58-61]. The specific direction of the associations between airway bacterial carriage and respiratory tract infections, as well as the associations of airway bacterial carriage with respiratory health outcomes at school age has not been established yet.

Rather than a focus on specific bacteria, recent developments enable analysis of the complete microbiome in large observational studies, [55]. Shifts in microbiome composition are suggested to have localized but also systemic effects on maturation of the immune system, and may promote a more inflammatory state by evoking T-helper mediating response that suppresses T-helper 2 activity[62] . In children, the gut microbiome has been associated with the risk of respiratory morbidity[53 63 64]. Specifically, the gut microbiome seems to be important for the development of respiratory health in preschool children. The role of the gut microbiome in later childhood in relation to atopic disease and respiratory health remains unclear.

HYPOTHESIS

The main hypothesis for this thesis is that factors in early-life, especially infections and bacteria, induce developmental adaptations in the pulmonary and immune system, which subsequently lead to an increased risk of lower lung function and asthma in childhood (Figure 1.1).

OBJECTIVES

The major aims of this thesis are:

1. To assess whether fetal and infant exposures, such as maternal psychological distress during pregnancy and breastfeeding, are associated with school-age lung function and asthma, and whether these associations are mediated by lower respiratory tract infections.
2. To assess whether fetal and childhood respiratory and other infections are associated with childhood respiratory health.
3. To assess whether childhood airway bacterial carriage and the gut microbiome are associated with childhood respiratory health.

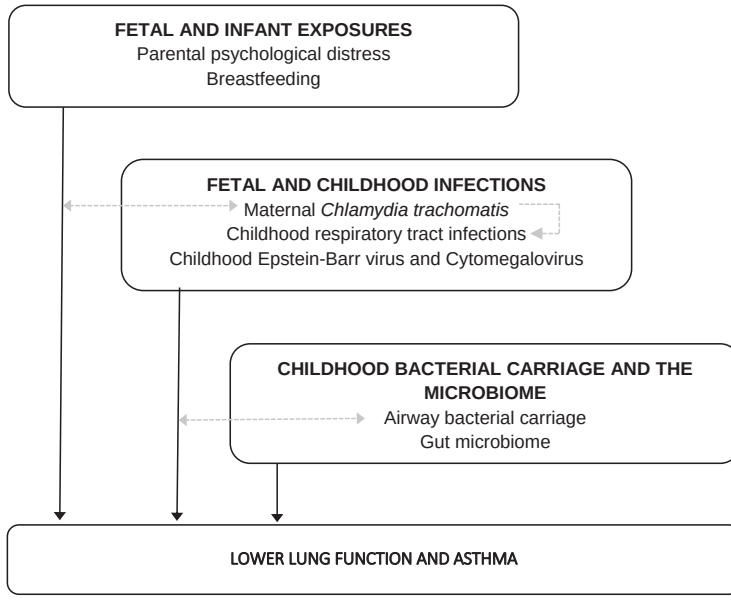

Figure 1.1. Overview of exposures influencing childhood asthma development studied in this thesis.

GENERAL DESIGN

The studies presented in this thesis were embedded in a population-based prospective cohort study, the Generation R Study, and an international collaboration of different cohort studies.

The Generation R Study

The Generation R Study is a population-based prospective cohort study from fetal life onwards[65]. The study is designed to identify early environmental and genetics causes and causal pathways leading to normal and abnormal growth, development and health from fetal life, into childhood and adulthood. Enrolment was aimed in the first trimester, but was allowed until birth of the child. In total, 9,778 mothers with a delivery date from April 2002 until January 2006 were enrolled in the study. A total of 1,232 pregnant women and their children form the focus cohort, a subgroup of Dutch children for additional detailed studies[66]. At enrolment during pregnancy, urine samples were collected and tested for *Chlamydia trachomatis* in a subgroup of 4,055 women. During the 2nd trimester, maternal and paternal psychological distress was assessed by questionnaires. Information on birth characteristics such as gestational age at birth and birth weight was obtained from midwife and hospital registries. During the preschool period, until age 4 years, information was mostly obtained from postal questionnaires, and included questions on wheezing and respiratory tract infections. In the focus cohort, detailed measurements were performed in the preschool period, including repeated assessment of airway bacterial carriage. At age 6 years, IgG antibodies against Cytomegalovirus and Epstein-Barr virus were measured. At age 10 years, lung function was measured by spirometry ac-

cording to the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines at our research center[67]. Information on wheezing and ever diagnosis of asthma was obtained by parental questionnaires based on the International Study on Asthma and Allergy in Childhood (ISAAC)[68]. Current asthma, defined as ever diagnosis of asthma with either wheezing or asthma medication use in the past 12 months. Information on inhalant allergic sensitization was obtained by skin-prick tests. Stool samples were collected for assessment of the gut microbiome (Figure 1.2).

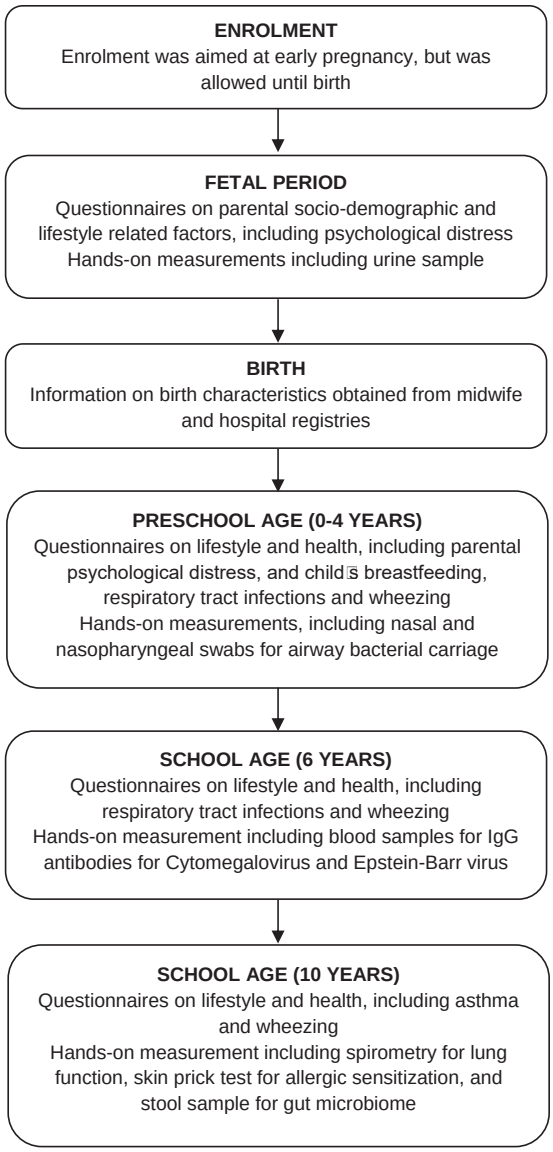

Figure 1.2 Overview of data collection of the Generation R Study used in this thesis

EU Child Cohort Network

A meta-analysis was conducted within the framework of the LifeCycle Project. European pregnancy and birth cohorts were identified from existing collaborations and literature and together form the EU Child Cohort Network. Inclusion criteria were cohorts that had included children born between 1989 and 2013, had available data on respiratory tract infections in early life and childhood lung function and/or asthma, and were willing and able to exchange original data. We selected European cohorts from both the LifeCycle project and other existing collaborations.

OUTLINE OF THIS THESIS

Chapter 2 focuses on the association of fetal and infant exposures with childhood respiratory health. In *Chapter 2,1*, the influence of parental psychological distress during pregnancy on school-age lung function and asthma is examined and discussed. The association of breastfeeding with school-age lung function and asthma is the focus of *Chapter 2.2* In **Chapter 3**, associations of early-life respiratory and other infections with later-life childhood respiratory health are analysed. The association of respiratory tract infections with lung function and asthma in the Generation R Study, and in an international collaboration, are presented in *Chapters 3.1 and 3.2*, respectively. C*hapter 3.3* focuses on the association of maternal *Chlamydia trachomatis* infection during pregnancy with childhood lower respiratory tract infections and wheezing, and school-age lung function and asthma. The associations of childhood Epstein-Barr virus and Cytomegalovirus infection with school age-lung function and asthma are described in *Chapter 3.4.* **Chapter 4** examines the effects of airway bacterial carriage and the gut microbiome on respiratory health. *Chapter 4.1* focuses on the bidirectional associations of airway bacterial carriage with childhood lower respiratory tract infections, and the associations with childhood wheezing, and school-age lung function and asthma. The cross-sectional association of the gut microbiome with atopic and respiratory disease is reported in *Chapter 4.2*. In *Chapter 4.3*, an overview is given on the role of respiratory tract infections and the microbiome in the development of asthma. The main findings of this thesis and their possible implications are discussed in the general discussion in **Chapter 5**, followed by the summaries in both English and Dutch in **Chapter 6**.

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Chapter 2

Fetal and infant exposures

Chapter 2.1

Parental psychological distress during pregnancy and the risk of childhood lower lung function and asthma

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ABSTRACT

Background

Although maternal psychological distress during pregnancy is associated with increased risks of respiratory morbidity in preschool children, it is unknown whether this association persists into later childhood.

Objective

To examine the association of parental psychological distress during pregnancy with lung function and asthma at school-age.

Methods

This study of 4,231 children was embedded in a population-based prospective cohort. Parental psychological distress were assessed by the Brief Symptom Inventory during and 3 years after pregnancy, and in mothers also at 2 and 6 months after pregnancy. At age 10 years, lung function was obtained by spirometry, and asthma by questionnaire.

Results

Prevalence of asthma was 5.9%. Maternal overall psychological distress during pregnancy was associated with a lower forced vital capacity (FVC) (z-score difference (95% CI): -0.10 (-0.20, -0.01) per 1-unit increase), maternal depressive symptoms during pregnancy with a lower forced expiratory volume in the first second (FEV_1) and FVC (-0.13 (-0.24, -0.01) and -0.13 (-0.24, -0.02) when using clinical cut-offs) in their children. All maternal psychological distress measures during pregnancy were associated with an increased risk of asthma (range OR (95% CI): 1.46 (1.12, 1.90) to 1.91 (1.26, 2.91)). Additional adjustment for paternal psychological distress during pregnancy, and parental psychological distress after pregnancy did not materially change the associations. Paternal psychological distress during pregnancy was not associated with childhood respiratory morbidity.

Conclusion

Maternal, not paternal, psychological distress during pregnancy is associated with an increased risk of asthma, and partly lower lung function in children. This suggests intrauterine programming for the risk of later-life respiratory disease.

INTRODUCTION

Early life is a sensitive period for the development of respiratory health[1]. We, and others, previously showed that maternal psychological distress during pregnancy is associated with increased risks of wheezing and asthma in their preschool-aged children[2-7]. This suggests a potential role of intrauterine mechanisms, such as altered programming of the fetal hypothalamic–pituitary–adrenal (HPA) axis, leading to adaptive airway and lung development and asthma[8-10]. The association of maternal psychological distress during pregnancy with childhood asthma might also be explained by residual confounding factors, such as unmeasured genetic, social, behavioral or environmental factors. Since these residual confounding factors are shared by mother and father, while only maternal psychological distress might have intra-uterine effects, examining paternal psychological distress during pregnancy in relation to childhood asthma is of importance to account for these residual confounders[3 11]. Only few studies measured paternal psychological distress, and observed that this is not associated with an increased risk of respiratory symptoms in early childhood, suggesting an intra-uterine effect[2 3 7]. To date, the associations of maternal and paternal psychological distress during pregnancy with asthma in later childhood, and the association with lung function are not fully clear[12 13]. We hypothesize that maternal psychological distress during pregnancy, but not paternal psychological distress, is associated with school-age lower lung function and asthma, suggesting intrauterine programming for the risk of respiratory disease.

Therefore, we examined the association of maternal psychological distress during pregnancy with lung function and asthma in children aged 10 years in a population-based prospective study. Further, we assessed whether these associations were independent of paternal psychological distress during pregnancy, and parental psychological distress after pregnancy.

METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards in Rotterdam, the Netherlands[14]. The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Centre in Rotterdam. Written informed consent was obtained from all parents or legal guardians of the participants. Children from twin pregnancies (n=185), with missing information on maternal psychological distress during pregnancy (n=2,595) or missing information on both lung function and asthma (n=382) were excluded, which left a total of 4,231 subjects at age 10 years for the current analyses.

Maternal and paternal psychological distress

Maternal and paternal psychological distress were assessed by questionnaires completed in the 2nd trimester of pregnancy and 3 years after pregnancy. Both parents were asked to complete their own questionnaires. At 2 and 6 months after pregnancy, only maternal psychological distress was assessed. We used the Brief Symptom Inventory, a validated 53-item self-report questionnaire covering a broad spectrum of psychological distress experienced in the last 7 days[15 16]. A global scale (Global Severity Index (GSI)) and nine symptom scales were defined[17 18]. The GSI is a measure of the current level or the depth of symptoms and denotes overall psychological distress. Of the symptom scales, we used the depressive and anxiety symptom scale only since in general, depression and anxiety are considered the most common psychological distress during and after pregnancy. Other subscales encompass less prevalent disorders as somatization, obsessive-compulsion, interpersonal sensitivity, hostility, phobia, paranoia and psychoticism. All scales were repeated at 2 months after pregnancy, and 6 months and 3 years after pregnancy, only depressive and anxiety symptoms were used. All items (53 for the GSI and 6 for both depressive and anxiety symptoms) were rated on a 5-point unidimensional scale ranging from 0 ('not at all') to 4 ('extremely'). Total scores for each scale were calculated by summing the item scores and dividing this by the number of endorsed items. Based on Dutch cut-off values, mothers and fathers were categorized as having clinically relevant psychological distress (no; yes) when having a score of ≥ 0.71 or ≥ 0.66 on the global scale, ≥ 0.80 or ≥ 0.71 on the depressive symptom scale and ≥ 0.71 or ≥ 0.65 on the anxiety symptom scale, respectively[19]. The Cronbach's alpha, reflecting internal consistency of the different scales ranged from 0.72 to 0.93, which is considered acceptable to excellent. Spearman correlations between parental psychological distress during and after pregnancy varied between 0.29 and 0.45 (weak to moderate), and between maternal and paternal psychological distress between 0.12 and 0.23 (very weak to weak).

School-age lung function and asthma

Lung function was measured by spirometry at the age of 10 years (median 9.7 (5-95% range 9.5-10.3)), as reported earlier[20]. In short, forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC, and forced expiratory flow after exhaling 75% of FVC (FEF_{75}) were measured in accordance with the American Thoracic Society (ATS) and European Respiratory Society (ERS) recommendations, and converted into sex-, height-, age-, and ethnicity adjusted z-scores according to the Global Lung Initiative reference data[21 22]. Additionally, we included 281 children whose spirometry did not meet formal reproducibility criteria, but who had at least one adequate curve with respect to reach and duration of plateau according to ATS/ERS criteria. Observed sizes and direction of effect estimates were similar as to when these children were excluded[23]. Current asthma was defined as ever diagnosis of asthma, with either wheezing or any asthma medication use in the past 12 months at the age of 10 years. Information on asthma diagnosis and wheezing was obtained by a parental questionnaire, based on the International Study on Asthma and Allergy in Childhood (ISAAC) Questionnaire ("Has your child ever had asthma diagnosed by a doctor?" and "Has your

child suffered from attacks of wheezing in the chest in the past 12 months?").[24]Information on asthma medication use was obtained during the visit to our research center.

Covariates

Information on maternal characteristics included age, parity, ethnicity, educational level, smoking during pregnancy, body mass index at enrollment, history of asthma and atopy, and pet keeping, and were obtained from multiple questionnaires during pregnancy. Information on paternal characteristics included age, ethnicity, educational level, smoking before pregnancy, body mass index at enrolment, and history of asthma and atopy, and were obtained by a questionnaire during pregnancy. Information on child's sex, gestational age at birth, and birthweight were obtained from midwife and hospital records. Information on child's ethnicity was based on questionnaires during pregnancy, and information on breastfeeding and daycare attendance were obtained by questionnaires in the first year of life. Information on childhood lower respiratory tract infections (<3 years and >3-6 years) was assessed by yearly questionnaires, and included pneumonia, bronchitis and bronchiolitis.

Statistical analysis

First, we compared the characteristics of children included in and excluded from our study due to loss to follow-up by using Man-Whitney U tests, T-tests and Chi square tests. Second, we used linear and logistic regression models to study the associations of maternal psychological distress during pregnancy with lung function and current asthma, respectively. Overall psychological distress, depressive and anxiety symptoms were studied separately, and both continuous measures and clinical cut-offs were used. Analyses were adjusted for confounders, which were first selected from the literature, and subsequently included in the model if they were associated with maternal psychological distress and lung function or asthma, or if the effect sizes of unadjusted analyses changed 10% or more after adding a confounder (main model). All confounders selected from literature met these criteria, and we therefore included all confounders in the model. Additionally, we added paternal psychological distress during pregnancy to the model, to minimize the potential correlated effect of maternal and paternal psychological distress during pregnancy on childhood lung function and asthma, and assess the individual effect of maternal psychological distress during pregnancy. Next, we added maternal psychological distress after pregnancy to the main model as a mediator to disentangle the individual effect of maternal psychological distress during pregnancy on respiratory morbidity of the child. We assessed the change in effect estimates for the associations of maternal psychological distress during pregnancy with lung function and asthma, after additional adjustment for maternal psychological distress after pregnancy, by using the following formulas for percentage change: and 100 * (Effect estimate$_{\text{full model}}$ – Effect estimate$_{\text{original model}}$)/(Effect estimate$_{\text{original model}}$) for continuous lung function measures, and 100 * (Effect estimate$_{\text{full model}}$ – Effect estimate$_{\text{original model}}$)/(Effect estimate$_{\text{original model}}$ – 1) for categorical asthma. All other confounders were kept similar to the model with only maternal distress during

pregnancy, to ascertain that the change in effect estimate is only due to the additional adjustment for parental psychological distress after pregnancy.

To assess residual confounding effects of unmeasured genetic, social, behavioral or environmental factors, we studied the associations of paternal psychological distress during pregnancy with lung function and asthma. Additionally, we adjusted the associations for paternal psychological distress after pregnancy including change in effect estimates similar as for maternal psychological distress. Last, we assessed whether associations of maternal psychological distress during pregnancy with lung function and asthma were mediated by childhood lower respiratory tract infections.

Since parental psychological distress after pregnancy is likely to be correlated to parental distress during pregnancy, adding psychological distress after pregnancy to the model could lead to multicollinearity. Correlations between parental psychological distress during and after pregnancy were however below 0.5. Additionally, we assessed the Variance Inflation Factor and tolerance statistics, which are measures for multicollinearity, of the models were parental psychological distress after pregnancy was added to parental psychological distress during pregnancy. Both these measured showed no indication for multicollinearity and inflation of the model. Lastly, we performed a sensitivity analysis focusing on patterns of depressive and anxiety symptoms. In this analysis, depressive and anxiety symptoms during and after pregnancy were combined into the following groups: never, symptoms during pregnancy only, symptoms after pregnancy only, and both symptoms during and after pregnancy. Symptoms after pregnancy reflect psychological distress at either 2, 6 or 36 months after pregnancy.

Missing data in covariates (range between 0 and 33%), maternal psychological distress after pregnancy and paternal psychological distress during and after pregnancy (range between 26 and 41%) were imputed by multiple imputation using the Markov Chain Monte Carlo method[25]. We only present the pooled results based on the imputed datasets, since we observed no major differences in the magnitude or direction of the effect when compared to complete case analysis. All measures of association are presented with their 95% Confidence Intervals (95% CI). Statistical analyses were performed using SPSS version 24.0 for Windows software (SPSS Inc.) and R version 3.4.1.

RESULTS

Subject's characteristics

Parental and child characteristics are presented in Table 2.1.1 and 2.1.2, respectively. Of the participating mothers, 8.6% (n = 362) had clinically relevant overall psychological distress, and of the participating fathers, 3.9% (n = 167). The mean (SD) values for FEV_1, FVC, FEV_1 /FVC and FEF_{75} were 2.02 L (0.30), 2.33 L (0.36), 86.72% (5.66) and 1.15 L/s (0.35), respectively. The prevalence of current asthma in children aged 10 years was 5.9% (n = 213). Most prominently, children lost to follow up had more often lower educated, non-European parents, and a lower gestational age at birth and birthweight (Supplementary Table S2.1.1).

Table 2.1.1. Characteristics of mothers and fathers

	n= 4,231	
	Mothers	**Fathers**
Age, years	30.9 (4.8)	33.4 (5.6)
Parity, nulliparous	59.5 (2,516)	--
Ethnicity, non-European	31.4 (1,329)	35.6 (1,507)
Education, lower	48.1 (2,034)	48.9 (2,068)
Smoking during pregnancy, yes	26.5 (1,122)	--
Smoking before pregnancy, yes	--	41.6 (1,761)
Body mass index at enrolment, kg/m^2	24.5 (4.2)	25.3 (3.4)
History of asthma or atopy, yes	37.7 (1,594)	33.7 (1,425)
Pet keeping, yes	35.9 (1,520)	--
Psychological distress during pregnancy		
Overall psychological distress, continuously[1]	0.15 (0.00, 0.91)	0.08 (0.00, 0.55)
Overall psychological distress, yes	8.6 (362)	3.9 (167)
Depressive symptoms, continuously[1]	0.00 (0.00, 1.00)	0.00 (0.00, 0.67)
Depressive symptoms, yes	8.2 (348)	4.3 (182)
Anxiety symptoms, continuously[1]	0.17 (0.00, 1.00)	0.00 (0.00, 0.75)
Anxiety symptoms, yes	9.3 (395)	8.0 (337)
Psychological distress at 2 months after pregnancy		
Overall psychological distress, continuously[1]	0.13 (0.00, 0.93)	--
Overall psychological distress, yes	7.9 (333)	--
Depressive symptoms, continuously[1]	0.00 (0.00, 1.08)	--
Depressive symptoms, yes	8.2 (349)	--
Anxiety symptoms, continuously[1]	0.00 (0.00, 1.00)	--
Anxiety symptoms, yes	8.3 (350)	--
Psychological distress at 6 months after pregnancy		
Depressive symptoms, continuously[1]	0.17 (0.00, 1.17)	--
Depressive symptoms, yes	8.5 (358)	--
Anxiety symptoms, continuously[1]	0.00 (0.00, 1.17)	--
Anxiety symptoms, yes	9.9 (417)	--
Psychological distress at 36 months after pregnancy		
Depressive symptoms, continuously[1]	0.00 (0.00, 0.78)	0.00 (0.00, 0.68)
Depressive symptoms, yes	5.1 (217)	4.4 (187)
Anxiety symptoms, continuously[1]	0.00 (0.00, 0.80)	0.00 (0.00, 0.68)
Anxiety symptoms, yes	5.1 (214)	7.7 (324)

Values are means (SD), [1]medians (5-95% range) or valid percentages (absolute numbers). Data on maternal depressive (n=5) and anxiety (n=6) symptoms during pregnancy was missing and not imputed. Not all information was available for both mothers and fathers, as denoted by --.

Table 2.1.2. Characteristics of the children

	n = 4,231
Sex, female	51.2 (2,166)
Gestational age at birth, weeks[1]	40.1 (37.1, 42.1)
Birthweight (grams)	3,450 (545)
Ethnicity, non-European	29.1 (1,233)
Ever breastfeeding, yes	90.9 (3,847)
Day care attendance 1st year, yes	57.4 (2,428)
$FEV_{1,}$ L	2.02 (0.30)
FVC, L	2.33 (0.36)
FEV_1/FVC, %	86.72 (5.66)
$FEF_{75,}$ L/s	1.15 (0.35)
$FEV_{1,}$ z-score	0.17 (0.97)
FVC, z-score	0.21 (0.93)
FEV_1/FVC, z-score	-0.10 (0.94)
$FEF_{75,}$ z-score	0.04 (0.91)
Current asthma, yes	5.9 (213)

Values are means (SD), [1]medians (5-95% range) or valid percentages (absolute numbers). Lung function measures (n=474) and current asthma (n=591) were missing and not imputed. Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}).

Maternal psychological distress, lung function and asthma

Unadjusted associations of maternal psychological distress during pregnancy with school-age lung function and asthma can be found in Supplementary Table S2.1.2, After adjustment for confounders, and when studied continuously, only maternal overall psychological distress during pregnancy was associated with a lower FVC (z-score difference (95% CI): -0.10 (-0.20, -0.01)) (Table 2.1.3) in their children. When using clinical cut-offs, only maternal depressive symptoms during pregnancy were associated with a lower FEV_1 and FVC (-0.13 (-0.24, -0.01) and -0.13 (-0.24, -0.02), respectively). Additional adjustment for paternal psychological distress during pregnancy did not change the direction or magnitude of the effect (Table 2.1.4). When we additionally adjusted for maternal psychological distress at 2, 6 and 36 months after pregnancy, and paternal psychological distress 36 months after pregnancy, the size and direction of the associations of depressive symptoms with a lower FEV_1 and FVC remained unchanged, except when adjusting for maternal psychological distress at 2 months (Table 2.1.5). The percentages change in effect estimates were, however, non-significant (data not shown).

Maternal overall psychological distress, and depressive and anxiety symptoms during pregnancy were all associated with an increased risk of current asthma in their children, both continuously and when using clinical cut-offs (range odds ratios (95% CI): 1.46 (1.12, 1.90) to 1.91 (1.26, 2.91)) (Table 2.1.3). When we additionally adjusted for paternal psychological distress during pregnancy, the associations of maternal psychological distress with asthma remained essentially

Table 2.1.3. Associations of maternal psychological distress during pregnancy with lung function and asthma at age 10 years

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Maternal psychological distress						
Overall psychological distress	4,231					
Per 1-unit increase		-0.07 (-0.17, 0.03)	**-0.10 (-0.20, -0.01)***	0.04 (-0.06, 0.13)	0.01 (-0.08, 0.10)	**1.83 (1.30, 2.59)****
Clinical cut-off		-0.02 (-0.14, 0.10)	-0.07 (-0.14, 0.01)	0.03 (-0.05, 0.10)	0.02 (-0.05, 0.09)	**1.91 (1.26, 2.91)****
Depressive symptoms	4,225					
Per 1-unit increase		-0.05 (-0.12, 0.03)	-0.07 (-0.14, 0.01)	0.03 (-0.04, 0.11)	0.02 (-0.05, 0.09)	**1.46 (1.12, 1.90)****
Clinical cut-off		**-0.13 (-0.24, -0.01)***	**-0.13 (-0.24, -0.02)***	-0.01 (-0.12, 0.11)	-0.03 (-0.14, 0.08)	**1.84 (1.21, 2.80)****
Anxiety symptoms	4,226					
Per 1-unit increase		-0.01 (-0.09, 0.07)	-0.03 (-0.10, 0.05)	0.01 (-0.07, 0.09)	-0.00 (-0.07, 0.07)	**1.55 (1.19, 2.02)****
Clinical cut-off		-0.01 (-0.12, 0.10)	0.01 (-0.09, 0.11)	-0.05 (-0.16, 0.06)	-0.05 (-0.15, 0.05)	**1.64 (1.09, 2.47)***

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI) from linear or logistic regression models, respectively. Maternal psychological distress is treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). The main models were adjusted for maternal age, parity, education level, smoking during pregnancy, body mass index at enrolment, history of asthma or atopy and pet keeping, and child's sex, gestational age at birth, birthweight, ethnicity, breastfeeding and daycare attendance. *p-value <0.05, **p-value <0.01.

Table 2.1.4. Associations of maternal psychological distress during pregnancy with lung function and asthma at age 10 years, adjusted for paternal psychological distress during pregnancy.

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Main model + paternal psychological distress						
Overall psychological distress	4,231					
Per 1-unit increase		-0.07 (-0.17, 0.03)	**-0.10 (-0.19, -0.00)***	0.03 (-0.07, 0.13)	0.01 (-0.09, 0.10)	**1.79 (1.26 2.56)****
Clinical cut-off		-0.02 (-0.14, 0.10)	-0.05 (-0.16, 0.06)	0.02 (-0.10, 0.13)	0.04 (-0.08, 0.15)	**1.92 (1.26, 2.92)****
Depressive symptoms	4,225					
Per 1-unit increase		-0.04 (-0.12, 0.03)	-0.06 (-0.13, 0.01)	0.02 (-0.05, 0.10)	0.02 (-0.05, 0.09)	**1.45 (1.10, 1.91)****
Clinical cut-off		**-0.12 (-0.24, -0.00)***	**-0.12 (-0.23, -0.01)***	-0.01 (-0.12, 0.11)	-0.03 (-0.14, 0.08)	**1.82 (1.19, 2.80)****
Anxiety symptoms	4,226					
Per 1-unit increase		-0.01 (-0.09, 0.07)	-0.02 (-0.10, 0.05)	0.01 (-0.07, 0.08)	0.00 (-0.07, 0.07)	**1.57 (1.20, 2.06)****
Clinical cut-off		0.00 (-0.11, 0.11)	0.02 (-0.09, 0.12)	-0.05 (-0.16, 0.06)	-0.04 (-0.15, 0.06)	**1.64 (1.09, 2.47)***

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI) from linear or logistic regression models, respectively. Maternal psychological distress is treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). The main models were adjusted for maternal age, parity, education level, smoking during pregnancy, body mass index at enrolment, history of asthma or atopy and pet keeping, and child's sex, gestational age at birth, birthweight, ethnicity, breastfeeding and daycare attendance. Additionally, models were adjusted for paternal psychological distress during pregnancy. *p-value <0.05, **p-value <0.01.

Table 2.1.5. Associations of maternal psychological distress with lung function and asthma at age 10 years, adjusted for maternal psychological distress at 2, 6 and 36 months after pregnancy, and paternal psychological distress at 36 months after pregnancy, respectively.

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Main model + maternal psychological distress at 2 mo						
Overall psychological distress	4,231					
Per 1-unit increase		-0.03 (-0.15, 0.09)	-0.06 (-0.18, 0.05)	0.05 (-0.08, 0.17)	0.05 (-0.07, 0.18)	**2.01 (1.26, 3.22)****
Clinical cut-off		0.01 (-0.12, 0.14)	-0.02 (-0.15, 0.10)	0.02 (-0.11, 0.14)	0.06 (-0.06, 0.18)	**2.11 (1.30, 3.43)****
Depressive symptoms	4,226					
Per 1-unit increase		-0.02 (-0.11, 0.06)	-0.05 (-0.13, 0.03)	0.04 (-0.05, 0.12)	0.04 (-0.04, 0.13)	1.37 (1.00, 1.88)
Clinical cut-off		-0.12 (-0.23, 0.01)	-0.10 (-0.21, 0.02)	-0.04 (-0.16, 0.09)	-0.04 (-0.16, 0.08)	**1.87 (1.18, 2.97)****
Anxiety symptoms	4,225					
Per 1-unit increase		0.00 (-0.09, 0.09)	-0.01 (-0.10, 0.08)	0.00 (-0.09, 0.10)	0.00 (-0.09, 0.09)	**1.70 (1.20, 2.41)****
Clinical cut-off		-0.01 (-0.12, 0.11)	0.02 (-0.09, 0.13)	-0.06 (-0.18, 0.05)	-0.05 (-0.16, 0.06)	**1.72 (1.09, 2.72)***
Main model + maternal psychological distress at 6 mo						
Depressive symptoms	4,226					
Per 1-unit increase		-0.05 (-0.14, 0.04)	-0.06 (-0.14, 0.03)	0.00 (0.08, 0.09)	0.01 (-0.08, 0.09)	1.29 (0.93, 1.78)
Clinical cut-off		**-0.13 (-0.26, -0.01)***	**-0.13 (-0.24, -0.01)***	-0.02 (-0.15, 0.10)	-0.04 (-0.16, 0.08)	1.58 (0.99, 2.54)
Anxiety symptoms	4,225					
Per 1-unit increase		-0.04 (-0.13, 0.04)	-0.04 (-0.13, 0.04)	-0.02 (-0.11, 0.07)	-0.02 (-0.11, 0.06)	**1.61 (1.16, 2.22)****
Clinical cut-off		-0.02 (-0.13, 0.09)	-0.01 (-0.11, 0.12)	-0.07 (-0.18, 0.05)	-0.06 (-0.17, 0.05)	**1.59 (1.02, 2.48)***

Table 2.1.5. Associations of maternal psychological distress with lung function and asthma at age 10 years, adjusted for maternal psychological distress at 2, 6 and 36 months after pregnancy, and paternal psychological distress at 36 months after pregnancy, respectively. (continued)

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Main model + maternal psychological distress at 36 mo						
Depressive symptoms	4,226					
Per 1-unit increase		-0.05 (-0.13, 0.04)	-0.07 (-0.15, 0.01)	0.03 (-0.05, 0.11)	0.03 (-0.06, 0.11)	**1.38 (1.02, 1.86)***
Clinical cut-off		**-0.12 (-0.24, -0.00)***	**-0.12 (-0.24, -0.01)***	-0.01 (-0.13, 0.11)	-0.02 (-0.14, 0.09)	**1.78 (1.14, 2.78)***
Anxiety symptoms	4,225					
Per 1-unit increase		-0.03 (-0.11, 0.06)	-0.03 (-0.12, 0.05)	-0.00 (-0.09, 0.08)	-0.01 (-0.09, 0.07)	**1.43 (1.05, 1.95)***
Clinical cut-off		-0.01 (-0.12, 0.10)	0.01 (-0.10, 0.12)	-0.06 (-0.17, 0.06)	-0.05 (-0.15, 0.06)	1.52 (0.99, 2.34)
Main model + paternal psychological distress at 36 mo						
Depressive symptoms	4,226					
Per 1-unit increase		-0.05 (-0.12, 0.03)	-0.07 (-0.14, 0.00)	0.03 (-0.05, 0.11)	0.02 (-0.05, 0.10)	**1.41 (1.07, 1.86)***
Clinical cut-off		**-0.13 (-0.24, -0.01)***	**-0.13 (-0.24, -0.02)***	-0.00 (-0.12, 0.11)	-0.03 (-0.14, 0.08)	**1.78 (1.16, 2.74)***
Anxiety symptoms	4,225					
Per 1-unit increase		-0.02 (-0.09, 0.06)	-0.03 (-0.11, 0.04)	0.01 (-0.07, 0.09)	-0.00 (-0.08, 0.07)	**1.51 (1.15, 1.99)****
Clinical cut-off		-0.01 (-0.12, 0.10)	0.01 (-0.10, 0.11)	-0.05 (-0.16, 0.06)	-0.05 (-0.15, 0.05)	**1.60 (1.06, 2.41)***

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI) from linear or logistic regression models, respectively. Maternal psychological distress is treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). The main models were adjusted for maternal age, parity, education level, smoking during pregnancy, body mass index at enrolment, history of asthma or atopy and pet keeping, and child's sex, gestational age at birth, birthweight, ethnicity, breastfeeding and daycare attendance. Additionally, models were adjusted for maternal psychological distress at 2, 6 or 36 months after pregnancy, or for paternal psychological distress at 36 months after pregnancy. At 6 and 36 months after pregnancy, not all subscales were measured, and therefor overall psychological distress could not be included at these time points. *p-value <0.05, **p-value <0.01.

Table 2.1.6. Associations of paternal psychological distress with lung function and asthma at age 10 years.

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Paternal psychological distress						
Overall psychological distress	4,231					
Per 1-unit increase		0.00 (-0.18, 0.18)	-0.05 (-0.23, 0.13)	0.07 (-0.08, 0.22)	0.03 (-0.13, 0.19)	1.13 (0.56, 2.26)
Clinical cut-off		-0.05 (-0.30, 0.20)	-0.08 (-0.31, 0.14)	0.04 (-0.17, 0.24)	-0.04 (-0.25, 0.17)	0.98 (0.44, 2.18)
Depressive symptoms	4,231					
Per 1-unit increase		-0.02 (-0.18, 0.14)	-0.04 (-0.19, 0.11)	0.02 (-0.10, 0.14)	-0.01 (-0.16, 0.13)	1.04 (0.59, 1.85)
Clinical cut-off		-0.07 (-0.30, 0.16)	-0.07 (-0.30, 0.16)	-0.02 (-0.20, 0.17)	-0.06 (-0.25, 0.13)	1.04 (0.43, 2.53)
Anxiety symptoms	4,231					
Per 1-unit increase		-0.01 (-0.17, 0.15)	-0.03 (-0.19, 0.14)	0.02 (-0.10, 0.14)	-0.01 (-0.14, 0.13)	0.85 (0.46, 1.58)
Clinical cut-off		-0.13 (-0.28, 0.02)	-0.13 (-0.27, 0.01)	0.01 (-0.14, 0.15)	-0.06 (-0.21, 0.09)	0.99 (0.51, 1.92)

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI) from linear or logistic regression models, respectively. Paternal psychological distress is treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). The models were adjusted for maternal age, parity, education level, smoking during pregnancy, body mass index at enrolment, history of asthma or atopy and pet keeping, and child's sex, gestational age at birth, birthweight, ethnicity, breast-feeding and daycare attendance and maternal psychological distress during pregnancy. *p-value <0.05, **p-value <0.01.

unchanged (Table 2.1.4). Additional adjustment for maternal psychological distress at 2, 6 and 36 months after pregnancy did not change the direction or magnitude of the effect estimates for the association with asthma, including a non-significant percentage change in effect estimates (Table 2.1.5). Analysis of patterns of psychological distress demonstrated that mostly depressive or anxiety symptoms both during and after pregnancy are associated with an increased risk of asthma (Supplementary Table S2.1.3). Separating the confounders into three different groups, including lifestyle and health-related factors, socio-economic factors and birth and early childhood factors, showed no differences in size or direction of effect estimates of the associations of maternal psychological distress with asthma (Supplementary Table S2.1.4). However, lifestyle and health-related factors seemed to account for attenuation of the effect of maternal psychological distress with FEF_{75} only. Additional adjustment for childhood lower respiratory tract infections did not change the direction or magnitude of the effect estimates for the association with asthma, including a non-significant percentage change in effect estimates (data not shown).

Paternal psychological distress, lung function and asthma

Paternal psychological distress during pregnancy was not associated with lung function or asthma in their children (Table 2.1.6). The associations of paternal psychological distress during pregnancy with lung function and asthma remained essentially unchanged after additional adjustment for paternal psychological distress at 36 months after pregnancy (Supplementary Table S2.1.5). Only the associations of clinical cut-offs of anxiety with FEV_1 and FVC became significant, as opposed to before adjustment for paternal psychological distress after birth (-0.15 (-0.29, -0.00) and -0.14 (-0.29, -0.00), respectively). However, the percentages change for these, and other associations, when adding paternal psychological distress after pregnancy to the model, were non-significant (Supplementary Table S2.1.6).

DISCUSSION

The results of this prospective population-based cohort study showed that children of mothers with overall psychological distress, and depressive or anxiety symptoms during pregnancy had an increased risk of asthma at the age of 10 years. Children of mothers with overall psychological distress, when measured continuously, had a lower FVC, and children of mothers with depressive symptoms, when using clinical cut-offs, had a lower FEV_1 and FVC. These findings were mostly independent of paternal psychological distress during pregnancy, and maternal and paternal psychological distress after pregnancy. The strongest effects were found for mothers that experienced psychological distress both during and after pregnancy. Although effects of distress during pregnancy only were not significant, this is most likely due to power, given that the effects estimates are in the same range as for the main analysis. Paternal psychological distress during pregnancy was not associated with an increased risk of lower lung function or asthma. Our results

may indicate an intrauterine effect of maternal psychological distress during pregnancy on fetal lung development and respiratory morbidity rather than an effect of unmeasured genetic, social, behavioral or environmental factors.

Comparison with previous studies

Studies examining the association of maternal psychological distress with childhood lung function are scarce. One study found that high prenatal, but also postnatal maternal psychological distress, measured as negative life events, was associated with a lower FEV_1, FVC and mid-expiratory flow (FEF_{25-75}), but not FEV_1/FVC at age 7 years [26]. This is in line with our study, were we demonstrate that the association of maternal psychological distress with a lower FEV_1 and FVC remains present in later childhood. These significant findings, compared to the more robust findings for the asthma outcome, are few and effect sizes are small from a clinical perspective but of importance from an etiological perspective. Studies examining the association of maternal psychological stress during pregnancy on wheezing and asthma have been summarized in two recent meta-analyses[5 6]. Both meta-analyses focused mostly on wheezing and asthma until preschool age, with only few studies included that measured respiratory outcomes after the age of 6 years. One meta-analysis including 10 studies, found that maternal psychological stress during pregnancy was associated a 1.56-fold increased risk of any respiratory morbidity[5]. When the associations with asthma and wheezing were studied separately, the effect sizes were approximately in the same range, although the latter showed high heterogeneity. The other, more recent, meta-analysis including 24 studies demonstrated that any maternal psychological stress during pregnancy was associated with a 1.13-fold increased risk of asthma[6]. This was most likely driven by anxiety during pregnancy, since only this association was significant when different types of psychological distress were studied separately. Anxiety, depression and negative life events during pregnancy were all associated with an increased risk of wheezing (OR or RR 1.19, 1.74 and 1.23, respectively). Our study demonstrates that maternal stress during pregnancy is associated with an increased risk of asthma even at a later age, with effect sizes within the same range. Additionally, we demonstrated that these results are independent of paternal psychological distress during pregnancy and parental psychological distress after pregnancy, which was not studied in these meta-analyses.

Possible mechanisms

Our results suggest that intrauterine mechanisms may underlie the associations of maternal psychological distress with childhood lung function and asthma in their children, rather than unmeasured genetic, social, behavioral or environmental factors. One potential intrauterine mechanism is excess of glucocorticoid production due to maternal psychological distress, which could lead impaired development of the fetal HPA axis[8]. Additionally, maternal psychological distress could influence the stimulation of corticotrophin releasing hormone (CRH) secretion, which results in increased CRH levels in the foetal circulation and could overstimulate the foetal HPA axis. Moreover, glucocorticoid-regulated genes are key to fetal lung development, especially

during the first and second trimesters of pregnancy[9]. Any disruption in this process could lead to developmental adaptations of the lungs and hence, altered lung function.

Further, epigenetics has been proposed as a possible mechanism. It has been demonstrated that maternal depression and anxiety are associated with methylation of the glucocorticoid receptor gene NR3C1 in cord blood[27]. Additionally, one study demonstrated an association of prenatal maternal stress with differently methylated regions (DMRs) related to the HPA axis, immune responses, and lung organogenesis[28]. Lastly, oxidative stress and the microbiome have been speculated as possible underlying mechanisms for the association of maternal psychological distress during pregnancy with offspring lung function and asthma[29-31]. However, these mechanisms should be studied in more detail.

Strengths and limitations

The major strength of this study is the use of a large population-based prospective cohort with follow-up until school age. In our study, we used a well-known measure of psychological distress, frequently used in epidemiological studies to assess psychological distress at multiple time points, both continuously and with clinical cut-offs. Additionally, we adjusted for multiple possible confounding factors, and considered paternal psychological distress to assess unmeasured confounding factors. Some methodological limitations should be discussed. As in any prospective cohort study, our population was subject to loss to follow-up. Of those with singleton live-born children with consent at age 10 years, 73% responded to the questionnaire measuring psychological distress. This loss to follow-up could lead to bias if the associations of maternal psychological distress during pregnancy with lung function and asthma were different between those included and not included in the study. This could lead to both an under- or overestimation of the association and, although this is unlikely it cannot be excluded[32]. Additionally, self-reporting of exposure and outcomes could potentially lead to information bias which could lead to misclassification. The use of validated questionnaires could have minimized the information bias. The prevalence of asthma seems relatively low, but lies within national and worldwide prevalence rates[33 34]. We consider the self-reported asthma as truly asthma because questions were based on the validated ISAAC Questionnaire, and wheezing or asthma medication use in the past 12 months were used to better define current asthma. Additionally, results were similar when we used persistent asthma as an outcome (data not shown). Moreover, psychological distress was measured only once during pregnancy. Hence, we do not know whether maternal psychological distress varied in intensity or persistence throughout pregnancy. However, one study that measured maternal anxiety at 2 time points during pregnancy demonstrated associations with asthma that were comparable for both time points, suggesting that timing in pregnancy seems to have a less prominent role in prenatal programming[2]. Still, future studies need to confirm these results. Lastly, although we adjusted for numerous confounders, we might not have had information on all possible confounding factors, such as maternal or child genetic predisposition for psychological stress disorders, or childhood exposures such as air pollution. Although some of their effects might be minimal and are highly

correlated with presently used confounders, they could potentially have a substantial effect given the relatively small prevalence of asthma.

In conclusion, our results suggest a possible intrauterine effect of maternal psychological distress during pregnancy on the risk of asthma and partly lower lung function in children at the age of 10 years. Results were independent of maternal psychological distress after pregnancy and paternal psychological distress during pregnancy and after pregnancy. Further studies are needed to explore underlying mechanisms.

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Supplementary Table S2.1.1. Subject characteristics for those included and not included in the study.

	Included n = 4,231	**Not included n = 3,162**	**P-value for difference**
Maternal characteristics			
Age (years)	30.9 (4.8)	30.1 (7.8)	**<0.001**
Parity, nulliparous (%)	59.5 (2,516)	47.1 (1,488)	**<0.001**
Ethnicity, non-European (%)	30.9 (1,289)	45.1 (1,325)	**<0.001**
Education, lower (%)	47.2 (1,937)	60.8 (1,587)	**<0.001**
Smoking during pregnancy, yes (%)	24.2 (941)	22.2 (366)	0.112
History of asthma or atopy, yes (%)	37.6 (1,434)	36.2 (591)	0.329
Pet keeping, yes (%)	34.9 (1,310)	29.6 (348)	**0.001**
Body mass index at enrolment (kg/m^2)	24.5 (4.17)	25.2 (4.7)	**<0.001**
Maternal overall psychological distress during pregnancy[1]	0.15 (0.00, 0.91)	0.15 (0.00, 1.21)	0.268
Paternal Characteristics			
Age (years)	33.4 (5.4)	33.3 (5.5)	0.790
Ethnicity, non-European (%)	25.1 (865)	34.5 (496)	**<0.001**
Education, lower (%)	54.7 (1,743)	49.6 (598)	**0.003**
Smoking before pregnancy, yes (%)	42.2 (1,639)	42.5 (517)	0.307
History of asthma or atopy, yes (%)	32.2 (1,005)	32.6 (360)	0.803
Body mass index at enrollment (kg/m^2)	25.2 (3.3)	25.4 (3.6)	0.070
Paternal overall psychological distress during pregnancy[1]	0.06 (0.00, 0.51)	0.08 (0.00, 0.54)	0.568
Child characteristics			
Sex, female (%)	51.2 (2,166)	48.1 (1,520)	**0.008**
Gestational age at birth (weeks)[1]	40.1 (37.1, 42.1)	39.9 (36.0, 42.0)	**<0.001**
Birth weight (grams)	3,450 (544)	3,354 (605)	**<0.001**
Ethnicity, non-European *(%)*	28.7 (1,207)	41.5 (1,220)	**<0.001**
Ever breastfeeding, yes (%)	92.6 (3,290)	91.7 (1,390)	0.252
Day care attendance 1st year, yes (%)	63.3 (1,791)	58.1 (548)	**0.005**

Values are means (SD), [1]medians (5-95% range) or valid percentages (absolute numbers) based on observed data. P values for difference are calculated by independent sample T-test for continuous variables with a normal distribution, the Mann-Whitney U-test for continuous variables with a skewed distribution, and Pearson's Chi-square test for categorical variables.

Supplementary Table S2.1.2. Unadjusted associations of maternal psychological distress during pregnancy with lung function and asthma at age 10 years

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Maternal psychological distress						
Overall psychological distress	4,231					
Per 1-unit increase		0.07 (-0.03, 0.16)	0.03 (-0.06, 0.12)	0.07 (-0.02, 0.16)	**0.16 (0.07, 0.24)****	**2.47 (1.82, 3.34)****
Clinical cut-off		0.010 (-0.01, 0.22)	0.06 (-0.05, 0.17)	0.06 (-0.05, 0.17)	**0.18 (0.07, 0.29)****	**2.78 (1.89, 4.07)****
Depressive symptoms	4,225					
Per 1-unit increase		0.05 (-0.02, 0.12)	0.20 (-0.05, 0.09)	0.06 (-0.02, 0.13)	**0.12 (0.05, 0.19)****	**1.79 (1.41, 2.26)****
Clinical cut-off		-0.01 (-0.12, 0.10)	-0.02 (-0.13, 0.09)	0.03 (-0.08, 0.14)	0.10 (-0.01, 0.21)	**2.58 (1.74, 3.83)****
Anxiety symptoms	4,226					
Per 1-unit increase		0.06 (-0.01, 0.14)	0.05 (-0.03, 0.12)	0.02 (-0.05, 0.10)	**0.08 (0.01, 0.15)***	**1.92 (1.50, 2.46)****
Clinical cut-off		0.08 (-0.03, 0.18)	0.09 (-0.01, 0.19)	-0.03 (-0.13, 0.08)	0.05 (-0.05, 0.15)	**2.18 (1.48, 3.22)****

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI) from linear or logistic regression models, respectively. Maternal psychological distress is treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). *p-value <0.05, **p-value <0.01.

Supplementary Table S2.1.3. Association of patterns of parental psychological distress with lung function and asthma at age 10 years.

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Maternal psychological distress						
Depressive symptoms patterns						
Never	3,457	Reference	Reference	Reference	Reference	Reference
Prenatal only	142	-0.15 (-0.35, 0.04)	-0.13 (-0.31, 0.07)	-0.05 (-0.24, 0.14)	-0.04 (-0.22, 0.14)	1.31 (0.61, 2.79)
Postnatal only	421	-0.03 (-0.18, 0.11)	-0.06 (-0.18, 0.06)	0.05 (-0.08, 0.17)	-0.01 (-0.15, 0.13)	1.00 (0.55, 1.83)
Both pre- and postnatal	206	-0.09 (-0.25, 0.07)	-0.12 (-0.27, 0.04)	0.03 (-0.12, 0.18)	-0.02 (-0.17, 0.13)	**2.20 (1.31, 3.70)****
Anxiety symptoms patterns						
Never	3,399	Reference	Reference	Reference	Reference	Reference
Prenatal only	161	-0.01 (-0.18, 0.17)	-0.00 (-0.19, 0.18)	-0.01 (-0.20, 0.18)	0.01 (-0.16, 0.18)	1.28 (0.62, 2.65)
Postnatal only	431	0.00 (-0.13, 0.14)	-0.03 (-0.15, 0.09)	0.06 (-0.06, 0.17)	0.02 (-0.10, 0.14)	1.08 (0.66, 1.76)
Both pre- and postnatal	234	-0.01 (-0.15, 0.14)	0.02 (-0.13, 0.16)	0.07 (-0.23, 0.08)	-0.09 (-0.23, 0.05)	**1.92 (1.16, 3.20)***
Paternal psychological distress[1]						
Depressive symptoms patterns						
Never	3,907	Reference	Reference	Reference	Reference	Reference
Prenatal only	136	-0.06 (-0.33, 0.22)	-0.05 (-0.31, 0.22)	-0.04 (-0.26, 0.17)	-0.07 (-0.31, 0.18)	0.91 (0.28, 3.03)
Postnatal only	142	0.07 (-0.14, 0.27)	0.10 (-0.12, 0.32)	-0.08 (-0.32, 0.16)	-0.09 (-0.33, 0.14)	1.26 (0.50, 3.18)
Both pre- and postnatal	46	-0.13 (-0.48, 0.22)	-0.18 (-0.52, 0.16)	0.06 (-0.31, 0.43)	-0.05 (-0.41, 0.30)	1.28 (0.21, 7.84)
Anxiety symptoms patterns						
Never	3,666	Reference	Reference	Reference	Reference	Reference
Prenatal only	241	-0.15 (-0.31, 0.00)	-0.14 (-0.29, 0.01)	-0.02 (-0.20, 0.16)	-0.09 (-0.28, 0.09)	1.09 (0.52, 2.28)
Postnatal only	228	0.06 (-0.14, 0.27)	0.08 (-0.10, 0.26)	-0.02 (-0.18, 0.13)	0.01 (-0.14, 0.16)	1.44 (0.81, 2.57)
Both pre- and postnatal	96	-0.06 (-0.33, 0.22)	-0.09 (-0.33, 0.15)	0.07 (-0.19, 0.33)	0.03 (-0.25, 0.31)	0.82, 0.25, 2.70)

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI) from linear or logistic regression models, respectively. Parental psychological distress is treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Postnatal distress reflect psychological distress at either 2, 6 or 36 months after pregnancy. Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). The models were adjusted for maternal age, parity, education level, smoking during pregnancy, body mass index at enrolment, history of asthma or atopy and pet keeping, and child's sex, gestational age at birth, birthweight, ethnicity, breastfeeding and daycare attendance. [1]Additionally, models were adjusted for maternal psychological distress. *p-value <0.05, **p-value <0.01

Supplementary Table S2.1.4. Association of maternal psychological distress with lung function and asthma at age 10 years, adjusted for different groups of confounders.

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Lifestyle and health-related factors[1]						
Overall psychological distress	4,231					
Per 1-unit increase		-0.09 (-0.19, 0.00)	**-0.11 (-0.20, 0.01)***	0.01 (-0.09, 0.11)	-0.01 (-0.11, 0.08)	**2.01 (1.45, 2.80)****
Clinical cut-off		-0.05 (-0.17, 0.06)	-0.07 (-0.17, 0.04)	0.00 (-0.11, 0.11)	0.01 (-0.10, 0.12)	**2.12 (1.41, 3.19)****
Depressive symptoms	4,225					
Per 1-unit increase		-0.06 (-0.12, 0.02)	-0.07 (-0.14, 0.00)	0.01 (-0.06, 0.09)	0.00 (-0.07, 0.07)	**1.53 (1.19, 1.98)****
Clinical cut-off		**-0.14 (-0.26, -0.03)***	**-0.13 (-0.24, -0.02)***	-0.23 (-0.14, 0.09)	-0.05 (-0.15, 0.06)	**2.04 (1.35, 3.08)****
Anxiety symptoms	4,226					
Per 1-unit increase		-0.03 (-0.11, 0.05)	-0.03 (-0.10, 0.04)	-0.01 (-0.08, 0.07)	-0.02 (-0.09, 0.06)	**1.67 (1.29, 2.17)****
Clinical cut-off		-0.03 (-0.14, 0.08)	-0.00 (-0.10, 0.10)	-0.07 (-0.17, 0.04)	-0.07 (-0.17, 0.04)	**1.76 (1.18, 2.63)****
Socio-economic factors[2]						
Overall psychological distress	4,231					
Per 1-unit increase		0.06 (-0.04, 0.16)	0.01 (-0.08, 0.04)	0.09 (-0.00, 0.18)	**0.16 (-0.07, 0.25)****	**2.18 (1.59, 2.99)****
Clinical cut-off		0.10 (-0.01, 0.22)	0.05 (-0.06, 0.16)	0.08 (-0.04, 0.19)	**0.18 (0.07, 0.29)****	**2.42 (1.63, 3.58)****
Depressive symptoms	4,225					
Per 1-unit increase		-0.02 (-0.09, 0.06)	0.01 (-0.06, 0.08)	0.07 (-0.00, 0.14)	**0.12 (0.05, 0.19)****	**1.66 (1.30, 2.13)****
Clinical cut-off		-0.01 (-0.13, 0.10)	-0.04 (-0.15, 0.07)	0.05 (-0.07, 0.16)	0.10 (-0.01, 0.21)	**2.26 (1.51, 3.39)****
Anxiety symptoms	4,226					
Per 1-unit increase		0.06 (-0.02, 0.14)	0.04 (-0.04, 0.11)	0.03 (-0.04, 0.11)	**0.08 (0.09, 0.15)****	**1.74 (1.35, 2.24)****
Clinical cut-off		0.07 (-0.04, 0.18)	0.08 (-0.03, 0.18)	-0.02 (-0.12, 0.09)	0.05 (-0.05, 0.15)	**1.96 (1.32, 2.91)****
Birth and early childhood factors[3]						
Overall psychological distress	4,231					

Supplementary Table S2.1.4. Association of maternal psychological distress with lung function and asthma at age 10 years, adjusted for different groups of confounders. (continued)

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Per 1-unit increase		0.08 (-0.02, 0.17)	0.02 (-0.07, 0.12)	0.09 (-0.00, 0.19)	**0.16, 0.07, 0.25)***	**2.32 (1.69, 3.19)****
Clinical cut-off		**0.12 (0.00, 0.23)***	0.06 (-0.05, 0.17)	0.08 (-0.03, 0.19)	**0.18 (0.07, 0.29)****	**2.56 (1.73, 3.81)****
Depressive symptoms	4,225					
Per 1-unit increase		0.06 (-0.12, 0.33)	0.02 (-0.05, 0.09)	0.07 (-0.01, 0.14)	**0.12 (0.05, 0.19)***	**1.72 (1.35, 2.19)****
Clinical cut-off		-0.00 (-0.12, 0.11)	-0.03 (-0.14, 0.08)	0.05 (-0.07, 0.16)	-0.10 (-0.01, 0.21)	**2.39 1.59, 3.57)****
Anxiety symptoms	4,226					
Per 1-unit increase		0.07 (-0.00, 0.28)	0.05 (-0.02, 0.12)	0.04 (-0.02, 0.11)	**0.08 (0.01, 0.15)***	**1.84 (1.42, 2.37)****
Clinical cut-off		0.09 (-0.02, 0.20)	0.09 (-0.01, 0.20)	-0.02 (-0.12, 0.09)	0.05 (-0.05,0.15)	**2.06 (1.39, 3.06)****

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI) from linear or logistic regression models, respectively. Maternal psychological distress is treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). The models were adjusted for [1]smoking during pregnancy, body mass index at enrolment and history of asthma or atopy, [2]maternal age, parity, education level and pet keeping, and child's sex and ethnicity, and [3]child's gestational age at birth, birthweight, breastfeeding and daycare attendance. *p-value <0.05, **p-value <0.01.

Supplementary Table S2.1.5. Associations of paternal psychological distress during pregnancy with lung function and asthma at age of 10 years adjusted for paternal psychological distress at 36 months after pregnancy.

	n	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Paternal psychological distress during pregnancy + at 36 mo						
Depressive symptoms	4.231					
Per 1-unit increase		-0.04 (-0.20, 0.12)	-0.07 (-0.22, 0.09)	0.04 (-0.09, 0.16)	0.01 (-0.15, 0.16)	1.04 (0.56, 1.93)
Clinical cut-off		-0.10 (-0.33, 0.14)	-0.10 (-0.33, 0.13)	-0.01 (-0.20, 0.17)	-0.06 (-0.25, 0.14)	1.07 (0.43, 2.65)
Anxiety symptoms	4,231					
Per 1-unit increase		-0.03 (-0.19, 0.13)	-0.06 (-0.21, 0.10)	0.03 (-0.10, 0.17)	-0.01 (-0.16, 0.14)	0.81 (0.43, 1.55)
Clinical cut-off		**-0.15 (-0.29, -0.00)***	**-0.14 (-0.29, -0.00)***	0.00 (-0.15, 0.15)	-0.07 (-0.22, 0.08)	0.94 (0.48, 1.84)

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI) from linear or logistic regression models, respectively. Maternal psychological distress are treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). The models were adjusted for maternal age, parity, education level, smoking during pregnancy, body mass index at enrolment, history of asthma or atopy and pet keeping, and child's sex, gestational age at birth, birthweight, ethnicity, breastfeeding and daycare attendance, and paternal psychological distress 36 months after pregnancy. At 36 months after pregnancy, not all subscales were measured, and therefor overall psychological distress could not be included at this time point. * p-value <0.05

Supplementary Table S2.1.6. Percentage change for associations of paternal psychological distress with lung function and asthma at age 10 years, adjusted for paternal psychological distress at 36 months after pregnancy.

	FEV_1 Z-score (95% CI) n = 3,757	FVC Z-score (95% CI) n = 3,757	FEV_1/FVC Z-score (95% CI) n = 3,757	FEF_{75} Z-score (95% CI) n = 3,757	Current asthma OR (95% CI) n = 3,640
Main model + paternal psychological distress at 36 mo					
Depressive symptoms					
Per 1-unit increase	9.8 (-366.8, 374.0)	-62.1 (-703.5, 713.1)	52.4 (-508.9, 537.9)	-179.3 (-579.4, 633.6)	-73.0 (-586.6, 619.1)
Clinical cut-off	6.0 (-156.0, 182.5)	7.3 (-158.3, 176.0)	-34.1 (-242.0, 260.7)	-20.0 (-270.3, 258.9)	-55.2 (-316.3, 306.5)
Anxiety symptoms					
Per 1-unit increase	201.4 (-581.6, 611.3)	84.2 (-706.2, 619.0)	29.3 (-628.5, 604.8)	35.4 (-635.3, 619.8)	193.1 (-846.1, 758.8)
Clinical cut-off	9.6 (-31.3, 93.5)	10.0 (-28.3, 102.1)	-2.9 (-320.1, 315.8)	9.8 (-231.8, 227.7)	-381.9 (-425.0, 371.1)

Values are percentage change (95% CI) between the main model, and the model additionally adjusted for paternal psychological distress at 36 months after pregnancy. Paternal psychological distress is treated as continuous variables (per 1-unit increase) or dichotomous variables based on clinical cut-offs (no; yes, where 'no' was the reference category). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 95% of FVC (FEF_{75}). The main models were adjusted for maternal age, parity, education level, smoking during pregnancy, body mass index at enrolment, history of asthma or atopy and pet keeping, and child's sex, gestational age at birth, birthweight, ethnicity, breastfeeding and daycare attendance. Additionally, models were adjusted for paternal psychological distress at 36 months after pregnancy. At 36 months after pregnancy, not all subscales were measured, and therefor overall psychological distress could not be included at this time point.

Chapter 2.2

Duration and exclusiveness of breastfeeding and school-age lung function and asthma

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ABSTRACT

Background

Breastfeeding reduces the risk of asthma in early childhood, but it is not clear whether its effect on respiratory morbidity is still present in later childhood.

Objective

We examined the associations of any breastfeeding, breastfeeding duration and exclusiveness with lung function and asthma in school-aged children, and whether associations were influenced by respiratory tract infections and maternal or child's atopic status.

Methods

This study among 4,464 children was embedded in a population-based prospective cohort study. Information on breastfeeding was obtained by multiple questionnaires from birth until age 1 year. At age 10 years, lung function was measured by spirometry, and information on asthma was obtained by questionnaire. Adjusted linear and logistic regression models were used to examine the associations.

Results

Shorter duration of breastfeeding was associated with a lower Forced Expiratory Volume in 1 second (FEV_1) only (Z-score change (95% CI): -0.01 (-0.02, -0.00) per month shorter breastfeeding), but not asthma. When categorized, breastfeeding for 2-4 months was associated with a lower Forced Vital Capacity (FVC) (Z-score change (95% CI): -0.11 (-0.20, -0.03), compared with breastfeeding for ≥6 months. Non-exclusive breastfeeding for 4 months was associated with a lower FVC (Z-score change (95% CI) -0.08 (-0.16, -0.01), compared with exclusive breastfeeding for 4 months. Results did not materially change after additional adjustment for lower respiratory tract infections, and were not modified by maternal history of asthma or atopy, child's eczema or inhalant allergic sensitization.

Conclusion

Shorter duration and non-exclusivity of breastfeeding were associated with a lower FEV_1 and FVC, but not asthma, at school-age.

INTRODUCTION

Early life exposures may affect the development and maturation of the respiratory system in early childhood and influence later respiratory health [1]. Our [2 3] and other previous studies [4-10], suggest that prolonged and exclusive breastfeeding reduces the risk of wheezing and asthma in infancy and early childhood, whereas the beneficial effect of breastfeeding on lung function [11-18] and asthma in later childhood remains unclear [19]. In a recent meta-analysis [8] of 42 observational studies, ever and prolonged breastfeeding were associated with an up to 12% and 10% reduced risk of asthma at age 5-18 years, respectively, while exclusive breastfeeding for longer than 3-4 months was not. A systematic review of 5 birth cohorts and 3 cross-sectional studies [20] suggested that prolonged and exclusive breastfeeding improved lung function in children until age 18 years. However, categorization of breastfeeding duration and exclusiveness differed between studies, effects could not be examined in detail, and heterogeneity was large. More recent studies [16 17 21] were performed mostly in high risk populations [16 21] with relatively small sample sizes. Not only are population-based studies on the effect of breastfeeding on lung function and asthma in later childhood scarce, most studies lack prospectively collected, detailed information on breastfeeding. Additionally, it has been shown that breastfeeding is associated with a reduced prevalence of lower respiratory tract infections[22 23]. Subsequently, it is suggested that a reduced prevalence of respiratory tract infections may result in less lung damage and a better lung function and less respiratory morbidity later in life[24]. This proposes a possible mediating role for lower respiratory tract infections in the association of breastfeeding with lung function and asthma. Previously, we showed that shorter and less exclusive breastfeeding was associated with an increased risk of early wheezing or asthma at age 6 years, which was partly explained by respiratory tract infections in early and to a lesser extent in later life[2]. The effect of respiratory tract infections on associations of breastfeeding with lung function and asthma at older ages are less known. Lastly, the role of maternal and child's atopic status on the association of breastfeeding with lung function and asthma is not fully clear[10 11 20].

Therefore, we examined in a population-based prospective cohort study, with detailed information on breastfeeding and frequently measured over time, the associations of any breastfeeding, breastfeeding duration and exclusiveness of breastfeeding with lung function and asthma in school-age children. Additionally, we examined if associations were mediated by lower respiratory tract infections or modified by maternal or child's atopic status.

METHODS

Design and cohort

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life until young adulthood in Rotterdam, the Netherlands. A detailed description

of the study design has been published previously [25]. The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Centre in Rotterdam (MEC-2012-165). Written informed consent was obtained from parents or legal representatives of all participants. Children were excluded for the current analyses if the parents or legal representatives did not give consent for participation in this phase, if they are twins, if information on breastfeeding was missing and if information on both lung function and asthma were missing. As a result, a total of 4,464 mothers and their children were included for the current analyses.

Breastfeeding duration and exclusiveness

Data on breastfeeding was collected using questionnaires administered at 2, 6, and 12 months after birth. Response rates varied between 71-82%. The duration of breastfeeding was assessed by asking whether mothers ever breastfed their child and at what age they stopped breastfeeding. Children were classified as 'never breastfed' and 'ever breastfed'. Among those who were breastfed, duration of breastfeeding was categorized into four groups: '<2 months', '2-4 months', '4-6 months', and '≥6 months'. Breastfeeding duration was also measured continuously by using the number of months a child was breastfed as a continuous variable. Exclusivity of breastfeeding was defined by the age of introduction of infant formula, other drinks, or food, and categorized into 'non-exclusive breastfeeding for 4 months' and 'exclusive breastfeeding for 4 months'. Analyses that focused on breastfeeding duration and exclusiveness were performed among children that were ever breastfed.

School-age lung function and asthma

At age 10 years (mean 9.8, SD 0.3), children visited our research center. Spirometry was performed according to the American Thoracic Society and European Respiratory Society recommendations [26]. Lung function measures included Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC), FEV_1/FVC and Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}), and were converted into sex-, height-, age-, and ethnicity-adjusted z-scores according to the Global Lung Initiative reference data [27]. Children were asked to stop the use of short- and/or long acting bronchodilators 8 to 48 hours before spirometry, respectively, if they did not suffer from asthma symptoms. A current acute asthma attack or respiratory tract infection were contra-indications for spirometry. Questionnaires based on the International Study on Asthma and Allergy in Childhood (ISAAC) Questionnaire [28] at age 10 years provided information on ever asthma (no; yes) and current wheezing (no; yes), and information on asthma medication use (no; yes) was obtained during the visit at the research center. Current asthma (no; yes) was defined as ever diagnosis of asthma with either wheezing or medication use in the past 12 months at age 10 years.

Covariates

Information on maternal characteristics included educational level, history of asthma or atopy, pet keeping, damp patches or mold in the house, parity, psychiatric symptoms defined using the

Global Severity Index (GSI)[29 30], body mass index (BMI) at intake, and smoking during pregnancy, and were obtained from multiple questionnaires completed by the mother during pregnancy. Midwife and hospital registries at birth provided information on child's sex, gestational age at birth, and birth weight. We collected information about child's ethnicity and daycare attendance by questionnaires during the first year of life. Information on physician-attended lower respiratory tract infections was obtained by multiple questionnaires at age 6 months to 6 years. Lower respiratory tract infections included bronchitis, bronchiolitis and pneumonia and were categorized into early (≤3 years) and late (3-6 years) lower respiratory tract infections. At age 10 years, information on ever diagnosis of eczema was obtained by questionnaire. Allergic sensitization for the five most common inhalant allergens (house dust mite, grass, birch, cat and dog; ALK-Abelló B.V., Almere, The Netherlands) was determined by a skin prick test at the age of 10 years, using the 'scanned area method' [31].

Statistical analysis

First, for the loss to follow-up analysis, we compared characteristics of children included and not included in the study using T-tests for normal distributed continuous variables, Mann-Whitney tests for non-normal distributed continuous variables, and Chi-square tests for categorical variables. Second, we used linear and logistic regression models to examine the associations of ever breastfeeding and duration and exclusiveness of breastfeeding with lung function and asthma, respectively. Analyses were adjusted for potential confounders, which were 1) selected from literature, or 2) if they were related to breastfeeding and asthma or lung function, or 3) if the effect estimate of the unadjusted analyses changed ≥10% when we additionally adjusted for a confounder. Third, we examined if the associations of breastfeeding with lung function or asthma were explained by lower respiratory tract infections by additionally adjusting for these variables in a mediation analysis. We examined effect modification by maternal history of asthma or atopy, and child's eczema or inhalant allergic sensitization by testing their interactive effect. We did not apply multiple testing due to strong correlations between different breastfeeding and lung function measures, which could potentially lead to overadjustment and, as an effect, possible false negative findings. Missing data for covariates were <20% except for day care attendance (25.6%). Missing data in covariates were imputed using the multiple imputation method using chained equations to select the most likely value for a missing response. Ten new datasets were created by imputation. All analyses were performed with both the original data (complete case analysis) and with imputed missing data. No major differences in the magnitude or direction of the effect estimates were observed between analyses with imputed missing data and complete cases only. We only present the results based on imputed datasets. All measures of association are presented with their 95% Confidence Intervals (95% CI). Statistical analyses were performed using SPSS version 21.0 for Windows software (SPSS Inc).

RESULTS

Subject characteristics

Maternal and child characteristics are presented in Table 2.2.1. Of the 4,464 included children, 7.4% (n=331) were never breastfed. Of the children who were ever breastfed, 26.6% (n=865) were breastfed for <2 months, 23.6% (n=767) for 2-4 months, 13.7% (n=446) for 4-6 months and 36.1% (n=1,175) for ≥6 months. 71.0% (n=2,370) of the children were non-exclusively breastfed for 4 months. Mean FEV_1 (SD) was 2.02 L (0.30), FVC 2.34 L (0.36), FEV_1/FVC 86.64% (5.66) and FEF_{75} 1.14 L/s (0.35) at the age of 10 years. The prevalence of current asthma at age 10 years was 5.6% (n=221). Supplementary Table S2.2.1 shows the same characteristics for children that were ever breastfed versus never breastfed separately. Children not included in the analysis had mothers who had a lower education, a higher prevalence of multiparity and smoking during pregnancy, a higher psychiatric symptom score and a higher BMI at intake. Children were born younger, had a lower birth weight, were less often from European ethnicity, and had a lower prevalence of day care attendance (Supplementary Table S2.2.2).

Breastfeeding and lung function or asthma

Ever breastfeeding was not associated with lung function or asthma, when compared with never breastfeeding (Table 2.2.2). When measured continuously, shorter duration of breastfeeding was associated with a lower FEV_1 (Z-score change (95% CI): -0.01 (-0.02, -0.00) per month shorter breastfeeding). When breastfeeding was categorized, results showed that only children who were breastfed for 2-4 months had a lower FVC (-0.11 (-0.20, -0.03)), compared with children that were breastfed for ≥6 months. Children who were not exclusively breastfed for 4 months had a lower FVC (-0.08 (-0.16, -0.01)), compared with children who were exclusively breastfed for 4 months. Breastfeeding duration and exclusiveness were not associated with FEV_1/FVC, FEF_{75}, or current asthma at age 10 years. We observed small changes in effect estimates between the crude and adjusted model (range change: Z-score change 0.00 to 0.06; OR 0.00 to 0.32).

Additional adjustment for early and late lower respiratory tract infections did not materially change the effect estimates of the associations of any breastfeeding, breastfeeding duration and exclusiveness with lung function and current asthma at age 10 years (Table 2.2.3). This suggests that there is no mediating effect of lower respiratory tract infections in the association of breastfeeding with lung function and asthma. Associations were not modified by maternal atopic status reflected by maternal history of asthma or atopy (p-values ranging from 0.118 to 0.986 when added to the model with confounders). Additionally, associations were not consistently modified by child's atopic status reflected by child's eczema or inhalant allergic sensitization (p-values for interaction 0.014, 0.019 and 0.034, and other 57 ranging from 0.064 to 0.986, when added to model with confounders).

Table 2.2.1. Characteristics of children and their mothers

	n = 4,464
Maternal characteristics	
Education, lower (%)	44.8 (1,999)
History of asthma or atopy, yes (%)	37.5 (1,673)
Pet keeping, yes (%)	36.4 (1,627)
Damp patches or mold in house, yes (%)	11.3 (504)
Parity, nullipara (%)	57.4 (2,562)
Psychiatric symptoms during pregnancy (GSI)[1]	0.15 (0.00-0.87)
Body mass index at intake (kg/m^2)	24.44 (4.19)
Smoking during pregnancy, yes (%)	22.8 (1,019)
Children's characteristics	
Female sex (%)	50.8 (2,268)
Gestational age at birth (weeks)[1]	40.1 (37.1-42.1)
Birth weight (grams)	3,459 (541)
Ethnicity, European (%)	71.9 (3,211)
Day care attendance 1st year, yes (%)	59.6 (2,660)
Early lower respiratory tract infections, yes (%)	25.6 (1,143)
Late lower respiratory tract infections, yes (%)	10.2 (457)
Ever eczema at age 10 years, yes (%)	23.4 (1,043)
Inhalant allergic sensitization at age 10 years, yes (%)	34.3 (1,531)
Never breastfeeding (%)	7.4 (331)
Breastfeeding duration (%)	
<2 months	26.6 (865)
2-4 months	23.6 (767)
4-6 months	13.7 (446)
≥6 months	36.1 (1,175)
Non-exclusive breastfeeding for 4 months (%)	71.0 (2,370)
FEV_1 (L)	2.02 (0.30)
FVC (L)	2.34 (0.36)
FEV_1/FVC (%)	86.64 (5.66)
FEF_{75} (L/s)	1.14 (0.35)
Current asthma at age 10 years, yes (%)	5.6 (221)

Values are means (SD), valid percentages (absolute numbers) or [1]medians (95% range), based on imputed data. Data on breastfeeding duration (n=1,211), breastfeeding exclusiveness (n=1,126), Forced Expiratory Flow in 1 second (FEV_1) (n=470), Forced Vital Capacity (FVC) (n=466), FEV_1/FVC ratio (n=470), Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}) (n=467) and current asthma (n=543) was missing, and not imputed.

Table 2.2.2. Associations of breastfeeding with lung function and asthma at age 10 years

		FEV_1 Z-score change (95% CI) n = 3,994	**FVC Z-score change (95% CI) n = 3,998**	**FEV_1/FVC Z-score change (95% CI) n = 3,994**	**FEF_{75} Z-score change (95% CI) n = 3,997**	**Current asthma OR (95% CI) n = 3,921**
Ever	**n = 4,464**					
No	n = 331	-0.06 (-0.18, 0.05)	-0.09 (-0.20, 0.02)	0.05 (-0.06, 0.17)	0.01 (-0.10, 0.12)	0.96 (0.56, 1.63)
Yes	n = 4,133	Reference	Reference	Reference	Reference	Reference
Duration	**n = 3,253**					
Per month shorter breastfeeding	n = 3,253	**-0.01 (-0.02, -0.00)***	-0.01 (-0.02, 0.00)	-0.00 (-0.01, 0.01)	-0.00 (-0.01, 0.01)	1.02 (0.97, 1.07)
<2 months	n = 865	-0.08 (-0.17, 0.01)	-0.06 (-0.14, 0.03)	-0.03 (-0.13, 0.06)	-0.05 (-0.14, 0.04)	1.21 (0.79, 1.86)
2-4 months	n = 767	-0.08 (-0.17, 0.01)	**-0.11 (-0.20, -0.03)***	0.06 (-0.03, 0.15)	0.00 (-0.09, 0.09)	0.98 (0.62, 1.55)
4-6 months	n = 446	-0.09 (-0.20, 0.02)	-0.07 (-0.18, 0.03)	-0.04 (-0.15, 0.07)	-0.05 (-0.16, 0.05)	0.75 (0.41, 1.38)
≥6 months	n = 1,175	Reference	Reference	Reference	Reference	Reference
Exclusivity	**n = 3,338**					
Non-exclusive 4 months	n = 2,370	-0.06 (-0.14, 0.01)	**-0.08 (-0.16, -0.01)***	0.04 (-0.04, 0.12)	-0.01 (-0.08, 0.07)	0.99 (0.69, 1.43)
Exclusive 4 months	n = 968	Reference	Reference	Reference	Reference	Reference

Values are change in Z-scores or odds ratios (OR) with 95% confidence interval (95% CI), derived from linear or logistic regression models, respectively. * p-value <0.05. Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}). Models were adjusted for maternal educational level, history of asthma or atopy, pet keeping, damp patches or mold in the house, parity, psychiatric symptoms, BMI at intake and smoking during pregnancy and child's sex, gestational age at birth, birthweight, ethnicity and day care attendance. Analyses that focused on breastfeeding duration and exclusiveness were performed among children that were ever breastfed.

Table 2.2.3. Associations of breastfeeding with lung function and asthma at age 10 years, additionally adjusted for lower respiratory tract infections.

		FEV_1 Z-score change (95% CI) n = 3,994	FVC Z-score change (95% CI) n = 3,998	FEV_1/FVC Z-score change (95% CI) n = 3,994	FEF_{75} Z-score change (95% CI) n = 3,997	Current asthma OR (95% CI) n = 3,921
Ever	**n = 4,464**					
No	n = 331	-0.05 (-0.17, 0.06)	-0.08 (-0.19, 0.03)	0.07 (-0.05, 0.18)	0.02 (-0.09, 0.13)	0.74 (0.42, 1.32)
Yes	n = 4,133	Reference	Reference	Reference	Reference	Reference
Duration	**n = 3,253**					
Per month shorter breastfeeding	n = 3,253	-0.01 (-0.02, 0.00)	-0.01 (-0.02, 0.00)	-0.00 (-0.01, 0.01)	-0.00 (-0.01, 0.01)	1.00 (0.95, 1.05)
<2 months	n = 865	-0.06 (-0.15, 0.03)	-0.05 (-0.14, 0.04)	-0.02 (-0.11, 0.07)	-0.04 (-0.12, 0.05)	1.04 (0.66, 1.65)
2-4 months	n = 767	-0.07 (-0.16, 0.02)	**-0.11 (-0.20, -0.02)***	0.07 (-0.02, 0.16)	0.01 (-0.08, 0.10)	0.89 (0.55, 1.44)
4-6 months	n = 446	-0.09 (-0.20, 0.02)	-0.07 (-0.18, 0.03)	-0.04 (-0.15, 0.07)	-0.05 (-0.16, 0.05)	0.78 (0.42, 1.48)
≥6 months	n = 1,175	Reference	Reference	Reference	Reference	Reference
Exclusivity	**n = 3,338**					
Non-exclusive 4 months	n = 2,370	-0.05 (-0.13, 0.02)	**-0.08 (-0.15, -0.01)***	0.05 (-0.03, 0.12)	0.00 (-0.07, 0.08)	0.88 (0.60, 1.30)
Exclusive 4 months	n = 968	Reference	Reference	Reference	Reference	Reference

Values are change in Z-scores or odds ratios (OR) with 95% confidence interval (95% CI), derived from linear or logistic regression models, respectively. * p-value <0.05. Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}). Models were adjusted for maternal educational level, history of asthma or atopy, pet keeping, damp patches or mold in the house, parity, psychiatric symptoms, BMI at intake and smoking during pregnancy and child's sex, gestational age at birth, birthweight, ethnicity, day care attendance, and early and late lower respiratory tract infections. Analyses that focused on breastfeeding duration and exclusiveness were performed among children that were ever breastfed.

DISCUSSION

We observed in a large population-based prospective cohort study that a shorter duration of breastfeeding was associated with a lower FEV_1 and FVC, and non-exclusive breastfeeding with a lower FVC in school-aged children. The z-score changes relate to a mean decrease in lung function of circa 3 to 40 mL or a 0.1 to 1.7% change. Associations were not explained by lower respiratory tract infections, or modified by maternal history of asthma or atopy, child's eczema or inhalant allergic sensitization. Breastfeeding duration and exclusiveness were not associated with other lung function outcomes or asthma.

Comparison with previous studies

A meta-analysis of 117 observational studies showed that prolonged and exclusive breastfeeding was associated with a decreased risk of asthma in early childhood but not consistently in later childhood [19]. Results of our previous [2 3] and current studies comprising detailed breastfeeding categorization suggest that the protective effect of breastfeeding on asthma in early childhood does not prolong into school-age. A possible explanation is a diminishing effect of breastfeeding over time due to other factors such as environmental stimuli that exceed the protective effect of breastfeeding on respiratory morbidity in later childhood [32]. Additionally, asthma at a later age could more likely be atopic asthma [5]. Since we found no interactive effect of atopy, this might explain the difference between the effects on asthma and lung function. Some studies even observed an increased asthma risk at school-age in prolonged and exclusive breastfed children [33 34]. However, we found that shorter duration of breastfeeding and less exclusive breastfeeding were associated with lower FEV_1 or FVC [12-14 18] at school-age. Results of our population based cohort study are consistent with the conclusions of a recent systematic review [20], where the strongest association was found for duration of breastfeeding for more than 4 months with an up to 40 ml increase in FEV_1 and an up to 100 ml increase in FVC. Exclusive breastfeeding for more than 4 months was not associated with FEV_1, but showed beneficial effects on Peak Expiratory Flow rates (PEF). However, later studies performed in high risk populations failed to find an association between prolonged and exclusive breastfeeding with FEV_1, FVC and other lung function measurements such as Forced Expiratory Flow after exhaling 50% (FEF_{50}) and between 25 and 75% (FEF_{25-75}) of the FVC, probably due to the relatively small sample sizes [16 21]. One study found an association of exclusive breastfeeding for 6 months with FEV_1/FVC, in asthmatic children only [21]. Studies among adults up to 79 years of age showed that shorter duration of breastfeeding was associated with a lower FEV_1 [35], while any breastfeeding was not associated with FEV_1 and FVC [36]. These results suggest that use of detailed breastfeeding measures is important, and that the effect of prolonged and exclusive breastfeeding on lung growth and airway obstruction might even persist into adulthood. More population-based cohort studies with detailed measurements of breastfeeding and respiratory morbidity at older ages are needed to fully disentangle the effect of breastfeeding on lung function and asthma across the life course.

Our mediation analysis showed that the observed associations were not explained by early or late lower respiratory tract infections, while previous studies suggested a potential mediating effect of lower respiratory tract infections [2 3 22] in early childhood. Possible explanations could be a misclassification of asthma at an early age (due to symptoms of lower respiratory tract infections or transient wheezing) or a diminishing effect over time of lower respiratory tract infections on lung function or asthma. We found no modifying effect of maternal and child's atopic status, which suggest no deleterious effect of breastfeeding by mothers with asthma or atopy. This is in line with most cohort studies [5 10 13 15 17 37], although some studies suggest that breastfeeding by asthmatic mothers is associated with an increased risk of asthma [6 14] and with a reduction in FEV_1/FVC and FEF_{25-75}/FVC in their children [14]. However, these deleterious associations were found in atopic children only [6] and in relatively small sample sizes [14], which limits the generalizability of the results. No previous study explored child's atopy as an effect modifier in the associations of breastfeeding with lung function, and our results are therefore difficult to compare.

Possible mechanisms

Various underlying mechanisms of the effect of breastfeeding on lung function outcomes or asthma have been proposed. Breast milk is immunologically complex, as it can be protective but could also possibly induce allergies [4]. Breastfeeding and its components, such as secretory IgA, cytokines, long chain fatty acids and oligosaccharides, might affect the developing immune system of the child. This could subsequently have a beneficial effect on development of asthma and allergy [4 22 38]. Breastfeeding might also alter the gut microbiota and promote gut maturation, which could mediate the development of asthma [22]. Also, breastfeeding might have a beneficial effect on lung development [38-40] by stimulating lung growth through cytokines and growth factors such as TGF-β1 and IGF-I in breast milk [38 39]. Although lower respiratory tract infections have been suggested as a mediating factor in the association of breastfeeding with lung function and asthma, we found no evidence for this hypothesis. Therefore other mechanisms might be more relevant. Additionally, new hypotheses suggest that breastfeeding has an impact on DNA methylation and thereby leads to differences in gene expression profiles [41 42]. An understanding of (epi) genetic factors may further clarify the underlying mechanisms of the effect of breastfeeding on respiratory morbidity.

Strengths and limitations

This study was embedded in a population-based prospective cohort study with a large number of participants and detailed information on breastfeeding, lung function and asthma. However, some limitations do apply to this study. First, selection bias towards a more affluent and healthy population might have occurred due to differences in characteristics between those lost to follow-up and included in the study [43]. Second, misclassification of breastfeeding categorization might have occurred, but since we expect this misclassification to be random, meaning irrespective of lung function or asthma, this would have only led to an underestimation of our results.

Third, information on current asthma and eczema was obtained by questionnaires. Validated questions were adapted from the ISAAC Core Questionnaires, which is considered a reliable instrument and sufficient for studying these outcomes in epidemiological studies[44]. However, misclassification due to self-report might be present. Also, with these questionnaires it is not possible to distinguish different phenotypes of asthma. However, no strong relationship between phenotypes and clinical patterns or response to treatment has been found previously, potentially limiting the usefulness of phenotype distinction[45]. Fourth, information on lower respiratory tract infections was also obtained by questionnaire. The possibility of misclassification and recall bias were reduced by using only physician-attended lower respiratory tract infections and by annual collection, respectively. Fifth, it was not possible to study any changes in the association of breastfeeding with lung function between the age of 6 and 10 years because different lung function tests, Rint and fractional exhaled nitric oxid (FeNO) versus spirometry, were applied. At the age of 6 years, children that were not breastfed had lower FeNO levels, most likely due to an increased prevalence of lower respiratory tract infections[2]. Last, although we adjusted for many essential confounders, residual confounding due to unmeasured or insufficient measured confounders might have influenced our results.

In conclusion, we demonstrated that shorter duration of breastfeeding was associated with a lower FEV_1 and FVC, and non-exclusive breastfeeding for 4 months with a lower FVC among school-age children. Results were not mediated by lower respiratory tract infections and not modified by maternal asthma or atopy, child's eczema or inhalant allergic sensitization. Breastfeeding duration and exclusiveness were not associated with asthma at school-age. Further studies are needed to examine the underlying mechanisms of breastfeeding that lead to respiratory changes and the effect of breastfeeding on lung function and asthma at older ages.

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Supplementary Table S2.2.1. Characteristics of children and their mothers for ever and never breastfeeding

	Ever breastfeeding n = 4,133	Never breastfeeding n = 331
Maternal characteristics		
Education, lower (%)	43.0 (1,779)	66.5 (220)
History of asthma or atopy, yes (%)	37.4 (1,544)	39.0 (129)
Pet keeping, yes (%)	35.7 (1,475)	46.2 (153)
Damp patches or mold in house, yes (%)	11.9 (490)	4.2 (14)
Parity, nullipara (%)	58.0 (2,397)	49.5 (164)
Psychiatric symptoms during pregnancy (GSI)[1]	0.15 (0.00-0.87)	0.19 (0.00-1.01)
Body mass index at intake (kg/m^2)	24.34 (4.08)	25.3 (5.25)
Smoking during pregnancy, yes (%)	21.9 (905)	34.4 (114)
Children's characteristics		
Female sex (%)	50.9 (2,105)	49.2 (163)
Gestational age at birth (weeks)[1]	40.1 (37.1-42.1)	40.0 (37.1-42.0)
Birth weight (grams)	3,462 (539.7)	3,421 (557.0)
Ethnicity, European (%)	69.0 (2,851)	78.9 (261)
Day care attendance 1st year, yes (%)	60.5 (2,501)	48.0 (159)
Early lower respiratory tract infections, yes (%)	25.3 (1,044)	29.9 (99)
Late lower respiratory tract infections, yes (%)	9.8 (405)	15.7 (52)
Ever eczema at age 10 years, yes (%)	23.2 (957)	26.3 (87)
Inhalant allergic sensitization at age 10 years, yes (%)	34.5 (1,427)	31.4 (104)
FEV_1 (L)	2.02 (0.30)	1.97 (0.28)
FVC (L)	2.34 (0.36)	2.28 (0.33)
FEV_1/FVC (%)	86.62 (5.66)	86.80 (5.62)
FEF_{75} (L/s)	1.14 (0.35)	1.12 (0.34)
Current asthma at age 10 years, yes (%)	5.6 (204)	5.7 (17)

Values are means (SD), valid percentages (absolute numbers) or [1]medians (95% range), based on imputed data. Data on breastfeeding duration (n=1,211), breastfeeding exclusiveness (n=1,126), Forced Expiratory Flow in 1 second (FEV_1) (n=470), Forced Vital Capacity (FVC) (n=466), FEV_1/FVC ratio (n=470), Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}) (n=467) and current asthma (n=543) was missing, and not imputed.

Supplementary Table S2.2.2. Characteristics of children included and not included in the study.

	Included n = 4,464	Not included n = 5,437	Test for difference p-value
Maternal characteristics			
Education, primary or secondary (%)	43.4 (1,835)	70.2 (3,113)	< 0.001
History of asthma or atopy, yes (%)	37.5 (1,471)	36.4 (554)	N.S.
Pet keeping, yes (%)	34.1 (1,211)	32.4 (447)	N.S.
Damp patches or mould in house, yes (%)	10.0 (363)	11.5 (358)	0.043
Parity, nullipara (%)	58.1 (2,542)	52.4 (2,697)	< 0.001
Psychiatric symptoms during pregnancy (GSI)[1]	0.13 (0.00-0.85)	0.19 (0.00-1.17)	< 0.001
Body mass index at intake (kg/m^2)	24.42 (4.17)	25.31 (4.83)	< 0.001
Smoking during pregnancy, yes (%)	22.2 (887)	27.2 (420)	< 0.001
Children's characteristics			
Female sex (%)	50.8 (2,268)	48.1 (2,541)	0.008
Gestational age at birth (weeks)[1]	40.1 (37.1-42.1)	39.9 (35.9-42.0)	< 0.001
Birth weight (grams)	3,459 (541)	3,322 (616)	< 0.001
Ethnicity, European (%)	72.8 (3,209)	51.5 (2,476)	< 0.001
Day care attendance 1st year, yes (%)	63.2 (2,096)	53.4 (243)	< 0.001
Early lower respiratory tract infections, yes (%)	31.0 (793)	40.8 (137)	< 0.001
Late lower respiratory tract infections, yes (%)	8.6 (284)	18.8 (101)	< 0.001
Ever eczema at 10 years, yes (%)	22.6 (882)	23.5 (217)	N.S.
Inhalant allergic sensitization at 10 years, yes (%)	31.8 (1,044)	33.6 (297)	N.S.

Values are means (SD), valid percentages (absolute numbers) or [1]medians (5-95% range). Test for difference is chi-squared test for categorical values, t-test for continuous variables, and Mann Whitney test for non-normal distributed variables. N.S. (non-significant).

Chapter 3

Fetal and childhood infections

Chapter 3.1

A population-based prospective cohort study examining the influence of early-life respiratory tract infections on school-age lung function and asthma.

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ABSTRACT

Background

Early-life respiratory tract infections could affect airway obstruction and increase asthma risk in later life. However, results from previous studies are inconsistent.

Objective

We examined the associations of early life respiratory tract infections with lung function and asthma in school-aged children.

Methods

This study among 5,197 children born between April 2002 and January 2006 was embedded in a population-based prospective cohort study. Information on physician-attended upper and lower respiratory tract infections until age 6 years (categorized into ≤3 and >3-6 years) was obtained by annual questionnaires. Spirometry measures and physician-diagnosed asthma were assessed at age 10 years.

Results

Upper respiratory tract infections were not associated with adverse respiratory outcomes. Compared with children without lower respiratory tract infections ≤3 years, children with lower respiratory tract infections ≤3 years had a lower forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC and forced expiratory flow at 75% of FVC (FEF_{75}) (Z-score (95% CI): ranging from -0.22 (-0.31, -0.12) to -0.12 (-0.21, -0.03)), and an increased risk of asthma (odds ratio (95% CI): 1.79 (1.19, 2.59)). Children with lower respiratory tract infections >3-6 years had an increased risk of asthma (3.53 (2.37, 5.17)) only. Results were not mediated by antibiotic or paracetamol use, and not modified by inhalant allergic sensitization. Cross-lagged modeling showed that results were not bidirectional and independent of preschool wheezing patterns.

Conclusion

Early-life lower respiratory tract infections ≤3 years are most consistently associated with lower lung function and increased risk of asthma in school-aged children.

INTRODUCTION

It has been hypothesized that respiratory tract infections in early life influence the risk of lower lung function[1-4] and asthma[5-10] in later childhood and adulthood. Respiratory tract infections could lead to higher airway sensitization leading to airway obstruction and hyperreactivity[11], and subsequently asthma. Respiratory tract infections might lead to persistent respiratory morbidity since both the immune and respiratory system are under development in early life. In observational studies, findings on the association of respiratory tract infections with lung function or asthma have been inconclusive[1-10]. Differences in results might be explained by power issues, ascertainment of respiratory tract infections, use of different lung function measurements, definitions of asthma, age of the outcome, and use of covariates. Most studies that examined associations of respiratory tract infections with asthma focused on children at high risk for atopy[5-7 9 10], which leads to limited external validity. Additionally, child's antibiotic[12-17] and paracetamol[17-19] use are suggested to be associated with increased risks of childhood asthma, and their effect on the associations of respiratory tract infections with lung function and asthma is unclear. Antibiotic or paracetamol use might have an effect on the risk of lower lung function and asthma, by influencing the microbiome or glutathione levels, respectively. However, any found association could also be the result of confounding by indication or reverse causality. Additionally, parents might change smoking behavior as a result of respiratory infections, which could subsequently influence the risk of asthma. Therefore, second-hand smoke exposure might act as a mediator in this association. Associations of respiratory tract infections with asthma seem different between subgroups of children with or without allergic sensitization, which suggests a modifying effect of allergy[6 7]. Last, it is unclear whether early respiratory tract infections lead to lower lung function and asthma or that lower lung function and asthma, represented by wheezing at preschool age, lead to vulnerability for respiratory tract infections.

Therefore, we examined in a population-based prospective cohort study the association of early-life respiratory tract infections with lung function and asthma in school-aged children, taking bidirectional associations and preschool wheezing into account. Next, we examined if these associations are explained by children's paracetamol and antibiotic use or modified by current inhalant allergic sensitization status, and if findings were specific for respiratory tract infections. This study adds to the current literature by examining the association of respiratory tract infections with lung function and asthma on a population-based level, using detailed and longitudinally measured information on both upper and lower respiratory tract infections, and respiratory outcomes measures, applying a unique approach to identify bidirectional associations, and taking the effect of infections in general into account. With this, we aim to provide new evidence for a possible direct effect of respiratory tract infections on lung function and development.

METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards in Rotterdam, The Netherlands. Women with a delivery data between April 2002 and January 2006 living in Rotterdam were eligible for participation in the study, as described previously[20]. The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands (MEC-2012-165). Written informed consent was obtained from parents or legal representatives of all participants. A total of 5,197 children were included for the current analyses.

Early life respiratory tract infections

Information on parental reported physician-attended upper and lower respiratory tract infections was obtained by questionnaires at the ages of 6 months and 1, 2, 3, 4 and 6 years. Response rates ranged from 71 to 80%. Questions about physician-attended upper respiratory tract infections included ear and throat infections, false croup and whooping cough, and about lower respiratory tract infections bronchitis, bronchiolitis and pneumonia in the past 6 or 12 months (no or yes, not physician-attended; yes, physician-attended). We combined each individual respiratory tract infection into groups of upper and lower respiratory tract infections. For the cross-lagged analyses, respiratory tract infections were first analyzed per year, and to improve readability subsequently combined into early (≤3 years: at least 1 infection in this period vs. no infections or late infections only) and late (3-6 years: at least 1 infection in this period vs. no infections or early infections only) upper and lower respiratory tract infections. The cut-off point of 3 years was chosen to obtain sufficient power, based on end of highest velocity of maturation of lungs and the immune system until this age[21-23], and for consistency with categorization of wheezing patterns.

School-age lung function and asthma

At age 10 years, Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC), FEV_1/FVC and Forced Expiratory Flow after expiring 75% of FVC (FEF_{75}) were measured by spirometry (MasterScreen-Pneumo, Jaeger Toennies (Viasys) CareFusion Netherlands). All curves were scored by two trained researchers according to the ERS/ATS guidelines[24] and when necessary discussed with a senior researcher. Reproducible curves were converted into sex-, age-, height- and ethnicity-adjusted z-scores[25]. Current asthma was defined as 1) ever diagnosis of physician-diagnosed asthma with 2) either wheezing or any asthma medication use in the past 12 months. Questions on asthma and wheezing were adapted from the International Study on Asthma and Allergy in Childhood (ISAAC), and information on medication use was obtained at the research center. Wheezing was reported by annual parental questionnaires from birth to age 4 years, and at age 6 years. We combined preschool wheezing patterns into early (≤3 years) and late (>3-6 years) wheezing, similar to groups of respiratory tract infections.

Covariates

Information on maternal characteristics included educational level, body mass index at intake, parity, smoking during pregnancy, psychiatric symptoms, pet keeping, and history of asthma and atopy, and were obtained from multiple questionnaires during pregnancy. Mode of delivery and child's sex, gestational age at birth, and birth weight were obtained from midwife and hospital records at birth. Information on country of birth, breastfeeding, day care attendance, environmental tobacco smoke exposure, antibiotic and paracetamol use were obtained by multiple questionnaires at age 6 months to 2 years. Ethnicity was based on country of birth of both parents. Inhalant allergic sensitization at the age of 10 years was measured by skin prick test using the 'scanned area method'[26].

Other infections

Information on infections other than respiratory tract infections were collected by similar questionnaires as respiratory tract infections and included physician-diagnosed gastro-enteritis and urinary tract infection (no or yes, not physician-attended; yes physician-attended). Similarly to respiratory tract infections, other infections were combined into early (≤3 years) and late (3-6 years) infections.

Statistical

analysis First, we examined associations of respiratory tract infections with lung function measures and asthma using linear and logistic regression models, respectively. Analyses were adjusted for potential confounders, which were selected from literature[8 9 27-29], if they were related to respiratory tract infections and the outcomes of interest, or if the effect estimate of the unadjusted analyses changed ≥10% when we additionally adjusted for a confounder. Missing data of covariates were imputed by the multiple imputation method using chained equations to select the most likely value for a missing response. Ten new datasets were created by imputation. Additionally, to take correlations between respiratory tract infections into account, both any and individual upper respiratory tract infections were adjusted for any preceding upper respiratory tract infections, and any and individual lower respiratory tract infections for any preceding lower respiratory tract infections. We did not apply multiple testing due to strong correlations between the different infections and lung function measures, since that could potentially lead to false negative findings. Second, we examined if associations were mediated by children's antibiotic or paracetamol use or environmental tobacco smoke exposure by additionally adjusting for these variables, or were modified by inhalant allergic sensitization by testing their interactive effects. Statistical analyses were performed with the Statistical Package of Social Sciences version 21.0 for Windows (SPSS Inc). Third, we applied a cross-lagged model using Mplus version 7.11 for Windows (Muthén & Muthén). With a cross-lagged model, bidirectional associations between the exposure, respiratory tract infections until age 5 years, and the outcome, wheezing until age 5 years, and lung function or asthma at age 10 years, can be studied within the same model. In this model, logistic

or linear regression models are used to study the associations of early respiratory tract infections with early and late wheezing and lung function or asthma, of late respiratory tract infections with late wheezing and lung function or asthma, and of early wheezing with late respiratory tract infections, while taking associations over time between respiratory tract infections, and wheezing and lung function or asthma into account[30 31]. These models enable us to disentangle the direction of the observed associations, such as whether respiratory tract infections influence the risk of lower lung function and asthma, or vice versa. Last, sensitivity analyses were performed to examine the associations of gastro-enteritis and urinary tract infection with lung function and asthma. These sensitivity analyses could prove insight to whether any associations of respiratory tract infections with lung function and asthma are due to a specific respiratory tract infection effect, or due to a general infection status. All measures of association are presented with their 95% Confidence Intervals (95% CI).

RESULTS

Subject characteristics

Maternal and child characteristics are shown in Table 3.1.1. The highest prevalence of upper and lower respiratory tract infections was at the age of 2 years (24.0% and 8.5%, Figure 3.1.1A and B, respectively). At age 10 years, mean FEV_1 (SD) was 2.02 L (0.30), FVC 2.33 L (0.36), FEV_1/FCV 86.6% (5.7) and FEF_{75} 1.14 L/s (0.35) (Table 3.1.1). Current asthma was present in 5.5% of the children. Current asthma was defined as ever asthma (9.4%), combined with wheezing (4.4%) or medication use (17.4%) in the past 12 months. Those not included in the analysis had, among others, mothers who were lower educated and had a higher prevalence of smoking during pregnancy. These children were more often born younger, had a lower birth weight, and a lower prevalence of breastfeeding (Supplementary Table S3.1.1).

Respiratory tract infections and lung function or asthma

Results from models on crude associations of upper and lower respiratory tract infections with lung function and asthma are shown in Supplementary Table S3.1.2 Results of per year analyses showed that upper respiratory tract infections were not consistently associated with lung function measures or asthma (Table 3.1.2 and Supplementary Table S3.1.2). Lower respiratory tract infections at ages 6 months, 1, 2, 3 and 6 years were associated with lower FEV_1, FVC, FEV_1/FVC or FEF_{75}, with Z-score differences (95% CI) ranging from -0.19 (-0.32, -0.05) to -0.34 (-0.50, -0.18) (Table 3.1.2). Lower respiratory tract infections at ages 2 to 6 years were associated with an increased risk of current asthma at 10 years, with odds ratios (95% CI) ranging from 4.19 (2.65, 6.64) to 13.45 (7.22, 25.05). Associations of individual upper and lower respiratory tract infections with lung function and current asthma are given in Supplementary Table S3.1.3 and S3.1.4. After adjustment for antibiotic and paracetamol use, and environmental tobacco smoke exposure,

Table 3.1.1. Characteristics of children and their mothers

	n = 5,197
Maternal characteristics	
Education, higher (%)	51.6 (2,685)
Body mass index at intake (kg/m^2)	24.6 (4.25)
Parity, nullipara (%)	56.6 (2,942)
Smoking during pregnancy, yes (%)	14.0 (729)
Psychiatric symptoms during pregnancy (GSI)[1]	0.17 (0.06,0.38)
Pet keeping, yes (%)	38.1 (1,982)
History of asthma or atopy, yes (%)	37.2 (1,936)
Mode of delivery, caesarian section (%)	13.6 (709)
Children's characteristics	
Female sex (%)	50.5 (2,626)
Gestational age at birth (weeks)[1]	40.0 (39.1, 41.0)
Preterm birth <37 weeks (%)	4.3 (223)
Birth weight (grams)	3,444 (550)
Breastfeeding never (%)	8.1 (419)
Day care attendance 1st year, yes (%)	58.2 (3,025)
Environmental tobacco smoke exposure first 2 years, yes (%)	19.3 (1,005)
Use of antibiotics 1st year, yes (%)	63.0 (3,278)
Use of paracetamol 1st year, yes (%)	87.8 (4,569)
Inhalant allergic sensitization at age 10 years, yes (%)	31.8 (1.172)
FEV_1 (L)	2.02 (0.30)
FEV_1 (Z-score)	0.17 (0.97)
FVC (L)	2.33 (0.36)
FVC (Z-score)	0.21 (0.93)
FEV/FEV (%)	86.6 (5.7)
FEV_1/FVC (Z-score)	-0.11 (0.95)
FEF_{75} (L/s)	1.14 (0.35)
FEF_{75} (Z-score)	0.00 (0.91)
Current asthma, yes (%)	5.5 (252)
Early wheezing, yes (%)	59.5 (1,828)
Late wheezing, yes (%)	20.0 (690)

Values are means (SD), valid percentages (absolute numbers) or [1]medians (25-75% range) based on imputed data. Global Severity Index (GSI). Data on Forced Vital Capacity (FVC) (available n=4,672), Forced Expiratory Flow in 1 second (FEV_1) (n=4,672), FEV1/FVC ratio (n=4,672), Forced Expiratory Flow when 75% of the FVC is exhaled (FEF_{75}) (n=4,672), current asthma (n=4,556) and early (n=3,074) and late (n=3,443) wheezing was not imputed.

the strength and direction of the effect estimates did not materially change (data not shown). We observed no consistent interactive effect of upper and lower respiratory tract infections and inhalant allergic sensitization for the associations with lung function and asthma, (p-values>0.05).

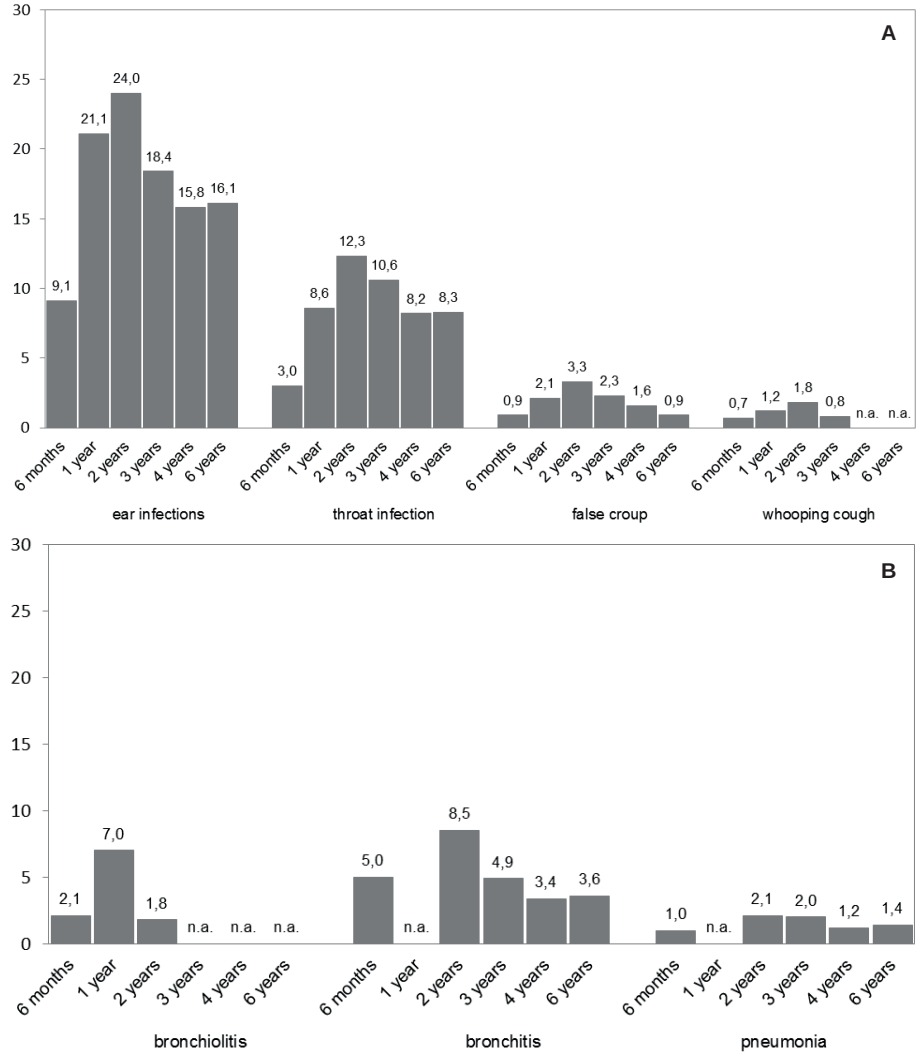

Figure 3.1.1. Prevalence of upper (A) and lower (B) respiratory tract infections.
Values represent % of specific upper and lower respiratory tract infections per age, and were not imputed. Not available – no data on this specific infection at this timepoint (n.a.)

Cross-lagged modelling

Cross-lagged modelling showed no association of upper respiratory tract infections ≤3 years with lung function measures (Figure 3.1.2A, B and C) or asthma (Figure 3.1.2D). Compared with no upper respiratory tract infections >3-6 years, upper respiratory tract infections >3-6 years were associated with a higher FEV_1/FVC and FEF_{75} (0.08 (0.02, 0.15) and 0.10 (0.04, 0.17), respectively, but not FEV_1. Upper respiratory tract infections >3-6 years were associated with a decreased risk of current asthma (0.69 (0.49, 0.97)) (Figure 3.1.2D). Lower respiratory tract infections ≤3 years

Table 3.1.2. Associations of any upper and lower respiratory tract infections with lung function and asthma at age 10 years

		FEV_1 Z-score (95% CI) n = 4,672	FVC Z-score (95% CI) n = 4,672	FEV_1/FVC Z-score (95% CI) n = 4,672	FEF_{75} Z-score (95% CI) n = 4,672	Current asthma OR (95% CI) n = 4,556
Any URTI						
Age 6 months	n = 2,930	-0.02 (-0.13, 0.10)	-0.04 (-0.15, 0.07)	0.02 (-0.09, 0.14)	0.00 (-0.11, 0.11)	1.46 (0.93, 2.28)
Age 1 year	n = 3,601	-0.03 (-0.12, 0.07)	-0.02 (-0.11, 0.07)	-0.02 (-0.11, 0.08)	-0.03 (-0.12, 0.05)	1.42 (0.96, 2.12)
Age 2 years	n = 3,768	0.06 (-0.03, 0.16)	0.04 (-0.05, 0.12)	0.04 (-0.05, 0.13)	0.05 (-0.04, 0.14)	**1.56 (1.04, 2.34)***
Age 3 years	n = 3,645	0.03 (-0.08, 0.13)	0.06 (-0.04, 0.16)	-0.07 (-0.18, 0.03)	-0.05 (-0.15, 0.05)	1.04 (0.65, 1.66)
Age 4 years	n = 3,658	0.01 (-0.11, 0.14)	-0.02 (-0.13, 0.10)	0.06 (-0.06, 0.18)	0.07 (-0.05, 0.18)	0.84 (0.48, 1.46)
Age 6 years	n = 4,681	0.03 (-0.09, 0.16)	0.02 (-0.10, 0.13)	0.03 (-0.09, 0.15)	0.03 (-0.09, 0.15)	1.01 (0.57, 1.79)
Any LRTI						
Age 6 months	n = 2,996	**-0.21 (-0.35, -0.07)****	-0.12 (-0.26, 0.01)	-0.12 (-0.26, 0.02)	-0.11 (-0.24, 0.02)	1.66 (0.97, 2.82)
Age 1 year	n = 3,623	**-0.34 (-0.50, -0.18)****	**-0.25 (-0.40, -0.10)****	-0.14 (-0.29, 0.02)	**-0.25 (-0.40, -0.10)****	1.27 (0.86, 2.87)
Age 2 years	n = 3,828	**-0.19 (-0.32, -0.05)****	-0.03 (-0.16, 0.10)	**-0.25 (-0.38, -0.12)****	**-0.28 (-0.40, -0.15)****	**4.19 (2.65, 6.64)****
Age 3 years	n = 3,678	**-0.21 (-0.41, -0.01)***	-0.07 (-0.26, 0.11)	**-0.22 (-0.41, -0.03)***	-0.16 (-0.34, 0.03)	**4.15 (2.33, 7.42)****
Age 4 years	n = 3,649	-0.08 (-0.32, 0.16)	0.03 (-0.20, 0.25)	-0.17 (-0.41, 0.07)	-0.13 (-0.35, 0.10)	**7.80 (4.13, 14.71)****
Age 6 years	n = 4,649	0.08 (-0.15, 0.30)	**0.23 (0.02, 0.45)***	**-0.27 (-0.49, -0.05)***	-0.15 (-0.36, 0.07)	**13.45 (7.22, 25.05)****

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI), derived from linear or binomial logistic regression models. * p-value <0.05, ** p-value <0.01. Models are adjusted for maternal education, body mass index, parity, smoking during pregnancy, psychiatric symptoms during pregnancy, pet keeping, history of asthma or atopy, mode of delivery, and child's sex, gestational age at birth, birth weight corrected for gestational age at birth, breastfeeding and day care attendance. Additionally, upper respiratory tract infections were adjusted for any preceding upper respiratory tract infections, and lower respiratory tract infections for any preceding lower respiratory tract infections. Upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI), Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow when 75% of the FVC is exhaled (FEF_{75}).

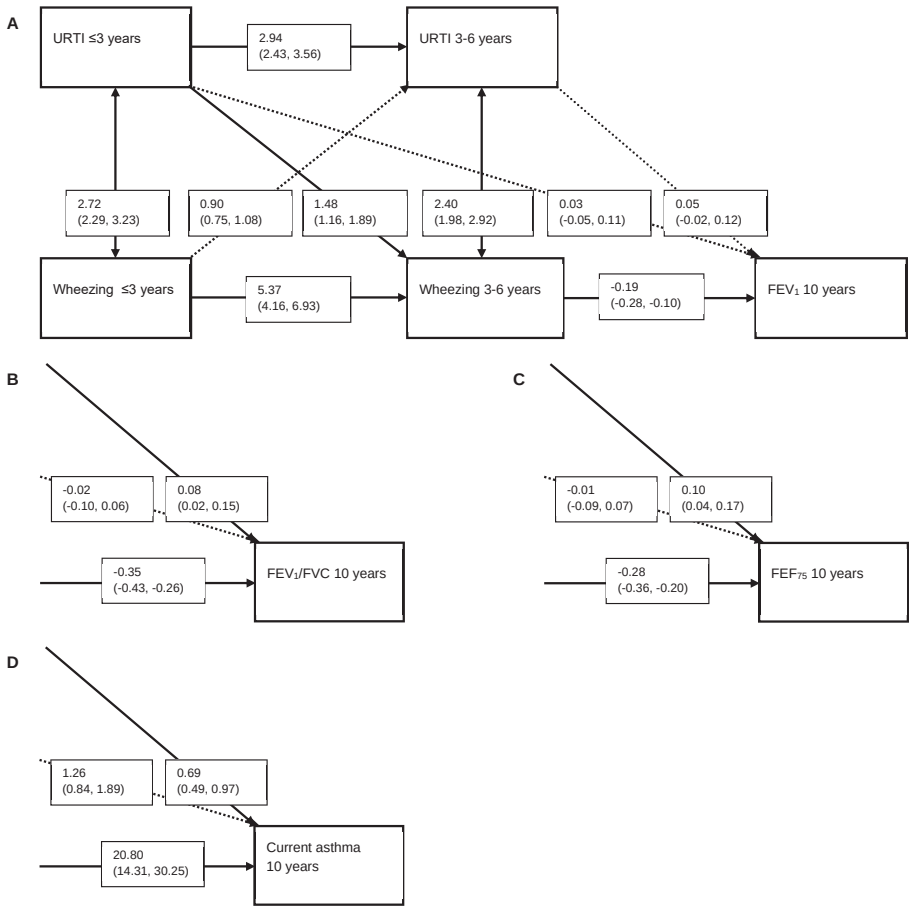

Figure 3.1.2. Direction of associations of upper respiratory tract infections (URTI) with wheezing and FEV_1 (A), FEV_1/FVC (B) FEF_{75} (C) and current asthma (D) at age 10 years. Values are odds ratios (OR) or change in Z-scores with their corresponding 95% confidence interval (95% CI) derived from binomial logistic or linear regression models, respectively, using cross-lagged modeling which takes bidirectional associations into account. Models are adjusted for maternal education, body mass index, parity, smoking during pregnancy, psychiatric symptoms during pregnancy, pet keeping, history of asthma or atopy, mode of delivery, and child's sex, gestational age at birth, birth weight corrected for gestational age at birth, breastfeeding and day care attendance. Arrows indicate the direction of the associations and if they are significant (bold) or non-significant (dashed). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow when 75% of the FVC is exhaled. For Figure 3.1.2B, C and D only the right lower quadrant of the figure is presented. All other directions and effect estimates of the associations were approximately the same as presented in Figure 3.1.2A.

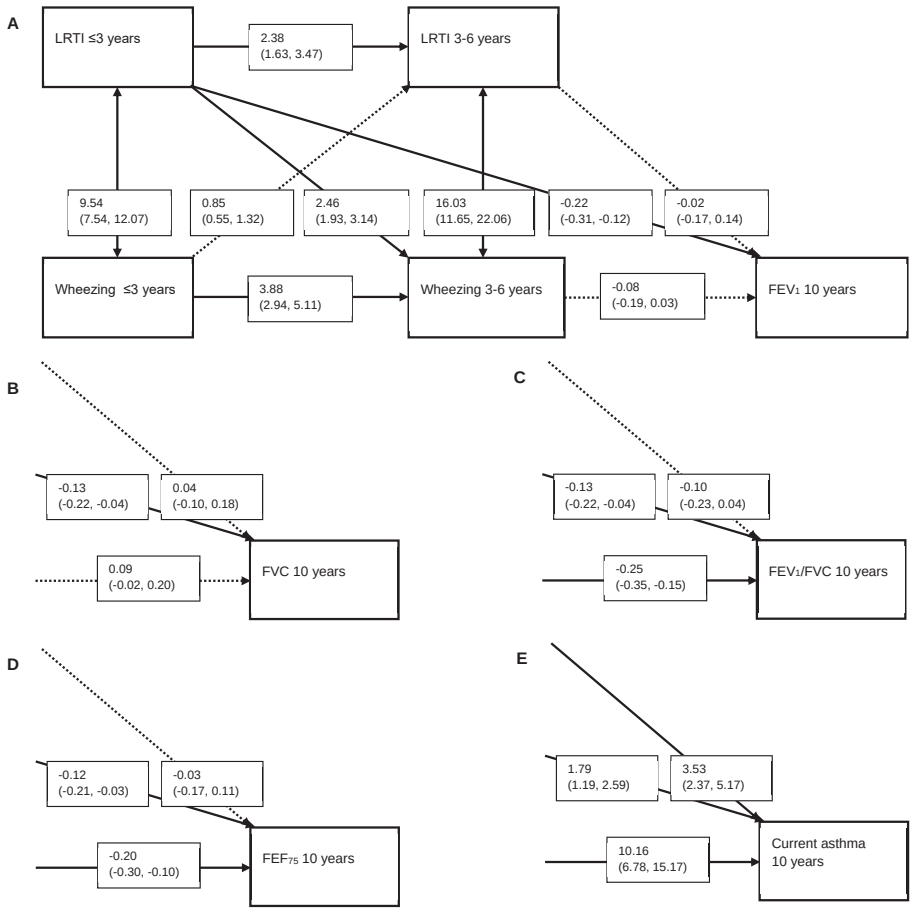

Figure 3.1.3. Direction of associations of lower respiratory tract infections (LRTI) with wheezing patterns and FEV_1 (A), FVC (B), FEV_1/FVC (C), FEF_{75} (D) and current asthma (E) at age 10 years. Values are odds ratios (OR) or Z-scores with their corresponding 95% confidence interval (95% CI) derived from binomial logistic or linear regression models, respectively, using cross-lagged modeling which takes bidirectional associations into account. Models are also adjusted for maternal education, body mass index, parity, smoking during pregnancy, psychiatric symptoms during pregnancy, pet keeping, history of asthma or atopy, mode of delivery, and child's sex, gestational age at birth, birth weight corrected for gestational age at birth, breastfeeding and day care attendance. Arrows indicate the direction of the associations and if they are significant (bold) or non-significant (dashed). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow when 75% of the FVC is exhaled. For Figure 3.1.3B, C, D and E, only the right lower quadrant of the figure is presented. All other directions and effect estimates of the associations were approximately the same as presented in Figure 3.1.3A.

were associated with lower FEV_1, FVC, FEV_1/FVC and FEF_{75} (range -0.12 (-0.21, -0.03) to -0.22 (-0.31, -0.12) (Figure 3.1.3A, B, C and D). Lower respiratory tract infections ≤3 and >3-6 years were also associated with an increased risk of current asthma (1.79 (1.19, 2.59) and 3.53 (2.37, 5.17), respectively) (Figure 3.1.3E). Lower respiratory tract infections >3-6 years were not associated with any lung function measurements. The directions from preschool wheezing patterns to later upper and lower respiratory tract infections were not significant.

Other infections

Cross-lagged modelling showed that gastro-enteritis was not associated with lung function measures (data not shown). Gastro-enteritis ≤3 years, but not >3-6 years, was associated with an increased risk of current asthma (1.68 (1.13, 2.47)) (Supplementary Figure 3.1.1SA). Urinary tract infections ≤3 year were associated with a higher FEV_1 (0.18 (0.00, 0.35), but not with current asthma (Supplementary Figure S3.1.1B).

DISCUSSION

We observed in large population-based prospective cohort study that lower respiratory tract infections ≤3 years were most strongly and consistently associated with lower lung function and an increased risk of current asthma at school-age. These associations were not mediated by use of antibiotics or paracetamol and environmental tobacco smoke exposure, and not modified by inhalant allergic sensitization. Additionally, we observed that these associations were not bidirectional, independent of preschool wheezing patterns, and not due to a general infection status. Upper respiratory tract infections at any age were not associated with lower lung function, and only upper respiratory tract infections at the age of 2 years were associated with an increased risk of asthma.

Comparison with previous studies

Previous prospective cohort studies observed inconclusive associations of respiratory tract infections with lung function or asthma, both in children and in adulthood[1-10]. Some studies that used viral sampling found that specific viral triggers, such as respiratory syncytial virus[6-9] or rhinovirus[6 9] were associated with an 2.1 to 3.9-fold increased risk of wheeze or 2.6 to 9.8-fold increased risk of asthma in children aged 5 to 10 years. However, other studies showed that not a specific viral trigger, but the number of infections was associated with an increased risk of asthma, current wheeze or bronchial hyperreactivity at age 7 years[5 10]. Two previous studies among children age 5[6] and 10 years[7] showed that associations of specific lower respiratory tract infections with asthma and wheeze were only present in children who were sensitized before the age of 2 years. Others showed no modifying effect of sensitization for the associations of respiratory syncytial virus, rhinovirus infections or both with the risk of asthma at age 6 years[9]. Some differences could be

explained by differences in the size of study groups[4-7 9]. Also, most studies examined children at high risk for atopy, which reduces generalizability[4-7 9 10]. Most studies were focused on respiratory tract infections in either the first year or the first 3 years of life[4-7 9 10 32]. Our findings are consistent with previous findings, and now show that results are applicable to a general population, are not mediated by use of antibiotics or paracetamol and environmental tobacco smoke exposure, and not modified by inhalant allergic sensitization. Our sensitivity analysis for non-respiratory tract infections showed an association of gastro-enteritis ≤3 years with asthma only. Since urinary tract infections at any age were not associated with any adverse respiratory outcome, the effect of gastro-enteritis might be specific, and we speculate that this could be mediated by the intestinal microbiome [33]. Another possibility however is that infections in general could have an effect on the immune system, and thereby influence the risk of asthma.

When we took possible bidirectional associations into account, cross-lagged modeling showed that lower respiratory tract infections ≤3 years were associated with lower lung function and current asthma, and lower respiratory tract infections at 3-6 years only with current asthma. By using cross-lagged models, we confirmed the direction of the association of respiratory tract infections with lung function and asthma, and not vice versa. These findings are supported by a randomized clinical trial, which observed that vaccination for respiratory syncytial virus infections led to a reduction of the number of wheezing days in the first year of life, suggesting causality from viral infection to wheeze[34].

We observed that lower respiratory tract infections, specifically bronchitis, bronchiolitis and pneumonia, were associated with lower lung function and an increased risk of asthma. Previous studies suggested that childhood respiratory tract infections influence lung function at school age and in adulthood[1-3 35 36]. In adults up to 70 years of age, early life bronchitis[3 36] and pneumonia[1-3 36] were associated with a lower FEV_1, FVC and, in lesser extent, a lower FEV_1/FVC. Together with the present finding, these findings suggest that exposure to respiratory tract infections in early life might affect respiratory health during the life course.

Potential mechanisms

Lung development starts in utero, and continues during childhood[21 37], similar to the development of the immune system[22 37 38]. In early life, the developing respiratory and immune system could be affected by lower respiratory tract infections leading to persistent adverse adaptations and subsequently lower lung function and an increased risk of asthma. Non-specific immune responses mediated by epithelial cells and phagocytes, and adaptive immune responses mediated by T cells, could contribute to airway inflammation in response to viral triggers[38]. Early airway infections could lead to changes in endothelial cell physiology, such as increased vascular permeability and hence, bronchial wall edema[39 40]. Because of the immaturity of the respiratory and immune systems at a young age, these processes with potential persistent consequences are more likely to occur when lower respiratory tract infections occur early in life. Our findings are in line with this hypothesis because we observed that mainly lower respiratory tract infections ≤3 years were

associated with lower lung function and increased risk of asthma. The effect of upper respiratory tract infections on the lungs seems to be less distinct, most probably because upper respiratory tract infections do not affect the lungs directly.

The immune system is also important for the resolution of airway inflammation after infections and a less developed immune system could potentially lead to persistent changes of airway physiology and function after infections[39]. To date, the immunological pathways responsible for the persistence of structural and functional abnormalities after respiratory tract infections have not yet been identified. The immune response to infections and the risk of developing lower lung function and asthma could both be dependent on common factors, such as the microbiome[41] or (epi)genetic factors[39], and need to be examined in future studies.

Strengths and limitations

This study was embedded in a population-based, prospective cohort study with a large number of participants, and detailed and longitudinally measured information on both upper and lower respiratory tract infections, and respiratory outcomes measures. Furthermore, we applied a unique method to identify bidirectional associations using cross-lagged modeling. Since the study was not limited to a high-risk population, the findings are applicable to the general population. However, some limitations apply to our study. First, selection bias towards a more affluent and healthy population might have been present. Also, we used multiple imputation to reduce potential bias due to missing data in covariates. By using this method we assumed that data in covariates was missing at random. However, it might be possible that some data was missing not at random, which may have led to bias [42]. Second, information on respiratory tract infections was parental based information, collected with questionnaires, which might have led to recall or misclassification bias. If so, we expect this possible misclassification to be random because information on infections was collected before the outcome was known, and would most probably have led to an underestimation of our observed effect estimates. Our study lacked viral or bacteriological sampling at the time of symptoms of respiratory tract infections, although it is suggested that not the specific microbial trigger but respiratory tract infections in general are important for the risk of asthma[5]. Third, information on asthma and wheezing was obtained by questionnaires. Although these questionnaires were adapted from ISAAC[43], which was validated in various age groups and is considered as a reliable measure in epidemiological studies, misclassification due to self-report might still have been present. Prevalences of early and late wheezing in our study slightly differ from previous birth cohort studies[44] due to the definition used for optimal cross-lagged modeling. Fourth, we did not have lung function measurements before the occurrence of respiratory tract infections. Therefore, we cannot distinguish whether the lower lung function is a result of the respiratory tract infections only, or whether it was already present before the infections occurred[45]. It has been suggested however, that lower respiratory tract infections impair lung function independent of lung function at younger age[46]. We partly addressed this issue by our statistical effort using cross-lagged modeling. Fifth, inhalant allergic sensitization was measured

at the age of 10 years. Early measurements or longitudinal patterns of allergic sensitization might be needed to better examine the intermediating role of allergy. Last, even though we corrected for numerous confounders, residual confounding due to unmeasured confounders, for example genetic susceptibility[47] or maternal infections during pregnancy[48], could have affected our results.

In conclusion, our results suggest that lower respiratory tract infections ≤3 years are most strongly and consistently associated with lower lung function and increased risk of asthma at school-age, while lower respiratory tract infections at 3-6 years were associated with asthma only. Upper respiratory tract infections were not associated with lower lung function or an increased risk of asthma, except at the age of 2 years with asthma. Cross-lagged modeling showed that observed associations were not bidirectional, independent of wheezing in early life and most likely not due to infections in general. Therefore, our findings support the hypothesis that early-life respiratory tract infections might have a direct effect on lung development and the risk of asthma. Further studies are needed to explore the possible underlying immunological and pathophysiological pathways.

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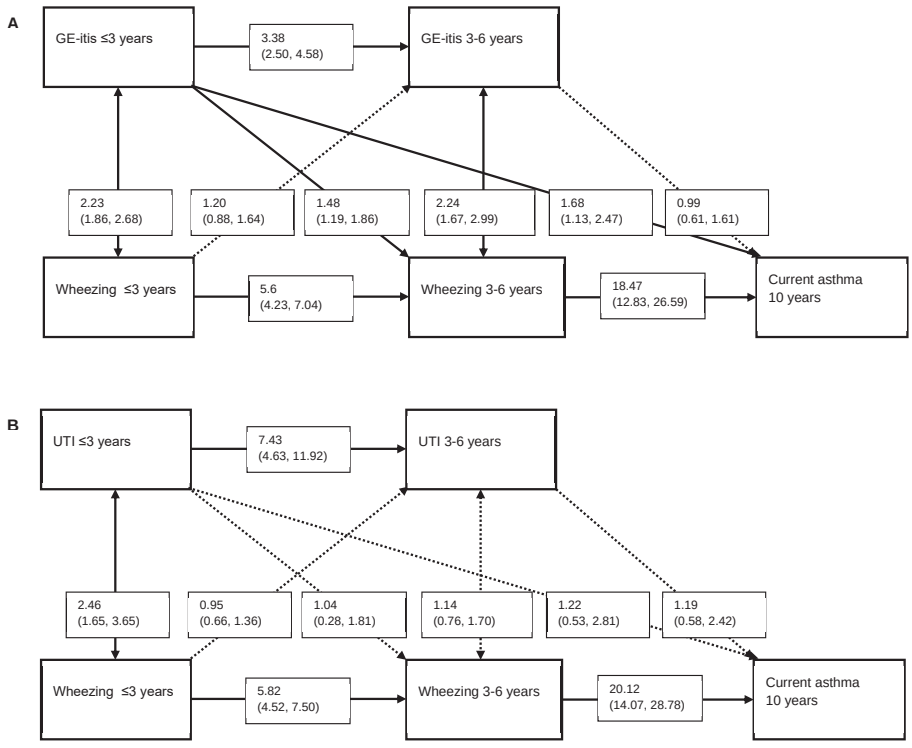

Supplementary Figure S3.1.1. Direction of associations of gastro-enteritis (A) and urinary tract infections (B) with wheezing patterns and current asthma, at age 10 years. Values are odds ratios (OR) with their corresponding 95% confidence interval (95% CI) derived from binomial logistic regression models, using cross-lagged modeling which takes bidirectional associations into account. Models are also adjusted for maternal education, body mass index, parity, smoking during pregnancy, psychiatric symptoms during pregnancy, pet keeping, history of asthma or atopy, mode of delivery, and child's sex, gestational age at birth, birth weight corrected for gestational age at birth, breastfeeding and day care attendance. Arrows indicate the direction of the associations and if they are significant (bold) or non-significant (dashed). Gastro-enteritis (GE-itis), urinary tract infection (UTI).

Supplementary Table S3.1.1. Characteristics of those included and not included in the study.

	Included subjects	Not included subjects	Test for difference
	n = 5,197	**n = 4,704**	**p-value**
Maternal characteristics			
Education, higher (%)	53.2 (2,603)	39.4 (368)	0.000
Body mass index at intake (kg/m^2)	24.5 (4.2)	25.3 (4.9)	0.000
Parity, nullipara	56.4 (2,935)	48.4 (554)	0.000
Smoking during pregnancy, yes (%)	13.7 (637)	20.5 (184)	0.000
Psychiatric symptoms during pregnancy (GSI)[1]	0.15 (0.00, 0.88)	0.19 (0.00, 1.30)	0.000
Pet keeping, yes (%)	34.4 (1,425)	29.4 (233)	0.006
History of asthma or atopy, yes (%)	37.1 (1,694)	37.4 (331)	N.S.
Mode of delivery, caesarian section (%)	12.9 (602)	13.0 (494)	N.S.
Children's characteristics			
Female sex (%)	50.5 (2,626)	48.0 (2,183)	0.015
Gestational age at birth (weeks)[1]	40.1 (37.0, 42.0)	39.9 (35.9, 42.0)	0.000
Birth weight (grams)	3,445 (549)	3,316 (620)	0.000
Breastfeeding ever, no (%)	7.3 (319)	9.9 (69)	0.015
Day care attendance 1st year, yes (%)	62.9 (2,123)	53.9 (216)	0.000

Values are means (SD), valid percentages (absolute numbers) or [1]medians (5-95% range). Differences between groups were evaluated using t-tests for continuous variables, chi-square tests for categorical variables or Mann-Whitney U tests for non-normal distributed variables. Non-significant (N.S.)

Supplementary Table S3.1.2. Unadjusted associations of any upper and lower respiratory tract infections with lung function and asthma at age 10 years

		FEV_1 Z-score (95% CI) n = 4,672	FVC Z-score (95% CI) n = 4,672	FEV_1/FVC Z-score (95% CI) n = 4,672	FEF_{75} Z-score (95% CI) n = 4,672	Current asthma OR (95% CI) n = 4,556
Any URTI						
Age 6 months	n = 2,930	-0.01 (-0.12, 0.10)	-0.03 (-0.14, 0.07)	0.04 (-0.08, 0.15)	0.02 (-0.08, 0.13)	**1.67 (1.08, 2.58)***
Age 1 year	n = 3,601	-0.05 (-0.12, 0.03)	-0.04 (-0.11, 0.03)	-0.01 (-0.08, 0.07)	-0.04 (-0.11, 0.03)	1.37 (0.99, 1.89)
Age 2 years	n = 3,768	0.01 (-0.06, 0.08)	-0.01 (-0.07, 0.06)	0.03 (-0.04, 0.10)	0.02 (-0.05, 0.09)	**1.87 (1.38, 2.53)****
Age 3 years	n = 3,645	-0.01 (-0.09, 0.07)	0.02 (-0.05, 0.09)	-0.06 (-0.13, 0.02)	-0.03 (-0.10, 0.04)	**1.49 (1.08, 2.06)***
Age 4 years	n = 3,658	-0.01 (-0.09, 0.07)	-0.01 (-0.08, 0.07)	-0.01 (-0.08, 0.07)	-0.00 (-0.08, 0.07)	1.20 (0.83, 1.73)
Age 6 years	n = 4,681	0.03 (-0.04, 0.10)	0.03 (-0.04, 0.10)	0.00 (-0.07, 0.07)	0.04 (-0.03, 0.11)	1.20 (0.87, 1.65)
Any LRTI						
Age 6 months	n = 2,996	**-0.21 (-0.35, -0.07)****	-0.13 (-0.26, 0.01)	-0.12 (-0.26, 0.02)	-0.11 (-0.24, 0.03)	**1.82 (1.09, 3.04)***
Age 1 year	n = 3,623	**-0.33 (-0.46, -0.20)****	**-0.24 (-0.37, -0.11)****	-0.12 (-0.25, 0.01)	**-0.21 (-0.34, -0.09)****	**1.99 (1.25, 3.17)****
Age 2 years	n = 3,828	**-0.21 (-0.30, -0.10)****	-0.08 (-0.18, 0.02)	**-0.18 (-0.28, -0.08)****	**-0.21 (-0.31, -0.12)****	**3.93 (2.80, 5.52)****
Age 3 years	n = 3,678	**-0.26 (-0.40, -0.13)***	-0.10 (-0.22, 0.03)	**-0.25 (-0.38, -0.12)****	**-0.23 (-0.36, -0.10)****	**6.22 (4.31, 8.98)****
Age 4 years	n = 3,649	**-0.21 (-0.37, -0.04)***	-0.04 (-0.20, 0.12)	**-0.25 (-0.42, -0.09)****	**-0.26 (-0.41, -0.10)****	**11.75 (7.84, 17.62)****
Age 6 years	n = 4,649	-0.07 (-0.21, 0.07)	0.11 (-0.02, 0.24)	**-0.30 (-0.43, -0.16)***	**-0.18 (-0.31, -0.05)****	**12.50 (8.85, 17.66)****

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI), derived from linear or binomial logistic regression models. * p-value <0.05, ** p-value <0.01. Models are unadjusted. Upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI), Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow when 75% of the FVC is exhaled (FEF_{75}).

Supplementary Table S3.1.3. Associations of individual upper respiratory tract infections with lung function and asthma at age 10 years

		FEV_1 Z-score (95% CI) n = 4,672	FVC Z-score (95% CI) n = 4,672	FEV_1/FVC Z-score (95% CI) n = 4,672	FEF_{75} Z-score (95% CI) n = 4,672	Current asthma OR (95% CI) n = 4,556
Age 6 months						
Ear infection	n = 2,958	-0.04 (-0.17, 0.09)	-0.07 (-0.19, 0.05)	0.04 (-0.09, 0.16)	0.01 (-0.11, 0.13)	1.25 (0.74, 2.09)
Throat infection	n = 2,963	-0.01 (-0.23, 0.21)	-0.03 (-0.24, 0.17)	0.01 (-0.21, 0.22)	-0.01 (-0.22, 0.19)	**2.19 (1.05, 4.53)***
False croup	n = 2,970	0.19 (-0.20, 0.58)	0.20 (-0.17, 0.56)	0.02 (-0.36, 0.40)	0.03 (-0.33, 0.40)	1.17 (0.27, 5.08)
Whooping cough	n = 2,967	-0.17 (-0.62, 0.27)	-0.04 (-0.46, 0.38)	-0.17 (-0.61, 0.27)	-0.11 (-0.53, 0.32)	0.58 (0.06, 5.68)
Age 1 years						
Ear infection	n = 3,573	-0.03 (-0.13, 0.07)	-0.04 (-0.14, 0.05)	0.01 (-0.08, 0.11)	-0.02 (-0.11, 0.08)	1.37 (0.90, 2.08)
Throat infection	n = 3,565	-0.04 (-0.18, 0.10)	0.05 (-0.08, 0.18)	-0.12 (-0.26, 0.02)	-0.11 (-0.24, 0.02)	**1.86 (1.09, 3.15)***
False croup	n = 3,582	-0.00 (-0.30, 0.30)	-0.08 (-0.37, 0.20)	0.14 (-0.16, 0.43)	0.02 (-0.27, 0.30)	1.39 (0.84, 2.30)
Whooping cough	n = 3,574	-0.44 (-0.92, 0.04)	-0.42 (-0.88, 0.03)	0.03 (-0.45, 0.50)	-0.22 (-0.67, 0.24)	1.28 (0.22, 7.56)
Age 2 years						
Ear infection	n = 3,756	0.08 (-0.02, 0.18)	0.04 (-0.05, 0.14)	0.07 (-0.03, 0.16)	0.09 (-0.01, 0.18)	1.25 (0.81, 1.93)
Throat infection	n = 3,774	0.05 (-0.08, 0.18)	0.02 (-0.10, 0.14)	0.05 (-0.07, 0.18)	0.03 (-0.09, 0.15)	1.34 (0.81, 2.23)
False croup	n = 3,777	0.01 (-0.19, 0.22)	0.06 (-0.14, 0.25)	-0.08 (-0.28, 0.12)	-0.04 (-0.23, 0.16)	1.69 (0.78, 3.68)
Whooping cough	n = 3,775	-0.11 (-0.45, 0.24)	-0.12 (-0.44, 0.21)	0.01 (-0.33, 0.35)	-0.11 (-0.43, 0.22)	2.06 (0.59, 7.22)
Age 3 years						
Ear infection	n = 3,622	0.03 (-0.09, 0.15)	0.05 (-0.06, 0.16)	-0.06 (-0.17, 0.06)	-0.06 (-0.17, 0.05)	1.07 (0.65, 1.78)
Throat infection	n = 3,616	0.10 (-0.05, 0.26)	0.12 (-0.02, 0.27)	-0.04 (-0.19, 0.12)	0.01 (-0.13, 0.16)	1.17 (0.63, 2.17)
False croup	n = 3,608	-0.15 (-0.43, 0.14)	0.01 (-0.26, 0.28)	**-0.28 (-0.56, -0.00)***	-0.13 (-0.40, 0.14)	0.64 (0.15, 2.80)
Whooping cough	n = 3,612	0.12 (-0.56, 0.79)	0.21 (-0.43, 0.85)	-0.19 (-0.85, 0.48)	-0.27 (-0.91, 0.37)	n.a.

Supplementary Table S3.1.3. Associations of individual upper respiratory tract infections with lung function and asthma at age 10 years (continued)

		FEV_1 Z-score (95% CI) n = 4,672	FVC Z-score (95% CI) n = 4,672	FEV_1/FVC Z-score (95% CI) n = 4,672	FEF_{75} Z-score (95% CI) n = 4,672	Current asthma OR (95% CI) n = 4,556
Age 4 years						
Ear infection	n = 3,599	0.05 (-0.09, 0.18)	0.01 (-0.12, 0.13)	0.08 (-0.05, 0.21)	0.07 (-0.05, 0.20)	1.20 (0.67, 2.13)
Throat infection	n = 3,601	-0.02 (-0.21, 0.17)	-0.03 (-0.21, 0.15)	0.02 (-0.16, 0.21)	0.07 (-0.12, 0.25)	0.51 (0.18, 1.47)
False croup	n = 3,576	-0.21 (-0.58, 0.17)	-0.32 (-0.67, 0.03)	0.20 (-0.17, 0.56)	0.09 (-0.26, 0.44)	n.a.
Age 6 years						
Ear infection	n = 4, 600	-0.01 (-0.14, 0.13)	-0.02 (-0.15, 0.10)	0.05 (-0.09, 0.18)	0.04 (-0.08, 0.17)	0.98 (0.53, 1.82)
Throat infection	n = 4,618	**0.26 (0.04, 0.47)***	**0.24 (0.05, 0.44)****	0.00 (-0.21, 0.21)	0.04 (-0.16, 0.24)	1.15 (0.50, 2.65)
False croup	n = 4,557	0.04 (-0.43, 0.51)	0.20 (-0.24, 0.64)	-0.29 (-0.75, 0.18)	-0.29 (-0.73, 0.16)	3.05 (0.89, 10.44)

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI), derived from linear or binomial logistic regression models. * p-value <0.05, ** p-value <0.01. Not available (n.a.). Models are adjusted for maternal education, body mass index, parity, smoking during pregnancy, psychiatric symptoms during pregnancy, pet keeping, history of asthma or atopy, mode of delivery, and child's sex, gestational age at birth, birth weight corrected for gestational age at birth, breastfeeding and day care attendance. Additionally, upper respiratory tract infections were adjusted for any preceding upper respiratory tract infections. Upper respiratory tract infections (URTI), Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow when 75% of the FVC is exhaled (FEF_{75}).

Supplementary Table S3.1.4. Associations of individual lower respiratory tract infections (LRTI) with lung function and asthma at age 10 years

		FEV_1 Z-score (95% CI) n = 4672	FVC Z-score (95% CI) n = 4,672	FEV_1/FVC Z-score (95% CI) n = 4,672	FEF_{75} Z-score (95% CI) n = 4,672	Current asthma OR (95% CI) n = 4,556
Age 6 months						
Bronchitis	n = 2,963	**-0.22 (-0.39, -0.05)***	-0.12 (-0.29, 0.04)	-0.15 (-0.32, 0.02)	-0.14 (-0.30, 0.03)	**1.91 (1.05, 3.48)***
Pneumonia	n = 2,961	-0.04 (-0.42, 0.33)	0.01 (-0.35, 0.36)	-0.07 (-0.43, 0.30)	-0.04 (-0.39, 0.31)	2.00 (0.57, 7.00)
Bronchiolitis	n = 2,959	-0.24 (-0.50, 0.01)	-0.22 (-0.46, 0.02)	-0.02 (-0.27, 0.23)	-0.10 (-0.34, 0.13)	1.23 (0.43, 3.52)
Age 1 year						
Bronchiolitis	n = 3,560	**-0.34 (-0.50, -0.18)****	**-0.25 (-0.40, -0.10)****	-0.14 (-0.29, 0.02)	**-0.25 (-0.40, -0.10)****	1.57 (0.86, 2.87)
Age 2 years						
Bronchitis	n = 3,777	**-0.18 (-0.33, -0.03)****	-0.00 (-0.15, 0.14)	**-0.28 (-0.42, -0.13)****	**-0.30 (-0.44, -0.16)****	**4.37 (2.68, 7.12)****
Pneumonia	n = 3,767	-0.26 (-0.55, 0.03)	**-0.32 (-0.59, -0.04)***	0.13 (-0.15, 0.42)	0.01 (-0.26, 0.29)	1.25 (0.37, 4.24)
Bronchiolitis	n = 3,776	-0.00 (-0.28, 0.28)	0.07 (-0.20, 0.33)	-0.13 (-0.40, 0.15)	-0.17 (-0.43, 0.09)	**2.63 (1.07, 6.46)***
Age 3 years						
Bronchitis	n = 3,615	-0.10 (-0.33, 0.13)	0.02 (-0.20, 0.23)	-0.17 (-0.39, 0.05)	-0.11 (-0.32, 0.11)	**4.91 (2.63, 9.15)****
Pneumonia	n = 3,620	**-0.33 (-0.64, -0.02)***	-0.18 (-0.48, 0.11)	-0.26 (-0.57, 0.04)	-0.25 (-0.55, 0.04)	**5.21 (2.33, 11.65)****
Age 4 years						
Bronchitis	n = 3,584	-0.15 (-0.42, 0.11)	-0.05 (-0.30, 0.19)	-0.16 (-0.42, 0.10)	-0.15 (-0.40, 0.09)	**9.20 (4.67, 18.11)****
Pneumonia	n = 3.579	0.27 (-0.23, 0.76)	0.37 (-0.09, 0.84)	-0.17 (-0.66, 0.32)	0.04 (-0.43, 0.51)	2.83 (0.73, 11.04)
Age 6 years						
Bronchitis	n = 4,592	0.12 (-0.13, 0.37)	0.26 (0.02, 0.49)	**-0.25 (-0.49, -0.00)***	-0.09 (-0.32, 0.15)	**13.34 (6.74, 26.42)****
Pneumonia	n = 4,568	0.18 (-0.22, 0.57)	**0.40 (0.03, 0.77)***	-0.36 (-0.74, 0.03)	-0.22 (-0.59, 0.15)	**10.55 (3.91, 28.44)****

Values are Z-scores or odds ratios (OR) with 95% confidence interval (95% CI), derived from linear or binomial logistic regression models. * p-value <0.05, ** p-value <0.01. Models are adjusted for maternal education, body mass index, parity, smoking during pregnancy, psychiatric symptoms during pregnancy, pet keeping, history of asthma or atopy, mode of delivery, and child's sex, gestational age at birth, birth weight corrected for gestational age at birth, breastfeeding and day care attendance. Additionally, lower respiratory tract infections were adjusted for any preceding lower respiratory tract infections. Lower respiratory tract infections (LRTI), Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow when 75% of the FVC is exhaled (FEF_{75}).

Chapter 3.2

Early-life respiratory tract infections and the risk of school-age lower lung function and asthma: a meta-analysis of 150,000 European children

van Meel ER, Mensink-Bout SM, den Dekker HT, Ahluwalia T, Anessi-Maesona A, Arshad SH, Baiz N, Barros H, von Berg A, Bisgaard H, Bønnelykke K, Carlsson CJ, Cases M, Chatzi L, Chevrier C, Dalmeijer G, Dezateux C, Duchen K, Eggesbø M, van der Ent C, Fantini M, Flexeder C, Frey U, Forastiere F, Gehring U, Gori D, Granell R, Griffiths LJ, Inskip H, Jerzynska K, Karvonen AM, Keil T, Kelleher C, Kogevinas M, Koppe G, Kuehni CE, Lambrechts N, Lau S, Lehmann I, Ludvigsson J, Magnus MC, Mélen E, Mehegan J, Mommers M, Nybo Andersen A, Nystad W, Pedersen ESL, Pekkanen J, Peltola V, Pike KC, Pizzi C, Polanska K, Popovic M, Porta D, Roberts G, Santos AC, Schultz ES, Smit J, Standl M, Sunyer J, Thijs C, Toivonen L, Uphoff E, Usemann J, Vafeidi M, Wright J, de Jongste JC, Jaddoe VWV, Duijts L.

Submitted.

Chapter 3.3

Chlamydia trachomatis infection during pregnancy and childhood asthma-related morbidity: a population-based prospective cohort study.

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ABSTRACT

Background

Chlamydia trachomatis is the most commonly reported sexually transmitted disease, and although infection during pregnancy is associated with neonatal complications, long-term respiratory consequences are unknown. We aimed to determine whether *Chlamydia trachomatis* infection during pregnancy is associated with asthma related symptoms across childhood

Methods

This study among 2,475 children and their mothers was embedded in a population-based prospective cohort study. Maternal urine samples were tested for *Chlamydia trachomatis* infection during pregnancy. Questionnaires provided information on childhood physician-attended lower respiratory tract infections and wheezing, and current asthma at age 10 years. Lung function was measured by spirometry at age 10 years.

Results

The prevalence of *Chlamydia trachomatis* infection during pregnancy was 3.2% (78/2,475). *Chlamydia trachomatis* infection during pregnancy was not associated with lower respiratory tract infections until age 6 years, but was associated with a higher odds of wheezing in children until age 10 years (odds ratio (OR) (95% confidence interval (CI)) 1.50 (1.10, 2.03)). *Chlamydia trachomatis* infection during pregnancy was associated with an increased odds of asthma (OR (95% CI): 2.29 (1.02, 5.13)), and with a lower FEV_1/FVC and FEF_{75} (Z-score difference (95% CI): -0.28 (-0.52, -0.04) and -0.24 (-0.46, -0.01), respectively) in children at age 10 years. The observed associations were only partly explained by mode of delivery, gestational age at birth or birth weight.

Conclusion

Chlamydia trachomatis infection during pregnancy is associated with increased odds of wheezing, asthma, and impaired lung function. The causality of the observed associations and potential underlying mechanisms need to be explored.

INTRODUCTION

Chlamydia trachomatis is the most commonly reported sexually transmitted disease in Western countries[1-3]. It is hypothesized that *Chlamydia trachomatis* during pregnancy could affect childhood respiratory health through direct or indirect effects. *Chlamydia trachomatis* during pregnancy could directly lead to placental inflammation, potentially leading to altered immune and atopic development, and persistent risks of respiratory morbidity of the child [4 5]. *Chlamydia trachomatis* infection during pregnancy is known to be associated with a 1.3 to 4-fold increased risk of neonatal complications such as perinatal mortality, preterm birth, low birth weight and pneumonia [4 6-10]. Preterm birth, low birth weight and neonatal respiratory disease are known to predispose to respiratory morbidity at later ages [11-13]. Therefore, *Chlamydia trachomatis* during pregnancy might also be associated with respiratory health in later life indirectly through these altered birth characteristics. Previous small sample-sized observational studies focused mostly on the association of *Chlamydia trachomatis* infection in children with their respiratory health, and suggested that an infection in infancy is associated with adverse respiratory health [14 15]. Only one prospective cohort study was performed, studying the association between *Chlamydia trachomatis* and asthma in a sub-analysis only, and reported that treatment for *Chlamydia trachomatis* during pregnancy was associated with a 3-fold increased risk of asthma in the offspring at age 7 years [16]. However, no studies have examined the association of *Chlamydia trachomatis* during pregnancy with respiratory health or with objective lung function measurements later in childhood.

We hypothesize that *Chlamydia trachomatis* during pregnancy leads to fetal respiratory and immunological developmental adaptations, predisposing children to long-term asthma related morbidity. We examined in a population-based prospective cohort study the associations of *Chlamydia trachomatis* infection during pregnancy with the odds of lower respiratory tract infections, wheezing, asthma and impaired lung function in children. We also examined whether any associations were explained by mode of delivery, gestational age at birth or birth weight.

METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, the Netherlands comprising 9,901 children and their mothers [17]. The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands (MEC-2012-165). Written informed consent was obtained from mothers, parents or legal representatives of all children. The *Chlamydia trachomatis* substudy was performed between February 2003 and January 2005, and comprised 4,055 mothers and children [6]. Non-singleton or non-live born children and children

without information on at least one of the respiratory outcomes were excluded, which left a total of 2,475 children and their mothers for the current analysis.

Chlamydia trachomatis infection during pregnancy

Women provided a first-void urine specimen to test for Chlamydia trachomatis at enrolment, as described previously [6]. In summary, urine samples were stored at 4°C, transported the same or following working day, and processed within 24 hours of receipt by the laboratory. DNA was isolated from five pooled urine specimens using the MagNA Pure LC Bacterial DNA isolation Kit III (Roche Molecular Systems, Alameda, USA) and amplified by polymerase chain reaction (PCR) (Cobas Amplicor, Roche Molecular Diagnostics, Branchburg, USA). Urines from positive pools were individually re-tested, and reported as negative or positive. Due to the observational nature of this study and the fact that screening for *Chlamydia trachomatis* during pregnancy was not part of standard care, results were not reported back to the women.

Asthma and asthma related morbidity in childhood

We obtained information on lower respiratory tract infections (physician-attended pneumonia, bronchitis or bronchiolitis) by annual questionnaires from birth to the age of 4 years, and at the age of 6 years. Information on wheezing was obtained at similar ages, and at age 10 years. Information on asthma medication use in the past 12 months was obtained during the visit at the research center (median age 9.8 years, 5-95% range 9.5-10.4 years). Asthma was defined as ever diagnosis of asthma, obtained by questionnaire at the age 10 years, with either wheezing or medication use in the past 12 months. All questions on wheezing and asthma were based on the International Study on Asthma and Allergy in Childhood (ISAAC) Questionnaire [18].

We performed spirometry according to the American Thoracic Society (ATS) and European Respiratory Society (ERS) recommendations during the visit at the research center at the age of 10 years. Children (n=154) with a >5% deviation in Forced Expiratory Volume in 1 second (FEV_1) or Forced Vital Capacity (FVC), but with at least one blow with adequate reach and duration of plateau according to ATS/ERS criteria were additionally included [19 20]. Lung function measures included FEV_1, FVC, FEV_1/FVC and Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}), and were converted into sex-, height-, age-, and ethnicity-adjusted z-scores according to the Global Lung Initiative reference data [21].

Covariates

Information on maternal age, educational level, risk behavior including ever treatment for sexually transmitted diseases and multiple sexual partners in the year before pregnancy, psychiatric symptoms, smoking during pregnancy, and alcohol use during pregnancy, was obtained by multiple questionnaires during pregnancy [17]. Midwife and hospital registries provided information on mode of delivery, child's sex, gestational age at birth, and birth weight [17]. Child's ethnicity was based on questionnaires during pregnancy. Questionnaires after birth provided information about child's breastfeeding and day care attendance.

Statistical analysis

First, we performed a loss-to-follow-up analysis by comparing characteristics of children included in our study and those lost to follow-up by using independent samples T-tests, Mann-Whitney U tests, and Pearson's Chi-square tests. Second, we used generalized estimating equation (GEE) models with an unstructured correlation matrix to examine the associations of C*hlamydia trachomatis* infection during pregnancy with the odds of lower respiratory tract infections and wheezing of the child until age 6 and 10 years, respectively. These models take the correlations between repeated measurements of either lower respiratory tract infections or wheezing within the same subject into account. Both an unstructured and first order autoregressive correlation structure was tested, and the difference between these matrices was assessed by means of the Quasilikelihood under the Independence model Criterion (QIC) statistic [22 23]. Given that both correlation structures yielded similar results, but with a lower QIC for the first order autoregressive correlation structure, this structure was used in the final model. Third, we used logistic and linear regression models to examine the associations of *Chlamydia trachomatis* infection during pregnancy with the odds of asthma and lung function measures, respectively. All multivariable analyses were adjusted for maternal age, educational level, psychiatric symptoms, and smoking and alcohol use during pregnancy, and child's ethnicity, breastfeeding and day care attendance in the first year of life (confounder model). Confounders were selected from literature first, and were subsequently tested for their association with both the determinant and the outcome, , or a change of the unadjusted effect estimates with 10% or more when added to the univariate model [24-28]. A directed acyclic graph (DAG) was created to visualize the relationship between exposure, outcome and possible covariates . Confounders were included in the final model if they were either associated with determinant and outcome, and not in the causal pathway, or if the effect estimate changed with more than 10% when they were included. To address potential residual confounding related to *Chlamydia trachomatis* infection during pregnancy, we additionally examined whether risk behavior including ever treatment for a sexually transmitted disease (yes/no) and multiple sexual partners in the year before pregnancy (yes/no) differed among women with and without Chlamydia trachomatis during pregnancy by means of a Fisher's exact test.

We examined if the associations of *Chlamydia trachomatis* during pregnancy with respiratory morbidity were explained by mode of delivery, gestational age or birth weight by additionally adjusting for these variables (full model). We assessed the change in effect estimates after additional adjustment for these variables, by using the following formulas for percentage change: 100 * (Effect estimate$_{\text{full model}}$ – Effect estimate$_{\text{original model}}$)/(Effect estimate$_{\text{original model}}$ – 1) for categorical lower respiratory tract infections, wheezing and asthma, and 100 * (Effect estimate$_{\text{full model}}$ – Effect estimate$_{\text{original model}}$)/(Effect estimate$_{\text{original model}}$) for continuous lung function measures [11]. Additionally, we tested possible effect-modification by gestational age at urine sampling for *Chlamydia* diagnosis by adding an interaction term to the model. Missing data in covariates, and in repeated measures of lower respiratory tract infections and wheezing, was imputed by the multiple imputation method using chained equations to select the most likely value for a missing response [29],

creating ten new datasets. Since we observed no major differences in the magnitude or direction of the effect estimates between analyses with imputed missing data and complete cases only, we only present the results based on imputed datasets. All measures of association are presented as odds ratios (OR) or Z-score differences and their corresponding 95% Confidence Intervals (95% CI). Statistical analyses were performed using SPSS version 24.0 for Windows software (IBM Corp), SAS version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA) and R version 3.4.1.

RESULTS

Subject characteristics

Maternal and child characteristics are presented in Table 3.3.1. The prevalence of *Chlamydia trachomatis* infection during pregnancy was 3.1% (78/2,475). The prevalence of childhood lower respiratory tract infections decreased after age 2 years, while the prevalence of childhood wheezing decreased gradually until age 10 years. Among the children born to mothers without versus with a *Chlamydia trachomatis* during pregnancy, the prevalence of asthma at age 10 years was 5.3% (101/1,893), versus 14.5% (8/55) (p-value for difference 0.004). Main results of the loss-to-follow-up analysis showed that children not included in the study had mother who more often had *Chlamydia trachomatis* during pregnancy, and had a lower gestational age at birth and birth weight (Supplementary Table S3.3.1).

Chlamydia trachomatis, asthma and asthma related morbidity

None of the children with a lower respiratory tract infection at age 6 months, was born to a mother with *Chlamydia trachomatis* infection during pregnancy, and therefore only lower respiratory tract infections from age 1 year onwards were included in the model. *Chlamydia trachomatis* infection during pregnancy was not associated with annual or overall odds of lower respiratory tract infections in childhood (Figure 3.3.1). Compared to children born to mothers without *Chlamydia trachomatis* infection during pregnancy, those born to mothers with a *Chlamydia trachomatis* infection during pregnancy had an increased odds of wheezing until age 10 years (odds ratio (OR) (95% confidence interval (CI)) 1.50 (1.10 to 2.03)) (Figure 3.3.1), and an increased odds of asthma (OR (95% CI): 2.29 (1.02 to 5.13)) (Table 3.2.2). Additionally, these children had a lower FEV_1/FVC (Z-score difference (95% CI): -0.28 (-0.52 to -0.04)) and FEF_{75} (Z-score difference (95% CI): -0.24 (-0.46 to -0.01)) at age 10 years, but not a lower FEV_1 or FVC (Table 3.3.2). The proportion of women ever treated for a sexually transmitted disease did not differ between the groups with and without *Chlamydia trachomatis* infection during pregnancy (12.1% vs 11.6%, p-value 0.84). The proportion of women having multiple sexual partners in the year before pregnancy in the group with *Chlamydia trachomatis* during pregnancy was lower than in the group without (0% vs 7.6%, p-value 0.02).

Table 3.3.1. Characteristics of children and their mothers

	Full group **n = 2,475**	**Chlamydia negative** **n = 2,397**	**Chlamydia positive** **n = 78**
Maternal characteristics			
Age, mean (SD), y	30.76 (4.8)	30.86 (4.7)	27.58 (6.2)
Caesarian section, No. (%)	361 (14.6)	309 (12.9)	12 (15.4)
Low/middle education level, No. (%)	1,218 (49.2)	1,161 (48.4)	56 (71.8)
Maternal psychiatric symptoms, median (IQR)	0.16 (0.07, 0.37)	0.15 (0.03, 0.31)	0.27 (0.12, 0.45)
Smoking during pregnancy, No. (%)	611 (24.7)	589 (24.6)	23 (29.5)
Alcohol use during pregnancy, No. (%)	1,417 (57.3)	1,373 (57.3)	44 (56.4)
Chlamydia trachomatis infection during pregnancy, No. (%)	78 (3.1)	N/A	N/A
Child's characteristics			
Female sex, No. (%)	1,230 (49.7)	1,193 (49.8)	37 (47.4)
Gestational age at birth, median (IQR), weeks	40.1 (39.3, 41.0)	40.1 (39.3, 41.0)	40.0 (39.1, 41.0)
Birth weight, mean (SD), grams	3,446 (546)	3,450 (545)	3,327 (627)
European ethnicity, No. (%)	1,556 (62.9)	1,591 (67.3)	30 (41.0)
Ever breastfeeding, No. (%)	2,301 (93.0)	2,229 (93.0)	71 (91.0)
Day care attendance 1st year, No. (%)	1,350 (54.5)	1,325 (55.3)	25 (32.1)
Lower respiratory tract infections, No. (%)			
Age 1 year	210 (8.5)	202 (8.4)	8 (10.2)
Age 2 years	317 (12.8)	310 (12.9)	7 (9.0)
Age 3 years	203 (8.2)	193 (8.1)	10 (12.8)
Age 4 years	159 (6.4)	149 (6.2)	10 (12.8)
Age 6 years	145 (5.8)	139 (5.8)	6 (7.6)
Wheezing, No. (%)			
Age 1 year	773 (31.2)	741 (30.9)	32 (41.0)
Age 2 years	541 (21.9)	519 (21.7)	22 (28.2)
Age 3 years	377 (15.2)	354 (14.8)	23 (29.5)
Age 4 years	381 (15.4)	363 (15.1)	18 (23.1)
Age 6 years	294 (11.9)	281 (11.7)	13 (16.7)
Age 10 years	162 (6.5)	152 (6.3)	11 (14.1)
Asthma, No. (%)	109 (5.6)	101 (5.3)	8 (14.5)
Lung function measures			
FEV_1 (L)	2.02 (0.30)	2.03 (0.30)	1.97 (0.32)
FVC (L)	2.34 (0.37)	2.34 (0.37)	2.27 (0.39)
FEV_1/FVC (%)	86.94 (5.68)	86.9 (5.7)	85.9 (5.1)
FEF_{75} (L/s)	1.16 (0.35)	1.16 (0.35)	1.04 (0.32)

Abbreviations: IQR, interquartile range; N/A, not applicable; FEV_1, Forced Expiratory Volume in the first second; FVC, Forced Vital Capacity; FEF_{75}, Forced Expiratory Flow after exhaling 75% of FVC. Data was missing and not imputed for asthma (n=527) and lung function measures (n=433).

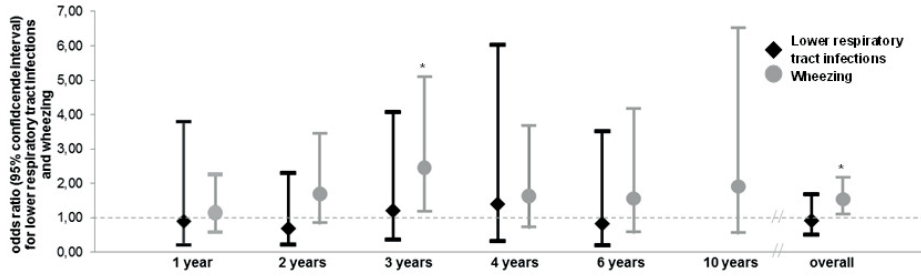

Figure 3.3.1. Associations of Chlamydia trachomatis infection during pregnancy with lower respiratory tract infections from age 1 to 6 years, and wheezing from age 1 to 10 years. Odds ratios with 95% confidence interval from generalized estimating equation models. †p-value 0.028, ‡p-value 0.013. Models are adjusted for maternal age, educational level, psychiatric symptoms, and smoking and alcohol use during pregnancy, and child's ethnicity, breastfeeding and day care attendance in the first year of life.

Additional adjustment for mode of delivery, gestational age at birth or birth weight did not materially change the effect estimates of the association of *Chlamydia trachomatis* infection during pregnancy with childhood respiratory health (Table 3.3.1 for asthma and lung function, Supplementary Table S3.3.2 and S3.3.3 for lower respiratory tract infections and wheezing, respectively). The percentual changes in the association of *Chlamydia trachomatis* during pregnancy with lung function and asthma after additional adjustment for birth characteristics ranged from -6.2 to 1.1 (Table 3.3.1), and were non-significant. The percentual changes for the overall odds of lower respiratory tract infection and wheezing were –2.7% (95% CI: -156 to 175%) and -5.0% (-24.7 to -0.2%), respectively. There was no effect modification by gestational age at diagnosis for associations of *Chlamydia trachomatis* with respiratory health (p-values for interaction between 0.434 and 0.887).

DISCUSSION

We observed that children of mothers with a *Chlamydia trachomatis* infection during pregnancy had increased odds of wheezing, asthma and impaired lung function in childhood. These observed associations with asthma and lung function were not explained by mode of delivery, gestational age at birth or birth weight, while the associations with overall wheezing were partly explained by these birth characteristics. *Chlamydia trachomatis* infection during pregnancy was not associated with lower respiratory tract infections across childhood.

Strengths and limitations

The major strength of this study is that it is embedded in a large prospective population-based cohort with longitudinal measurement of multiple respiratory outcomes, and adjustment for relevant confounders. Additionally, we used a highly sensitive microbiological method to diagnose

Table 3.3.2. Associations of *Chlamydia trachomatis* during pregnancy with asthma and lung function

		FEV_1 Z-score difference (95% Confidence Interval) n = 2,402	FVC Z-score difference (95% Confidence Interval) n = 2,402	FEV_1/FVC Z-score difference (95% Confidence Interval) n = 2,402	FEF_{75} Z-score difference (95% Confidence Interval) n = 2,402	Asthma Odds ratio (95% Confidence Interval) n = 1,948
***Chlamydia trachomatis* infection during pregnancy (yes versus no)**						
Crude model	n = 2,475	-0.16 (-0.39, 0.08)	-0.04 (-0.27, 0.18)	**-0.24 (-0.47, -0.10)**	-0.15 (-0.37, 0.07)	**3.02 (1.39, 6.56)**
p-value		*0.198*	*0.700*	*0.041*	*0.176*	*0.005*
Confounder model[†]	n = 2,475	-0.23 (-0.47, 0.01)	-0.11 (-0.34, 0.12)	**-0.28 (-0.52, -0.04)**	**-0.24 (-0.46, -0.01)**	**2.29 (1.02, 5.13)**
p-value		*0.059*	*0.366*	*0.021*	*0.040*	*0.045*
Full model[‡]	n = 2,475	-0.23 (-0.47, 0.02)	-0.11 (-0.34, 0.13)	**-0.27 (-0.51, -0.02)**	**-0.23 (-0.45, -0.00)**	**2.27 (1.01, 5.15)**
p-value		*0.070*	*0.359*	*0.032*	*0.049*	*0.049*
Percentage change (95% CI)		-3.9 (-28.8, 51.4)	1.1 (-160.5, 208.1)	-6.2 (-35.5, 16.5)	-3.3 (-42.1, 31.0)	-0.6 (-47.8, 53.3)

Values are changes in Z-score or odds ratios with 95% confidence interval, derived from linear and logistic regression models, respectively. Abbreviations: FEV_1, Forced Expiratory Volume in the first second; FVC, Forced Vital Capacity; FEF_{75}, Forced Expiratory Flow after exhaling 75% of FVC. [†]Adjusted for maternal age, educational level, psychiatric symptoms, and smoking and alcohol use during pregnancy, and child's ethnicity, breastfeeding and day care attendance in the first year of life. [‡]Additionally adjusted for mode of delivery, gestational age at birth and birth weight. The percentage change is between the full model and the confounder model.

Chlamydia trachomatis [30]. The study was performed in a low-risk population, which increases generalizability to the general population. Some limitations need to be discussed. As in most prospective cohorts, loss to follow-up might have led towards selection of a more healthy population. The prevalence of *Chlamydia trachomatis* infection during pregnancy was higher in those lost to follow-up, which most likely might have led to an underestimation of the observed associations. The low absolute numbers of *Chlamydia trachomatis* infection during pregnancy and of specific diagnoses of lower respiratory tract infections might have led to lack of power to show associations. A previous study did observe a prevalence of 7% of *Chlamydia trachomatis* in children until age 6 months with respiratory tract infections [31]. However, the study population comprised children presenting with respiratory complaints to a specialised university medical center, which is not comparable to our general more healthy study population. Our results might suggest that the risk of overall wheezing is driven by the significant association of *Chlamydia trachomatis* with wheezing at the age of 3 years. However, the effects estimates for wheezing at all other ages are also taken into account and were moderate to strong (1.22 to 2.06). Their lack of significance was most probably due to a low prevalence of *Chlamydia trachomatis*. The confidence intervals of the percentage change due to mediators were large, which could be due to the large confidence intervals of the original effect estimates. Information on lower respiratory tract infections, wheezing and asthma was obtained by questionnaire, which might have led to reporting bias. For wheezing and asthma however, validated and frequently used ISAAC questionnaires were used. Last, we did not observe increased risk behavior, such as multiple sexual partners or treatment for previous STDs of women with *Chlamydia trachomatis* infection during pregnancy. Although we took various potential confounders and mediators into account, residual confounding might be an issue, as in any observational study. As our study was largely focused on subclinical chlamydia trachomatis infection, we assume the large majority of women was not treated with antibiotics.

Comparison with previous studies

Chlamydia trachomatis infection during pregnancy is associated with neonatal complications such as preterm birth and neonatal respiratory morbidity [5 7 8]. Only one prospective birth cohort study among 8,088 mothers and children demonstrated that treatment for Chlamydia during pregnancy, as reported by the mother as treatment for a gynaecological infection, was associated with a 3-fold increased risk of asthma in the offspring at age 7 years [16]. These women were most likely symptomatic, while due to non-selective urine screening women in our study were most likely asymptomatic. Additionally, in this study treatment for Chlamydia was self-reported, and studied in a sub-analysis only, which could have led to bias. Our findings suggest that *Chlamydia trachomatis* infection during pregnancy is also associated with asthma in later childhood and impaired lung function measurements reflecting airflow limitation and obstruction in the small airways. To our knowledge, the current study is the first study that examined the associations of *Chlamydia trachomatis* infection during pregnancy with various types of respiratory morbidity and objective lung function measurements in childhood.

Other studies focused on the associations of Chlamydial infections in infancy with childhood outcomes. An observational study compared 18 children hospitalized for Chlamydial pneumonia with 19 controls and reported that infants with Chlamydial pneumonia were more likely to have asthma at age 7 years, and more often had an impaired lung function and bronchial hyperreactivity [14]. Findings from another study comparing 40 children hospitalized for pneumonia or bronchitis in infancy with 71 healthy controls suggest that the association of *Chlamydia* infection in infancy with respiratory diseases is not solely explained by respiratory tract infections [15]. Children with Chlamydial lower respiratory tract infections in infancy had an increased risk of cough and were more likely to have an abnormal functional residual capacity until age 5 years than children with non-Chlamydial lower respiratory tract infections. Overall, we did not observe associations of *Chlamydia trachomatis* infection during pregnancy with lower respiratory tract infections. This might be due to the low prevalence of lower respiratory tract infections in this general, non-hospital based population. These findings suggest that *Chlamydia trachomatis* infection during pregnancy specifically predisposes children for asthma related symptoms, but not to general respiratory tract infections.

Explanation and implications of the findings

Because of the observational design of the study, we cannot draw conclusions on the underlying causality and mechanisms. In vitro studies have elucidated possible direct mechanisms underlying the association between *Chlamydia trachomatis* and respiratory health. An in vitro study among neonatal and adult mice showed that mice infected as neonates with *Chlamydia muridarim,* the murine biovar of *Chlamydia trachomatis,* were not able to clear the infection, had *Chlamydia muridarum* specific IgE in BAL fluid and serum, and had an increased production of IL-4 and IL-10, while this was not observed for mice infected in adulthood [32]. Both IgE and IL-4 are of importance in asthma pathogenesis, which might explain our observed association with asthma. Additionally, a related in vitro study demonstrated that alveolar diameter was increased in mice that were infected in the neonatal period, but not in mice infected in infancy or adulthood, which might imply that *Chlamydia trachomatis* directly affects the lungs [33]. These findings suggest that *Chlamydial* infection during early life specifically might lead to developmental lung and immune system adaptations and consequently an increased risk of wheezing, asthma and lower lung function in later life, but not lower respiratory tract infections. We did not observe any modifying effect of gestational age at diagnosis of *Chlamydia trachomatis* on respiratory health. However, *Chlamydia trachomatis* could have been asymptomatically present for a longer duration of time [34 35]. *Chlamydia trachomatis* infection during pregnancy may lead to chorioamnionitis [5 6]. Chorioamnionitis itself is associated with an increased risk of asthma or impaired lung function especially in children born preterm [36-39], possibly through immune changes in the child as a result of the chorioamnionitis [40]. In our study, the associations of *Chlamydia trachomatis* infection during pregnancy with school-age lung function and asthma were not explained by preterm birth, while the association with childhood wheezing was. This might imply that this mediation has only

short-term effects. Further studies are needed to assess whether chorioamnionitis explains the observed associations. Lastly, co-infections during pregnancy might play a role in the association of *Chlamydia trachomatis* with respiratory health. Previous studies demonstrated that *Chlamydia trachomatis* can co-occur with for example *Neisseria gonorrhoeae* and *Trichomonas vaginalis*, mostly in high-risk populations, but their association with respiratory health is unclear [41-44].

Our findings are important from both an etiological and population-health perspective. They suggest that a *Chlamydia trachomatis* infection during pregnancy leads to fetal developmental adaptations, which predispose children to asthma related morbidity, rather through lower lung function than susceptibility for lower respiratory tract infections. Population attributable fractions (calculated as prevalence of exposure among cases*(1-1/RR)) of *Chlamydia trachomatis* for asthma and overall wheezing in this study were 4.2 and 1.2%, respectively [45]. We only observed associations of *Chlamydia trachomatis* on FEV_1/FVC and FEF_{75}, and not the other lung function measures. Although the effect sizes are important from an etiological perspective, they should be carefully considered from a clinical perspective. *Chlamydia trachomatis* infections are common, and infections are mostly asymptomatic. Screening of pregnancy women for *Chlamydia trachomatis* is recommended within the United States for all women under the age of 25, or women with risk factors for a sexually transmitted disease [46 47]. According to local practice, screening for *Chlamydia trachomatis* is not routine practice, although it has been suggested to be cost-effective [48]. *Chlamydia trachomatis* can be treated easily and effectively with a single-dose therapy of azithromycin, even during pregnancy [46 49]. Implementation of screening and treatment of pregnant women in the United States has led to a decrease of *Chlamydia trachomatis* infection of the newborn, and morbidity such as preterm rupture of the membranes and preterm birth [47 50]. Additionally, prevention due to vaccination could be considered in the future [51-53]. Replication of these findings would be of interest, and future studies should focus on the effects of treatment during pregnancy on intra-uterine transmission, chorioanmionitis and long term offspring health.

Conclusion and future research

Our results suggest that *Chlamydia trachomatis* infection during pregnancy is associated with increased odds of wheezing, asthma, and impaired lung function across childhood. The causality of the observed associations and potential underlying biological mechanisms need to be explored.

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Supplementary Table S3.3.1. Differences between those included and not included in the study

	Included n = 2,475	Not included n = 1,580	P-value
Maternal characteristics			
Age, mean (SD), y	30.76 (4.82)	27.96 (5.49)	<0.001
Caesarian section, No. (%)	275 (12.3)	170 (13.1)	0.523
Low/middle education level, No. (%)	1,117 (47.7)	947 (72.2)	<0.001
Maternal psychiatric symptoms, median (IQR)	0.15 (0.06, 0.33)	0.25 (0.10, 0.52)	0.006
Smoking during pregnancy, No. (%)	520 (24.0)	24 (32.4)	0.098
Alcohol use during pregnancy, No. (%)	1,239 (58.2)	492 (39.0)	<0.001
Chlamydia trachomatis infection, No. (%)	78 (3.2)	79 (5.0)	0.003
Child's characteristics			
Female sex, No. (%)	1,230 (49.7)	713 47.4)	0.155
Gestational age at birth, median (IQR), weeks	40.1 (39.3, 41.0)	39.9 (38.9, 40.9)	<0.001
Birth weight, mean (SD), grams	3,447 (543)	3,344 (612)	<0.001
European ethnicity, No. (%)	1,621 (66.5)	636 (45.3)	<0.001
Ever breastfeeding, No. (%)	1,977 (93.5)	41 (91.1)	0.527
Day care attendance 1st year, No. (%)	1,021 (61.8)	11 (52.4)	0.377

Abbreviation: IQR, interquartile range. Differences between groups were evaluated using t-tests for continuous variables, chi-square tests for categorical variables or Mann-Whitney U tests for non-normal distributed variables. Values are based on observed data.

Supplementary Table S3.3.2. Associations of *Chlamydia trachomatis* infection during pregnancy with lower respiratory tract infections from age 1 to 6 years

	Lower respiratory tract infections Odds Ratio (95% Confidence Interval)					
	1 year	**2 years**	**3 years**	**4 years**	**6 years**	**Overall**
***Chlamydia trachomatis* infection during pregnancy (yes versus no)**						
Crude model	1.24 (0.56, 2.75)	0.6 (0.31, 1.47)	1.15 (0.52, 2.52)	**2.25 (1.17, 4.34)**	1.51 (0.71, 3.18)	0.85 (0.54, 1.35)
p-value	*0.589*	*0.320*	*0.737*	*0.016*	*0.283*	*0.500*
Confounder model[a]	1.00 (0.33, 3.04)	0.49 (0.17, 1.38)	1.29 (0.46, 3.60)	1.50 (0.37, 6.14)	0.77 (0.19, 3.20)	0.95 (057, 1.59)
p-value	*0.996*	*0.191*	*0.633*	*0.576*	*0.723*	*0.855*
Full model[b]	0.95 (0.57, 1.59)	1.03 (0.18, 1.42)	1.32 (0.47, 3.69)	1.54 (0.38, 6.25)	0.79 (0.19, 3.27)	0.95 (0.57, 1.59)
p-value	*0.961*	*0.197*	*0.601*	*0.550*	*0.749*	*0.735*

Values are odds ratios with 95% confidence interval from generalized estimating equation models. [a]Adjusted for maternal age, educational level, psychiatric symptoms, and smoking and alcohol use during pregnancy, and child's ethnicity, breastfeeding and day care attendance in the first year of life. [b]Additionally adjusted for mode of delivery, gestational age at birth and birth weight.

Supplementary Table S3.3.3. Associations of *Chlamydia trachomatis* infection during pregnancy with wheezing from age 1 to 10 years

	Wheezing Odds Ratio (95% Confidence Interval)						
	1 year	**2 years**	**3 years**	**4 years**	**6 years**	**10 years**	**Overall**
***Chlamydia trachomatis* infection during pregnancy (yes versus no)**							
Crude model	1.24 (0.77, 1.99)	1.14 (0.67, 1.95)	**3.30 (2.05, 5.31)**	**2.36 (1.43, 3.89)**	1.13 (0.56, 2.29)	1.58 (0.90, 2.77)	**2.03 (1.50, 2.75)**
p-value	*0.378*	*0.624*	*<0.001*	*0.001*	*0.734*	*0.112*	*<0.001*
Confounder model[a]	1.31 (0.72, 2.38)	1.22 (0.66, 2.24)	**2.09 (1.10, 3.96)**	1.41 (0.64, 3.12)	1.23 (0.56, 2.69)	2.06 (0.88, 4.83)	**1.50 (1.10, 2.03)**
p-value	*0.374*	*0.529*	*0.028*	*0.399*	*0.551*	*0.100*	*0.013*
Full model[b]	1.31 (0.72, 2.38)	1.22 (0.66, 2.24)	**2.09 (1.10, 3.96)**	1.41 (0.64, 3.12)	1.27 (0.58, 2.79)	2.06 (0.88, 4.83)	**1.47 (1.09, 1.99)**
p-value	*0.374*	*0.529*	*0.028*	*0.399*	*0.551*	*0.100*	*0.015*

Values are odds ratios with 95% confidence interval from generalized estimating equation models. [a]Adjusted for maternal age, educational level, psychiatric symptoms, and smoking and alcohol use during pregnancy, and child's ethnicity, breastfeeding and day care attendance in the first year of life. [b]Additionally adjusted for mode of delivery, gestational age at birth and birth weight.

Chapter 3.4

The influence of Epstein-Barr virus and Cytomegalovirus on childhood respiratory health: a population-based prospective cohort study.

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ABSTRACT

Background

Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) infection are common in early childhood. CMV infection favors a T-helper-1 and EBV infection a T-helper-2 cell response, possibly leading to disbalanced T-helper cell response, and subsequent risk of asthma or atopy.

Objective

To study the associations of EBV and CMV with lung function, asthma and inhalant allergic sensitization at school age.

Methods

This study among 3,546 children was embedded in a population-based prospective cohort. At age 6 years, serum IgG levels against EBV and CMV were measured by ELISA. At age 10 years, lung function was measured by spirometry, asthma by questionnaire and inhalant allergic sensitization by skin prick test.

Results

Unadjusted models showed that seropositivity for EBV was associated with a higher FEV_1 and FEF_{75} (Z-score difference (95% CI): 0.09 (0.02, 0.16) and 0.09 (0.02, 0.15)), while seropositivity for CMV was not. Specific combinations of viruses showed that seropositivity for EBV was only associated with FEV_1 and FEF_{75} in the presence of seropositivity for CMV (0.12 (0.04, 0.20)) and 0.08 (0.01, 0.15)). Seropositivity for CMV in the absence of seropositivity for EBV was associated with an increased risk of inhalant allergic sensitization (OR (95% CI): 1.31 (1.02, 1.68)). All effect estimates attenuated into non-significant mainly after adjustment for child's ethnicity. Seropositivity for EBV or CMV was not associated with asthma.

Conclusion

Associations of EBV and CMV infections in early childhood with school-age lung function and inhalant allergic sensitization are explained by ethnicity, or sociodemographic and lifestyle related factors.

INTRODUCTION

Infectious diseases in early life are suggested to influence the risk of lower lung function and asthma in later childhood and adulthood[1-4]. Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) are herpesviridae that are commonly present. Between 60 and 90% of the adults are seropositive with the peak of infections occurring in childhood, and result in life-long viral persistence[5-7]. Successful viral suppression is dependent on expanded T-cell memory population. Both viruses primarily affect immune cells, but do not have the same effects. EBV favors a T-helper-2 cell mediated response and mostly seems to affect B-cells[8 9]. CMV has a T-helper-1 cell mediated response, and impacts on T-cell and natural killer (NK) cell differentation[10 11]. Moreover, both viruses drive memory T-cell expansions in young children[12 13]. Therefore, infections with these viruses could lead to a disbalance in immune responses, specifically T-helper cell mediated responses, and subsequently an increased risk of asthma[2-4]. A prospective cohort study demonstrated that EBV coinfection enhances immune maturation driven by CVM[14]. Two other studies found that children with EBV had a reduced antibody response to measles or rubella vaccination, while children co-infected with CMV had not[15 16]. This further suggests that these herpesviridae might have interacting effects on the immune system. Results from previous studies on the associations of seropositivity for EBV or CMV with asthma and atopy are inconsistent[2 4 17]. This might be explained by the examination of EBV or CMV solely, while co-infections of these viruses might be more important given their difference in immune response[3 4]. This is supported by a cohort study that demonstrated that CMV in the absence of EBV at the age of 4 years was associated with an increased risk of specific IgE of inhalant allergens, or inhalant and food allergens combined, but not asthma[3]. Studies on associations of EBV or CMV with atopic outcomes at older ages in childhood, including more objective respiratory measures such as lung function, are lacking.

Therefore, we examined the associations of childhood EBV or CMV, solely and in combination, with school-age lung function, asthma and inhalant allergic sensitization in a population-based prospective cohort study among 3,546 children.

METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, the Netherlands [18]. The study is designed to identify early environmental and genetic causes and causal pathways leading to normal and abnormal growth, development and health from fetal life to childhood and young adulthood. In total, 9,778 mothers with a delivery date from April 2002 until January 2006 were enrolled in the study. Response at baseline was 61%, and general follow-up rates until the age of 10 years were around 80%. The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands (MEC-2012-165). Written informed consent was obtained from parents or legal representatives of all children. Of the 7,393 children with participation at age 10 years, we excluded twins (n=185), children without information on EBV or CMV (n=3,709), and without information on respiratory health (n=484), which left 3,546 children for the current analysis .

EBV and CMV

Venous blood samples were obtained by antecubital venipuncture during a visit at the research center (median age 6.0 years, 5-95% range 5.7-7.2 years). The response rate for serum samples was 69%. Samples were stored for a maximum of 4 hours at 4 ^{o}C, and transported twice daily for further processing and storage. Serum samples were analyzed using ELISA for IgG antibodies against EBV capsid antigen (native mixture of several viral capsid antigens) and CMV (purified native antigens strain 'AD169') (EUROIMMUN, Lübeck, Germany). As described earlier, seropositivity was defined as a sample-treshold ratio above 0.8 for EBV and 0.6 for CMV[12]. The presence of seropositivity for the viruses were further combined into neither, EBV only, CMV only, and both EBV and CMV.

Respiratory health

We performed spirometry according to the American Thoracic Society (ATS) and European Respiratory Society (ERS) recommendations during a visit at the research centre (median age 9.7 years, 5-95% range 9.5-10.3 years). Lung function measures included Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC), FEV_1/FVC and Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}), and were converted into sex-, height-, age-, and ethnicity-adjusted z-scores according to the Global Lung Initiative reference data[19]. Children (n=239) with a >5%, instead of ≤5%, deviation in FEV_1 and FVC, and at least one blow with adequate reach and duration of plateau according to ATS/ERS criteria were also included. The effect estimates for the associations did not differ when we in- or excluded those children [20 21].. Information on asthma medication use in the past 12 months was obtained during the research centre visit. Asthma was defined as ever diagnosis of asthma, obtained by questionnaire at the age 10 years, with either wheezing or

asthma medication use in the past 12 months. Questions on wheezing and asthma were based on the International Study on Asthma and Allergy in Childhood (ISAAC) Questionnaire obtained by post ("Has your child suffered from attacks of wheezing in the chest in the past 12 months?" (no/yes, <4 attacks/yes ≥4 attacks) and "Has your child ever had asthma diagnosed by a doctor?" (no/yes <3 years/yes 3-6 years/yes >6 years of age))[22]. Information on medication use was obtained by a short questionnaire during the visit at the research center ("Did your child receive any prescribed medication in the past 12 months for complaints of the airways, lungs, allergy or skin? If yes, which medication was this?" (no/yes: <name prescribed drug>)). Median time between the questionnaire and visit to the research center was 1.4 (5-95% range -6-12) weeks. As described earlier, inhalant allergic sensitization at the age of 10 years was measured by skin prick test (SPT), using the scanned are method[23]. Inhalant allergens included house dust mite, 5-grass mixture, birch, cat and dog (ALK-Albelló B.V., Almere, the Netherlands). Children were considered to have inhalant allergic sensitization when they were sensitized to at least one inhalant allergen.

Covariates

At enrollment, information on maternal age, ever history of asthma and atopy (no/yes), educational level (low or middle/high), body mass index, parity, and current pet keeping (no/yes) was obtained by questionnaires during pregnancy. Information on maternal smoking during pregnancy was obtained in early, mid and late pregnancy by questionnaires, and combined into no smoking or quitting in early pregnancy/continued smoking. Psychological distress during mid pregnancy was measured by the Brief Symptom inventory[24]. Child's gestational age at birth and birth weight were obtained from midwife and hospital records at birth. Child's ethnicity was based on country of birth of the parents from parental questionnaires at enrolment, and combined (European/non-European). Questionnaires in the first year of life provided information on ever breastfeeding (no/yes) and daycare attendance in the first year of life (no/yes).

Statistical analysis

First, we compared characteristics of mothers and children included in the study to those lost to follow-up. Next, we studied the associations of both EBV and CMV solely and combined (neither, EBV only, CMV only or both EBV and CMV) with lung function, and asthma and inhalant allergic sensitization by using linear and logistic regression models, respectively. All models were adjusted for confounders, which were selected from literature, and were associated with the exposure and the outcome, or changed the effect estimates of univariate analyses with 10% or more when added to the crude model. Confounders were grouped into sociodemographic and health-related factors (maternal age, history of asthma and atopy, educational level, parity, and psychological distress during pregnancy), lifestyle related factors (maternal body mass index, smoking during pregnancy and pet keeping, and child's birth weight adjusted for gestational age, breastfeeding and daycare attendance) and child's ethnicity. Models were adjusted for each group of confounders separately, and finally for all groups of confounders. The percentage of missing data in

confounders was between 1.7 and 24.8%, except for daycare attendance (37.9%). Missing data was imputed by multiple imputation using chained equations to select the most likely value for a missing response, creating ten new datasets. Since we observed no major differences in the magnitude or direction of the effect estimates between analyses with imputed missing data and complete cases only, we only present the results based on imputed datasets. All measures of association are presented as odds ratios (OR) or Z-score differences and their corresponding 95% Confidence Intervals (95% CI). Statistical analyses were performed using SPSS version 25.0 for Windows software (IBM Corp).

RESULTS

Subject characteristics

Characteristics of children and their mothers are presented in Table 3.4.1. At the age of 6 years, 50.4% (n=1,787) of the children were seropositive for EBV, and 41.5% (n=1,470) for CMV. When we combined the viruses, 32.6% (n=1,155) of the children was seropositive for none of the viruses, 26.0% (n=921) for EBV only, 17.0% (n=604) for CMV only, and 24.4% (n=866) for both. The prevalence of current asthma at the age of 10 years was 5.8% (n=172), and of inhalant allergic sensitization 31.7% (n=818). Children not included in the analysis had mothers who were younger and lower educated, had a higher body mass index and had more psychological distress during pregnancy. Children were more often of non-European ethnicity, had a lower gestational age and birth weight, and were more likely to be seropositive for EBV, but not CMV (Supplementary Table S3.4.1).

EBV, CMV and respiratory health

In the unadjusted analyses, EBV, but not CMV, was associated with a higher FEV_1 and FEF_{75} (Z-score difference (95% Confidence Interval): 0.09 (0.02, 0.16) and 0.09 (0.02, 0.15), respectively) (Table 3.4.2). Combinations of seropositivity for the viruses showed that only seropositivity for EBV in the presence of seropositivity for CMV was associated with higher FEV_1, FVC and FEF_{75} (Z-score difference (95% CI): 0.12 (0.04, 0.20), 0.10 (0.02, 0.17) and 0.08 (0.01, 0.15), respectively)). Seropositivity for EBV or CMV, or combinations of seropositivity for the viruses were not associated with asthma. Seropositivity for EBV or CMV solely were not associated with inhalant allergic sensitization. Combinations of seropositivity for the viruses demonstrated that only seropositivity for CMV in the absence of seropositivity for EBV was associated with an increased risk of inhalant allergic sensitization (OR (95% CI): 1.29 (1.01, 1.65). After adjustment for confounders, all effect estimates attenuated into non-significant (Table 3.4.3 and Supplementary Table S3.4.2). The associations of the viruses with lung function were mainly explained by ethnicity of the child, while the association with inhalant allergic sensitization was explained by sociodemographic and health-related factors, lifestyle related factors, and ethnicity.

Table 3.4.1. Characteristics of children and their mothers

	n = 3,546
Maternal characteristics	
Age (years)	31.2 (4.9)
History of asthma or atopy, yes (%)	36.2 (1,285)
Educational level, low/middle (%)	50.3 (1,751)
Body mass index at intake (kg/m^2)	24.6 (4.2)
Parity, nulliparous (%)	55.5 (1,967)
Psychological distress during pregnancy[1]	0.17 (0.00, 0.93)
Smoking during pregnancy, yes (%)	14.4 (512)
Pet keeping, yes (%)	36.4 (1,290)
Child's characteristics	
Female sex (%)	48.8 (1,729)
Gestational age at birth (weeks)[1]	40.1 (37.0, 42.0)
Birth weight (grams)	3,447 (546)
Ethnicity, European (%)	67.7 (2,399)
Ever breastfeeding, yes (%)	91.7 (3,252)
Day care attendance 1st year, yes (%)	55.4 (1,966)
EBV IgG at 6 years, positive (%)	50.4 (1,787)
CMV IgG at 6 years, positive (%)	41.5 (1,470)
Combination of infections (%)	
Neither	32.6 (1,155)
EBV only	26.0 (921)
CMV only	17.0 (604)
Both	24.4 (866)
Lung function measures	
FEV_1 (L)	2.02 (0.30)
FVC (L)	2.34 (0.36)
FEV_1/FVC (%)	87.0 (5.6)
FEF_{75} (L/s)	1.15 (0.35)
Asthma, yes (%)	5.8 (172)
Inhalant allergic sensitization, yes (%)	31.7 (818)

Values are means (SD), valid percentages (absolute numbers) or [1]medians (5-95th percentiles). Values are based on imputed data. Data was missing and not imputed for lung function measures (n=382), asthma (n=561), and inhalant allergic sensitization (n=965). Epstein-Barr virus (EBV). Cytomegalovirus (CMV). Forced Expiratory Volume in the first second (FEV_1). Forced Vital Capacity (FVC). Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}).

Table 3.4.2. Unadjusted associations of EBV and CMV with respiratory health.

	FEV_1 Z-score difference (95% Confidence Interval) n = 3,164	**FVC Z-score difference (95% Confidence Interval) n = 3,164**	**FEV_1/FVC Z-score difference (95% Confidence Interval) n = 3,164**	**FEF_{75} Z-score difference (95% Confidence Interval) n = 3,164**	**Asthma Odds ratio (95% Confidence Interval) n = 2,985**	**Inhalant allergic sensitization Odds ratio (95% Confidence Interval) n = 2,581**
EBV						
Seronegative	Reference	Reference	Reference	Reference	Reference	Reference
Seropositive	**0.09 (0.02, 0.16)***	0.06 (-0.00, 0.13)	0.06 (-0.01, 0.12)	**0.09 (0.02, 0.15)****	1.13 (0.83, 1.54)	1.05 (0.89, 1.23)
CMV						
Seronegative	Reference	Reference	Reference	Reference	Reference	Reference
Seropositive	0.07 (-0.00, 0.14)	0.06 (-0.00, 0.13)	0.01 (-0.06, 0.08)	0.02 (-0.04, 0.09)	0.96 (0.70, 1.32)	1.11 (0.94, 1.31)
Combination of viruses						
Neither	Reference	Reference	Reference	Reference	Reference	Reference
EBV only	0.01 (-0.07, 0.08)	-0.02 (-0.09, 0.06)	0.04 (-0.04, 0.12)	0.04 (-0.04, 0.11)	1.20 (0.81, 1.78)	1.17 (0.94, 1.65)
CMV only	-0.04 (-0.13, 0.05)	-0.02 (-0.11, 0.07)	-0.02 (-0.11, 0.07)	-0.07 (-0.15, 0.02)	1.02 (0.64, 1.62)	**1.29 (1.01, 1.65)***
Both	**0.12 (0.04, 0.20)****	**0.10 (0.02, 0.17)***	0.04 (-0.04, 0.11)	**0.08 (0.01, 0.15)***	1.07 (0.70, 1.62)	1.12 (0.90, 1.40)

Values are Z-score differences or odds ratios with 95% confidence interval, derived from unadjusted linear or logistic regression models, respectively. * p-value <0.05, ** p-value <0.01. Epstein-Barr virus (EBV). Cytomegalovirus (CMV). Forced Expiratory Flow in 1 second (FEV_1). Forced Vital Capacity (FVC). Forced Expiratory Flow when 75% of the FVC is exhaled (FEF_{75}).

Table 3.4.3. Adjusted associations of combinations of EBV and CMV with respiratory health.

	FEV_1 Z-score difference (95% Confidence Interval) n = 3,164	FVC Z-score difference (95% Confidence Interval) n = 3,164	FEV_1/FVC Z-score difference (95% Confidence Interval) n = 3,164	FEF_{75} Z-score difference (95% Confidence Interval) n = 3,164	Asthma Odds ratio (95% Confidence Interval) n = 2,985	Inhalant allergic sensitization Odds ratio (95% Confidence Interval) n = 2,581
Sociodemographic and health-related factors[1]						
Neither	Reference	Reference	Reference	Reference	Reference	Reference
EBV only	-0.00 (-0.08, 0.07)	-0.02 (-0.10, 0.05)	0.03 (-0.05, 0.11)	0.02 (-0.05, 0.09)	1.10 (0.74, 1.64)	1.14 (0.92, 1.43)
CMV only	-0.03 (-0.12, 0.06)	-0.01 (-0.10, 0.07)	-0.01 (-0.10, 0.07)	-0.05 (-0.13, 0.04)	0.99 (0.62, 1.59)	1.30 (1.00, 1.64)
Both	**0.12 (0.04, 0.20)****	**0.10 (0.03, 0.18)***	0.02 (-0.05, 0.10)	0.06 (-0.01, 0.14)	0.95 (0.61, 1.47)	1.10 (0.88, 1.38)
Lifestyle related factors[2]						
Neither	Reference	Reference	Reference	Reference	Reference	Reference
EBV only	0.01 (-0.07, 0.09)	-0.01 (-0.09, 0.06)	0.04 (-0.03, 0.12)	0.04 (-0.04, 0.11)	1.13 (0.68, 1.77)	1.12 (0.90, 1.41)
CMV only	-0.04 (-0.13, 0.05)	-0.01 (-0.10, 0.07)	-0.03 (-0.12, 0.06)	-0.07 (-0.15, 0.02)	1.13 (0.76, 1.68)	1.24 (0.97, 1.59)
Both	**0.11 (0.03, 0.19)****	**0.09 (0.01, 0.16)***	0.03 (-0.05, 0.11)	0.07 (-0.00, 0.15)	1.08 (0.70, 1.66)	1.06 (0.85, 1.33)
Ethnicity[3]						
Neither	Reference	Reference	Reference	Reference	Reference	Reference
EBV only	0.01 (-0.07, 0.09)	-0.01 (-0.08, 0.06)	0.04 (-0.03, 0.12)	0.04 (-0.03, 0.11)	1.10 (0.74, 1.63)	1.11 (0.89, 1.39)
CMV only	-0.04 (-0.13, 0.04)	-0.02 (-0.11, 0.06)	-0.03 (-0.07, 0.02)	0.11 (-0.15, 0.01)	0.94 (0.59, 1.48)	1.23 (0.96, 1.56)
Both	0.02 (-0.06, 0.10)	0.01 (-0.07, 0.08)	0.01 (-0.07, 0.09)	-0.01 (-0.07, 0.06)	0.89 (0.58, 1.37)	0.99 (0.79, 1.25)
Fully adjusted model[4]						
Neither	Reference	Reference	Reference	Reference	Reference	Reference
EBV only	0.02 (-0.06, 0.09)	-0.01 (-0.08, 0.07)	0.04 (-0.04, 0.12)	0.04 (-0.03, 0.12)	1.04 (0.69, 1.56)	1.09 (0.87, 1.37)
CMV only	-0.05 (-0.14, 0.04)	-0.03 (-0.11, 0.06)	-0.03 (-0.12, 0.06)	-0.07 (-0.16, 0.01)	0.99 (0.61, 1.60)	1.18 (0.92, 1.52)
Both	0.03 (-0.05, 0.11)	0.03 (-0.05, 0.10)	0.00 (-0.08, 0.08)	-0.01 (-0.09, 0.06)	0.90 (0.57, 1.41)	0.97 (0.77, 1.23)

Values are Z-score differences or odds ratios with 95% confidence interval, derived from linear or logistic regression models, respectively. * p-value <0.05, ** p-value <0.01. Epstein-Barr virus (EBV). Cytomegalovirus (CMV). Forced Expiratory Flow in 1 second (FEV_1). Forced Vital Capacity (FVC). Forced Expiratory Flow when 75% of the FVC is exhaled (FEF_{75}). [1]Adjusted for maternal age, history of asthma and atopy, educational level, parity, and psychological distress during pregnancy. [2]Adjusted for maternal body mass index, smoking during pregnancy and pet keeping, and child's birth weight adjusted for gestational age, breastfeeding and daycare attendance. [3]Adjusted for child's ethnicity. [4]Adjusted for all confounders.

DISCUSSION

Principal findings

In this population-based prospective cohort study we observed that seropositivity for EBV in early childhood was associated with higher lung function at school age. This association was driven by seropositivity for EBV in in the presence of seropositivity for CMV. Seropositivity for EBV or CMV were not associated with asthma. Only seropositivity for CMV in the absence of seropositivity for EBV was associated with an increased risk of inhalant allergic sensitization. The associations of the viruses with respiratory health were fully explained by ethnicity, or sociodemographic and health-related factors, and lifestyle related factors.

Comparison with previous studies

We observed that EBV and CMV in early childhood were not independently associated with respiratory health in later childhood life. To our knowledge, this is the first study to associate EBV or CMV with lung function measures, and respiratory health later in childhood. A cross-sectional analysis within a prospective cohort study demonstrated that seropositivity for EBV was not associated with asthma or allergic sensitization at age 4 years[2]. Additionally, they demonstrated that CMV solely was not associated with asthma, allergy or allergic sensitization[3]. Two studies have shown that CMV in the absence of EBV was associated with an increased risk of allergic sensitization measured by IgE, at either 2 or 4 years of age[3 4]. Our result that CMV in the absence of EBV is associated with an increased risk of inhalant allergic sensitization is in line with these findings. However, we additionally found that these associations were explained by confounding factors. The composition of the study population, difference in confounders or measurement of allergic sensitization by skin prick test as opposed to specific serum IgE might explain this difference in results. We could not replicate the finding that EBV was associated with a decreased risk of allergic sensitization[4]. Similar to previous findings, we showed that EBV and CMV, solely or combined, were not associated with asthma[2 3]. A case-control study comparing CMV DNA between adults with and without asthma, demonstrated that those with asthma were more likely to be positive for CMV DNA[25]. Additionally, the number of CMV copies was higher in elderly, defined as age above 65 years, with asthma as compared to elderly without asthma. The number of CMV copies was not different in non-elderly with and without asthma. The number of CMV copies was also higher in elderly with asthma as compared to non-elderly with asthma. This suggests that age might be of importance, although due to the cross-sectional nature of the study it was not possible to disentangle the direction of the effect. Further studies are needed to examine the role of age of the child at virus infection, and the role of ethnicity and other confounding factors in the association of EBV and CMV with respiratory health across the life course.

Strengths and limitations

This study was embedded in a population-based cohort study, and detailed measurements of respiratory health, and information on numerous confounders are important strengths. Some limitations should be discussed. First, prospective cohort studies have suggested that early infection with herpesviridae might lead to a decreased risk of atopy, measured by SPT or IgE, and late infections to an increased risk[17 26]. However, we measured seropositivity for IgG only and were not able to determine age of seroconversion. Additionally, children in our study with an infection occurring between 6 and 10 years of age would be classified as seronegative, leading to dilution of the effect. Second, it has been suggested that immune responses might differ between asymptomatic and mononucleosis-like infections. Unfortunately, we did not have detailed information on the clinical course of the herpesviridae infections. However, given that we study a childhood cohort with infections before 6 years of age, these infections are most likely mild or asymptomatic[15]. Third, information on asthma was obtained by questionnaire, which might have led to misclassification although a validated questionnaire was used to minimize bias. Last, selection bias due to loss to follow-up might have occurred. Most importantly, those lost to follow-up were more likely to be of non-European ethnicity, and more likely to be seropositive for EBV. This might have led to either an over- or underestimation of the effect, if the associations of those lost to follow-up with respiratory health would be different from those included in the study. This is unlikely, but cannot be excluded[27 28].

Underlying mechanisms

Some evidence on possible underlying mechanisms for the associations of herpesviridae with the immune and pulmonary system are provided in murine studies. One murine study demonstrated that mice infected in early life with murid herpesvirus4, the murine equivalent of EBV, were protected against housedustmite-induced airway allergy, compared to uninfected mice[29]. These infected mice had a reduced peribronchial infiltration by inflammatory cells, and lower numbers of eosinophils and interleukin-4. 5 and 6 in broncho-alveolar fluid. Additionally, the T-helper-2 polarisation of housedustmite-specific T-cells, but not T-helper-1 polarisation, was suggested to be impaired among infected mice. Another murine study found that after infection with murid herpesvirus4, or murine cytomegalovirus, more secretion of T-helper-1 cytokines was present, which might inhibit the T-helper-2 pathway[30]. In our study, we found that mainly ethnicity explained the association of EBV with a higher lung function. The association of CMV in the absence of EBV with inhalant allergic sensitization was explained by ethnicity, and also sociodemographic and health-related, and lifestyle factors. A previous study within our cohort demonstrated that non-Dutch children were more likely to be seropositive for EBV and CMV[31]. These ethnic differences were partly explained by socio-economic factors and factors related to crowding for EBV, but not for CMV. Other studies also demonstrated an association of ethnicity with the prevalence of herpesviridae, although the role of other factors explaining the association such as socio-economic or lifestyle-related factors in these studies were less prominent[32 33]. Another prospective

cohort study demonstrated that maternal age was associated with time to infection for EBV and CMV, regardless of other factors such as daycare and siblings[34]. Additionally, they demonstrated that CMV is often acquired earlier than EBV, which might be explained by the immune response after CMV infection. Our findings suggest that there might be no causal association of EBV and CMV with respiratory health, but that ethnicity and possible other confounding factors are the underlying explaining mechanisms.

Conclusion

Seropositivity for EBV and CMV at the age of 6 years were associated with a higher lung function and inhalant allergic sensitization, respectively, but not asthma at school-age. However, the association of seropositivity for EBV with school-age lung function was explained by child's ethnicity, while the association of seropositivity for CMV in the absence of EBV with inhalant allergic sensitization was additionally explained by sociodemographic and health-related, and lifestyle related factors. Future studies should focus on the role of age of the child at primaryinfection, specifically in relation to lung function and asthma, and the possible explaining role of ethnicity.

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Supplementary Table S3.4.1. Loss to follow-up analysis

	Included n = 3,546	Not included n = 3,847	p-value for difference
Maternal characteristics			
Age (years)	31.2 (4.9)	30.0 (5.2)	**<0.001**
History of asthma or atopy, yes (%)	1,118 (36.4)	907 (38.2)	0.166
Educational level, low/middle (%)	1,595 (48.5)	1,929 (56.3)	**<0.001**
Body mass index at intake (kg/m^2)	24.6 (4.2)	24.9 (4.5)	**0.009** …
Parity, nulliparous (%)	1,948 (56.9)	2,056 (55.0)	0.096
Psychological distress during pregnancy[1]	0.13 (0.00, 0.88)	0.15 (0.00, 0.98)	**0.018**
Smoking during pregnancy, yes (%)	440 (14.2)	381 (15.7)	0.116
Pet keeping, yes (%)	938 (33.7)	720 (33.5)	0.834
Child's characteristics			
Female sex (%)	1,729 (48.8)	1,957 (50.9)	0.068
Gestational age at birth (weeks)[1]	40.1 (37.0, 42.0)	40.0 (36.3, 42.0)	**<0.001**
Birth weight (grams)	3,448 (544)	3,374 (596)	**<0.001**
Ethnicity, European (%)	2,395 (68.7)	2,323 (63.5)	**<0.001**
Ever breastfeeding, yes (%)	2,693 (92.8)	1,987 (91.7)	0.132
Day care attendance 1st year, yes (%)	1,385 (62.9)	954 (60.7)	0.168
EBV IgG at 6 years, positive (%)	1,787 (50.4)	351 (58.6)	**<0.001**
CMV IgG at 6 years, positive (%)	1,470 (41.5)	261 (43.2)	0.418

Values are means (SD), valid percentages (absolute numbers) or [1]medians (5-95th percentiles). Values are based on observed data. Epstein-Barr virus (EBV). Cytomegalovirus (CMV).

Supplementary Table S3.4.2. Adjusted associations of EBV and CMV separately with respiratory health.

	FEV_1 Z-score difference (95% Confidence Interval) n = 3,164	FVC Z-score difference (95% Confidence Interval) n = 3,164	FEV_1/FVC Z-score difference (95% Confidence Interval) n = 3,164	FEF_{75} Z-score difference (95% Confidence Interval) n = 3,164	Asthma Odds ratio (95% Confidence Interval) n = 2,985	Inhalant allergic sensitization Odds ratio (95% Confidence Interval) n = 2,581
EBV, seropositive						
Sociodemographic and health- related factors[1]	**0.08 (0.02, 0.15)***	0.06 (-0.01, 0.13)	0.04 (-0.03, 0.13)	0.06 (-0.00, 0.13)	1.03 (0.75, 1.42)	1.03 (0.87, 1.22)
Lifestyle related factors[2]	**0.09 (0.02, 0.16)***	0.06 (-0.01, 0.12)	0.06 (-0.01, 0.12)	**0.08 (0.02, 0.14)***	1.07 (0.78, 1.46)	1.01 (0.85, 1.20)
Ethnicity[3]	0.02 (-0.05, 0.09)	-0.00 (-0.07, 0.06)	0.04 (-0.03, 0.11)	0.03 (-0.04, 0.09)	1.02 (0.75, 1.40)	0.98 (0.82, 1.16)
Fully adjusted model[4]	0.04 (-0.03, 0.10)	0.01 (-0.05, 0.08)	0.03 (-0.03, 0.10)	0.03 (-0.04, 0.09)	0.98 (0.71, 1.35)	0.97 (0.82, 1.15)
CMV, seropositive						
Sociodemographic and health-related factors[1]	**0.07 (0.00, 0.14)***	**0.07 (0.00, 0.14)***	0.01 (-0.06, 0.08)	0.02 (-0.05, 0.08)	0.93 (0.67, 1.28)	1.10 (0.93, 1.31)
Lifestyle related factors[2]	0.06 (-0.01, 0.13)	0.06 (-0.01, 0.13)	0.01 (-0.06, 0.08)	0.02 (-0.05, 0.08)	1.03 (0.74, 1.42)	1.07 (0.91, 1.28)
Ethnicity[3]	0.06 (-0.01, 0.13)	0.06 (-0.00, 0.13)	-0.00 (-0.07, 0.07)	0.00 (-0.07, 0.07)	0.92 (0.71, 1.37)	1.03 (0.87, 1.26)
Fully adjusted model[4]	-0.01 (-0.08, 0.07)	0.00 (-0.06, 0.07)	-0.01 (-0.08, 0.06)	-0.05 (-0.12, 0.01)	0.92 (0.66, 1.28)	1.02 (0.85, 1.21)

Values are Z-score differences or odds ratios with 95% confidence interval, derived from linear or logistic regression models, respectively. All models have seronegativity as the reference. * p-value <0.05. Epstein-Barr virus (EBV). Cytomegalovirus (CMV). Forced Expiratory Flow in 1 second (FEV_1), Forced Vital Capacity (FVC), Forced Expiratory Flow when 75% of the FVC is exhaled (FEF_{75}). [1]Adjusted for maternal age, history of asthma and atopy, educational level, parity, and psychological distress during pregnancy. [2]Adjusted for maternal body mass index, smoking during pregnancy and pet keeping, and child's birth weight adjusted for gestational age, breastfeeding and daycare attendance. [3]Adjusted for child's ethnicity. [4]Adjusted for all confounders.

Chapter 4

Childhood bacterial carriage and the microbiome

Chapter 4.1

Airway bacterial carriage and childhood respiratory health: a population-based prospective cohort study.

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ABSTRACT

Background

Airway bacterial carriage might play a role in respiratory disease. We hypothesize that nasal carriage with *Staphylococcus aureus,* or nasopharyngeal carriage with *Haemophilus influenzae, Moraxella catarrhalis* and *Streptococcus pneumonia* predispose individuals to adverse respiratory health.

Objective

To examine the association of early-life airway bacterial carriage with respiratory tract infections and vice-versa, and of early-life airway bacterial carriage with wheezing, lung function and asthma in later childhood.

Methods

We collected upper airway swabs for bacterial culturing for *S. aureus, H. influenzae, M. catarrhalis* and *H. influenzae* at six timepoints between the ages of 6 weeks to 6 years among 945 children participating in a population-based prospective cohort study. Information on respiratory tract infections and wheezing until age 6 years, and asthma at age 10 years was obtained by questionnaires. Lung function at age 10 years was measured by spirometry. We tested possible bidirectional associations between airway bacterial carriage and respiratory tract infections by cross-lagged models, and associations of repeatedly measured airway bacterial carriage with wheezing, lung function and asthma by generalized estimating equations models and regression models.

Results

Cross-lagged modelling showed that early-life airway bacterial carriage was not consistently associated with upper and lower respiratory tract infections, or vice-versa. Nasopharyngeal carriage with any bacteria in infancy was associated with an increased risk of wheezing (OR (95% CI): 1.66 (1.31, 2.10)). Airway bacterial carriage was not consistently associated with school-age lung function or asthma.

Conclusion

Nasopharyngeal carriage with any bacteria is associated with wheezing, but not respiratory tract infections, asthma or lung function.

INTRODUCTION

Respiratory tract infections in early life have been associated with the development of chronic obstructive respiratory disease in childhood and adulthood[1 2]. Previous studies showed that hypopharyngeal bacterial carriage of *Haemophilus influenzae, Moraxella catarrhalis* and *Staphylococcus pneumonia* in infancy is associated with an increased risk of lower respiratory tract infections in the first three years of life[3]. Alternatively, respiratory tract infections might also influence airway bacterial carriage through bacterial superinfection or changed bacterial colonization after a viral infection[4 5]. Therefore, it is difficult to disentangle the direction of the association between airway bacterial carriage and respiratory tract infections. Additionally, previous studies have shown that airway bacterial carriage with these pathogens in infancy is associated with increased risks of wheezing and asthma in childhood[6-9]. Most observational studies have focused on the association of airway bacterial carriage in infancy with wheezing or asthma in early childhood, but its persistence into later childhood and the relation with lung function is not known.

We hypothesized that airway bacterial carriage in early childhood is more prominently associated with an increased risk of respiratory tract infections than vice-versa, and that bacterial carriage in early childhood is associated with wheezing, lower lung function and asthma in later childhood. Therefore, we examined in a population-based prospective cohort study among 945 children the associations of airway bacterial carriage of *H. influenzae, M. catarrhalis, S. pneumonia* or *Streptococcus aureus* from age 6 weeks to 6 years with respiratory tract infections taking possible bidirectional associations into account. Next, we examined whether airway bacterial carriage with these pathogens was associated with wheezing from age 1 to 6 years, and lung function and asthma at age 10 years.

METHODS

Design

This study was embedded in a subcohort of 1,232 Dutch mother and their children of the Generation R Study, a population-based prospective cohort from early fetal life onwards in Rotterdam, the Netherlands[10 11]. The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands (MEC-2012-165). Written informed consent was obtained from parents or legal representatives of all children. After exclusion of twins, children without upper airway swabs, and children without any information on respiratory health, 945 children were included in the analysis.

Bacterial carriage

At the age of 6 weeks, 6 and 14 months, and 2, 3 and 6 years after birth, upper airway swabs of the nose and nasopharyngeal area were taken at the research center, as described earlier[12 13]. Swabs

were classified as either negative or positive for *S. aureus, H. influenzae, M. catarrhalis* or *S. pneumoniae*. Nasopharyngeal carriage of *H. influenza, M. catarrhalis* or *S. pneumoniae* was studied separately, and additionally nasopharyngeal carriage with any bacteria was classified as positive when either of these three pathogens was positive, and negative if all three were negative.

Respiratory health

We obtained information on physician-attended upper (ear infection, throat infection, croup or whooping cough) and lower (pneumonia, bronchitis or bronchiolitis) respiratory tract infections by questionnaires at the age of 2, 6 and 12 months, and 2, 3, 4 and 6 years. Information on wheezing was obtained by questionnaire annually from 1 to 4 years, and at age 6 years. Lung function measures were obtained by spirometry during a visit at the research centre at the mean age of 9.8 (SD 0.34) years, and converted into sex-, height-, age-, and ethnicity-adjusted z-scores according to the Global Lung Initiative reference data[14-16]. Asthma was defined as ever diagnosis of asthma, obtained by questionnaire at the age 10 years, with either wheezing or asthma medication use in the past 12 months. Questions on wheezing and asthma were based on the International Study on Asthma and Allergy in Childhood (ISAAC) Questionnaire, a validated questionnaire for respiratory epidemiological research[17].

Covariates

Information on maternal history of asthma and atopy, education level, smoking during pregnancy, psychological distress during pregnancy, parity and pet keeping was obtained by questionnaires during pregnancy. Maternal history of asthma and atopy included diagnosis of asthma, house-dustmite allergy, hay fever or eczema. Maternal psychological distress was measured by the Global Severity Index[18]. Child's gestational age at birth was obtained from midwife and hospital records. Questionnaires in the first year of life provided information on breastfeeding and daycare attendance, and questionnaires until age 6 years on antibiotic use.

Statistical analysis

First, we compared characteristics of mothers and children included in the study to those eligible at baseline but not included using t-tests, Mann-Whitney U tests and chi-squared tests. Next, we analysed the associations of airway bacterial carriage with respiratory tract infections using cross-lagged models. Cross-lagged models take potential bidirectional associations between the exposure and the outcome into account . With this method, we were able to disentangle the directions of the observed associations of airway bacterial carriage with respiratory tract infections[2 19]. Next, we analysed the associations of airway bacterial carriage at 6 weeks, 6 and 14 months, and 2, 3 and 6 years separately, with childhood wheezing, and school-age lung function and asthma. Since we did not expect bidirectional associations between airway bacterial carriage and wheezing until the age of 10 years, we used generalized estimating equation (GEE) models with a toeplitz correlation matrix to examine these associations. Last, we studied the association of airway bacterial

carriage with lung function and asthma at school-age using linear and logistic regression models, respectively. All models are adjusted for confounders, with missing data imputed by multiple imputation. All measures of association are presented as odds ratios (OR) or Z-score differences and their corresponding 95% Confidence Intervals (95% CI). Statistical analyses were performed using Mplus version 7.4 (Muthén & Muthén), SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and SPSS version 25.0 for Windows software (IBM Corp).

RESULTS

Subject characteristics

Characteristics of children and their mothers, and of those not included, are presented in Table 4.1.1, Figures 4.1.1 and 4.1.2, and Supplementary Table S4.1.1. Nasal bacterial carriage with *S. aureus* decreased from 54.0% at age 6 weeks to 13.5-15.3% at age 2-3 years, and thereafter increased to 27.8% at age 6 years (Figure 4.1.1). Nasopharyngeal carriage with any of *H. influenzae, M. catarrhalis* or *S. pneumoniae* increased from 22.9% at age 6 weeks to 66.5% at age 2 years,

Table 4.1.1. Characteristics of children and their mothers

	n = 945
Maternal characteristics	
History of asthma or atopy, yes (%)	39.6 (374)
Educational level, low/middle (%)	34.0 (321)
Smoking during pregnancy, yes (%)	12.1 (114)
Maternal psychiatric symptoms[1]	0.12 (0.00, 0.54)
Parity, nulliparous (%)	63.1 (596)
Pet keeping, yes (%)	43.7 (413)
Child's characteristics	
Female sex (%)	49.1 (464)
Gestational age at birth (weeks)[1]	40.3 (37.3, 42.1)
Ever breastfeeding, yes (%)	90.8 (858)
Day care attendance 1st year, yes (%)	68.3 (645)
Current asthma age 10 years, yes (%)	4.0 (33)
Lung function measures age 10 years	
FEV_1 (L)	2.05 (0.29)
FVC (L)	2.38 (0.35)
FEV_1/FVC (%)	86.33 (5.58)
FEF_{75} (L/s)	1.15 (0.34)

Values are means (SD), valid percentages (absolute numbers) or [1]medians (5-95th percentiles). Forced Expiratory Volume in the first second (FEV_1). Forced Vital Capacity (FVC). Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}). Data was missing and not imputed for asthma (n=121) and lung function measures (n=172).

and thereafter decreased to 37.2% at age 6 years. The prevalences of these three pathogens separately showed a similar pattern, but with lower prevalence rates. The prevalences of upper and lower respiratory tract infections increased until age 2 years and decreased thereafter, while the prevalence of wheezing decreased from age 1 to 6 years (Figure 4.1.2). The prevalence of asthma was 4.0% at age 10 years (Table 4.1.1).

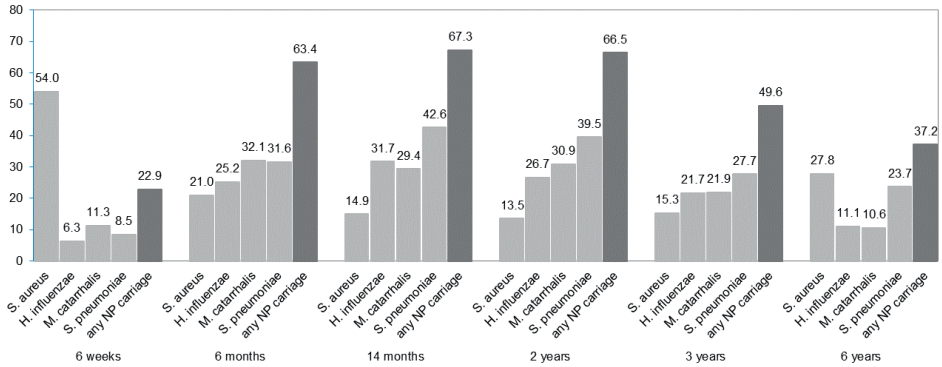


Figure 4.1.1. Prevalence (%) of bacterial carriage of the upper airways in childhood. Nasopharyngeal (NP). Nasopharyngeal carriage of any bacteria was classified as positive when either of H. influenza, M. catarrhalis or S. pneumoniae was positive, and negative if all three were negative.

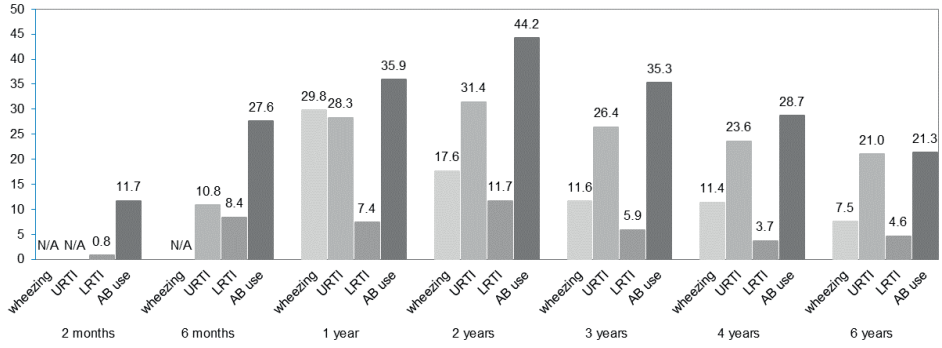


Figure 4.1.2. Prevalence (%) of wheezing, upper and lower respiratory tract infections and antibiotic use in childhood. Upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI), antibiotics (AB).

Early-life airway bacterial carriage and respiratory tract infections

For upper respiratory tract infections, cross-sectional correlations and cross-lagged associations showed that both nasal and nasopharyngeal bacterial carriage were not associated with upper respiratory tract infections, or vice-versa (Supplementary Figure S4.1.1). For lower respiratory tract infections, cross-sectional correlations showed that only *M. catarrhalis* carriage was associated with an increased risk of lower respiratory tract infections at 6 months, but a decreased risk

at age 14 months and similarly vice-versa (OR (95% CI): 2.78 (1.30, 5.93) and 0.24 (0.10, 0.59), respectively) (Table 4.1.2 and Supplementary Figure S4.1.2). Cross-lagged associations showed that only *H. influenza* at age 6 weeks was associated with an increased risk of lower respiratory tract infections at age 6 months (OR (95% CI): 5.77 (2.80, 11.72)), while only lower respiratory tract infections at 6 months were associated with an increased risk of carriage with *M. catarrhalis* at age 14 months (OR (95% CI): 2.69 (1.21, 5.83)).

Early-life bacterial carriage and wheezing

S. aureus was not associated with overall wheezing (Figure 4.1.3A). Nasopharyngeal carriage of any bacteria at 6 months was associated with an increased risk of overall wheezing (1.66 (1.31, 2.10)) (Figure 4.1.3B). Per year, nasopharyngeal carriage of any bacteria at the age of 6 months was associated with an increased risk of wheezing at the age of 1 year (1.81 (1.23, 2.66)), and at age 1 year with an increased risk of wheezing at 2 years (1.72 (1.02, 2.90)). Associations of nasopharyngeal carriage of *H. influenzae, M. catarrhalis* and *S. pneumoniae* individually with wheezing showed similar tendencies (Supplementary Figure S4.1.3). *M. catarrhalis* at age 6 months was associated with an increased risk of overall wheezing (1.33 (1.07, 1.65)), while *M. catarrhalis* at age 6 weeks and 2 years was associated with a decreased risk of overall wheezing (0.65 (0.42, 1.00) and 0.66 (0.47, 0.91) respectively).

Early-life bacterial carriage, lung function and asthma

Overall, no consistent associations of early-life airway bacterial carriage with lung function or asthma in later childhood were observed (Table 4.1.3). Only H. influenza and any nasopharyngeal carriage at age 14 months were most prominently associated with a decreased risk of current asthma at age 10 years (OR (95% CI): 0.28 (0.08, 0.99) and 0.38 (0.15, 0.94), respectively). For all analyses, effect estimates did not materially change when excluding antibiotic use as a confounder (data not shown).

DISCUSSION

Principal findings

Within this population-based prospective cohort study, studying the effect of airway bacterial carriage with *S. aureus, H. influenzae, M. catarrhalis* and *S. pneumonia*, we observed no consistent directions of associations between airway bacterial carriage and respiratory tract infections during childhood. Nasopharyngeal carriage of any bacteria at the age of 6 months was associated with an increased risk of wheezing in childhood, especially until age 2 years. We did not find any consistent associations of airway bacterial carriage with lung function and asthma in later childhood. This suggest that airway bacterial carriage might have a short-term effect only.

Table 4.1.2. Direction of associations between airway bacterial carriage and lower respiratory tract infections from age 6 weeks until 10 years

	S. aureus OR (95% CI)	*H. influenzae* OR (95% CI)	*M. catarrhalis* OR (95% CI)	*S. pneumonia* OR (95% CI)	Any NP carriage OR (95% CI)
Cross-lagged effects					
Carriage 6 weeks → LRTI 6 months	0.92 (0.42, 1.98)	**4.79 (1.50, 15.21)***	0.76 (0.21, 2.71)	1.47 (0.47, 4.57)	2.16 (0.88, 5.31)
Carriage 6 months → LRTI 1 year	1.73 (0.81, 3.70)	1.32 (0.66, 2.64)	0.78 (0.36, 1.68)	0.82 (0.40, 1.70)	1.14 (0.57, 2.30)
Carriage 1 year → LRTI 2 years	1.39 (0.65, 2.96)	1.47 (0.83, 2.59)	1..31 (0.72, 2.37)	0.81 (0.46, 1.41)	1.44 (0.81, 2.56)
Carriage 2 years → LRTI 2 years	0.18 (0.02, 1.67)	0.44 (0.16, 1.22)	1.29 (0.56, 3.01)	1.07 (0.44, 2.57)	0.70 (0.28, 1.73)
Carriage 3 years → LRTI 4 years	0.50 (0.13, 1.92)	1.63 (0.41, 6.40)	1.35 (0.32, 5.65)	2.73 (0.84, 8.88)	1.27 (0.45, 3.62)
LRTI 6 weeks → carriage 6 months	2.64 (0.30, 22.81)	--	0.74 (0.07, 9.93)	--	--
LRTI 6 months → carriage 1 year	1.04 (0.37, 2.89)	1.64 (0.76, 3.47)	**2.69 (1.21, 5.83)****	1.47 (0.65, 3.26)	2.26 (0.77, 6.51)
LRTI 1 year → carriage 2 years	0.96 (0.36, 2.60)	0.47 (0.21, 1.05)	1.40 (0.74, 2.66)	0.73 (0.38, 1.41)	1.07 (0.53, 2.16)
LRTI 2 years → carriage 3 years	1.07 (0.50, 2.29)	0.53 (0.25, 1.14)	0.95 (0.48, 1.90)	0.98 (0.52, 1.83)	0.75 (0.43, 1.30)
LRTI 4 years → carriage 6 years	0.61 (0.21, 1.78)	2.11 (0.56, 7.72)	0.66 (0.08, 5.66)	2.19 (0.87, 5.46)	2.11 (0.85, 5.21)
Cross-sectional effects					
Carriage ↔ LRTI 6 weeks	--	--	--	--	--
Carriage ↔ LRTI 6 months	0.53 (0.19, 1.47)	0.90 (0.38, 2.16)	**2.78 (1.30, 5.93)***	1.73 (0.75, 4.01)	1.89 (0.78, 4.50)
Carriage ↔ LRTI 1 year	0.60 (0.24, 1.50)	0.96 (0.49, 1.90)	**0.24 (0.10, 0.59)****	0.69 (0.36, 1.33)	0.50 (0.24, 1.05)
Carriage ↔ LRTI 2 year	1.12 (0.45, 2.82)	0.97 (0.50, 1.89)	0.88 (0.47, 1.65)	1.65 (0.88, 3.10)	1.10 (0.55, 2.21)
Carriage ↔ LRTI 3 year	1.31 (0.43, 4.03)	1.10 (0.39, 3.09)	0.70 (0.24, 2.01)	1.25 (0.50, 1.22)	1.14 (0.48, 2.69)
Carriage ↔ LRTI 6 year	1.42 (0.64, 3.13)	0.32 (0.06, 1.62)	0.26 (0.03, 2.31)	0.68 (0.25, 1.85)	0.47 (0.19, 1.16)

Associations of *S. aureus*, *H. influenzae*, *M. catarrhalis*, *S. pneumonia* and any nasopharyngeal carriage with lower respiratory tract infections, and vice-versa. Values are odds ratios (95% confidence interval) from cross-lagged models. Models are adjusted for maternal history of asthma and atopy, education level, smoking during pregnancy, stress during pregnancy, parity and pet keeping, and child's gestational age at birth, breastfeeding and daycare attendance. Bold values indicate significant associations. For some associations, no effect estimate could be given due to limited power, as indicated by --. Lower respiratory tract infections (LRTI). Nasopharyngeal (NP).

Comparison with previous studies

Studies examining longitudinal associations of bacterial carriage and respiratory tract infections on a population-based level are scarce. A prospective cohort study among 411 children in Denmark studied the associations of hypopharyngeal carriage with *H. influenzae, M. catarrhalis* or *S. pneumoniae* at the age of 4 weeks with respiratory health up to age 5 years[3 6 8]. This study demonstrated that hypopharyngeal carriage with any of these bacteria was associated with an increased risk of pneumonia or bronchiolitis in the first three years of life[3]. We only found few associations between airway bacterial carriage and lower respiratory tract infections. This is most probably due to insufficient power, since the prevalence of lower respiratory tract infections in our study was maximum 11.7%, while in this study this was 58%. However, we did observe that *H. influenzae* at age 6 weeks was associated with an increased risk of lower respiratory tract infections at age 6 months, and that *M. catarrhalis* at the ages of 6 months is associated with an increased risk, and at 14 months with a decreased risk of lower respiratory tract infections at those ages, and vice-versa. A case-control study assessing the airway microbiome also showed that children with lower respiratory tract infections had a low abundance of *M. catarrhalis* compared to age-, sex- and time-matched healthy controls[20]. These findings, combined with ours, suggest that mostly *M. catarrhalis* and lower respiratory tract infections in early life seem to influence each other. Why specifically *M. catarrhalis* seems to have an effect remains open for discussion, given the different findings in other studies[20 21]. The Danish study also demonstrated that hypopharyngeal carriage is associated with an increased risk of wheezing especially in the first three years of life, and an increased risk of asthma at age 5 years[6 8]. In line with these findings, we observed that nasopharyngeal carriage with any bacteria at age 6 months was associated with an increased risk of overall wheezing until age 10 years, mostly with wheezing in the first 2 years. We however did not observe an association of airway bacterial carriage with asthma at school-age. Prospective cohort studies that used microbiome analyses showed an association of a high abundance of *Streptococcus,* but not *Moraxella* with wheeze at age 5 or 10 years[21 22]. Differences in findings, especially with regards to the association of hypopharyngeal bacterial carriage with lower respiratory tract infections and asthma between our study and the Danish study, might be explained by the higher prevalence of lower respiratory tract infections in their cohort, and assessment of asthma at an earlier age. Additionally, effects might differ between the high-risk and our general population, although we did not observe interaction of maternal asthma or atopy. Lastly, results may differ when focusing on individual pathogens, as compared to more recent studies that focuses on the entire microbiome. To date, findings suggest that bacterial carriage in infancy is associated with short-term, but not long-term respiratory health.

Underlying mechanisms

The hygiene hypothesis postulates that early-life exposure to certain micro-organisms might be protective for the risk of later-life atopic diseases[23 24]. Micro-organisms might influence the immune system, and specifically the type 1 and 2 helper T-cell balance, and regulatory T-cells,

Table 4.1.3. Associations of bacterial carriage of the upper airways with lung function and asthma at age 10 years.

	FEV_1 Z-score difference (95% Confidence Interval) n = 719	FVC Z-score difference (95% Confidence Interval) n = 719	FEV_1/FVC Z-score difference (95% Confidence Interval) n = 719	FEF_{75} Z-score difference (95% Confidence Interval) n = 719	Current asthma Odds ratio (95% Confidence Interval) n = 762
2 months					
S. aureus	0.05 (-0.12, 0.21)	0.03 (-0.13, 0.19)	0.02 (-0.16, 0.21)	0.01 (-0.16, 0.17)	0.75 (0.27, 2.03)
H. influenza	0.01 (-0.37, 0.40)	0.15 (-0.23, 0.52)	-0.28 (-0.71, 0.15)	-0.30 (-0.70, 0.07)	--
M. catarrhalis	0.01 (-0.25, 0.28)	-0.00 (-0.26, 0.26)	0.01 (-0.29, 0.31)	0.04 (-0.23, 0.30)	0.55 (0.07, 4.62)
S. pneumoniae	0.09 (-0.23, 0.41)	-0.01 (-0.32, 0.30)	0.19 (-0.16, 0.54)	0.10 (-0.21, 0.41)	0.70 (0.08, 6.14)
Any nasopharyngeal carriage	0.03 (-0.20, 0.25)	0.00 (-0.21, 0.22)	0.03 (0.21, 0.28)	-0.02 (-0.24, 0.20)	0.45 (0.09, 2.20)
6 months					
S. aureus	-0.01 (-0.18, 0.16)	0.01 (-0.15, 0.18)	-0.04 (-0.23, 0.15)	-0.06 (-0.23, 0.11)	0.84 (0.30, 2.39)
H. influenza	0.03 (-0.15, 0.20)	0.02 (-0.14, 0.19)	-0.02 (-0.21, 0.17)	-0.06 (-0.23, 0.11)	0.59 (0.19, 1.86)
M. catarrhalis	-0.02 (-0.18, 0.13)	-0.03 (-0.17, 0.12)	-0.02 (-0.19, 0.15)	-0.00 (-0.16, 0.16)	1.53 (0.65, 3.61)
S. pneumoniae	0.02 (-0.14, 0.17)	-0.01 (-0.16, 0.14)	0.04 (-0.13, 0.21)	0.04 (-0.12, 0.20)	0.42 (0.14, 1.32)
Any nasopharyngeal carriage	-0.09 (-0.24, 0.07)	-0.07 (-0.22, 0.08)	-0.02 (-0.19, 0.15)	-0.05 (-0.21, 0.10)	0.81 (0.33, 1.98)
14 months					
S. aureus	-0.13 (-0.34, 0.08)	-0.05 (-0.25, 0.15)	-0.13 (-0.35, 0.10)	-0.17 (-0.38, 0.04)	1.65 (0.58, 4.71)
H. influenza	**-0.19 (-0.35, -0.02)***	-0.14 (-0.30, 0.02)	-0.05 (-0.23, 0.13)	-0.13 (-0.30, 0.03)	**0.28 (0.08, 0.99)***
M. catarrhalis	0.12 (-0.04, 0.29)	0.10 (-0.06, 0.26)	0.01 (-0.17, 0.19)	0.06 (-0.10, 0.22)	0.91 (0.34, 2.43)
S. pneumoniae	0.07 (-0.09, 0.24)	0.07 (-0.09, 0.22)	-0.03 (-0.20, 0.15)	0.07 (-0.09, 0.23)	0.47 (0.18, 1.22)
Any nasopharyngeal carriage	0.03 (-0.14, 0.21)	0.05 (-0.12, 0.21)	-0.04 (-0.23, 0.15)	-0.02 (-0.20, 0.15)	**0.38 (0.15, 0.94)***
2 years					
S. aureus	0.13 (-0.11, 0.37)	-0.02 (-0.25, 0.21)	**0.26 (0.01, 0.51)***	0.21 (-0.02, 0.44)	1.46 (0.46, 4.63)
H. influenza	0.18 (-0.01, 0.37)	0.13 (-0.06, 0.32)	0.05 (-0.15, 0.26)	0.18 (-0.01, 0.36)	0.63 (0.20, 1.97)

Table 4.1.3. Associations of bacterial carriage of the upper airways with lung function and asthma at age 10 years. (continued)

	FEV_1 Z-score difference (95% Confidence Interval) n = 719	FVC Z-score difference (95% Confidence Interval) n = 719	FEV_1/FVC Z-score difference (95% Confidence Interval) n = 719	FEF_{75} Z-score difference (95% Confidence Interval) n = 719	Current asthma Odds ratio (95% Confidence Interval) n = 762
M. catarrhalis	0.06 (-0.12, 0.24)	0.00 (-0.17, 0.18)	0.08 (-0.11, 0.27)	0.15 (-0.02, 0.33)	0.45 (0.15, 1.39)
S. pneumoniae	0.16 (-0.02, 0.33)	0.12 (-0.05, 0.28)	0.06 (-0.13, 0.24)	0.14 (-0.03, 0.31)	0.86 (0.34, 2.19)
Any nasopharyngeal carriage	0.17 (-0.01, 0.35)	0.10 (-0.07, 0.27)	0.10 (-0.09, 0.29)	**0.21 (0.04, 0.39)***	0.68 (0.27, 1.71)
3 years					
S. aureus	0.10 (-0.12, 0.31)	0.07 (-0.15, 0.25)	0.13 (-0.09, 0.35)	0.11 (-0.10, 0.32)	1.79 (0.62, 5.17)
H. influenza	0.01 (-0.18, 0.20)	0.00 (-0.18, 0.19)	0.03 (-0.17, 0.22)	0.00 (-0.18, 0.18)	1.61 (0.57, 4.54)
M. catarrhalis	0.10 (-0.09, 0.28)	0.14 (-0.05, 0.32)	-0.04 (-0.23, 0.15)	0.05 (-0.13, 0.23)	1.38 (0.51, 3.70)
S. pneumoniae	0.09 (-0.09, 0.27)	0.10 (-0.07, 0.28)	-0.00 (-0.18, 0.18)	-0.00 (-0.17, 0.17)	1.66 (0.65, 4.25)
Any nasopharyngeal carriage	0.15 (-0.01, 0.30)	**0.15 (0.01, 0.31)***	-0.00 (-0.16, 0.16)	0.07 (-0.09, 0.22)	1.65 (0.65, 4.14)
6 years					
S. aureus	-0.01 (-0.16, 0.14)	0.02 (-0.12, 0.16)	-0.01 (-0.17, 0.15)	0.05 (-0.10, 0.19)	0.95 (0.39, 2.32)
H. influenza	0.03 (-0.17, 0.23)	0.05 (-0.15, 0.24)	-0.04 (-0.26, 0.19)	-0.03 (-0.22, 0.17)	0.96 (0.28, 3.30)
M. catarrhalis	0.10 (-0.10, 0.31)	0.13 (-0.07, 0.33)	-0.08 (-0.30, 0.15)	-0.08 (-0.28, 0.12)	1.08 (0.31, 3.73)
S. pneumoniae	-0.06 (-0.22, 0.09)	-0.12 (-0.26, 0.03)	0.10 (-0.06, 0.27)	0.08 (-0.07, 0.23)	1.10 (0.45, 2.69)
Any nasopharyngeal carriage	0.01 (-0.13, 0.14)	-0.00 (-0.13, 0.13)	0.00 (-0.14, 0.15)	0.01 (-0.13, 0.14)	1.38 (0.63, 3.03)

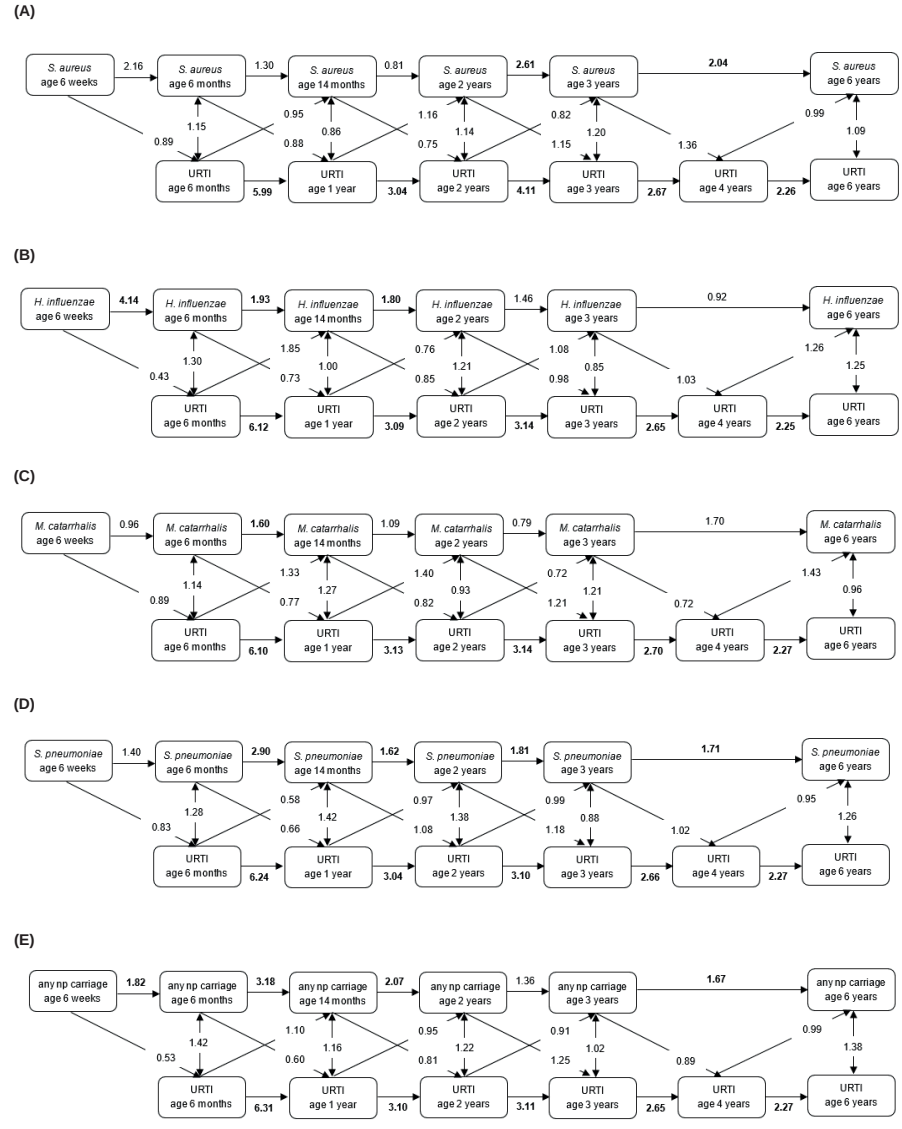
Values are odds ratios (OR) or changes in Z-score with 95% confidence interval, derived from logistic and linear regression models, respectively. *p-value <0.05, **p-value <0.01. Models are adjusted for maternal history of asthma and atopy, education level, smoking during pregnancy, stress during pregnancy, parity and pet keeping, and child's gestational age at birth, breastfeeding, daycare attendance and antibiotic use in the past 2 months (2 months), 6 months (6 months) or year (1 to 6 years). For the association of H. influenza at the age of 6 weeks with current asthma, not effect estimate could be given due to limited power, as indicated by ---. Forced Expiratory Volume in 1 second (FEV_1). Forced Vital Capacity (FVC), Forced Expiratory Flow after exhaling 75% of FVC (FEF_{75}). Information on lung function and asthma was obtained at age 10 years.

which could subsequently influence the risk of atopic diseases such as asthma[25]. Additionally, the immune system might also affect the risk of bacterial airway colonization with micro-organisms[26]. Airway bacterial carriage in early life has been associated with a low-grade systemic inflammation at age 6 months, including C-reactive protein and Interleukin-6, which could also affect the risk of wheezing or asthma[27]. Bacterial carriage might influence the risk of lower respiratory tract infections, and vice-versa[21]. However, we observed limited evidence for this. Bacterial carriage in infancy seems most prominently associated with adverse respiratory health, which might be explained by the high dynamics of bacterial carriage between 6 weeks and 6 months. Future large-scale prospective studies are needed to explore whether assessment of the microbiome as opposed to specific bacterial culturing is more informative in the association with respiratory health, and whether airway bacterial carriage and the microbiome only affect short-term or also long-term respiratory health.

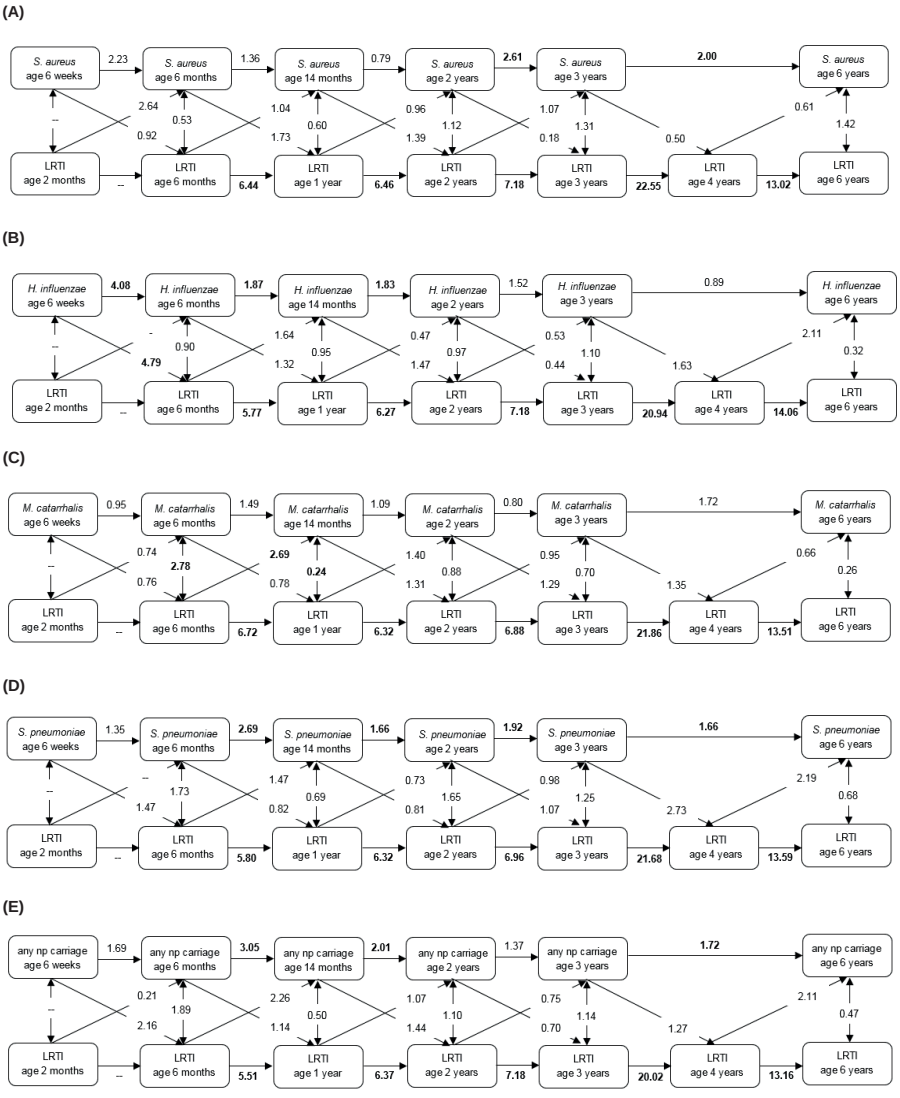
Strengths and limitations

The main strength of our study is that it is embedded in a population-based prospective cohort study with repeated assessment of airway bacterial carriage and respiratory health. Limitations include the low prevalences of a positive culture and lower respiratory tract infections in the first 6 months of life, leading to limited power. Second, only a small group of children had airway swabs taken at every visit. Therefore, we were not able to study the effect of cumulative or persistent positive swabs on respiratory health. Third, questionnaire data was used to obtain information on respiratory tract infections, wheezing and asthma. We only used information on doctor-diagnosed respiratory tract infections, and validated questionnaires for wheezing and asthma. However, variability in agreement between parents' and clinicians' report on respiratory health cannot be fully excluded, which might have led to non-differential misclassification and therefore dilution of the effect estimates[28]. Fourth, although we used standard material and commonly used methods, sensitivity of culturing might be affected. This will however most likely be operator dependent, and lead to random misclassification[29]. Lastly, we should consider the possibility that any found associations are due to chance. Although this might be the case for the associations with lung function and asthma, we believe this to be less likely for the associations with lower respiratory tract infections and wheezing, given the pattern of effects, with mostly low p-values.

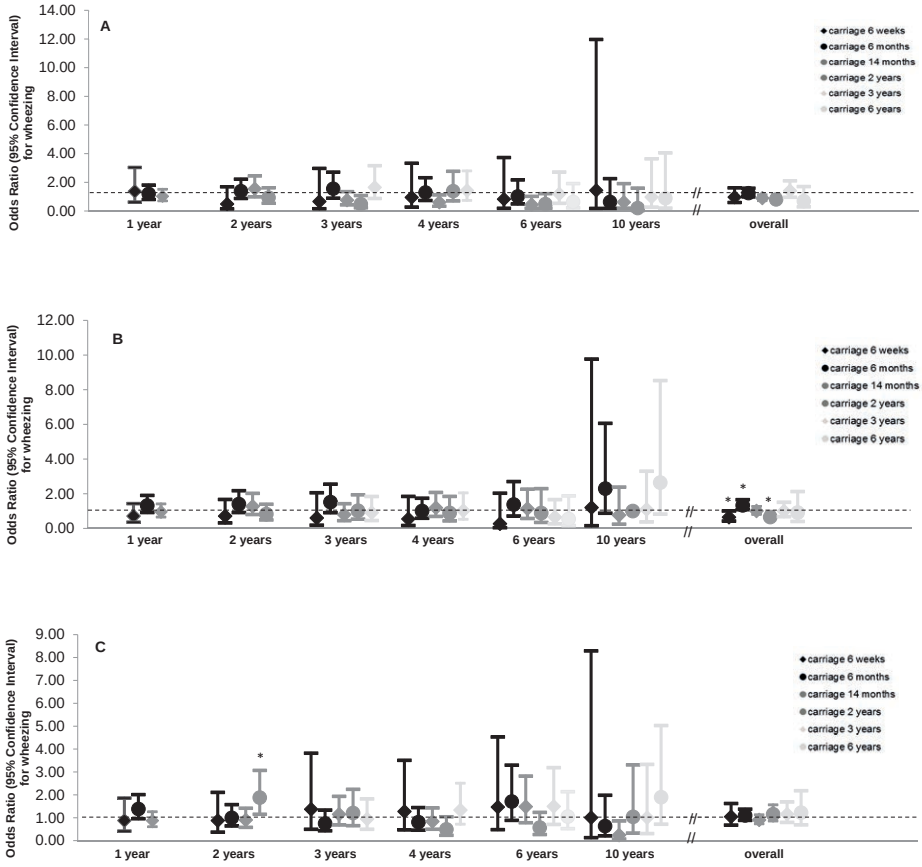
In conclusion, we observed no consistent associations in either direction between airway bacterial carriage and respiratory tract infections during childhood, or of airway bacterial carriage with lung function and asthma in later childhood. Only nasopharyngeal carriage of any bacteria at the age of 6 months was associated with an increased risk of wheezing in childhood, especially with wheezing in the first 2 years of life. This implies that airway bacterial carriage seems to have an effect on short-term effect on respiratory health only. Future studies should focus on the effect of the airway microbiome, which comprises more than individual pathogens, on short and long-term respiratory health.



Supplementary Figure S4.1.1. Associations of S. aureus (A), H. influenzae (B), M. catarrhalis (C), S. pneumoniae (D) and any nasopharyngeal carriage (E) with upper respiratory tract infections, and vice-versa. Values are odds ratios from cross-lagged models. Models are adjusted for maternal history of asthma and atopy, education level, smoking during pregnancy, stress during pregnancy, parity and pet keeping, and child's gestational age at birth, breastfeeding and daycare attendance. Bold values indicate significant associations. Upper respiratory tract infections (URTI). Nasopharyngeal (NP).



Supplementary Figure S4.1.2. Associations of S. aureus (A), H. influenzae (B), M. catarrhalis (C), S. pneumoniae (D) and any nasopharyngeal carriage (E) with lower respiratory tract infections, and vice-versa. Values are odds ratios from cross-lagged models. Models are adjusted for maternal history of asthma and atopy, education level, smoking during pregnancy, stress during pregnancy, parity and pet keeping, and child's gestational age at birth, breastfeeding and daycare attendance. Bold values indicate significant associations. For some associations, no effect estimate could be given due to limited power, as indicated by --. Lower respiratory tract infections (LRTI). Nasopharyngeal (NP).



Supplementary Figure S4.1.3. Associations of H. influenzae (A), M. catarrhalis (B) and S. pneumoniae (C) with wheezing from age 1 to 10 years. Values are odds ratios with 95% confidence interval from generalized estimating equation models. *p-value <0.05. Models are adjusted for maternal history of asthma and atopy, education level, smoking during pregnancy, stress during pregnancy, parity and pet keeping, and child's gestational age at birth, breastfeeding, daycare attendance, and antibiotic use in the past 2 months (2 months), 6 months (6 months) or year (1 to 6 years). Only airway bacterial carriage before or at the age of assessment of wheezing was included in the models, and models were additionally adjusted for any wheezing in the preceding period if the first age at which wheezing was assessed was later than 1 year of age.

Supplementary Table S4.1.1. Differences between those included and not included in the study

	Included n = 945	Not included n = 301	p-value
Maternal characteristics			
History of asthma or atopy, yes (%)	39.1 (329)	37.1 (23)	0.758
Educational level, low/middle (%)	33.7 (315)	49.8 (144)	<0.001
Smoking during pregnancy, yes (%)	12.0 (102)	17.7 (11)	0.181
Maternal psychiatric symptoms[1]	0.12 (0.00, 0.54)	0.15 (0.00, 0.88)	0.219
Parity, nulliparous (%)	63.1 (595)	52.3 (157)	0.001
Pet keeping, yes (%)	58.0 (488)	59.7 (37)	0.799
Child's characteristics			
Female sex (%)	50.9 (481)	55.7 (167)	0.150
Gestational age at birth (weeks)[1]	40.3 (37.3, 42.1)	39.9 (33.7, 41.9)	<0.001
Ever breastfeeding, yes (%)	91.0 (838)	87.1 (54)	0.306
Day care attendance 1st year, yes (%)	70.5 (577)	66.7 (34)	0.566
Antibiotic use, yes (%)			
Age 2 months	9.6 (74)	18.4 (9)	0.048
Age 6 months	23.1 (146)	30.0 (15)	0.272
Age 1 year	35.5 (308)	42.6 (23)	0.294
Age 2 years	43.3 (375)	48.1 (25)	0.500
Age 3 years	33.4 (280)	38.5 (20)	0.451
Age 4 years	26.3 (221)	27.5 (14)	0.861
Age 6 years	19.9 (176)	22.2 (12)	0.680

Values are valid percentages (absolute numbers) or [1]medians (5-95th percentiles). Differences between groups were evaluated using chi-square tests for categorical variables or Mann-Whitney U tests for non-normal distributed variables. Values are based on observed data.

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Chapter 4.2

A population-based study on associations of stool microbiota with atopic diseases in school-age children.

Hu C, van Meel ER, Medina-Gomez C, Kraaij R, Barossa M, Kiefte-de Jong J, Radjabzadeh D, Pasmans SGMA, de Jong NW, de Jongste JC, Moll HA, Nijsten TEC, Rivadeneira F, Pardo LM, Duijts L.

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ABSTRACT

Background

Infants with less diverse gut microbiota seem to have higher risks of atopic diseases in early life, but any associations at school age are unclear.

Objective

To examine the associations of diversity, relative abundance and functional pathways of stool microbiota with atopic diseases in school-aged children.

Methods

We performed a cross-sectional study within an existing population-based prospective cohort among 1,440 children aged 10 years. On stool samples, 16S rRNA gene sequencing was performed, and taxonomic and functional tables were produced. Physician-diagnosed eczema, allergy and asthma were measured by questionnaires, allergic sensitization by skin prick tests, and lung function by spirometry.

Results

Alpha diversity of stool microbiota was associated with a decreased risk of eczema (OR (95%CI): 0.98 (0.97, 1.00)), and beta diversity was associated with physician-diagnosed inhalant allergy (R2 (p-value): 0.001 (0.047)). *Lachnospiraceae*, *Ruminococcaceae_UCG-005* and *Christensenellaceae_R-7_group* species were associated with decreased risks of eczema, inhalant allergic sensitization, and physician-diagnosed inhalant allergy (OR range (95%CI): 0.88 (0.79, 0.96) – 0.94 (0.88, 0.98)), while *Agathobacter* species was associated with an increased risk of physician-diagnosed inhalant allergy (1.23 (1.08, 1.42)). Functional pathways related to heme and terpenoid biosynthesis were associated with decreased risks of physician-diagnosed inhalant allergy and asthma (OR range (95%CI): 0.89 (0.80, 0.99) - 0.86 (0.73, 1.02)). No associations of stool microbiota with lung function were observed.

Conclusions

The diversity, relative abundance and functional pathways of stool microbiota were most consistently associated with physician-diagnosed inhalant allergy in school-aged children, and less consistent with other atopic diseases.

INTRODUCTION

Atopic diseases such as eczema, allergy and asthma are a major public health concern. At school age, up to 30% of children is affected by at least one of these atopic diseases globally[1 2]. Genetic susceptibility alone does not explain the high prevalence of atopic diseases as environmental exposures, partly through changes in the developing immune system, are likely to have major influence on the development of atopic diseases[3 4]. The hygiene hypothesis suggests that the urbanization and modern public health practices lead to less microbial exposure, and thereby a less stimulated immune system, and subsequently an increased risk of eczema, allergy and asthma[4]. The gut microbiota has a prominent role in the development and regulation of the immune system[5]. Previous cohort and case-control studies have demonstrated that the diversity and relative abundance of stool microbiota, as proxy for gut microbiota, in early life are associated with the risk of atopic diseases[6]. Specifically, children with a less diverse stool microbiota before the age of 1 year, and with greater relative abundance of *Bacteroidaceae*, *Clostridiaceae* and *Enterobacteriaceae*, and lower relative abundance of *Bifidobacteriaceae* and *Lactobacillaceae*, have a higher risk of eczema, allergy or asthma until age 3 years[6]. Whether stool microbiota is also associated with atopic diseases in later childhood is less clear. Only few studies have been performed with limited power to detect the effects of diversity and differential relative abundance of stool microbiota on the risks of eczema, allergy and asthma on a population-based level. Lastly, information on the association of stool microbiota with lower lung function, one of the underlying mechanism in asthma, is lacking.

Therefore, we aimed to examine among 1,440 children participating in a prospective population-based cohort study the associations of diversity, relative abundance, and functional pathways of stool microbiota with eczema, allergic sensitization, allergy, lung function and asthma at school age.

METHODS

Design

This cross-sectional study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards in Rotterdam, the Netherlands[7]. The study has been approved by the Medical Ethical Committee of the Erasmus MC University Medical Centre Rotterdam, in Rotterdam, The Netherlands. Written informed consent was obtained from both parents or legal guardians. A total of 1,440 children with relevant data for stool microbiota and atopic diseases were available at a mean age of 9.8 years (SD: 0.27) for the current analysis.

Stool microbiota

Stool samples of children participating in the Generation R Study were collected at home using a Commode Specimen Collection System (Covidien, Mansfield, MA) and a feces collection tube (Minigrip Nederlands, Lelystad, The Netherlands) without preserving agent. Stool samples were stored at 4°C until mailing, and thereafter sent by post to the appropriate laboratory of the Erasmus MC, and locally stored at -20°C [8]. DNA was isolated in two batches from stool samples according to manufacturer's protocols (Arrow Stool DNA; Isogen Life Science; DiaSorin S.P.A.), utilizing bead beating and automated DNA isolation. Stool microbiota profiles were determined by Next Generation Sequencing (Illumina MiSeq) of the V3 and V4 variable regions of the 16S ribosomal RNA (rRNA) gene according to the protocol described by Fadrosh et. al.[9]. For current analyses, phylogenetic profiling and denoising was performed using DADA2 to produce amplicon- or exact sequence variants (ASVs)[10]. High-throughput sequencing produced compositional data, therefore, we used the term 'relative abundance' to refer to the counts of ASVs reads generated by 16S rRNA gene sequencing[11]. ASVs were assigned a taxonomy from the SILVA version 132 rRNA database using the RDP naïve Bayesian classifier[12 13]. The sequence data was then analysed for alpha diversity metrics (Chao richness index, Shannon diversity Index, and Inverse Simpson Index). Pair-wise beta diversities were calculated using Bray-Curtis dissimilarity metrics and Aitchison distance in centred log-ratio (CLR) transformed ASV relative abundances [11]. As a sensitivity analyses among the same subjects, we compared the observations of the current data processing approach using the DADA2 pipeline with the results obtained by our previously in-house developed wrapper pipeline wrapper pipeline (microRapTor) based on QIIME (version 1.9.0), TAGCleaner (version 0.16), PEAR (version 0.9.6), and UPARSE (version 8.1) software package [14-17]. Samples were subsampled at 10,000 reads and combined reads of all samples were clustered into operational taxonomic units (OTUs) using the UPARSE command *cluster_otus* at a minimum cluster identity of 97% [8]. Taxonomy was assigned to the representative read of each OTU using the SILVA rRNA database version 128 [12] and RDP Naïve Bayesian Classifier version 2.12 [13].

Atopic diseases

At age 10 years, information on current physician-diagnosed eczema, inhalant allergy (for pollen (hay fever), house dust mite, cat or dog), food allergy (for cashew nut or peanut) and asthma was obtained from a parental-reported questionnaire with questions adapted from the ISAAC core questionnaires[18 19]. Current asthma was defined as physician diagnosis of ever asthma, with asthma medication use and/or wheezing in the past 12 months. Sensitization to the most relevant inhalant and food allergens on a population level was examined by skin prick tests. Inhalant allergens included house dust mite, 5-grass mixture, birch, cat, and dog (ALK-Abelló B.V., Almere, The Netherlands), and food allergens included hazelnut, cashew nut, peanut, and peach[19 20]. Lung function was measured by spirometry at our research center according to ATS/ERS criteria, and values of forced expiratory volume in the first second (FEV_1) and forced vital capacity (FVC) were converted into sex-, height-, age-, and ethnicity-adjusted Z-scores according to GLI-reference

values[21-23]. The ratio FEV_1 and FVC was dichotomized into obstructive and non-obstructive lung function (Z-score cut-off of ≤-1.64 and >1.64, respectively)[23]. We assumed that children with two or more atopic diseases tend to have a more severe variant of their atopic diseases[24 25]. Therefore, we regrouped children into those with only one atopic disease (with any eczema, asthma or physician-diagnosed food or inhalant allergies), or two or more atopic diseases for sensitivity analyses.

Covariates

Information on maternal education (primary or secondary school; higher than secondary school) was available from parental questionnaires obtained at enrolment. Child's sex was obtained from midwives and hospital records, and ethnic origin (European; non-European) was defined based on the parents' country of birth[26]. Child's height and weight were measured during their visit to our research center to calculate their body mass index (BMI) (kg/m2). Information on the use of antibiotics in the past 3 months was derived from a questionnaire obtained during stool sample collection. We considered these covariates as most influential lifestyle and socioeconomic confounders based on literature and their associations with both the determinant and the outcome in our population [27]. Therefore, confounders such as diet, steroid medication use, recent probiotics use (past 3 days), inflammatory bowel syndrome, celiac disease, and travel activities in the past month were not included in the models. Confounders were missing in 7% or less in our population for analysis, and therefore not imputed. Furthermore, the general collection time and DNA isolation batch were considered as technical covariates as these are correlated with overall composition (beta-diversity) of stool microbiota[8].

Statistical analysis

We compared characteristics of those included and not included in our study using Pearson's Chi-square and independent sample *t*- tests. Analyzing high-throughput sequencing data is challenging, and rather than a golden standard, the use of multiple independent tools are recommended[28]. Therefore, we used both count-based and compositional approaches to examine the associations of stool microbiota with atopic diseases. All models were adjusted for lifestyle and socioeconomic confounders, and technical covariates. Unadjusted analyses are presented in Supplementary Tables 4.2.1, 4.2.2, and 4.2.3. We considered the adjusted model as our main model for interpretation of results. Functional pathways were predicted with PICRUSt2 [29 30]. The statistical analyses were performed in R version 4.0.0 [31] using the packages microbiome[32], zCompositions[33], vegan[34], and phyloseq[35], and analysis of composition of microbiomes (ANCOM) with a detection cut-off of 0.60 (Supplementary Figure 4.2.1)[36]. Measures of association are presented as odds ratios (OR) per unit increase of ASV relative abundance with their corresponding 95% confidence intervals (95%CI).

RESULTS

Subject characteristics

Table 4.2.1 shows the characteristics of the subjects. Compared with children included in the analysis, those not included had mothers who were lower educated, were more often of non-European ethnicity, and had a higher BMI (Table 4.2.1). Characteristics of the major phyla, families and genera of stool microbiota of the children included in the analyses are presented in Figure 4.2.1. Supplementary Figure S4.2.2 shows that the major phyla for children with any atopic disease and without any atopic diseases are similar.

Table 4.2.1. Characteristics of children and their mothers

	Included subjects n=1,440	**Not included subjects** n=3,187
Maternal characteristics		
Maternal education, higher % (n)	53.8 (719)	49.8 (1,436)*
Child characteristics		
Sex, female % (n)	50.3 (725)	50.4 (1,605)
Ethnicity, non-European % (n)	30.1 (430)	33.7 (1,039)*
Body mass index (BMI) at age 10 years, mean (SD)	17.4 (2.6)	17.7 (2.8)*
Antibiotic use in the past 3 months, yes % (n)	3.9 (56)	0.0 (0)
Stool sample		
Collection time in days, median (IQR)	2.00 (1.00-3.00)	1.50 (1.00-2.00)
DNA isolation batch, number 2 %(n)	89.0 (1,281)	100.0 (3)
Alpha diversity metrics, mean (SD)		
Chao index	150.6 (37.7)	150.2 (31.7)
Shannon Index	4.0 (0.41)	4.0 (0.4)
Inverse Simpson Index	31.9 (14.3)	30.1 (15.9)
Eczema, yes % (n)	7.0 (89)	7.0 (182)
Sensitization for food allergens, yes % (n)	6.4 (80)	7.2 (188)
Sensitization for inhalant allergens, yes % (n)	30.8 (385)	32.3 (842)
Physician-diagnosed food allergy, yes % (n)	2.3 (29)	2.1 (49)
Physician-diagnosed inhalant allergy, yes % (n)	11.8 (150)	12.4 (296)
Obstructive lung function, yes % (n)	5.1 (67)	4.5 (123)
Asthma, yes % (n)	5.0 (64)	6.0 (145)

Values are percentages (absolute values), mean (standard deviation (SD)) or median (interquartile range (IQR)) based on observed data. For the included subjects, data were missing for maternal education (n=103), ethnicity (n=13), BMI (n=5), eczema (n=147), sensitization for food (n=195) and inhalant allergens (n=189), physician-diagnosed food (n= 201) and inhalant allergy (n=170), lung function (n=118), and asthma (n= 172). *p-value for difference <0.05.

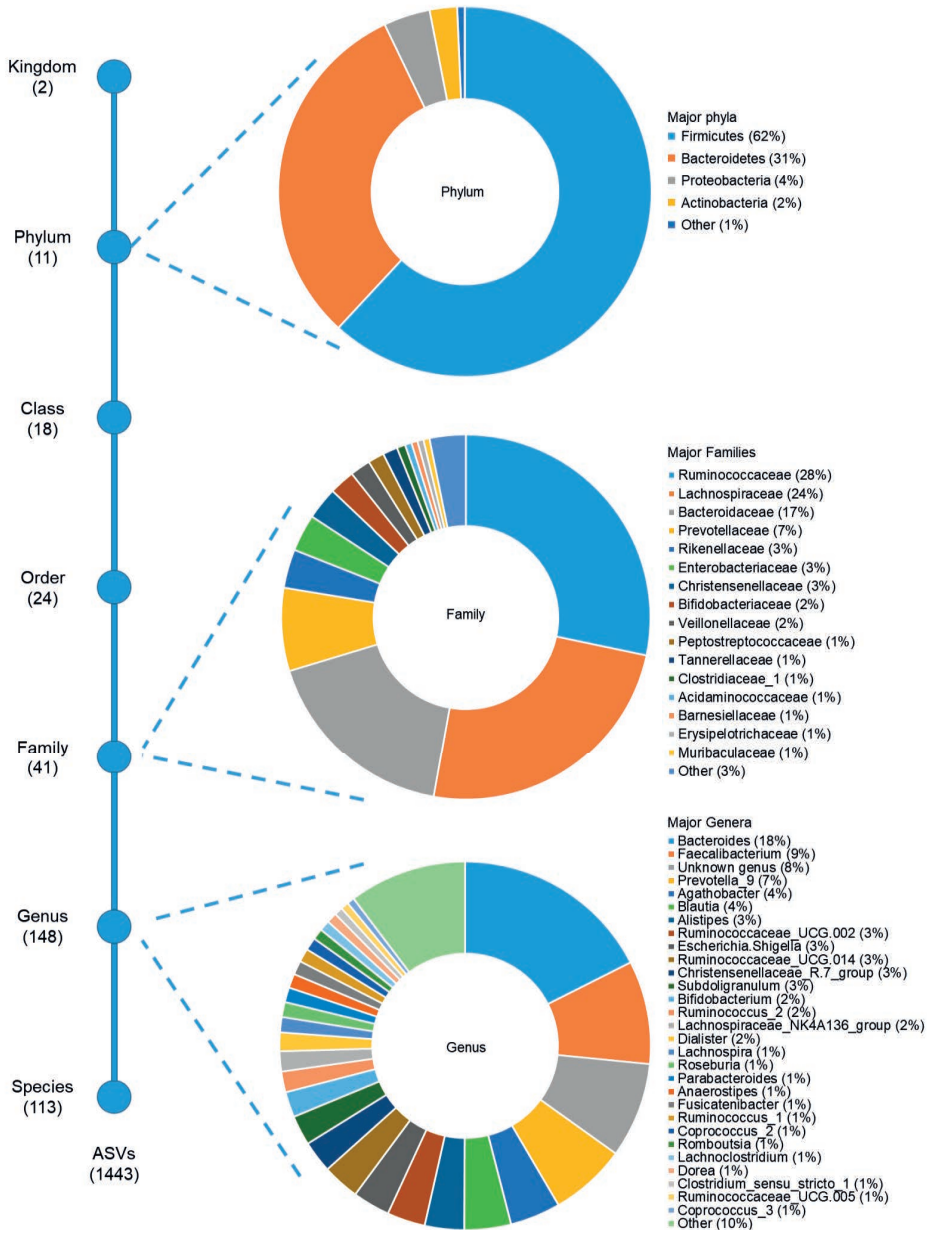


Figure 4.2.1. Characteristics of stool microbiota of the study population (n=1,440)
Left: Number of observed taxa at each taxonomy level. Within brackets represents the number of unique amplicon sequence variants (ASVs) identified in each taxonomic class.
Right: donut plots of average relative abundance of the top major phyla, families and genera and within brackets the percentage coverage of the total relative abundance.

Diversity of stool microbiota and atopic diseases

In the adjusted analyses, all alpha diversity indices and Aitchison beta diversity showed that the diversity, and overall compositional variation of stool microbiota was not different between those with and without atopic diseases, except for eczema, namely inverse Simpson index was associated with a decreased risk of eczema (OR (95%CI): 0.98 (0.97, 1.00)) (Table 4.2.2). The Bray-Curtis beta diversity showed that the overall compositional variation of stool microbiota was different between children with and without physician-diagnosed inhalant allergies only (Bray-Curtis beta-diversity R2 (adjusted p-value): 0.001 (0.047) (Table 4.2.2). We observed no associations of alpha and beta diversity of stool microbiota with two or more atopic diseases, compared to those with only one atopic disease (data not shown). Sensitivity analyses with previous in-house developed pipeline showed that alpha diversity indices and Bray-Curtis beta diversity were not associated with any atopic diseases. Aitchison beta diversity showed that overall compositional variation of stool microbiota was different between children with and without physician-diagnosed inhalant allergy (Aitchison beta-diversity R2 (adjusted p-value): 0.001 (0.014))

Differential relative abundance of stool microbiota and atopic diseases.

The associated ASVs with atopic diseases are depicted in Supplemental Figure S 4.2.3. In the adjusted ANCOM and logistic regression analyses, *Lachnospiraceae_unknowngenus_5* was associated with a decreased risk of eczema (OR (95%CI): 0.88 (0.79, 0.96)), and with a decreased risk of sensitization for inhalant allergens (0.94 (0.89, 0.98)) (Table 4.2.3). *Ruminococcaceae_UCG-005_unknownspecies_2* and *Christensenellaceae_R-7_group_unknownspecies_2* were associated with a decreased risk of physician-diagnosed inhalant allergy (0.89 (0.82, 0.96) and 0.94 (0.88, 0.99), respectively), while *Agathobacter_unknownspecies* was associated with an increased risk of physician-diagnosed inhalant allergy (1.23 (1.08, 1.42)). No associations were observed for specific ASVs of stool microbiota with sensitization for food allergens, physician-diagnosed food allergy, lung function, and asthma in the adjusted ANCOM analyses. No specific ASVs were associated with two or more atopic diseases compared to only one atopic disease in the adjusted ANCOM analyses (data not shown). Sensitivity analyses with the previous in-house developed pipeline showed similar results for *Lachnospiraceae* genus with sensitization for inhalant allergens (0.99 (0.99, 1.00)), and *Ruminococcaceae_UCG-005* species and *Christensenellaceae_R-7_group* species with physician-diagnosed inhalant allergy (0.99 (0.98, 1.00) and 1.00 (1.00, 1.00)).

Functional pathways of stool microbiota and atopic diseases

In the adjusted ANCOM and logistic regression analyses, N-acetylneuraminate catabolism was associated with a decreased risk of sensitization for food allergens (OR (95%CI): 0.71 (0.54, 0.92)) (Table 4.2.4). Ethylmalonyl-CoA, L-leucine degradation I, Adenosylcobalamin biosynthesis I (anaerobic) and Aerobic respiration I (cytochrome c) pathways were associated with a decreased risk of sensitization for inhalant allergens in the adjusted ANCOM analyses, but the associations attenuated to non-significant in the adjusted logistic regression analyses (OR range (95%CI: 0.80

Table 4.2.2. Associations of alpha and beta diversity with atopic diseases at age 10 years

	Eczema (n=1,217)	Sensitization for food allergens (n=1,156)	Sensitization for inhalant allergens (n=1,161)	Physician-diagnosed food allergy (n=1,169)	Physician-diagnosed inhalant allergy (n=1,197)	Obstructive lung function (n=1,229)	Asthma (n=1,192)
Alpha diversity	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***
Chao index	1.00 (0.99, 1.01)	1.00 (1.00,1.01)	1.00 (1.00, 1.00)	1.00 (0.99, 1.01)	1.00 (0.99, 1.00)	0.99 (0.99,1.00)	1.00 (0.99,1.01)
Shannon index	0.75 (0.45, 1.30)	1.46 (0.78,2.88)	1.03 (0.75, 1.42)	0.81 (0.35, 2.14)	0.78 (0.51, 1.20)	0.76 (0.44,1.40)	0.85 (0.46,1.68)
Inverse Simpson index	0.98 (0.97, 1.00)*	1.01 (0.99,1.03)	1.00 (0.99, 1.01)	0.99 (0.96, 1.02)	0.99 (0.98, 1.00)	0.99 (0.97,1.00)	0.99 (0.97,1.01)
Beta diversity	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***
Bray-Curtis	0.001 (0.552)	0.001 (0.964)	0.001 (0.683)	0.001 (0.230)	0.001 (0.047)*	0.001 (0.548)	0.001 (0.559)
Aitchison	0.001 (0.787)	0.001 (0.703)	0.001 (0.494)	0.001 (0.368)	0.001 (0.119)	0.001 (0.216)	0.001 (0.850)

Values are odds ratios (95% confidence intervals) from adjusted logistic regression models for alpha diversity, and R-squared (Benjamin Hochberg -adjusted p-values) from adjusted PERMANOVA analysis for 999 iterations for beta diversity. Models were adjusted for maternal education, child's sex, ethnicity, body mass index at age 10 years, antibiotic use in the past 3 months, DNA isolation batch and general collection time. *p-value <0.05, **p-value<0.01.

Table 4.2.3. Differential relative abundance of stool microbiota and atopic diseases at age 10 years

	Amplicon sequence variants (ASV)	Odds ratio (95%CI)
Eczema	Lachnospiraceae unknowngenus_5	0.88 (0.79, 0.96)**
Sensitization for inhalant allergens	Lachnospiraceae unknowngenus_5	0.94 (0.89, 0.98)**
Physician-diagnosed inhalant allergy	Ruminococcaceae_UCG-005 unknownspecies_2	0.89 (0.82, 0.96)**
	Christensenellaceae_R-7_group unknownspecies_2	0.94 (0.88, 0.99)*
	Agathobacter unknownspecies	1.23 (1.08, 1.42)**

The number of subjects included for the adjusted ANCOM and adjusted logistic regression analyses for eczema (n=1,217), sensitization for food allergens (n=1,156), sensitization for inhalant allergens (n=1,161), physician-diagnosed food allergy (n=1,169), physician-diagnosed inhalant allergy (n=1,197), lung function (n=1,229), and asthma (n=1,192). Models were adjusted for maternal education, child's sex, ethnicity, body mass index at age 10 years, antibiotic use in the past 3 months, DNA isolation batch and general collection time, and other associated Amplicon sequence variants (ASVs) with the outcome of interest. No associations were found for stool microbial ASVs with sensitization for food allergens, physician-diagnosed food allergy, lung function, and asthma at age 10 years. *p-value <0.05, **p-value<0.01.

Table 4.2.4. Associations of functional pathways of stool microbiota with atopic diseases at age 10 years

	Functional pathway	Odds ratio (95%CI)
Sensitization for food allergens	N-acetylneuraminate catabolism; sialic acid degradation	0.71 (0.54, 0.92)**
Sensitization for inhalant allergens	Ethylmalonyl-CoA pathway	0.80 (0.60, 1.08)
	L-leucine degradation I pathway	0.91 (0.75, 1.09)
	Adenosylcobalamin biosynthesis I (anaerobic) pathway	1.16 (0.98, 1.41)
	Aerobic respiration I (cytochrome c) pathway	1.01 (0.94, 1.07)
Physician-diagnosed food allergy	L-lysine biosynthesis II pathway	0.67 (0.52, 0.89)**
Physician-diagnosed inhalant allergy	Taxadiene biosynthesis	0.89 (0.78, 1.01)
	Geranylgeranyldiphosphate biosynthesis I (via mevalonate)	0.90 (0.83, 0.98)*
	Superpathway of heme b biosynthesis from glycine	0.92 (0.85, 0.99)*
	Methylphosphonate degradation I pathways	0.94 (0.85, 1.06)
Asthma	Superpathway of heme b biosynthesis from glycine	0.89 (0.80, 0.99)*
	L-glutamate degradation V (via hydroxyglutarate)	0.88 (0.79, 0.99)*
	Taxadiene biosynthesis	0.86 (0.73, 1.02)

The number of subjects included for the adjusted ANCOM and adjusted logistic regression analyses for eczema (n=1,217), sensitization for food allergens (n=1,156), sensitization for inhalant allergens (n=1,161), physician-diagnosed food allergy (n=1,169), physician-diagnosed inhalant allergy (n=1,197), lung function (n=1,229), and asthma (n=1,192). Models were adjusted for maternal education, child's sex, ethnicity, body mass index at age 10 years, antibiotic use in the past 3 months, DNA isolation batch and general collection time, and other associated pathways with the outcome of interest. No associations were found for stool microbial functional pathways with eczema, and lung function at age 10 years. *p-value <0.05, **p-value<0.01.

(0.60, 1.08) - 1.16 (0.98, 1.41)). L-lysine biosynthesis II pathway was associated with a decreased risk of physician-diagnosed food allergy (0.67 (0.52, 0.89)) in the adjusted ANCOM and logistic regression analyses. Geranylgeranyldiphosphate biosynthesis I (via mevalonate) and superpathway of heme b biosynthesis from glycine were associated with a decreased risk of physician-diagnosed inhalant allergy (0.90 (0.83, 0.98) and 0.92 (0.85, 0.99), respectively), while the identified pathways of Taxadiene biosynthesis and Methylphosphonate degradation I from adjusted ANCOM analyses attenuated to non-significant in the adjusted logistic regression model (0.89 (0.78, 1.01) and 0.94 (0.85, 1.06), respectively). The superpathway of heme b biosynthesis from glycine, and L-glutamate degradation V (via hydroxyglutarate) were associated with a decreased risk of asthma (0.89 (0.80, 0.99) and 0.88 (0.79, 0.99), respectively), while the association of Taxadiene biosynthesis with a decreased risk of asthma in adjusted ANCOM analyses attenuated to non-significant in the adjusted logistic regression (0.86 (0.73, 1.02)). No functional pathways of stool microbiota were associated with eczema or lung function. No specific functional pathways were associated with two or more atopic diseases compared to only one atopic disease (data not shown).

DISCUSSION

In this population-based prospective cohort study among children aged 10 years, we observed that the diversity, relative abundance, and functional pathways of stool microbiota were associated with physician-diagnosed inhalant allergy. Associations of diversity, relative abundance, and functional pathways of stool microbiota with other atopic outcomes were less consistent.

Comparison with previous studies

Compared to our previous study on diversity, compositional, and functional differences of stool microbiota of children and adults, the top four major phyla and the top 10 major families remained the same, and of the top most abundant genera 24 were also in the top 30 genera of the in the current study population[8]. In addition, we gained more unique ASV numbers on each taxonomic level using the DADA2 pipeline with the final dataset, including 1443 different ASVs in the current study compared to 661 operational taxonomic units in our previous study. We observed that inverse Simpson index (alpha diversity) of stool microbiota was associated with a decreased risk of eczema only, and not with other current atopic diseases at age 10 years. We observed that the overall composition of stool microbiota, based only on Bray-Curtis beta diversity, is different in children with and without physician-diagnosed inhalant allergy at the age of 10 years. A recent systematic review of previous cohort and case-control studies performed in children mostly before the age of 6 months showed associations of alpha diversity of stool microbiota with eczema, inhalant and food allergies, and asthma, while studies performed in older children using modern sequencing methods showed conflicting results[6]. Therefore, the relative abundance of stool microbiota might be more important in atopic diseases than the diversity at school age. The ASVs

that we identified as being associated with atopic diseases at 10 years were in line with results of previous studies among children younger than 5 years These demonstrated that genera from *Lachnospiraceae, Ruminococcaceae*, and *Prevotellaceae* families, and higher taxonomic order of *Clostridiaceae* measured at ages 0-2 years were associated with atopic diseases at ages 0-8 years[6]. However, the direction of associations between specific stool microbiota and atopic diseases was not consistent. For example, the family of *Lachnospiraceae* and the order of *Clostridiaceae* were associated with both increased and decreased risks of atopic diseases[6]. The differences in results of diversity and relative abundance of stool microbiota with atopic diseases in our and previous studies might be due to differences in characteristics of children included in the analysis, such as age and geographic location, and the prospective or cross-sectional design. It might be more challenging to find associations of stool microbiota with atopic diseases in older children, since the complexity of gut microbiota increases with age, despite its stabilization in later life[8]. Also, the heterogeneity of the bioinformatics and statistical approaches of the studies due to the compositional nature of stool microbiota data, and limitation of sequencing technique could explain the differences in results[11]. Homogenization of microbiota tables is needed to adequately compare results of gut microbiota with atopic diseases between different studies. Future studies should analyse gut microbiota at lower taxonomic levels, because specific microbial species within a genus may influence atopic diseases differently.

No specific functional pathways of stool microbiota were consistently associated with all atopic diseases, which might be explained by differences in the etiology of atopic diseases, and only a small proportion of children that follows the atopic march in our population-based cohort [37-39]. The most consistent observations included functional pathways related to heme and terpenoid biosynthesis, which were associated with decreased risks of physician-diagnosed inhalant allergy and asthma. Taxadiene biosynthesis, Geranylgeranyldiphosphate biosynthesis I (via mevalonate) and Mevalonate pathway I (eukaryotes and bacteria) are part of the pathways class of biosynthesis of terpenoids, which are a class of naturally occurring organic compounds, derived from five carbon isoprene units, and often used in cosmetics, pharmaceuticals, or biofuels[40]. Murine studies showed that mevalonate biosynthesis plays a role in T-helper 2 cell differentiation, and that inhibiting mevalonate pathway can lower the allergic inflammation and airway hyperreactivity, making it a possible novel therapeutic target for atopic diseases[41 42]. This is in line with findings of a recent review suggesting that terpenoids have anti-inflammatory effects, and might be effective in treating respiratory inflammation and atopic dermatitis[43]. Although the specific superpathway of heme b biosynthesis from glycine has not been previously found to be associated with atopic diseases, hemeoxygenase-1 (HO-1) protein, which catabolizes heme to biliverdin, free iron and carbonoxide, is increased in murine lung tissue in allergic airway inflammation and in skin lesions of patients with eczema[44 45]. In addition, iron-deficiency has also been related to increased risk of atopic diseases, possibly through changes in the gut microbiome[46]. Interestingly, we observed that N-acetylneuraminate catabolism (also known as sialic acid degradation) pathway was associated with a decreased risk of sensitization of food allergens. Further, this finding might explain

the increased sialic acid content that a recent study found on total IgE from individuals with a peanut-allergy as compared to those without any allergies[47]. We did not observe an association of N-acetylneuraminate catabolism with physician-diagnosed food allergy, which might be due to the used definition of allergy. Allergic sensitization measured by skin prick tests reflects children who are sensitized but partly do not experience symptoms of food allergy. Another interesting observation included the association of L-glutamate degradation V (via hydroxyglutarate) with a decreased risk of current asthma. Via this pathway, bacteria are able to ferment amino acids into short chain fatty acids (SCFA) among other products. This observation supports the hypothesis of SCFA having anti-inflammatory properties, and its association with decreased risks of atopic diseases[48 49].

Interpretation of results

To the best of our knowledge, our study is one of the few studies to examine the associations of stool microbiota with atopic diseases in school-age children from the general population. Our results contribute to the body of knowledge on the role of gut microbiota in the development of atopic diseases in later childhood. We observed no consistent associations of gut microbiota with eczema, allergy and asthma using DADA2 pipeline and our previously in-house developed pipeline, which suggests that the role of gut microbiota on atopic diseases might be limited in later childhood. The relation between gut microbiota and atopic diseases might be influenced by the severity of the condition, steroid use, and persistence of atopic diseases. Therefore, observations might be different in hospital-population or at an individual based level, when children have multiple and persistent atopic diseases, and greater disease severity. Furthermore, due to the cross-sectional design of our study, we could not examine the influence of early life gut microbiota on later life gut microbiota, and subsequently atopic diseases in later life. Our observations that diversity and relative abundance of stool microbiota were not prominently associated with atopic diseases at school age support the hypothesis of an 'early window of opportunity' in which the gut microbiota plays an more important role in the maturation of the immune system in early childhood, and to a lesser extent in later childhood[50]. This has also been suggested by murine studies that showed that changes in gut microbiota in neonatal mice, but not in adult mice are associated with the of development of atopic diseases[51 52]. Gut microbiota protects the host from potential pathogenic colonization, contributes to the intestinal barrier function, and helps develop and regulate the immune system[5]. Especially in early childhood, when maturation of the gut and immune system is still ongoing, disruption of systems contributing to the maturation of the gut and immune system might increase the risk of developing atopic diseases. T-regulatory cells play an important role in the immune responses to allergens, the regulation of type 2 T-helper cells, and the production of immunoglobulin type E[53]. Gut microbiota can induce the differentiation of T-regulatory cells, and reduce pro-inflammatory cytokines through different pathways, such as via the production of short-chain fatty acids leading to activation of G protein–coupled receptors signaling pathways and epigenetic changes[50]. Also, gut microbiota might affect atopic diseases

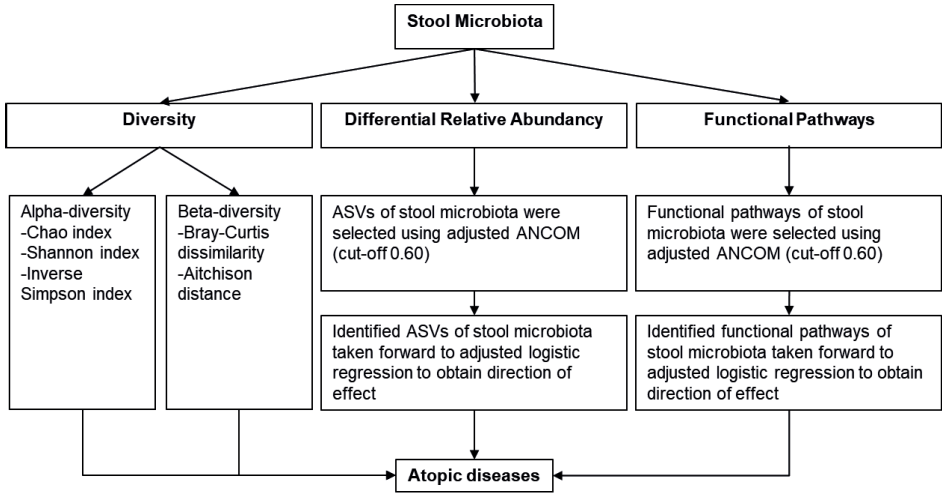
through interaction with other microbiota, such as on the lung or skin[54 55]. Gut bacteria and their metabolites can migrate to the blood, the lymphatic system, skin, and lung via an impaired intestinal barrier, and can produce short chain fatty-acids that can exhibit antimicrobial effects against for example Staphylococcus aureus, which on the skin and nose has been associated with increased risk of eczema and eczema severity[54 55]. Our study was designed cross-sectionally and prospective cohort studies should examine the role of early life microbiome and its longitudinal development on later life atopic diseases.

Strengths and limitations

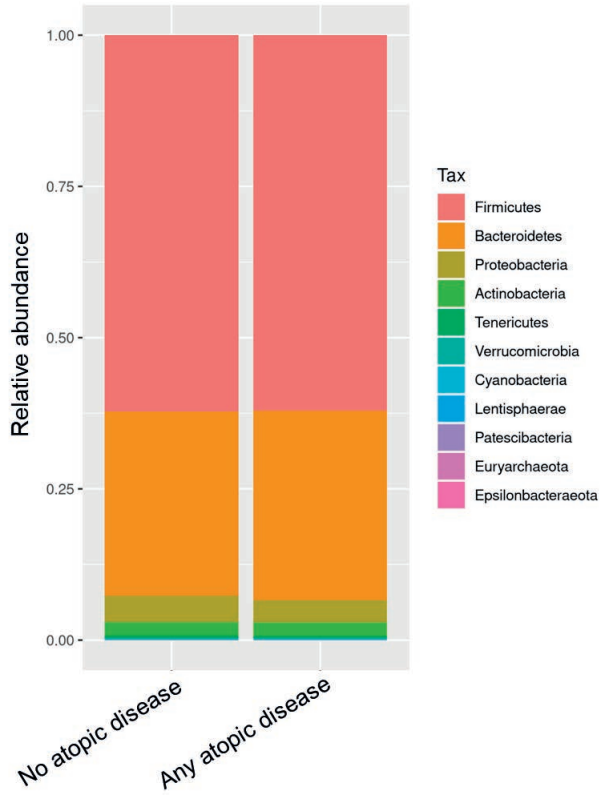
The strengths of this study include its cross-sectional design within a well-characterized population-based prospective study, a large number of subjects, and examining stool microbiota with atopic diseases at school age using a combination of compositional and non-compositional approaches. Also, we used novel and open source microbiome analyses tools such as DADA2 and PICRUSt2, which enables better comparison with other (future) studies, since the used DADA2 pipeline produced sequences reads with counts that can be compared directly across studies[10 29]. However, limitations of this study should also be considered in the interpretation of the results. Children not included in the analyses had less favourable socio-economic factors, were more often of non-European ethnicity, and had a higher BMI, which could have resulted in selection bias if the associations of gut microbiota with atopic diseases would have been different in children included and not included in the analysis. Non-differential misclassification of eczema, asthma and physician-diagnosed allergies remains possible due to collection via self-reported questionnaires and might have led to dilution of results. We used validated questionnaires to minimize this bias[18 56]. In addition, residual confounding might still be present as in any observational study. Besides inhaled asthma medication, no information was available on steroid use. However, on a population level most atopic diseases are of mild severity, and therefore the effect of steroid use on the associations of stool microbiota with atopic disease will most likely be constrained. Further, although the 16S rRNA gene amplification and shotgun metagonomics approaches provide different information, the phylogeny and biomolecular function have been shown to be strongly correlated[30]. Shotgun metagenomic sequencing would allow greater precision in the functional predictions obtained from PICRUSt2 (28). Unfortunately, metagenomic profiling was not available considering the high costs of performing shotgun sequencing in our large population-based cohort._Also, high inter-individual variability of the gut microbiota and high-dimensionality of the microbiota data can hinder the power of analysis. Uniform and harmonized methodological approaches for better identification and comparison of results between large-scale studies are urgently needed[57]. Lastly, although we consider the applied methods as the most appropriate following the current standards set in the field, analytical tools in the field of microbiota are still developing and as such results may depend on the method applied. While microbiome profiling methods continue to evolve further, our study already provides some leads that await replication to confirm the involvement of the gut microbiota in the etiology of atopic diseases.

Conclusion

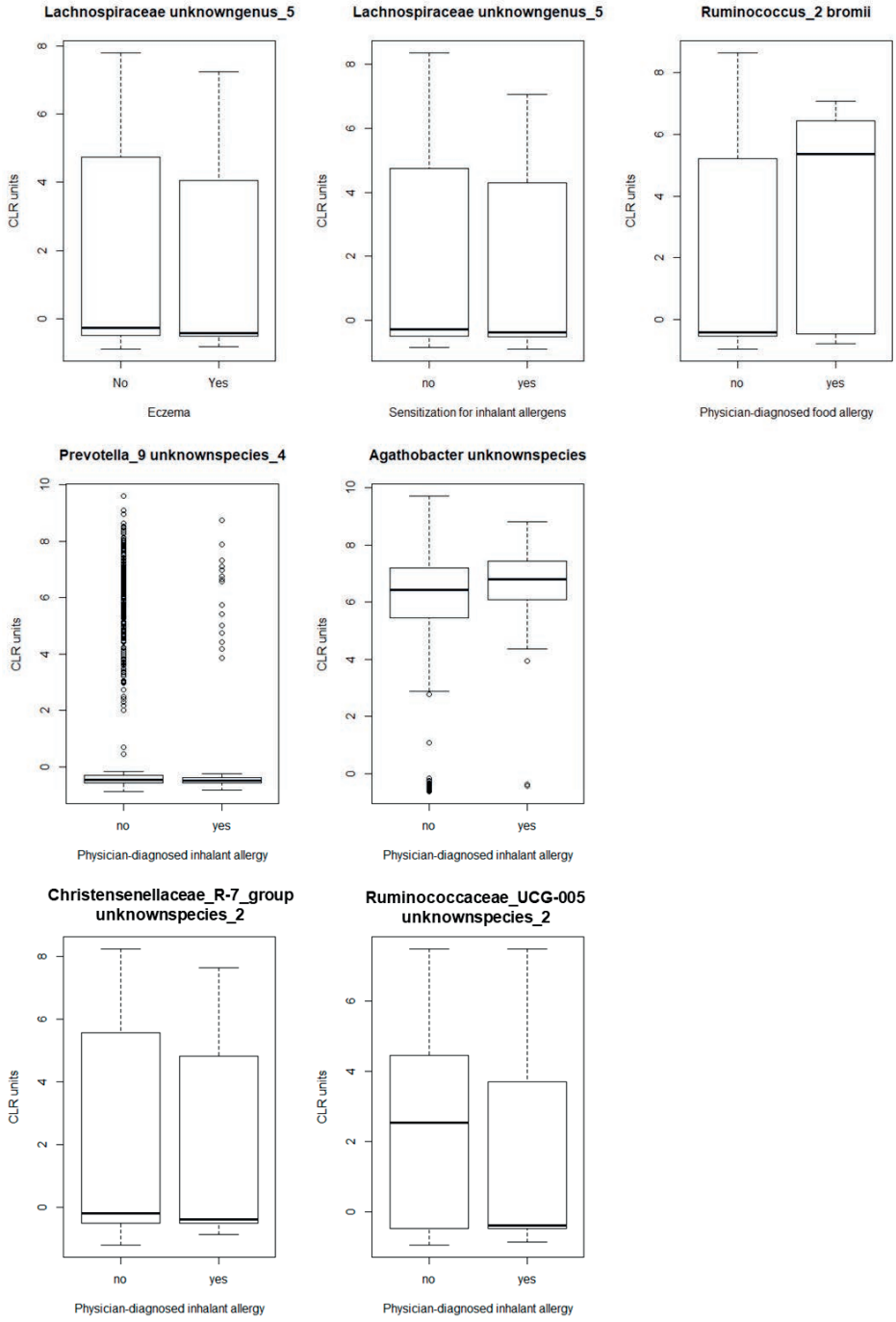
We observed that the diversity, relative abundance and pathways derived from stool microbiota were consistently associated with physician-diagnosed inhalant allergy, and less consistent with current eczema, food allergy outcomes and asthma at school age. The role of stool microbiota on atopic diseases therefore seems limited in later childhood. Despite the relatively large sample of our study, we cannot exclude that weak associations with other atopic diseases were not detected given the large dimensionality and heterogeneity of the stool microbiome data, and the low prevalence of some of the atopic diseases. Additionally, the effect of the stool microbiota on atopic diseases with a different etiology might differ. Future large-scale studies should repeatedly examine the longitudinal associations of stool microbiota and atopic diseases from infancy to school-age. This might clarify any age specific influences of gut microbiota on the development of atopic diseases throughout childhood.



Supplementary Figure S4.2.1. Study design



Supplementary Figure S4.2.2. Relative abundance of phyla for children with any atopic disease outcome compared to those without any atopic diseases.



Supplementary Figure S4.2.3. Associated ASVs from adjusted ANCOM analyses for each atopic disease

Supplementary Table S4.2.1. Unadjusted associations of alpha and beta diversity with atopic diseases at age 10 years

	Eczema	Sensitization for food allergens	Sensitization for inhalant allergens	Physician-diagnosed food allergy	Physician-diagnosed inhalant allergy	Obstructive lung function	Asthma
	(n=1,293)	(n=1,245)	(n=1,251)	(n=1,239)	(n=1,270)	(n=1,322)	(n=1,268)
Alpha diversity	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***	***Odds Ratio (95%CI)***
Chao index	1.00 (0.99, 1.00)	1.00 (1.00,1.01)	1.00 (1.00, 1.00)	1.00 (0.99, 1.01)	1.00 (0.99, 1.00)	0.99 (0.99,1.00)	1.00 (0.99,1.00)
Shannon index	0.69 (0.44, 1.14)	1.12 (0.65,2.04)	0.98 (0.73, 1.32)	0.95 (0.42, 2.47)	0.79 (0.54, 1.19)	0.90 (0.52,1.64)	0.63 (0.38,1.11)
Inverse Simpson index	0.98 (0.96, 1.00)*	1.01 (1.00,1.01)	1.00 (0.99, 1.01)	0.99 (0.97, 1.02)	0.99 (0.98, 1.00)	0.99 (0.97,1.01)	0.98 (0.96,1.00)*
Beta diversity	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***	***R^2 (p-value)***
Bray-Curtis	0.001 (0.453)	0.001 (0.988)	0.001 (0.434)	0.001 (0.283)	0.001 (0.014)*	0.001 (0.843)	0.001 (0.473)
Aitchison	0.001 (0.375)	0.001 (0.756)	0.001 (0.400)	0.001 (0.401)	0.001 (0.057)	0.001 (0.645)	0.001 (0.381)

Values are odds ratios (95% confidence intervals) from unadjusted logistic regression models for alpha diversity, and R-squared (Benjamin Hochberg -adjusted p-values) from unadjusted PERMANOVA analysis for 999 iterations for beta diversity. *p-value <0.05, **p-value<0.01. n=number of children included in the analysis.

Supplementary Table S4.2.2. Unadjusted differential relative abundance of stool microbiota and atopic diseases at age 10 years

	Amplicon sequence variants (ASVs)	Odds ratio (95%CI)
Eczema	Lachnospiraceae unknowngenus_5	0.86 (0.79, 0.94)**
Sensitization for inhalant allergens	Lachnospiraceae unknowngenus_5	0.93 (0.89, 0.97)**
Physician-diagnosed food allergy	Ruminococcus_2 bromii	1.16 (1.03, 1.30)*
Physician-diagnosed inhalant allergy	Ruminococcaceae_UCG-005 unknownspecies_2	0.87 (0.81, 0.93)**
	Christensenellaceae_R-7_group unknownspecies_2	0.92 (0.87, 0.97)**
	Agathobacter unknownspecies	1.26 (1.11, 1.45)**
	Prevotella_9 unknownspecies_4	0.88 (0.80, 0.95)**

The number of subjects included for the unadjusted ANCOM and unadjusted logistic regression analyses were for eczema (n=1,293), sensitization for food allergens (n=1,245), sensitization for inhalant allergens (n=1,251), physician-diagnosed food allergy (n=1,239), physician-diagnosed inhalant allergy (n=1,270), lung function (n=1,322), and asthma (n=1,268). No associations were found for stool microbial Amplicon sequence variants (ASVs) with sensitization for food allergens, lung function, and asthma at age 10 years. *p-value <0.05, **p-value<0.01.

Supplementary Table S4.2.3. Unadjusted analysis of functional pathways of stool microbiota with atopic diseases at age 10 years

	Functional pathway	Odds ratio (95%CI)
Sensitization for inhalant allergens	Ethylmalonyl-CoA pathway	0.91 (0.85, 0.96)**
	L-leucine degradation I pathway	0.91 (0.86, 0.97)*
	Adenosylcobalamin biosynthesis I (anaerobic) pathway	0.94 (0.90, 0.98)**
	Aerobic respiration I (cytochrome c) pathway	0.95 (0.91, 0.99)*
	Glycerol degradation to butanol pathway	1.07 (1.01, 1.14)*
Physician-diagnosed food allergy	L-lysine biosynthesis II pathway	0.67 (0.52, 0.89)**
Physician-diagnosed inhalant allergy	Taxadiene biosynthesis	0.82 (0.73, 0.92)**
	Geranylgeranyldiphosphate biosynthesis I (via mevalonate)	0.92 (0.86, 0.99)*
	Mevalonate pathway I (eukaryotes and bacteria)	0.92 (0.85, 0.99)*
	Peptidoglycan biosynthesis V (β-lactam resistance)	0.95 (0.90, 1.00)
	(S)-propane-1,2-diol degradation	1.09 (1.01, 1.19)*
Asthma	Superpathway of heme b biosynthesis from glycine	0.87 (0.79, 0.96)**
	L-glutamate degradation V (via hydroxyglutarate)	0.87 (0.79, 0.97)*
	4-aminobutanoate degradation V	0.76 (0.61, 0.97)*

The number of subjects included for the unadjusted ANCOM and unadjusted logistic regression analyses were for eczema (n=1,293), sensitization for food allergens (n=1,245), sensitization for inhalant allergens (n=1,251), physician-diagnosed food allergy (n=1,239), physician-diagnosed inhalant allergy (n=1,270), lung function (n=1,322), and asthma (n=1,268). No associations were found for stool microbial functional pathways with eczema, sensitization for food allergens, and lung function at age 10 years. *p-value <0.05, **p-value<0.01.

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Chapter 4.3

The role of respiratory tract infections and the microbiome in the development of asthma: A narrative review.

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ABSTRACT

Asthma is a common disease in childhood, and might predispose for chronic obstructive respiratory morbidity in adolescence and adulthood. Various early-life risk factors might influence the risk of wheezing, asthma and lower lung function in childhood. Cohort studies demonstrated that lower respiratory tract infections in the first years of life are associated with an increased risk of wheezing and asthma, while the association with lung function is less clear. Additionally, the gut and airway microbiome might influence the risk of wheezing and asthma. The interaction between respiratory tract infections and the microbiome complicates studies of their associations with wheezing, asthma and lung function. Furthermore, the causality behind these observations is still unclear, and several other factors such as genetic susceptibility and the immune system might be of importance. This review is focused on the association of early-life respiratory tract infections and the microbiome with wheezing, asthma and lung function, it's possible influencing factors and perspectives for future studies.

INTRODUCTION

Asthma is a common disease in childhood and has a worldwide prevalence of 5-10%[1]. In preschool children, the prevalence of asthma related symptoms, such as wheezing and shortness of breath, is even higher[1]. Results of birth cohort studies of the last decades suggest that part of the origins of childhood asthma occur in early life[2-10]. Lower lung function or asthma in childhood might predispose to chronic obstructive respiratory diseases, including COPD, in adolescence and adult life[11 12]. Therefore, identifying risk factors in early life is important to understand the development of lower lung function and asthma and allow prevention of disease. Recent studies suggest that early-life respiratory tract infections could lead to airway obstruction and hyperreactivity[13], and subsequently increased risk of persistent lower lung function and asthma. Also, exposure to microorganisms seems associated with either an increased risk or lower risk of atopic diseases, depending on the type of bacteria, the composition of the microbiome, or both.

In this review, we describe the current knowledge from cohort studies on the role of early-life respiratory tract infections and the microbiome in the development of asthma.

RESPIRATORY TRACT INFECTIONS

Respiratory tract infections occur most frequently in early childhood, which is also the age with the most rapid development of the immune and respiratory system[14-16]. The prevalence varies per type of respiratory tract infection. In developed countries, the incidence of pneumonia in children until the age of 5 years is 0.05 episodes per child year[17]. Symptoms of an upper respiratory tract infection, such as sore throat and rhinorrhea, in children until the age of 5 years are more common, with a reported prevalence of 5.06 episodes per child year in the USA[18].

The diagnosis of early-life respiratory tract infections in studies that examine the relation of respiratory tract infections with wheezing, asthma or lung function varies largely, and is mainly divided into respiratory tract infections diagnosed in the laboratory or physician-diagnosed. For laboratory proven respiratory tract infections, nasal samples are taken either during symptoms of an acute respiratory tract infection[19-27], or at scheduled time points[19 24 28]. These are mainly analyzed for either human rhinovirus (HRV) or respiratory syncytial virus (RSV), the most common viral pathogens, and more rarely for other viruses, such as adenovirus, coronavirus or influenza virus. The difference in timing of sampling could potentially lead to biased results, since the prevalence of a virus during an acute respiratory infection is most likely not comparable to the prevalence of a virus during an asymptomatic period. Studies that used physician-diagnosed respiratory tract infections in early-life as outcomes used either questionnaires or standardized registries. Some prospective cohort studies used questionnaires in which parents were asked whether their child has had a specific infection in a specific period of time[29-31]. Alternatively, studies use ICD-codes or hospital (admission) registries to identify children with respiratory tract

infections[32-37]. Such differences in definitions could lead to differences in effect sizes, which is also illustrated by a cohort study that compared RSV infections in children that visited the outpatient clinic, the emergency department or who were hospitalized[34] in relation to asthma diagnosis. Children with RSV infections in these 3 different clinical groups had a 1.86, 2.41 and 2.82 fold increased risk for asthma, respectively, compared with children without a hospital visit for RSV infections. This is in line with another retrospective cohort study that showed that children with RSV infections who were at the outpatient clinic or prolonged hospitalized had a 1.38 vs 2.59[21] increased odds for recurrent wheeze at the age of 5 years, respectively, compared with those without an RSV infection. The use of questionnaires or registries has the advantage of easily accessible data at relatively low cost, but could possibly lead to misclassification. Viral sampling is more reliable in terms of accurate diagnosis, but whether sampling during symptoms of an acute respiratory tract infection is comparable to sampling at scheduled times is unclear. Additionally, costs will be higher and logistics are more complex. Thus, in large observational population-based cohort studies, questionnaires or registries are cost-effective methods to assess respiratory tract infections, while smaller studies using viral sampling provide information on specific agents.

In preschool children, asthma is difficult to diagnose and mainly based on the occurrence of wheezing. Previous population-based or high risk cohorts showed that HRV, RSV, or bronchiolitis in the first 1-3 years of life are associated with an up to 13-fold increased risk of preschool wheezing[20-23 28 30 38]. One cohort study showed that hospitalization for RSV was associated with an increased risk of recurrent wheezing at the age of 18 years[39]. Cohort studies or case-control studies that focused on childhood asthma at age 4 to 13 years showed that HRV, RSV or bronchiolitis in the first year, the first two or the first three years of life were associated with an increased risk of asthma with odds ratios ranging from 1.39 to 13.55[19 29 32-37 40]. Some studies examined a group of, and not individual, respiratory tract infections only. A case-control study showed that hospitalization for any respiratory tract infection in the first year of life was associated with a 1.5-fold increased risk of asthma at the age of 5 years[35], while a high-risk cohort study demonstrated that wheezy and febrile lower respiratory tract infections in the first year of life were associated with an increased risk of asthma at the age of 5 of 10 years. However, these latter associations seemed only present when the child had allergic sensitization before the age of 2 years[41 42]. It has also been suggested that the number of respiratory tract infections, rather than a specific infection, is associated with an increased risk of asthma[43]. Thus, it might be that individual and type of respiratory infections are less important for the development of preschool wheezing or childhood asthma than groups and number of respiratory tract infections.

Only a few studies have examined the effect of early-life respiratory tract infections on lung function. Population-based cohorts, but also a high-risk cohort and a case-control study showed that respiratory tract infections in the first or the first three years of life were associated with a lower lung function, specifically Forced Expiratory Volume in 1 second (FEV_1), Forced Expiratory Flow after exhaling 50% of the Forced Vital Capacity (FEF_{50}) , FEV_1/FVC, Midexpiratory Flow (FEF_{2575}) or Peek Expiratory Flow (PEF) mostly at age 6-8 years[24-26], although one prospective

cohort study assessed lung function multiple times between the ages of 11 to 26 years[44]. The decrease in lung function varied between –62.8 to –80 mL for absolute values, and –2.5 to –20% for percentage predicted values. However, two population-based cohorts found no associations of lower respiratory tract infections before the age of 2 years or pneumonia or whooping cough in the first 7 years of life with lung function at the age of 10 years[31], or decline in FEV_1 between the age of 35 and 45[45], respectively. It could be argued that respiratory tract infections in later childhood might not have the same adverse effect as in earlier childhood, explaining why no association was found in the latter study. Further studies on the role of early respiratory tract infections with later life wheezing, asthma and lung function are warranted.

The vast majority of the studies focused on respiratory tract infections in the first 1-3 years of life. It is speculated that respiratory tract infections in early life most probably have the greatest adverse effect, since both the immune system and the respiratory system are still under development in this stage of life[15,16]. Early-life respiratory tract infections could lead to a disturbance in the development of both systems leading to persistent adaptations, and risk of respiratory morbidity in later life.

It remains unclear whether lower respiratory tract infections influence the risk of lower lung function, asthma and wheezing, or vice versa. Some evidence on causality is provided by two randomized controlled trials (RCT) comparing palivizumab with placebo in preterm infants. Both demonstrated that compared with the placebo, palivizumab decreased the risk of later wheezing, either in the first year of life[46] or at the age of 2 to 5 years[47]. However, some studies that measured early-life lung function suggest the opposite direction of causality. One prospective cohort study showed that increased bronchial responsiveness in infancy was associated with increased risk of severe bronchiolitis[48] and another showed that children with a lower respiratory system compliance, and higher resistance at the age of 2 months, were at greater risk for hospitalization for an RSV infection and wheeze after the infection[25]. However, the latter study group demonstrated that the association of HRV in the first year of life with wheezing at the age of 4 years remained significant after adjusting for lung function measurements at the age of 2 months[28]. Additionally, another cohort study demonstrated that lower respiratory tract infections in the first year of life were associated with an increased respiratory rate at 1 year of age, and that recurrent lower respiratory tract infections were associated with a lower tidal volume and increased lung clearance index, irrespective of lung function measured at the age of 6 weeks[49]. To examine the direction of causality, further longitudinal studies with detailed information on respiratory tract infections and lung function measures early and later in life are urgently needed to provide more insight in the causal direction of these associations.

THE MICROBIOME

The microbiome could be defined as the community of micro-organisms living in or on the human body[50]. Two locations of the microbiome are of great interest in relation to wheezing, asthma and lung function, namely the airway and gut microbiome. Analyses of bacterial communities are mostly performed by 16S rRNA gene sequencing, identifying different bacteria. Challenges in measuring the airway microbiome lie in the relative low density of bacterial communities when compared to the gut microbiome. Additionally, the lower airway microbiome is difficult to sample and carries the risk of contamination of the upper respiratory tract microbiome[51]. When studying the microbiome, it is possible to focus on microbiome diversity or composition, or on specific bacteria. For microbiome diversity, the richness and evenness of species is estimated, for example by using the Shannon index or the Simpson's diversity index[52]. Here, richness reflects the number of different species, while evenness reflects how even these different species are distributed. Another approach is to model bacterial community compositions, to form distinguishable groups[53]. If specific bacteria are used as the exposure, it is possible to focus on bacterial groups or specific bacteria. Some of these bacteria might have a beneficial effect on wheezing, asthma and lung function,, such as *Bifidobacterium* spp, whilst others might have a negative effect, such as *Clostridium difficile*. The use of different methods for the determination of the microbiome makes it difficult to compare studies. Also, the question remains whether the focus should be on specific bacteria, or rather on the total composition of the microbiome. Characterizing specific bacteria is used more commonly, and is less complicated in terms of both analysis and interpretation but is likely to be an over simplification of the health effects of the entire microbial composition.

Airway microbiome

The composition of the airway microbiome changes in the first years of life. Nasopharyngeal samples in healthy subjects around the age of 2 months are mostly dominated by *Staphylococcus* and *Corynebacterium*, but this frequency decreases with increasing age. In contrast, *Alloicoccus* and *Moraxella* colonization is low at the age of 2 months, but is increased at the age of 12 months[54]. Interestingly, when an acute respiratory illness had occurred in between the two sample periods, a transition to *Moraxella* or a stable colonization with *Moraxella* was most commonly present. Other than respiratory illness, exposure to pets, daycare attendance, siblings and antibiotic use in the four weeks prior to sampling were associated with nasopharyngeal colonization, showing mostly higher rates of *Haemophilus* and *Moraxella* colonization. Additionally, a prospective cohort study collected samples of the hypopharyngeal microbiome at the age of 1 week, 1 month and 3 months, and showed that the microbiome at the age of 1 week represented over 60% of the microbiome at 3 months[55]. Thus, although various factors can influence the airway microbiome, it is also likely that the microbiome formation in later life is determined by early colonization.

Various airway microbiota have been linked to later life asthma and wheezing. A prospective high-risk cohort study showed that *Streptococcus* colonization at the age of 2 months was as-

sociated with an increased risk of chronic wheeze at the age of 5 years[54], while another prospective cohort study showed that in neonates, *S. pneumoniae, M. catharallis* and *H. influenza* were associated with an increased risk of wheezing at the age of 5 years[56]. Additionally, some cross-sectional studies have compared the lung microbiome obtained by bronchoalveolar lavage or broncho-epithelial brush of asthmatics and healthy controls. Those studies showed that the bronchi of both children and adults with asthma contained more *Haemophilus spp*[57], and that that the bacterial concentration and diversity were higher in adults with suboptimally controlled asthma[58], compared with children or adults without asthma. To date, it is nclear whether differences in the microbiome of the airways and lungs precede asthma, or whether the disease itself is the cause of these changes.

Gut microbiome

The composition of the gut microbiome changes during the first years of life, mostly as an effect of the changing diet in the same period[59]. The introduction of supplementary feeding, the introduction of solids and the start of weaning[60] are important time periods in which the microbial composition changes. These time periods should be taken into account when deciding at which age to sample feces for measuring the gut micriobiome, or when interpreting results of different studies.

Previously published human studies have linked the gut microbiome with the development of atopic diseases such as asthma. A prospective cohort study showed that *C difficile* colonization at the age of 1 month was associated with an increased risk of recurrent wheeze until the age of 2 years, but also with eczema and atopic sensitization at the same age[61]. However, only the presence of *C difficile*, not the concentration in the feces was associated with these outcomes. Other bacteria, such as bifobacteria, *B fragilis* species, *E coli* and lactobacilli were not associated with recurrent wheeze. A birth cohort study characterized the bacterial composition at the age of 1 month and 6 months within three distinct groups[53]. The group with a lower abundance of bacteria such as Bifidobacteria and Lactobacillus, but a higher abundance of fungi such as Candida had the highest risk of asthma, predominantly multisensitized atopy at the age of 2 years, and asthma at the age of 4 years. No difference was observed between the three groups in the risk of atopy at the age of 2 years, defined as an IgE level above 0.35 IU ml^{-1}. A comparison of stool samples at the age of 4 years between non-wheeze, non-sensitized controls and wheezy-sensitized cases showed no difference in the microbiome composition[62]. Both a prospective cohort and a substudy of an RCT showed that the gut microbiome until the age of 1 month is also associated with asthma development at the age of 6 or 7 years[63 64]. The microbiome at the age of 12 months however was not associated with asthma. In summary, previous studies support the hypothesis that mostly the microbiome in early life is important for the development of wheezing and asthma at a later age[63].

RESPIRATORY TRACT INFECTIONS AND THE AIRWAY MICROBIOME

It is likely that respiratory tract infections and the airway microbiome influence each other. Respiratory tract infections could have an effect on the microbiome both during, and after the infection[65]. Additionally, it is possible that the microbiome colonization of the airway could increase the risk of a subsequent respiratory tract infection[66]. Hypopharyngeal colonization with *S. pneumoniae, H. influenza or M.* catharralis in the first 3 years of life is associated with an increased risk of pneumonia and bronchitis at the age of 4 years, providing evidence for the latter. It has also been shown that early *Streptococcus* colonization is associated with an increased risk of a lower respiratory tract infection at an earlier age, while *Moraxella* colonization is associated with an upper respiratory tract infection at an earlier age[54]. These findings suggest that respiratory tract infections might not only influence the airway microbiome, but also vice versa. This further complicates the relationship of either with wheezing, asthma and lung function, and the understanding of this association.

INFLUENCING FACTORS

Several factors could be of influence in the associations of respiratory tract infections or the microbiome with wheezing, asthma and lung function. The difference between the influencing factors is of importance in the matter of possible prevention or treatment strategies.

Environmental factors

Mode of delivery has been suggested as a possible intermediate factor in the relationship between the microbiome and risk of asthma[67-72]. Children born through a caesarian section are likely to have a lower gut microbiome diversity, especially in the first three months of life. Mode of delivery might also influence the airway microbiome, although the association of mode of delivery with the airway microbiome has been studied less. Antibiotic use could be related to both the associations of respiratory tract infections with wheezing, asthma and lung function, and to the associations of the microbiome with these outcomes. Respiratory tract infections could lead to increased antibiotic use, which thereby has as an intermediate effect on wheezing, asthma and lung function[73 74]. The use of antibiotics could also have an effect on the composition of the gut microbiome, and therefore act as a confounder in the associations of the microbiome with asthma or wheezing[75-77]. Confounding by indication or reverse causation can complicate studies on the effect of the microbiome. For example, it has been suggested that the use of antibiotics is a results of confounding by indication, meaning that only respiratory tract infections themselves have an effect on wheezing, asthma and lung function, and that a observed adverse effect of antibiotic use on these outcomes is solely because of the direct relation between the use of antibiotics and respiratory tract infections[74].

Intrinsic factors

An explaining factor for the associations of respiratory tract infections with wheezing, asthma and lung function is genetic susceptibility. The 17q21 locus, the strongest known susceptibility locus for asthma, was demonstrated to be associated with HRV wheezing in early life as well, but not with RSV wheezing illness[78]. Similarly, CDHR3 gene variation increases risk of childhood asthma with severe exacerbations[79], with an increased susceptibility to rhinovirus C infections as a possible underlying mechanism[80]. Genetic differences in immune response to infections are also of interest. Some single nucleotide polymorphisms (SNPs) have been identified to be associated with respiratory tract infections and asthma or airway hyperreactivity, including picornavirus with atopic asthma and airway hyperreactivity, and RSV with atopic asthma and airway hyperreactivity[81]. A cohort study of children with RSV demonstrated that genes coding for the Interleukin (IL) pathway, specifically IL4 which might promote allergic inflammation and asthma, are associated with both RSV infection and wheezing, suggesting a potential role for genetic differences in immune responses to infections[82]. The immune system could also be an explaining factor in the associations of the microbiome with asthma and wheezing. The gut-associated lymphoid tissue is an important factor in the immune system, and plays a role in the development of the gastrointestinal immune system[83]. Additionally, the birth cohort study that demonstrated that one of three distinct groups of bacterial composition of the gut microbiome was associated with a higher risk of asthma and atopy, also showed that this same group was associated with $CD4^+$ cell dysfunction. Specifically, $CD4^+IL4^+$ cells are upregulated, as is the concentration of IL-4 released, which could contribute to airway inflammation[53]. This could possibly mean that the risk of asthma and wheezing due to differences in the gut microbiome might be mediated by differences in the immune system. The immune system could be a true underlying causal factor in these associations, meaning that the immune system could influence both the risk of respiratory tract infections or alter the microbiome, and influence the risk of wheezing, asthma and lung function separately. Alternatively, the immune system might be a mediating factor in the association of respiratory tract infections or the microbiome with wheezing, asthma and lung function. Further studies on the role of the immune system are needed to disentangle these associations.

The associations between respiratory tract infections and wheezing, asthma and lung function might be modified by some factors such as the atopic status characterized by sensitization or paternal asthma or atopy. In a prospective cohort study, associations of wheezy or febrile lower respiratory tract infections was only found if children were sensitized by the age of 2 years, defined as a positive skin prick test for either food or inhalant allergens[41 42]. Some differences in the effect estimates for bronchiolitis with asthma were also found when children with and without atopic parents were compared, although for both groups the effect estimates were significant (odds ratios 3.11 versus 1.66)[40]. The role of genetics, the immune system and atopy in the associations of the microbiome or respiratory tract infections with wheezing, asthma and lung function should be explored further.

FUTURE RESEARCH

The role of respiratory tract infections and the microbiome has been of increasing interest in the past decade, but population-based cohort studies on its associations with wheezing, asthma and lung function are scarce. Population-based prospective cohort studies with frequent measures of respiratory tract infections and the microbiome in early life are needed to better understand the causal pathway between respiratory tract infections and the microbiome, and the development of chronic obstructive respiratory diseases in later life. Although the associations of respiratory tract infections with wheezing, asthma and lung function has been studied more commonly, the question still remains whether observed associations are causal, or whether children with a lower lung function at birth or at risk of asthma are more likely to develop respiratory tract infections before asthma occurs. Additional to frequent early-life measures of respiratory tract infections and the microbiome, population-based prospective cohort studies with lung function measurements in infancy could provide more insight. Large studies or meta-analyses might be of interest to take heterogeneity or small effect estimates into account. Studies should also focus on the relationship between the microbiome and respiratory tract infections, and their combined associations with chronic obstructive respiratory diseases. Additional to bacteria and viruses influencing each other, it is also possible that there is an interaction between bacteria and fungi in the airway. Studies on this subject, as well as the role of fungi alone, are scarce and should be addressed in future studies. Also, contamination of lower respiratory tract sampling with bacteria from the upper respiratory tract remains a problem. However, it has also been suggested that in healthy persons, the bacterial communities in the upper and lower respiratory tract resemble each other. RCT's within this research area are scarce, but could provide more insight in the causal pathway. Identifying true causal factors, as opposed to confounding and intermediating factors, is the first step towards development of preventive strategies or specific treatment options.

CONCLUSION

Results of cohort studies of the last decades show that mostly lower respiratory tract infections in the first years of life are associated with an increased risk of wheezing and asthma and lower lung function in later life. The presence of specific bacteria in the airway and gut in the first years of life, or the composition of the gut microbiome seem associated with an increased risk of wheezing and asthma only. The airway microbiome and respiratory tract infections could influence each other, which complicates their relation as well as the understanding of their associations with later life chronic obstructive respiratory diseases (Figure 4.3.1). More detailed population-based, prospective cohort studies taking influencing factors into account, as well as RCT's, are needed to further study the causal effect of respiratory tract infections and the microbiome in early life on later life wheezing, asthma and lung function. Specifically, longitudinal analysis of both the

gut and airway microbiome to identify critical periods, early lung function measurements to establish causality in the association of respiratory tract infections with wheezing, asthma and lung function, and the relation between the microbiome, respiratory tract infections and the immune system are needed. This could ultimately allow targeted treatment and prevention strategies aiming at reducing respiratory morbidity at the long term.

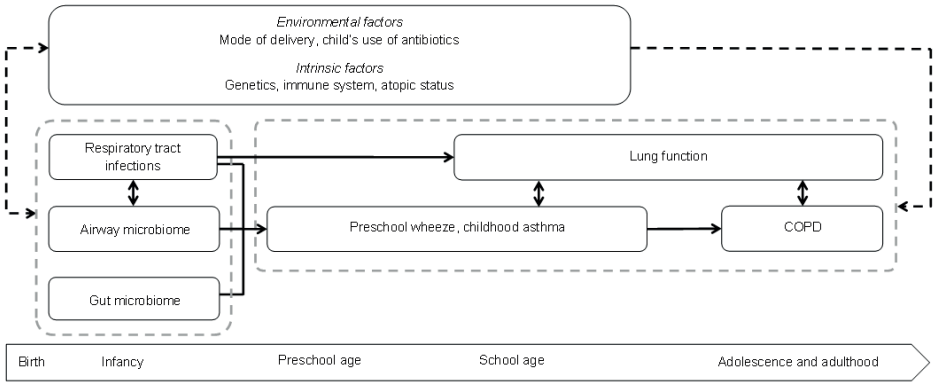

Figure 4.3.1. Pathways leading from respiratory tract infections and the microbiome in early life, to chronic obstructive respiratory diseases across the life course, and influencing factors. Chronic obstructive pulmonary disease (COPD).

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Chapter 5

General discussion and summary

INTRODUCTION

It is suggested that asthma has its origins in early life[1-4]. Early life exposures could lead to developmental adaptations of the immune and respiratory system, that increase the risk of lower lung function and asthma.

The main aim of this thesis was to identify early-life exposures leading to an increased risk of adverse respiratory health in childhood. The main results, strengths and limitations of these studies have been discussed in the previous chapters. In this chapter, a general discussion of the main findings of this thesis and their interpretation will be given. Additionally, this chapter will provide a discussion on general methodological issues, and will conclude with the clinical implications and suggestions for future research.

MAIN FINDINGS AND THEIR INTERPRETATION

Fetal and infant exposures

Maternal psychological distress during pregnancy has previously been associated with an increased risk of wheezing and asthma in early childhood[5-7]. These findings might reflect intra-uterine mechanisms as well as unmeasured confounding factors. Additionally, it is unclear whether these effects persist in later childhood. Therefore, we assessed the association of both maternal and paternal psychological distress during pregnancy with school-age lung function and asthma. We demonstrated that maternal psychological distress during pregnancy was consistently associated with a 1.5 to 2-fold increased risk of school-age asthma in the offspring, while paternal psychological distress during pregnancy was not (Table 5.1). Additionally, maternal overall psychological distress and maternal depressive symptoms were associated with a lower FEV_1 and FVC in the children. The observed associations were not mediated by parental psychological distress after pregnancy, or childhood lower respiratory tract infections. These findings suggest that maternal psychological distress during pregnancy might have direct intra-uterine effects on fetal lung development and later-life respiratory morbidity. Possible underlying biological mechanisms could be an impaired development of the fetal HPA axis or increased corticotrophin releasing hormone secretion due to maternal psychological distress, leading to developmental adaptations of the lungs[8 9].

Previous studies have shown an association of breastfeeding with respiratory tract infections, wheezing and asthma[10-12]. In this thesis, we examined the association of breastfeeding with lung function and asthma at school age. We demonstrated that a shorter duration and non-exclusive breastfeeding were associated with a lower FEV_1 and FVC (Table 5.1). Breastfeeding was not associated with asthma at school age. This might indicate that breastfeeding is only associated with short-term respiratory health, such as preschool wheezing and early-life respiratory tract infections. This could be explained by a diminishing protective effect of breastfeeding over time,

or a different effect for atopic and non-atopic asthma, since asthma at a later age is more likely to be atopic. Additionally, these findings suggests that the use of detailed breastfeeding measures, as opposed to examining ever breastfeeding only, is of importance. Although previous studies suggested that the association between breastfeeding and childhood asthma might be mediated by lower respiratory tract infections, our study showed that the observed associations were not. Differences in these findings might potentially be due to misclassification of asthma at an earlier age .

Thus, these findings suggest that both maternal psychological distress during pregnancy and duration and exclusivity of breastfeeding are of importance in the development of childhood respiratory health. These associations are not mediated by lower respiratory tract infections, suggesting that these exposures have a direct effect on respiratory health rather than an indirect effect mediated by increased susceptibility to respiratory infections. Future studies might specifically focus on the role of preventive strategies to improve mental health during pregnancy, and the association with offspring health.

Table 5.1. Overview of the results presented in this thesis on fetal and infant exposures and school-age lung function and asthma.

	Lung function				
	FEV_1	**FVC**	**FEV_1/FVC**	**FEF_{75}**	**Asthma**
Maternal stress					
Overall	=	=	=	=	↑
Anxiety	=	↓	=	=	↑
Depression	↓	↓	=	=	↑
Breastfeeding					
Never	=	=	=	=	=
Shorter	↓	↓	=	=	=
Non-exclusive	=	↓	=	=	=

Lung function was measured by spirometry, and asthma by questionnaire at age 10 years. FEV_1: Forced Expiratory Volume in the first second. FVC: Forced Vital Capacity. FEF_{75}: Forced Expiratory Flow after exhaling 75% of the FVC. . A ↑ represents a positive association, a ↓ a negative association, and a = that no association was observed.

Fetal and childhood infections

Respiratory tract infections could lead to airway sensitization, and subsequently airway hyperreactivity and adverse respiratory health[13-15]. We studied the associations of respiratory tract infections with school-age asthma and lung function in two studies using data of one population-based prospective cohort, the Generation R study, and of 38 cohorts of an international collaboration. In the Generation R Study, we found that lower respiratory tract infections in early childhood were associated with an increased risk of lower lung function and asthma (Table 5.2). By using

cross-lagged models, we demonstrated that lower respiratory tract infections were associated with wheezing, but not vice-versa. By performing a meta-analysis, we were able to use individual participant data from the general European population. This method allowed harmonization of the data, usage of the same set of confounders and therefore more powerful analyses. We demonstrated that early-life upper respiratory tract infections were associated with an increased risk of school-age asthma, and early-life lower respiratory tract infections with increased risks of school-age lower FEV_1, FEV_1/FVC and FEF_{75}, and asthma (Table 5.2). Results were not modified by wheezing in early-life suggesting that these associations are present irrespective of possible early-life susceptibility to asthma. It is likely that both upper and lower respiratory tract infections have an effect on the immune system through adapted T-helper-2 and regulatory T-cell responses, which could subsequently lead to an increased risk of asthma[16]. Additionally, lower respiratory tract infections might have a more direct effect on the lungs through disruption of the normal lung development and growth, specifically in the small airways. This could in its turn lead to a lower lung function, predominantly airway obstruction and airflow limitation. Within the Generation R Study, we were also able to study possible bidirectional associations of upper and lower respiratory tract infections with wheezing, asthma and lung function. We observed that lower respiratory tract infection until the age of 3 years were most strongly and consistently associated with and increased risk of lower lung function and asthma at school age (Table 5.2). These associations were not found for lower respiratory tract infections after the age of 3 years, or upper respiratory tract infections at any age. Additionally, due to cross-lagged modelling, we observed that these associations were not bidirectional, i.e. wheezing was not associated with an increased risk of lower respiratory tract infections at a later age. This concurs with the finding from the meta-analysis that lower respiratory tract infections might influence the risk of lower lung function and asthma, irrespective of possible early-life susceptibility to asthma.

Specific infections might, next to respiratory tract infections, in specific periods influence the immune system, and subsequently lead to an increased risk of respiratory disease. First, we studied the association of maternal *Chlamydia trachomatis* infection during pregnancy, the most common sexually transmitted disease, with childhood respiratory health. *Chlamydia trachomatis* during pregnancy has been associated with an increased risk of prematurity and short-term respiratory health such as neonatal pneumonia[17 18]. However, the association with long-term respiratory health is unknown. We observed that maternal *Chlamydia trachomatis* infection during pregnancy was associated with an 1.5-fold increased risk of childhood wheezing, lower lung function, and a 2-fold increased risk of asthma at school age (Table 5.2). *Chlamydia trachomatis* infection during pregnancy was not associated with an increased risk of childhood lower respiratory tract infections. The association of maternal *Chlamydia trachomatis* during pregnancy with wheezing was partly explained by birth characteristics, such as gestational age at birth, while the association with lung function and asthma was not. This could indicate that the mediating effect of birth characteristics is only present on short-term respiratory health. Other mechanisms explaining the association of *Chlamydia trachomatis* during pregnancy with

wheezing, asthma and lung function could be an intra-uterine effect on the immune system of the fetus, leading to increased production of interleukins[19]. Additionally, *Chlamydia trachomatis* during pregnancy might have a direct effect on the lung, as shown by an increase in alveolar diameter in mice infected during the neonatal period[20]. Lastly, maternal *Chlamydia trachomatis* infection during pregnancy might cause chorio-amnionitis, which is associated with both preterm birth and an increased risk of asthma and lower lung function[21 22]. Further studies are needed to assess whether screening and treatment for *Chlamydia trachomatis* during pregnancy influences short- and long-term respiratory health.

Table 5.2. Overview of the results of studies presented in this thesis on fetal and childhood infections and childhood respiratory health.

	Lung function				Symptoms and disease		
	FEV_1	**FVC**	**FEV_1/FVC**	**FEF_{75}**	**LRTI**	**Wheezing**	**Asthma**
Respiratory infections							
Generation R Study							
URTI	=	=	=	=	N.A.	↑	=
LRTI	↓	↓	↓	↓	N.A.	↑	↑
Meta-analysis							
URTI	=	=	=	=	N.A.	n.s.	↑
LRTI	↓	=	↓	↓	N.A.	n.s.	↑
Maternal Chlamydia	=	=	↓	↓	=	↑	↑
Herpesviridae							
EBV	=	=	=	=	n.s.	n.s.	=
CMV	=	=	=	=	n.s.	n.s.	=
EBV and CMV combined	=	=	=	=	n.s.	n.s.	=

Lung function was measured by spirometry at age 10 years. Lower respiratory tract infections and wheezing were measured by repeated questionnaires from age 2 months to 6 years, and age 1 to 10 years, respectively. Asthma was measured by questionnaire at age 10 years. FEV_1: Forced Expiratory Volume in the first second. FVC: Forced Vital Capacity. FEF_{75}: Forced Expiratory Flow after exhaling 75% of the FVC. URTI: upper respiratory tract infections. LRTI: lower respiratory tract infections. EBV: Epstein-Barr virus. CMV: Cytomegalovirus N.A.: not applicable. n.s.: not studied in this thesis. . A ↑ represents a positive association, a ↓ a negative association, and a = that no association was observed.

Second, we assessed the association of Epstein-Barr virus and Cytomegalovirus infection with school-age lung function and asthma. Infections with Epstein-Barr virus and Cytomegalovirus are common in childhood. Both have a different immune response, and therefore infection with specific combinations of viruses might lead to disbalanced T-helper-cell mediated responses[23]. Subsequently, this could affect the risk of respiratory diseases. This is supported by a study that demonstrated that Cytomegalovirus in the absence of Epstein-Barr virus only is associated with an increased risk of atopy, but not asthma[24]. We assessed the association of Epstein-Barr virus, Cytomegalovirus and specific combinations of these viruses with lung function, asthma

and inhalant allergic sensitization at school age. In the unadjusted analyses only, seropositivity for Epstein-Barr virus was associated with a higher FEV_1 and FEF_{75}, while seropositivity for both Epstein-Barr virus and Cytomegalovirus only was associated with a higher FEV_1, FVC and FEF_{75} . Only Cytomegalovirus in the absence of Epstein-Barr virus was associated with an increased risk of inhalant allergic sensitization. However, after adjustment for confounders, all these associations attenuated into non-significant (Table 5.2). This was mostly explained by ethnicity of the child for associations of Epstein-Barr virus and Cytomegalovirus with lung function measures, and by all confounders for the association of Cytomegalovirus only with inhalant allergic sensitization. The results indicate no associations of Epstein-Barr virus and Cytomegalovirus with lung function, asthma and inhalant allergic sensitization. Rather, confounding factors such as socio-demographic and health-related factors, lifestyle-related factors and ethnicity are the explanation. Further studies should focus on the role of the age at acquired EBV and CMV infection, and specifically co-infections, on their association with lung function, asthma and inhalant allergic sensitization.

By studying these exposures, we were able to demonstrate the importance of early-life infections in the development of respiratory health. Future studies should focus on underlying mechanisms, and targets for possible preventive strategies.

Childhood bacterial carriage and the microbiome

Airway bacterial carriage during infancy with *Haemophilus influenzae, Moraxella catarrhalis* and *Streptococcus pneumonia* has been associated with an increased risk of lower respiratory tract infections, wheezing and asthma, specifically until 5 years of age[25 26]. However, airway bacterial carriage might have bidirectional associations with both upper and lower respiratory tract infections in the relation with childhood asthma. In this thesis, we demonstrated that airway bacterial carriage and upper respiratory tract infections do not influence each other (Table 5.3). Also, airway bacterial carriage and lower respiratory tract infections do not have consistent bidirectional associations, although *M. catarrhalis* and *H. influenzae* show some bidirectional associations with lower respiratory tract infections in the first year of life. Nasopharyngeal carriage with any bacteria was associated with an increased risk of wheezing, especially in the first year of life, while no consistent association of any airway bacterial carriage with school-age lung function and asthma was found. These findings, combined with previous studies, suggest that airway bacterial carriage might have an effect on short-term respiratory health only, and not on long-term respiratory health. This could suggest that bacterial carriage, as opposed to the entire microbiome does not appropriately reflect the complexity of the association of bacteria with respiratory health.

Recently, advanced laboratory analyses have enabled us to study the composition of the entire microbiome, as opposed to single pathogens. The gut microbiome in infancy has been associated with childhood respiratory health, but the role of the gut microbiome at a later age in relation to respiratory health is not known[27]. We therefore assessed the association of the gut microbiome with lung function and asthma cross-sectionally in children aged 10 years. Both alpha and beta-diversity were not associated with lung function and asthma (Table 5.3). Only one specific OTU

was borderline significant associated with a lower FEV_1/FVC, while none OTUs were associated with asthma. The diversity and abudance of stool microbiota were associated with the risk of sensitization for inhalant allergens and psyhician-diagnosed inhalant allergy, and stool microbiota lipid metabolism pathways with physician-diagnosed inhalant allergy. These findings suggest that the effect of the gut microbiome on asthma might be limited in later childhood, although it might have an effect on allergic conditions.

Our findings indicate that mostly airway bacterial carriage and the microbiome in infancy are of importance in the development of respiratory health. Future studies using repeatedly measures of the airway and gut microbiome and the presence of lower respiratory tract infections and wheezing in early-life, are needed in order to better understand the role of the early-life influences and possible bidirectional associations.

Table 5.3. Overview of the results of studies presented in this thesis on childhood bacterial carriage and the microbiome and childhood respiratory health.

	Lung function				Symptoms and disease			
	FEV_1	**FVC**	**FEV_1/FVC**	**FEF_{75}**	**URTI**	**LRTI**	**Wheezing**	**Asthma**
Airway bacterial carriage								
Nasal *s. aureus*	=	=	=	=	=	=	=	=
Any NP carriage	=	=	=	=	=	=	↑	=
Gut microbiome								
Alpha diversity	n.s.	n.s.	=	n.s.	n.s.	n.s.	n.s.	=
Beta diversity	n.s.	n.s.	=	n.s.	n.s.	n.s.	n.s.	=
Specific OTUs	n.s.	n.s.	=	n.s.	n.s.	n.s.	n.s.	=

Lung function was measured by spirometry at age 10 years. Lower respiratory tract infections and wheezing were measured by repeated questionnaires from age 2 months to 6 years, and age 1 to 10 years, respectively. Asthma was measured by questionnaire at age 10 years. FEV_1: Forced Expiratory Volume in the first second. FVC: Forced Vital Capacity. FEF_{75}: Forced Expiratory Flow after exhaling 75% of the FVC. LRTI: lower respiratory tract infections. NP: nasopharyngeal. OTUs: operational taxonomic units. n.s.: not studied in this thesis. A ↑ represents a positive association, a ↓ a negative association, and a = that no association was observed.

METHODOLOGICAL CONSIDERATIONS

Most of the studies presented in this thesis were embedded in the Generation R Study, a prospective population-based cohort study from fetal life onwards. Additionally, an individual participant meta-analysis was performed, using data from 38 birth cohorts from Europe. Specific methodological considerations of the separate studies have been discussed in their respective chapters in this thesis. Some general methodological considerations, including selection bias, information bias, confounding and external validity will be discussed in the following section.

Selection bias

Selection bias occurs when the likelihood of being selected for a study, or being retained in the study during follow-up leads to a result that is different from the result when the entire target population is selected. The percentage of pregnant women eligible for enrolment in the Generation R Study that are actually included in the study is not estimable. However, the response rate based on the number of children at birth is 61%[28]. Household income and highest education of the parents suggest a selection towards the more affluent population, since both income and education are higher than in the whole study area. Loss to follow-up might be related to either exposure or outcome, and could therefore potentially lead to biased results. This bias could cause either over- or underestimation of the effect. Of the original participating cohort, 82% were able to be invited, and of those 93% participated in the study at school-age[28]. Those not lost to follow-up were older, more frequently Dutch and higher educated. This selections toward a relatively healthy population could have led to biased effect estimates. This might have led to either an over- or underestimation of the effect, if the associations of those lost to follow-up with respiratory health would be different from those included in the study. This is unlikely, but cannot be excluded.

Information bias

Information bias, or misclassification, is defined as incorrect classification of the information about participants. This misclassification is non-differential if it is random, i.e. not related to exposure or outcome, and differential if it is non-random, i.e. related to exposure or outcome. While differential misclassification could lead to either over- or underestimations of the effect, non-differential misclassification usually leads to an underestimation of the effect. Various of the exposures in this thesis, including parental psychological distress, maternal Chlamydia trachomatis during pregnancy, breastfeeding, and child's airway bacterial carriage, respiratory tract infections and herpesviridae have been collected before outcome assessments. Additionally, both parents and researchers involved in the initial data collections were not aware of the research questions, which makes differential misclassification less likely. Maternal Chlamydia trachomatis infection during pregnancy, childhood airway bacterial carriage, microbiome composition and herpesviridae have been measured by objective tests and therefore misclassification is less likely to occur. Breastfeeding and respiratory tract infections were self-reported by the parents, which could lead to differential misclassification. Mothers might have overreported breastfeeding practices, since breastfeeding is deemed as the most healthy choice. This would most likely have led to an underestimation of the effect of breastfeeding on lung function or asthma. For respiratory tract infections, only physician-attended infections were taken into account, in order to minimize bias.

Although lung function measured by spirometry does depend on maximal performance of the participants, researchers were unaware of lung function or asthma status of the participant making differential misclassification less likely. Wheezing and asthma were measured by validated

questionnaires, but since these were self-reported by the parents differential misclassification might have occurred. This could have led to both over- and underestimation of the effect. Thus, although misclassification might have occurred, we suspect this effect to be minimal.

Confounding

A confounder can be characterized as a variable that influences both exposure and outcome, and is no intermediary in the causal chain. If confounders are not taken into account in the analysis, this may lead to biased effect estimates. The studies presented in this thesis were all adjusted for many potential confounders, which have been selected from literature and tested in our population. However, we cannot fully exclude the possibility of residual confounding, due to unmeasured variables or variables that are not known as a possible confounder.

External validity

External validity reflects the generalizability of the results of a study to other populations. The Generation R Study is based on the general population in Rotterdam, the Netherlands, with a distribution of different ethnic groups comparable to the city. When compared to the characteristics at enrolment, mothers who still participated when their children were adolescents were older, more frequently Dutch and higher educated[28]. This pattern of loss to follow-up is comparable to other population-based prospective cohort studies. However, this selection towards a more affluent population should be kept in mind when results are applied to other populations.

The meta-analysis was performed with data from 38 different cohort studies from across Europe. Although some Eastern European countries were included, these were underrepresented. Additionally, as within the Generation R Study, it could be assumed that those still participating in these studies during follow-up were likely higher educated and more frequently Caucasian. However, the results from this study are most likely a fair representation of the general European population.

Methodological issues in meta-analyses

In an individual participant data (IPD) meta-analysis, the original data from participants is collected and analysed, as opposed to a meta-analysis in which the results from published studies are combined. The main advantages of an IPD meta-analysis is that it allows for better harmonisation of the data and that it is not subject to publication and reporting bias, therefore minimizing the risk of biased results. By using the raw data, we were able to better harmonize data and perform sensitivity analyses to account for possible differences in results due to different methods of data ascertainment. We found similar results for cohorts using ISAAC –based questionnaires for asthma, which are validated for epidemiological research, when compared to the results of the full meta-analysis. However, there were substantial differences between cohorts in terms of prevalence of respiratory tract infections, which might not only reflect true differences in prevalence between countries, but also differences in data ascertainment. This would most likely

reflect non-differential misclassification, leading to an underestimation of the effect. Additionally, in an IPD meta-analysis, both a one-stage and two-stage approach can be used for the analyses. In a one-stage approach, all data is synthesized while taking account of clustering of data within the same cohort, while in a two-stage approach, data is analysed per cohort and effect estimates are then combined while taking account of the relative size of the cohort. In general, a one-stage model yields more reliable results in the case of heterogeneity or smaller numbers, while two-stage models are less computationally difficult and can easily visualize heterogeneity by means of forests plots[29 30]. In our meta-analysis, we used both methods in order to test the robustness of the results. Both methods yielded similar results.

CAUSALITY

The findings from this thesis are based on observational studies, which makes it difficult to establish causality. Randomized clinical trials could not be conducted for ethical or practical reasons. However, the Bradford Hill criteria can be useful in establishing evidence for a possible causal relationship. These include strength, consistency, specificity and temporality of the associations, a dose-response relationship, a plausible mechanism, coherence with for example animal studies and experimental evidence. For the associations of parental psychological distress, early-life respiratory tract infections and *Chlamydia trachomatis* during pregnancy with respiratory health, we found consistent associations for similar exposures and outcomes. For all associations except microbiota and respiratory health, the exposure was measured before the outcome, indicating causality. For all observations plausible mechanisms, partly supported by animal studies, are available. Thus, causal inference could largely be drawn from our observational studies since certain criteria have been met.

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The results presented in this thesis are based on observational data, which limits the possibility to draw conclusions about underlying mechanisms. Still, some clinical implications and future perspectives can be discussed.

We demonstrated the importance of fetal and infant exposures in the development of respiratory health. The association of maternal psychological distress with offspring asthma demonstrates the importance of attention for mental health during pregnancy. Further studies could focus on the effect of preventive strategies to improve mental health before and during pregnancy, and the effect on offspring health. For example, a randomized clinical trial of pregnant woman giving one group regular care and one group mind-body therapy using attractive group educational sessions by well-trained nurses during pregnancy could be of benefit. Additionally, studies focusing on

the underlying mechanisms, such as cortisol levels during pregnancy or epigenetics could be of interest.

In this thesis, fetal and childhood infections were identified, which might contribute to the development of respiratory health. Hypothetically, prevention of infections could lead to improvement of respiratory health. One example of this is a trial, in which preterm born children are randomized to receive either a monoclonal antibody (palivizumab) which protects against respiratory syncytial virus, or placebo. Palivizumab seemed protective against wheezing in the first year of life, although the effects at preschool age seem more limited with only a decreased risk of current asthma, but not ever asthma or lung function[31 32]. This is however limited to preterm infants, and future studies could focus on the effect in other risk groups, or the full population. Additionally, respiratory tract infections might be used in risk stratification or predictive tools in the identification of those at risk for adverse respiratory health. Future studies might also focus on the mechanisms underlying the association of *Chlamydia trachomatis* infection during pregnancy with asthma-related morbidity, and the possible effect of early identification of those infected. Since *Chlamydia trachomatis* is easily and effectively treated, even during pregnancy, screening programs might lead to improved offspring health[33].

In this thesis, we found a minimal association of airway bacterial carriage in infancy, and the gut microbiome at school-age, with asthma-related morbidity only. However, bacterial carriage identified by culturing may underestimate the importance of the full range of the bacterial composition and the interaction between bacteria, and these are better reflected by the airway microbiome. There are suggestions that the airway microbiome in infancy is associated with short term respiratory health, including wheezing and lower respiratory tract infections[34-36]. The association with long-term respiratory health, including school-age lung function and asthma, as well as the possible bidirectional relationship between the airway microbiome and respiratory tract infections is less clear, and would be an interesting target for future studies. Lastly, we did not find a consistent relationship between the gut microbiome and respiratory health at school-age. The gut microbiome in infancy does seem to impact respiratory health, but similar to the airway microbiome, data on long-term effects of the gut microbiome on respiratory health is lacking[27 37]. Shifts in microbiome composition have a local but also a strong systemic effect such as altering maturation of the immune system into a more inflammatory state by altered T-helper 2 (Th2) activity [38 39]. Also, low-grade, chronic inflammation is suggested to lead to tissue and organ alterations potentially increasing the risk of chronic lung diseases [40-45]. An integrative approach using the microbiome and its relation with different biomarkers for chronic systemic inflammation and its effects on chronic lung disease outcomes at a population-based level would be of interest for future studies, as well as preventive strategies to influence the gut microbiome composition in early life.

CONCLUSION

Most likely, an important part of the origins of asthma lie in early life. In this thesis, we identified early-life exposures, specifically infections and bacteria, leading to an increased risk of adverse respiratory health in childhood (Figure 5.1). Further studies are needed to explore possible underlying mechanisms for these associations. Future studies could focus specifically on prevention and risk prediction of early-life infections, the role of the airway and gut microbiome in infancy and the possible bidirectional relationship between infections and the microbiome in the development of respiratory health. Additionally, future preventive strategies could be developed to improve childhood respiratory health.

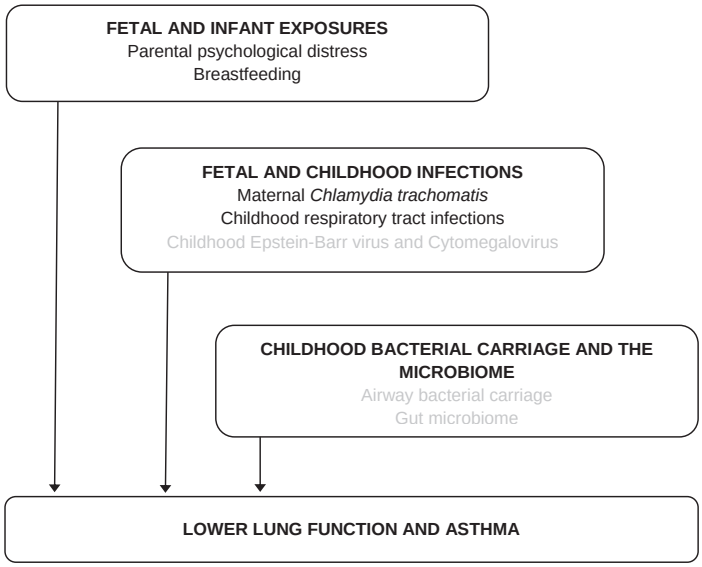

Figure 5.1. Overview of exposures influencing childhood asthma development studied in this thesis. Grey font denotes that the exposure is not associated with lower lung function and asthma.

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Chapter 6

Samenvatting

SAMENVATTING

In dit proefschrift onderzochten we de hypothese dat factoren vroeg in het leven, en meer specifiek luchtweg- en specifieke infecties en bacteriën, kunnen zorgen voor veranderingen in de ontwikkeling van het respiratoire en immuunsysteem, wat vervolgens kan leiden tot een hoger risico op een lagere longfunctie en astma op de kinderleeftijd. De identificatie van deze blootstellingen en infectieziekten vroeg in het leven die kunnen bijdragen aan de ontwikkeling van een slechtere gezondheid van de longen kunnen helpen bij het begrijpen van de ontwikkeling van een lagere longfunctie en astma. Hiermee kan de eerste stap genomen worden voor het ontwikkelen van preventie strategieën.

In **Hoofdstuk 1** wordt de achtergrond en de rationale van de studies die beschreven worden in dit proefschrift gegeven. Tevens worden de onderzoeksdoelen van de studies beschreven en wordt de indeling van het proefschrift toegelicht.

Hoofdstuk 2 beschrijft de associatie van blootstellingen in het foetale leven en op de zuigelingenleeftijd met longfunctie en astma op de schoolleeftijd, en of deze associaties gemedieerd worden door lage luchtweginfecties. In *Hoofdstuk 2.1* beschrijven we dat psychische klachten van moeder tijdens de zwangerschap geassocieerd zijn met een hoger risico op astma en deels ook lagere longfunctie bij hun kinderen op de schoolleeftijd. Psychische klachten van de partner tijdens de zwangerschap zijn niet geassocieerd met lagere longfunctie of astma. Dit impliceert dat psychische klachten van moeder tijdens de zwangerschap een direct effect zouden kunnen hebben op de longontwikkeling van het kind, en niet zozeer dat dit verklaard zou kunnen worden door bijvoorbeeld leefstijlfactoren die moeder en partner delen. *Hoofdstuk 2.2* beschrijft dat een kortere duur van borstvoeding en niet-exclusieve borstvoeding deels geassocieerd zijn met een lagere longfunctie op de schoolleeftijd. Borstvoeding was niet geassocieerd met astma. Dit geeft aan dat borstvoeding waarschijnlijk alleen een effect heeft op gezondheid van de longen in de eerste levensjaren, en dat het van belang is om gedetailleerdere maten van borstvoeding te gebruiken in onderzoek naar de relatie van borstvoeding met longfunctie of astma. Zowel de associatie van psychische klachten van moeder tijdens de zwangerschap als borstvoeding met longfunctie en astma worden niet verklaard door lage luchtweginfecties bij het kind. Hoogstwaarschijnlijk hebben deze blootstellingen in het vroege leven een direct effect op longfunctie en astma, in plaats van dat deze zorgen voor een verhoogde gevoeligheid voor lage luchtweginfecties.

Samenvattend tonen we een associatie aan van psychische klachten van de moeder tijdens de zwangerschap met een lagere longfunctie en astma, en van duur en exclusiviteit van borstvoeding met een lagere longfunctie bij het kind.

In **Hoofdstuk 3** beschrijven we de associaties van infecties tijdens de zwangerschap en op de kinderleeftijd met gezondheid van de longen op kinderleeftijd. Zowel *Hoofstuk 3.1* als *3.2* richten zich op het effect van luchtweginfecties met longfunctie en astma. In *Hoofdstuk 3.1* laten we zien dat lage luchtweginfecties in de eerste drie levensjaren geassocieerd zijn met een lagere longfunctie en een verhoogd risico op astma op schoolleeftijd. Tevens tonen we aan dat deze

associaties onafhankelijk zijn van een piepende ademhaling op kinderleeftijd en dat een piepende ademhaling niet geassocieerd is met een hoger risico op lage luchtweginfecties op latere leeftijd. *Hoofdstuk 3.2* richt zich tevens op de associatie van luchtweginfecties met longfunctie en astma, waarbij er gebruik gemaakt wordt van data uit de algemene Europese populatie. We laten zien dat bovenste luchtweginfecties geassocieerd zijn met een hoger risico op astma, terwijl lage luchtweginfecties geassocieerd zijn met zowel een lagere longfunctie als een hoger risico op astma. Deze associaties blijven bestaan wanneer we stratificeren voor een piepende ademhaling ten tijde van de luchtweginfectie. Deze bevindingen impliceren dat luchtweginfecties het risico op een lagere longfunctie en astma ten minste deels onafhankelijk van enige predispositie voor astma beïnvloeden. In *hoofdstuk 3.3* bekijken we de associatie van maternale *Chlamydia trachomatis* tijdens de zwangerschap met gezondheid van de longen op kinderleeftijd. Maternale *Chlamydia trachomatis* tijdens de zwangerschap is geassocieerd met een lagere longfunctie en een verhoogd risico op astma op schoolleeftijd, en met een piepende ademhaling op kinderleeftijd. Maternale *Chlamydia trachomatis* tijdens de zwangerschap is echter niet geassocieerd met lage luchtweginfecties op kinderleeftijd. *Hoofdstuk 3.4* beschrijft dat Epstein-Barr virus en Cytomegalovirus voor de leeftijd van 6 jaar niet geassocieerd zijn met een lagere longfunctie, astma en allergische sensitisatie wanneer andere verklarende factoren zoals etniciteit en leefstijl in acht genomen worden.

Samenvattend tonen we aan dat infecties tijdens de zwangerschap en op kinderleeftijd, zoals luchtweginfecties en maternale *Chlamydia trachomatis* tijdens de zwangerschap, geassocieerd zijn met een verhoogd risico op een lagere longfunctie en astma op kinderleeftijd. Epstein-Barr virus en Cytomegalovirus zijn niet geassocieerd met gezondheid van de longen indien er rekening gehouden wordt met leefstijl, sociaal-economische factoren en ethniciteit.

Hoofstuk 4 beschrijft de associatie van bacterieel dragerschap en het microbioom met gezondheid van de longen. In *Hoofdstuk 4.1* tonen we aan dat bacterieel dragerschap van de luchtwegen geassocieerd is met een piepende ademhaling in de eerste levensjaren. Bacterieel dragerschap van de luchtwegen is niet geassocieerd met longfunctie en astma op schoolleeftijd. Tevens laten we zien dat bacterieel dragerschap van de luchtweg en lage luchtweginfecties elkaar niet lijken te beïnvloeden. *Hoofdstuk 4.2* richt zich op de cross-sectionele associatie van het microbioom van de darm met longfunctie en astma op schoolleeftijd, en laat zien dat het darmmicrobioom niet geassocieerd is met longfunctie of astma, maar wel met inhalatie allergie en sensitisatie voor inhalatieallergenen. *Hoofdstuk 4.3* geeft een overzicht van de rol van luchtweginfecties en het microbioom in de ontwikkeling van een lagere longfunctie, astma en een piepende ademhaling.

Samenvattend tonen we aan dat bacterieel dragerschap in het vroege leven geassocieerd is met een piepende ademhaling, terwijl zowel bacterieel dragerschap van de luchtwegen in het vroege leven als het microbioom van de darm op schoolleeftijd niet geassocieerd zijn met longfunctie of astma.

Tot slot wordt in **Hoofdstuk 5** een algemeen overzicht en interpretatie van de studies die beschreven worden in dit proefschrift gegeven. Tevens worden methodologische overwegingen, klinische implicaties en suggesties voor toekomstig onderzoek besproken.

Chapter 7

Appendices

LIST OF PUBLICATIONS

van Meel ER*, Saharan G*, Jaddoe VWV, de Jongste JC, Reiss IKM, Tiemeier H, El Marroun H, Duijts L. Parental psychological distress during pregnancy and the risk of childhood lower lung function and asthma: a population-based prospective cohort study. *Thorax. 2020 Oct 12;thoraxjnl-2019-214099. Epub ahead of print.* *These authors contributed equally.

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PHD PORTFOLIO

Name:	Evelien van Meel
Department:	the Generation R Study Group, and Pediatrics, division of Respiratory Medicine and Allergology, Erasmus University Medical Center
Research School:	Netherlands Institute for Health Sciences (NIHES), 2011 – 2016
PhD Period:	June 2016 – December 2019
Promotors:	Prof. Dr. V.W.V. Jaddoe, prof. dr. J.C. de Jongste
Co-promotor:	Dr. L. Duijts

PhD training	Years	ECTS
Specific courses		
Master of Science in Clinical Epidemiology, NIHES, Rotterdam Including courses at Johns Hopkins Bloomberg School of Public Health (USA) and Cambridge Institute of Public Health (UK)	2011-2016	120
General Academic courses		
MRI Safety training	2016	0.3
Stralingshygiëne voor röntgentoestellen	2016	0.3
Scientific Integrity	2017	0.2
Seminars and workshops		
Research meetings Generation R Study	2016-2019	4.0
Maternal and Child Health meetings	2016-2019	4.0
Research meetings Pediatric Pulmonology	2016-2019	4.0
VENA workshops	2016-2019	1.0
NRS Young Investigators Day	2019	0.6
Health Sciences Research day, Erasmus MC	2019	0.6
Presentations		
National conferences		
Pediatrics Research day, Erasmus MC	2016	0.6
International conferences		
European Respiratory Society Congress, London	2016	1.0
European Respiratory Society Congress, Milan	2017	1.0
DOHaD World Congress, Rotterdam	2017	1.0
Conference on Epidemiological Birth Cohort and Longitudinal Studies , Oulu	2018	1.0

European Respiratory Society Congress, Paris	2018	1.0
European Respiratory Society Congress, Madrid	2019	1.0

Invited Speaker

European Birth Cohorts Network Meeting, Barcelona	2017	1.0
Refeeravond POAG-JGZ Maastricht, Maastricht	2019	1.0
LOVAH Wetenschapsdag, Utrecht	2019	1.0
European Respiratory Society Congress, Madrid	2019	1.0

Chairing

European Birth Cohorts Network Meeting, Barcelona	2017
Chair Parallel Session Respiratory Health	
European Respiratory Society Congress, Paris	2018
Chair Thematic Poster Sessions	
European Respiratory Society Congress, Madrid	2019
Chair Guideline Session	

Teaching activities

Supervising students

M. de Jong, Master thesis Medicine	2016	3.0
M. Attanasi, Research visit	2017	3.0
G. Saharan, Master thesis Health Sciences	2016-2018	3.0

Supervising practicals

NIHES: Principles of Research in Medicine and Epidemiology	2017	1.0

Other activities

- Organizational committee DOHaD World Congress 2017
- Organizational committee Health Sciences Research Day 2019
- Member workpackage Respiratory Health of the LifeCycle Project
- Working group member ERS Taskforce on long-term management of BPD

Grants/prices

- Vereniging Trustfonds Erasmus Universiteit Rotterdam: several travel grants
- European Respiratory Society Congress 2017: Best abstract from a young investigator submitted to the Pediatrics Assembly
- DOHAD 2017: Young Investigator Award

Peer review

- Peer review of articles for various scientific journals (American Journal of Respiratory and Critical Care Medicine, European Respiratory Journal, European Respiratory Journal Open Research, Pediatrics, Pediatric Allergy and Immunology)
- Peer review of abstracts for scientific congresses (DOHaD World Congress 2017, European Respiratory Society Congress 2019)

1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours

ABOUT THE AUTHOR

Evelien van Meel was born on January 31st 1989 in Alkmaar, the Netherlands. In 2007, she graduated from the Murmellius Gymnasium in Alkmaar. Because she was not selected for medical school, she started studying Health Policy and Management at the Erasmus University Rotterdam. Fortunately, in 2008 she was admitted to the Medicine study at VU Medical Center in Amsterdam. After successful completion of the first year of Medicine, she transferred back to Rotterdam where she completed both her bachelor in Medicine and in Health Policy and Management in 2012. On top of the medical curriculum, she started with the master Clinical Epidemiology for which she attended a summer programme at Johns Hopkins Bloomberg School of Public Health in the United States of America. After completing her clinical rotations in 2015, she commenced with the research project for her master Clinical Epidemiology at the Generation R Study. In retrospect, this formed the start of a PhD-project focusing on the early origins of childhood respiratory health (promotores prof. dr. V.W.V. Jaddoe and prof.dr. J.C. de Jongste, co-promotor dr. L. Duijts). Evelien is currently working as a resident (not in training) at the Department of Internal Medicine of Franciscus Gasthuis & Vlietland. She aspires to become a General Practitioner, and combine clinical practice with epidemiological research. Together with her partner Hans-Willem and daughters Sophie and Liselotte, she is living in Capelle aan den IJssel