Early Environmental and Epigenetic Influences on Respiratory Health

H.T. den Dekker

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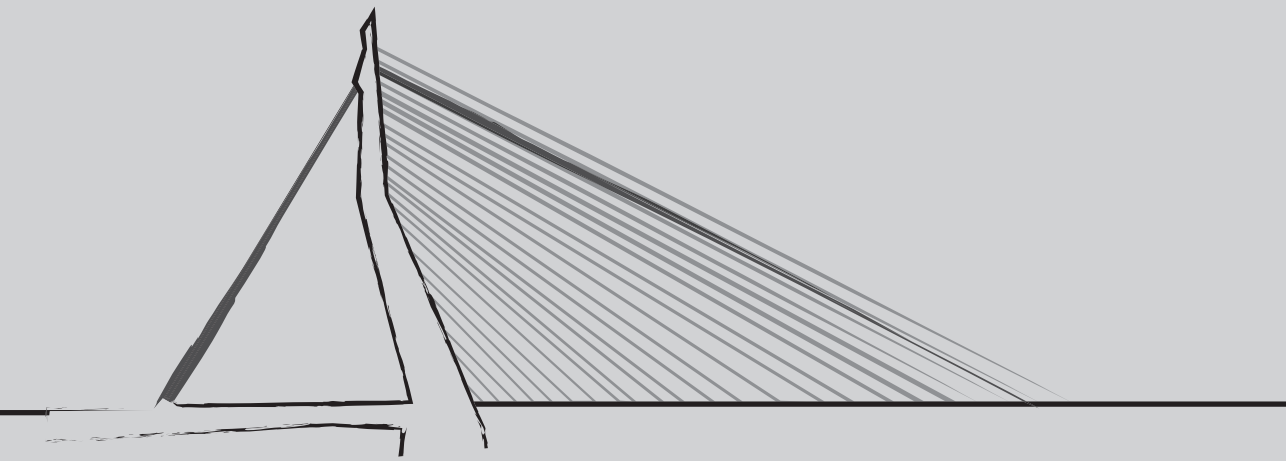

Chapter 2.1

Den Dekker HT, Sonnenschein-van der Voort AM, de Jongste JC, Anessi-Maesano I, Arshad SH, Barros H, Beardsmore CS, Bisgaard H, Craig L, Devereux G, van der Ent CK, Esplugues A, Fantini MP, Flexeder C, Frey U, Forastiere F, Gehring U, Gori D, van der Gugten AC, Henderson AJ, Heude B, Ibarluzea J, Inskip HM, Keil T, Kogevinas M, Kreiner-Møller E, Kuehni CE, Lau S, Mélen E, Mommers M, Morales E, Penders J, Pike KC, Porta D, Reiss IK, Roberts G, Schmidt A, Schultz ES, Schulz H, Sunyer J, Torrent M, Vassilaki M, Wijga AH, Zabaleta C, Jaddoe VWV, Duijts L. Early growth characteristics and the risk of reduced lung function and asthma: A meta-analysis of 25,000 children. *J Allergy Clin Immunol 2016;137(4):1026-35*.

Chapter 2.2

Den Dekker HT, Jaddoe VWV, Reiss IK, de Jongste JC, Duijts L. Fetal and infant growth patterns and risk of lower lung function and asthma. The Generation R Study. *Submitted*

Chapter 2.3

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Chapter 3.1

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Chapter 3.2

Den Dekker HT, Jaddoe VWV, Reiss IK, de Jongste JC, Duijts L. Maternal folic acid use during pregnancy, *MTHFR* polymorphism, and child's lung function and asthma. *Submitted*

Chapter 3.3

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Chapter 4.2

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Chapter 4.3

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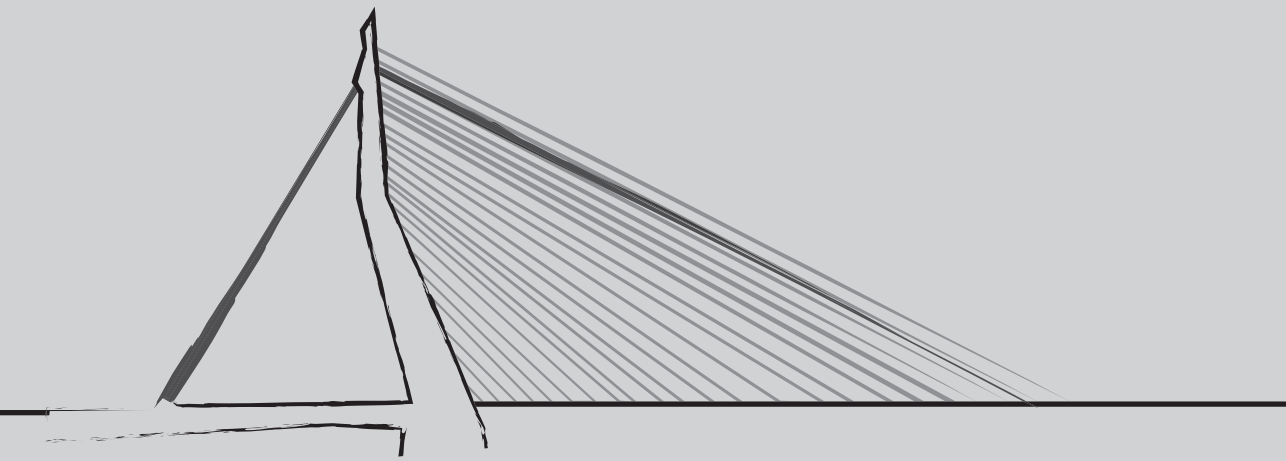
Chapter 1

General introduction



INTRODUCTION

Background

Asthma is the most common chronic disease in childhood. Worldwide, 14% of all children aged 6 to 14 years report asthmatic symptoms in the last year.[1] In the Netherlands, the estimated cumulative asthma prevalence in children aged 5-18 years is 8.1%.[2] Severe childhood asthma is associated with an up to 32-fold increased risk of chronic obstructive pulmonary disease (COPD) in later life.[3] Asthma and COPD account for 6% of global mortality.[4]

Asthma is an inflammatory disease that most commonly develops in childhood and affects both the large and small airways. Children with asthma have periods of generalized airway obstruction, caused by smooth-muscle spasms, increased mucus secretion and inflammation of the airways.[5] Clinical presentation of childhood asthma comprises periods of combined coughing, wheezing and breathlessness, which are reported in 39% of all children on the day before an asthma exacerbation.[6] In most cases, the first asthma-symptoms occur from a young age onwards[7], and symptom severity and lung function deficits track with age.[8] Lower lung function or asthma in childhood might predispose for chronic obstructive respiratory diseases, including COPD, in adolescence and adult life.[9, 10]

The strongest predictor of asthma is chronic airflow limitation.[11, 12] Children are able to perform spirometry from approximately 6 years onwards.[13] Spirometry measurements in children with asthma show airway obstruction characterized by lower expiratory airflow rates with a lower forced expiratory volume in 1 second (FEV_1) and FEV_1/forced vital capacity (FVC).[14] The measurement of respiratory resistance using the interrupter technique (Rint) is a method that could successfully be applied to children below age 6 years. The Rint-technique has good repeatability and biological validity for the detection of airway obstruction.[15] Additionally, markers of inflammation could be measured in exhaled air of children of all ages. Fractional Exhaled Nitric Oxide (FeNO) is a marker of eosinophilic inflammation in the airways, and is one of the strongest single biomarkers associated with childhood wheezing and asthma.[16, 17] Although effective treatments are available for asthma symptoms, the morbidity of asthma and COPD remains high. The lack of curative options is mainly due to the largely unknown pathophysiological mechanisms.[5] Therefore, it is important to identify risk factors and mechanisms which may lead to lower lung function, predisposing the individual for an increased risk of chronic obstructive respiratory diseases across the life course.

Over the past decades, multiple factors in early life, such as growth, environmental exposures and genetic susceptibility have been associated with respiratory symptoms in early childhood, and asthma and COPD at older ages.[18] Epigenetic mechanisms, which refer to changes in gene expression that does not involve changes to the underlying DNA-sequence, have been suggested as a potential underlying mechanism.[18, 19]

Growth

Children born extremely preterm or with a low birth weight have high rates of neonatal respiratory diseases, such as infant respiratory distress syndrome and bronchopulmonary dysplasia.[20] Recent prospective studies in children suggest that also preterm birth, small size for gestational age at birth, and accelerated weight growth in the first months of infancy are associated with increased risks of childhood wheezing and asthma.[21] The associations of early growth characteristics with chronic obstructive respiratory diseases might be explained by developmental adaptations of the lungs and airways, leading to relatively small airways and hence a reduction in expiratory flows reflected by lower lung function values.[18] Previous studies examining associations between early growth characteristics and childhood lung function have reported inconsistent results.[22-24] To further understand the causal pathways between early growth characteristics and childhood asthma, it is important to unravel whether lower lung function measures explain these associations.

Birth weight is the result of fetal weight growth, and the starting point for infant weight growth. Detailed information about growth throughout pregnancy and infancy enables identification of periods in early life, which might be critical for development of respiratory morbidity in later life.[18] Most studies that focused on the associations of fetal growth with childhood lung function or asthma did not take infant growth into account, and vice versa. This limits conclusions because fetal and infant growth are correlated.[25] Studies focused on combined fetal and infant growth patterns in relation to childhood respiratory morbidity are scarce, limited to young ages only and differ in definitions of fetal and infant growth patterns or asthma-related outcomes.[26, 27] Additionally, children's current body mass index (BMI) and atopy might affect associations of fetal and infant growth with childhood lung function and asthma. This warrants further studies.

Early growth characteristics are associated with an increased risk of obesity in later life [28] and obesity is hypothesized to be associated with asthma.[29] An increased BMI could reduce the pulmonary vital capacity by mechanical pressure, and increase obstruction-related respiratory resistance and the risk of asthma symptoms.[29] Alternatively, a higher BMI could lead to an increased production of systemic pro-inflammatory mediators by fat tissue, with subsequent airway inflammation.[29] The major limitation of BMI is that it does not distinguish fat mass from free-fat mass, while it is suggested that specific fat mass distribution is more strongly associated with adverse health risks. Studies that assessed detailed adiposity measures are scarce and are mainly performed in adult populations. Further understanding of the associations of childhood BMI and detailed body fat measures with childhood lung function and asthma will provide further insight on pathophysiological mechanisms.

By exploration of potential pathways explaining the associations between early growth characteristics with childhood asthma, identification of specific growth periods

in early life which might be critical for development of respiratory morbidity in later life, and the effects of body composition in later life, we will expand current knowledge on the pathophysiological mechanisms linking growth and development of childhood lung function and asthma.

Environmental exposures

The knowledge on the role of environmental exposures in the developmental origins of health and disease emerges quickly.[30] Insight into environmental exposures during pregnancy and early childhood that affect lung development and asthma provides an opportunity for interventions at the time when they have their greatest effect. Maternal smoking during pregnancy is strongly associated with fetal growth retardation, preterm birth and lower birth weight.[31, 32] Evidence suggests that fetal tobacco smoke exposure might also have a direct effect on lung development, which may include suboptimal development of the respiratory tract, resulting in impaired lung growth with smaller airway diameters leading to a higher respiratory resistance.[33] Maternal smoking during pregnancy has been associated with wheezing up to age 4 years.[34, 35] Previous studies on the adverse effect of maternal smoking during pregnancy on childhood lung function and asthma at older ages are inconsistent.[36, 37] Furthermore, it is unknown whether associations could be explained by early growth characteristics.

Folic acid supplement use during pregnancy is recommended to prevent neural tube defects.[38] Folic acid supplement use and related folate concentrations in blood, but also vitamin B_{12} and homocysteine, are involved in the one-carbon metabolism, necessary for multiple physiological processes, including biosynthesis, amino acid homeostasis and epigenetic changes to the DNA.[39, 40] The genetic variant *C677T* in the methylenetetrahydrofolate reductase gene (*MTHFR*) is known to affect circulating folate, vitamin B_{12} and homocysteine concentrations.[41] Increased intake of folic acid supplements during pregnancy has been associated with increased risks of asthma and allergic diseases in the offspring in animal studies.[42] Human studies show conflicting results [43], and the modifying effects of maternal or child's *MTHFR-C677T* variants on the associations of maternal folic acid supplement use, folate, vitamin B_{12} and homocysteine blood concentrations during pregnancy with lung function and asthma are unclear.[44-46]

Prolonged and exclusive breastfeeding have been suggested to be associated with a decreased risk of asthma symptoms in early childhood with a possible diminishing effect over time.[47] Underlying mechanisms for the association of breastfeeding with asthma symptoms might include secretory factors in breast milk that stimulate the neonatal immune system, and change the balance between pro- and anti-inflammatory mechanisms.[48] This might lead to altered airway inflammation or airway resistance. Previous studies suggest a potential mediating role of inhalant allergies and respiratory tract infections.[47] More detailed asthma phenotyping and use of objective measure-

ments, such as asthma related lung function tests, might improve the understanding of the potential protective effect of breastfeeding. Furthermore, observing dose-response relationships based on breastfeeding duration or exclusivity would support the causality of the association of breastfeeding with childhood asthma.

Thus, exploration of the associations of maternal tobacco smoke exposure during pregnancy, use of folic acid supplements during pregnancy and lack of breastfeeding might provide new insights on pathophysiological pathways of environmental exposures on development of childhood lung function and asthma, and could enable targeted interventions.

Genetic susceptibility

Family studies have shown that lung function is a heritable trait for 30-50% [49], and asthma for up to 75%.[50] However, the role of genetic factors on respiratory morbidity on an individual population-based level is unclear. In the past decade a methodology has been introduced to study the genetics of complex non-Mendelian diseases, the genome-wide association (GWA) study. GWA studies test associations between a large number (~10 million) of genetic variants and predefined phenotypes in a hypothesis-free manner. Recent GWA studies identified many common genetic variants associated with asthma-related outcomes, including childhood onset asthma [51, 52], adult asthma [52-54], impaired lung function [55-57] and atopy.[58-60] Overall, current studies have identified 313 different genetic variants associated with asthma (Figure 1.1). However, asthma is unlikely to be a single disease but rather a series of complex, overlapping individual diseases or phenotypes, each defined by its unique interaction between genetic and environmental factors.[61] This suggests that a more detailed defined phenotype might represent a specific pathogenic mechanism, and thus, focusing on a more specific phenotype may increase the power of genetic studies. As individuals with severe asthma-exacerbations at young age are highly susceptible to lower lung function and increased risk of chronic obstructive respiratory diseases in later life [3], identification of genetic variants associated with severe asthma exacerbations in childhood will provide new knowledge on the genetic mechanisms affecting respiratory morbidity and mortality across the life course.

Previously, three meta-analyses of GWA studies have identified 35 genetic variants to be associated with lung function in adults of European ancestry.[55-57, 62] Adult asthma and COPD are partly the result of lung development in childhood and adolescence, in which the pattern of lung function growth and decline is an important determinant of lung function and respiratory health in adulthood.[10] Both reduced growth resulting in a low maximal level of lung function and early decline are associated with the subsequent development of chronic airflow obstruction.[10] It is not known whether the identified genetic variants associated with adult lung function already affect lung function in

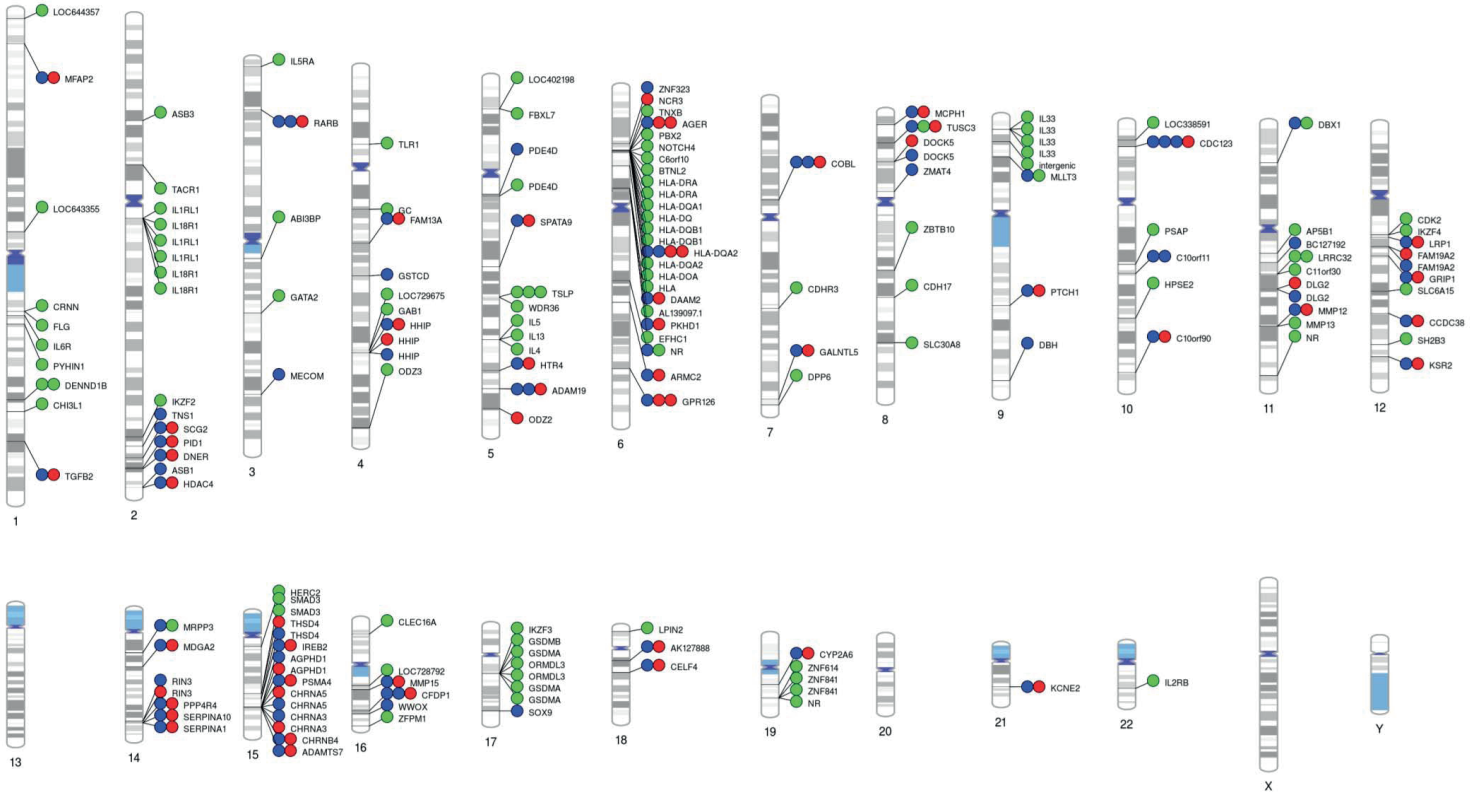


Figure 1.1. Overview of the genetic variants currently known to be associated with Asthma (green), Forced Expiratory Volume in 1 second (FEV_1) (blue) and FEV1/Forced Vital Capacity (FVC) (red). The National Human Genome Research Institute catalogue of published GWAS was searched using asthma, FEV_1 and FEV_1/FVC as traits.

childhood, thereby predisposing the individual for chronic obstructive respiratory diseases at an older age.

Epigenetics

Asthma is explained by both environmental exposures and genetic susceptibility.[63] Pathways of environmental exposures, such as tobacco smoke exposure during pregnancy, lack of breastfeeding and folic acid that affect lung development and risk of chronic obstructive respiratory diseases might be modified by genetic susceptibility. Identified genetic variants associated with childhood asthma in large-scale GWA studies only account for a low fraction of variance, up to 7.5%.[64] Epigenetic mechanisms could link the role of environmental exposures with the unexplained heritability for childhood asthma.[65, 66] The term epigenetics literally translates into "outside conventional genetics", and refers to changes in DNA structure that does not involve changes to the underlying DNA-sequence.[19] Multiple epigenetic mechanisms, including DNA-methylation, histone modification, and non-coding RNA-associated gene silencing are considered to initiate and sustain epigenetic changes.[67] Current advances in assays to assess DNA-methylation have enabled the study of methylation status of >480,000 sites (CpGs) in the genome with a good genomic coverage and requirement of low amounts of DNA, making it ideal for use in large cohorts. DNA-methylation of genetic regions could modify the expression of nearby genes.[68]

DNA-methylation occurs by the addition of a methyl-group to DNA and is a natural occurrence, but is also influenced by several environmental exposures, such as tobacco smoke and folic acid.[69] Folate provides methyl groups for a range of biochemical mechanisms, including DNA-methylation.[70] Fetal development is characterized by high rates of DNA-methylation changes and rapid organ development.[18] Whether periconceptional maternal folate levels may alter fetal DNA-methylation levels reflected at birth needs to be studied.

Subsequently, altered DNA-methylation at birth may affect gene expression and related respiratory tract development, predisposing individuals for obstructive airway diseases in later life.[9, 71] Studies that examined associations of DNA-methylation with lung function, asthma or COPD are scarce, limited to candidate genes or high-risk populations and lack replication. Identification of genomic regions with altered DNA-methylation levels related to lung function and respiratory diseases across the life course is important to understand underlying mechanisms of environmental and genetic factors that influence the development of lower lung function and risk of respiratory diseases.

HYPOTHESIS

The hypothesis of this thesis is that early growth and adverse environmental exposures in fetal life and infancy, in combination with genetic susceptibility, lead to structural and functional adaptations in early lung development, and eventually lower lung function and higher risk of chronic obstructive respiratory diseases in later life (Figure 1.2). These pathways might be explained by epigenetic mechanisms.

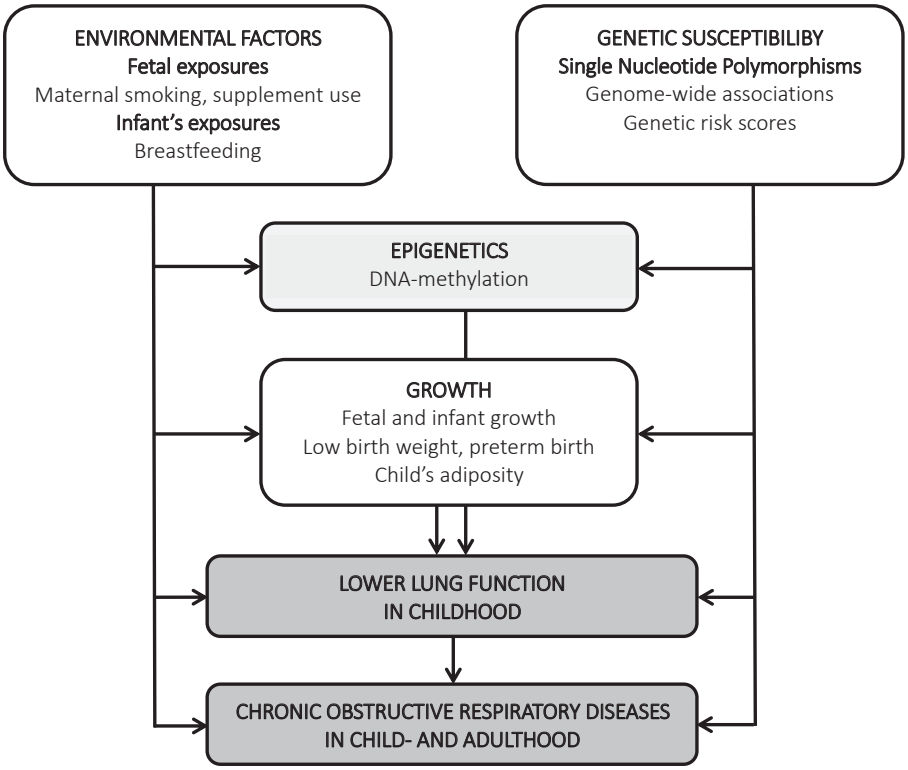


Figure 1.2. Overview of the early origins of chronic obstructive respiratory diseases and its potential underlying early growth, environmental and (epi)genetic mechanisms studies in this thesis.

OBJECTIVES

The major aims of this thesis are:

1. To identity which fetal, birth, infant, and child's growth or body composition characteristics are associated with childhood lung function and asthma.

2. To assess whether early life environmental exposures, including maternal tobacco smoking, folate, vitamin B_{12} and homocysteine concentrations, and child's breastfeeding, are related to childhood lung function and asthma.
3. To identify genetic and epigenetic variants related to childhood lung function and asthma, and epigenetics variants explaining the associations of environmental exposures with chronic obstructive respiratory disease outcomes across the life course.

GENERAL DESIGN

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

The Generation R Study

The Generation R Study is a population-based prospective cohort study in Rotterdam, the Netherlands, following pregnant women and their children from fetal life onwards (www. generationr.nl). The study is designed to identify early environmental and genetic causes and causal pathways leading to normal and abnormal growth, development and health during fetal life, childhood and adulthood. Enrolment was aimed in first trimester, but was allowed until birth of the child (Figure 1.3). In total, 9,778 mothers with a delivery date from April 2002 until January 2006 were enrolled in the study, and response at baseline was 61%.[72] Data collection for the current thesis included fetal ultrasounds examinations during each trimester of pregnancy for fetal growth, detailed infant physical examinations, questionnaires for environmental exposures, and biological samples including cord blood for DNA and DNA- methylation.[73]

Child's DNA and DNA-methylation was extracted from white cells in cord blood. DNA Samples were genotyped using Illumina Infinium II HumanHap610-660 Quad Arrays following standard manufacturer's protocols. Bisulfite conversion in DNA-methylation samples was performed using the EZ-96 DNA Methylation kit (Zymo Research Corporation, Irvine, USA), after which samples were processed with the Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, USA) followed quality control, probe exclusion and data normalization.

Information on birth characteristics was obtained from midwife and hospital registries. During the preschool years (from birth until the age of 4 years) information was mainly obtained from postal questionnaires including questions adapted from the International Study on Asthma and Allergy in Childhood (ISAAC). Growth data was collected at community health centers. At the age of 6 years, asthma diagnosis was obtained by questionnaire. Additional detailed hands-on assessments were performed in a dedicated research center to measure length, weight, body composition and body

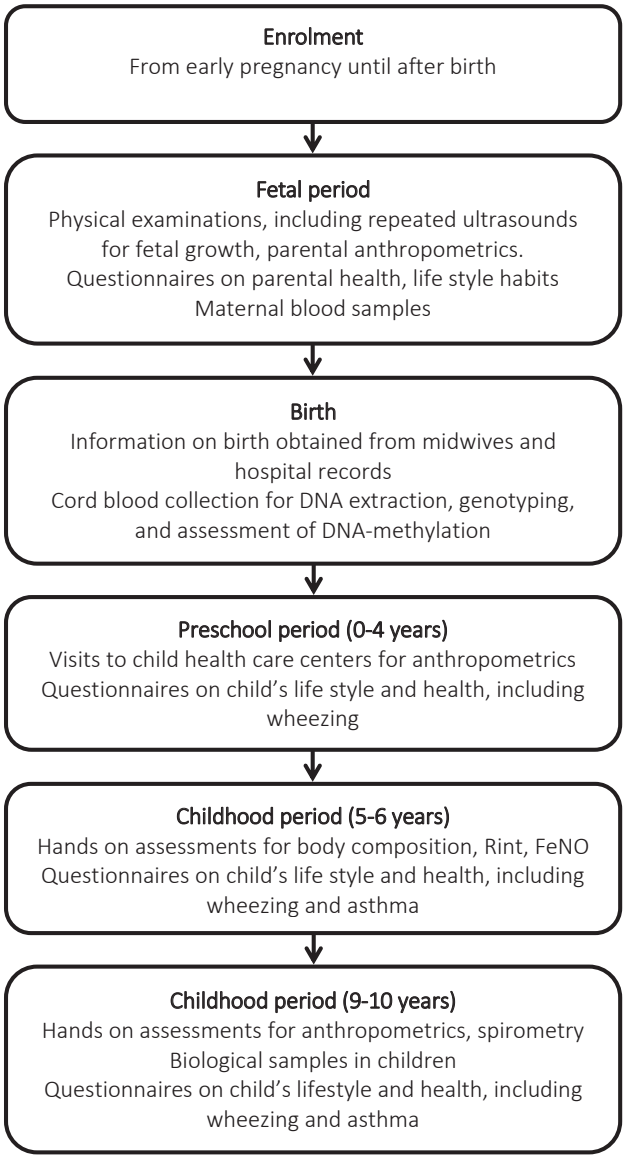


Figure 1.3. Design and data collection in the Generation R Study.

fat distribution, Fractional exhaled Nitric Oxide (FeNO) and airway resistance (Rint). At age 10 years, length and weight were measured, lung function was assessed using spirometry according to the American Thoracic Society / European Respiratory Society (ATS/ERS) guidelines, and inhalant allergic sensitization was measured using skin prick testing. Furthermore, parental questionnaires provided information on ever physician-

diagnosed asthma, and respiratory symptoms and use of inhalant medication in the past 12 months (Figure 1.3). With the available data in Generation R, we assessed the associations of early growth, early environmental exposures in fetal life and early childhood, and genetics and epigenetics with childhood lung function and asthma outcomes at ages 6 and 10 years.

CHICOS Consortium

We conducted a meta-analysis focused on the associations of early growth characteristics with childhood lung function and asthma with partners participating in the framework of the European consortium CHICOS (Child Cohort Research Strategy for Europe, www.chicosproject.eu). The overall aim of CHICOS is to improve child health across Europe by developing an integrated strategy for mother-child cohort research in Europe. European population-based birth- and mother-child cohorts were able to participate if they included children born between 1989 and 2011, had information available on at least gestational age and weight at birth and lung function measurements and asthma in childhood (until age 18 years), and were willing and able to exchange original data. We selected cohorts from both the CHICOS Consortium and other existing collaborations or birth cohorts (www.birthcohorts.net).

PACE Consortium

We conducted two Epigenome Wide Association Studies (EWAS) with partners collaborating in the Pregnancy and Child Epigenetics (PACE) Consortium. The aim of the PACE consortium is to facilitate joint analyses of DNA-methylation data in relation to a wide range of exposures and outcomes pertinent to health in pregnancy and childhood by bringing together researchers and by leveraging existing knowledge, skills and data. We first assessed the association of maternal folate levels during pregnancy with DNA-methylation in cord blood in collaboration with a Norwegian cohort and investigators of the National Institute of Environmental Health Sciences (NIEHS). Next, we assessed the associations of DNA-methylation in cord blood with lung function in childhood. Population-based birth- and mother-child cohorts were able to participate in the meta-analysis if they had information available on DNA-methylation in cord blood assessed with the Illumina Infinium HumanMethylation450 BeadChip array and lung function measurements in childhood (until age 18 years). Replication of the findings in older subjects, associations with asthma and COPD and differential expression was sought in existing collaborations with infant-, adolescent- and adult cohorts.

OUTLINE OF THIS THESIS

Chapter 2 focuses on associations of early growth with childhood lung function and asthma. *Chapter 2.1*. presents a meta-analysis on the associations of preterm birth, birth weight and infant growth with childhood lung function and asthma. In *Chapter 2.2*, the associations of fetal and infant growth patterns with lung function and school-age respiratory morbidity are presented. The associations of detailed body fat measures with lung function, wheezing and asthma at age 6 years are explored in *Chapter 2.3*. In **Chapter 3**, the effect of early exposures on childhood lung function and asthma are described. *Chapter 3.1* presents the influence of maternal smoking during pregnancy on lung function, wheezing and asthma at school-age. The associations of maternal folic acid supplement use, and folate, vitamin B_{12} and homocysteine levels in pregnancy and at birth with lung function and asthma in childhood are presented in *Chapter 3.2*. The associations of breastfeeding duration and exclusivity with lung function, wheezing and asthma at school-age are reported in *Chapter 3.3*. **Chapter 4** focuses on associations of genetic variants with lung function and asthma in childhood, and the role of DNA-methylation on the association of environmental exposures and genetic variants with lung function and chronic obstructive respiratory diseases. In *Chapter 4.1*, the discovery of a new genetic locus associated with severe childhood asthma is described. *Chapter 4.2* presents the associations of a genetic risk score based on genetic variants for adult lung function with childhood lung function and asthma. *Chapter 4.3* presents the associations of maternal folic acid levels during pregnancy with child's epigenome-wide DNA-methylation status at birth, measured in cord blood. In *Chapter 4.4*, the associations of epigenome-wide DNA-methylation at birth with lung function and chronic obstructive respiratory diseases throughout the life course is described. The main findings and implications described in this thesis are discussed in the general discussion in **Chapter 5**, followed by a English and Dutch summary in **Chapter 6**.

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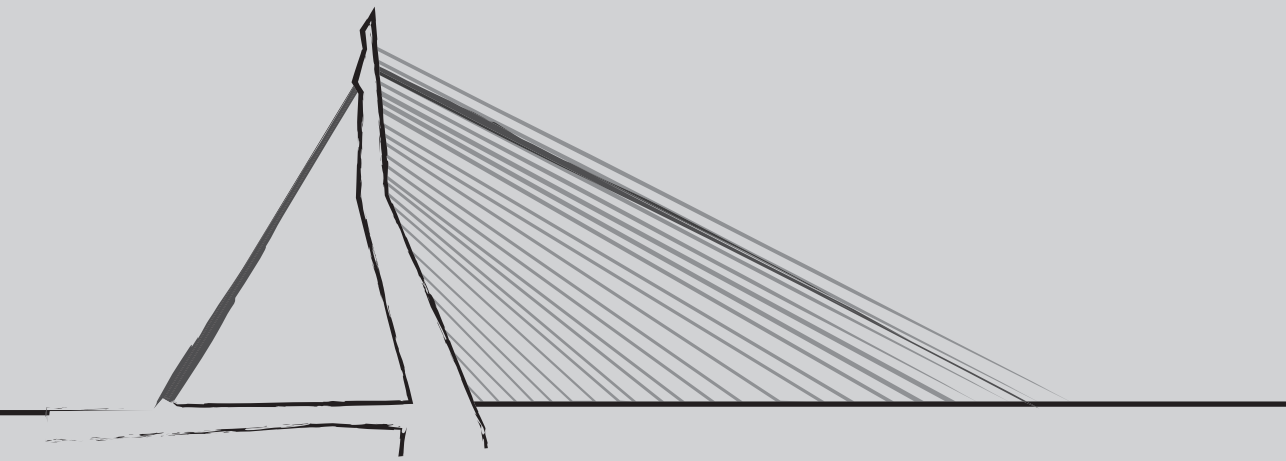
Chapter 2

Early growth, childhood lung function and asthma



Chapter 2.1

Early growth characteristics and the risk of reduced lung function and asthma

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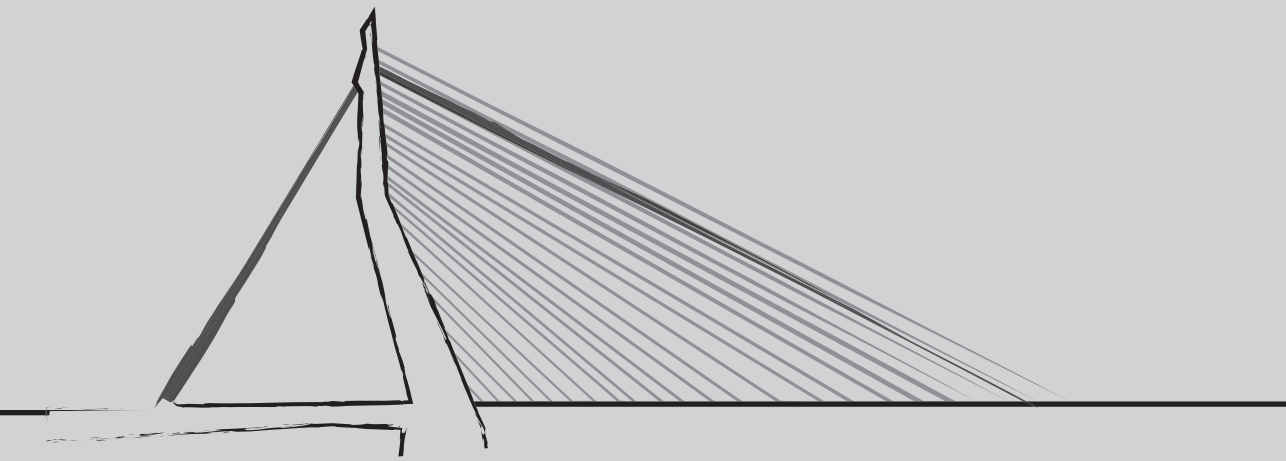


ABSTRACT

Background Children born preterm or with a small-size-for-gestational-age are at increased risk for childhood asthma.

Objective To assess the hypothesis that these associations are explained by reduced airway patency.

Methods We used individual participant data of 24,938 children from 24 birth cohorts to examine and meta-analyze the associations of gestational age, size-for-gestational-age, and infant weight gain with childhood lung function and asthma (age range 3.9 – 19.1 years). Second, we explored whether these lung function outcomes mediated the associations of early growth characteristics with childhood asthma.

Results Children born with a younger gestational age had a lower forced expiratory volume in 1 second (FEV_1), FEV_1/forced vital capacity (FEV_1/FVC), and forced expiratory volume after exhaling 75% of vital capacity (FEF_{75}), whereas those born with a smaller size-for-gestational-age at birth had lower FEV_1 but higher FEV_1/FVC (p-values<0.05). Greater infant weight gain was associated with higher FEV_1, but lower FEV_1/FVC and FEF_{75} in childhood (p-values<0.05). All associations were present across the full range and independent of other early life growth characteristics. Preterm birth, low birth weight and greater infant weight gain were associated with an increased risk of childhood asthma (pooled odds ratio (95% CI): 1.34 (1.15, 1.57), 1.32 (1.07, 1.62) and 1.27 (1.21, 1.34), respectively). Mediation analyses suggested that FEV_1, FEV_1/FVC and FEF_{75} may explain 7 (2, 10)% to 45 (15, 81)% of the associations between early growth characteristics and childhood asthma.

Conclusions Younger gestational age, smaller size-for-gestational-age, and greater infant weight gain were across the full ranges associated with childhood lung function. These associations explain to a substantial extent the risk of childhood asthma.

INTRODUCTION

Children born extremely preterm or with a low birth weight have high rates of neonatal respiratory diseases such as infant respiratory distress syndrome and bronchopulmonary dysplasia.[1] An accumulating body of evidence suggests that these children also have an increased risk of chronic obstructive respiratory diseases in adulthood.[2] More recent, prospective studies in children suggest that preterm birth and small size for gestational age at birth increase the risk of childhood asthma.[3] Recent results of a meta-analysis of individual participant data of 147,000 children participating in prospective birth cohort studies showed consistent associations of younger gestational age at birth and greater infant weight gain with childhood asthma.[4] The associations of lower birth weight with childhood asthma seem to be largely explained by gestational age at birth.[4] The mechanisms underlying the associations of early growth characteristics with childhood asthma are not known yet. Airway caliber is a key determinant of total airway resistance. A reduced airway caliber could result in airway obstruction that predisposes to asthma and chronic obstructive pulmonary diseases.[5-7] Therefore, we hypothesized that the associations of early growth characteristics with childhood asthma might be explained by developmental adaptations of the lungs and airways, leading to relatively small airways and, hence, a reduction in expiratory flows reflected by lower lung function values.[8] Thus far, previous studies focused on the associations of birth weight and infant weight gain with childhood lung function have reported inconsistent results.[9-16] These inconsistent results might be due to the different ages at which spirometry was performed, and not taking other early growth characteristics or potential confounders into account.

To test the hypothesis that the associations of early life growth characteristics with childhood asthma are explained by reduced airway patency, we performed an individual participant data meta-analysis of 24,938 children from 24 birth cohort studies. We examined the strength, consistency, and independence of the associations of gestational age at birth, birth weight and infant weight gain with lung function outcomes in childhood and whether these lung function outcomes explain the previously reported associations of early growth characteristics with risk of childhood asthma.

METHODS

Sources of data

European population-based birth- and mother-child cohorts participated if they included children born between 1989 and 2011, had information available on at least gestational age and weight at birth and lung function measurements in childhood (until age 18 years), and were willing and able to exchange original data.[4] We identi-

fied 50 European cohorts selected from existing collaborations on childhood health or asthma-related outcomes (www.chicosproject.eu, www.birthcohortsenrieco.net, www.ga2len.org, and www.birthcohorts.net) accessed until May 29, 2012). In total, 24 cohorts, comprising data on 24,938 children, fulfilled the criteria (S-figure 2.1.1).

Information about gestational age and weight at birth and weight in the first year of life was obtained by measurements, medical registries or parental questionnaires (S-table 2.1.1). We created gestational age-adjusted birth weight standard deviation scores (birth weight SDS) based on European reference values.[17] Infant weight gain in the first year was defined as the difference between weight at age 1 year (range 6-18 months) and weight at birth, divided by the number of months between these two measurements. Standard deviation scores (SDS) for age-specific infant weight gain were derived by intra-cohort means and standard deviations.[18] Cohort specific growth characteristics are given in the Supplemental Material (S-table 2.1.2).

All cohorts obtained lung function measurements by spirometry, of which 22 according to the recent guidelines of the American Thoracic Society / European Respiratory Society (ATS/ERS)[19-21], and 2 according to earlier guidelines of the ATS [22] or ERS and European Coal and Steel Community [23] (S-table 2.1.1). If cohorts had collected lung function data at multiple time points (n = 6 cohorts), we used the measurement closest to the mean age of children (8.5 years) in the full meta-analysis. Variables for analyses were forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), forced mid-expiratory flow (FEF_{25-75}) and forced expiratory flow after exhaling 75% of the vital capacity (FEF_{75}). We mainly focused on FEV_1, FEV_1/FVC, and FEF_{75}, which reflect reduced airway patency in obstructive lung diseases such as asthma or bronchopulmonary dysplasia due to preterm birth or low birth weight.[24, 25] All lung function variables were converted into sex-, height-, age-, and ethnicity (Caucasian versus non-Caucasian) -adjusted Z-scores based on the Global Lung Initiative reference values.[26] Asthma (yes / no) was defined as ever physician diagnosed asthma, and was obtained by medical registries (2 cohorts) or parental questionnaires adapted from the International Study on Asthma and Allergy in Childhood (ISAAC) [27] (22 cohorts) at the age of spirometry (S-table1). Cohort specific characteristics of lung function measurements and asthma are given in the Supplemental Material (S-table 2.1.3).

We included covariates based on known associations with childhood lung function from previous studies.[28, 29] Information on covariates was mainly assessed by questionnaires (S-table 2.1.1). Potential confounders included maternal educational level, smoking during pregnancy, smoking during infancy of their offspring, history of asthma or atopy, child's sex, siblings, day care attendance in the first 2 years of life, breastfeeding, lower respiratory tract infections in the first 2 years of life, eczema, inhalant allergies, and body mass index (BMI) at the moment of lung function measurement. Cohort specific characteristics of all covariates are given in the Supplemental Material (S-tables 2.1.4-5).

Table 2.1.1. Characteristics of Participating Cohorts.

Cohort name (country)	N	Birth years	Gestational age at birth (weeks)	Birth weight (gram)	FVC	FEV_1	FEV_1/ FVC	FEF_{25-75}	FEF_{75}	Childhood asthma
			Median (5-95% range)	Mean (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Yes, % (N)
ALSPAC (United Kingdom)	6,873	1991-1992	39.5 (1.9)	3,424 (543)	0.49 (1.28)	0.44 (1.17)	-0.07 (1.15)	0.04 (1.08)	0.30 (1.06)	17.9 (1,231)
BAMSE (Sweden	2,042	1994-1996	39.9 (1.8)	3,537 (551)	0.65 (0.93)	0.45 (0.96)	-0.37 (0.89)	-	-	14.8 (303)
BILD (Switzerland)	159	1999-ongoing	39.7 (1.3)	3,367 (441)	-0.23 (0.98)	0.02 (0.89)	0.33 (0.95)	-0.06 (0.87)	-	-
CONER (Italy)	217	2004-2005	39.2 (1.4)	3,335 (457)	-1.76 (0.82)	-1.04 (0.90)	0.51 (1.65)	0.45 (1.00)	-	6.0 (13)
COPSAC2000 (Denmark)	314	1998-2001	40.0 (1.6)	3,529 (531)	-0.53 (0.98)	-0.11 (1.03)	0.47 (0.95)	-	-	18.8 (59)
EDEN (France)	897	2003-2005	39.3 (1.7)	3,284 (514)	-1.08 (1.05)	-0.77 (1.03)	0.21 (0.97)	-0.39 (1.01)	0.16 (0.88)	18.1 (162)
GASPII (Italy)	453	2003-2004	39.2 (1.8)	3,314 (530)	0.06 (0.76)	-0.01 (0.88)	-0.15 (0.97)	-0.30 (0.90)	-	6.6 (30)
GENERATION R (The Netherlands)	1,927	2002-2006	39.7 (1.9)	3,392 (576)	0.23 (0.92)	0.15 (0.95)	-0.19 (0.92)	0.15 (1.05)	-0.09 (0.89)	5.5 (106)
GENERATION XXI (Portugal)	1,562	2005-2006	38.4 (2.1)	3,152 (551)	0.41 (0.95)	0.59 (0.98)	0.21 (0.82)	0.12 (0.85)	0.44 (0.80)	6.5 (102)
GINI (Germany)	707	1995-1998	-	3,493 (479)	-	0.02 (0.92)	-	-	-	5.9 (49)
INMA Gipuzkoa (Spain)	277	2006-2008	39.7 (1.4)	3,284 (436)	-0.54 (1.16)	-0.59 (1.17)	-0.05 (0.91)	-0.45 (0.99)	-0.16 (1.00)	5.4 (15)

Table 2.1.1. Characteristics of Participating Cohorts. (continued)

Cohort name (country)	N	Birth years	Gestational age at birth (weeks)	Birth weight (gram)	FVC	FEV_1	FEV_1/ FVC	FEF_{25-75}	FEF_{75}	Childhood asthma
			Median (5-95% range)	Mean (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Yes, % (N)
INMA Menorca (Spain)	367	1997-1998	39.2 (1.8)	3,200 (493)	0.01 (1.13)	-0.16 (1.07)	-0.24 (1.19)	-0.42 (1.29)	-0.06 (1.32)	4.9 (18)
INMA Sabadell (Spain)	408	2004-2007	39.8 (1.3)	3,261 (404)	-0.47 (1.38)	-0.57 (1.30)	-0.08 (1.03)	-0.61 (1.00)	-0.25 (1.12)	0.7 (3)
INMA Valencia (Spain)	455	2003-2005	39.6 (1.7)	3,227 (491)	0.30 (1.10)	0.30 (1.08)	-0.04 (0.95)	-0.13 (0.91)	-0.04 (0.90)	-
ISLE OF WIGHT (United Kingdom)	1,030	1989-1990	39.9 (1.5)	3,411 (510)	0.24 (0.91)	0.39 (1.01)	0.22 (1.03)	0.04 (0.99)	-	21.5 (221)
KOALA (The Netherlands)	438	2000-2003	40.0 (1.2)	3,552 (467)	0.15 (0.94)	-0.13 (0.95)	-0.55 (0.84)	-	-	8.0 (35)
LEICESTER 1990 (United Kingdom)	290	1985-1990	39.0 (2.2)	3,373 (599)	-0.33 (1.11)	-0.38 (1.12)	-0.76 (0.90)	-0.62 (1.01)	-	37.2 (108)
LEICESTER 1998 (United Kingdom)	1,476	1993-1997	39.2 (2.0)	3,314 (592)	-0.41 (1.04)	-0.39 (1.05)	0.01 (1.03)	-	0.05 (0.94)	36.4 (538)
MAS (Germany)	641	1990	40.0 (1.4)	3,414 (460)	-0.06 (0.97)	0.24 (1.00)	0.41 (1.00)	1.15 (0.14)	-	5.0 (32)
PIAMA (The Netherlands)	1,767	1996-1997	39.9 (1.7)	3,526 (540)	0.04 (0.95)	0.07 (1.04)	-0.04 (1.01)	-1.67 (1.21)	-0.21 (0.95)	10.0 (176)
RHEA (Greece)	666	2007-2008	38.1 (1.7)	3,175 (506)	-0.25 (1.09)	-0.33 (1.14)	-0.10 (0.94)	-0.38 (0.96)	-0.17 (1.05)	5.9 (39)
SEATON (United Kingdom)	578	1997	39.5 (1.8)	3,488 (563)	-0.12 (1.08)	-0.06 (1.08)	-0.04 (0.96)	-0.27 (0.98)	-	20.1 (116)

Table 2.1.1. Characteristics of Participating Cohorts. (continued)

Cohort name (country)	N	Birth years	Gestational age at birth (weeks)	Birth weight (gram)	FVC	FEV_1	FEV_1/ FVC	FEF_{25-75}	FEF_{75}	Childhood asthma
			Median (5-95% range)	Mean (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Mean Z-score (SD)	Yes, % (N)
SWS (United Kingdom)	803	1998-2007	39.7 (1.9)	3,447 (548)	0.13 (1.01)	0.03 (0.95)	-0.18 (1.05)	-0.28 (0.94)	-	15.1 (121)
WHISTLER (The Netherlands)	591	2001-2012	40.0 (1.3)	3,553 (499)	0.16 (1.11)	0.46 (1.14)	0.31 (0.93)	-0.04 (1.23)	0.12 (1.07)	9.3 (55)

N = number of participants with information on at least gestational age or birth weight, and a lung function outcome. Lung function outcomes are forced vital capacity (FVC), force expiratory volume in 1 second (FEV_1), mid forced expiratory flow (FEF_{25-75}) and force expiratory flow at 75% of the exhaled FVC (FEF_{75}). Values are means (standard deviations) and percentages (absolute numbers) for the information on asthma. Additional information on data collection (S-Table 2.1.1), determinants (S-Table 2.1.2), outcomes (S-Table 2.1.3), and maternal and child related covariates (S-Tables 2.1.4, 2.1.5) is provided in the Supplemental Material.

Statistical analysis

First, we conducted 1-stage random effect regression analyses to study the separate and combined associations of gestational age, birth weight and infant weight gain with FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75}. For these analyses, individual participant data from all cohorts were combined and modeled simultaneously taking into account clustering of participants within studies.[30] To prevent multicollinearity in our regression models, we initially assessed the separate associations of gestational age and birth weight with lung function. Thereafter, we assessed whether the associations of birth weight with lung function was driven by gestational age by creating gestational age adjusted birth weight standard deviation scores. The models focused on the associations of infant weight gain with lung function outcomes were adjusted for gestational age and weight at birth. For these analyses, we used early growth characteristics as continuous variables in the models providing p-values for trend. To test non-linear and dose-response associations, we categorized gestational age, birth weight SDS and infant weight gain SDS. As a sensitivity analysis, we conducted a 2-stage random effect meta-analysis to study the associations of gestational age, birth weight, and infant weight gain, and dichotomized preterm birth and low birth weight with each lung function outcome. For this analysis, we used linear regression models per cohort, after which pooled regression coefficients (β's) from the per cohort effect estimates were calculated. We tested for heterogeneity between effect estimates using I^2.[31, 32] For all analyses, the first model was adjusted for child's sex (crude model), the second model was additionally adjusted for potential confounders (full model). To determine interactive effects between gestational age, birth weight and infant weight gain we added the corresponding multiplicative terms in the full model. Since we used Northern-European reference curves for birth weight SDS, we performed a sensitivity analysis to explore whether the associations were different in North-Western European subjects only. Numbers were too small to perform these analyses separately in other European regions. To assess differences in results related to pubertal growth changes, we repeated our analyses is strata of children aged < 11 years and ≥11 years.[33] We also performed a complete-case sensitivity analysis to explore any differences between complete and non-complete-case analyses, and sensitivity analyses in which we excluded cohorts that used parental report of early growth characteristics or that did not perform spirometry measurements according to the ATS/ERS guidelines.

Second, we conducted a 1-stage random effect regression analysis to assess the associations of early growth characteristics with asthma, and observed whether changes in the effect estimates occurred after additional adjustment for lung function measures (FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75}) as potential mediators (mediator model). The difference between the original effect estimates and the effect estimates after additional adjustment for potential mediators was expressed as percentage change. The percentage change was calculated by the formula: 100 x (effect $estimate_{mediator}$ - effect

estimate$_{original\ model}$)/(effect estimate$_{original\ model}$- 1). A 95% confidence interval for the percentage change of the effect estimate was calculated using a bootstrap method with 1,000 resamplings.[34-36]

For all analyses, missing values in covariates were used as an additional group in the categorical variables to prevent exclusion of non-complete cases. Statistical analyses were performed with R version 3.0.0 (libraries rmeta and metafor; The R foundation for Statistical Computing), and Comprehensive Meta-Analysis (Biostat, US).

RESULTS

Subject characteristics

Information about the main characteristics of the cohorts are given in Table 2.1.1. Detailed information about determinants, outcomes and covariates is given in the Supplemental Material (S-tables 2.1.1-5). Of all participants, 8.2% (n = 2,053) was born preterm (<37 weeks of gestational age), and 4.8% (n = 1,191) was born with a low birth weight (<2,500 gram). The mean age at which spirometry assessments were performed was 8.5 (range 3.9 - 19.1) years. The proportion of children aged ≥11 years was 11.9% (n = 2,972).

Early growth measures and lung function outcomes

Results from the 1-stage random effect models showed that younger gestational age at birth was, across the full range, associated with lower FEV_1, FEV_1/FVC and FEF_{75} in childhood (p-values for trend <0.01) (Figures 2.1.1A-C). A smaller size-for-gestational-age at birth across the full range was associated with lower FEV_1 and higher FEV_1/FVC (p-values for trend <0.01) (Figures 2.1.1D-E). Small size-for-gestational-age at birth was not associated with FEF_{75} (Figure 2.1.1F). Greater infant weight gain was associated with a higher FEV_1, but with a lower FEV_1/FVC and FEF_{75} (p-values for trend <0.01; Figures 2.1.1G-I). Most associations showed a linear trend, except for the associations of birth weight with FEV_1/FVC and infant weight gain with FEV_1 and FEV_1/FVC which were non-linear (Figures 2.1.1E, G, H).

To explore the combined effects of gestational age, birth weight SDS and infant weight gain SDS, we performed tests for interaction between these early growth characteristics. These tests for interaction were significant for gestational age and birth weight SDS in relation to FEV_1, FEV_1/FVC, FEF_{25-75} and FEF_{75} (p-values for interaction <0.01; Figure 2.1.2, S-table 2.1.9). Stratified analyses showed that a lower birth weight was associated with lower FEV_1 and FEV_1/FVC among children born after ≥ 32 weeks only, whereas higher birth weight was associated with FEF_{75} only among term born children (p-values for strata <0.05).

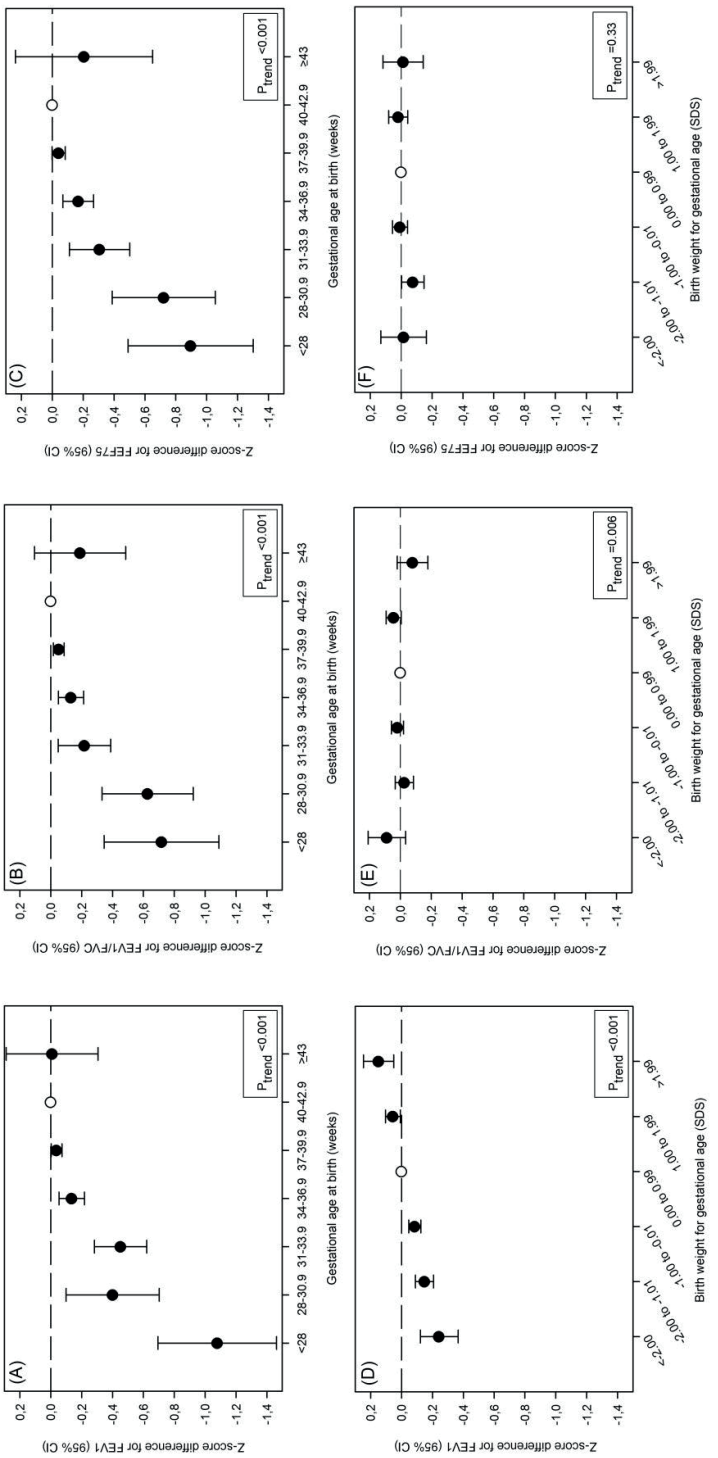


Figure 2.1.1. Associations of Gestational Age, Birth Weight and Infant Weight Gain with FEV_1, FEV_1/FVC and FEF_{75}.

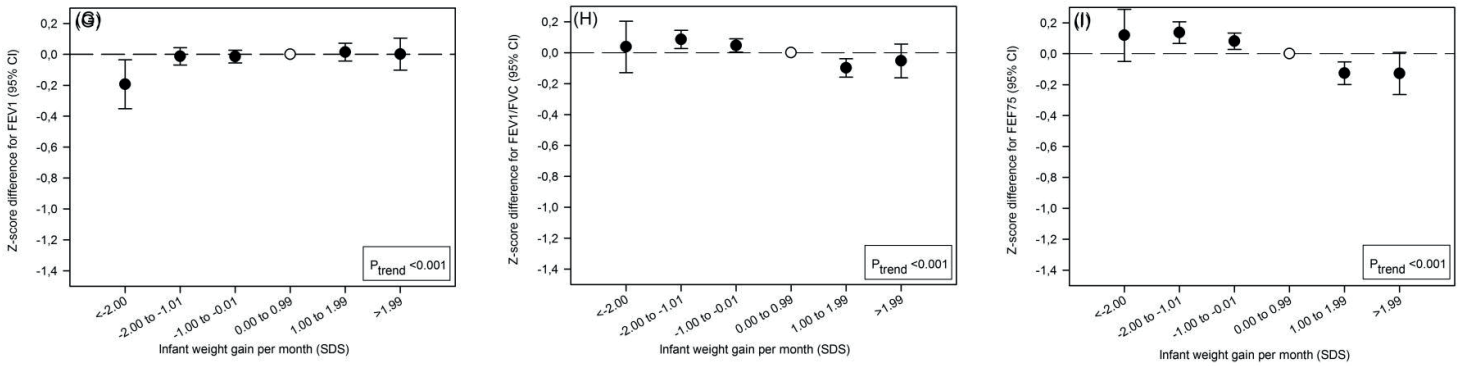


Figure 2.1.1. Associations of Gestational Age, Birth Weight and Infant Weight Gain with FEV_1, FEV_1/FVC and FEF_{75}. (continued)
Values represent Z-scores differences (95% confidence interval) from multi-level random effect models for the associations of gestational age at birth (A, B, C), gestational age adjusted birth weight (birth weight SDS) (D, E, F), and infant weight gain (SDS) (G, H, I) with lung function outcomes, compared with reference groups. Reference groups were 40-42.9 weeks of gestational age, 0-0.99 birth weight SDS and 0.00 – 0.99 infant weight gain (SDS) (largest groups), and represented by an open bullet. Lung function outcomes are forced expiratory volume in 1 second (FEV_1), FEV_1/forced vital capacity (FVC) ratio, and forced expiratory flow at 75% of the exhaled FVC (FEF_{75}). Models are adjusted for maternal education, smoking during pregnancy, smoking during childhood, atopy, asthma and child's sex, number of siblings, daycare attendance, breastfeeding, respiratory tract infections, childhood eczema, inhalant allergies and body mass index. Infant weight gain SDS was additionally adjusted for birth weight and gestational age at birth.

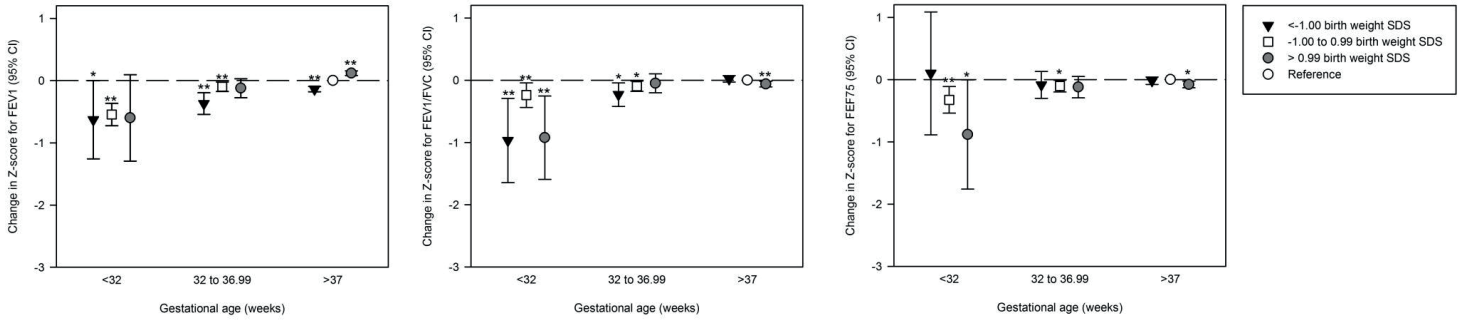


Figure 2.1.2. Combined Associations of Gestational Age and Birth Weight with FEV_1, FEV_1/FVC and FEF_{75}.

Values are Z-score differences (95% confidence interval) from multi-level models for the combined associations of gestational age at birth and birth weight SDS (A, B, C) with lung function outcomes, compared with reference groups. Reference groups were >37 weeks of gestational age with -1.00 to 0.99 birth weight SDS (largest group), and represented by a bullet. Lung function outcomes are forced expiratory volume in 1 second (FEV_1), FEV_1/forced vital capacity (FVC) ratio, and forced expiratory flow at 75% of the exhaled FVC (FEF_{75}). Models are adjusted for maternal education, smoking during pregnancy, smoking during childhood, atopy, asthma and child's sex, number of siblings, daycare attendance, breastfeeding, respiratory tract infections, childhood eczema, inhalant allergies and body mass index. *P-value < 0.05. **P-value < 0.01. Given p-values reflect differences between birth weight SDS groups (A, B, C) within strata of gestational age using -1.00 to 0.99 birth weight SDS as reference group. P_{int}: p-values of multiplicative interaction terms.

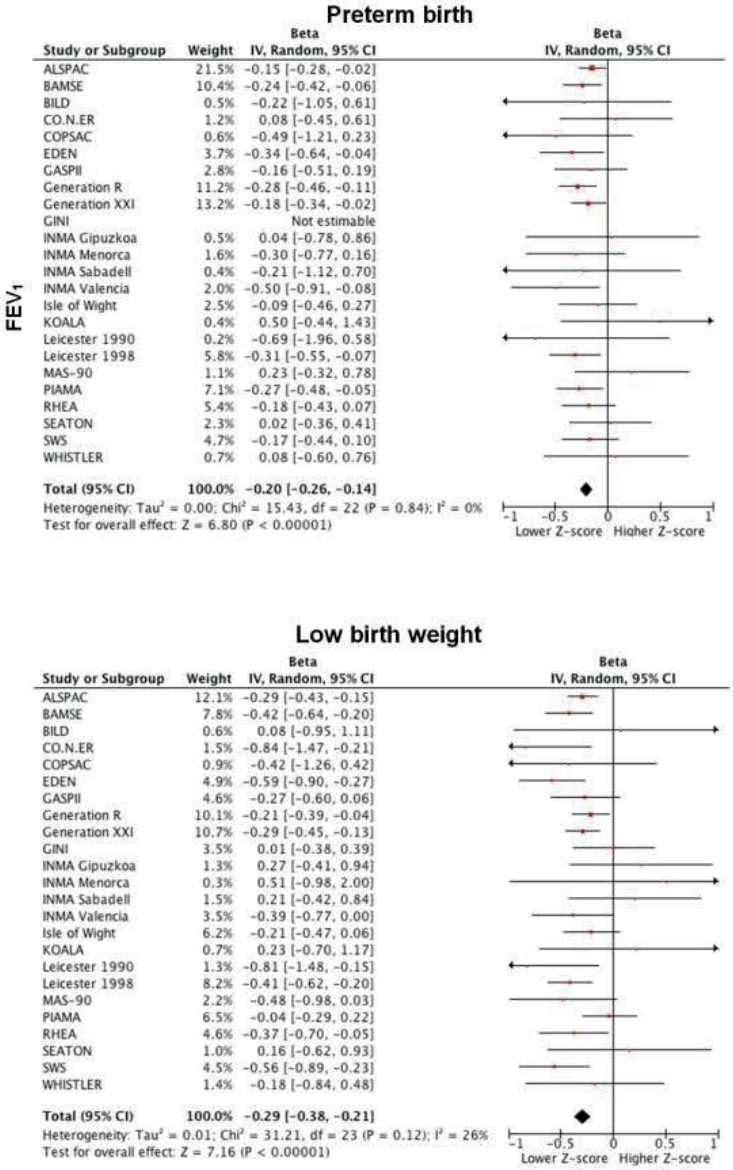


Figure 2.1.3. Forest Plots of the Associations between Preterm Birth and Low Birth Weight with FEV_1, FEV_1/FVC and FEF_{75}.

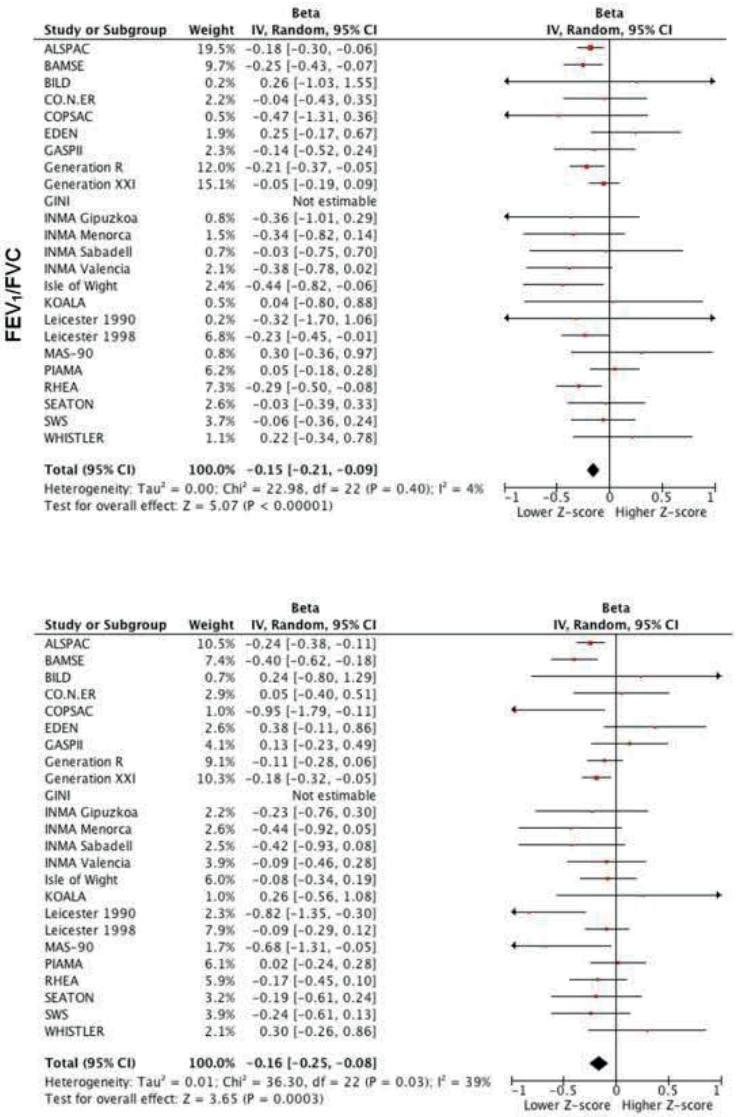
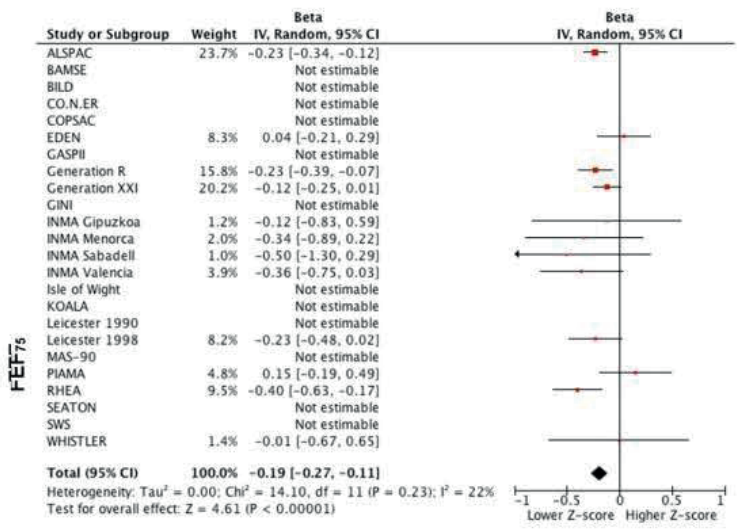


Figure 2.1.3. Forest Plots of the Associations between Preterm Birth and Low Birth Weight with FEV_1, FEV_1/FVC and FEF_{75}. (continued)



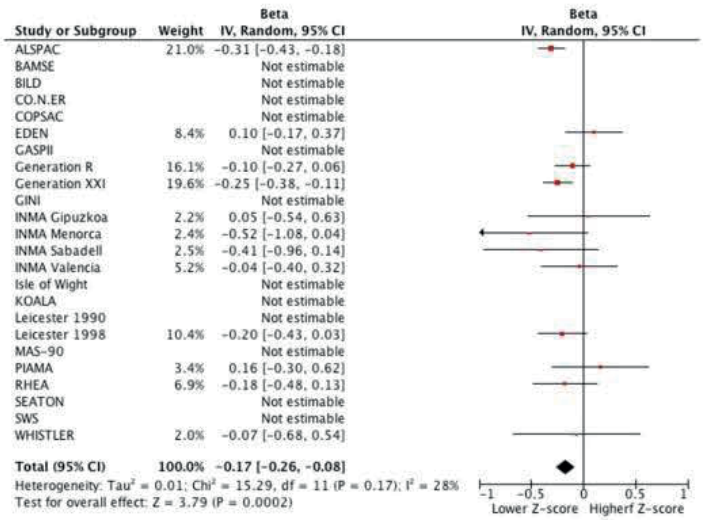


Figure 2.1.3. Forest Plots of the Associations between Preterm Birth and Low Birth Weight with FEV_1, FEV_1/FVC and FEF_{75}. (continued)

Values are pooled Z-score differences (95% confidence interval) from random effect meta-analysis for the associations of preterm birth vs. term birth (A, B, C) and low birth weight vs. normal birth weight (D, E, F) with lung function outcomes. Lung function outcomes are forced expiratory volume in 1 second (FEV_1), FEV_1/forced vital capacity (FVC) ratio, and forced expiratory flow at 75% of the exhaled FVC (FEF_{75}). Models are adjusted for maternal education, smoking during pregnancy, smoking during childhood, atopy, asthma and child's sex, number of siblings, daycare attendance, breastfeeding, respiratory tract infections, childhood eczema, inhalant allergies and body mass index. Low birth weight was adjusted for gestational age.

No differences in results were observed when we used 2-stage random effect models of combined effect estimates (S-tables 2.1.6-7). Also, the results from the sensitivity analyses showed similar results when we used cohorts with North-Western European subjects only, when we excluded cohorts that did not perform spirometry measurements according to the recent ATS/ERS guidelines, when we performed stratified analyses for children aged < 11 years or ≥ 11 years (S-table 2.1.8), or when we excluded cohorts that used parental report of early growth characteristics (data not shown).

Figure 2.1.3 shows that compared to term born children, those born preterm had a lower FEV_1, FEV_1/FVC and FEF_{75}, (pooled Z-score (95% CI): -0.20 (-0.26, -0.14), -0.15 (-0.21, -0.09) and -0.19 (-0.27, -0.11), respectively). Also, compared to normal birth weight children, those with a low birth weight had lower FEV_1, FEV_1/FVC and FEF_{75} (-0.29 (-0.38, -0.21) and -0.16 (-0.25, -0.08) and -0.17 (-0.26, -0.08) respectively), independent of gestational age. Results of associations of growth characteristics with all lung function outcomes, including FVC and FEF_{25-75} are given in the Supplemental Material: S-tables 2.1.6-8.

Early growth, lung function and asthma

Preterm birth, low birth weight and greater weight gain were all associated with an increased risk of childhood asthma (OR (95% CI): 1.34 (1.15, 1.57), 1.32 (1.07, 1.62) and 1.27 (1.21, 1.34), respectively. Mediation analyses suggested that FEV_1, FEV_1/FVC and FEF_{75} may explain 7 (2, 10)% to 45(15, 81)%. Specifically, after additional adjustment for FEV_1, FEV_1/FVC or FEF_{75}, the associations of preterm birth with asthma attenuated with -7 (-19, -1)%, -14 (-40, -3)% and -39 (-69, -3)%, respectively. Similarly, the associations of low birth weight with asthma attenuated with -19 (-37, -12)%, -22 (-47, -11)% and -222 (-47, -11)%, respectively (Table 2.1.2). The strongest mediating effect was observed for FEF_{75} for the association between gestational age and asthma (-45 (-81, -15)%). Similar trends were observed for greater weight gain, although the associations did not attenuate into non-significant.

DISCUSSION

In this meta-analysis of individual participant data of 24,938 children from 24 birth cohorts, we observed that lower gestational age, smaller size at birth and greater infant weight gain were all associated with lower childhood FEV_1. The positive associations of birth weight and infant weight gain with FVC were larger than the positive associations of birth weight and infant weight gain with FEV_1. This combination resulted in associations of higher birth weight and infant weight gain with lower FEV_1/FVC. Also, a lower gestational age at birth was associated with a lower FEF_{75} in childhood, suggesting persistent reduction of small airways patency. A greater infant weight gain was associ-

Table 2.1.2. Associations of Birth Weight, Gestational Age and Infant Weight Gain with Childhood Asthma, Additionally Adjusted for Lung Function.

	Risk of childhood asthma Odds ratio (95% Confidence Interval)						
	Full model	**Full model + FEV_1**	**% change (95% CI)**	**Full model + FEV_1/FVC**	**% change (95% CI)**	**Full model + FEF_{75}**	**% change (95% CI)**
Gestational age (weeks)	0.94 (0.92, 0.97)** n = 15,019	0.95 (0.93, 0.97)** n = 14,832	-9.8% (- 16.4, - 5.3)**	0.95 (0.93, 0.97)** n = 14,017	-13.5% (-21.0, -7.3)**	0.97 (0.94, 1.00) n = 9,177	-44.6% (-81.1, -14.6)**
Preterm birth (<37 weeks)	1.34 (1.15, 1.57)** n = 15,019	1.30 (1.11, 1.53)** n = 14,832	-7.3% (-18.8, -0.9)*	1.27 (1.08, 1.49)** n = 14,017	-14.4% (-39.6, -2.8)*	1.20 (0.99, 1.47) n = 9,177	-39.0% (-69.3, -3.4)*
Birth weight (500 grams)	0.94 (0.90, 0.97)** n = 15,547	0.95 (0.91, 0.99)* n = 15,360	-18.9% (-37.0, -11.2)**	0.94 (0.90, 0.98)** n = 13,985	-10.5% (-21.9, -3.4)**	0.96 (0.92, 1.02) n = 9,135	-17.8 (-50.6, -9.0)**
Low birth weight (<2,500 grams)	1.32 (1.07, 1.62)** n = 15,547	1.25 (1.02, 1.54)* n = 15,360	-19.0% (-37.3, -11.8)**	1.23 (0.99, 1.52) n = 13,985	-21.6% (-47.3, -11.4)**	1.05 (0.81, 1.36) n = 9,135	-82.5% (-149, 10.3)
Birth weight (SDS)	0.98 (0.94, 1.03) n = 14,947	1.00 (0.96, 1.05) n = 14,760	-83.8% (-950, 825)	0.98 (0.94, 1.03) n =13,946	-14.0% (-247, 281)	0.99 (0.93, 1.04) n = 9,122	-15.8% (-158, 169)
Small for gestational age (<10th percentile)	1.18 (1.01, 1.37)* n = 14,947	1.13 (0.97, 1.32) n = 14,760	-28.9% (-253, 108)	1.16 (0.99, 1.36) n = 13,946	-18.8% (-123, 164)	1.20 (1.00, 1.44) n = 9,122	10.2% (-8.3, 26.2)
Infant weight gain in first year (SDS), adjusted for gestational age and weight at birth	1.27 (1.21, 1.34)** n = 12,511	1.28 (1.22, 1.35)** n = 12,511	6.5% (2.3, 9.9)**	1.25 (1.18, 1.31)** n = 11,780	-8.4% (-16.1, -3.2)**	1.13 (1.06, 1.20)** n = 7,969	-60.8 (-115, 39.5)

*p<0.05 **p<0.01. Values are odds ratios or percentage change in odds ratios (95% confidence interval) from random effect models and represent the risk of asthma per week, 500 grams or SDS increase in gestational age, birth weight, gestational age adjusted birth weight (birth weight SDS), or infant weight gain (SDS), respectively, or represent odds ratios or percentage change in odds ratios (95% confidence interval) in risk of asthma for preterm birth vs. term birth, low birth weight vs. normal birth weight or small for gestational age vs. normal and large for gestational age ($<10^{th}$ percentile vs $>10^{th}$ percentile). Percentage change in odds ratio (OR) is calculated using the formula (100 x ($OR_{mediator}$ - $OR_{model\ 1}$)/($OR_{model\ 1}$ - 1)), with corresponding 95% confidence interval obtained by bootstrap procedures. To enable comparison of effect estimates, results for gestational age adjusted birth weight and infant weight gain are presented as per SDS. Models are adjusted for maternal education, smoking during pregnancy, smoking during childhood, atopy, asthma and child's sex, number of siblings, daycare attendance, breastfeeding, respiratory tract infections, childhood eczema, inhalant allergies and body mass index (full model), and additionally for lung function outcomes (mediator model).

ated with lower FEF_{75}. Remarkably, these associations were present across the full-range of early growth and not restricted to clinically diagnosed preterm- or low birth weight children. Also, the observed associations of the early life growth characteristics with lung function outcomes were independent of each other. Stratified analyses showed that children born very preterm with a relatively low birth weight had the lowest FEV_1 and FEV_1/FVC. The associations of early growth characteristics with childhood asthma were partly explained by lung function adaptations.

Whereas lung growth continues until the early adulthood, the most rapid development of airways and alveoli occurs in early life.[37] Developmental adaptations in fetal life and infancy due to early life adverse exposures might result in impaired lung growth with smaller airways, decreased lung volume, and subsequently to an increased risk of bronchopulmonary dysplasia, asthma or COPD.[9, 14, 38] Previous studies suggest that children with asthma already have a reduced lung function in the first months of life, and that this deficit progresses into childhood and early adulthood.[39, 40] Airway caliber is a key determinant of total airway resistance and reduced caliber is a prominent feature of asthma and chronic obstructive pulmonary diseases.[5-7] Lower lung function in early life is likely to lead to lower peak lung function in early adulthood, and the natural decline in FEV_1 from that point onwards will be accelerated by any additional adverse exposures.[41] Thus, lung function during the life course seems to be programmed at least partly in early life.

Children born preterm or with a very low birth weight are at increased risk of neonatal respiratory diseases.[1] We observed that children born at a younger gestational age had a lower FEV_1, even after taking FVC into account, and a lower FEF_{75} in childhood. These associations were not only present among children born very preterm, but across the full range of gestational age at birth. Moreover, the associations of preterm birth with childhood asthma were partly explained by lung function. These findings are in line with previous studies showing persistent lung function adaptions in children and adults born preterm. A recent meta-analysis of 28 published studies showed that children born between 24 and 36 weeks had a lower FEV_1 at ages 5 up to 23 years.[42] These and other studies suggest that preterm birth has adverse effects on lung function, persisting into adulthood.[42-44]

In the present study, a lower birth weight was associated with lower FEV_1 in childhood. This suggests that a lower birth weight leads to a persistent reduction of airway patency. A previous study analyzed 10 studies examining the associations of birth weight with FEV_1 in adults (range 19 – 70 years).[10] The authors reported a modest positive association between FEV_1 and birth weight. Two recent studies from longitudinal birth cohorts among adults reported strong positive associations of birth weight with FEV_1 and FEF_{25-75} in young adults aged 21 and 31 years.[9, 11] The effect of birth weight was independent of preterm birth in both studies. However, studies among children showed

conflicting results.[12, 13] We observed an association of lower birth weight with lower FEV_1, independent of gestational age at birth. We previously reported that the effect of lower birth weight on asthma was largely explained by gestational age.[4] Therefore, although gestational age-adjusted birth weight is associated with lower lung function this seems not related to the risk of clinically manifest childhood asthma.

Previous studies examining associations between infant weight gain and childhood lung function have reported inconsistent results.[14-16] Differences might be due to different ages at which spirometry was performed, not taking other weight characteristics into account, such as birth weight or current body mass index, and possible hidden bias due to the use of mL instead of Z-scores for lung function.[45] In line with the findings for birth weight, we observed that lower infant weight gain was associated with a lower childhood FEV_1 (p-value for trend <0.01). Alternatively, a greater infant weight gain was associated with a higher childhood FEV_1. This association was fully explained by FVC. These results suggest dysanapsis, in which FVC was higher relative to FEV_1 as a result of possible disproportional growth of lung volume and airways. Dysanapsis is commonly used to indicate relatively narrow airways for lung volume, but here a relatively higher lung volume for airways applies.[46] Greater infant weight gain was also associated with a lower FEF_{75}, which is in line with previous studies reporting associations of body mass index or adiposity with reduced expiratory flows and asthma.[47, 48] A suggested mechanism is leptin release from adipose tissue, which might have pro-inflammatory effects in the airways [49], or a direct effect of increased body weight on lung function.[50] However, our analyses were adjusted for childhood body mass index. Further studies are needed to explore whether the associations of infant weight gain with end-expiratory flows are explained by specific adiposity-related measures or biomarkers.

To the best of our knowledge this is the first study that examines the individual and combined associations of the main early growth characteristics with childhood lung function outcomes, and whether lung function adaptations explain the previously reported associations of early growth characteristics with childhood asthma. Our results suggest that respiratory consequences of preterm birth and a low birth weight present across the full range. This observation might have important population effects, since the largest majority of children are in the less extreme ranges of gestational age and weight at birth. Furthermore, our results suggest that the associations of gestational age, birth weight and infant weight gain with childhood asthma are at least partly explained by adaptions in airway caliber. We observed strong effect estimates with wide confidence intervals which limit the precision. Therefore, these mediation effects should be interpreted carefully. The effect estimates for the observed associations could be considered as small and without clinical relevance for individuals. However, the associations may be important from an etiological respiratory developmental perspective and may be important on a population-level. The associations of early growth characteristics

with lung function outcomes seemed already established before the pubertal growth spurt. The largest lung and airway growth occurs before pubertal growth spurt [37, 51], with FVC increasing proportionately more than the FEV_1.[33] Lung and airway growth is proportionally less after start of the pubertal growth spurt [33], which might explain the similar effect estimates before and after the pubertal growth spurt. Further studies are needed to identify the developmental adaptations of the lungs and immune system that might explain the mediating effect of lung function on the associations of early growth characteristics with childhood asthma. Identification of modifiable exposures may lead to development of future preventive strategies.

Some methodological limitations need to be discussed. We used data from 24 ongoing cohort studies. Missing values always occur in these studies. Since we did not have additional data on patterns of missing values in all 24 cohorts, we were not able to perform multiple imputation. Data on childhood asthma was mainly obtained by parental questionnaires adapted from the International Study on Asthma and Allergy in Childhood (ISAAC).[27] This questionnaire has been validated in various age groups in many countries against measurements of bronchial hyperresponsiveness and doctor-diagnosed asthma, and is widely accepted in epidemiological studies. We did not have information on use of asthma medication, which might have influenced the lung function values in asthmatic patients. This missing information on asthma medication may have influenced our effect estimates. We would expect that asthmatic children who use asthma medication would in general have had a higher lung function values in case of good adherence and inhaler technique. We used GLI reference data to convert lung function values into Z-scores. These prediction equations were based on 74,187 individuals including 31,840 individuals aged <20 years, of whom 58% were assessed before, and 42% were assessed during pubertal growth spurt.[26] To date, the GLI normal values are considered the most accurate reference values for all age ranges, and have been adopted by both the ATS and ERS. For the covariates, we imputed missing values as additional category to prevent exclusion of non-complete cases. No differences in results were observed in complete case analyses. No direct clinical and laboratory information about pubertal growth was available. Also, although we took major potential confounders into account, residual confounding may still be an issue. No information was available about e.g. exposure to environmental micro-organisms or asthma severity. Exploring mediation of lung function for the association of early growth characteristics with asthma using the method proposed by Baron and Kenny might have been limited by misclassification of lung function measurements or asthma diagnosis although we aimed to reduce this issue by multi-level modelling.[52] Most of the participating studies had measured childhood lung function and asthma at the same age. Therefore, further follow-up studies with longitudinally measured detailed data on lung function and asthma or related symptoms from birth onwards are needed to disentangle the direction of causality.

In conclusion, younger gestational age, lower birth weight and lower infant weight gain were independently associated with persistent changes in childhood lung function. These associations were present across the full spectrum of these early growth characteristics. Stratified analyses showed that children born very preterm with a relatively low birth weight had the lowest FEV_1 and FEV_1/FVC. Our results suggest that associations of early growth with the risk of childhood asthma were partly explained by lung function adaptations. Thus, fetal and infant growth patterns may persistently affect lung function, and thereby contribute to the risk of respiratory diseases in later life.

Detailed acknowledgements and online resources can be found in the published article online: http://www.sciencedirect.com/science/article/pii/S0091674915013615

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Chapter 2.2

Fetal and infant growth patterns and risk of lower lung function and asthma

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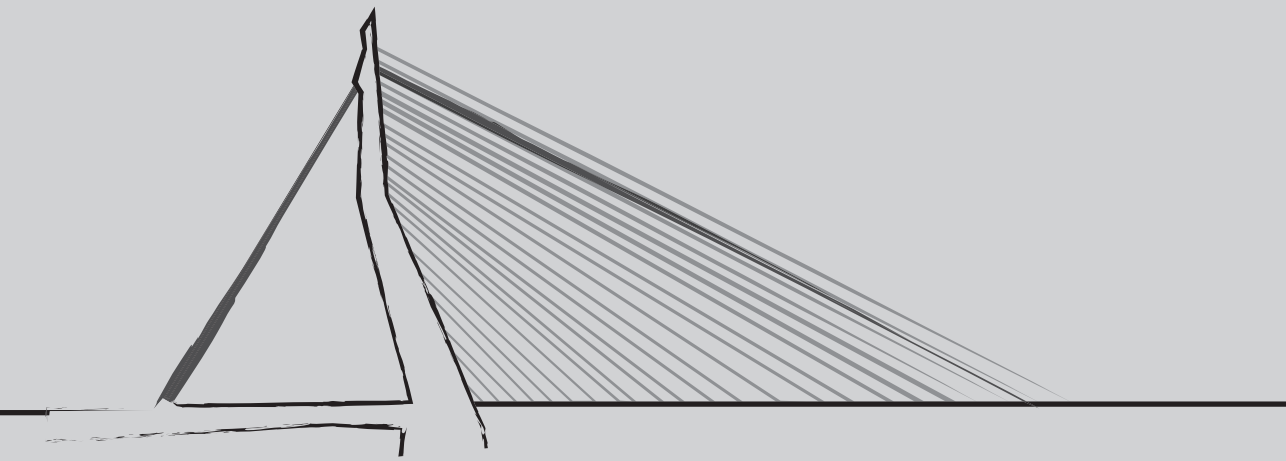
Submitted



Chapter 2.3

Body fat mass distribution and asthma at school-age

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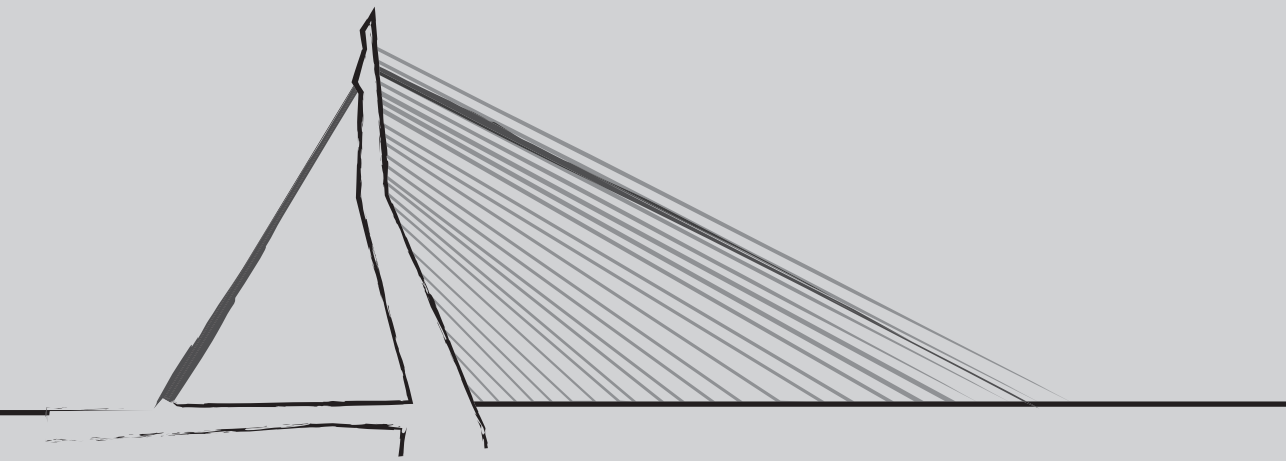

ABSTRACT

Background Obesity and asthma often coexist. We hypothesized that detailed body fat distribution measures might be more strongly associated with childhood asthma than body mass index (BMI).

Objective We examined the associations of total body and abdominal fat measures with respiratory resistance (Rint), fractional exhaled nitric oxide (FeNO), and the risks of wheezing and asthma in school-aged children.

Methods In a population-based prospective cohort study among 6,178 children aged 6 years, we measured BMI, fat mass index, android/gynoid ratio and pre-peritoneal and subcutaneous fat mass by physical examinations, Dual-energy X-ray absorptiometry and ultrasound, respectively. We performed Rint and FeNO measurements, and assessed physician-diagnosed wheezing and asthma by questionnaires.

Results A higher BMI was associated with a higher Rint (Z-score (95% CI): 0.06 (0.01, 0.12)) and increased risk of wheezing (OR (95% CI): 1.07 (1.00, 1.14), per Z-score BMI increase), but not with FeNO or asthma. A high fat mass index was associated with a higher Rint (Z-score (95% CI): 0.40 (0.13, 0.68)). A high android/gynoid fat mass ratio was associated with a lower FeNO (Sym% (95% CI): -9.8 (-16.3, -3.4)), whereas a high pre-peritoneal fat mass was associated with a higher FeNO (Sym% (95% CI): 6.5 (0.1, 12.9)). Subcutaneous fat mass was not associated with any respiratory outcome.

Conclusion Studying detailed body fat distribution measures might provide better insight of the obesity-asthma paradigm.

INTRODUCTION

Asthma and obesity are two of the leading chronic childhood morbidities in developed countries with reported prevalences up to 10% and 25%, respectively.[1,2] It has been hypothesized that obesity leads to asthma. A proposed underlying mechanism is that a higher body mass index (BMI) leads to an increased production of systemic pro-inflammatory mediators by fat tissue, with subsequent airway inflammation and an increased risk of asthma.[3] Alternatively, an increased BMI might lead to increased intrathoracic and abdominal fat deposition. This might reduce the pulmonary vital capacity and increase obstruction-related respiratory resistance and the risk of asthma symptoms.[3] Most studies that assessed the obesity-asthma link use BMI as a proxy for body fat and body composition. The major limitation of BMI is that it does not distinguish fat mass from free-fat mass [4], while it is suggested that fat mass distribution is more strongly associated with adverse health risks.[5] Higher visceral fat mass has been associated with cardiometabolic diseases, which might be due to an increased secretion of leptin.[3, 6] Therefore, more detailed measurements of total and abdominal fat mass distribution might be better predictors for the development of asthma.[4] Studies that assessed detailed adiposity measures are scarce, are mainly performed in adults and show inconsistent results. The use of dual-energy X-ray absorptiometry (DXA) and ultrasound allows a more accurate, fast and precise measurement of body fat composition, including regional fat measurements [7], and has been proven to be highly reproducible in children.[8]

To test the hypothesis that fat mass distribution leads to higher obstruction related respiratory resistance and airway inflammation, and subsequently increased risk of childhood asthma, we assessed the associations of BMI, total body and abdominal fat mass measures with respiratory resistance (Rint), fractional exhaled nitric oxide (FeNO), wheezing and asthma among 6,178 school-aged children participating in a population-based prospective cohort study.

METHODS

This study was embedded in the Generation R Study, a population-based prospective cohort study of pregnant women and their children from fetal life onwards in Rotterdam, The Netherlands.[9] The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam (MEC-2007-413-NL21545.078.0), and written informed consent of parents / legal guardians of participants was obtained.

Childhood body fat profile

At age 6 years, height and weight were measured. Body fat distribution was measured with a DXA scanner (GE-Lunar, 2008, Madison, WI, USA), and analyzed with enCORE v.12.6.[10] Total body fat mass (kg) was calculated as percentage of total body weight (kg) measured by DXA. Fat mass index (total body fat mass/height^2) and android/gynoid fat mass ratios were calculated and used as total and regional fat measures, respectively. The android/gynoid fat ratio reflects the central body fat mass distribution in the abdominal (android) and hip (gynoid) region, and is generally used as a marker for waist/hip fat mass distribution.[11] Abdominal ultrasounds were used to measure subcutaneous fat and pre-peritoneal fat, a measure of visceral abdominal fat, as described in detail before.[7] We constructed Z-scores [(observed value – mean)/standard deviation] for all body fat measures, using known prevalences of overweight and obesity in children aged 6 years in the Netherlands (12.7% and 2.9%, respectively) [12] and to enable comparison of the effect sizes of different outcome measures. To examine non-linear associations and for clinical interpretation, we categorized body fat measures Z-score into "low" (Z-score < -1.00), "normal" (Z-score ≥-1.00 and ≤1.00) and "high" (Z-score >1.00), which reflect the 16th and 84th percentiles. Categorization of body fat measures into tertiles resulted in similar results (data not shown). Additional information on body fat profile measures is provided in the Supplemental Material.

Childhood lung function, wheezing and asthma

At age 6 years, lung function measurements were performed according to European Respiratory Society (ERS) and American Thoracic Society (ATS) recommendations.[13, 14]

Rint was measured during expiration with occlusion of the airway at peak expiratory flow, and converted into sex- and height-adjusted Z-scores.[15] Fractional exhaled nitric oxide (FeNO) was measured using the NIOX Flex chemiluminescence analyser (Aerocrine AB, Solna, Sweden). Additional information on Rint and FeNO-measures is provided in the Supplemental Material. Information on the prevalences of wheezing in the past 12 months (no; yes) and ever physician-diagnosed asthma (no; yes) was collected by questionnaires adapted from the International Study on Asthma and Allergy in Childhood (ISAAC).[16]

Covariates

Potential covariates included early growth, and socio-economic and lifestyle factors. In detail, maternal characteristics included age (years), pre-pregnancy BMI (kg/m^2), educational level (low; middle; high), history of asthma and atopy (no; yes), psychological distress during pregnancy (no; yes), parity (nulli-; multiparous), and maternal smoking during pregnancy (no; yes), and were obtained from questionnaires during pregnancy. Maternal psychological distress in the second trimester of pregnancy was defined using

the global severity index (GSI), a measure of current level or depth of the symptoms, and denotes overall psychological distress.[17] High scores represent an increased occurrence of overall distress, based on Dutch cut-offs.[17] We used parity as a proxy for siblings, and the correlation between those variables was high (κ = 0.87). Sex (female; male), gestational age (weeks) and birth weight (grams) of the children were obtained from midwife and hospital registries at birth. Information about ethnicity (European; non-European, and pet keeping (no; yes, cat, dog or bird) in the first year were obtained by questionnaires. Postal questionnaires at 6 and 12 months provided information about ever breastfeeding (no; yes). Information on ever physician diagnosed inhalant allergy (no; yes, pollen, house dust mite, or pets), physical activity (hours per day) and lower respiratory tract infections (no; yes, but not physician attended; yes, physician attended for bronchitis and/or pneumonia) was obtained by parental questionnaires at age 6 years.

Statistical analysis

We used linear and logistic regression models to examine the associations of body fat profile measures with Rint, FeNO, wheezing and asthma in children aged 6 years. We constructed age- and sex-adjusted Z-scores for BMI using Dutch reference data (Growth Analyzer 3.5, Dutch Growth Research Foundation). Sex-specific weights were classified by the International Obesity Task Force cut offs into underweight, normal, overweight and obesity.[18] Due to low numbers of underweight children (n=55), we excluded these individuals from the analyses. First, models were adjusted for child's sex only. Second, to assess the influence of possible confounders, models were adjusted for growth related factors (maternal pre-pregnancy BMI, child's gestational age at birth and birth weight) and socio-economic and lifestyle related factors (maternal age, educational level, maternal smoking during pregnancy, history of asthma and atopy, psychological distress during pregnancy, parity, child's sex, ethnicity, breastfeeding, pet keeping, physical activity and lower respiratory tract infections). Confounders were included in our models based on the literature, if they were associated with both the determinant and the outcome and were not in the causal pathway, or if they changed the effect estimates with ≥10%. Additionally, associations of total body and abdominal fat mass measures with Rint, FeNO, wheezing and asthma were adjusted for BMI. Missing data were <25%, except for maternal stress during pregnancy (26.5%). To reduce potential bias due to missing data on covariates, we performed multiple imputation generating 10 datasets by Markov Chain Monte Carlo and used the pooled estimates.[19] We log-transformed FeNO due to skewed distribution and present those results in sympercents (sym%).[20] All other measures of association are presented as odds ratios (OR) or Z-score differences with their corresponding 95% Confidence Intervals (95% CI). Statistical analyses were performed using SPSS version 21.0 for Windows (SPSS Inc).

Table 2.3.1. Maternal and Child Characteristics.

	Subjects n = 6,178	**Never asthma-diagnosed (n = 4,140)**	**Ever asthma-diagnosed (n = 298)**
Maternal characteristics			
Age (years)[#]	30.6 (5.1)	31.4 (4.7)	31.2 (5.1)
Pre-pregnancy body mass index (kg/m^2)[‡]	23.8 (18.9,36.0)	23.4 (4.0)	23.6 (4.5)
Education (higher)	46.0 (2,843)	53.8 (2,228)	44.3 (132)
History of asthma or atopy (yes)	42.8 (2,645)	41.2 (1,706)	54.4 (162)
Maternal psychological distress (yes)	10.1 (625)	7.6 (313)	12.1 (36)
Parity (≥1)	43.6 (2,695)	41.1 (1,701)	45.6 (136)
Smoking during pregnancy (yes)	26.8 (1,658)	23.4 (968)	27.9 (83)
Birth and infant characteristics			
Sex (female)	50.0 (3,090)	50.4 (2,088)	39.6 (118)
Gestational age at birth (weeks)[‡]	40.1 (35.7, 42.3)	40.1 (36.0, 42.3)	39.9 (33.1, 42.0)
Birth weight (gram)[#]	3,428 (556)	3,453 (553)	3,359 (624)
Ethnicity (European)	65.7 (4,061)	73.2 (3,032)	66.8 (199)
Breastfeeding (ever)	91.8 (5,674)	92.1 (3,815)	87.2 (260)
Pet keeping 1st year (yes)	37.9 (2,342)	38.5 (1,595)	39.9 (119)
School-age characteristics			
Age at follow-up measurements (years) [#]	6.2 (0.5)	6.2 (0.5)	6.3 (0.5)
Physical activity per day (hours)[‡]	1.4 (0.1, 4.5)	1.4 (0.0, 4.3)	1.4 (0.1, 4.6)
Lower respiratory tract infections age 6 years (yes)	5.8 (358)	3.2 (133)	25.5 (76)
Current height (cm)[#]	119.5 (6.0)	119.1 (5.6)	119.2 (6.2)
Missing	0.1 (7)	0.1 (6)	-
Current weight (kg)[#]	22.3 (17.3, 33.5)	22.6 (3.7)	23.0 (4.0)
Missing	1.4 (88)	1.3 (54)	1.7 (5)
Body mass index (kg/m^2)[‡]	15.7 (13.4, 20.9)	15.6 (13.4, 20.2)	15.8 (13.4, 21.7)
Underweight	0.9 (55)	0.9 (35)	1.0 (3)
Normal weight	83.6 (5,079)	85.5 (3,484)	81.6 (239)
Overweight	12.0 (728)	10.3 (418)	13.3 (39)
Obese	4.5 (271)	3.4 (139)	4.1 (12)
Missing	1.6 (100)	1.6 (64)	1.7 (5)
Total body and regional fat mass			
Fat mass index (kg/cm^2)[‡]	0.37 (0.24, 0.79)	0.37 (0.23, 0.74)	0.38 (0.24, 0.81)
Missing	1.5 (94)	1.5 (60)	1.7 (5)
Android/Gynoid ratio[‡]	0.24 (0.16, 0.42)	0.24 (0.16, 0.40)	0.24 (0.14, 0.48)
Missing	1.4 (87)	1.3 (54)	1.7 (5)
Abdominal fat mass			
Subcutaneous area (mm^2)[‡]	48.0 (18.0, 191.9)	46.0 (17.0, 171.0)	46.0 (14.2, 201.8)
Pre-peritoneal area (mm^2)[‡]	39.0 (16.0,120.0)	38.0 (16.0, 109.6)	38.0 (13.2, 111.5)

Table 2.3.1. Maternal and Child Characteristics. (continued)

	Subjects n = 6,178	Never asthma-diagnosed (n = 4,140)	Ever asthma-diagnosed (n = 298)
Missing	18.3 (1,131)	18.7 (773)	17.7 (52)
Rint ($kPa.L^{-1}.s$)	0.84 (0.29)	0.85 (0.29)	0.92 (0.30)
Missing	29.0 (1,794)	31.4 (1,301)	30.1 (89)
FeNO (ppb)	7.3 (2.8, 30.5)	7.2 (2.7, 26.4)	8.3 (2.9, 40.2)
Missing	36.0 (2,221)	37.9 (1,570)	38.2 (113)
Wheezing (yes)	9.4 (426)	6.3 (254)	54.1 (160)
Missing	26.3 (1,626)	0.8 (32)	0
Asthma (yes)	6.1 (298)	-	-
Missing	28.2 (1,745)	-	-

Values are #means (SD), ‡medians (2.5-97.5th percentile) or percentages (absolute numbers) based on multiple imputation except for main risk factors and outcomes under study (Current height and weight, body mass index, total body and regional fat mass, abdominal fat mass, Rint, FeNO, wheezing and asthma).

RESULTS

General

A total of 6,178 children were included for the current analyses (S-Figure 2.3.1). Loss to follow-up was mainly due to non-response to questionnaires, and due to technical issues for Rint and FeNO. Maternal and child characteristics are presented in Table 2.3.1 and S-Table 2.3.1. Of all children, 83.6% had a normal weight, 12.0% had overweight, and 4.5% were obese. Of all children, 9.4% wheezed in the past year, and 6.1% were ever diagnosed by a physician with asthma. All characteristics, except for maternal history of asthma or atopy and parity, and child's sex and breastfeeding, differed between subjects included and those lost to follow-up (S-Table 2.3.2).

Body mass index, lung function and asthma

In the full model, a higher BMI was associated with a higher Rint and increased risk of wheezing (Z-score (95% CI) 0.06 (0.01, 0.12)), and OR (95% CI) 1.07 (1.00, 1.14)), respectively, per Z-score increase in BMI) but not with FeNO or asthma (Figures 2.3.1A-D). Compared with normal weight, overweight was associated with a higher Rint (Z-score (95% CI): 0.36 (0.07, 0.65)) (Figure 2.3.1A). Obesity was associated with an increased risk of wheezing (OR (95% CI): 2.09 (1.22, 3.57)) (Figure 2.3.1C). Underweight was not associated with Rint (S-Table 2.3.3).

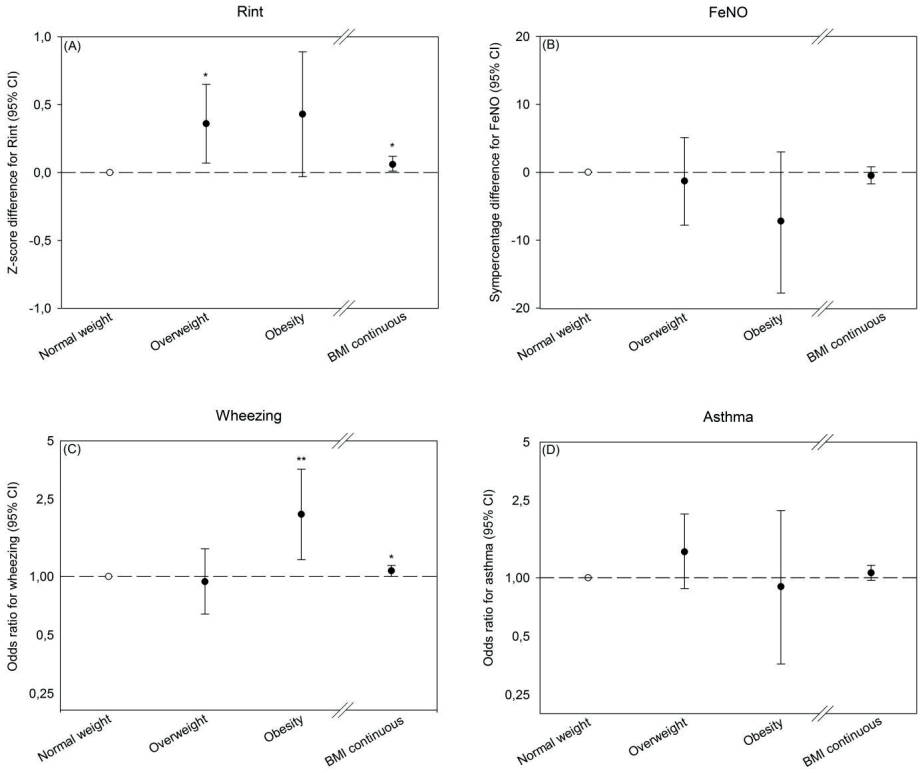

Figure 2.3.1. Association of Body Mass Index in Categories and Continuously with Rint, FeNO, Wheezing and Asthma in Children at Age 6 Years.
Values are changes in Z-score for Rint (A) or in sympercent for FeNO (B), and odds ratios for wheezing (C) and asthma (D) (95% confidence interval presented as error bars) of overweight and obesity compared with normal weight or per kg/m^2 increase of BMI, from logistic or linear regression models, *p < 0.05 and **p < 0.01. Models were adjusted for maternal age, pre-pregnancy BMI, educational level, history of asthma and atopy, psychological distress during pregnancy, parity, smoking during pregnancy, and child's sex, gestational age at birth, birth weight, ethnicity, breastfeeding, pet keeping, physical activity, lower respiratory tract infections and current height.

Total and abdominal fat measures, lung function and asthma

Fat mass index was not linearly associated with Rint, FeNO, wheezing or asthma. When categorized, we observed that a high fat mass index was associated with a higher Rint (Z-score 0.40 (0.13, 0.68)), compared to a normal fat mass index. Continuous regional android/gynoid fat mass ratio was associated with a lower FeNO (sym% difference: -2.8 (-4.9, -0.6) per Z-score increase of android/gynoid fat mass ratio), but not with Rint, wheezing or asthma. A high android/gynoid fat mass ratio, compared with normal android/gynoid fat mass ratio, was associated with a lower FeNO (sym% -9.8 (-16.3, -3.4)). Subcutaneous fat area was not associated with any asthma-related outcome (Table 2.3.2). Also, we

did not observe associations of pre-peritoneal fat area with Rint, wheezing or asthma. Higher pre-peritoneal fat area was associated with a higher FeNO (sym% difference: 2.6 (0.3, 5.0) per Z-score increase of pre-peritoneal fat area. When categorized in 3 groups, high pre-peritoneal fat area was associated with higher FeNO (sym% difference: 6.5 (0.1, 12.9)) compared with normal pre-peritoneal fat area. When we additionally adjusted the associations of total body and abdominal fat mass measures with Rint, FeNO, wheezing and asthma for BMI, only the association of fat mass index with Rint attenuated (Z-score (95%CI): 0.30 (-0.05, 0.65) (S-Table 2.3.4). When we mutually adjusted for android/gynoid ratio and pre-peritoneal fat area and their associations with FeNO, the size and the directions of the effect estimates remained (Z-scores (95% CI): 4.4 (1.7, 7.1) and -3.9 (-6.6, -1.2), respectively).

Table 2.3.2. Association of Total Body and Abdominal Fat Mass Measures with Rint, FeNO, Wheezing and Asthma in Children at Age 6 Years.

	Rint	**FeNo**	**Wheezing**	**Asthma**
	Z-score (95% CI)	**Sym% (95% CI)**	**Odds Ratio (95% CI)**	**Odds Ratio (95% CI)**
	n = 4,384	n = 3,957	n = 4,554	n = 4,433
Fat mass index (Z-score)				
N	4,283	3,896	417 / 4,484	264 / 4,375
Basic model	-0.03 (-0.12, 0.07)	0.5 (-1.6, 2.6)	**1.17 (1.05, 1.30)****	1.16 (1.00, 1.34)
Full model	0.08 (-0.02, 0.19)	-1.4 (-3.8, 0.1)	1.09 (0.96, 1.25)	1.09 (0.92, 1.29)
Low	0.15 (-0.12, 0.42) (n = 605)	-4.3 (-10.0, 1.6) (n = 557)	1.07 (0.78, 1.46) (n = 65 / 679)	0.90 (0.60, 1.35) (n = 38 / 665)
Normal	Reference (n = 3,030)	Reference (n = 2,767)	Reference (n = 290 / 3,246)	Reference (n = 193 / 3,220)
High	**0.40 (0.13, 0.68)**** (n = 648)	-5.8 (-12.0, 0.4) (n = 572)	1.20 (0.84, 1.70) (n = 62 / 559)	1.09 (0.69, 1.72) (n = 33 / 490)
Android/gynoid fat mass ratio (Z-score)				
N	4,279	3,897	418 / 4,486	264 / 4,376
Basic model	0.03 (-0.07, 0.12)	-1.7 (-3.9, 0.5)	**1.14 (1.01, 1.27)***	1.04 (0.89, 1.21)
Full model	0.06 (-0.03, 0.15)	**-2.8 (-4.9, -0.6)***	1.07 (0.94, 1.21)	0.97 (0.82, 1.15)
Low	-0.15 (-0.44, 0.15) (n = 444)	-3.1 (-10.0, 3.2) (n = 436)	1.05 (0.74, 1.51) (n = 51 / 543)	0.99 (0.63, 1.56) (n = 32 / 538)
Normal	Reference (n = 3,299)	Reference (n = 2,996)	Reference (n = 314 / 3,429)	Reference (n = 207 / 3,375)
High	0.10 (-0.19, 0.38) (n = 536)	**-9.8 (-16.3, -3.4)*** (n = 465)	1.00 (0.70, 1.44) (n = 53 / 518)	0.85 (0.52, 1.39) (n = 25 / 463)

Table 2.3.2. Association of Total Body and Abdominal Fat Mass Measures with Rint, FeNO, Wheezing and Asthma in Children at Age 6 Years. (continued)

	Rint	**FeNo**	**Wheezing**	**Asthma**
	Z-score (95% CI)	**Sym% (95% CI)**	**Odds Ratio (95% CI)**	**Odds Ratio (95% CI)**
	n = 4,384	n = 3,957	n = 4,554	n = 4,433
Subcutaneous area (Z-score)				
N	3,503	3,363	355 / 3,729	225 / 3,606
Basic model	-0.05 (-0.15, 0.06)	2.1 (-0.1, 4.4)	1.02 (0.91, 1.15)	1.04 (0.88, 1.21)
Full model	0.06 (-0.14, 0.26)	2.7 (-1.6, 7.0)	1.08 (0.85, 1.37)	1.04 (0.77, 1.41)
Low	-0.02 (-0.30, 0.26) (n = 536)	-3.6 (-10.0, 2.8) (n = 450)	0.97 (0.70, 1.36) (n = 58 / 590)	0.91 (0.60, 1.40) (n = 44 / 599
Normal	Reference (n = 2,454)	Reference (n = 2,408)	Reference (n = 245 / 2,662)	Reference (n = 154 / 2,622
High	0.10 (-0.19, 0.39) (n = 542)	1.8 (-4.5, 8.1) (n = 530)	1.14 (0.78, 1.67) (n = 52 / 477)	0.97 (0.59, 1.61) (n = 27 / 385)
Pre-peritoneal area (Z-score)				
N	3,466	3,363	353 / 3,704	223 / 3,593
Basic model	**-0.11 (-0.21, -0.01)***	**4.0 (1.8, 6.2)****	1.01 (0.89, 1.13)	1.03 (0.87, 1.21)
Full model	-0.03 (-0.14, 0.08)	**2.6 (0.3, 5.0)***	0.96 (0.84, 1.09)	0.98 (0.83, 1.16)
Low	-0.17 (-0.45, 0.11) (n = 528)	-4.9 (-11.2, 1.4) (n = 462)	1.10 (0.80, 1.52) (n = 64 / 602)	1.23 (0.83, 1.83) (n = 43 / 595)
Normal	Reference (n = 2,455)	Reference (n = 2,413)	Reference (n = 247 / 2,648)	Reference (n = 154 / 2,617)
High	-0.27 (-0.57, 0.03) (n = 483)	**6.5 (0.1, 12.9)*** (n = 488)	0.84 (0.56, 1.26) (n = 42 / 454)	1.15 (0.69, 1.91) (n = 26 / 381)

Values are changes in Z-score for Rint or in sympercent for FeNO, and odds ratios for wheezing and asthma (95% confidence interval) from linear and logistic regression models, *p < 0.05 and **p < 0.01. "n =" represents number of total group (Rint, FeNO) or number of cases per total group (wheezing, asthma). Z-score was categorized into "low" (Z-score < -1.00), "normal" (Z-score ≥-1.00 and ≤1.00) and "high" (Z-score >1.00). Full models were adjusted for maternal age, pre-pregnancy BMI, educational level, history of asthma and atopy, psychological distress during pregnancy, parity, smoking during pregnancy, and child's sex, gestational age at birth, birth weight, ethnicity, breastfeeding, pet keeping, physical activity, lower respiratory tract infections and current height.

DISCUSSION

We observed that a higher BMI was associated with a higher Rint and an increased risk of wheezing in school-aged children. Detailed assessment of total body fat mass distribution showed that a higher fat mass index was associated with a higher Rint, whereas a higher android/gynoid fat mass ratio was associated with a lower FeNO. A higher pre-peritoneal fat mass, a measure of visceral abdominal fat, was associated with a higher

FeNO. No other associations of specific childhood body fat mass measures with Rint, FeNO, wheezing or asthma were observed.

Comparison with previous studies

A recent meta-analysis of 6 cohort studies comprising 25,000 children aged 5-14 years examined the association between obesity and physician-diagnosed asthma, at least one year after BMI assessment, and observed that overweight and obese children had a 1.35 and 1.50-fold increased risk of asthma , respectively.[21] The use of BMI as Z-scores led to weaker and non-significant effect estimates. We observed no associations between overweight and obesity with childhood asthma. This difference might be explained by the young age of our subjects and our broad definition of ever asthma, which might have comprised multiple asthma phenotypes including those with asthma symptoms at a young age due to respiratory infections. A recent multi-center cross-sectional study comprising 10,652 children aged 8 – 12 years reported that overweight and obese children had a 1.14 and 1.67 fold increased risk of wheezing, respectively.[22] We observed similar effect sizes, and additionally took growth and socio-economic and lifestyle-related factors into account.

Previous studies that examined the association of BMI with lung function obtained by spirometry in children reported inconsistent results. Among 8-12 year-old children, cross-sectional analyses showed that childhood obesity was associated with a 2.5% lower Forced Expiratory Volume in 1 second / Forced Vital Capacity (FEV_1/FVC).[22, 23] Others reported that obesity was not associated with changes in FVC[24], or with higher FEV_1 and FVC.[25, 26] In the latter studies the effect estimates for the association of obesity with FEV_1 was twice smaller than with FVC, which implies that higher BMI was associated with more obstruction if FEV_1/FVC was used.[25, 26] These results are in line with our observed association of BMI with increased respiratory resistance when we used Rint.

Studies that examined associations between adiposity and FeNO are scarce and showed that higher BMI, percent body fat and waist circumference were associated with an increased risk of current asthma in individuals with a low to normal FeNO [25], or showed no associations between BMI and FeNO.[27, 28] Differences in results with our study might be explained by the limitations of BMI, which does not distinguish fat mass from free-fat mass, the use of different obesity measures such as waist circumference, and age at time of measurement.

Of the specific body fat mass measures, we observed that a high fat mass index and a high android/gynoid fat mass ratio were associated with a higher Rint and lower FeNO, respectively. In contrast with the android/gynoid fat mass ratio, a measure of waist-hip ratio, a high pre-peritoneal fat mass was not associated with Rint, but was with a high FeNO. When we mutually adjusted android/gynoid ratio and pre-peritoneal fat area and their associations with FeNO the size and the directions of the effect estimates remained,

suggesting independent opposite effects of android/gynoid fat mass ratio and pre-peritoneal fat. Also, we did not observe any interaction between physician-diagnosed inhalant allergies of the child and any fat measure for the associations with Rint, FeNO, wheezing and asthma. Several studies assessed the associations of specific body fat measures with lung function and asthma. In a study among 327 asthmatic children aged 10 years and 351 matched controls in Puerto Rico, a higher BMI was associated with a higher FEV_1 and FVC, and increased risk of asthma (OR (95%CI): 1.27 (1.1-1.5), per z-score increase in BMI).[29] A greater waist-hip ratio, waist circumference and percentage body fat were associated with a higher FVC, and a greater waist circumference with a lower FEV_1/FVC. Three central obesity indicators (waist-hip ratio, waist circumference and waist-to-height ratio) consistently showed stronger dose-dependent associations with active asthma in a population of 2,758 schoolchildren than BMI.[30] Besides age of participants, differences in study designs and methods of fat mass distribution measurements could explain differences with our results. We additionally assessed associations of pre-peritoneal and subcutaneous fat mass with lung function and asthma.

Interpretation of results

We observed that higher BMI was associated with higher Rint and increased risk of wheezing, high fat mass index with higher Rint, and android/gynoid fat mass ratio and pre-peritoneal fat mass with lower and higher FeNO, respectively. A higher BMI and obesity were also associated with current wheezing, but not with ever physician diagnosed asthma. This difference could be explained by the differences in definition of current wheezing and ever physician diagnosed asthma. Current wheezing reflects obstructive respiratory symptoms most commonly due to asthma at age 6 years, whereas ever physician diagnosed asthma could partly reflect respiratory symptoms due to recurrent lower respiratory tract infections in early life. Respiratory symptoms due to recurrent lower respiratory tract infections are difficult to clinically distinguish from respiratory symptoms due to asthma at younger ages.

Because of the observational design of our study, we cannot draw conclusions about causality. However, support for our findings comes from animal studies, which have shown that inflammatory adipokines, mainly leptin and adiponectin, are secreted by adipose tissue.[31] Leptin increases airway inflammation and responsiveness [32], whereas adiponectin reduces airway hyperreactivity and inflammation.[33] Leptin is a member of the interleukin (IL)-6 family of cytokines and elevated levels affect allergen-induced T-cells and bronchial epithelial cells through TNF-α related IL-4 an IL-5 [34], and histamine release.[35] Leptin decreases the airway diameter in mice through the inhibition of central cholinergic tone [36], a parasympathetic effect. Visceral fat, as opposed to subcutaneous fat, exhibits relatively higher levels of adiponectin production in lean animals.[37] Higher adiponectin levels lead to lower inflammatory responses reflected by decreased

neutrophil recruitment and lower expression of inflammatory markers in mice when exposed to ozone.[38] Obese mice have reduced serum adiponectin levels and increased eosinophil levels in bronchoalveolar lavage fluids and the peribronchovascular space.[39] Thus, animal studies show that adiposity could affect airway obstruction and inflammation. Human studies on the adiposity-asthma relation through leptin and adiponectin are scarce, mostly performed in adults, and show conflicting results. A recent study in mainly asthmatic children aged 8 years showed that higher leptin plasma concentrations were associated with a lower FEV_1, but not with FeNO and sputum eosinophils.[40]

We observed that regional fat measures were associated with changes in FeNO. An underlying mechanism could be that larger fat depots lead to an increase of arginase relative to L-arginine concentration, and lower FeNO. This is because NO is produced from *L*-arginine by the NO synthase (NOS) family of enzymes and arginase is a biological competitor with NOS for L-arginine, which could lead to changes in FeNO levels, as known in children with asthma.[41, 42] In humans, an increase in BMI has been associated with lower FeNO.[43, 44] Also, lower arginine levels and higher arginase activity have been associated with lower FEV_1, FEV_1/FVC and FeNO.[45]

We observed that a higher pre-peritoneal fat area was associated with a lower Rint and higher FeNO, whereas subcutaneous fat area was not associated with any outcome. Visceral fat is known to be a better marker for inflammatory status, and risk factor for metabolic and cardiovascular outcomes, than subcutaneous fat mass.[46] Also, animal studies showed that increased nutrient-intake in lean rats increased expression of leptin more in visceral fat and plasma than in subcutaneous fat.[47] In a study among 799 11-year old children, a higher trunk-to-extremity fat ratio, which is a surrogate of visceral fat, was associated with lower serum adiponectin levels.[47] In a study amongst 394 adults both leptin and adiponectin were associated with higher central body fat distribution, and serum adiponectin concentrations seemed determined predominantly by the visceral fat compartment.[48] Regarding the L-arginine metabolism, a recent study showed that the addition of arginase in subcutaneous fat deposits of morbid obese individuals reduced vasodilation, whereas this was not observed in the visceral tissues of these individuals. Furthermore, their visceral fat tissue exerted greater expression of pro-inflammatory oxidative-stress related, hypoxia-induced and proangiogenic genes with increased macrophage populations. This suggests that the visceral microenvironment, as opposed to subcutaneous fat tissue, may affect systemic health.[49] Also, in mice models, L-arginine decreased fat accumulation in visceral fat [50], even in rats with high BMI.[51] Thus, a local fat depot-specific expression of adipokines and increased arginase levels might be contributing to the pathogenesis of asthma.

The different directions for the associations of android/gynoid fat mass ratio and abdominal pre-peritoneal fat area with FeNO could be explained by their different locations. The android area contains both pre-peritoneal and subcutaneous fat deposits. The

android/gynoid ratio, a proxy of waist-hip ratio, is a practical index of central adipose tissue distribution.[11] In a study amongst 30 girls, waist-hip ratio was not associated with visceral or subcutaneous fat deposits.[52] In adults, waist-hip ratio is a poor predictor of the distribution of adipose tissues among several fat compartments in the abdominal region.[53] Waist-hip ratio does not account for large variations in the level of total fat and abdominal visceral adipose tissues.[54] Among children, pre-peritoneal fat mass represents less than 10% of total abdominal fat. Therefore, android/gynoid fat mass ratio reflects fat depositions of different locations in the body, whereas the pre-peritoneal and subcutaneous fat areas reflect the effects of two different fat tissue types localized in the abdomen. Thus, our results suggest that the associations between obesity and asthma might be driven by local fat deposits, and not by general adiposity. Specific underlying pathophysiological mechanisms for differences in direction of android/gynoid fat mass ratio and abdominal pre-peritoneal fat area with FeNO need to be explored.

An alternative hypothesis proposes that a higher central body fat mass distribution exerts mechanical effects on the lungs, such as diminished tidal lung volumes, due to deposition of adipose tissue in the chest and abdomen, and around the airways, which might result in lower functional vital capacity.[55] In the present study, a higher total fat mass index was associated with a higher respiratory resistance. We did not observe associations of a higher android/gynoid fat mass ratio with higher respiratory resistance. Studies focused on persistent or increasing total and abdominal fat mass and the risk of higher respiratory resistance and subsequently risk of wheezing or asthma among children in later life are needed.

Strengths and limitations

A strength of this cross-sectional study in a population-based cohort is the use of detailed respiratory outcomes and adjustment for many relevant growth, socio- demographic and lifestyle covariates. However, some methodological considerations need to be discussed. Biased effect estimates in longitudinal studies mainly occur due to loss to follow-up. We observed that children lost to follow-up more often had unfavorable growth, socio-economic and lifestyle factors, which suggests that follow-up was selective. We did not have data on forced oscillation which is suggested to provide a more reliable evaluation of bronchial obstruction compared to the interrupter technique.[56] We used the interrupter technique to assess bronchial patency, as this is one of the very few methods that can successfully be applied to young children in the setting of an epidemiological study. When used in a standardized way according to recommendations, the expiratory interrupter resistance has good feasibility, repeatability and biological validity for the detection of airways obstruction [57], and appropriate reference data for commercial Rint devices are available.[58] Its diagnostic value compared to forced oscillation and multiple breath washout, two other techniques that can be used in young chil-

dren, is still debated as very few comparative studies have been published, that widely differ in patient selection criteria.[59] Although the use of ISAAC-based questionnaires is a standardized and widely accepted method to identify children at risk for asthma, reporting bias might have occurred[16], resulting in either overestimations or underestimations of the true associations. When absolute values of BMI (kg/m^2) and Rint ($kPa.L^{-1}.s$) were used, an increase of 1 kg/m^2 in BMI was associated with an increase of 0.005 (95%CI: 0.001, 0.010) $kPa.L^{-1}.s$ in Rint. This effect estimate is small and replication studies using Rint as outcome at this age are therefore needed. Also, the prevalence of children with underweight and obesity was low (0.9 and 4.5%, respectively), which could have affected the power of our study. We categorized fat measures to assess possible non-linear associations and for easier clinical interpretation, although methods of cut-offs and the use of categorization could be argued.[60] We additionally adjusted associations of total body and abdominal fat mass measures with Rint, FeNO, wheezing and asthma for BMI. The correlation between total body and abdominal fat mass measures and BMI was moderate (Pearson correlation 0.46 to 0.63) and might have introduced multicollinearity. Despite this, most effect estimates remained similar in size and direction. Last, we assessed both body fat profile and Rint, FeNO, wheezing and asthma in a cross-sectional setting, and therefore we cannot distinguish the direction of causation between body fat profile measures, Rint, FeNO, wheezing and asthma.

In conclusion, a higher BMI was associated with higher Rint and increased risk of wheezing in school-age children. Abdominal body fat mass distribution may affect Rint and FeNO. Detailed body fat distribution measures might be better measures to understand the obesity-asthma paradigm.

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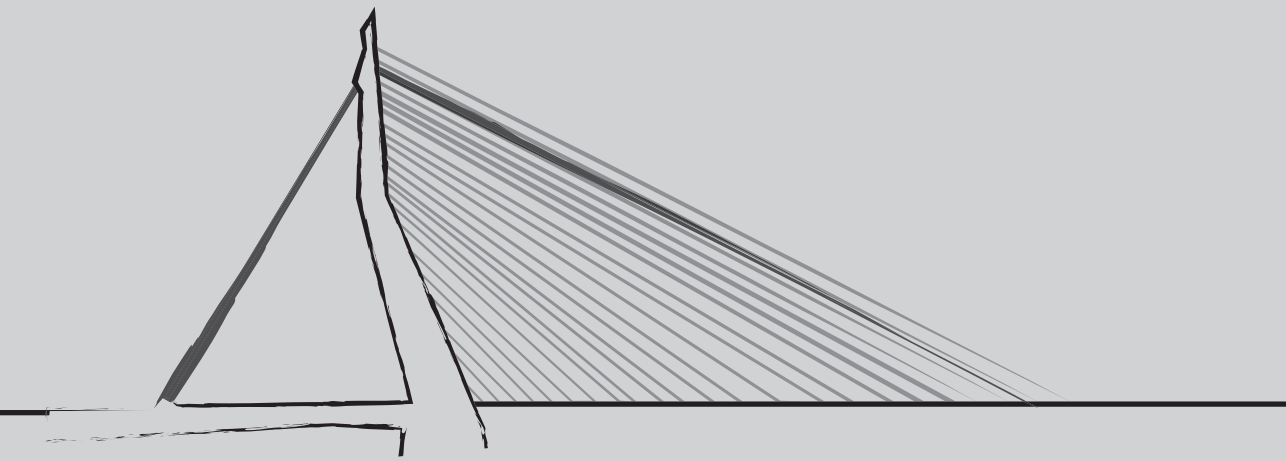
Chapter 3

Early environmental exposures, childhood lung function and asthma



Chapter 3.1

Tobacco smoke exposure, airway resistance and asthma in school-age children

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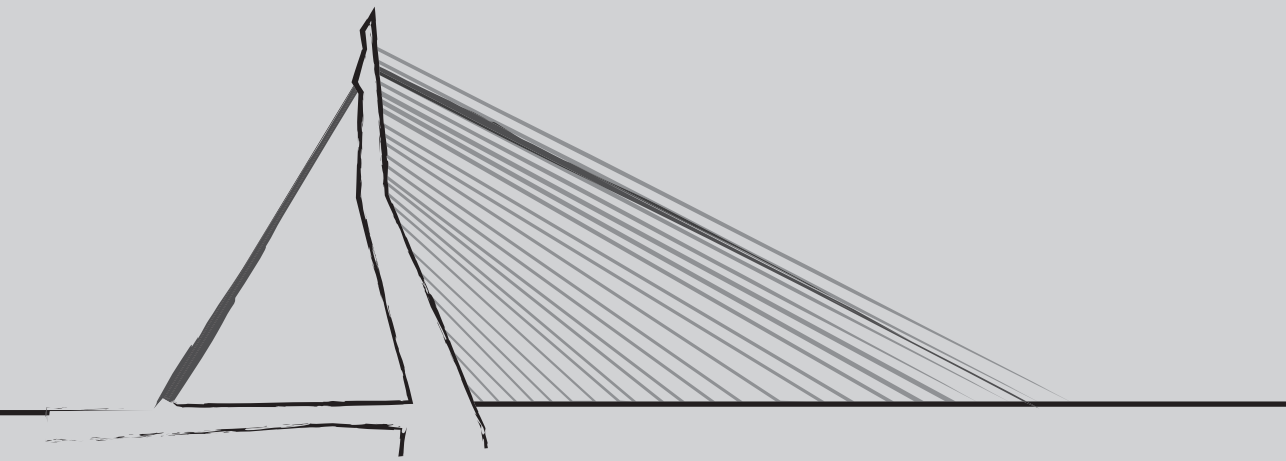

ABSTRACT

Background Tobacco smoke exposure has been associated with early childhood asthma symptoms. We assessed the associations of tobacco smoke exposure during pregnancy and childhood with wheezing patterns, asthma, airway interrupter resistance (Rint) and Fractional exhaled Nitric Oxide (FeNO) in school-age children, and whether birth characteristics explained the associations.

Methods This study was embedded in a population-based prospective cohort study among 6,007 children. Paternal and maternal smoking during pregnancy (never, first trimester only, continued), secondhand tobacco smoke exposure during childhood, wheezing patterns and asthma were prospectively assessed by questionnaires. Wheezing patterns were defined as never, early (≤3 years only), late (>3 years only) and persistent (≤3 and >3 years) wheezing. Rint and FeNO were measured at age 6 years. Birth characteristics were available from registries.

Results Continued maternal smoking during pregnancy was associated with increased risks of early and persistent wheezing (OR: 1.24 (1.01, 1.52); 1.48 (1.13, 1.95)), and asthma (1.65 (1.07, 2.55), for ≥5 cigarettes per day), but not with Rint or FeNO. Birth characteristics did not explain these associations. Childhood tobacco smoke exposure was associated with higher Rint (difference Z-score 0.45 (0.00, 0.90)), but this effect attenuated after adjustment for birth characteristics. Maternal smoking during first trimester only or paternal smoking during pregnancy was not associated with Rint, FeNO, wheezing or asthma.

Conclusion Continued maternal smoking during pregnancy was associated with increased risks of asthma outcomes in school-age children, whereas childhood tobacco smoke exposure was associated with higher Rint. Birth characteristics may explain part of these associations.

INTRODUCTION

Toxic environmental exposures in fetal life and infancy, including secondhand tobacco smoke exposure, are associated with an increased risk of childhood asthma.[1-6] Recently, we have observed that continued maternal smoking throughout pregnancy was associated with an increased risk of preschool wheezing.[7] These associations were independent of paternal smoking, smoke exposure in childhood and being small for gestational age, and suggest a direct adverse effect of fetal tobacco smoke exposure on lung development. Direct intrauterine mechanisms in response to fetal smoke exposure may include suboptimal development of the respiratory tract system, which results in impaired lung growth with smaller airways and airway diameters leading to a higher airway resistance.[7-9] Previous studies on the adverse effect of maternal smoking during pregnancy on childhood asthma at older ages are inconsistent.[1,10-13] This might be due to socio-economic or life style related factors, or to not taking current tobacco smoke exposure in childhood or important birth outcomes such as gestational age and weight at birth into account.[14] To disentangle the effects of direct intra-uterine adaptation mechanisms from unknown socio-economic, or life style related factors on childhood asthma, information on paternal smoking during pregnancy of the mother can be used.[15] If stronger associations of maternal smoking during pregnancy with asthma or related outcomes is observed than for paternal smoking, taking secondhand tobacco smoke exposure in childhood into account, this would support the hypothesis that intra-uterine adaptation mechanisms underlie the observed associations. Similar associations for maternal and paternal smoking with asthma or related outcomes would suggest that common and shared socio-economic or life style related factors within families explain these associations.[15-17] Additionally, the effects of secondhand tobacco smoke exposure in childhood on airway resistance and asthma outcomes, and the roles of being born early or small for gestational age in the association of maternal smoking during pregnancy with childhood asthma are not clear.[7,18,19]

Therefore, we first aimed to examine the associations of maternal and paternal smoking in different periods of pregnancy with airway resistance, airway inflammation, wheezing patterns and physician-diagnosed asthma in school-aged children participating in a large population-based prospective cohort study. Second, we examined the associations of secondhand tobacco smoke exposure during childhood with lung function and asthma outcomes, taking account for parental smoke exposure during pregnancy. Third, we examined whether the associations of tobacco smoke exposure with the lung function and asthma outcomes were modified by gestational age and weight at birth or atopy.

MATERIALS AND METHODS

General design

This study was embedded in the Generation R Study, a population-based prospective cohort study of pregnant women and their children in Rotterdam, The Netherlands. In each trimester of pregnancy assessments were performed, including physical examination, fetal ultrasound examination, and questionnaires.[20,21] All children were born between April 2002 and January 2006. Of all eligible children in the study area, 61% participated in the study at birth.[20] The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam (MEC 217.595/2002/20). Written informed consent was obtained from all participants. A total of 6,007 children were included for the current analyses (Figure 3.1.1).

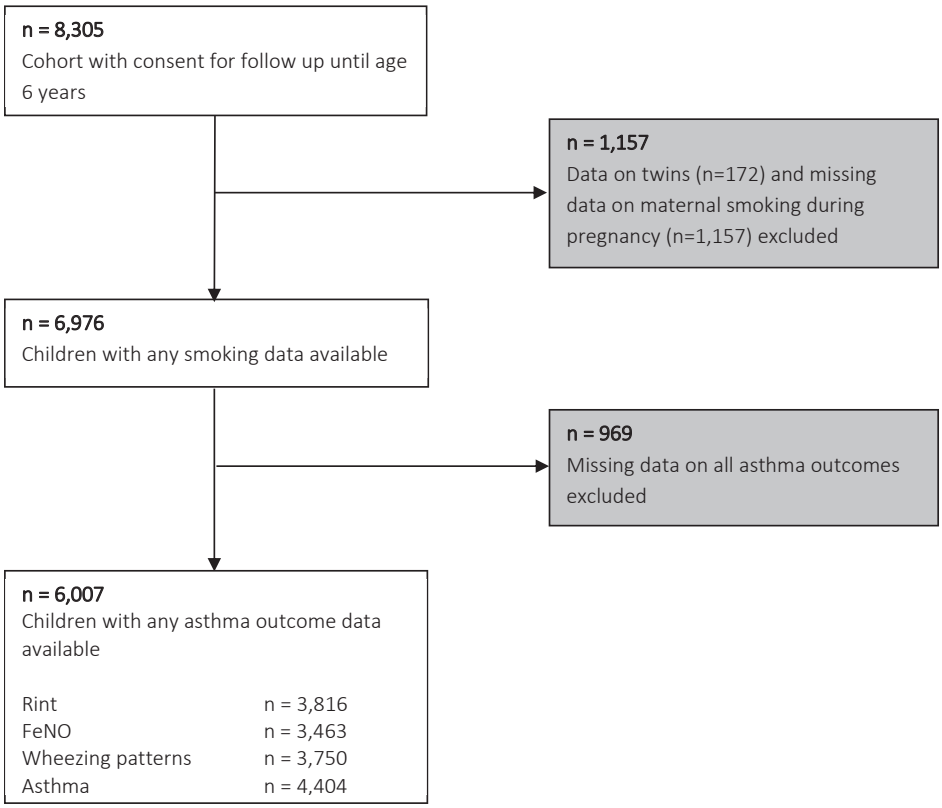

Figure 1. Flowchart of Participants.

Fetal and childhood smoke exposure

As previously described in detail [7], mothers reported their active tobacco use by 3 questionnaires during pregnancy. We grouped mothers' tobacco use into three categories based on the first questionnaire: 1) never during pregnancy, 2) first trimester only, and 3) continued during pregnancy. Reported tobacco use in the second and third trimester were used to reclassify maternal smoking, when appropriate. Active paternal smoking was assessed in the first questionnaire by asking the mother whether the father smoked during her pregnancy (n=5,411). The number of cigarettes smoked daily was classified as none, ≤4 cigarettes per day, and ≥5 cigarettes per day. Information about any in-house secondhand tobacco smoke exposure in childhood at age 6 years, irrespective whether this source was the mother, father or anyone else, was obtained by a questionnaire at the age of 6 years (response rate 76%;" Was the child exposed to any in-house tobacco smoke (never / yes, ≤ 1 time per week / yes, ≥ 2 times per week").

Childhood asthma outcomes

At age 6 years, airway interrupter resistance (Rint) was measured in kPa/L (MicroRint, MicroMedical, Rochester, Kent, UK) during tidal expiration, with occlusion of the airway at peak expiratory flow, according to ERS and ATS guidelines. Fractional exhaled nitric oxide (FeNO) in ppb was measured using the NIOX chemiluminescence analyzer (Aerocrine AB, Solna, Sweden) according to ERS and ATS guidelines. FeNO levels were natural log-transformed to obtain normality. Wheezing was reported by parental questionnaires annually from birth to age 4 years and at age 6 years. Wheezing patterns were defined in 4 categories as previously proposed [22] and commonly used in epidemiological studies: 1) no wheezing: no recorded wheezing at any age; 2) early wheezing: at least 1 wheezing episode during the first 3 years of life but no wheezing episodes at 4 and 6 years of age; 3) late wheezing: no wheezing episodes during the first 3 years of age but at least 1 wheezing episode at 4 or 6 years of age; and 4) persistent wheezing: at least 1 wheezing episode in the first 3 years of life and 1 episode of wheezing at 4 or 6 years of age.[22,23] Physician-diagnosed ever asthma was assessed using questions adapted from the International Study on Asthma and Allergy in Childhood (ISAAC) at age 6 years.[24]

Covariates

We obtained information on maternal age, anthropometrics, socio-economic status, history of asthma and atopy, parity, child's ethnicity and pet keeping by questionnaires, completed by the mother at enrollment. Data on gestational age and birth weight was obtained by midwife and hospital registries. Detailed information on fetal and childhood smoke exposure, childhood asthma outcomes, and covariates, including child's inhalant allergies, is provided in the Supplemental Material.

Statistical analysis

First, we used multivariate logistic and linear regression analyses to examine the associations of maternal and paternal smoking during pregnancy, including reported number of cigarettes, with Rint, FeNO and increased risk of asthma at age 6 years. These models were adjusted for maternal age, pre-pregnancy body mass index, educational level, history of asthma or atopy, psychological distress during pregnancy, parity, and child's sex, ethnicity, breastfeeding, pet keeping, and secondhand tobacco smoke exposure at age 6 years (see Supplemental Material). Differences in prevalences of wheezing patterns in strata of maternal and paternal smoking during pregnancy were tested using univariate and multivariate polynomial regression models, with "no maternal smoking" and "never wheezing" as reference. The associations of paternal smoking with Rint, FeNO, wheezing patterns and asthma were assessed among mothers who did not smoke during pregnancy (n = 4,504). Second, we used similar regression analyses to examine the associations of secondhand tobacco smoke exposure, including secondhand tobacco smoke exposure per week, with Rint, FeNO and asthma at age 6 years. These associations were adjusted the same covariates as the maternal model, and maternal and paternal smoking during pregnancy. Third, to assess whether these were explained by birth outcomes, we additionally adjusted the models for gestational age and weight at birth (birth outcome model). Also, we additionally adjusted models for atopy (inhalant allergy or eczema) at age 6 years to assess the potential confounding or mediating roles of atopy (Supplemental Material). Finally, we categorized tobacco smoke exposure into 4 categories (never, smoke exposure during pregnancy only, smoke exposure in childhood only, smoke exposure during pregnancy and in childhood) and examined their associations with Rint, FeNO, wheezing and asthma using the birth outcome model.

Additional information on used methods is provided in the Supplemental Material. Measures of association are presented in Odds Ratios (ORs) for wheezing and asthma, in sympercents (symmetric percentage difference = regression coefficients of ^{e}log transformed FeNO*100%) for FeNO measurements [25] and in standardized z-score differences for Rint measurements, all with their 95% Confidence Interval (95% CI). Statistical analyses were performed using SPSS version 21.0 for Windows software (SPSS Inc).

RESULTS

Of the population for the current analysis, 67.6% (n = 4,063) was of European origin. Those of non-European ethnicity were mainly of Turkish (7.3%), Surinamese (6.8%), Moroccan (5.1%) or Dutch Antilles (2.5%) origin. Mean maternal age at inclusion was 30.6 years. Of all mothers, 25.0% (n = 1,503) reported to smoke during pregnancy of which 8.8% (n = 528) smoked during the first trimester only, and 16.2% (n = 975) smoked

continuously during pregnancy (Table 3.1.1). Of all fathers, 44% (n = 2,383) smoked during pregnancy of their partners. Children were classified as never wheezing (45.6%, n = 2,149), early wheezing (28.6%, n = 1,266), late wheezing (7.4%, n = 324), and persistent wheezing (18.5%, n = 765). At age 6 years of the children, average airway resistance (Rint) was 0.84 (SD 0.29) kPa/L/s, and median FeNO was 7.5 (SD 8.5) ppb. Current wheezing was reported for 8.7% (n = 305), and physician-diagnosed asthma for 6.5% (n = 213) of the children. Other characteristics of parents and their children are given in Table 3.1.1 and S-Table 3.1.1. Participants without follow-up data at age 6 years had younger, lower educated, more smoking parents, mothers with a higher pre-pregnancy body mass index, and higher prevalence of parity and psychological distress, and had a lower birth weight and more often were of non-European ethnicity than those participants with follow-up data (S-Table 3.1.2).

Smoking exposure and Rint, FeNO, wheezing patterns and asthma

As compared with no maternal smoking, maternal smoking in the first trimester only was not associated with a higher mean Rint and FeNO, or increased risks of wheezing patterns and asthma in childhood (Table 3.1.3, confounder model).

Continued maternal smoking during pregnancy was not associated with Rint or FeNO. Continued maternal smoking of ≥ 5 cigarettes per day was associated with an increased risk of physician diagnosed asthma (OR 1.65 (1.07, 2.55)). The effect estimate did not materially change when we additionally adjusted for gestational age and birth weight (Table 3.1.3, birth outcome model). The effect estimate became stronger after adjustment for inhalant allergies and eczema (OR 1.77 (1.13, 2.79) (S-Table 3.1.3). The distribution of wheezing patterns was not different between children from mothers who did or did not smoke during first trimester only. As compared with children from mothers who did not smoke, those from mothers who continued smoking during pregnancy showed a higher prevalence of early wheezing (29.9% vs. 28.1%, respectively) and persistent wheezing (25.2% vs. 17.0%, respectively). Similarly, continued maternal smoking during pregnancy showed increased odds for early and persistent wheezing when taking confounders and birth outcomes into account (Table 3.1.2).

Among children of mothers who did not smoke during pregnancy, paternal smoking was not associated with childhood Rint or FeNO (Z-score difference -0.4 (-0.34, 0.26) and sympercent change -0.2 (-6.9, 6.5), respectively). In contrast to maternal smoking of ≥ 5 cigarettes per day, paternal smoking of ≥ 5 cigarettes per day during pregnancy was not associated with physician diagnosed asthma (OR 1.01 (0.58, 1.75); Table 4). No differences in risk for wheezing patterns were observed between children from fathers who did not smoke or fathers who smoked during pregnancy (Table 3.1.2).

Table 3.1.1. Characteristics of Parents and their Children

	Maternal smoking during pregnancy (n = 6,007)		
	No smoking n = 4,504 (75.0%)	**First trimester only smoking n = 528 (8.8%)**	**Continued smoking n = 975 (16.2%)**
Maternal characteristics			
Age (years)	31.0 (4.8)	30.3 (5.0)	29.2 (5.8)
Pre-pregnancy body mass index (kg/m2)	24.6 (4.2)	24.4 (4.1)	25.1 (4.6)
Education, higher (%)	53.3 (2,401)	47.0 (248)	23.6 (230)
History of asthma or atopy (%)	38.3 (1,716)	34.7 (183)	36.3 (354)
Psychological distress during pregnancy (%)	16.5 (745)	16.7 (88)	25.7 (251)
Parity ≥1 (%)	45.0 (2,029)	32.0 (169)	44.0 (429)
Paternal characteristics			
Age (years)	33.5 (5.4)	32.9 (5.8)	32.0 (6.0)
Education, higher (%)	58.7 (2,685)	49.6 (282)	27.9 (241)
History of asthma or atopy (%)	34.4 (1,597)	34.2 (190)	25.6 (208)
Smoking during pregnancy of partner (%)	34.9 (1,428)	65.4 (320)	76.6 (635)
Child characteristics			
Sex, female (%)	50.6 (2,281)	52.1 (275)	45.3 (442)
Gestational age at birth (weeks)	39.9 (1.7)	39.9 (0.6)	39.7 (1.9)
Birth weight (grams)	3,466 (549)	3,466 (188)	3,285 (557)
Ethnicity, non-European (%)	34.0 (1,532)	23.5 (124)	37.7 (368)
Breastfeeding until 1st^{t} year (%)	92.2 (4,151)	90.3 (477)	83.9 (818)
Pet keeping until 1st^{t} year (%)	31.1 (1,399)	40.7 (215)	44.2 (431)
Inhalant allergies age 6 years (%)	12.4 (557)	12.3 (65)	13.1 (128)
Eczema ever until 6 year (%)	21.7 (976)	21.8 (115)	24.4 (975)
Secondhand tobacco smoke exposure age 6 years (%)			
No	92.7 (3,271)	83.9 (324)	49.9 (308)
Yes, ≤ 1 time / week	2.4 (86)	4.9 (19)	9.1 (56)
Yes, ≥ 2 times / week	4.8 (171)	11.1 (43)	41.0 (253)
Wheezing patterns until 6 years (%)			
Never	47.7 (2,149)	43.6 (230)	36.7 (358)
Early	28.1 (1,266)	30.1 (159)	29.7 (290)
Late	7.2 (324)	7.8 (41)	8.3 (81)
Persistent	17.0 (765)	18.6 (98)	25.2 (246)
Rint age 6 years (kPa/L/s)	0.84 (0.29)	0.81 (0.28)	0.86 (0.31)
FeNO age 6 years(ppb)	9.5 (8.5)	8.6 (6.0)	8.9 (7.4)
Asthma age 6 years (%)	6.2 (213)	5.3 (20)	8.8 (53)

Values are means (SD), medians (2.5-97.5th percentile) or percentages (absolute numbers) based on imputed data. Missing data on paternal smoking during pregnancy (9.9%), secondhand tobacco smoke exposure (22.3%), child's Rint (36.5%), FeNO (23.1%), and asthma (26.7%) were not imputed.

Table 3.1.2. Associations of Maternal Smoking during Pregnancy with Rint, FeNO and Asthma of Children at Age 6 Years.

	Confounder model			**Birth outcome model**		
	Rint	**FeNO**	**Asthma**	**Rint**	**FeNO**	**Asthma**
	Difference (95% CI)	**Difference (95% CI)**	**Odds Ratio (95% CI)**	**Difference (95% CI)**	**Difference (95% CI)**	**Odds Ratio (95% CI)**
	n = 3,816	n = 3,463	n = 4,404	n = 3,816	n = 3,463	n = 4,404
Maternal smoking n=6,007						
No smoking (n = 4,504)	*Reference* n = 2,195	*Reference* n = 1,764	*Reference* n = 212 / 3,410	*Reference* n = 2,195	*Reference* n = 1,764	*Reference* n = 212 / 3,410
First trimester only smoking (n = 528)	-0.24 (-0.64, 0.16) n = 248	-5.3 (-13.9, 3.3) n = 219	0.91 (0.56, 146) n = 20 / 377	-0.21 (-0.61, 0.19) n = 248	-6.6 (-15.5, 2.2) n = 219	0.93 (0.57, 1.50) n = 20 / 377
Continued smoking (n = 975)	-0.01 (-0.37, 0.35) n = 381	-2.3 (-10.6, 6.0) n = 289	1.32 (0.92, 1.90) n = 53 / 598	-0.07 (-0.44, 0.30) n = 381	-1.5 (-9.9, 7.0) n = 289	1.32 (0.92, 1.90) n = 53 / 598
≤4 cigarettes/day (n = 439)	-0.23 (-0.72, 0.27) n = 152	0.3 (-11.8, 11.2) n = 113	1.01 (0.60, 1.72) n = 17 / 252	-0.28 (-0.78, 0.21) n = 152	-0.3 (-11.8, 11.3) n = 113	1.00 (0.59, 1.70) n = 17 / 252
≥5 cigarettes/day (n = 531)	0.25 (-0.21, 0.71) n = 221	-3.3 (-13.7, 7.1) n = 176	**1.65 (1.07, 2.55)*** n = 36 / 341	0.18 (-0.28, 0.64) n = 221	-2.5 (-13.0, 8.1) n = 176	**1.66 (1.07, 2.58)*** n = 36 / 341
P for trend	*0.69*	*0.28*	*0.19*	*0.51*	*0.45*	*0.19*

Values are z-score differences in Rint, sympercent changes in FeNO, and odds ratios for asthma (95% confidence interval) from linear and logistic regression models. "n =" represents number of total group (Rint, FeNO) or number of cases per total group (asthma). *p < 0.05. Models were adjusted for maternal age, pre-pregnancy body mass index, educational level, history of asthma or atopy, psychological distress during pregnancy, parity, and child's gender, ethnicity, breastfeeding, pet keeping, and secondhand tobacco smoke exposure at age 6 years. The birth outcome adjusted model was additionally adjusted for gestational age and size at birth.

Table 3.1.3. Multivariate Analysis of the Association between Parental Smoking during Pregnancy and Wheezing Patterns in Childhood.

	Confounder model			**Birth outcome model**		
	Wheezing patterns			**Wheezing patterns**		
	Early	**Late**	**Persistent**	**Early**	**Late**	**Persistent**
	Odds Ratio (95% CI)	**Odds Ratio (95% CI)**	**Odds Ratio (95% CI)**	**Odds Ratio (95% CI)**	**Odds Ratio (95% CI)**	**Odds Ratio (95% CI)**
	n=1,715	n=446	n=1,108	n=1,715	n=446	n=1,108
Maternal smoking						
No smoking (n = 4,504)	*Reference n = 1,267*	*Reference n = 324*	*Reference n = 765*	*Reference n = 1,267*	*Reference n = 324*	*Reference n = 765*
First trimester only smoking (n = 528)	1.18 (0.92, 1.52) n = 157	1.18 (0.71, 1.98) n = 41	1.26 (0.88, 1.79) n = 98	1.17 (0.89, 1.52) n = 157	1.18 (0.70, 1.98) n = 41	1.28 (0.90, 1.83) n = 98
Continued smoking (n = 975)	**1.24 (1.01, 1.52)*** n = 292	1.15 (0.74, 1.78) n = 81	**1.48 (1.13, 1.95)**** n = 246	1.20 (0.98, 1.48) n = 292	1.19 (0.77, 1.83) n = 81	**1.46 (1.11, 1.92)*** n = 246
Paternal smoking						
No smoking (n = 2,649)	*Reference n = 719*	*Reference n = 183*	*Reference n = 436*	*Reference n = 719*	*Reference n = 183*	*Reference n = 436*
Paternal smoking (n = 1,428)	1.18 (0.91, 1.53) n = 420	1.19 (0.89, 1.57) n = 113	1.09 (0.87, 1.36) n = 256	1.18 (0.91, 1.54) n = 420	1.19 (0.90, 1.58) n = 113	1.12 (0.82, 1.55) n = 256

Values are odds ratios (95% confidence interval) from multivariate polynomial regression models. "n =" represents number of cases per group. *p < 0.05, ** p < 0.01. Both models were adjusted for maternal age, pre-pregnancy body mass index, educational level, history of asthma or atopy, psychological distress during pregnancy, parity, child's gender, ethnicity, breastfeeding, pet keeping, and environmental smoke exposure at age 6 years. The birth outcome adjusted model was additionally adjusted for gestational age and weight at birth.

Table 3.1.4. Associations of Paternal Smoking during Pregnancy with Rint, FeNO and Asthma of Children at Age 6 Years.

	Confounder model			**Birth outcome model**		
	Rint	**FeNO**	**Asthma**	**Rint**	**FeNO**	**Asthma**
	Difference (95% CI)	**Difference (95% CI)**	**Odds Ratio (95% CI)**	**Difference (95% CI)**	**Difference (95% CI)**	**Odds Ratio (95% CI)**
	n = 3,816	n = 3,463	n = 4,404	n = 3,816	n = 3,463	n=4,404
Paternal smoking (n= 4,077)						
No smoking (n = 2,649)	*Reference* n = 1,284	*Reference* n = 1,194	*Reference* n = 129 / 2,025	*Reference* n = 1,284	*Reference* n = 1,194	*Reference* n = 129 / 2,025
Smoking (n = 1,428)	-0.04 (-0.34, 0.26) n = 657	-0.2 (-6.9, 6.5) n = 570	0.98 (0.64, 1.50) n = 62 / 1,011	-0.09 (-0.43, 0.24) n = 657	0.00 (-6.7, 6.7) n = 570	0.97 (0.63, 1.49) n = 62 / 1,011
≤4 cigarettes/day (n = 435)	-0.23 (-0.66, 0.20) n = 222	-3.1 (-12.8, 6.7) n = 188	1.26 (0.75, 2.12) n = 22 / 340	-0.26 (-0.70, 0.18) n = 222	-3.7 (-13.5, 6.0) n = 188	1.25 (0.74, 2.11) n = 22 / 340
≥5 cigarettes/day (n = 523)	-0.14 (-0.58, 0.29) n = 264	-0.7 (-10.3, 8.9) n = 218	1.01 (0.58, 1.75) n = 22 / 404	-0.12 (-0.55, 0.31) n = 264	-0.9 (-10.5, 8.7) n = 218	1.00 (0.58, 1.76) n = 22 / 404
P for trend	*0.37*	*0.95*	*0.78*	*0.42*	*0.99*	*0.79*

Values are z-score differences in Rint, sympercent changes in FeNO, and odds ratios for asthma (95% confidence interval) from linear and logistic regression models. "n =" represents number of total group (Rint, FeNO) or number of cases per total group (asthma). *p < 0.05 and **p < 0.01. Models were adjusted for paternal age, educational level, history of asthma or atopy, and child's gender, ethnicity, breastfeeding, pet keeping, and secondhand tobacco smoke exposure at age 6 years. The birth outcome adjusted model was additionally adjusted for gestational age and size at birth. Analyses on paternal smoking were restricted to mothers who did not smoke during pregnancy (n = 4,504).

Table 3.1.5. Associations of Secondhand Tobacco Smoke Exposure with Rint, FeNO and Asthma of Children at Age 6 Years.

	Confounder model			**Birth outcome model**		
	Rint	**FeNO**	**Asthma**	**Rint**	**FeNO**	**Asthma**
	Difference (95% CI)	**Difference (95% CI)**	**Odds Ratio (95% CI)**	**Difference (95% CI)**	**Difference (95% CI)**	**Odds Ratio (95% CI)**
	n = 2,824	n = 2,272	n = 4,385	n = 2,824	n = 2,272	n = 4,385
Secondhand tobacco smoke exposure (n = 4,531)						
No smoking (n = 3,903)	*Reference* n = 2,425	*Reference* n = 1,983	*Reference* n = 236 / 3,752	*Reference* n = 2,425	*Reference* n = 1,983	*Reference* n = 236 / 3,752
Smoking (n = 628)	0.32 (-0.07, 0.72) n = 372	-9.5 (-19.7, 0.8) n = 289	0.83 (0.55, 1.25) n = 42 / 588	0.28 (-0.11, 0.68) n = 372	-9.3 (-19.5, 0.9) n = 289	0.83 (0.48, 1.40) n = 42 / 588
≤ 1 time / week (n = 161)	0.03 (-0.61, 0.67) n = 101	-8.4 (-22.6, 5.7) n = 79	0.95 (0.49, 1.84) n = 13 / 149	-0.03 (-0.66, 0.61) n = 101	-7.8 (-22.0, 6.4) n = 79	1.02 (0.42, 2.46) n = 13 / 149
≥ 2 times / week (n = 467)	**0.45 (0.00, 0.90)*** n = 271	-10.2 (-22.5, 2.2) n = 210	0.78 (0.49, 1.25) n = 29 / 439	0.41 (-0.03, 0.86) n = 271	-10.4 (-22.8, 2.0) n = 210	0.76 (0.42, 1.39) n = 29 / 439
P for trend	*0.06*	*0.08*	*0.32*	*0.09*	*0.08*	*0.40*

Values are z-score differences in Rint, sympercent changes in FeNO, and odds ratios for asthma (95% confidence interval) from linear and logistic regression models. "n =" represents number of total group (Rint, FeNO) or number of cases per total group (asthma). *p < 0.05 and **p < 0.01. Models were adjusted for maternal age, pre-pregnancy body mass index, educational level, history of asthma or atopy, psychological distress during pregnancy, parity, maternal and paternal smoking during pregnancy, and child's gender, ethnicity, breastfeeding, and pet keeping. The birth outcome adjusted model was additionally adjusted for gestational age and size at birth.

Compared with children not exposed to secondhand tobacco smoke , those who were exposed had a higher Rint (difference in Z-score 0.45 (0.00, 0.90)), but no difference in FeNO, or increased risk of asthma (Table 3.1.5). Additional adjustment for gestational age and size at birth did attenuate the size of the effect estimate for Rint (0.41 (-0.03, 0.86).

We observed no statistical significant interactions between gestational age or size at birth with maternal of paternal smoking during pregnancy, or secondhand tobacco smoke exposure in childhood (all p-values for interaction > 0.05).

When we used the combined tobacco smoke exposure variable, we observed that smoke exposure during pregnancy only was associated with persistent wheezing (OR 1.32 (1.00, 1.74) (S-Table 3.1.4). We did not observe associations of smoke exposure in childhood only or smoke exposure both during pregnancy and in childhood with Rint, FeNO and asthma (S-Table 3.1.4).

DISCUSSION

We observed, in a large population-based prospective cohort study from early pregnancy onwards, that children of mothers who continued smoking ≥5 cigarettes per day during pregnancy had an increased risk of early and persistent wheezing, and asthma at school-age. These associations were not explained or modified by gestational age or birth weight. Maternal smoking during first trimester only and paternal smoking were not associated with childhood Rint, FeNO, or asthma. This implies that the observed associations are due to continued intra-uterine adverse effects, and not by unmeasured socio-economic, behavioral or genetic factors. Associations of smoke exposure with airway resistance were present for childhood secondhand tobacco smoke exposure, independently of any tobacco smoke exposure during pregnancy. This association seemed partly explained by gestational age and weight at birth.

Comparison of main findings with other studies

Many previous studies suggest a direct adverse effect of tobacco smoke exposure on lung development, although disentanglement of exposure to maternal smoking during pregnancy and secondhand tobacco smoke remains difficult.[1-3,7,26,27] Our study is a follow-up of a study previously performed in the same population at younger age, in which we observed that fetal exposure to continued maternal smoking is associated with increased risks of wheezing in preschool children.[7] We do now show that the adverse effects of maternal smoking during pregnancy on wheezing patterns and asthma extends into school-age, independent of paternal smoking, smoke exposure in childhood and birth characteristics. A large meta-analysis performed by Burke et al. observed

that postnatal passive smoke exposure was associated with a 30% to 70% increased risk of incident wheezing and that prenatal maternal smoking was associated with a 21% to 85% increase in incidence of asthma in children aged ≤2 years.[1] A recent pooled analysis focused on wheezing and asthma at older ages, and showed an 1.4 and 1.6-fold independent effect of maternal smoking during pregnancy on wheezing and asthma in children aged 4 to 6 years who were not exposed to secondhand tobacco smoke in their first year of life.[2] Also, a linear dose-dependent association of maternal daily cigarette consumption during pregnancy with wheezing and asthma was observed. The sizes of these effect estimates were similar to those observed in our study. We additionally took other important confounders such as parental history of asthma and atopy into account.[28] Younger gestational age and weight at birth might be associated with smaller airways and could subsequently lead to lower lung function, in particular lower airway patency.[29, 30] It is known that these birth characteristics play an important role in the development of respiratory symptoms and lower lung function in childhood and adulthood.[19,30,31] We observed that the associations between maternal smoking with wheezing and asthma were not explained by gestational age and birth weight. We additionally observed that socio-economic or life style related factors, using the paternal smoking during pregnancy as a proxy[15], did not explain the associations of maternal smoking during pregnancy with Rint, wheezing and asthma.

Burke et al. observed that secondhand tobacco smoke exposure in childhood was associated with childhood asthma (age 5 to 18 years) with approximately similar effect estimates (OR 1.20 (0.98-1.46)) as maternal smoke exposure during pregnancy.[1] We only observed an association of secondhand tobacco smoke exposure with a higher Rint. This is consistent with earlier studies [32,33], although these studies did not take smoke exposure during pregnancy into account, did not use asthma as a separate outcome or were performed in asthma-suspected children only. Additionally, we explored the role of birth characteristics and observed that the association between secondhand tobacco smoke exposure and Rint was partly explained by gestational age and size at birth.

Interpretation of results

We observed that the associations of maternal smoking during pregnancy with Rint, FeNO, wheezing patterns and physician diagnosed asthma were not explained or modified by gestational age or weight at birth. Thus, despite the strong associations between maternal smoking during pregnancy with birth characteristics, the pathways leading from fetal smoke exposure to physician diagnosed asthma might be independent of early body growth. The effects of maternal smoking during pregnancy on airway remodeling, hyper-responsiveness and inflammation in offspring was recently assessed in mice models.[34] Smoking during pregnancy induced airway remodeling including increased airway smooth muscle layer, collagen III deposition and house dust mite-induced goblet

cell numbers, which may contribute to increased methacholine responsiveness. This remodeling was irrespective of allergen exposure, although allergen exposure resulted in higher methacholine responsiveness in house dust mite-exposed offspring from smoking mothers when compared to non-smoking mothers. Other pathways that have been suggested are adverse effects of nicotine leading to a reduced blood flow and decreased delivery of oxygen and nutrients to the fetus, a reduction in fetal breathing movements or a reduction in number and metabolism of alveolar type II cells, which can affect abnormal growth and maturation of the airways and lungs independent of body size.[35-37] However, we did not observe associations of maternal smoking during pregnancy with Rint. Alternatively, recent studies propose that maternal smoking during pregnancy changes the expression of asthma susceptibility genes by a reduction of histone deacetylase activity and changes in methylation patterns.[38-40] Thus far it is not known to what extent these epigenetic changes persist throughout life course or which specific critical periods for epigenetic changes are important to have an effect on the risk of later lung disease.

Strengths and weaknesses

The major strength of this study is that we used a population-based prospective cohort design, with detailed information about maternal and paternal smoking during pregnancy, and secondhand tobacco smoke exposure in childhood. Some methodological considerations need to be discussed. First, follow-up data was available in 70% of our original study population. This non-response could have led to biased effect estimates, if associations of Rint, wheezing patterns or asthma would be different between children included and not included in the analyses. Second, information about parental smoking during pregnancy was prospectively collected. Reporting bias by underreporting of the participants might have occurred although assessing smoke exposure by questionnaires is valid in epidemiologic studies.[41] Assessing smoke exposure by biomarkers (cotinine, nicotine) in urine, blood and air has not been proven to enhance the quality of smoking data when studying asthma or asthma-related outcomes.[41,42] We had no objectively measured data on inhalant allergy such as specific IgE sensitization measured with serum or skin prick tests. Third, we did not have data on spirometry, the preferred measure in asthma assessment. Since lung function measurements using spirometry in children aged 6 years are only successful in approximately 50%, we did not perform these measurements at this age.[43] The Rint technique showed a high feasibility in this age group, and is known to detect small changes in proximal and more distal airway function with good within- and between-occasion reproducibility.[44] Previous studies have shown that Rint is able to identify differences in baseline and change in airway caliber. The discriminating capacity of Rint to identify asthma was found to be useful with positive predictive values of 82%.[44-46] Also, Rint is associated with clinically relevant

endpoints including asthma diagnosis or wheezing and is able to distinguish between groups of symptomatic and healthy young children.[46] Fourth, asthma is a difficult diagnosis in young children. Both wheezing patterns and asthma were self-reported outcome measures. Although using validated questionnaires based on international guidelines[24], underreporting or over-reporting might have occurred, which might have led to misclassification of the outcomes resulting in either overestimations or underestimations of the true associations. Finally, although we took account for many potential confounders, residual confounding might still be an issue, as in any observational study.

In conclusion, our results suggest that maternal smoking during pregnancy leads to increased risks of early and persistent wheezing and asthma in school-aged children. Secondhand tobacco smoke exposure in childhood is associated with higher Rint but this effect is partly explained by gestational age and weight at birth.

Detailed acknowledgements and online resources can be found in the published article online: http://www.sciencedirect.com/science/article/pii/S0012369215506387

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Chapter 3.2

Maternal folic acid use during pregnancy, *MTHFR* polymorphisms, and child's lung function and asthma

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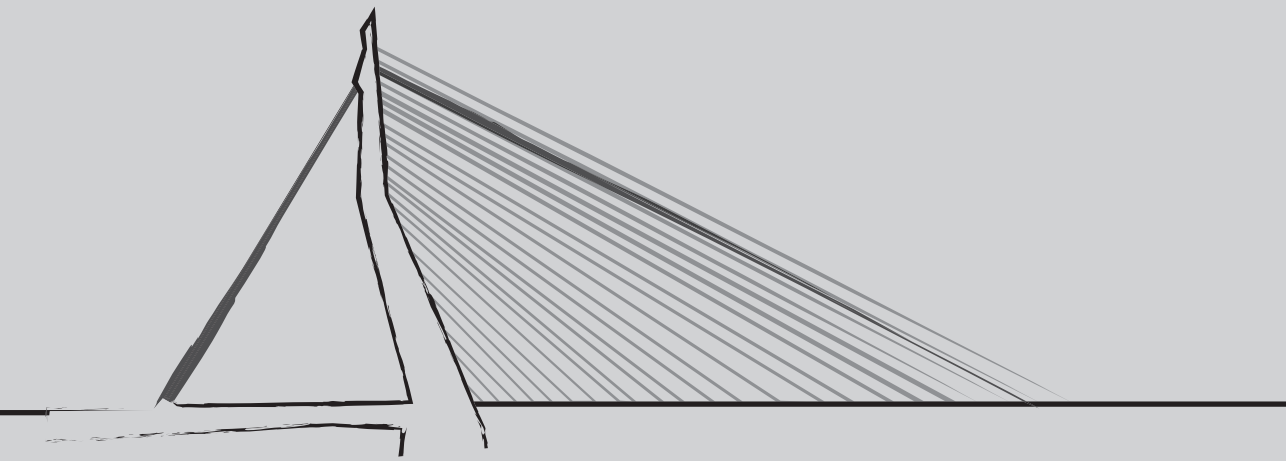
Submitted



Chapter 3.3

Duration and exclusiveness of breastfeeding and outcome in asthma

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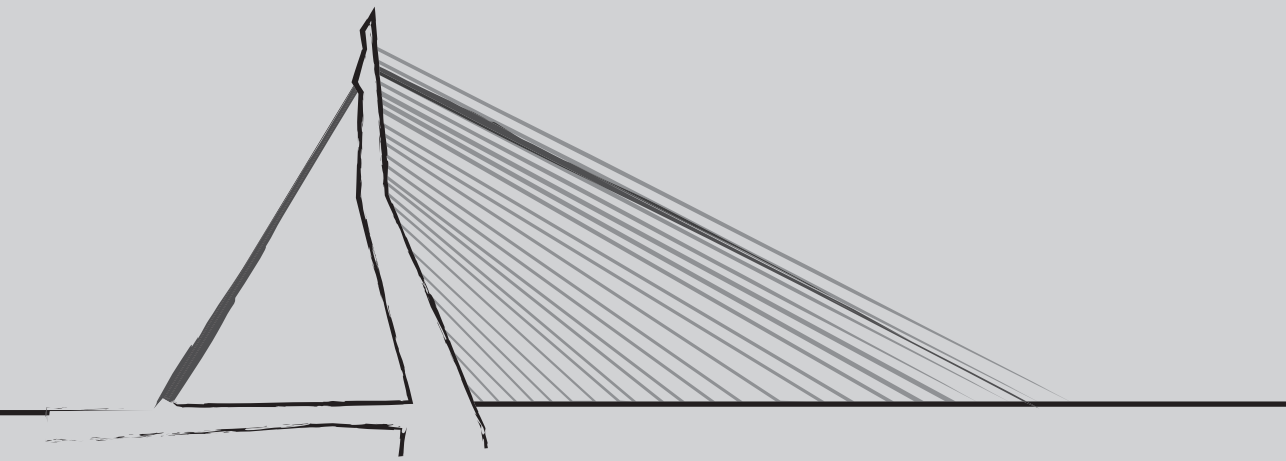

ABSTRACT

Background: Breastfeeding is associated with a lower risk of asthma symptoms in early childhood, but its effect at older ages remains unclear. We examined the associations of duration and exclusiveness of breastfeeding with asthma outcomes in children aged 6 years, and whether these associations were explained by atopic or infectious mechanisms.

Methods: We performed a population-based prospective cohort study among 5675 children. Information about breastfeeding was collected by questionnaires. At age 6 years, we measured interrupter resistance (Rint) and fractional exhaled nitric oxide (FeNO). Information about wheezing patterns (early (≤3 years only), late (>3 years only), persistent (≤3 and >3 years)), and current asthma at 6 years was derived from repeated questionnaires.

Results: Compared to children who were ever breastfed, those who were never breastfed had lower FeNO levels (sympercent (95% CI): -16.0 (-24.5, -7.5)) and increased risks of late and persistent wheezing (OR(95% CI): 1.69 (1.06, 2.69) and 1.44 (1.00, 2.07), respectively). Shorter duration of breastfeeding was associated with early wheezing and current asthma (1.40 (1.14, 1.73) and 2.19 (1.29, 3.71), respectively). Less exclusive breastfeeding was associated with early wheezing (1.28 (1.08, 1.53)). Breastfeeding duration and exclusiveness were not associated with FeNO or Rint. The associations were not explained by inhalant allergies, partly by lower respiratory tract infections in early life, and to a lesser extent by lower respiratory tract infections in later life.

Conclusions: Breastfeeding patterns may influence wheezing and asthma in childhood, which seems to be partly explained by infectious mechanisms.

INTRODUCTION

Breastfeeding may influence the development of childhood asthma.[1] Prolonged and exclusive breastfeeding has been associated with a decreased risk of asthma symptoms in early childhood with a possible diminishing effect over time.[2-5] Underlying mechanisms might involve secretory IgA, cytokines, and long-chain fatty acids in breast milk that stimulate the immune system [6] and change the balance between pro- and anti-inflammatory mechanisms.[7] This might lead to altered airway inflammation or airway resistance. Previous studies suggest a potential mediating role of inhalant allergies and respiratory tract infections.[8]

In a recent meta-analysis using data of 775,718 children from 117 observational studies [5], breastfeeding was associated with a decreased risk of asthma, regardless of asthma definition or the age at which asthma was measured (0–2 years, 3–6 years, or 7 years and older). The authors observed a large heterogeneity, which might partly be explained by the high variability of the asthmatic phenotypes used by the individual studies.[9] Furthermore, in a randomized trial amongst 13,889 children followed up until age 6.5 years receiving an experimental breastfeeding intervention, no differences in allergic symptoms or asthma prevalence were observed.[3] More detailed asthma phenotyping and use of objective measurements, such as lung function tests, might improve the understanding of the protective effect of breastfeeding. Furthermore, observing dose-response relationships based on breastfeeding duration or exclusivity would support the causality of the association of breastfeeding with childhood asthma.

Therefore, we aimed to examine among 5,675 children participating in a population-based prospective cohort study the associations of breastfeeding duration and exclusiveness with airway resistance, airway inflammation, and the risks of wheezing and asthma in children up to age 6 years, and to explore whether these associations were mediated by atopic or infectious mechanisms.

METHODS

Design and cohort

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in Rotterdam, The Netherlands. A detailed description of the study design has been published previously.[10] The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam (MEC-2007-413-NL21545.078). Written informed consent was obtained from all participants. A total of 5,675 children were included for the current analyses (S-Figure 3.3.1).

Breastfeeding duration and exclusiveness

In the Netherlands, use of breastfeeding until age 6 months is encouraged and supported. Detailed information about breastfeeding was collected using questionnaires administered at 2, 6 and 12 months after birth. Children were classified as 'never breastfed' and 'ever breastfed'. Duration of breastfeeding was categorized into 4 groups: '<2 months', '2-4 months', 4-6 months and '≥6 months'. Exclusivity of breastfeeding was defined by at which age infant formula, other drinks or food was introduced. Exclusivity of breastfeeding was categorized into 'non-exclusive breastfeeding for 4 months', and 'exclusive breastfeeding for 4 months'.

Asthma outcomes

Children visited the research center at a mean age of 6.1 (SD 0.4) years. Lung function tests were performed according to international guidelines.[11,12] Airway resistance (Rint) was measured in kPa/L (MicroRint, MicroMedical, Rochester, Kent, UK) during tidal expiration, and sex- and height-adjusted z-scores were calculated.[12] We corrected for a stepwise variation due to technical issues that required replacement of the Rint device. Fractional exhaled Nitric Oxide (FeNO) was used as a measure of eosinophilic airway inflammation and measured online (NIOX chemiluminescence analyzer; Aerocrine AB, Solna, Sweden). Questionnaires adapted from the International Study on Asthma and Allergy in Childhood (ISAAC) provided information on wheezing (no, yes) at ages 1 to 4 and 6 years.[13] Wheezing patterns were characterized by time of onset and subsequent absence or persistence into 'never', 'early' (wheezing ≤3 years only), 'late' (wheezing >3 to 6 years only), or 'persistent wheezing (wheezing ≤3 years and >3 to 6 years) in children with information on wheezing for at least two time points.[13] We defined 'current asthma' (no, yes) based on information on ever physician-diagnosed asthma (no, yes) and presence of wheezing in the past 12 months obtained at age 6 years.

Covariates

We obtained information on maternal age, pre-pregnancy body mass index (BMI), educational level, parity, history of asthma or atopy, and pet keeping by questionnaires completed by the mother at enrolment. Information about active maternal smoking was obtained by postal questionnaires during the first, second and third trimester of pregnancy and combined into smoking during pregnancy (no, yes). Maternal psychological distress was defined using the global severity index (GSI), a measure of current level or depth of the symptoms, which denotes overall psychological distress.[14,15] Midwife and hospital registries at birth provided information on sex, gestational age and birth weight. Ethnicity of the child was based on country of birth of the parents. Information on inhalant allergies (pollen, house dust mite, pets) and lower respiratory tract infections

(pneumonia, bronchitis) was obtained by questionnaire at age 6 years. More detailed information on covariates is provided in the Supplementary Material.

Data analysis

We used multivariate regression models to examine the associations between duration and exclusiveness of breastfeeding with Rint, FeNO, wheezing patterns, and current asthma at 6 years. Detailed information on covariates is given in the supplementary material. Missing data of covariates and wheezing were imputed to reduce bias and improve efficiency. The final models were adjusted for maternal BMI, educational level, parity, smoking, and child's sex, birth >37 weeks of gestation, birth weight and ethnicity. We additionally adjusted our models for inhalant allergies and respiratory tract infections as they are hypothesized to be within the causal pathway, and calculated the percentage change of the effect estimate by the formulas: 100x(effect estimate$_{mediator}$-effect estimate$_{model1}$)/(effect estimate$_{model1}$) for Rint and FeNO, and 100x(effect estimate$_{mediator}$-effect estimate$_{model1}$)/(effect estimate$_{model1}$-1) for wheezing patterns and current asthma. A 95% confidence interval for the percentage change of the effect estimate was calculated using a bootstrap method with 1,000 resamplings.[16] FeNO levels were natural log-transformed to obtain normality and presented as sympercent difference (sym%), which represents the regression coefficient of ^{e}log transformed FeNO*100%, and can be interpret as percentage change.[17] All measures of association are presented with their 95% confidence intervals (95% CI). Statistical analyses were performed using the Statistical Package of Social Sciences version 21.0 (IBM Corp., Armonk, NY, USA), and R version 3.0.0 (The R foundation for Statistical Computing).

RESULTS

Detailed characteristics of children and their mothers are presented in Table 3.3.1 and S-Table 3.3.1. Of the children, 92.2% were ever breastfed, 20.1% was breastfed <2 months, 16.5% for 2-4 months, 9.4% for 4-6 months and 24.7% for ≥6 months. Mean Rint was 0.84 (SD 0.29) kPa/l/s and median FeNO was 9.2 (range 0.10– 19) ppb. Of the children, 54.1% were categorized as never, 28.0% as early, 4.8% as late, and 13.0% as persistent wheeze. Current asthma was reported for 3.2%. Non-responders and participants without follow-up data had younger, lower educated mothers with a higher pre-pregnancy BMI and higher prevalence of smoking and psychological distress. Children were more often non-from European ethnicity, born younger, had a lower birth weight, and more often had respiratory tract infections than those included in the study (S-Table 3.3.2).

Table 3.3.1. Characteristics of Mothers and Their Children.

	Imputed data (n = 5,675)
Maternal characteristics	
Age (years)	31.1 (4.9)
Body mass index (kg/m^2)	
<20	8.6 (487)
20-25.0	55.0 (3,121)
25-30.0	26.5 (1,504)
≥30	9.9 (562)
Higher educational level (%)	53.0 (3,009)
Multi-parous (%)	42.9 (2,431)
History of asthma or atopy (%)	37.8 (2,142)
Pet keeping (%)	32.9 (1,897)
Smoking during pregnancy (%)	14.3 (812)
Psychological distress during pregnancy (%)	7.8 (444)
Child characteristics	
Female sex (%)	50.1 (2,845)
Gestational age at birth (weeks)	40.1 (26.7, 42.9)
Birth weight (grams)	3,459 (545)
European ethnicity (%)	70.8 (4,018)
Inhalant allergy (%)	6.9 (391)
Lower respiratory tract infections at 6 years (%)	5.1 (290)
Breastfeeding ever (%)	92.2 (5,231)
Breastfeeding duration (%)	
Never	9.9 (444)
<2 months	20.1 (1,140)
2-4 months	16.5 (939)
4-6 months	9.4 (531)
≥6 months	24.7 (1,404)
Breastfeeding exclusiveness (%)	
Never	9.6 (444)
Non-exclusive for 4 months	65.0 (2,993)
Exclusive for 4 months	25.4 (1,171)
Rint (kPa/L/s)	0.84 (0.29)
FeNO (ppb)	9.2 (0.1, 119)
Wheezing	
Never	54.1 (3,072)
Early	28.0 (1,590)
Late	4.8 (274)
Persistent	13.0 (739)
Current asthma (%)	3.2 (132)

Values are means (SD), medians (range) or percentages (absolute numbers). Data on breastfeeding duration and exclusiveness, Rint, FeNO and current asthma were not imputed.

Breastfeeding and asthma outcomes

In crude analyses, breastfeeding was associated with FeNO, wheezing patterns and asthma, but not with Rint (S-Tables 3.3.3). Results did not materially change after adjustment for confounders (Tables 2 and 3). Compared with children who were ever breastfed, those never breastfed had lower FeNO levels (sym% (95% CI): -16.0 (-24.5, -7.5) (Table 3.2.2). The duration and exclusivity of breastfeeding was not associated with FeNO. Never breastfed children had increased risks of late and persistent wheezing (Odds Ratio (OR) (95% CI): 1.69 (1.06, 2.69) and 1.44 (1.00, 2.07), respectively) (Table 3.3.3). Among breastfed children, those breastfed for <2 months had increased risks of early wheeze and current

Table 3.3.2. Associations of Breastfeeding with Childhood Rint and FeNO

	Rint	**FeNO**
	Z-score difference	**Sympercent difference**
	n = 3,422	n = 3,150
Breastfeeding (n = 5,675)		
Never (n = 444)	-0.11 (-0.51, 0.29) n = 248	**-16.0 (-24.5, -7.5)**** n = 241
Ever (n = 5,231)	Reference n = 3,174	Reference n = 2,906
Duration of breastfeeding (n = 4,023)		
0.1 - 2 months (n = 1,138)	-0.7 (-0.38, 0.25) n = 677	-1.1 (-7.8, 5.6) n = 621
2 - 4 months (n = 941)	0.07 (-0.25, 0.40) n = 556	-4.5 (-11.3, 2.4) n = 535
4 - 6 months (n = 540)	0.20 (-0.19, 0.59) n = 320	0.20 (-4.3, -12.5, 3.9) n = 304
≥6 months (n = 1,404)	Reference n = 848	Reference n = 776
Duration (per month) (n = 4,023)	0.01 (-0.03, 0.04) n = 2,401	0.2 (-0.5, 0.9) n = 2,236
Exclusivity of breastfeeding (n = 4,164)		
Non-exclusive for 4 months (n = 2,993)	-0.10 (-0.37, 0.17) n = 1,784	-2.8 (-8.7, 3.1) n = 1,672
Exclusive for 4 months (n = 1,171)	Reference n = 718	Reference n = 663

Values are z-scores differences (95% confidence intervals) or sympercent differences (95% confidence intervals). "n =" represents number of total group. Models were adjusted for maternal body mass index, educational level, parity, smoking, and child's sex, gestational age at birth, weight at birth and ethnicity. **p < 0.01.

asthma (OR (95% CI): 1.40 (1.14, 1.73) and 2.19 (1.29, 3.71), respectively) compared with those breastfed for ≥6 months. Longer duration of breastfeeding was associated with early wheezing and current asthma (OR (95% CI): 0.97 (0.94, 1.00) and 0.92 (0.87, 0.98), respectively) (Tables 3.3.2 and 3.3.3). Similarly, non-exclusively breastfed children had an increased risk of early wheezing (OR (95% CI): 1.28 (1.08, 1.53)), compared with those breastfed exclusively for 4 months. Additional adjustment for inhalant allergies did not materially change the effect estimates (S-Table 3.3.4a). After additional adjustment for

Table 3.3.3. Associations of Breastfeeding with Wheezing Patterns and Current Asthma.

	Wheezing patterns			Current asthma
	Early Wheezing Odds Ratio	**Late Wheezing Odds Ratio**	**Persistent Wheezing Odds Ratio**	**Odds Ratio**
	n = 1,590	n = 274	n = 739	n = 4,093
Breastfeeding (n = 5,675)				
Never (n = 444)	1.31 (0.98, 1.75) n = 141	**1.69 (1.06, 2.69)*** n = 28	**1.44 (1.00, 2.07)*** n = 76	1.57 (0.90, 2.74) n = 17 / 317
Ever (n = 5,231)	Reference n = 1,449	Reference n = 246	Reference n = 663	Reference n = 115 / 3,776
Duration of breastfeeding (n = 4,023)				
0.1 - 2 months (n = 1,138)	**1.40 (1.14, 1.73)**** n = 344	1.13 (0.72, 1.77) n = 62	1.24 (0.94, 1.65) n = 156	**2.19 (1.29, 3.71)**** n = 41 / 806
2 - 4 months (n = 941)	1.20 (0.97, 1.48) n = 266	0.80 (0.49, 1.32) n = 37	1.14 (0.86, 1.52) n = 119	1.27 (0.69, 2.31) n = 20 / 695
4 - 6 months (n = 540)	1.14 (0.89, 1.46) n = 153	0.55 (0.26, 1.14) n = 15	0.90 (0.63, 1.30) n = 53	0.86 (0.39, 1.93) n = 8 / 420
≥6 months (n = 1,404)	Reference n = 355	Reference n = 65	Reference n = 157	Reference n = 25 / 1,130
Duration (per month) (n = 4,023)	**0.96 (0.94, 0.98)**** n = 1,118	0.99 (0.95, 1.04) n = 179	**0.97 (0.94, 1.00)*** n = 485	**0.92 (0.87, 0.98)*** n = 94 / 3,051
Exclusivity of breastfeeding (n = 4,164)				
Non-exclusive for 4 months (n = 2,993)	**1.28 (1.08, 1.53)**** n = 858	1.23 (0.81, 1.86) n = 146	1.23 (0.97, 1.56) n = 391	1.48 (0.89, 2.47) n = 76 / 2,200
Exclusive for 4 months (n = 1,171)	Reference n = 295	Reference n = 45	Reference 126	Reference n = 20 / 941

Values are odds ratios (95% confidence intervals). "n =" represents number of cases (wheezing patterns) and number of cases per total group (current asthma). Models were adjusted for maternal body mass index, educational level, parity, smoking, and child's sex, gestational age at birth, weight at birth and ethnicity. *p < 0.05, **p < 0.01

early respiratory tract infections the effect estimates most prominently and significantly attenuated for children breastfed <2 months with early and persistent wheezing and current asthma, and for children non-exclusively breastfed for 4 months with early and persistent wheezing (range %change -8.8 to -66.4)). After additional adjustment for late respiratory tract infections, only the effect estimate for children breastfed <2 months with persistent wheezing attenuated (%change (95% CI): -33.5 (-82.8, -17.6)) (S-Table 3.3.4b).

DISCUSSION

We observed that children who were never breastfed had lower FeNO levels and increased risks of late and persistent wheezing. Those who were shorter breastfed had increased risks of early wheezing and current asthma. Less exclusive breastfeeding was associated with an increased risk of early wheezing. The associations were partly explained by lower respiratory tract infections in early life, and to a lesser extent by lower respiratory tract infections in later life. Inhalant allergies did not explain the associations. Breastfeeding was not associated with Rint.

Comparison of main findings with other studies

Recently, 117 studies that examined the associations between breastfeeding and asthma were meta-analysed.[5] The effect of breastfeeding on asthma was most pronounced in children aged 0–2 years and decreased with age, but seemed still evident at school age. The size and the directions of our effect estimates were similar when we used the same definition of breastfeeding duration (ever vs. never, ≥3-4 months vs <3-4 months; and ≥6 months vs < 6 months) (data not shown). It has also been reported that children who were breastfed longer [18-21] or more exclusive [19] had a higher forced expiratory volume in 1 second (FEV_1) at ages 8-18 years, although not all studies observed positive effects.[22] We observed no association between breastfeeding and Rint. Besides different lung function test, differences in results might be explained by different definitions of duration of exclusiveness of breastfeeding, the age at which lung function measurements were performed, adjustment for confounders and sample sizes. Further studies on the associations between breastfeeding and lung function are needed. Only one study examined the association of breastfeeding duration with FeNO levels, and among asthmatic children only. Children who were never breastfed or breastfed for <6 months had no difference in FeNO level at age 8 years, compared with children who were breastfed ≥6 months.[23] We observed that children who were never breastfed had lower FeNO levels, compared to children who were ever breastfed. The duration and exclusiveness

of breastfeeding was not associated with FeNO. Further studies are needed to replicate our findings before any strong conclusion might be drawn.

Previous studies suggested a mediating effect of allergies and respiratory tract infections.[5, 19, 20, 24-26] We applied thorough mediation analyses, and observed that the associations were not explained by inhalant allergies. Lower respiratory tract infections in early life, and to a lesser extent lower respiratory tract infections in later life, did partly explain the associations. Other potential underlying mechanisms such as the impact of breastmilk on the microbiome need to be explored.

Interpretation of results

Underlying mechanisms for the associations of breastfeeding and asthma might include secretory factors in breast milk such as IgA, cytokines, and long-chain fatty acids [6] which stimulates the development of the infants immune system. Also, breastmilk stimulates the intestinal microbiota, which influences the developing immune system and activates T-regulatory cells.[27] Opposite of expected, we observed that children who were never breastfed had lower FeNO levels than children who were longer or more exclusively breastfed, and thus might have less eosinophillic airway inflammation. Based on previous findings, we speculate that children who were never, shorter or less exclusively breastfed more often had respiratory tract infections in early life.[28, 29] This is supported by our results that shorter or less exclusive breastfeeding was associated with increased risks of early wheezing, which is more commonly induced by respiratory tract infections [13]. Respiratory tract infections usually lead to high amounts of neutrophillic granulocytes in the airways.[30] The presence of numerous neutrophillic granulocytes might suppress the production of eosinophils [31], and lead to less eosinophillic airway inflammation. However, asthma phenotypes based on cell type might not be consistent over time.[32] Furthermore, we observed that results changed less when we additionally adjusted for respiratory tract infections in later life, as compared to when respiratory tract infections in early life. This implies that the associations between breastfeeding and asthma-related outcomes are partly explained by the protective effect of breastfeeding on lower respiratory tract infections in early life. Finally, as the child develops, more factors influence respiratory morbidity, making it difficult to identify the specific role of breastfeeding. In later childhood, associations of atopic mechanisms with persistent wheezing and asthma seem stronger than the associations of infectious mechanisms with persistent wheezing and asthma.[13, 33] The dose-dependent effect of breastfeeding on asthma in atopic children remains under debate as earlier studies observed more [26] or no [34, 35] protective effects of breastfeeding on asthma in school-aged children. In the current study, the associations between breastfeeding and asthma-related outcomes were not mediated by inhalant allergies, which might be limited by the unavailability of objective allergy measures.

Strengths and limitations

This study was embedded in a population-based prospective cohort study with detailed data on breastfeeding status, lung function and asthma outcomes. However, some limitations do apply. First, characteristics of non-responders at baseline and those lost to follow-up differed from those included in the study. This could have led to biased results if associations of breastfeeding status with asthma-related outcomes would be different between those included and not included. Second, we did not perform spirometry. It is known that spirometry is feasible and acceptable for approximately 50% of children performing spirometry for the first time.[36] Rint is more feasible at this age, and can detect small differences in airway resistance with good within- and between-occasion reproducibility. The biological validity of increased airway resistance has been extensively demonstrated and airway resistance is associated with clinically relevant endpoints.[37] Also, Rint can distinguish between groups of symptomatic and healthy young children.[38] Third, exploring mediation using the method proposed by Baron and Kenny is limited by assumptions of causality, absence of mediator-outcome confounding and absence of exposure-mediator interaction.[39] Objective measures of inhalant allergies and respiratory tract infections were not available. Although questionnaires are efficient tools in epidemiological studies [40, 41], lack of objective measures could have affected our results. Further studies with longitudinally and objective measured data on inhalant allergies and respiratory tract infections are needed to disentangle the direction of causality and possible mediating effects. Current asthma was defined as ever physician-diagnosed asthma (5.9%) and presence of wheezing symptoms in the past 12 months at age 6 years (9.0%), which led to a relatively low prevalence. This might have been an underestimation of true asthma cases, as asthmatic children with proper treatment might not have had any wheezing symptoms. Furthermore, the Generation R study is a multi-ethnic population–based birth cohort. Of the population for the current analysis, those of non-European ethnicity were mainly of Turkish (6.4%), Surinamese (6.2%), Moroccan (4.5%) or Dutch Antilles (2.2%) origin with current asthma prevalences of 3.7%, 4.8%, 3.4%, and 11.1%, respectively. Europeans (76.6%) had a current asthma prevalence of 3.1%. Last, as in any observational study, residual confounding due to unmeasured or insufficiently measured confounders might be an issue.

In conclusion, never breastfeeding was associated with lower FeNO levels, and increased risks of persistent wheezing. A shorter duration or non-exclusiveness of breastfeeding was associated with an increased risk of wheezing and asthma, providing evidence for a dose-response relationship. Results were independent of atopic mechanisms, but were partly explained by infectious mechanisms in early life. Further studies using detailed information on allergies and respiratory tract infections throughout life are needed to explore the underlying pathophysiological mechanisms.

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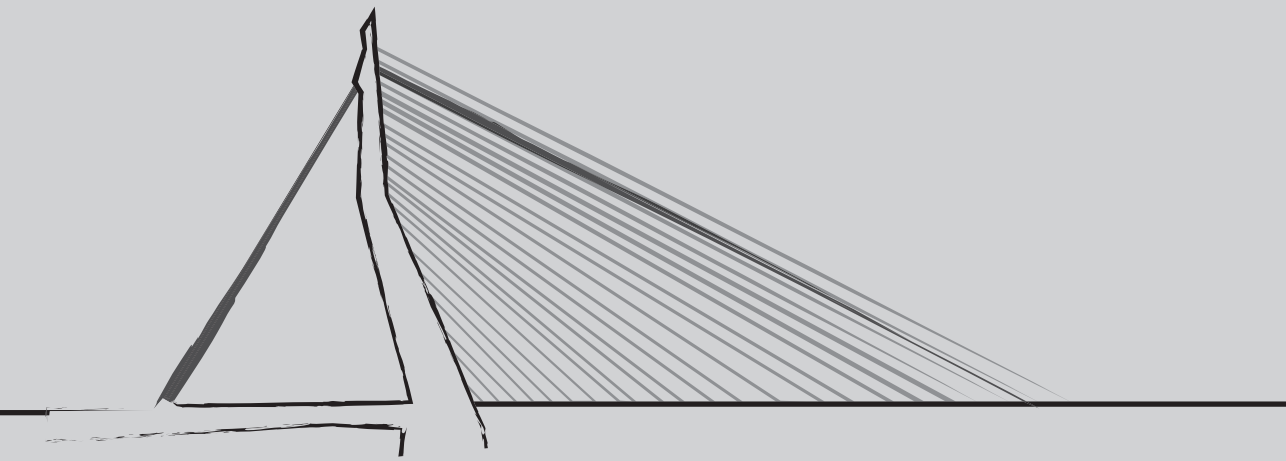
Chapter 4

Genetics and epigenetics of childhood lung function and asthma



Chapter 4.1

A genome-wide association study identifies *CDHR3* as a susceptibility locus for early childhood asthma with severe exacerbations

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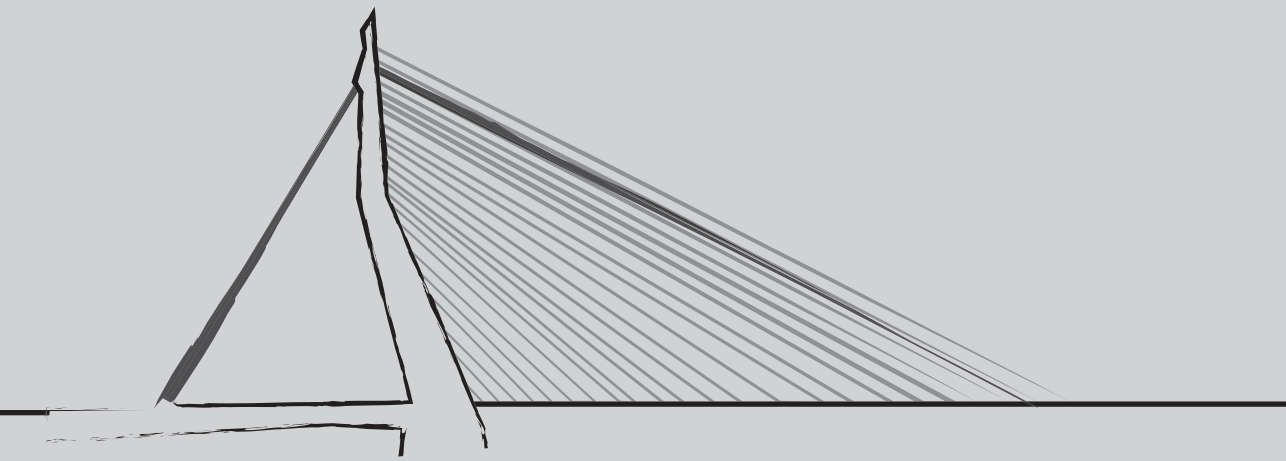
Nat Genet. 2014;46:51-55



ABSTRACT

Asthma exacerbations are among the most frequent causes of hospitalization during childhood, but the underlying mechanisms are poorly understood. We performed a genome-wide association study of a specific asthma phenotype characterized by recurrent, severe exacerbations occurring between 2 and 6 years of age in a total of 1,173 cases and 2,522 controls. Cases were identified from national health registries of hospitalization, and DNA was obtained from the Danish Neonatal Screening Biobank. We identified five loci with genome-wide significant association. Four of these, *GSDMB*, *IL33*, *RAD50* and *IL1RL1*, were previously reported as asthma susceptibility loci, but the effect sizes for these loci in our cohort were considerably larger than in the previous genome-wide association studies of asthma. We also obtained strong evidence for a new susceptibility gene, *CDHR3* (encoding cadherin-related family member 3), which is highly expressed in airway epithelium. These results demonstrate the strength of applying specific phenotyping in the search for asthma susceptibility genes.

MAIN

Acute asthma exacerbations are among the most frequent causes of hospitalization during childhood and are responsible for large health-care expenditures.[1-4] Available treatment options for prevention and treatment of asthma exacerbations are inadequate [5], suggesting that asthma with severe exacerbations may represent a distinct subtype of disease and demonstrating a need for improved understanding of its pathogenesis.

Asthma heritability is estimated to be 70–90% [6, 7], but only a limited number of susceptibility loci have been verified in genome-wide association studies (GWAS).[8-13] Larger GWAS may identify new susceptibility loci with smaller effects, but, owing to the large heterogeneity in asthma [14], an alternative strategy is to increase phenotype specificity in genome-wide analyses. A specific phenotype is likely to be more closely related to a specific pathogenic mechanism, and focusing on a particular phenotype may increase the power of genetic studies.

We aimed to increase understanding of the genetic background of early childhood asthma with severe exacerbations by conducting a GWAS of this particular asthma phenotype. We identified children with recurrent acute hospitalizations for asthma occurring between 2 and 6 years of age (cases) from the Danish National Patient Register. We then extracted and amplified DNA from dried blood spot samples isolated from the Danish Neonatal Screening Biobank, as previously described [15, 16], before genome-wide array genotyping (Affymetrix Axiom CEU array). Case criteria were fulfilled for 2,029 of 1.7 million children born in Denmark between 1982 and 1995 (1.1/1,000 children). The final case cohort (Copenhagen Prospective Studies on Asthma in Childhood exacerbation cohort, $COPSAC_{exacerbation}$) after genotyping and quality control comprised 1,173 children (S-Figure 4.1.1). Compared to the general population, cases were more often boys (67 versus 51%) and more often had mothers who smoked during pregnancy (32 versus 15%) (S-Tables 4.1.1 and 4.1.2). Controls consisted of 2,511 individuals of Danish descent without asthma who were previously genotyped (Illumina Human610-Quad v1.0 BeadChip). We analyzed association between disease and 124,514 SNPs genotyped in both cases and controls, and we accounted for population stratification by multidimensional scaling. The genomic inflation factor was 1.04. The genome-wide association analysis detected an excess of association signals beyond those expected by chance (S-Figure 4.1.2), and SNPs from five regions reached genome-wide significance ($P < 5 \times 10^{-8}$; Figure 4.1.1 and S-Figure 4.1.3). The top SNPs from the five loci were rs2305480 in *GSDMB* (odds ratio (OR) = 2.28, $P = 1.3 \times 10^{-48}$), rs928413 near *IL33* (OR = 1.50, $P = 4.2 \times 10^{-13}$), rs6871536 in *RAD50* (OR = 1.44, $P = 1.7 \times 10^{-9}$), rs1558641 in *IL1RL1* (OR = 1.56, $P = 6.6 \times 10^{-9}$) and rs6967330 in *CDHR3* (OR = 1.45, $P = 1.4 \times 10^{-8}$) (Table 4.3.1). Validation of results for the top SNPs by re-genotyping of cases and use of an alternative control population gave similar results (S-Tables 4.1.3 and 4.1.4). Association analyses in

the discovery cohort stratified on number of asthma- related hospitalizations showed higher OR with increasing number of hospitalizations for all five SNPs (Table 4.1.2). There was no significant interaction between the top SNPs and no effect modification by sex.

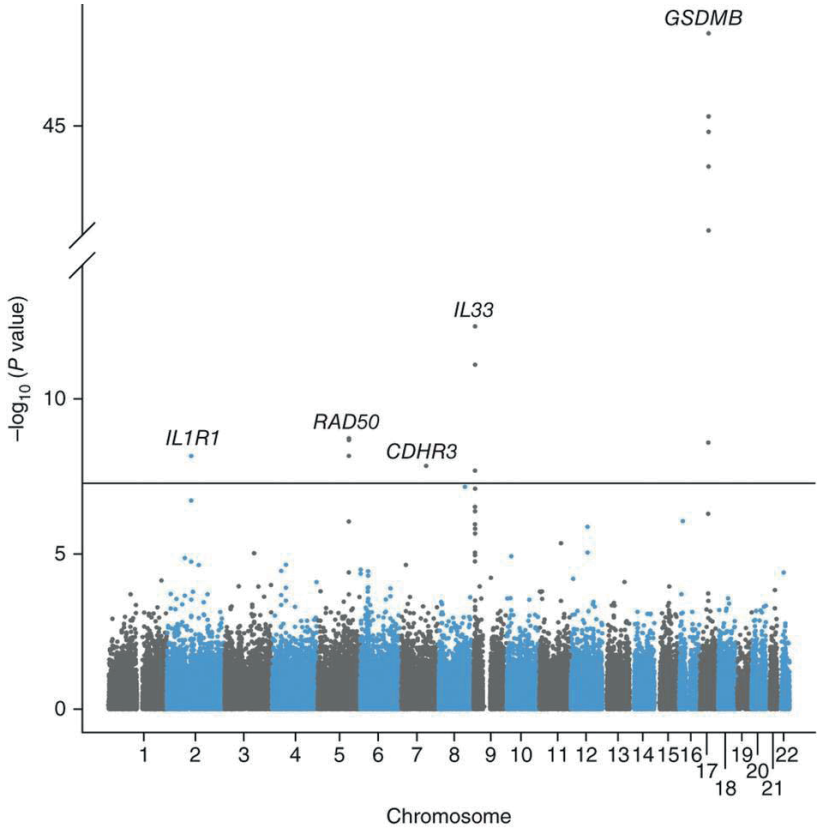


Figure 4.1.1. Manhattan Plot for the Discovery Genome-wide Association Analysis. The Horizontal Line indicates the Genome-wide Significance Threshold ($P < 5 \times 10^{-8}$).

We first sought replication in the childhood-onset stratum (with onset before 16 years of age) from a previous GWAS of asthma including 14,503 individuals conducted by the GABRIEL Consortium11 (S-Table 4.1.5), which showed evidence of association for all 5 of the genome- wide significant loci reported here (Table 4.1.1). The *CDHR3* locus was the only locus that had not previously been associated with asthma or any other atopic trait. We therefore followed up the top SNP from this locus (rs6967330) by further replication in a total of 3,975 children from 2 birth cohorts of European ancestry (COPSAC2000 and the Manchester Asthma and Allergy Study (MAAS)) and in 1 cohort with a population of mixed ancestry (Generation R). There was evidence for association with asthma before

Table 4.1.1. Discovery and Replication Results for the Five Genome-wide Significant Loci in the Discovery Analyses.

Chr.	SNP effect allele	Nearest gene	Distance to gene (bp)	Effect allele frequency	Stage	OR (95% CI)	*P* value (fixed- effects model)[a]	*P* value (random effects model)	*P* heterogeneity
17	rs2305480[G]	*GSDMB*	0	0.60	Discovery	2.28 (2.04–2.55)	1.3×10^{-48}	–	–
					Replication 1	1.32 (1.23–1.39)	6.4×10^{-23}	6.4×10^{-23}	0.86
9	rs928413[G]	*IL33*	2,418	0.28	Discovery	1.50 (1.34–1.67)	4.2×10^{-13}	–	–
					Replication 1	1.24 (1.17–1.32)	8.8×10^{-13}	2.5×10^{-6}	0.007
5	rs6871536[C]	*RAD50*	0	0.22	Discovery	1.44 (1.28–1.62)	1.8×10^{-9}	–	–
					Replication 1	1.17 (1.10–1.25)	7.6×10^{-7}	7.6×10^{-7}	0.54
2	rs1558641[G]	*IL1R1*	0	0.85	Discovery	1.56 (1.34–1.81)	6.6×10^{-9}	–	–
					Replication 1	1.11 (1.04–1.19)	0.003	0.003	0.75
7	rs6967330[A]	*CDHR3*	0	0.19	Discovery	1.45 (1.28–1.66)	1.4×10^{-8}	–	–
					Replication 1	1.18 (1.10–1.27)	3.0×10^{-6}	1.3×10^{-4}	0.04
					Replication 2	1.40 (1.16–1.67)	3.2×10^{-4}	3.2×10^{-4}	0.87
					Replications 1 + 2	1.21 (1.13–1.29)	1.6×10^{-8}	2.6×10^{-6}	0.05
					Discovery + replications 1 + 2	1.26 (1.18–1.33)	2.7×10^{-14}	2.7×10^{-7}	0.02

Replication *P* values are shown in bold if significant after Bonferroni correction for the five loci tested ($P < 0.01$). Replication 1 results are from a previously published large-scale GWAS of asthma (asthma onset before 16 years; sub-analysis of Moffatt et al [11]. Replication 2 results are from the $COPSAC_{2000}$, MAAS and Generation R cohorts (asthma onset before 6 years). Chr., chromosome. [a]A fixed-effects model was not applied in the discovery analysis.

the age of 6 years in combined analyses of the three birth cohorts and in the combined replication sets (Table 4.1.1 , S-Figure 4.1.4 and S-Table 4.1.6), as well as in a subsample including the 980 individuals with non-European ancestry (S-Table 4.1.6). Phenotype-specific replication was possible in the COPSAC2000 and MAAS birth cohorts with prospective registration of acute asthma hospitalizations and exacerbations from birth to 6 years of age in a total of 1,091 children. The rs6967330 risk allele (A) was associated with greater risk of asthma hospitalizations (hazards ratio (HR) = 1.7 (95% confidence interval (CI) = 1.2–2.4), P = 0.002) and severe exacerbations (HR = 1.4 (95% CI = 1.1–1.9), P = 0.007) in combined analyses (Figure 4.1.2, S-Figure 4.1.5 and S-Table 4.1.6).

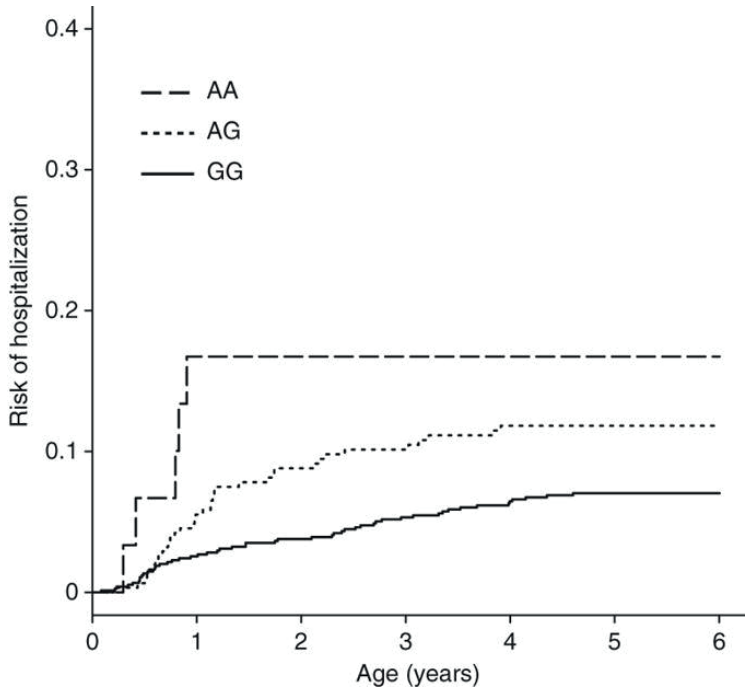


Figure 4.1.2. Cumulative Risk of Asthma Hospitalization during the First 6 Years of Life Stratified on *CDHR3* (rs6967330) Genotype.
Data are from combined analysis of the $COPSAC_{2000}$ and MAAS birth cohorts (replication), including a total of 1,091 children, of whom 92 were hospitalized for asthma. Genotype distribution was as follows: AA, 30 individuals; AG, 312 individuals; GG, 749 individuals. The *P* value for the association between genotype and risk of hospitalization was 0.002 (Cox regression analysis using an additive genetic model).

In COPSAC2000, we observed a trend in the direction of increased neonatal bronchial responsiveness associated with the rs6967330 risk allele (P = 0.10) (S-Table 4.1.7). There was no association with eczema in any of the three birth cohorts, and data on allergic sensitization were inconsistent (S-Table 4.1.6).

The top SNP at the *CDHR3* locus (rs6967330) is a nonsynonymous coding SNP, where the risk allele (A), corresponding to the minor allele, results in an amino acid change from cysteine to tyrosine at position 529. This SNP is the only known nonsynonymous variant in this linkage disequilibrium (LD) region, but there are other variants located within Encyclopedia of DNA Elements (ENCODE)-predicted regulatory regions that are in moderate to high LD ($r^2 > 0.5$) with the sentinel SNP (S-Table 4.1.8). Two SNPs with partial LD ($r^2 = 0.71$ and 0.58) were also associated with asthma in the discovery analysis but with less statistical significance. A similar association pattern with rs6967330 as the top SNP was observed in the GABRIEL (replication) study (S-Figure 4.1.6) and in the Generation R (replication) subsample of individuals with non-European ancestry (S-Figure 4.1.7), suggesting that rs6967330 might be the causal gene variant at this locus.

We investigated the potential functional consequences of the top variant in *CDHR3* (rs6967330; p.Cys529Tyr) by generating an expression construct encoding tagged human *CDHR3* and introducing the mutation encoding p.Cys529Tyr (A allele at rs6967330 resulting in mutation of cysteine 529 to tyrosine) by site- directed mutagenesis. We transfected the constructs for wild-type and mutant *CDHR3* into 293T cells. Consistent results from six independent experiments involving flow cytometry (n = 3) (S-Figure 4.1.8) and immunofluorescence staining (n = 3) (S-Figure 4.1.9) showed that the wild-type protein was expressed at very low levels at the cell surface, whereas the Cys529Tyr mutant showed a marked increase in cell surface expression (Supplementary Note). These results support the possibility that rs6967330 represents the causal variant at this locus. A recent study [17] reported that a SNP (rs17152490) in high LD ($r^2 = 0.69$) with our top SNP was associated with lung expression of *CDHR3*, further supporting a functional role for this locus.

CDHR3 is a transmembrane protein with six extracellular cadherin domains. Protein structure modeling showed that the risk-associated alteration (p.Cys529Tyr) was located at the interface between two membrane-proximal cadherin domains, D5 and D6 (Figure 4.1.3). Interestingly, Cys592 and Cys566, which are expected to form a disulfide bridge within D6, are close to Cys529 in D5, and the short distance between them could allow disulfide rearrangement (for the wild-type, non-risk cysteine variant). The location of the variant residue at the domain interface suggests that the variant residue may interfere with interdomain stabilization, overall protein stability, folding or conformation, in agreement with the observation in our experimental studies of altered cell surface expression.

The biological function of *CDHR3* is unknown, but it belongs to the cadherin family of transmembrane proteins involved in homologous cell adhesion and important for several cellular processes, including epithelial polarity, cell-cell interaction and differentiation.[18] Other members of the cadherin family have been associated with asthma and related traits, including E-cadherin [19] and protocadherin-1.[20]

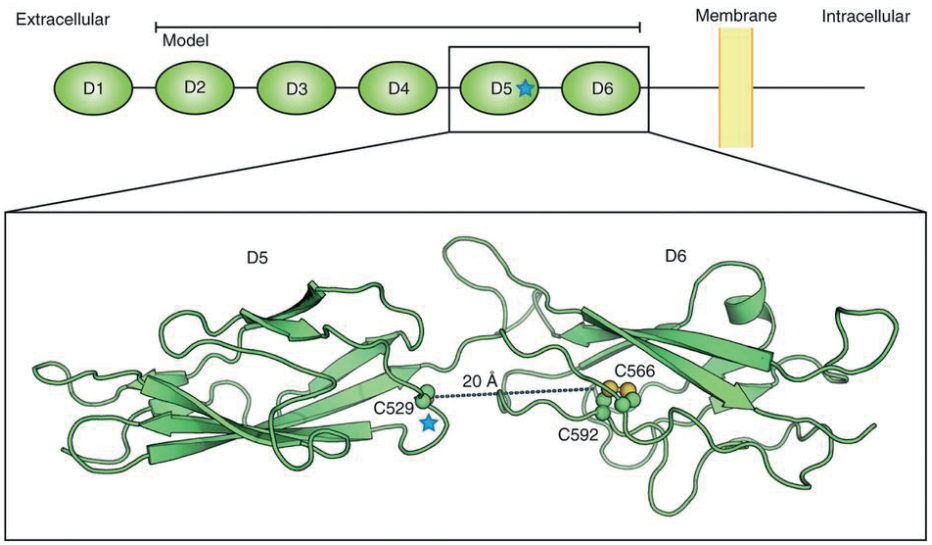


Figure 4.1.3. Overview of the *CDHR3* Protein Model.
The model covers cadherin domains 2–6 (D2–D6) and is based on the structure of the entire mouse N-cadherin ectodomain (Protein Data Bank (PDB) 3Q2W; domains 1–5). The location of the alteration at position 529 is indicated with a blue star. The distance between residue 529 and the disulfide bridge in D6 (between residues 566 and 592) is approximately 20 Å.

We demonstrated protein expression of *CDHR3* in bronchial epithelium from adults and in fetal lung tissue (S-Figure 4.1.10). *CDHR3* was previously found to be highly expressed in normal human lung tissue [21] and specifically in the bronchial epithelium.[22]*CDHR3* (probe 235650_at) was upregulated by tenfold in differentiating epithelial cells (with a rank of 123 out of more than 47,000 transcripts ranked by magnitude of upregulation)[23] and seems to be highly expressed in the developing human lung.[24]

There is an increasing focus on the role of the airway epithelium in asthma pathogenesis. Structural or functional abnormalities in the epithelium may increase susceptibility to environmental stimuli by exaggerating immune responses and structural changes in underlying tissues and increasing airway reactivity.[25] Epithelial integrity is dependent on the interaction of proteins in cell-cell junction complexes, including adhesion molecules. Studies have shown impaired tight junction function [26] and reduced E-cadherin expression [27] in the airway epithelium of individuals with asthma. *CDHR3* is a plausible candidate gene for asthma because of its high level of expression in the airway epithelium and the known role of cadherins in cell adhesion and interaction. Most asthma exacerbations in children are caused by respiratory infections, predominantly common viral infections such as rhinovirus [28], but bacterial infection may also have a role [29], as well as exposure to air pollution.[30] It is therefore plausible that *CDHR3* variation increases

susceptibility to respiratory infections or other airway irritants through impaired epithelial integrity and/or disordered repair processes.

Interestingly, the *CDHR3* asthma risk allele is the ancestral allele. Public data from protein databases suggest that humans are unique among 36 other vertebrate species in having the derived (non-risk) allele resulting in a cysteine at position 529 (S-Table 4.1.9), which is now the wild-type allele in most human populations (Human Genome Diversity Project (HGDP) selection browser). This finding suggests that the risk (ancestral) allele, associated with increased surface expression of *CDHR3*, may have been advantageous during early human evolution. This phenomenon in which the ancestral allele is the risk allele is known for other common diseases and may reflect a shift from a beneficial to a deleterious effect for a particular allele as a result of a changing environment.[31]

The *CDHR3* variant seems to be associated with an asthma phenotype of early onset, as demonstrated by the strongest replication of association in the GABRIEL stratum with asthma onset before 16 years of age (S-Table 4.1.10) and in the second replication including children with asthma onset before 6 years of age (Table 4.1.1). Increased risk was already demonstrated in the first year of life (Figure 4.1.2), particularly in children who were homozygous for the risk allele (A). This finding is in line with the tendency toward association of increased airway reactivity in neonates with the risk allele and findings of *CDHR3* expression in the fetal lung. *CDHR3* variation also seems to be more strongly associated with an asthma phenotype with exacerbations (Supplementary Table 4.1.6), particularly with recur-rent exacerbations (Table 4.1.2 and S-Table 4.1.6).

The top locus in this study, on chromosome 17q12-21, has consistently been associated with childhood-onset asthma.[11, 13] The effect size in the present study is remarkably high, with an OR of 2.3 that increases to 2.7 for the children with the highest number of exacerbations. This finding suggests a key role for this locus in severe exacerbations in early childhood, in line with a previous report from the COPSAC2000 birth cohort study.[32]

Genome-wide significant association with asthma has previously been shown for variants in or near *IL33*, *RAD50-IL13* and *IL1RL1*.[11, 33] The fact that the top loci in our study were generally shared with previous GWAS of asthma suggests that early-onset asthma with severe exacerbations is at least partly driven by multiple common variants in the same genes that contribute to asthma without severe exacerbations.

The sample size of the present GWAS was less than one-fifth that of the largest published GWAS of asthma (GABRIEL) [11], and, yet, we found a similar number of genome-wide significant loci, similar statistical significance and considerably larger effect estimates. Further increasing phenotypic specificity by stratified analysis in the 358 children with the highest number of exacerbations resulted in an additional increase in effect estimates, with ORs between 1.6 and 2.7 per risk allele, and strong statistical significance. Effect estimates were also higher than previously reported when replicating the exact

Table 4.1.2. Association Results for the Five Genome-wide Significant and Replicated Top SNPs Stratified on Number of Hospitalizations for Asthma or Acute Bronchitis from 0–6 Years of Age in the Discovery Cohort.

		Number of asthma-related hospitalizations				Association between number of hospitalizations and genotype
		2 *n* = 272	3 *n* = 228	4–5 *n* = 277	6 or more *n* = 358	
SNP effect allele	Nearest gene	OR (95% CI) *P* value	OR (95% CI) *P* value	OR (95% CI) *P* value	OR (95% CI) *P* value	*P* value[a]
rs2305480[G]	*GSDMB*	1.87 (1.54–2.26)	2.24 (1.81–2.78)	2.24 (1.83–2.73)	2.72 (2.26–3.28)	0.002
		1.5×10^{-10}	2.1×10^{-13}	1.7×10^{-15}	3.5×10^{-27}	
rs928413[G]	*IL33*	1.32 (1.09–1.61)	1.22 (0.98–1.50)	1.47 (1.21–1.79)	1.91 (1.61–2.26)	2.4×10^{-4}
		0.005	0.07	8.5×10^{-5}	6.2×10^{-14}	
rs6871536[C]	*RAD50*	1.31 (1.06–1.61)	1.26 (1.00–1.59)	1.45 (1.18–1.78)	1.58 (1.31–1.89)	0.09
		0.01	0.05	3.6×10^{-4}	1.3×10^{-6}	
rs1558641[G]	*IL1R1*	1.53 (1.16–2.02)	1.20 (0.91–1.57)	1.32 (1.02–1.71)	2.19 (1.66–2.90)	0.02
		0.002	0.20	0.04	3.2×10^{-8}	
rs6967330[A]	*CDHR3*	1.23 (0.98–1,56)	1.37 (1.07–1.75)	1.42 (1.13–1.78)	1.63 (1.33–1.97)	0.04
		0.07	0.01	0.003	1.6×10^{-6}	

Only the 1,135 children with full follow-up were included. The number of controls was 2,511 for all analyses. [a]Mantel-Haenszel test for linear association.

top SNP from the GABRIEL study (S-Table 4.1.11). This finding demonstrates that specific phenotyping is a helpful approach in the search for asthma susceptibility genes. The narrow age criteria (2–6 years) for disease may be an important phenotypic characteristic, as heritability has been demonstrated to be higher for early-onset asthma.[33]

The method of case identification through national registries allowed us to define a specific and rare phenotype of repeated acute hospitalizations in young children from 2 to 6 years of age, which, to our knowledge, has not previously been done in a GWAS. One limitation of this study is that we had relatively poor genome-wide coverage (approximately 125,000 SNPs).

In conclusion, our results demonstrate the strength of specific phenotyping in genetic studies of asthma. Future research focusing on understanding the role of *CDHR3* variants in the development of asthma and severe exacerbations may increase understanding and improve treatment of this clinically important disease entity.

METHODS

$COPSAC_{exacerbation}$ cohort (GWAS)

This is a register-based cohort of children with asthma who were identified and characterized from national health registries. The study was approved by the Ethics Committee for Copenhagen (H-B-2998 -103) and the Danish Data Protection Agency (2008-41-2622). According to Danish law, research ethics committees can grant exemption from obtaining informed consent for research projects based on biobank material under certain circumstances. For this study, such an exemption was granted (H-B-2998-103).

Case selection

Children with repeated acute hospitalizations (cases) were identified in the Danish National Patient Register covering all diagnoses of discharges from Danish hospitals.[34] Information on birth-related events was obtained from the national birth register. Inclusion criteria were at least two acute hospitalizations for asthma (ICD8-codes 493, ICD- 10 codes J45-46) from 2 to 6 years of age (both years included). Duration of hospitalization had to be more than 1 day, and two hospitalizations had to be separated by at least 6 months. Exclusion criteria were side diagnosis during hospitalization, registered chronic diagnosis considered to affect risk of hospitalization for asthma, low birth weight (<2.5 kg) or gestational age of under 36 weeks at birth. Cases were further characterized with respect to the number of hospitalizations from asthma and acute bronchitis and for concurrent atopy.

DNA sampling and genotyping of cases

DNA was obtained from blood spots sampled as part of the Danish neonatal screening program and stored in the Danish Neonatal Screening Biobank.[35] Two disks, each 3.2 mm in diameter, were punched from each blood spot. DNA was extracted, and the whole genome for each individual sample was amplified in triplicate as previously described.[15, 16] Cases were genotyped on the Affymetrix Axiom CEU array (567,090 SNPs). Top SNPs from the five genome -wide significant loci were re-genotyped with the PCR KASPar genotyping system (KBiosciences) to validate the results (Supplementary Table 3). Two additional SNPs in the proximity of the newly discovered *CDHR3* variant were genotyped for further exploration of the region encompassing it.

Controls

The control population was randomly drawn from two large Danish cohorts: the Danish National Birth Cohort (females) and the Copenhagen draft board examinations (males). Individuals who indicated in a questionnaire that they had physician-diagnosed asthma were excluded. Genome-wide genotyping had previously been performed as part of the Genomics of Overweight in Young Adults (GOYA) study [36] on the Illumina Human610-Quad v1.0 BeadChip (545,350 SNPs). Potential bias introduced by differences in chemistry between the different platforms used for cases and controls (Affymetrix and Illumina, respectively) was investigated by also using control data from the Wellcome Trust Case Control Consortium 2 (WTCCC2) project that performed genotyping on an Affymetrix platform (Affymetrix 6.0) (S-Table 4.1.4).

Replication in a previously published GWAS

Replication of the five genome-wide significant loci from the discovery analysis was sought in publically available data from a GWAS performed by the GABRIEL Consortium. This replication included 19 studies of childhood-onset asthma (onset before 16 years of age) with a total of 6,783 cases and 7,720 controls.

Replication in birth cohorts for the CDHR3 top SNP

The COPSAC2000 replication cohort

Replication and phenotypic characterization of the *CDHR3* risk locus were sought in the COPSAC2000 cohort, a prospective clinical study of a birth cohort of 411 children. This cohort is not overlapping with the $COPSAC_{exacerbation}$ discovery study. The COPSAC2000 cohort study was approved by the Ethics Committee for Copenhagen (KF 01-289/96) and the Danish Data Protection Agency (2008-41-1754), and informed consent was obtained from both parents of each child. All mothers had a history of a doc-tor's diagnosis of asthma after 7 years of age. Newborns were enrolled in the first month of life, as previ-

ously described in detail.[37-39] This cohort is characterized by deep phenotyping during close clinical follow-up. Doctors employed in the clinical research unit were acting primary physicians for the children from the cohort and diagnosed and treated respiratory and skin symptoms, and asthmatic symptoms were recorded in daily diaries.[40] Acute, severe exacerbations from birth to 6 years of age were defined as requiring the use of oral prednisolone or high-dose inhaled corticosteroid for wheezy symptoms, prescribed at the discretion of the doctor in the clinical research unit, or by acute hospitalization at a local hospital for such symptoms.[41] Asthma from birth to 7 years of age was diagnosed on the basis of predefined algorithms of symptoms and response to treatment, as previously described.[39]

Neonatal spirometry and analysis of neonatal bronchial responsiveness to methacholine were carried out by 4 weeks of age, applying the raised volume, rapid thoracic compression technique. Lung function was measured by spirometry in the child's seventh year of life. Specific airway resistance (sRaw) was measured at 4 and 6 years by whole-body plethysmography. Bronchial responsiveness at ages 4 and 6 years was determined as the relative change in sRaw after hyperventilation of cold, dry air. Allergic sensitization against common inhalant allergens was determined at 6 years of age by measurement of serum-specific IgE levels. Atopic dermatitis was diagnosed using the Hanifin-Rajka criteria [42] from birth to 7 years of age.

High- throughput genome-wide SNP genotyping was performed using the Illumina Infinium II HumanHap550 v1, v3 or Quad BeadChip platform at the Children's Hospital of Philadelphia's Center for Applied Genomics. We excluded SNPs with call rate of <95%, minor allele frequency (MAF) of <1% or Hardy-Weinberg equilibrium P value of $<1 \times 10-5$. rs6967330 was a genotyped SNP on this array.

MAAS replication cohort

The Manchester Asthma and Allergy Study is a population-based birth cohort described in detail elsewhere.[43] Subjects were recruited prenatally and were followed prospectively. The study was approved by the local research ethics committee (South Manchester, reference 03/SM/400). Parents gave written informed consent. Participants attended follow-up at ages 1, 3 and 5 years of age.

For asthma, validated questionnaires were administered by interviewers to collect information on parentally reported symptoms, physician-diagnosed asthma and treatments received. 'Current wheeze and asthma treatment' was defined as parentally reported wheeze in the past 12 months. 'Asthma ever' was defined as positive if, at any given time point, two of three responses were positive to the following questions: "Has your child wheezed within the past 12 months?", "Does your child currently take asthma medication?" or "Has a doctor ever told you that your child has asthma?" Controls were defined as children with none of these symptoms.

For exacerbations, a pediatrician extracted data from primary-care medical records, including information on diagnosis with wheeze and/or asthma, all prescriptions (including inhaled corticosteroids (ICS) and β2 agonists), unscheduled visits and hospital admissions for asthma and/or wheeze during the first 8 years of life. Following American Thoracic Society guidelines, we defined asthma exacerbations by either admission to a hospital or an emergency department visit and/or by receipt of oral corticosteroids for at least 3 days.[44]

DNA samples were genotyped on the Illumina Human610-Quad BeadChip. Genotypes were called using the Illumina GenCall application, following the manufacturer's instructions. Quality control criteria for samples included call rate of greater than 97%, exclusion of samples with outlier autosomal heterozygosity and sex validation. We excluded SNPs with call rate of <95%, Hardy-Weinberg equilibrium P value of $>5.9 \times 10{-}7$ and MAF of <0.005. We then performed a look -up for SNP rs6967330, which showed a genotyping success rate of 100% and a Hardy-Weinberg equilibrium P value of 0.4164.

Generation R replication cohort

The Generation R Study is a population-based prospective cohort study of pregnant women and their children from fetal life onward in Rotterdam, The Netherlands.[45] The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam (MEC 217.595/2002/20). Written informed consent was obtained from all mothers and biological fathers or legal guardians. Information on wheezing, asthma and eczema was collected for the children by questionnaires at the ages of 1 to 4 and 6 years.[46] Questions about wheezing included: "Has your child had problems with a wheezing chest during the last year? (never, 1–3 times, >4 times) (age 1 to 4 years)" and "Did your child ever suffer from chest wheezing? (never, 1–3 times, >4 times) (age 6 years) ." Questions about asthma included: "Has a doctor diagnosed your child as having asthma during the past year? (yes, no) (age 2 and 4 years)" and "Was your child ever diagnosed with asthma by a doctor? (yes, no) (age 3 and 6 years)." On the basis of the last obtained questionnaire, we grouped children as having 'asthma ever before 6 years of age'. Reported asthma at 2, 3 or 4 years of age was used to reclassify children included in this group where appropriate. We then re-categorized children as those with an asthma diagnosis before 3 years of age and at 3 years of age or older. Reported numbers of wheezing episodes at 1 and 2 years of age and at 3 to 6 years of age, respectively, were used to reclassify asthma diagnosis before and at 3 years of age into 'asthma diagnosis or ≥3 episodes of wheezing before 3 years of age'. Questions about eczema included: "Has a doctor diagnosed your child as having eczema during the past year? (yes, no) (age 1 to 4 years)" and "Was your child ever diagnosed with eczema by a doctor? (yes, no) (6 years)." As with asthma, we grouped children into those with 'eczema ever before 6 years of age'

on the basis of the last obtained questionnaire and used reported eczema at 1 or 4 years of age to reclassify children included in this group where appropriate.

Samples were genotyped using Illumina Infinium II HumanHap610 Quad arrays, following standard manufacturer's protocols. Intensity files were ana-lyzed using BeadStudio Genotyping Module software v.3.2.32, and genotypes were called using default cluster files. Any sample with a call rate of less than 97.5%, excess autosomal heterozygosity (F < mean – 4 s.d.) or mismatch between called and phenotypic sex was excluded. rs6967330 was a genotyped SNP in this set. Individuals identified as genetic outliers by identity-by -state (IBS) clustering analysis (>3 s.d. away from the mean for the HapMap CEU population (Utah residents of Northern and Western European ancestry)) were considered to have non-European ancestry. Ancestry determination analysis included genomic data from all Generation R individuals merged with data for three reference panels from Phase 2 of the HapMap Project (YRI (Yoruba from Ibadan, Nigeria), CHB + JPT (Han Chinese in Beijing, China, and Japanese in Tokyo, Japan) and CEU). Analysis of association between an asthma or eczema phenotype and GWAS SNPs was carried out using a regression frame-work, adjusting for population stratification in the Generation R cohort using MACH2QTL, as implemented in GRIMP. Ten genomic principal components obtained after the application of SNP quality exclusion criteria and LD pruning were used to adjust for population substructure in the combined population, four principal components were used for the European subpopulation and eight principal components were used for the non- European subpopulation. Individuals were grouped as having European (n = 1,962; 64.5%) or non-European (n = 1,078; 35.5%) ancestry on the basis of genetic ancestry. On the basis of information on the country of birth of parents and grandparents obtained by questionnaires, the largest non-European ancestry groups included individuals of Turkish (5.4%), Surinamese (4.6%), Dutch Antillean (4.0%), Moroccan (2.9%) and Cape Verdean (2.3%) origin.

Genome-wide association analysis

Quality control was carried out separately on cases and controls. This included filtering on SNP call rate (>99%) and sample call rate (>98%) and tests for excess heterozygosity, deviation from Hardy-Weinberg equilibrium, sex mismatch and familial relatedness. Non-European individuals were excluded on the basis of deviation from the HapMap CEU reference panel (release 22). Indication of population stratification or genotyping bias was tested by multidimensional scaling (MDS) after quality control. This analysis showed evidence of association with disease status for the first seven MDS components, and these were therefore included as covariates in the association analysis. Additional analyses including the first 100 MDS components did not materially alter the results. Merged data for SNPs present on both arrays after quality control were used for association testing with PLINK (v. 1.07) using a logistic additive model, adjusting for the first

seven MDS components. Additional quality control was performed for genome-wide significant SNPs after association analysis, including a test for genotyping batch effects, resulting in the removal of one genome-wide significant SNP with strong evidence of batch-related genotyping error.

Functional annotation for the SNPs in LD (r2 > 0.5) with the *CDHR3* top SNP (rs6967330) was obtained from the RefSeq track downloaded from the UCSC Genome Browser. SNPs were associated with regulatory elements by HaploReg [47] in terms of predicted ENCODE chromatin state (promoter and enhancer histone modification signals) and DNase I hyper sensitivity (S-Table 4.1.8).

Regional imputation was performed to describe the identified loci from the discovery analysis (S-Figure 4.1.3) as well as reported loci from the previous largest published GWAS (GABRIEL) (S-Table 4.1.11).[11] We used two- step genotype imputation as described.[48] We used the SHAPEIT algorithm to pre-phase the haplotypes [49] and then used IMPUEv2 software for the imputation of unknown genotypes [50] separately in case and controls. We used the 1000 Genomes Project reference panel [51] (April 2012 version). We used a strict cutoff (info of 0.88), which, according to our analyses, pro-vides an allelic dosage R^2 correlation between real and imputed genotypes of greater than 0.8 and shows an optimal balance between sufficient accuracy and power.[52] We then compared the resulting allelic frequencies using SNPTEST 2.4.1.[53]

CDHR3 protein expression in experimental models

The top SNP at the *CDHR3* locus is a nonsynonymous SNP (encoding p.Cys529Tyr). To determine the functional consequences of the p.Cys529Tyr variant, we generated expression constructs encoding tagged human CDHR3 protein, and the mutation encoding the p.Cys529Tyr alteration was introduced by site-directed mutagenesis. Plasmids encoding wild- type or mutant *CDHR3* or empty vector were transfected into 293T cells, and cells were monitored for surface and intra-cellular expression of CDHR3 by flow cytometry. 293T cells were from the American Type Culture Collection (ATCC), catalog number CRL-3216. They were recently tested for mycoplasma contamination but were not authenticated. For protein blotting, cells expressing *CDHR3* proteins were lysed, and whole-cell lysates were separated by SDS-PAGE under reducing or non-reducing conditions, transferred to PVDF membranes and blotted for Flag (anti-Flag antibody, clone M2 (Agilent Technologies, 200470-21) at a dilution of 1:2,000). For immunofluorescence and confocal microscopy, 293T cells were grown on glass coverslips in DMEM with 3 mM glutamine and 10% heat-inactivated FBS at 37 °C and 5% CO2 before and for 2 d after transfection with expres-sion constructs for Flag-tagged wild-type *CDHR3* and *CDHR3* Cys529Tyr using TransIT 2020 reagent according to a standard protocol (Mirus Bio). Cells were obtained and used at a low passage from ATCC and had recently been tested for mycoplasma. Cells were incubated in 10% serum-containing culture medium

plus primary anti-Flag mouse antibodies (F3165, Sigma; 1:300 dilution) for 1 h at 37 °C before being washed briefly with culture medium. Cells were then stained with secondary rabbit anti-mouse antibodies (F0261, Daco; 1:600 dilution) conjugated with fluorescein isothiocyanate (FITC) with incubation at 37 °C for 30 min and washed with culture medium before PBS. Afterward, cells were fixed in 2% paraformaldehyde for 15 min, washed with PBS and permeabilized in 0.2% Triton X-100 in PBS for 5 min, washed and incubated with Cy3-conjugated mouse anti-Flag antibody (Cy3-labeled F3165, Sigma; 1:300 dilution). Finally, cells were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Images were acquired using a Leica DMI 6000-B confocal microscope (Leica Microsystems) with 40× magnifica-tion and were processed in Photoshop (Adobe Systems). Experiments were performed in triplicate (independent transfections) for both flow cytometry and immunofluorescence staining. Data presented (S-Figures 4.1.8 and 4.1.9) were chosen as being representative of the repeated experiments.

CDHR3 protein structure modeling

A homology model of *CDHR3* domains 2–6 (residues 141–681) was generated using the HHpred server.[54] The model was based on the structure of mouse N-cadherin (PDB 3Q2W) domains 1–5. A disulfide bridge was manually introduced in the final model between the structurally adjacent residues Cys566 and Cys592, as this corresponds to a disulfide bridge commonly observed in cadherin domains.

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Chapter 4.2

Influence of genetic variants for adult lung function on childhood lung function

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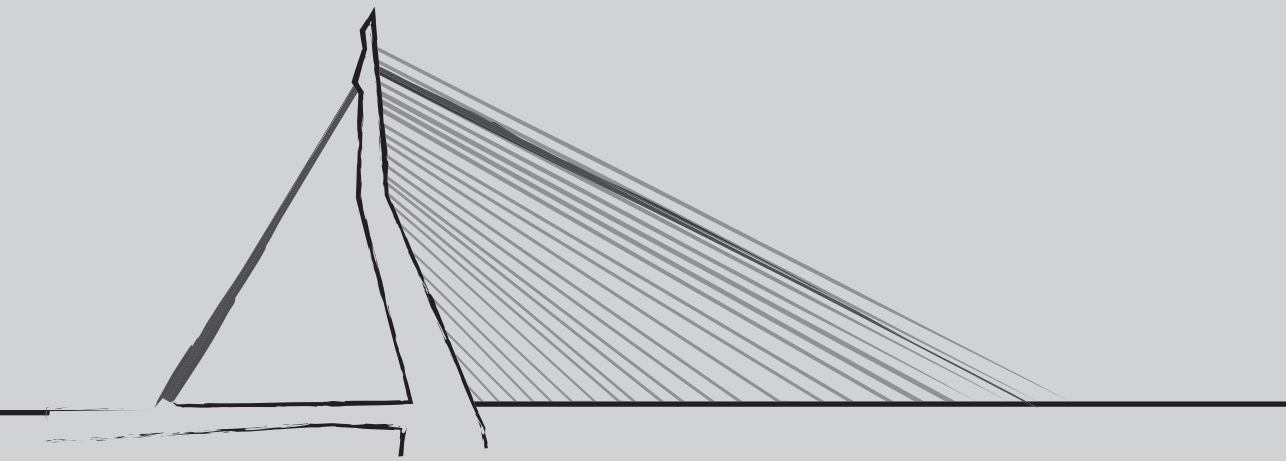
Submitted



Chapter 4.3

Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns

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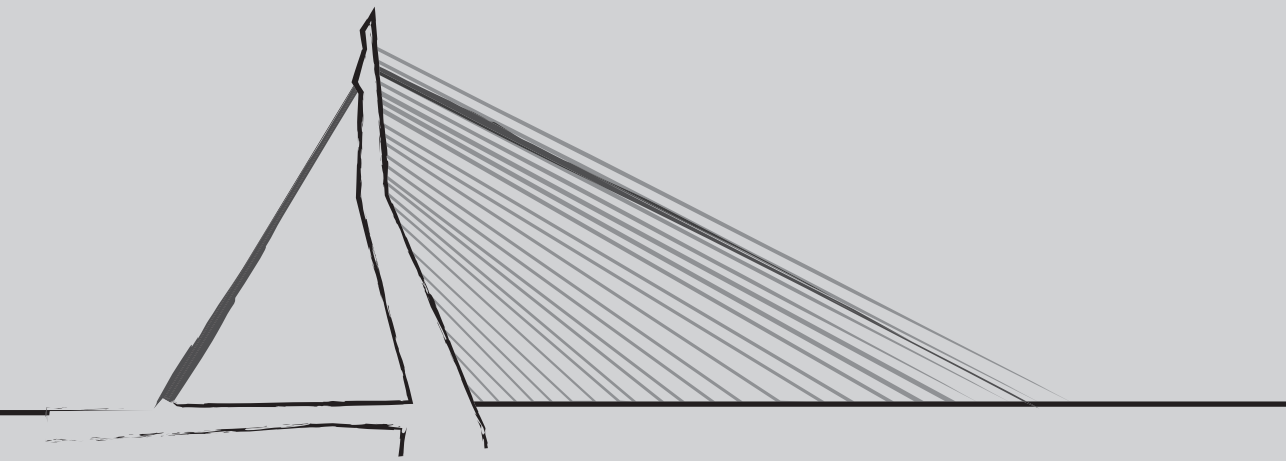

ABSTRACT

Folate is vital for fetal development. Periconceptional folic acid supplementation and food fortification are recommended to prevent neural tube defects. Mechanisms whereby periconceptional folate influences normal development and disease are poorly understood: epigenetics may be involved. We examined the association between maternal plasma folate during pregnancy and epigenome-wide DNA methylation using Illumina's HumanMethyl450 Beadchip in 1,988 newborns from two European cohorts. Here we report the combined covariate-adjusted results using meta-analysis and employ pathway and gene expression analyses. Four-hundred forty-three CpGs (320 genes) are significantly associated with maternal plasma folate levels during pregnancy (false discovery rate 5%); 48 are significant after Bonferroni correction. Most genes are not known for folate biology, including *APC2*, *GRM8*, *SLC16A12*, *OPCML*, *PRPH*, *LHX1*, *KLK4* and *PRSS21*. Some relate to birth defects other than neural tube defects, neurological functions or varied aspects of embryonic development. These findings may inform how maternal folate impacts the developing epigenome and health outcomes in offspring.

INTRODUCTION

Folate (vitamin B_9) is vital for fetal development. Folic acid supplementation at 0.4 mg per day or higher is recommended worldwide before and in the very early stages of pregnancy to reduce the incidence of neural tube defects (NTDs). Over 50 countries have introduced programs to fortify the food supply with folic acid to increase folate levels in women of childbearing age.[1] Rates of NTDs have clearly decreased following fortification[2] and there is increasing interest in the possibility that higher maternal folate prevents additional birth defects including oral clefts, cardiac defects and others.[3] A large international trial has been launched of supplementation with 4 mg versus the standard 0.4 mg to attempt to address these questions.[3]

Other beneficial effects of higher maternal folate levels have been reported in humans. These include reduced risk of low birth weight, pre-term delivery, language delay, leukaemia, childhood brain tumours and autism, although the evidence is inconsistent.[4,5] In the United States, food fortification has led to an increase in folate intake twice as large as anticipated[6], and therefore concern has been raised about possible adverse effects, such as cancer in adults, as a result of this population-wide intervention.[1] Furthermore, higher folic acid intake during pregnancy has been associated with an increased risk of childhood retinoblastoma and early respiratory illness.[4]

The mechanisms whereby folic acid prevents NTDs and potentially other birth defects and later health outcomes are poorly understood[7] but could involve epigenetic changes. Folate is a critical component in the one-carbon metabolism pathway providing methyl groups for a range of biochemical reactions, including methylation of DNA.[8] DNA methylation is an important epigenetic determinant of gene expression, and differential methylation has been associated with multiple diseases.[9] Periconceptional maternal folate levels may alter methylation patterns established *in utero* that are vital for fetal development, which could impact later health outcomes in the offspring.

In mouse models, *in utero* dietary methyl donor supplementation has been associated with altered methylation patterns and disease phenotypes.[4] The brains of human fetuses with NTDs had lower global methylation compared with controls, which was positively correlated with maternal folate levels.[10] With respect to gene-specific differential methylation, perinatal folate has also been associated with differential methylation in specific imprinted genes, such as *IGF2* and *H19*, in offspring, but reported results are inconsistent.[11] The only published study using a platform with reasonable genome-wide coverage, the Illlumina HumanMethyl450 Beadchip (450 K), investigated 23 subjects and reported that folic acid supplementation during pregnancy was related to differential methylation upstream of the gene *ZFP57*, which plays a central role in the regulation and maintenance of imprinting.[12]

Some countries, such as Norway and the Netherlands, do not fortify the food supply with folic acid. These populations may be particularly useful for examining the biological implications of periconceptional folic acid supplementation on offspring health, as greater variability in the dose and the source of folate may exist compared with fortified populations.

To better understand the biological implications of folate status on the developing fetus, we examine the association between maternal plasma folate during pregnancy and epigenome-wide differential DNA methylation in newborn cord blood using the Illumina HumanMethyl450 (450 K) Beadchip. We include 1,988 newborns from two European pregnancy cohorts of Caucasian ancestry, the Norwegian Mother and Child Cohort Study (MoBa), and the Generation R Study (Generation R). We combine results using meta-analysis. Secondary pathway analyses and gene expression analyses are also explored.

METHODS

Study populations

This analysis included participants of the Norwegian Mother and Child Cohort Study (MoBa)[13,14] and participants of the Generation R Study from the Netherlands. The study populations and cohort-specific methods described below are more extensively detailed in the Supplementary Information. The MoBa participants were mother–offspring pairs from a sub-study measuring maternal plasma folate during pregnancy.[15] The Generation R Study is a population-based prospective cohort study from fetal life onwards.[16,17] For this analysis, information on plasma folate and DNA methylation was available for 1,289 mothers and their children from the MoBa study (1,275 with complete covariate data) and 790 Caucasian mothers, and their children from the Generation R Study (713 with complete covariate data). The MoBa study was approved by the Regional Committee for Ethics in Medical Research, the Norwegian Data Inspectorate and the Institutional Review Board of the National Institute of Environmental Health Sciences, USA, and written informed consent was provided by all mothers participating. The Generation R Study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam, Netherlands and written consent was obtained from participating parents of their children.

Maternal plasma folate measurements

Both cohorts measured maternal plasma folate during pregnancy. For MoBa, maternal blood samples were drawn during pregnancy (median weeks gestation=18 weeks, 25–75th percentile=16–21 weeks) in EDTA-lined tubes, centrifuged within 30 min after

collection and stored at 4 °C in the hospital where they were collected. Samples were then shipped overnight to the Biobank of MoBa at the Norwegian Institute of Public Health in Oslo. Upon receipt (1–2 days after blood collection), plasma was aliquoted onto polypropylene microtiter plates, sealed with heat-sealing foil sheets and stored at −80 °C. Plasma folate concentration was measured at Bevital AS (www.bevital.no) by microbiological assay, using a chloramphenicol-resistant strain of *Lactobacillus casei*[18], which measures biologically active folate species, predominantly 5-methyl-tetrahydrofolate. The coefficient of variation (CV) for this assay corresponds to 4% within day and 5% between days, at population median.

For the Generation R cohort, venous blood samples were drawn at enrolment of the mothers in early pregnancy (median weeks gestation=12.9 weeks; 25–75th percentile=12.1–13.9 weeks) and stored at room temperature for a maximum of 3 h. Samples were transported to a laboratory facility of the regional laboratory in Rotterdam, Netherlands (Star-Medisch Diagnostisch Centrum) for additional processing and storage at −80 °C. The samples were analysed at the Department of Clinical Chemistry at the Erasmus MC, University Medical Center Rotterdam, Netherlands. After thawing, folate concentrations were analysed using an immunoelectrochemoluminence assay on the Architect System (Abbott Diagnostics BV). Between-run CVs for plasma measurements were 8.9% at 5.6 nmol /l, 2.5% at 16.6 nmol/ l and 1.5% at 33.6 nmol/ l with an analytic range of 1.8–45.3 nmol/ l for plasma folate.

Covariates

Each cohort had information on maternal age, education and parity from questionnaires completed by the mother or from birth registry records. Maternal smoking during pregnancy was ascertained with questionnaires (both cohorts) and cotinine levels (MoBa). Plasma levels of vitamin B_{12}, vitamin D and total homocysteine from samples taken during pregnancy were available for both cohorts. Mothers in both cohorts were genotyped for two SNPs in the (NAD(P)H) *MTHFR* gene, rs1801131 and rs1801133. Additional detail on these measurements is in the Supplementary Information.

DNA methylation measurements

DNA was extracted from cord blood and bisulfite conversion performed (EZ-96 DNA Methylation kit, Zymo Research Corporation, Irvine, USA). Samples were processed with Illumina's Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, USA) followed by cohort-specific laboratory quality control. Each cohort calculated the methylation betas, and normalized the betas using a published method.[19,20]

Estimation of cell-type proportions

Both the MoBa and Generation R studies estimated cell-type proportion with the Houseman method[21] as implemented in the *R minfi* package[22] using the Reinius *et al*. data set for reference.[23] Cell-type correction was applied by including the six estimated cell-type proportions as covariates in cohort-specific statistical models.

Cohort-specific statistical analyses

The cohort-specific statistical models were run independently. For each cohort, we used robust linear regression models in *R*[24] to evaluate the association between natural log-transformed maternal plasma folate and cord blood DNA methylation for each probe while accounting for potential heteroskedasticity and/or influential outliers. Models were adjusted for maternal age, education, smoking during pregnancy, parity and for batch effects (adjustment for plate in Generation R, correction using *ComBat*[19] in MoBa). Additional correction for study design was done in MoBa (whether the participant was in the MoBa1 or MoBa2 data set). Sex of the child was not expected to be associated with maternal plasma folate and was therefore not included as a covariate in the analyses. The adjustment variables were selected on *a priori* considerations and because they were also associated with maternal plasma folate levels at $P<0.05$.

Meta-analysis

The probe-specific quality control resulted in 473,731 CpGs in the MoBa cohort and 436,013 CpGs in the Generation R cohort. The meta-analysis was limited to the 425,749 CpGs common to both cohorts. An additional 5,844 CpGs were excluded for having a SNP mapping to the last five nucleotides of the probe sequence and with a minor allele frequency ≥5% in the CEU (Utah residents with North and Western European ancestry) population, curated by 1000G projects (http://www.1000genomes.org/, 06/2011 release, 87 individuals), HapMap project (http://hapmap.ncbi.nlm.nih.gov/, release 28, 8/2010, 174 individuals) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/, build 134, 8/2011, 116 individuals). This left 419,905 CpGs for the final meta-analyses.

Fixed-effect meta-analysis weighted by the inverse of the variance was completed using *METAL*.[25] Multiple testing was accounted for by using the FDR procedure by Benjamini and Hochberg (BH).[26] For each CpG, the resulting BH corrected *P* values are denoted by P_{BH}. CpGs with $P_{BH}<0.05$ were considered statistically significant. CpGs that were statistically significant based on the more stringent Bonferroni correction (uncorrected $P<1.19 \times 10^{-7}$ to account for 419,905 tests) were noted. We present the covariate-adjusted model without cell-type adjustment as the primary results. In the Supplementary Information, we present results additionally adjusted for cell type and results without covariate adjustment.

Sensitivity analyses

We performed sensitivity analyses to assess whether the associations observed between folate and methylation might be explained by levels of vitamin B_{12}, a dietary co-factor involved in regulating carbon unit bioavailability. Vitamin B_{12} is generally present in multivitamins that pregnant women in our studies may have taken in addition to, or in lieu of, separate folic acid supplements. Multivitamin supplements containing B_{12} typically contain other B vitamins including vitamin B_6, which is also involved in one-carbon metabolism. Because mothers with higher folate levels may have higher intakes of other vitamin supplements not involved in the one-carbon metabolism pathway, or healthier diets in general, we also performed separate analyses adjusting for maternal plasma vitamin D levels during pregnancy. We also examined two SNPs in *MTHFR* involved in modulation of one-carbon metabolism: rs1801131 and rs1801133.[27,28] We evaluated the impact of adjustment for total homocysteine on the association between maternal plasma folate and DNA methylation in newborns. Finally, we examined whether the associations with methylation seen for maternal folate levels are also seen for newborn folate levels in a subset of 572 subjects in Generation R.

Pathway analysis

To better understand the functional relationships between differentially methylated CpGs, we evaluated the FDR-significant CpGs with pathway analysis using three independent software programs. First, gene ontology analysis was performed using the IPA (www.ingenuity.com) based on the content version of 21249400 (release date: 22 September 2014). For a given category in IPA, Fisher's exact test was used to measure the probability that the category was randomly associated ($P<0.05$ defined as significantly enriched). Second, the NIAID's DAVID Bioinformatics Resources 6.7[29] was used to analyse enrichments in main categories: biological process, cellular component, molecular function and KEGG pathway. Third, we used gene ontology enrichment analysis and visualization tool[30] to identify the most informative terms that are significantly enriched.

Methylation expression analysis

We evaluated the association between methylation and quantitative levels of gene expression for our top CpGs. We used messenger RNA gene expression and 450 K methylation data both from white blood cells from adults over 45 years of age in the Rotterdam Study, a population-based prospective cohort study in Rotterdam, the Netherlands. Among the 443 FDR-significant CpGs associated with folate, we were able to match 365 CpGs to a gene transcript in our gene expression data set within a region of 250-kb upstream or downstream of the CpG (total region 500 kb). We analysed the associations of these CpGs with expression levels of the corresponding gene transcripts.

RESULTS

Study characteristics

In MoBa participants (N=1,275), maternal plasma folate levels ranged from 1.6 to 53.2 nmol/ l (mean=11.9). The maternal plasma folate levels in Generation R (N=713) ranged from 4.1 to 45.3 nmol/ l (mean=20.3; Table 4.3.1). The mean maternal age was ~30 years for both cohorts. Approximately, 15% of MoBa mothers and 25% of Generation R mothers smoked during the pregnancy and over 60% obtained college or more advanced levels of education in both studies (Table 4.3.1).

Meta-analysis

Our meta-analysis of the association between maternal plasma folate levels during pregnancy and differential DNA methylation in newborn cord blood, adjusted for covariates, resulted in 443 false discovery rate (FDR)-significant CpGs (Benjamini and Hochberg FDR-corrected *P* (P_{BH})<0.05; Figure 4.3.1). Genes with two or more FDR-significant CpGs, where at least one CpG was within the gene, were prioritized for further discussion (Table 4.3.2). Results for all FDR-significant CpGs are shown in Supplementary Data 1 (sorted by the uncorrected *P* value) and Supplementary Data 2 (sorted by chromosome and position). The vast majority of the FDR-significant CpGs were robust to covariate adjustment as well as adjustment for cell type; coefficients from the unadjusted, covariate-adjusted, and covariate- and cell-type-adjusted models were in the same direction and had a similar magnitude of effect (Supplementary Data 1 and 2). More detailed gene information is provided in S-Table 1. The genomic inflation factor (lambda)[31] values for the unadjusted, covariate-adjusted, and covariate- and cell-type-adjusted models were 0.96, 1.07 and 1.16, respectively (Supplementary Figures 1–3). Among the 443 FDR-significant CpGs in the covariate-adjusted meta-analysis model, increasing levels of maternal plasma folate during pregnancy were associated with decreased methylation of 416 (94%) and increased methylation of 27 (6%) CpGs. There were 48 CpGs that also met the strict Bonferroni threshold for statistical significance ($P<1.19 \times 10^{-7}$, correcting for 419,905 tests). The direction of effects for the statistically significant CpGs was largely consistent in the MoBa and Generation R populations (Table 4.3.2; Supplementary Data 1 and 2).

Sensitivity analyses

We considered whether vitamin B_{12}, a co-factor with folate in one-carbon metabolism, contained in most multivitamins, along with other B vitamins such as B_6 and riboflavin, might confound associations between folate and methylation. Vitamin B_{12} and folate levels were modestly positively correlated (Spearman correlation 0.11 in MoBa, 0.14 in Generation R, *P*<0.001 for both). When we adjusted for vitamin B_{12}, the coefficients for folate in relation to methylation changed only minimally (median change 4.9%, 25–75th

Table 4.3.1. Descriptive Characteristics of the MoBa and Generation R Study Populations.

Variable	Category	MoBa N (%)	Generation R N (%)
Maternal plasma folate (nmol l^{-1})	Min–max	1.6–53.2	4.1–45.3
	Mean	11.9	20.3
	Median	9.1	19.6
	25–75th percentile	6.2–16.0	13.3–26.4
Log-transformed maternal plasma folate	Min–max	0.48–4.0	1.4–3.8
	Mean	2.3	2.9
	Median	2.2	3.0
	25–75th percentile	1.8–2.8	2.6–3.3
Maternal age (years)	Mean (s.d.)	29.9 (4.3)	31.5 (4.1)
Maternal education level	Less than secondary school	96 (7.5)	14 (1.8)
	Secondary school completion	415 (32.3)	267 (34.3)
	Some college or university	566 (44.1)	203 (26.0)
	4 Years or more of college/university	206 (16.1)	296 (37.9)
	Missing	6	10
Parity*	0	537 (41.7)	479 (60.7)
	1	511 (39.6)	240 (30.4)
	2	179 (13.9)	63 (8.0)
	3+	62 (4.8)	7 (0.9)
	Missing	0	1
Maternal smoking during pregnancy	No	1098 (85.2)	541 (75.6)
	Yes	191 (14.8)	175 (24.5)
	Missing	0	74

N=1,289 MoBa and N=790 Generation R participants with maternal plasma folate and newborn DNA methylation data. N=1,275 MoBa and N=713 Generation R participants with complete data were included in the adjusted models.

*Parity was categorized as ≥1 versus 0 in the statistical models for the Generation R study.

percentile 2.3–8.2%, N=1,933 subjects). In addition to the consistency of effect estimates after adjustment, results remained statistically significant for 376 (85%) at Bonferroni correction for 443 tests, $P<1.13 \times 10^{-4}$, and all 443 CpGs had $P<9 \times 10^{-4}$ (Supplementary Data 3). Thus, vitamin B_{12} does not confound the folate–methylation associations we observed.

Women with higher folate levels, which largely reflect supplement use, might be more likely to take multivitamins and/or separate supplements such as cod liver or fish oils that are common in Norway. However, vitamin D (total of D_2 and D_3) levels were modestly correlated with folate levels (Spearman correlation coefficient=0.14 in MoBa, 0.23 in Generation R, $P<0.001$ for both cohorts). Adjustment for vitamin D only minimally altered effect estimates for folate in relation to methylation (median absolute value of

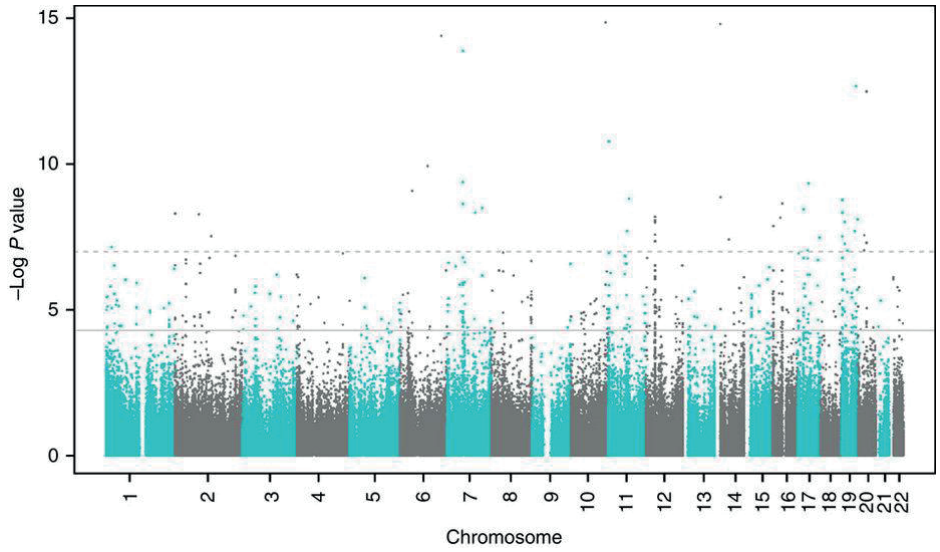

Figure 4.3.1. Association between Maternal Plasma Folate and DNA-methylation in Newborn Cord Blood: Meta-analysis Results for MoBa (n = 1,275) and Generation R (n = 713) Cohorts.
The uncorrected –log10(P values) are plotted by CpG genomic position. Multiple testing was accounted for using the false discovery rate (FDR) procedure by Benjamini and Hochberg. A total of 443 CpGs were considered FDR significant (solid horizontal line); 48 CpGs were also Bonferroni significant (dashed horizontal line)

change 7.3%, 25–75th percentile 3.3–12.3%). Despite the reduction in power due to the smaller sample size for these adjusted analyses (N=1,664), 70% of CpGs significantly related to folate in the main model remained Bonferroni significant after adjustment for vitamin D (308 with $P<1.13 \times 10^{-4}$; Supplementary Data 3).

We performed additional analyses adjusting for two single-nucleotide polymorphisms (SNPs) in the *MTHFR* gene that influence one-carbon metabolism and are correlated with plasma folate: rs1801133 and rs1801131.[27,28] These SNPs are in moderate linkage disequilibrium with each other (r^2=0.20–21 in the two studies). Adjustment for these two SNPs made little difference in the effect estimates compared with the main model; median change in coefficient=3.8% (25–75th percentile=2.0–6.9%) and 85% of CpGs remained statistically significant despite reduction in sample size to 1,880 ($P<1.13 \times 10^{-4}$, correction for 443 tests). Thus, these genetic variants do not confound the relationship between folate and methylation.

Homocysteine, unlike folate or vitamin B_{12}, is not a nutrient that plays a role as a methyl donor or carrier, but is a product formed during transmethylation in the one-carbon metabolism cycle. It could be regarded as an intermediate on the causal pathway between folate and methylation. In addition, like plasma folate, it is an excellent marker of folate status. Homocysteine was strongly correlated with maternal plasma folate in MoBa (Spearman correlation=–0.49, $P<0.001$) and moderately correlated in Generation

Table 4.3.2. Selected Loci with Differential Methylation in Cord Blood in Relation to Maternal Folate.

CHR	Position	CpG	Gene	Gene group	Meta-analysis		
					Coef	s.e.	*P*
7	126698829	cg15908975	*GRM8*	Body	−0.012	0.002	6.76E−07
7	126889015	cg18574254	*GRM8*	5′-UTR	−0.011	0.002	3.27E−09
10	91296252	cg22591480	*SLC16A12*	TSS1500	−0.008	0.002	1.34E−05
10	91296311	cg14920044	*SLC16A12*	TSS1500	−0.011	0.003	4.31E−06
11	132951838	cg24829292	*OPCML*	Body	0.010	0.002	6.60E−06
11	132951861	cg22629528	*OPCML*	Body	0.019	0.005	2.91E−05
11	132951950	cg26283170	*OPCML*	Body	0.009	0.002	1.30E−05
12	49689685	cg24804179	*PRPH*	Body	−0.007	0.002	8.05E−06
12	49690254	cg05775627	*PRPH*	Body	−0.007	0.002	1.01E−05
12	49692283	cg16010628	*PRPH*	3′-UTR	−0.005	0.001	1.73E−05
16	2866901	cg05635274	*PRSS21*	TSS1500	0.009	0.002	4.77E−06
16	2867051	cg02296564	*PRSS21*	TSS200	0.011	0.003	6.21E−06
16	2867434	cg22730830	*PRSS21*	Body	0.013	0.003	3.99E−06
16	2867446	cg01232511	*PRSS21*	Body	0.014	0.003	1.23E−05
17	35285205	cg10612259	*LHX1*		−0.011	0.002	9.10E−08
17	35285295	cg01965477	*LHX1*		−0.002	0.001	2.09E−05
19	1453909	cg11775595	*APC2*	Body	−0.015	0.003	1.64E−07
19	1456246	cg14907738	*APC2*	Body	−0.006	0.001	8.57E−06
19	1456337	cg27150178	*APC2*	Body	−0.009	0.002	5.81E−07
19	1456886	cg03165176	*APC2*	Body	−0.012	0.003	1.44E−05
19	1457211	cg14559388	*APC2*	Body	−0.003	0.001	4.98E−06
19	1465207	cg04624885	*APC2*	Body	−0.010	0.002	1.56E−05
19	1472936	cg19870717	*APC2*	3′-UTR	−0.009	0.002	4.64E−09
19	1473042	cg16613938	*APC2*	3′-UTR	−0.016	0.003	3.05E−08
19	1473179	cg23291200	*APC2*	3′-UTR	−0.010	0.002	1.72E−09
19	51415450	cg13793157	*KLK4*		−0.009	0.002	4.00E−05
19	51415452	cg10078829	*KLK4*		−0.007	0.002	1.84E−05

CHR, chromosome; Coef, regression coefficient from statistical model; gene, mapped or nearest gene (within 10 Mb) symbol using the UCSC database and Snipper software; gene group, gene region feature category (UCSC with verification); P, uncorrected P value from statistical model; UTR, untranslated region. Selection limited to genes with at least two CpGs at FDR significance that were prioritized for discussion. Meta-analysis of results for 1,275 MoBa participants and 713 Generation R participants. Robust linear regression models adjusted for maternal age, maternal education, maternal sustained smoking during pregnancy, parity and batch. Results sorted by the chromosome and position of the CpG sites listed. For complete list of CpGs differentially methylated in relation to maternal folate and for results from meta-analysis models unadjusted for covariates and adjusted for covariates and cell type see Supplementary Data 1 (sorted by P value) and 2 (sorted by chromosome, position). Supplementary Data include columns for mapped and nearest gene for each CpG.

R (Spearman correlation=−0.24, $P<0.001$), making it challenging to estimate independent effects. Given these various factors, inclusion of homocysteine in the model led to a moderate change in the coefficients for folate in relation to methylation (median change 10.7%, 25–75th percentile 5.8–17.2%. N=1,931 subjects) and only 137 (31%) CpGs remained statistically significant ($P<1.13 \times 10^{-4}$, correction for 443 tests).

We also examined whether the associations with methylation seen for maternal folate levels are also seen for newborn folate levels in a subset of 572 subjects in Generation R. Thus, this analysis is not well powered compared with our maternal folate analysis with 1,988 subjects. However, of the 443 FDR-significant findings for maternal folate in the meta-analysis there were 60 (14%) with nominal *P*values<0.05 for newborn folate which is higher than the 5% expected by chance alone (Kolmogorov $P<1.2 \times 10^{-13}$). This supports the interpretation that some similar loci are differentially methylated in response to infant folate, although we were severely underpowered to address this properly.

Pathway analysis

Pathway analysis with the FDR-significant CpGs showed strong and consistent enrichment of fundamental development pathways and of neurodevelopmental pathways (Supplementary Tables 2–4). The biological processes implicated from the DAVID pathway analysis included cell development, embryonic morphogenesis, development, regulation of multicellular organismal processes, cell–cell signalling, embryonic development, forebrain development and, notably, neural tube development (Supplementary Table 2). Ingenuity Pathway Analysis (IPA) results indicated pathways related to nervous system development and function, cell–cell signalling and basic developmental processes (Supplementary Table 3). Gene ontology enrichment analysis and visualization tool results included pathways related to the synaptic signalling, cell–cell signalling, regulation of cAMP biosynthetic process, single-organism behaviour, single-organism signalling, signalling, regulation of gastrulation and the regulation of nervous system development (Supplementary Table 4).

Methylation expression analysis

Of the 365 CpGs associated with folate that we were able to match to a gene transcript (±250 kb), 43 CpGs were significantly associated with altered expression of nearby genes ($P_{BH}<0.1$). For most CpGs, increased methylation was associated with decreased gene expression (Supplementary Table 5).

DISCUSSION

Our study is the largest to date using the Illumina 450 K epigenome-wide platform to evaluate the impact of maternal plasma folate levels during pregnancy on DNA methylation in newborns. We meta-analysed results from two population-based birth cohort studies in Northern Europeans that measured DNA methylation using the same platform. We observed epigenome-wide FDR-significant associations between maternal plasma folate and DNA methylation in cord blood at 443 CpGs.

It is notable that many of the implicated genes have functional relevance to various developmental pathways. Some are relevant to NTDs, the indication for maternal folic acid supplementation, and others to distinct developmental conditions that have not been previously associated with maternal folate levels. Additional genes we identified have been implicated in conditions where there is some concern about possible adverse effects of higher folate levels, such as breast cancer progression.[32] Due to the large number of genes significantly differentially methylated in relation to folate (Supplementary Data 1 and 2), we focus this discussion primarily on genes with two or more CpGs at genome-wide significance after FDR correction (P_{BH}<0.05) where at least one CpG is within the gene (Table 4.3.2).

We observed the largest number (nine) of statistically significant CpGs mapping to the gene adenomatosis polyposis coli 2 gene (*APC2*). *APC2* is expressed in both human fetal and adult brain17 and in the peripheral nervous system.[33] It plays a critical role in the brain development in several model systems.[34]*APC2* may also play a role in cancer aetiology. A homologue of the tumour suppressor gene *APC*, *APC2*, is involved in the regulation of the Wnt signalling pathway, which impacts both normal development and tumorigenesis.[35] Studies in mice have reported associations between periconceptional maternal folate and methylation of *APC* genes.[36] In two human breast cancer lines, folate leads to methylation-mediated silencing of *APC* and other tumour suppressor genes, raising concern about the risk of tumour progression.[37] Thus, folate-related methylation of *APC2* during fetal development could impact both pathways of neurodevelopment and carcinogenesis.

GRM8 encodes a glutamate receptor that interacts with L-glutamate, the major excitatory neurotransmitter in the central nervous system. Glutamatergic neurotransmission is ubiquitous in normal brain function[38] and is perturbed in various neuropathologies. In humans, copy-number variations of *GRM8* have been associated with neurodevelopmental disorders such as attention-deficit hyperactivity disorder[39] and autism spectrum disorder.[40]

A number of genes we identified as differentially methylated in newborns in relation to maternal folate are known to harbour mutations that have been causally implicated in various developmental abnormalities other than NTDs, the indication for folic acid

supplementation in pregnancy. These include several with two or more statistically significant CpGs (Table 4.3.2) such as *SLC16A12*, implicated in juvenile cataracts with microcornea and renal glucosuria[41]; and *KLK4*, implicated in the dental malformation amelogenesis imperfecta[42]. Mutations in *LHX1* have been associated with abnormalities in uterine development[43], and recent evidence suggests an important role in retinal development[44]. Several genes with one CpG at genome-wide statistical significance (Supplementary Data 1 and 2) also harbour mutations that are causal for various development malformations. These include *IHH* involved in skeletal malformations, *ROBO3* involved in horizontal gaze palsy with progressive scoliosis, *PCSK9* involved in familial hypercholesterolemia, *FAM83H* related to amelogenesis imperfecta type 3 and *GJA3* associated with congenital cataracts. Taken together, these findings suggest a role for periconceptional folate levels in birth defects not previously known to be related to this nutrient.

Our agnostic evaluation of maternal folate levels and DNA methylation in newborns also identified genes related to various neurologic diseases. Genetic variation in *OPCML* and *PRPH* has been associated with the neurodegenerative disease amyotrophic lateral sclerosis.[45,46] In genome-wide association studies, *CSMD1* has been associated with schizophrenia and autism.[47]

Some previous studies of folate and methylation have examined the *H19* imprinted region.[11,34] We identified three significant CpGs located 45–48-Kb upstream of *H19* among 77 CpGs on the platform that are within 48 up- or downstream of *H19*. The largest number of statistically significant associations at any locus, 31, are on chromosome 12 and, based on our extended annotation, are nearest to *ALG10*. Two CpGs are 262–573-kb upstream; the other 29 CpGs are 261–573-kb downstream. None are in *ALG10*. Most are in a CpG island near the centromere and there are no features that suggest functional impact.

In the only previous study using the 450 K platform, Amarasekera *et al*[12] reported differential methylation in relation to maternal folate in a 923-bp region on chromosome 6, 3-kb upstream of *ZFP57*. Our studies differ in sample sizes, design and analysis methods. However, when we evaluate the 20 CpGs that map to *ZFP57*, we find 5 with uncorrected *P* values of 0.05 or smaller—more than would be expected by chance alone. Thus, our data provide support for association at this locus.

From correlation analysis of 450 K methylation data and gene expression in white blood cells in adults, after correction for multiple testing, 43 CpGs that we implicated in relation to maternal folate were also related to expression of nearby genes (Supplementary Table 5). Although correlation of 450 K methylation with gene expression in the same newborn samples would have been preferable, we were only able to examine correlations in a population of Dutch adults. The most statistically significant correlation between methylation and gene expression was observed for the gene *PRSS21* (protease

serine 21 (testisin)); four CpGs were both significantly associated with maternal folate (Table 2) and expression of this gene (Supplementary Table 5). *PRSS21* is a tumour suppressor gene silenced by aberrant methylation in testicular germ cell tumours.[48] Testicular germ cell tumours are diagnosed in early adulthood and can manifest as early as 15 years of age. Prenatal origin of this tumour has been proposed[49]; perhaps, methylation *in utero*, influenced by maternal folate levels, could play a role in this pathogenesis.

Because other important factors in one-carbon metabolism could potentially explain associations between folate levels and DNA methylation in cord blood, we performed various sensitivity analyses (Supplementary Data 3). On the basis of these analyses, vitamin B_{12} does not confound the folate–methylation association. This lack of confounding by B_{12} should extend to other B vitamins such as B_6 and riboflavin that are present in multivitamins along with B_{12}. We did not have data in both studies on choline, a nutrient that can serve as a source of one-carbon units. However, in MoBa, where choline was measured, there was no correlation with folate levels (Spearman correlation=−0.034, P=0.23) and thus choline should not confound associations between folate and methylation. Vitamin D is not part of the one-carbon metabolism cycle but might impact methylation by other mechanisms.[50] We performed analyses in a subsample taking vitamin D into account as proxy for intake of other supplements or possibly healthy dietary patterns and observed no major differences in results. Adjustment of the folate–methylation association for homocysteine, a product formed in one-carbon metabolism that is itself an excellent marker of folate status, resulted in a substantial reduction in the number of statistically significant findings. Although caution is required, both because folate and homocysteine are correlated, and because they operate together in a cycle rather than a clear unidirectional pathway, this attenuation could be interpreted as homocysteine, at least in part, mediating some of the associations between folate and methylation.

Given the role of folate as a major provider of methyl groups in the one-carbon metabolism pathway, our finding of reduced methylation with higher folate at the majority of the implicated CpGs may seem counterintuitive. However, methyl groups from the one-carbon metabolism pathway are used in a range of biological processes and the complex interactions of these systems may not necessarily result in linear relationships. Indeed, there is evidence that effects of folate on folate-dependent enzymes may switch directions at the higher intracellular concentrations that may accompany folic acid supplementation.[38] Folic acid, in vitamin supplements or food fortification, is a synthetic folate with possible effects that differ from those of natural occurring folate species. There is recent evidence that folic acid interferes with the inhibitory effect of *S*-adenosylmethionine (SAM) on methylenetetrahydrofolate reductase (MTHFR)[51] and may inhibit MTHFR activity, thereby reducing the amount of 5-methyl-tetrahydrofolate, SAM and the SAM/*S*-adenosylhomocysteine (SAH) ratio.[52] The SAM/SAH ratio has been referred to as the methylation potential; low SAM/SAH ratio may decrease DNA methylation. This

may explain the inverse relationship we observe in our study but additional research is needed to more fully explain the complex biochemistry behind these observations. Of note, inverse correlations between prenatal folate status and DNA methylation at differentially methylated loci have been identified in the other population studies including Hoyo *et al*[53] and Amarasekera *et al*.[12]

Although the health outcomes that have been related to folic acid supplementation involve target tissues such as the nervous system, we only had cord blood available for assessment of methylation. We do not know whether differential methylation at the sites that we observed in cord blood would be observed in relevant target tissues. While divergence in epigenetic patterns is critical for cell-type regulation, there is also evidence of similarities in patterns among some tissues.[54-56] We do not have data on methylation at older ages and thus the question of whether the differential methylation at these loci seen at birth in relation to maternal folate persists to later childhood would need to be addressed in future studies.

We measured folate using two different platforms in the two studies. Both are valid methods for the measurements of folate. Levels were reasonably similar although slightly higher in Generation R, which could reflect a difference in the platforms, differences in folate intake or the earlier timing of measurements in Generation R (~12-week gestation in Generation R versus ~18-week gestation in MoBa). Nonetheless, the top findings were consistent in both cohorts and thus robust to differences in measurement platforms. This may increase their generalizability to other populations.

One-carbon metabolism is a complex pathway with influences from multiple genetic, hormonal and environmental factors. Despite our attempt to account for other important dietary intake involved in one-carbon metabolism, other supplementation and genetic variants, residual confounding could still be present and influence the observed associations of folate levels in pregnancy with methylation at birth.

The MoBa and Generation R cohorts offer a unique opportunity to study the epigenetic effect of folic acid supplementation in the absence of food supply fortification. It is possible that results may differ in populations exposed to fortification.

In conclusion, we identified multiple novel genes not previously implicated in biological responses to folate. Many of the implicated genes have functional relevance to various developmental pathways, including the nervous system. Some of these are relevant not only to NTDs, the indication for maternal folic acid supplementation, but also to other developmental abnormalities that have not been previously associated with maternal folate levels. The associations between periconceptional folate and these conditions are difficult to study because the abnormalities are rare and both supplementation and fortification are now widespread. Other genes identified are implicated in conditions where concern exists about possible adverse effects of higher folate levels, such as breast cancer progression16. These findings may provide new insights into

mechanisms for the associations between maternal folate status and health outcomes in the offspring. Given that food fortification programs have greatly increased the folate status of the population, greater understanding of the biological effects of this nutrient is important. The large number of novel genes identified using our genome-wide methylation approach may shed light on the protean effects of folate on human health.

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Chapter 4.4

Newborn DNA-methylation, childhood lung function, and the risk of asthma and COPD across the life course

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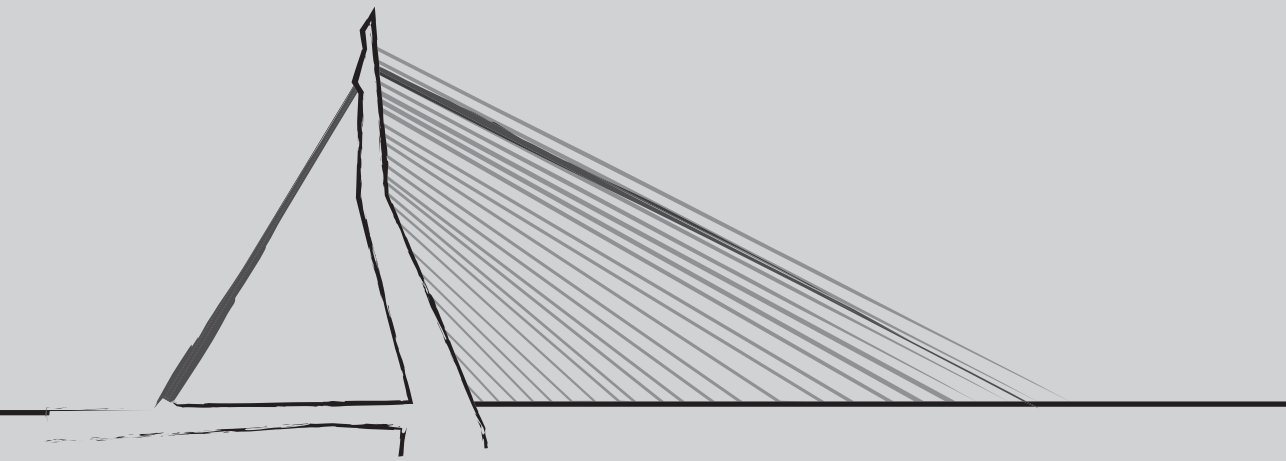
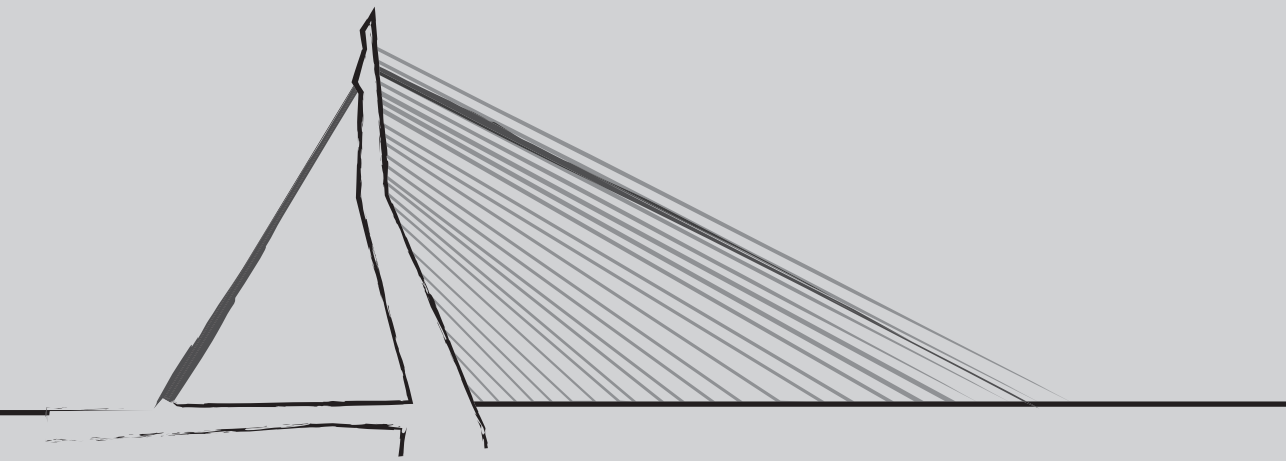
Submitted



Chapter 5

General discussion



INTRODUCTION

Early life factors are suggested to have an important role in the development of diseases in later life.[1, 2] A classic example is low birth weight, which has been associated with a wide range of adult morbidities, including chronic obstructive respiratory diseases.[2-4] These observations have resulted in the "Developmental Origins of Health and Disease" hypothesis, which proposes that the development of an organism is partly depending on the environment it is exposed to.[2] Adverse exposures may result in specific adaptations that benefit short term development and survival, but eventually lead to diseases in later life.

The main objective for this thesis was to identify early growth characteristics, environmental exposures in early life, and genetic and epigenetic variants that affect lung function and predispose the individual for chronic obstructive respiratory diseases in childhood and adulthood. The main results, merits and limitations of the studies presented in this thesis have been discussed in the previous chapters. This chapter provides a general overview and interpretation of the main findings presented in this thesis, considers general methodological issues in epidemiological studies, and suggests directions for further studies.

INTERPRETATION OF MAIN FINDINGS

Growth

Previous studies suggested that children born extremely preterm or with a low birth weight have high rates of neonatal respiratory diseases.[5] Furthermore, children born preterm, with a small size for gestational age at birth, or with accelerated weight growth in the first months of infancy have increased risks of childhood wheezing and asthma.[6] Not much is known about the longitudinal foetal and infant growth patterns predisposing to chronic obstructive respiratory diseases.

In this thesis, we examined the associations between early growth characteristics and childhood asthma using the individual data of 25,000 children participating in 24 European cohort studies. We observed that a younger gestational age, smaller size for gestational age and greater infant weight gain across the full ranges were associated with lower childhood lung function (Table 5.1). Mediation analyses suggested that lower lung function explained up to 45% of the associations between early growth characteristics and childhood asthma. Studies that examined the combined effects of growth in both fetal life and infancy are scarce, but suggest that increased infant growth is associated with lower lung function and an increased risk of wheezing and asthma in childhood.[7-9] In studies assessing weight gain from birth onwards, increased weight

growth between birth and 3 months was most consistently associated with lower FEV_1/FVC and an increased risk of asthma.[10] We assessed combined fetal and infant growth patterns and showed that smaller weight growth in late fetal life and greater weight growth in early infancy were independently associated with lower lung function measures and an increased risk of asthma in childhood. Both our and other studies support the hypothesis that early growth characteristics affect the development of the lungs and airways, and that adverse growth characteristics lead to relatively small airways, a reduction in expiratory flows reflected by lower lung function values, and an increased risk of childhood asthma.[11] The highest rate of airway and alveolar development occurs in early life.[12] Adaptations related to fetal and infant growth could affect lung function and the risk of respiratory diseases.[13] Other potential explanations include underdeveloped anatomical or immunological mechanisms, or interaction with environmental factors, such as tobacco smoke exposure, or genetic factors.[11] Further studies are needed to identify whether early developmental adaptations of the lungs and immune system explain the associations of fetal and infant growth characteristics with childhood asthma.

Next we examined the associations of detailed measured body fat composition with childhood lung function and asthma. We observed that a higher body mass index in children aged 6 years was associated with a higher respiratory resistance (Rint) and an increased risk of wheezing (Table 5.1). Detailed assessment of total body fat mass distribution showed that a higher fat mass index was associated with a higher Rint, whereas a higher android/gynoid fat mass ratio was associated with a lower Fractional exhaled Nitric Oxide (FeNO). A higher pre-peritoneal fat mass, a measure of visceral abdominal fat, was associated with a higher FeNO, whereas subcutaneous fat mass was not associated with any outcome. In a recent case-control study of asthma in 678 Puerto Rican children, waist circumference and percentage body fat were associated with a larger increased risk of exercise-induced asthma symptoms, lower FEV_1/FVC, higher total IgE levels and diagnosis of allergic rhinitis than BMI with these outcomes.[14] Similarly, the association of central obesity with asthma showed larger effect estimates than BMI with asthma in a survey of Taiwanese schoolchildren.[15] These and our findings suggest that specific adipose tissue distribution patterns are linked to pathways underlying the effect of obesity on asthma in childhood. One of these pathways could be the metabolic complications of obesity, rather than obesity itself, which might affect lung function and asthma risk in children.[16, 17]

Thus, preterm birth, low birth weight, infant growth, obesity and specific body fat mass distribution measures were associated with development of childhood lung function and asthma-related symptoms, and are potential targets for early prevention of impaired childhood lung function.

Table 5.1. Overview of the results of studies presented in this thesis on early growth and childhood lung function and asthma.

	Lung function							Symptoms and diseases	
	Rint	FeNO	FEV_1	FVC	FEV_1/FVC	FEF_{25-75}	FEF_{75}	Wheezing	Asthma
Birth characteristics									
Gestational age (weeks)	n.s.	n.s.	↑	↑	↑	↑	↑	n.s.	↓
Preterm birth (<37 weeks)	n.s.	n.s.	↓	↓	↓	↓	↓	n.s.	↑
Birth weight (SDS)	n.s.	n.s.	↑	↑	↑	↑	↑	n.s.	↓
Low birth weight (<2500 gram)	n.s.	n.s.	↓	↓	↓	↓	↓	n.s.	↑
Growth									
Fetal weight growth (SDS)	n.s.	n.s.	↑	↑	=	=	=	n.s.	=
Infant weight growth (SDS)	n.s.	n.s.	=	↑	↓	=	↓	n.s.	=
Adiposity									
BMI (SDS)	↑	=	n.s.	n.s.	n.s.	n.s.	n.s.	↑	=
Fat mass index	=	=	n.s.	n.s.	n.s.	n.s.	n.s.	=	=
Android/gynoid fat mass ratio	=	↓	n.s.	n.s.	n.s.	n.s.	n.s.	=	=
Subcutaneous area (SDS)	=	=	n.s.	n.s.	n.s.	n.s.	n.s.	=	=
Pre-peritoneal area	↓	↑	n.s.	n.s.	n.s.	n.s.	n.s.	=	=

Lung function was measured at age 6 (Respiratory resistance (Rint), Fractional exhaled Nitric Oxide (FeNO)) and 10 years (lung function measures by spirometry), and symptoms and diseases until 6 years (wheezing) and at 6 or 10 years (ever/current physician diagnosed asthma). FEV_1: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; FEF_{25-75}: Forced Expiratory Flow between 25 and 75% of FVC; FEF_{75}: Forced Expiratory Flow at 75% of FVC. Arrows represent directions of associations per increase of early growth measure. Up going arrows represent a positive association, down going arrows represent a negative association. The "=" sign represents that no association was observed. "n.s.": not studied in this thesis.

Environmental exposures

Various common environmental exposures have been linked to respiratory health.[11, 18] We focused on tobacco smoke exposure, which is the most important adverse exposure for the development of chronic obstructive lung diseases. Furthermore, our interested was on the use of folic acid supplements during pregnancy, which is highly recommended by the World Health Organization to prevent congenital anomalies. Intake of high dosages of folic acid supplements have been associated with increased risks of allergic airway diseases in animals, but results in human studies are scarce and conflicting.[19-22] Last, we focused on the role of breastfeeding on respiratory health. Breastfeeding dura-

tion and exclusiveness is known to be associated with a lower risk of asthma symptoms in early childhood, but its effect on respiratory health at older ages is unclear.

Maternal smoking during pregnancy has been associated with an up to 1.7 times increased risk of wheezing until age 2 years.[23] We observed that continued maternal smoking during pregnancy was associated with increased risks of wheezing and asthma up to age 6 years, but not with Rint or FeNO.[24] Gestational age and birth weight did not explain these associations (Table 5.2). In mice, nicotine exposure during pregnancy reduces forced expiratory flows, and increases airway reactivity in offspring, which suggests a direct intrauterine effect of tobacco smoke on respiratory morbidity in childhood.[25] Tobacco smoke exposure might also affect lung development through DNA-methylation. Maternal tobacco smoking during pregnancy has been associated with differential DNA-methylation of >6,000 loci across the newborn genome.[26] Further research is needed to identify the pathophysiologic mechanisms of intrauterine tobacco smoke exposure on childhood respiratory health. Additionally, prevention strategies such as smoke legislation programs should be further implemented.[27]

Maternal folic acid supplement use during pregnancy is strongly recommended to prevent congenital anomalies [28], while high dosages of folic acid supplement use are suggested to have adverse effects on respiratory health. We observed that maternal folic acid supplement use during pregnancy was associated with lower lung function measures in children, but only among mothers carrying*MTHFR-C677T* variants (Table 5.2). A higher vitamin B_{12} level at birth was associated with a lower FEV_1 and FVC, but only among children carrying *MTHFR-C677T* wildtype. *MTHFR* produces an enzyme that affects the one-carbon metabolism, a process that converts the amino acid homocysteine to methionine, which is an important methyl donor.[29, 30] The variant *C677T* is known to affect the activity of the *MTHFR* enzyme, leading to lower circulating folate and higher homocysteine concentrations.[31] Also, folate and vitamin B_{12} are important cofactors in the one-carbon metabolism.[32] Elevated folic acid exposure during pregnancy has been linked to cellular modifications associated with asthma and allergic diseases.[33] In animal models, maternal diet enriched with folic acid and vitamin B_{12} was associated with epigenetic changes in offspring, including immune functioning.[33, 34] Further research on the interactions between folic acid and genetic predisposition is needed to provide insight into mechanisms leading to chronic obstructive respiratory diseases.[35] The results of the first study examining associations of maternal plasma folate levels during pregnancy and epigenetic changes in neonatal cord blood are presented below. Because of the importance of folic acid supplement use during pregnancy to prevent congenital anomalies, the potential adverse effects of folic acid supplement use on childhood respiratory health need to be carefully evaluated before any changes in the current recommendations are considered.

Table 5.2. Overview of the results of studies presented in this thesis on environmental exposures in early life and childhood lung function and asthma.

	Lung function							Symptoms and diseases	
	Rint	FeNO	FEV_1	FVC	FEV_1/FVC	FEF_{25-75}	FEF_{75}	Wheezing	Asthma
Tobacco smoke exposure	=	=	n.s.	n.s.	n.s.	n.s.	n.s.	↑	↑
Folic acid supplement use during pregnancy									
Maternal *MTHFR-C677T* wildtype	n.s.	n.s.	↑	↑	=	↑	=	n.s.	=
Maternal *MTHFR-C677T* variants	n.s.	n.s.	=	=	↓	↓	=	n.s.	=
Vitamin B_{12} levels at birth									
Child *MTHFR-C677T* wildtype	n.s.	n.s.	↓	↓	=	=	=	n.s.	=
Child *MTHFR-C677T* variants	n.s.	n.s.	=	=	=	=	=	n.s.	=
Folate, vitamin B_{12} and homocysteine levels in early pregnancy	n.s.	n.s.	=	=	=	=	=	n.s.	=
Folate and homocysteine levels at birth	n.s.	n.s.	=	=	=	=	=	n.s.	=
Breastfeeding									
Never	=	↓	n.s.	n.s.	n.s.	n.s.	n.s.	↑	=
Shorter duration	=	=	n.s.	n.s.	n.s.	n.s.	n.s.	↑	↑
Less exclusive	=	=	n.s.	n.s.	n.s.	n.s.	n.s.	↑	=

Lung function was measured at age 6 (Respiratory resistance (Rint), Fractional exhaled Nitric Oxide (FeNO)) and 10 years (lung function measures by spirometry), and symptoms and diseases until 6 years (wheezing) and at 6 or 10 years (ever/current physician diagnosed asthma). FEV_1: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; FEF_{25-75}: Forced Expiratory Flow between 25 and 75% of FVC; FEF_{75}: Forced Expiratory Flow at 75% of FVC. Arrows represent directions of associations for each exposure. Up going arrows represent a positive association, down going arrows represent a negative association. The "=" sign represents that no association was observed. "n.s.": not studied in this thesis.

Prolonged and exclusive breastfeeding has been associated with a decreased risk of asthma symptoms up to age 2 years.[36] We observed that children who were never breastfed had a lower FeNO and an increased risk of wheezing until age 6 years (Table 5.2). Among breastfed children, a shorter duration and less exclusive breastfeeding were associated with an increased risk of wheezing in early life, and a breastfeeding duration less than 2 months was associated with a 2.2 times increased risk of asthma. The associations partly attenuated when lower respiratory tract infections in early life were taken into account. This suggests that the associations between breastfeeding and childhood asthma are partly explained by the protective effect of breastfeeding on lower respira-

tory tract infections. Breastfeeding might contain secretory factors in breastmilk that stimulate the developing immune system and protect for childhood asthma.[37-39] Also, a longer duration of breastfeeding has been associated with a lower methylation status of *Leptin*, a gene implicated in appetite regulation and fat metabolism.[40] Interestingly, a shorter duration of breastfeeding has been associated with higher central fat mass in early childhood.[41] Therefore, a shorter duration or less exclusive breastfeeding might lead to genome-wide or *Leptin*-specific epigenetic changes, resulting in specific adverse adipose tissue distributions, which subsequently affect childhood lung function and asthma.

In summary, we identified adverse effects of maternal smoking during pregnancy, of maternal folic acid supplements use during pregnancy when carrying *MTHFR-C677T* variants, and of shorter and less exclusive breastfeeding on childhood lung function, wheezing or asthma. Further research is needed to identify underlying mechanisms, including epigenetics changes.

Genetic and epigenetic studies

Genome-wide association (GWA) studies enable to examine the associations of millions of common genetic variants, known as Single Nucleotide Polymorphisms (SNPs). With recent advances in genetic technologies, currently the associations of >10 million SNPs with any phenotype can be explored. Only a limited number of loci associated with asthma have been verified in GWA studies, any many variants have not been replicated.[42-47]

We increased the understanding of the genetic background of early childhood asthma with severe exacerbations by conducting a GWA study of this specific asthma phenotype.[48] We identified 5 loci, 4 of which were previously associated with asthma. We also identified a new susceptibility gene, *CDHR3*, which encodes the gene cadherin-related family member 3 (Table 5.3). The detailed biological function of *CDHR3* is unknown. Variants in *CDHR3* may increase the risk of severe asthma exacerbations by altering the structural integrity of airway epithelium, thus promoting infection by respiratory microbes. The pathophysiologic mechanisms of variants in *CDHR3* in childhood asthma need to be further explored, which could potentially lead to prevention strategies and targeted therapies of the malfunctioning protein.

Three meta-analyses of GWA studies from population-based studies previously identified 35 SNPs associated with lung function in European adults.[49-52] We now showed that 2 genetic risk scores composed of SNPs associated with adult lung function were also associated with childhood lung function, but not with childhood asthma (Table 5.3). Exposure to maternal smoking and gestational age at birth modified some of the observed associations. These results suggest that SNPs associated with adult lung function already affect lung function in early life. Identification of abnormal lung function development

in early childhood life may help identify individuals at risk for chronic airflow obstruction in adulthood.[53, 54]

Genetics are unlikely to explain the quickly altering prevalence of asthma in the past decades, because any mutation would require multiple generations to occur on population level.[55] Epigenetic changes are influenced by environmental exposures and could exert population effects much more rapidly than genetic mutations.[56] DNA-methylation is currently the best understood epigenetic mechanism, and techniques have been developed to assess epigenome-wide DNA-methylation patterns in large population-based studies.

DNA-methylation affects fetal development through effects on gene transcription and expression.[57] The methyl groups required for DNA-methylation are mostly provided by the one-carbon metabolism.[32, 58]

Table 5.3. Overview of the results of studies presented in this thesis on genetic susceptibility and epigenetic factors associated with childhood lung function and asthma, and COPD in adults.

	Lung function					**Childhood diseases**		**Adult diseases**
	FEV_1	**FVC**	**FEV_1/FVC**	**FEF_{25-75}**	**FEF_{75}**	**Current asthma**	**Asthma exacerbations**	**COPD**
Genome-wide association study								
CDHR3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	+	n.s.
Genetic risk scores								
Based on SNPs associated with adult FEV_1	↓	=	↓	=	↓	=	n.s.	n.s.
Based on SNPs associated with adult FEV_1/FVC	↓	=	↓	=	↓	=	n.s.	n.s.
Differentially methylated regions in cord blood*	+	n.s.	+	n.s.	+	+	n.s.	+

Lung function was measured at age 10 years (lung function measures by spirometry), and symptoms and diseases until 6 years (asthma exacerbations), at 10 years (current physician diagnosed asthma) and in adulthood (COPD; mean age 65 years). FEV_1: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; FEF_{25-75}: Forced Expiratory Flow between 25 and 75% of FVC; FEF_{75}: Forced Expiratory Flow at 75% of FVC. Arrows represent directions of associations for each genetic or epigenetic variant. Up going arrows represent a positive association, down going arrows represent a negative association. The "+" sign represents that significant associations were observed, in which the direction of the association is depending on the direction of the genetic variant or methylation of the specific differentially methylated region. The "=" sign represents that no association was observed. "n.s.": not studied in this thesis. *Of all 59 identified differentially methylated regions related with childhood lung function, 18 (31%) were associated with childhood asthma, and 9 (15%) were associated with COPD.

Folate is an essential cofactor in this biological pathway. In this thesis, we presented a meta-analysis in which we observed that maternal plasma folate levels during pregnancy were associated with differential methylation of 443 CpGs located near 320 genes of the child at birth (Table 5.3). Most genes were not known for folate biology. Some were related to birth defects other than neural tube defects, neurological functions or varied aspects of embryonic development. These findings provide additional evidence that maternal folate impacts the developing epigenome of the child, which subsequently could affect the occurrence of chronic non-communicable diseases in later life.

We additionally reported the identification of 22, 15 and 22 differentially methylated regions of the child at birth to be associated with childhood FEV_1, FEV_1/FVC and FEF_{75}, respectively. Differentially methylated regions are genomic regions containing multiple correlated CpGs within a close distance, usually defined as 500 to 1000 base-pairs. Multiple of the identified differentially methylated regions were also associated with childhood asthma, adolescent and adult lung function, and adult COPD. Further analyses showed that the identified differentially methylated regions were related with differential expression of >100 genes, of which many have previously been related to lung function and chronic obstructive respiratory diseases. Also, genes related to the identified differentially methylated regions were expressed in adult lung tissue, and associated with respiratory developmental or pathogenic pathways. This supports the hypothesis that adverse exposures in fetal life impact DNA-methylation at birth, gene expression and subsequent respiratory development, predisposing individuals for obstructive airway diseases. Further studies on the identified differentially methylated regions and annotated genes might benefit strategies in early life to improve lung function and respiratory health in later life.

METHODOLOGICAL CONSIDERATIONS

Most studies presented in this thesis were performed within the Generation R Study, a prospective population-based cohort study with follow-up from fetal life onwards located in Rotterdam, The Netherlands. For meta-analyses on the associations of early growth characteristics with childhood lung function and asthma, and a genome wide association study on childhood asthma with severe exacerbations we used individual participant or effect estimate data from 24 and 23, respectively, European birth cohorts. For epigenome-wide studies on maternal folate levels during pregnancy and lung function and respiratory health across the life course, we used effect estimate data from 2 European birth cohorts, and 4 European and 2 American birth cohorts, respectively.

Specific methodological considerations of the presented studies have been discussed in the studies presented in this thesis. In the following paragraphs, some general meth-

odological issues with respect to the internal and external validity, and genetic and epigenetic methodological issues of epidemiological studies are discussed.

Selection bias

Selection bias could occur if the association between the exposure and outcome of interest is different in study participants and those who did not participate in the study, but were eligible for the study. The results obtained are thereby not representative for the population intended to be analyzed. Of all children eligible at birth, the overall response to participate in the Generation R Study was 61%.[59] This non-response at baseline is not likely at random. Non-participating parents and children more frequently were of non-Caucasian origin, had a lower socio-economic status, and more adverse birth outcomes, such as low birth weight, compared to mothers and children who did participate in the study, suggesting a selection toward a relative more healthy study population.[60]

Another source of selection bias is selective loss to follow-up, which occurs when the association between the exposure and outcome of interest is different between those participating in the studies described in this thesis and those lost to follow-up. Of all children (n = 9,901) originally included in the Generation R study, 85.2% (n = 8,305) participated in the follow-up studies at age 6 years, and 83.1% (n = 6,899) of them had information on airway resistance, airway inflammation, wheezing or asthma.[59] At age 10 years, 74.7% of all children (n = 7,393) originally participating in Generation R still participated, and 77.4% (n = 5,721) of them had information on lung function or physician-diagnosed asthma. Non-response and loss to follow-up may reduce statistical power, due to lower prevalence rates of exposures and outcomes. We performed non-response analyses to determine differences between participants and non-participating families. Overall, mothers from children who did not answer questionnaires related to r specific research questions or did not visit the research center for lung function measures of their child were more often of non-Caucasian origin, more frequently lower educated and smoked more during pregnancy, and their children were more often born with a low birth weight. Selection towards a relatively healthy population may have biased the observed effect estimates, despite its difficulty to quantify. Therefore, we applied multiple imputation, which limits the risk of selection bias due to missing values in covariates.[61, 62]

Information bias

Information bias is a systematic error due to misclassification of participant data. Misclassification is differential (non-random) when the misclassification is different for those with and without the exposure or outcome of interest. Misclassification is non-differential (random) when it is unrelated to the occurrence or the presence of the exposure or outcome of the study. Differential misclassification may lead to either

over- or underestimated effect estimates, whereas non-differential misclassification usually results in an underestimation of the true effect estimates. Data used in the studies presented in this thesis, including fetal and childhood length and weight at different time points, tobacco smoke exposure during pregnancy, maternal folic acid supplement use and breastfeeding, and genetic and epigenetic samples, were collected before assessment of the outcomes. All parents and researchers involved in data collection were unaware of specific research questions, which limits differential misclassification of the exposures. Lifestyle factors with potential adverse health effects, such as maternal smoking during pregnancy, are known to be underreported in epidemiologic studies. Differential misclassification may have therefore occur, because the difference in the risk of the outcome between those who smoke and those who do not smoke becomes smaller due to underreporting of mainly those who smoke, leading to an underestimation of effect estimates. Similarly, overreporting of lifestyle factors with potential beneficial effects could have influenced the observed associations. Mothers are likely to have overreported the use of folic acid supplements during pregnancy, and might have exaggerated the duration of breastfeeding. This would again lead to differential misclassification of the exposure, resulting in underestimations of effect sizes.

Lung function and asthma were the two main outcomes examined in this thesis. Lung function was measured by Rint, FeNO or spirometry. The quality and reliability of lung function measures mainly depend on optimal performance of the study participant.[63] It seems unlikely that lung function measurements were influenced by differential misclassification, because the researchers involved in data collection were unaware of the lung function or asthma-status of the participant. Asthma is a difficult diagnosis in young children. Both wheezing patterns and asthma were self-reported outcome measures. Underreporting or over-reporting might have occurred despite the use of validated questionnaires based on international guidelines [64], and this might have led to misclassification of respiratory disease diagnosis resulting in either overestimations or underestimations of the true associations.

Confounding

A confounding factor is a factor associated with both the exposure and the outcome, but not located in the causal pathway. If not taken into account, confounding may lead to biased effect estimates. We took well-known confounding variables as previously reported by literature into account, and examined the confounding effect of potential variables in our statistical models. Although we adjusted for many potential confounders in all studies presented in this thesis, we cannot exclude that results were affected by unmeasured variables or by variables not known to be confounders in specific analyses, such as specific viral or bacterial respiratory infections and antibiotic use in early life, the

pulmonary and gastro-intestinal microbiome, and interactions between the environment and genetic susceptibility.[65-68]

External validity

External validity is the extent to which results of a study can be applied to other populations. The Generation R study is based on the general population in Rotterdam, the Netherlands. The largest ethnic groups are from Dutch, Surinamese, Turkish and Moroccan origins, and is comparable to the general ethnic distribution of the population in the Rotterdam region.[60] Both household income and highest followed educational level in parents participating in the study suggest a selection towards a population with a higher socioeconomic status than the average in the whole study area. This pattern was similar in the follow-up assessments until age 10 years, and in line with other large scale prospective cohort studies. Although there is a selection towards a population with a higher socio-economic status in the general Generation R cohort, the population that was under study for the projects presented in this thesis contained representative ethnic and socio-economic subgroups of the general population. The results of this thesis could therefore presumably be applied to western populations with mixed ethnicities. The meta-analysis examining the associations of early growth characteristics and childhood lung function and asthma was based on individual participant data of 24 birth cohort studies from countries throughout Europe. Although countries from Eastern Europe were somewhat underrepresented, we assume that the overall study population was a good representation of the average European population. The genetic and epigenetic studies were performed in populations of mainly Caucasian origin. Genetic variants might have differential effects across different ethnic populations.[69] Replication of the associations of the identified genetic variants in non-Caucasian populations is needed before conclusions can be drawn on the global generalizability of these results.

Methodological issues in genetic and epigenetic studies

In the past decade, GWA studies have been the main approach to identify genetic variants associated with common non-communicable diseases.[70] Despite the potential of GWA-studies to examine the associations of a large number (currently >10 million) of genetic variants in a hypothesis-free manner, some methodological issues need to be considered. GWA studies will primarily detect small effect sizes for outcomes in relation to common SNPs, because the currently available genotyping platforms and imputation technologies mainly include SNPs with ≥1% minor allele frequencies. Current GWA studies are underpowered to detect associations of SNPs with low (<1%) minor allele frequencies to be associated with an outcome. We observed that the risk alleles associated with lung function, asthma and COPD by GWA studies accounted for small proportions of the variance explained by genetic susceptibility. Combined non-identified rare

variants might potentially have larger effects than common variants on disease risk.[71] Furthermore, outcome assessment might differ between different study populations in GWA meta-analyses. This could potentially lead to non-differential (random) misclassification, because the outcome assessment is unlikely to be related to the genotype of the examined individuals within participating studies. This non-differential misclassification may reduce the statistical power to detect associations of SNPs with the phenotype of interest. Also, population stratification may arise when genotype and outcome distribution is different between different participating study populations, although the selection of children for genetic studies in population-based cohorts usually is unrelated to any outcome. Population stratification may lead to false positive results.[72] To minimize this, we applied genomic control by using principal components in all genetic association studies presented in this thesis. Last, in GWA studies associations of a large number of SNPs with an outcome are tested, which might lead to false-positive findings when conventional statistical significance thresholds are used. Therefore, the statistical significance threshold of GWA studies has been set to $5x10^{-8}$, reflecting a Bonferroni correction of testing one million variants (0.05/1,000,000). Still, false-positive findings are likely to occur due to insufficient statistical power, because common genetic variants are likely to have small effects. Combining data from multiple study cohorts has increased power for identification of common genetic variants.

Epigenome-wide association (EWA) studies have the potential to examine associations of a large number (~485.000) of CpG-sites across the genome with exposures and outcomes. However, some limitations need to be addressed. The Infinium HumanMethylation450 BeadChip array currently provides the best coverage of the human genome and covers 96% of all known CpG-islands, but only about 1.6% of all CpGs located in the human genome.[73] Furthermore, the Illumina BeadChip technology relies on hybridization of genomic fragments to probes on the chip. Certain genomic factors, such as SNPs, may compromise the ability to measure DNA-methylation.[74] It is not known to what extend these genomic factors do impact association analyses of CpGs with any outcome. However, stringent exclusion of probes potentially containing these genomic factors would results in many false-negative results. Therefore, the exclusion of probes with potential irrelevant genomic variants should be applied as sensitivity analysis in any EWA study, and further research is needed to make any technical recommendations. Furthermore, knowledge about the methylation status of an individual CpG is usually of limited value unless it is contextualized by the methylation status of neighboring CpGs nearby. The use of differentially methylated regions increases power to identify regions of interest and is conceptually consistent with what is known about human DNA methylation patterns.[75-77] EWA studies are subject to confounding, including environmental, genetic and technical factors, which should be accounted for.[73, 78] Blood is an easy accessible tissue in large cohort studies, however, in epigenetic studies concerns

arise regarding confounding by differential cell types and the biological relevance of the tissues assessed. DNA-methylation differences between blood samples are strongly influenced by cellular heterogeneity.[79] Consequently, EWA studies need to adjust for cell type composition. The most common used reference dataset to estimate cell type composition is based on blood samples from adults and is not likely representative of the cell type composition in neonatal cord blood.[80] Recently, two new reference sets for cell type adjustment in cord blood have been published and are currently being validated. Future studies are needed to shed light on the differences between the available reference panels.[81, 82] Blood DNA-methylation does not necessarily reflect lung epithelial DNA-methylation but this is difficult to examine in living children. Asthma and COPD have systemic manifestations characterized by increased inflammatory blood markers, and therefore blood might be an appropriate proxy for the EWA studies presented in this thesis.[83, 84] Furthermore, we used publicly available databases to examine expression of the genes identified by cord blood DNA-methylation in lung tissue. Last, multiple testing correction in EWA studies is required similar to GWA studies to prevent false positive findings.

CAUSALITY

We assessed associations but not causal effects of early growth, environmental exposures, genetic susceptibility, and epigenetic mechanisms on childhood lung function and asthma due to the observational design of all studies reported in this thesis. The Bradford Hill criteria define specific criteria to determine the causality of observed associations between exposures and diseases.[85] These criteria include the strength, consistency, specificity and temporality of the observed associations, and causality is further strengthened by the observation of a biological gradient, a biologically plausibility, coherence with current knowledge of the biology, experimental evidence and analogy with comparable exposures. Taking the Bradford Hill criteria into account, we can conclude that the observed associations were in line with results reported by previous studies. Temporality indicates that the effect has to occur after the cause, which we observed in our studies assessing the associations of growth in early life, tobacco smoke exposure and folic acid supplement use during pregnancy, and breastfeeding in early life, with respiratory outcomes at ages 6 and 10 years. Additionally, we observed dose-response effects for tobacco smoke exposure during pregnancy and breastfeeding duration with respiratory outcomes. For all observed associations of early growth and environmental exposures, plausible underlying mechanisms with coherence in animal studies are available.[85] We could not fulfill the experimental criterion as defined by the Bradford Hill criteria, such as the use of animal models to prove associations of early

growth and environmental factors with childhood lung function and asthma. Studies that assessed analogue factors for the environmental factors assessed in this thesis provide further evidence for the observed associations. For example, low physical activity and higher waist circumference, which could be considered analogues for high BMI and adverse body fat distribution, have been associated with asthma in children and adolescents.[86, 87]

The causality of early growth and environmental exposures on lower lung function and increased risk of asthma can theoretically be proven using randomized controlled trials. However, randomized exposure to externally regulated growth patterns or environmental chemicals with proven adverse or beneficial effects on health, such as respectively maternal smoking and folic acid supplement use during pregnancy and breastfeeding, is unethical. Instead, experimental interventions promoting smoke cessation, the intake of folic acid and breastfeeding coherent to the current guidelines, might provide additional evidence for the causality of these exposures on childhood respiratory diseases. This is illustrated by the PROBIT-trial, in which children of mothers who were intensively promoted to breastfeed according to the newest WHO guidelines had decreased risks of gastrointestinal tract infections and atopic eczema in the first year of life, compared to children of mothers who were not promoted and continued usual infant feeding practices and policies.[88] To further explore causal effects of early growth and environmental factors on lung function and asthma, results from further studies according to the Bradford Hill criteria provide the highest likelihood of causality of the identified factors.[85]

GWA studies most often do not directly identify the causal genetic variant. GWA studies rely on linkage disequilibrium between genotyped SNPs and causal variants, which are most often not genotyped. If the identified variant has an effect on gene expression in selected cell types, this could support the finding that the identified variant is within a causal gene. As presented in this thesis, we assessed the functional consequences of the *CDHR3*-variant in T-cells, and we observed that the wildtype protein was expressed at very low levels, whereas the mutated protein showed an increase in cell surface expression. Furthermore, we observed a high expression of *CDHR3* in adult bronchial epithelium and in fetal lung tissue. Further evidence for causality could be derived from animal knockout models, in which the occurrence of the disease after removing the suspect gene suggests a causal effect. Last, Mendelian randomization offers a unique method to assess causality in observational studies.[89] Mendelian randomization is based on the principle that if a genetic variant is associated with an environmental exposure, and the environmental exposure itself is associated with the risk of a disease, then that genetic variant should also be associated with the disease.[89] A study that applied Mendelian randomization using a genetic risk score based on 32 SNPs associated with BMI and adiposity suggested a causal association between BMI and asthma up to age

7 years.[90] This is consistent with the observed associations of higher infant weight gain and obesity with childhood asthma, as presented in this thesis.

In contrast to GWA studies, EWA studies are subject to genetic, environmental and technical confounding. DNA-methylation is a dynamic biological process, with altering patterns across the human life course.[91] Differential methylation of a differentially methylated region might cause the disease, but development of the disease might also affect methylation at the same differentially methylated region. Therefore, interpretation of the directionality and causality of effects from an observational EWA studies is challenging. In the studies presented in this thesis, we observed that maternal folic acid levels in early pregnancy were associated with newborn DNA-methylation in cord blood. We also reported associations of newborn DNA-methylation in cord blood with lung function and respiratory outcomes across the life course. Due to the longitudinal aspect between the assessment of the exposures and outcomes in both studies, it is unlikely that reverse causation could have affect the observed results. Additionally to multiple studies with single observations, longitudinal studies with epigenome-wide DNA-methylation, confounding variables and outcomes measured at multiple time points are needed.[92-94] Longitudinal studies enable the identification of the direction and possibly the causality of EWA findings. Also, twin studies provide an additional and unique opportunity to eliminate confounding in EWA studies, because twins are matched controls for nearly all genetic variants and many environmental exposures. Last, epigenetic Mendelian randomization could be applied.[95] In this method, first associations of a genetic variant with an environmental exposure, which is known to associated with differential DNA-methylation, are assessed. Second, associations of another genetic variant with DNA-methylation, which is known to be associated with an outcome, are assessed. With this two-step method it could be examined whether DNA-methylation is within the causal pathway between the exposure and the disease.

CLINICAL IMPLICATIONS

The research we presented in this thesis is mainly based on observational collected data. As discussed previously, this limits the identification of causality and application in clinical situations. Still, our studies do have some important clinical implications:

– Our observations that children born with a younger gestational age, lower birth weight, and restricted or accelerated growth patterns in early life have lower lung function measures and risks of asthma-related symptoms in childhood could be used in clinical models to predict the probability of the development of childhood asthma [96]. An early risk prediction for the development of lower lung function and

asthma in childhood will support early identification and prevention of respiratory health problems in later life, such as COPD.
- We observed that specific body fat mass distributions were associated with childhood lung function and asthma. Public intervention programs should be further developed, aiming to reduce specific obesity phenotypes in childhood.[97]
- Our findings on the associations between tobacco smoke exposure during pregnancy with lung function and asthma in childhood provide additional support for nation-wide smoke legislation programs, which have shown strong beneficial effects on a population level.[27]
- Our study on the protective effect of breastfeeding on childhood asthma supports the current WHO-guidelines for prolonged and exclusive breastfeeding.[98]
- Our identification of *CDHR3* as a new asthma-susceptibility gene has increased understanding of severe childhood asthma, and has created a new potential target for therapy. Research focusing on understanding the role of *CDHR3* variants in the development of asthma and severe exacerbations is currently ongoing.

The other main results presented in this thesis require replication, and future research needs to shed light on underlying pathophysiological mechanisms.

FUTURE PERSPECTIVES

As discussed before, randomized controlled trials to assess the causal effects of the risk factors studied in this thesis have ethical limitations. Alternative designs, such as the PROBIT-trial described above, might provide additional evidence for causality of the observed associations.[88] We observed that maternal folic acid supplement use was associated with lower childhood lung function when mothers were carrier of *MTHFR-C677T* variants. Potential adverse effects of folate supplement use needs to be carefully evaluated before clinical implications can be determined and any changes in the current guidelines are considered. Our results on the associations between breastfeeding and childhood asthma suggest a mediating effect by lower respiratory tract infections. Additionally, *CDHR3* is a plausible candidate gene for asthma because of its high level of expression in the airway epithelium and the known role of cadherins in cell adhesion and interaction. There is an increasing focus on the role of the airway epithelium in asthma. Structural or functional abnormalities in the epithelium may increase susceptibility to environmental stimuli, such as respiratory viruses, by exaggerating immune responses and structural changes in underlying tissues.[99] Therefore, further insight on potential pathophysiological mechanisms of respiratory tract infections on long term lung function and asthma is needed.

Current results of GWA studies explain only a minor part of the heritability of chronic obstructive respiratory diseases.[50, 51, 100, 101] The effects of rare variants have been proposed as an explanation for the "missing genetic variance". The identification of functional rare variants is best carried out by DNA sequencing, since most rare variants will either not have been seen before or have such low allele frequencies that they are not included in genotyping arrays.[102] Current advances in inexpensive whole-genome and whole-exome sequencing and rare variants genotyping arrays will enable studies for the effects of rare variants. In adults, the environmental influence of e.g. tobacco smoke exposure on lung function may be more important than genetic factors or interact with genetic factors. Therefore, some phenotypes are particularly interesting to study in children since environmental exposures have been present for a relatively short period. Although the number of subjects available for GWA studies is increasing and genotyping techniques advance quickly, other options to identify common genetic variants should be considered. Within one locus, multiple common genetic variants are independently associated with the phenotype. These secondary signals can be found by conditioning on the identified variants. Also, as shown in this thesis, a more detailed definition of the asthmatic phenotype will benefit identification of genetic variants.

Genetics in general are unlikely to explain the quickly altering prevalence of asthma and COPD over the past decades[55, 103, 104] Therefore, it is likely that changes in environmental exposures in the last decades interact with susceptible genes, thereby affecting development and health.[56] The identification of environmental exposures that lead to specific changes in gene regulation has just recently been started. Functional studies including identification of functional elements in DNA using gene expression analysis, protein-protein interaction or RNA-methods should use the candidate genes identified by GWA- and EWA-studies, and aim to identify the underlying biological mechanisms. We need to determine whether epigenetics are an important underlying mechanism of the associations of environmental exposures and genetic susceptibility with respiratory health, and whether DNA-methylation acts differently on gene expression at various stages in human development. Future birth cohort studies should track DNA-methylation over time, and ideally over multiple generations. This will provide critical information about developmental phases in life which are most suitable to prevent adverse DNA-methylation patterns, or enables interventions to normalize DNA-methylation.[105] Given that DNA-methylation patterns are heritable, future research will provide a unique opportunity to not only predict and treat chronic obstructive respiratory diseases in the current generation, but also prevent it for generations to come.

CONCLUSION

We identified fetal and infant growth patterns, adverse environmental exposures, and genetic and epigenetic factors that are associated with changes in lung function and risk of chronic obstructive respiratory diseases across the life course. Functional studies are needed to evaluate the associations of the identified risk factors on lung function and asthma in later life, and potential underlying epigenetic mechanisms need to be further explored in detail. Ultimately, by identification of early life exposures, genetic susceptibility and their interactions, therapeutic and preventive strategies focused on pregnant women and young children could be developed to improve lung development in early life, which would benefit respiratory health across the life course.

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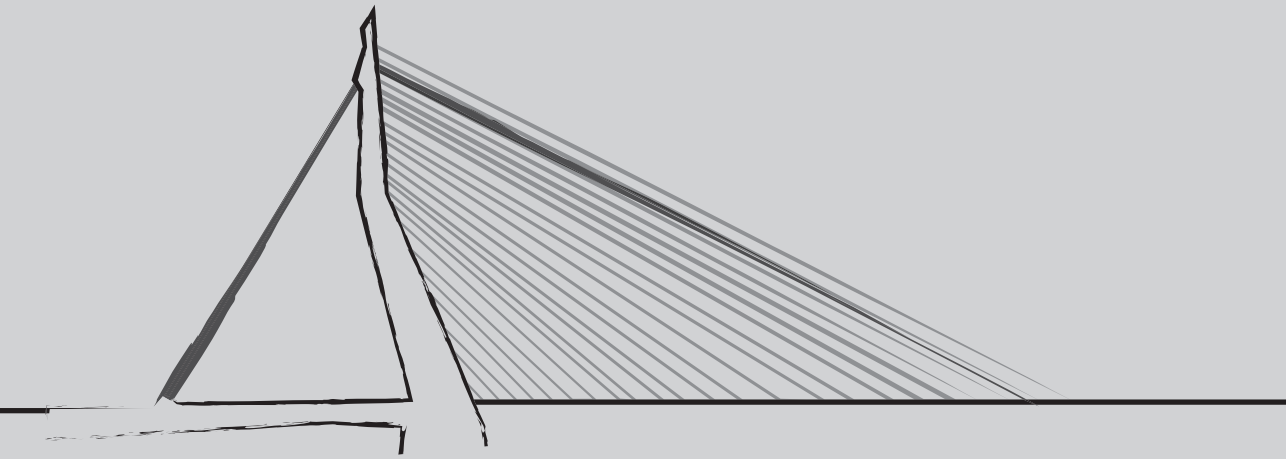
Chapter 6

Summary

Samenvatting



SUMMARY

In this thesis, we examined the hypothesis that early growth and adverse environmental exposures in fetal life and infancy, in combination with genetic susceptibility, lead to structural and functional adaptations in early lung development, with subsequently lower lung function and higher risk of chronic obstructive respiratory diseases in later life. Furthermore, we explored epigenetic mechanisms as a potential pathway explaining environmental and genetic factors that influence the development of lower lung function and risk of respiratory diseases. By identification of early life growth and environmental exposures, genetic variants, and DNA-methylation as underlying mechanism, we improve the understanding of the origins of chronic obstructive respiratory diseases in childhood and adulthood. Furthermore, we might be able to develop new preventive strategies and therapeutic interventions for pregnant women and young children, aiming at reducing the burden of later life chronic obstructive respiratory diseases.

In **Chapter 1**, the background and rationale of the studies presented in this thesis are given. We also provide the aims of the performed studies and describe the outline of this thesis.

Chapter 2 describes the associations of early growth characteristics with childhood lung function and asthma. In *Chapter 2.1*, we report that a younger gestational age, smaller size for gestational age and greater infant weight gain across the full ranges were associated with lower childhood lung function. Mediation analyses suggested that lower lung function explains 7% to 45% of the associations between early growth characteristics and childhood asthma. In *Chapter 2.2*, we report that smaller weight growth in late fetal life and greater weight growth in early infancy was associated with lower lung function and an up to 1.3-fold increased risk of childhood asthma. The results of *Chapters 2.1* and *2.2* support the hypothesis that changes in early growth characteristics lead to developmental adaptations of the lungs and airways, resulting in relatively small airways, and potential risk of childhood asthma. In *Chapter 2.3*, we show that a higher BMI was associated with higher respiratory resistance and increased risk of wheezing in school-age children. We also report that higher fat mass index was associated with higher respiratory resistance, and higher android/gynoid fat mass ratio and pre-peritoneal fat mass, a measure of visceral abdominal fat, with higher Fractional exhaled Nitric Oxide. These findings suggests that detailed body fat distribution measures might be better measures than BMI only to understand the obesity-asthma paradigm.

In conclusion, preterm birth, low birth weight, infant growth, obesity, and specific body fat distribution measures are associated with changes in lung function and risk of asthma in childhood, and could be used as potential targets for early prevention.

Chapter 3 describes the associations of adverse environmental exposures with childhood lung function and asthma. In *Chapter 3.1*, we report that continued maternal

smoking during pregnancy was associated with increased risks of early and persistent wheezing and asthma, but not with Rint or FeNO in children aged 6 years. Gestational age and birth weight did not explain these associations. In *Chapter 3.2*, we show that folic acid supplement use during pregnancy was associated with lower lung function measures, but only among mothers carrying variants of the *MTHFR-C677T* gene. A higher vitamin B_{12} level at birth was associated with a lower FEV_1 and FVC among children carrying *MTHFR-C677T* wildtype. In *Chapter 3.3*, we show that a shorter duration and less exclusive breastfeeding was associated with an increased risk of wheezing in early life, and that a breastfeeding duration less than 2 months was associated with a 2.2-fold increased risk of asthma up to age 6 years.

In conclusion, maternal smoking during pregnancy, the use of folic acid supplements in pregnancy in mothers carrying the *MTHFR-C677T* variants, and breastfeeding are suggested to affect lung function and the risk of asthma in childhood.

Chapter 4 describes the identification of genetic loci related to childhood lung function and asthma, and of epigenetic loci related to an adverse environmental exposures and chronic obstructive respiratory disease outcomes across the life course. In *Chapter 4.1*, we report the identification a new asthma-susceptibility gene, *CDHR3*. Variants in *CDHR3* may increase the risk of severe asthma exacerbations by altering the integrity of airway epithelium, thus promoting entry and replication of respiratory viruses. In *Chapter 4.2*, we show that genetic variants associated with adult lung function were also associated with childhood lung function, but not with childhood asthma. This suggests that genetic variants associated with adult lung function already affect lung function in early life. In *Chapter 4.3*, results of our meta-analysis showed an association between maternal plasma folate levels during pregnancy with differential methylation of 443 CpGs located near 320 genes across the human genome. These findings provide new insights in the impact of maternal folate levels during pregnancy on the developing epigenome of the newborn. We report the identification of 59 differentially methylated regions (DMRs) associated with childhood lung function in *Chapter 4.4*. Multiple of these DMRs were also associated with childhood asthma, adolescent and adult lung function, and COPD. This study supports the hypothesis that DNA-methylation at birth has impact on gene expression and subsequent respiratory development, predisposing individuals for obstructive airway diseases.

In conclusion, genetic variants affect lung function and respiratory health from early life onwards, maternal folate levels in pregnancy have a genome-wide impact on neonatal DNA-methylation, and neonatal DNA-methylation has impact on lung function and respiratory health across the life course.

Finally, in **Chapter 5** we provide a general overview and interpretation of the results of the studies presented in this thesis. Furthermore, methodological issues of the studies, causality of the observed associations, and directions for future research are discussed.

SAMENVATTING

In dit proefschrift hebben we de hypothese onderzocht dat groei in het vroege leven en blootstelling aan nadelige omgevingsfactoren tijdens de zwangerschap en op de vroege kindertijd, in combinatie met genetische predispositie, leiden tot een lagere longfunctie en een hoger risico op chronisch obstructieve respiratoire aandoeningen op oudere leeftijd. Daarnaast onderzochten we epigenetische mechanismen als een mogelijke verklaring voor de interacties tussen omgevingsfactoren en genetische predispositie. Door het identificeren van nadelige blootstellingen in het vroege leven, genetische predispositie en DNA-methylatie als mogelijk onderliggend mechanisme, kunnen nieuwe preventie-strategieën en therapeutische interventies worden ontwikkeld die toegepast kunnen worden tijdens de zwangerschap en op de kinderleeftijd. Dit kan uiteindelijk resulteren in een betere preventie en behandeling van chronisch obstructief longlijden op de oudere leeftijd.

In **Hoofdstuk 1** bespreken we de achtergrond en rationale van de studies die worden gepresenteerd in dit proefschrift. Ook geven we in dit hoofdstuk een overzicht van de onderzoeksdoelen van de verrichte studies, en wordt de indeling van dit proefschrift toegelicht.

Hoofdstuk 2 beschrijft de associaties van groei in het vroege leven met longfunctie en astma op de kinderleeftijd. In *Hoofdstuk 2.1* tonen we aan dat een kortere zwangerschapsduur, een lager geboortegewicht voor de zwangerschapsduur, en een grotere gewichtstoename in het eerste levensjaar geassocieerd zijn met een lagere longfunctie bij kinderen. Een mediatie-analyse toonde dat een lagere longfunctie 7% tot 45% van de associaties tussen vroege groei en astma op de kinderleeftijd verklaard. In *Hoofstuk 2.2* laten we zien dat een kleinere gewichtstoename tijdens de foetale periode, en een grotere gewichtstoename op de vroege kinderleeftijd zijn geassocieerd met een lagere longfunctie en een 1.3 maal verhoogd risico op astma. De resultaten van *Hoofdstukken 2.1* en *2.2* ondersteunen de hypothese dat veranderingen in vroege groei leiden tot aanpassingen in de ontwikkeling van de longen en luchtwegen, wat leidt tot relatief kleine luchtwegen, en een verhoogd risico op astma. In *Hoofstuk 2.3* tonen we aan dat een hogere BMI is geassocieerd met een hogere luchtwegweerstand en een hogere kans op een piepende ademhaling bij kinderen op de schoolgaande leeftijd. Ook tonen we aan dat een grotere vet massa is geassocieerd met een hogere luchtwegweerstand, en een hogere androïde/gynoide ratio en meer preperitoneaal vet, een maat van visceraal vet, met een hoger stikstofoxide in de uitademingslucht. Deze resultaten suggereren dat gedetailleerde metingen van lichaamsvet mogelijk betere maten zijn dan BMI om de complexe relatie tussen obesitas en astma te verklaren.

Samenvattend tonen wij dat vroeggeboorte, een laag geboortegewicht, obesitas, en vetmassa's op specifieke locaties in het lichaam zijn gerelateerd aan veranderingen in

longfunctie en het risico op astma op de kinderleeftijd. Deze factoren vormen dan ook potentiële aangrijpingspunten voor de preventie van astma.

In **Hoofdstuk 3** beschrijven we de associaties tussen blootstellingen in het vroege leven met longfunctie en astma in kinderen. In *Hoofdstuk 3.1* tonen we aan dat roken van de moeder tijdens de gehele zwangerschap is geassocieerd met een verhoogd risico op een piepende ademhaling en astma, maar niet met luchtwegweerstand of stikstofoxide in de uitademingslucht, bij 6-jarige kinderen. Deze associaties werden niet verklaard door de zwangerschapsduur of geboortegewicht. In *Hoofdstuk 3.2* beschrijven we dat het gebruik van foliumzuursupplementen tijdens de zwangerschap is geassocieerd met lagere longfunctie bij kinderen, maar alleen als moeder drager is van variant *C677T* in het *MTHFR*-gen. Een hogere bloedwaarde van vitamine B_{12} is geassocieerd met een lagere longfunctie bij kinderen die drager waren van het reguliere *MTHFR*-gen. In *Hoofdstuk 3.3* tonen we aan dat een kortere periode van borstvoeding en het eerder starten van kunstvoeding is geassocieerd met een verhoogde kans op een piepende ademhaling tot de leeftijd van 3 jaar, en dat een borstvoedingsduur korter dan 2 maanden is geassocieerd met een 2.2 maal verhoogd risico op astma tot de leeftijd van 6 jaar.

Samenvattend tonen wij in deze studies aan dat roken van de moeder tijdens de zwangerschap, het gebruik van foliumzuursupplementen tijdens de zwangerschap als moeder drager is van variant *C677T* in het *MTHFR*-gen, en borstvoedingspatronen geassocieerd zijn met veranderingen in longfunctie en het risico op astma op de kinderleeftijd.

Hoofdstuk 4 beschrijft de identificatie van een gen geassocieerd met long functie en astma op de kinderleeftijd, en van locaties in het DNA waar epigenetische factoren gerelateerd zijn aan omgevingsblootstellingen en chronisch obstructieve respiratoire aandoeningen gedurende het leven. In *hoofdstuk 4.1* beschrijven we de identificatie van een nieuw gen, *CDHR3*, dat geassocieerd is met astma. Varianten in het *CDHR3*-gen geven veranderingen in de integriteit van de luchtwegepitheelcellen, waardoor deze meer vatbaar worden voor virale infecties, en de kans op ernstige astma-aanvallen mogelijk verhoogd. In *hoofdstuk 4.2* tonen we aan dat genen die geassocieerd zijn met longfunctie in volwassenen ook geassocieerd zijn met longfunctie van kinderen. Dit suggereert dat deze genen al op de kinderleeftijd invloed hebben op longfunctie. In *hoofdstuk 4.3* tonen de resultaten van een meta-analyse de associaties van foliumzuurwaarden van de moeder tijdens de zwangerschap met verschillen in DNA-methylatie van het kind bij de geboorte, namelijk in 443 CpGs die gelokaliseerd zijn bij 320 verschillende menselijke genen. Deze resultaten leveren nieuw inzicht in de invloed van foliumzuurwaarden tijdens de zwangerschap op het ontwikkelende epigenoom van het kind. In *Hoofdstuk 4.4.*beschrijven we de identificatie van 59 regio's waarin verschillen in DNA-methylatie bij de geboorte zijn geassocieerd met longfunctie op de kinderleeftijd. Meerdere van deze regio's waren ook geassocieerd met astma op de kinderleeftijd, longfunctie op de

adolescente en volwassen leeftijd, en met COPD op de volwassen leeftijd. Dit onderzoek ondersteunt de hypothese dat DNA-methylatie patronen bij de geboorte invloed hebben op gen-expressie en daaropvolgende longontwikkeling, die de kans op chronische obstructieve respiratoire aandoeningen vergroot.

Samenvattend hebben wij aangetoond dat genetische varianten vanaf het vroege leven invloed hebben op longfunctie en de kans op respiratoire aandoeningen, dat foliumzuurwaarden tijdens de zwangerschap invloed hebben op DNA-methylatie veranderingen van het kind bij geboorte, en dat DNA-methylatie veranderingen van het kind bij de geboorte geassocieerd zijn met longfunctie en respiratoire aandoeningen gedurende het leven.

In **Hoofdstuk 5** geven een samenvatting en interpretatie van de bevindingen gerapporteerd in dit proefschrift. Ook bespreken we methodologische aspecten, causale verbanden van de gevonden associaties, en geven we suggesties voor toekomstig onderzoek.

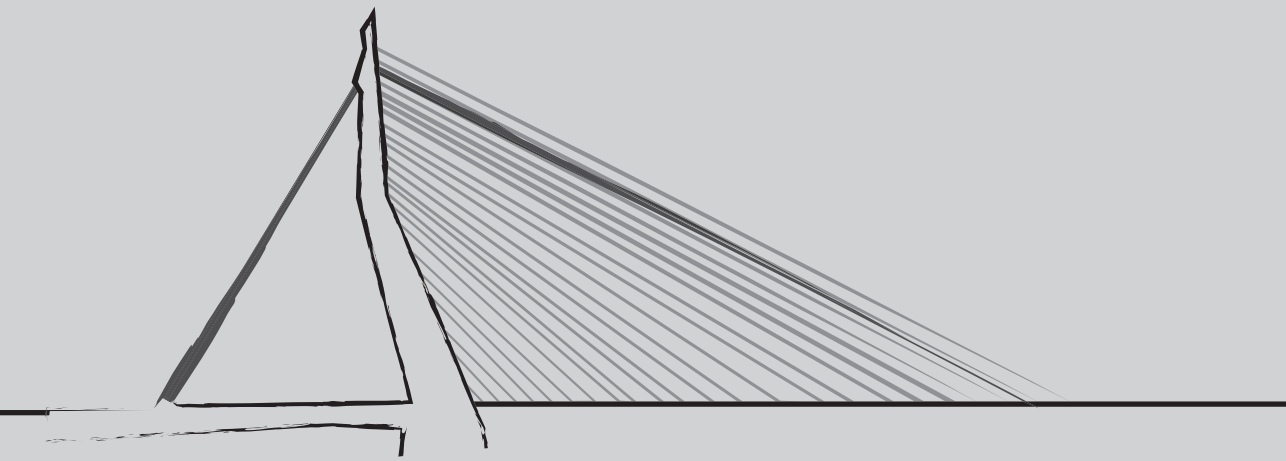
Chapter 7

List of publications

PhD portfolio

About the author

Dankwoord



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PHD PORTFOLIO

Name:	Herman Teun den Dekker
Department:	Epidemiology, Erasmus Medical Center, Rotterdam
	Pediatrics, Erasmus Medical Center, Rotterdam
Medical School:	University Medical Center Utrecht, 2005 – 2011
Research School:	Netherlands Institute for Health Sciences (NIHES), Rotterdam, 2013 – 2015
PhD Period:	2013 – 2016
Promotors:	Prof. Dr. V.W.V. Jaddoe, prof. dr. J.C. de Jongste
Co-promotor:	Dr. L. Duijts

	Year	Workload (ECTS)
PhD training		
Master of Science in Clinical Epidemiology, NIHES, Rotterdam, the Netherlands	2013-2015	
General courses		
Principles of Research in Medicine	2013	0.7
Clinical Decision Analysis	2013	0.7
Methods of Public Health Research	2013	0.7
Health Economics	2013	0.7
Genome Wide Association Analysis	2014	1.4
Conceptual Foundation of Epidemiologic Study Design	2014	0.7
Principles of Genetic Epidemiology	2014	0.7
Markers and Prognostic Research	2013	0.7
The Practice of Epidemiologic Analysis	2013	0.7
Introduction to Bayesian Methods in Clinical and Epidemiological Research	2014	1.4
Study Design	2014	4.3
Biostatistical Methods I: Basic Principles	2013	5.7
Clinical Epidemiology	2014	5.7
Methodologic Topics in Epidemiologic Research	2014	1.4
Biostatistical Methods II: Classical Regression Models	2014	4.3
English Language	2013	1.4
Introduction to Medical Writing	2013	1.1
Advanced courses		
Repeated Measurements in Clinical Studies	2015	1.4
Missing Values in Clinical Research	2014	0.7
Principles of Epidemiologic Data-analysis	2014	0.7
A first encounter with next-generation sequencing data	2014	1.4
General Academic courses		
Research Integrity	2015	2.0
MRI Safety training	2013	0.3

Seminars and workshops		
Seminars Epidemiology	2013 – 2016	4.0
Research meetings Generation R Study	2013 – 2016	4.0
Maternal and Child Health meetings	2013 – 2016	4.0
Research meetings Pediatric Pulmonology	2013 – 2016	4.0
Pediatrics Research day, Erasmus MC	2014	0.6
Pediatrics Research day, Erasmus MC	2015	0.6
Conferences		
Longdagen 2016, Ermelo	2016	0.7
ERS European Respiratory Society, Amsterdam	2015	1.4
ONS Obstetrie & Neonatologie, Veldhoven	2014	0.7
Developmental Origins of Health and Disease, Singapore	2013	1.4
Teaching activities		
A.C. van Berkel, MSc Student	2014	3.0
K.P.I. Ros, MSc Student	2014	3.0
S.N. Schipper, MSc Student	2014	3.0
E. Rucci, MSc thesis Clinical Epidemiology, NIHES	2014 - 2015	3.0
S. Balentin, MSc Student	2014	3.0
E. van Meel, MSc thesis Clinical Epidemiology, NIHES	2015 - 2016	3.0
S. Shagiwal, MSc thesis Clinical Epidemiology, NIHES	2015 - 2016	3.0
S. Brandt, MSc thesis Clinical Epidemiology, NIHES	2015 - 2016	3.0
S.D. Bahadoer, MSc Student	2016	1.5

Other

- Grant recipient; European Union 7th Framework Programme; grant agreement number 247642, GEoCoDE

- Peer review of articles for various scientific journals (American Journal of Respiratory Critical Care Medicine, Chest, European Journal of Epidemiology, European Respiratory Journal, Pediatric Allergy and Immunology, Pediatric Pulmonology, Respiration).

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

ABOUT THE AUTHOR

Herman Teun (Martijn) den Dekker was born on the 3th of August 1987 in Woudrichem, The Netherlands. In 2005, he graduated from the Gynmasium Camphusianum in Gorinchem. In the same year, he started to study Medicine at the University Medical Center Utrecht. As part of his medical training he performed research in the Department of Pediatric Cardiology supervised by dr. M.W. Freund which resulted in the publication of his first scientific paper on the hypoplastic left heart complex in 2013. He obtained his medical degree in 2011, and subsequently started working as a resident (ANIOS) at the Department of Pediatrics of the Albert Schweitzer Hospital in Dordrecht. In 2012, he continued his residency (ANIOS) at the Department of Neonatology of the Erasmus MC – Sophia Children's Hospital in Rotterdam. In 2013, he started with the current PhD-project at the Generation R Study, and the Departments of Pediatrics and Epidemiology (promotors: Prof. V.W.V Jaddoe and Prof. J.C. de Jongste; co-promotor: Dr. L. Duijts). His work focused on early growth and environmental, genetic and epigenetic factors that influence lung function, asthma and COPD in later life. During his PhD-project, he spent 3 months at the Telethon Kids Institute in Perth, Australia, to study the associations of interactions between genetic variants and environmental exposures with childhood lung function and asthma under supervision of Prof. G. Hall. From January 2017 onwards, Martijn has started his training in Pediatrics (AIOS) at the Erasmus MC - Sophia Children's Hospital in Rotterdam.

DANKWOORD

"If I have seen further, it is by standing on the shoulders of giants (Isaac Newton, 1676)."

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