Early Environmental and Epigenetic Influences on Respiratory Health

H.T. den Dekker

ACKNOWLEDGMENTS

The general design of the Generation R Study is made possible by financial support from the Erasmus Medical Center, Rotterdam, the Erasmus University Rotterdam, the Netherlands Organization for Health Research and Development (ZonMW), the Netherlands Organization for Scientific Research (NOW), the Ministry of Health, Welfare and Sport and the Ministry of Youth and Families. All work presented in this thesis was financially supported by the Lung Foundation Netherlands (Projectnr: 3.2.12.089).

The work presented in this thesis was conducted at the Department of Pediatrics, division of Respiratory Medicine and Allergology, the Generation R Study Group, and the Department of Epidemiology of the Erasmus Medical Center in Rotterdam.

The printing of this thesis has been financial supported by the Erasmus University Rotterdam and the Generation R Study. Further financial support for this dissertation was kindly provided by Nutricia.

ISBN / EAN: 978-94-92683-29-8

Cover design: Guus Gijben, proefschrift-AIO
Layout and printed by: Optima Grafische Communicatie, Rotterdam, the Netherlands

Early Environmental and Epigenetic Influences on Respiratory Health

Vroege omgevings- en epigenetische invloeden op respiratoire gezondheid

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de

rector magnificus

Prof. dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

Woensdag 14 juni 2017 om 13.30 uur

door

Herman Teun den Dekker

geboren te Woudrichem

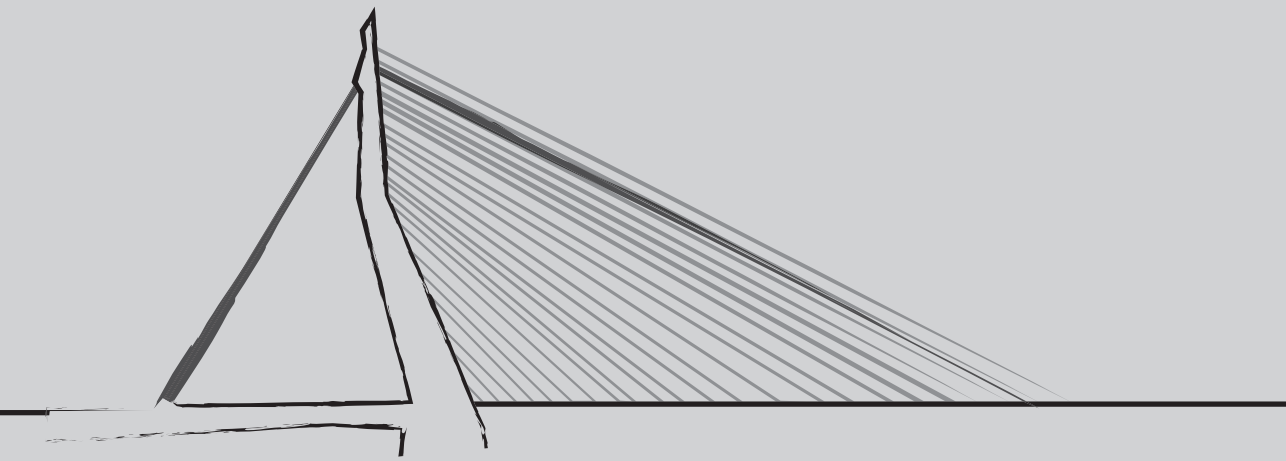
Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. V.W.V. Jaddoe
	Prof.dr. J.C. de Jongste
Overige leden:	Prof. dr. I.K. Reiss
	Prof. dr. G.G. Brusselle
	Prof. dr. A.G. Uitterlinden
Co-promotor:	Mw. Dr. L. Duijts

TABLE OF CONTENTS

Manuscripts that form the basis of this thesis



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

Den Dekker HT, Ros KPI, de Jongste JC, Reiss IK, Jaddoe VWV, Duijts L. Body fat mass distribution and interrupter resistance, fractional exhaled nitric oxide and asthma at school-age. *J Allergy Clin Immunol 2016 Jul 16. pii: S0091-6749(16)30625-X*.

Chapter 3.1

Den Dekker HT, Sonnenschein-van der Voort AM, de Jongste JC, Reiss IK, Hofman A, Jaddoe VWV, Duijts L. Tobacco smoke exposure, airway resistance, and asthma in school-age children: the Generation R Study. *Chest 2015;148(3):607-17.*

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

Den Dekker HT, Sonnenschein-van der Voort AM, de Jongste JC, Reiss IK, Jaddoe VWV, Duijts L. Breastfeeding and asthma outcomes at the age of 6 years: the Generation R Study. *Pediatr Allergy Immunol 2016;27(5):486-92*.

Chapter 4.1

Bønnelykke K, Sleiman P, Nielsen K, Kreiner-Møller E, Mercader JM, Belgrave D, **den Dekker HT**, Husby A, Sevelsted A, Faura- Tellez G, Mortensen LJ, Paternoster L, Flaaten R, Mølgaard R, Smart DE, Thomsen PF, Rasmussen MA, Bonàs-Guarch S, Holst C, Nohr EA,

Yadav R, March ME, Blicher T, Lackie PM, Jaddoe VWV, Simpson A, Holloway JW, Duijts L, Custovic A, Davies DE, Torrents D, Gupta R, Hollegaard MV, Hougaard DM, Hakonarson H, Bisgaard H. A genome-wide association study identifies *CDHR3* as a susceptibility locus for early childhood asthma with severe exacerbations. *Nat Genet 2014;46(1)51-1*.

Chapter 4.2

Shagiwal S, **den Dekker HT**, de Jongste JC, Jaddoe VW, Felix JF, Duijts L. Adult lung function susceptibility loci and childhood lung function. The Generation R Study. *Submitted*

Chapter 4.3

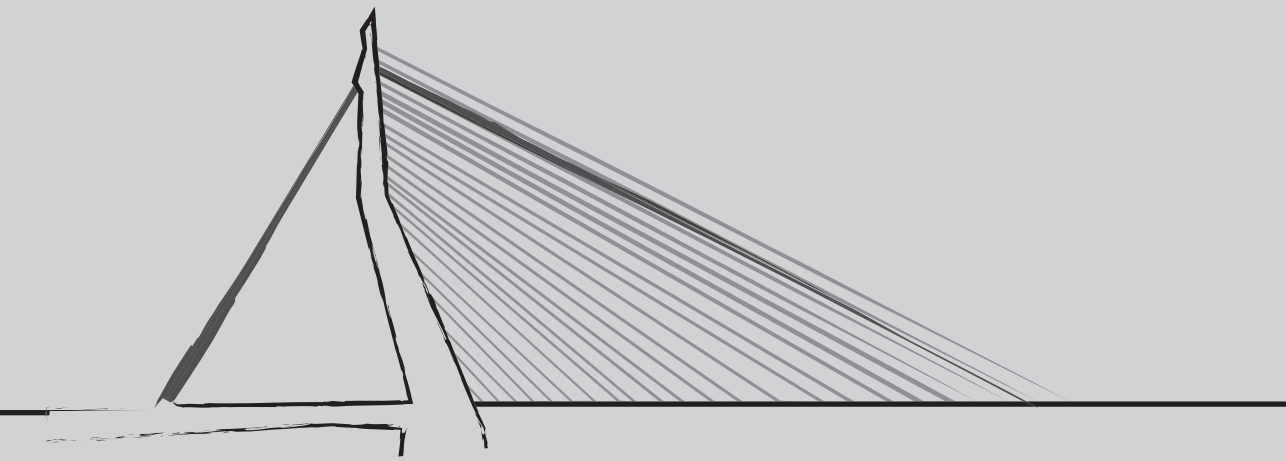
Joubert BR*, **den Dekker HT***, Felix JF, Bohlin J, Ligthart S, Beckett E, Tiemeier H, van Meurs JB, Uitterlinden AG, Hofman A, Håberg SE, Reese SE, Peters MJ, Kulle Andreassen B, Steegers EA, Nilsen RM, Vollset SE, Midttun Ø, Ueland PM, Franco OH, Dehghan A, de Jongste JC, Wu MC, Wang T, Peddada SD, Jaddoe VWV, Nystad W, Duijts L[#], London SJ[#]. Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. *Nat Commun. 2016;10;7:10577*.
*[#]Both authors contributed equally.

Chapter 4.4

Den Dekker HT, Burrows K, Felix JF, Salas LA, Nedeljkovic I, Yao J, Rifas-Shiman SL, Ruiz-Arenas C, DeMeo DL, Henderson AJ, Howe CG, Hivert M, Ikram MA, de Jongste JC, Lahousse L, Mandaviya P, van Meurs JB, Pinart M, Stolk L, Sunyer J, Uitterlinden AG, Anto JM, Litonjua AA, Breton CV, Brusselle GG, Bustamante M, Davey Smith G, Relton CL, Jaddoe VWV, Duijts L. Newborn DNA-methylation, childhood lung function, and the risk of asthma and COPD across the life course. *Submitted*

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

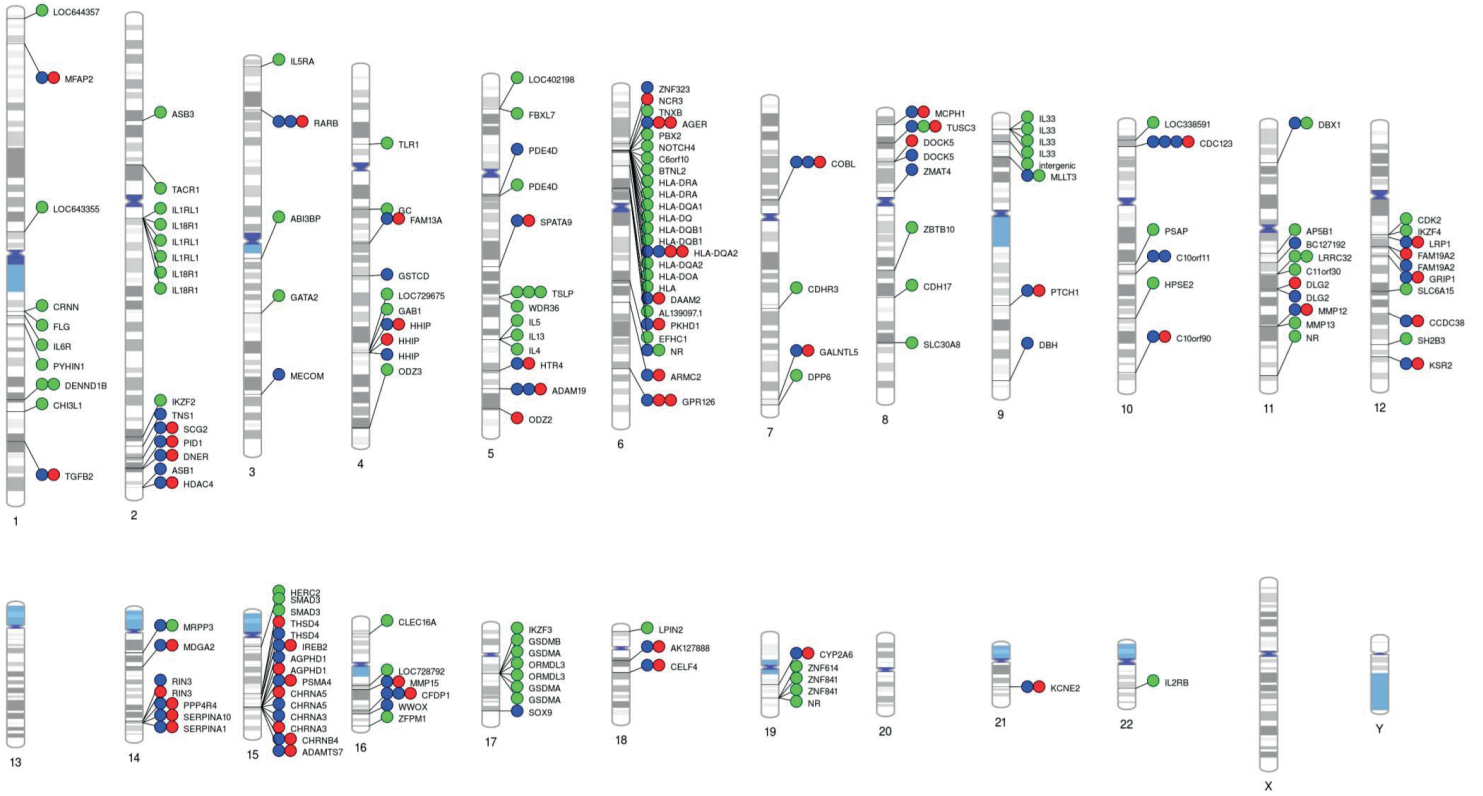


Figure 1.1. Overview of the genetic variants currently known to be associated with Asthma (green), Forced Expiratory Volume in 1 second (FEV_1) (blue) and FEV1/Forced Vital Capacity (FVC) (red). The National Human Genome Research Institute catalogue of published GWAS was searched using asthma, FEV_1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

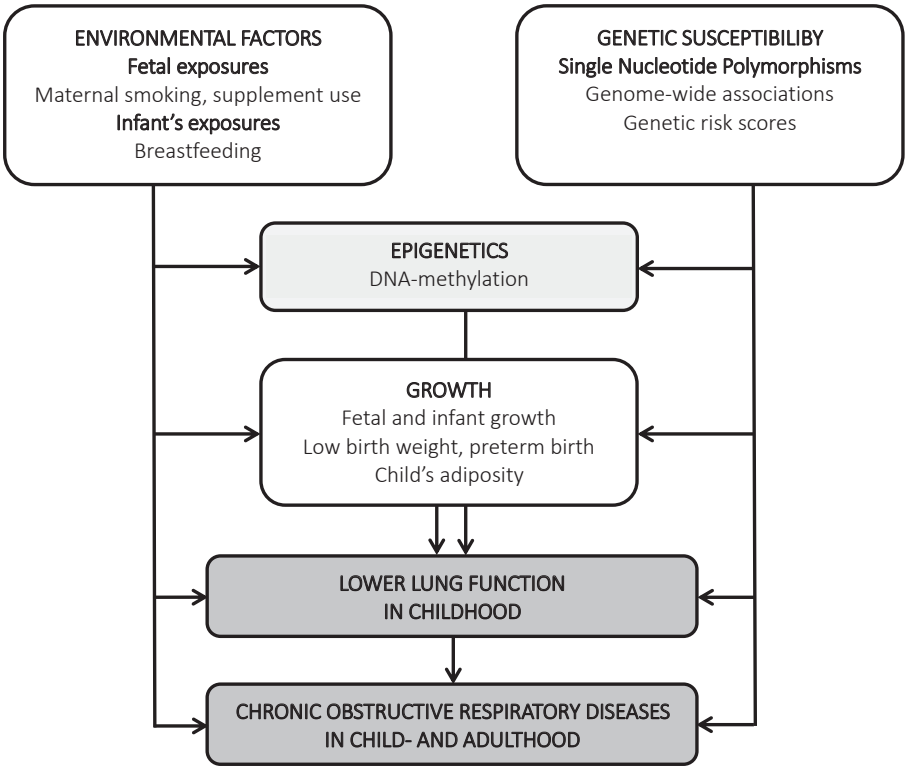


Figure 1.2. Overview of the early origins of chronic obstructive respiratory diseases and its potential underlying early growth, environmental and (epi)genetic mechanisms studies in this thesis.

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

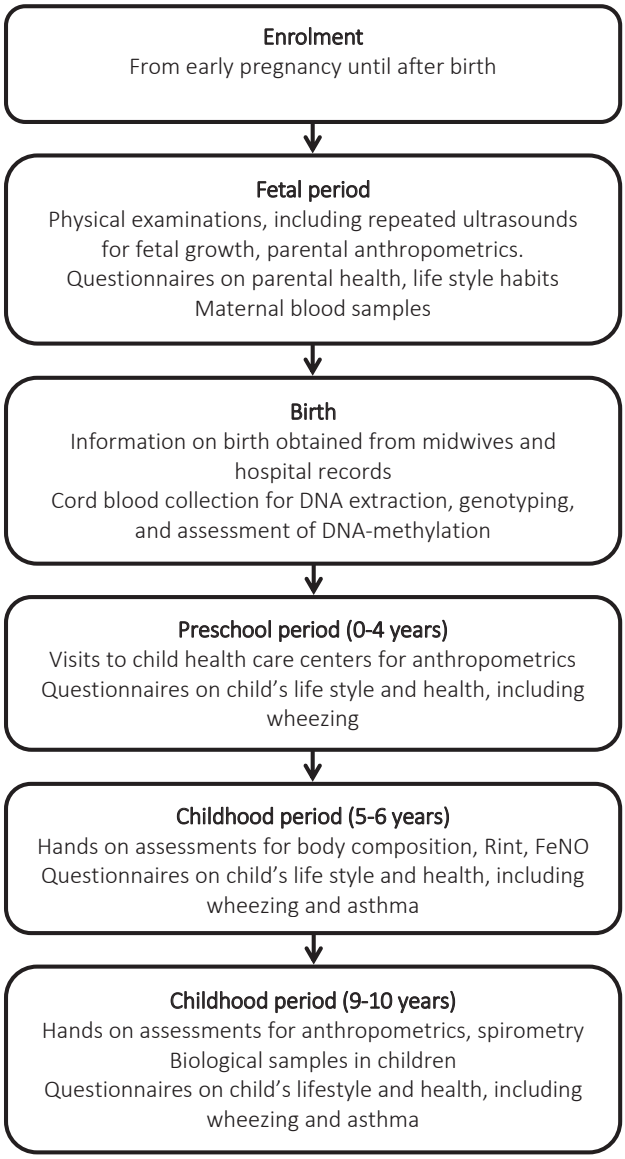


Figure 1.3. Design and data collection in the Generation R Study.

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**.

REFERENCES

1. Asher I, Pearce N. Global burden of asthma among children. International Journal of Tuberculosis and Lung Disease 2014; 18:1269-78.
2. Engelkes M, Janssens HM, de Ridder MAJ, de Jongste JC, Sturkenboom MCJM, Verhamme KMC. Time trends in the incidence, prevalence and age at diagnosis of asthma in children. Pediatric Allergy and Immunology 2015; 26:367-74.
3. Tai A, Tran H, Roberts M, Clarke N, Wilson J, Robertson CF. The association between childhood asthma and adult chronic obstructive pulmonary disease. Thorax 2014; 69:805-10.
4. Chronic obstructive pulmonary disease (COPD). World Health Organisation; 2015.] Available from http://www.who.int/mediacentre/factsheets/fs315/en/.
5. Martinez FD, Vercelli D. Asthma. Lancet 2013; 382:1360-72.
6. Bisgaard H, Swern AS, Knorr B. "To wheeze or not to wheeze": That is not the question-the sequel. Journal of Allergy and Clinical Immunology 2012; 130:531-2.
7. Duijts L, Granell R, Sterne JA, Henderson AJ. Childhood wheezing phenotypes influence asthma, lung function and exhaled nitric oxide fraction in adolescence. Eur Respir J 2016; 47:510-9.
8. Rasmussen F, Taylor DR, Flanneryb EM, Cowan JO, Greene JM, Herbison GP, et al. Risk factors for hospital admission for asthma from childhood to young adulthood: A longitudinal population study. Journal of Allergy and Clinical Immunology 2002; 110:220-7.
9. Postma DS, Rabe KF. The Asthma-COPD Overlap Syndrome. N Engl J Med 2015; 373:1241-9.
10. McGeachie MJ, Yates KP, Zhou X, Guo F, Sternberg AL, Van Natta ML, et al. Patterns of Growth and Decline in Lung Function in Persistent Childhood Asthma. New England Journal of Medicine 2016; 374:1842-52.
11. Vonk JM, Postma DS, Boezen HM, Grol MH, Schouten JP, Koeter GH, et al. Childhood factors associated with asthma remission after 30 year follow up. Thorax 2004; 59:925-9.
12. Covar RA, Strunk R, Zeiger RS, Wilson LA, Liu AH, Weiss S, et al. Predictors of remitting, periodic, and persistent childhood asthma. Journal of Allergy and Clinical Immunology 2010; 125:359-66.
13. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. European Respiratory Journal 2005; 26:319-38.
14. Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma - Summary Report (vol 120, pg S94, 2007). Journal of Allergy and Clinical Immunology 2008; 121:1330-.
15. Beydon N, M'Buila C, Bados A, Peiffer C, Bernard A, Zaccaria I, et al. Interrupter resistance short-term repeatability and bronchodilator response in preschool children. Respiratory Medicine 2007;101:2482
16. Konradsen JR, Skantz E, Nordlund B, Lidegran M, James A, Ono J, et al. Predicting asthma morbidity in children using proposed markers of Th2-type inflammation. Pediatric Allergy and Immunology 2015; 26:772-9.
17. van der Valk RJP, Caudri D, Savenije O, Koppelman GH, Smit HA, Wijga AH, et al. Childhood wheezing phenotypes and FeNO in atopic children at age 8. Clinical and Experimental Allergy 2012; 42:1329-36.
18. Duijts L, Reiss IK, Brusselle G, de Jongste JC. Early origins of chronic obstructive lung diseases across the life course. Eur J Epidemiol 2014; 29:871-85.
19. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003; 33 Suppl:245-54.
20. Jobe AH. Mechanisms of Lung Injury and Bronchopulmonary Dysplasia. Am J Perinatol 2016; 33:1076-8.

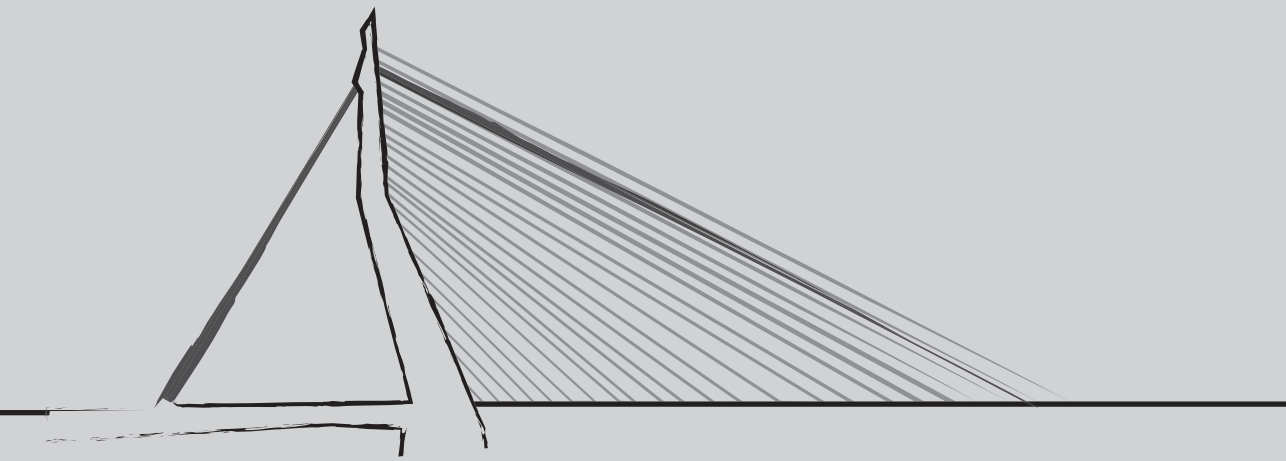
21. Sonnenschein-van der Voort AM, Arends LR, de Jongste JC, Annesi-Maesano I, Arshad SH, Barros H, et al. Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. J Allergy Clin Immunol 2014; 133:1317-29.
22. Hancox RJ, Poulton R, Greene JM, McLachlan CR, Pearce MS, Sears MR. Associations between birth weight, early childhood weight gain and adult lung function. Thorax 2009; 64:228-32.
23. Sherrill DL, Guerra S, Wright AL, Morgan WJ, Martinez FD. Relation of Early Childhood Growth and Wheezing Phenotypes to Adult Lung Function. Pediatric Pulmonology 2011; 46:956-63.
24. van der Gugten AC, Koopman M, Evelein AMV, Verheij TJM, Uiterwaal CSPM, van der Ent CK. Rapid early weight gain is associated with wheeze and reduced lung function in childhood. European Respiratory Journal 2012; 39:403-10.
25. Turner S, Zhang G, Young S, Cox M, Goldblatt J, Landau L, et al. Associations between postnatal weight gain, change in postnatal pulmonary function, formula feeding and early asthma. Thorax 2008; 63:234.
26. Pike KC, Crozier SR, Lucas JS, Inskip HM, Robinson S, Southampton Women's Survey Study G, et al. Patterns of fetal and infant growth are related to atopy and wheezing disorders at age 3 years. Thorax 2010; 65:1099-106.
27. Sonnenschein-van der Voort AM, Gaillard R, de Jongste JC, Hofman A, Jaddoe VW, Duijts L. Foetal and infant growth patterns, airway resistance and school-age asthma. Respirology 2016; 21:674-82.
28. Taal HR, Vander Heijden AJ, Steegers EAP, Hofman A, Jaddoe VWV. Small and Large Size for Gestational Age at Birth, Infant Growth, and Childhood Overweight. Obesity 2013; 21:1261-8.
29. Permaul P, Kanchongkittiphon W, Phipatanakul W. Childhood asthma and obesity-what is the true link? Annals of Allergy Asthma & Immunology 2014; 113:244-6.
30. Balbus JM, Barouki R, Birnbaum LS, Etzel RA, Gluckman PD, Sr., Grandjean P, et al. Early-life prevention of non-communicable diseases. Lancet 2013; 381:3-4.
31. Tobacco Advisory Group of the Royal College of Physicians. Report on passive smoking and children. RCP 2010.
32. Murphy DJ, Dunney C, Mullally A, Adnan N, Deane R. Population-based study of smoking behaviour throughout pregnancy and adverse perinatal outcomes. Int J Environ Res Public Health 2013; 10:3855-67.
33. Duijts L. Fetal and infant origins of asthma. European Journal of Epidemiology 2012; 27:5-14.
34. Duijts L, Jaddoe VW, van der Valk RJ, Henderson JA, Hofman A, Raat H, et al. Fetal exposure to maternal and paternal smoking and the risks of wheezing in preschool children: the Generation R Study. Chest 2012; 141:876-85.
35. Vardavas CI, Hohmann C, Patelarou E, Martinez D, Henderson AJ, Granell R, et al. The independent role of prenatal and postnatal exposure to active and passive smoking on the development of early wheeze in children. Eur Respir J 2016; 48:115-24.
36. Stein RT, Holberg CJ, Sherrill D, Wright AL, Morgan WJ, Taussig L, et al. Influence of parental smoking on respiratory symptoms during the first decade of life: the Tucson Children's Respiratory Study. Am J Epidemiol 1999; 149:1030-7.
37. Hollams EM, de Klerk NH, Holt PG, Sly PD. Persistent effects of maternal smoking during pregnancy on lung function and asthma in adolescents. Am J Respir Crit Care Med 2014; 189:401-7.
38. Wolff T, Witkop CT, Miller T, Syed SB, Force USPST. Folic acid supplementation for the prevention of neural tube defects: an update of the evidence for the U.S. Preventive Services Task Force. Ann Intern Med 2009; 150:632-9.
39. Sonnenschein-van der Voort AM, Howe LD, Granell R, Duijts L, Sterne JA, Tilling K, et al. Influence of childhood growth on asthma and lung function in adolescence. J Allergy Clin Immunol 2014.

40. Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease. Cell Metab 2016.
41. Tanaka T, Scheet P, Giusti B, Bandinelli S, Piras MG, Usala G, et al. Genome-wide Association Study of Vitamin B6, Vitamin B12, Folate, and Homocysteine Blood Concentrations. American Journal of Human Genetics 2009; 84:477-82.
42. Sharma S, Litonjua A. Asthma, allergy, and responses to methyl donor supplements and nutrients. J Allergy Clin Immunol 2014; 133:1246-54.
43. Brown SB, Reeves KW, Bertone-Johnson ER. Maternal folate exposure in pregnancy and childhood asthma and allergy: a systematic review. Nutr Rev 2014; 72:55-64.
44. Granell R, Heron J, Lewis S, Davey Smith G, Sterne JA, Henderson J. The association between mother and child MTHFR C677T polymorphisms, dietary folate intake and childhood atopy in a population-based, longitudinal birth cohort. Clin Exp Allergy 2008; 38:320-8.
45. van der Valk RJ, Kiefte-de Jong JC, Sonnenschein-van der Voort AM, Duijts L, Hafkamp-de Groen E, Moll HA, et al. Neonatal folate, homocysteine, vitamin B12 levels and methylenetetrahydrofolate reductase variants in childhood asthma and eczema. Allergy 2013; 68:788-95.
46. Kiefte-de Jong JC, Timmermans S, Jaddoe VW, Hofman A, Tiemeier H, Steegers EA, et al. High circulating folate and vitamin B-12 concentrations in women during pregnancy are associated with increased prevalence of atopic dermatitis in their offspring. J Nutr 2012; 142:731-8.
47. Dogaru CM, Nyffenegger D, Pescatore AM, Spycher BD, Kuehni CE. Breastfeeding and Childhood Asthma: Systematic Review and Meta-Analysis. American Journal of Epidemiology 2014; 179:1153-67.
48. Friedman NJ, Zeiger RS. The role of breast-feeding in the development of allergies and asthma. Journal of Allergy and Clinical Immunology 2005; 115:1238-48.
49. Palmer LJ, Knuiman MW, Divitini ML, Burton PR, James AL, Bartholomew HC, et al. Familial aggregation and heritability of adult lung function: results from the Busselton Health Study. Eur Respir J 2001; 17:696-702.
50. an Beijsterveldt CE, Boomsma DI. Genetics of parentally reported asthma, eczema and rhinitis in 5-yr-old twins. Eur Respir J 2007; 29:516-21.
51. Moffatt MF, Kabesch M, Liang LM, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature 2007; 448:470-U5.
52. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A Large-Scale, Consortium-Based Genomewide Association Study of Asthma. New England Journal of Medicine 2010; 363:1211-21.
53. Torgerson DG, Ampleford EJ, Chiu GY, Gauderman WJ, Gignoux CR, Graves PE, et al. Meta-analysis of genome-wide association studies of asthma in ethnically diverse North American populations. Nature Genetics 2011; 43:887-U103.
54. Hirota T, Takahashi A, Kubo M, Tsunoda T, Tomita K, Doi S, et al. Genome-wide association study identifies three new susceptibility loci for adult asthma in the Japanese population. Nature Genetics 2011; 43:893-U108.
55. Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat M, et al. Genome-wide association study identifies five loci associated with lung function. Nat Genet 2010; 42:36-44.
56. Hancock DB, Eijgelsheim M, Wilk JB, Gharib SA, Loehr LR, Marciante KD, et al. Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. Nat Genet 2010; 42:45-52.
57. Wilk JB, Chen TH, Gottlieb DJ, Walter RE, Nagle MW, Brandler BJ, et al. A genome-wide association study of pulmonary function measures in the Framingham Heart Study. PLoS Genet 2009; 5:e1000429.

58. Gudbjartsson DF, Bjornsdottir US, Halapi E, Helgadottir A, Sulem P, Jonsdottir GM, et al. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. Nature Genetics 2009; 41:342-7.
59. Weidinger S, Gieger C, Rodriguez E, Baurecht H, Mempel M, Klopp N, et al. Genome-Wide Scan on Total Serum IgE Levels Identifies FCER1A as Novel Susceptibility Locus. Plos Genetics 2008; 4.
60. Castro-Giner F, Bustamante M, Gonzalez JR, Kogevinas M, Jarvis D, Heinrich J, et al. A pooling-based genome-wide analysis identifies new potential candidate genes for atopy in the European Community Respiratory Health Survey (ECRHS). Bmc Medical Genetics 2009; 10.
61. Borish L, Culp JA. Asthma: a syndrome composed of heterogeneous diseases. Annals of Allergy Asthma & Immunology 2008; 101:1-8.
62. Artigas M, Loth DW, Wain LV, Gharib SA, Obeidat M, Tang W, et al. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. Nat Genet 2011; 43:1082-90.
63. Nystad W, Roysamb E, Magnus P, Tambs K, Harris JR. A comparison of genetic and environmental variance structures for asthma, hay fever and eczema with symptoms of the same diseases: a study of Norwegian twins. International Journal of Epidemiology 2005; 34:1302-9.
64. Soler Artigas M, Loth DW, Wain LV, Gharib SA, Obeidat M, Tang W, et al. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. Nat Genet 2011; 43:1082-90.
65. Cookson W, Moffatt M, Strachan DP. Genetic risks and childhood-onset asthma. J Allergy Clin Immunol 2011; 128:266-70; quiz 71-2.
66. English S, Pen I, Shea N, Uller T. The Information Value of Non-Genetic Inheritance in Plants and Animals. Plos One 2015; 10.
67. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nature Reviews Genetics 2012; 13:97-109.
68. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. Nature Reviews Genetics 2016; 17:487-500.
69. Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, et al. DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. American Journal of Human Genetics 2016; 98:680-96.
70. Fox JT, Stover PJ. Folate-Mediated One-Carbon Metabolism. Folic Acid and Folates 2008; 79:1-44.
71. Fu JJ, McDonald VM, Baines KJ, Gibson PG. Airway IL-1 beta and Systemic Inflammation as Predictors of Future Exacerbation Risk in Asthma and COPD. Chest 2015; 148:618-29.
72. Jaddoe VWV, van Duijn CM, Franco OH, van der Heijden AJ, van IIzendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. European Journal of Epidemiology 2012; 27:739-56.
73. Kruithof CJ, Kooijman MN, van Duijn CM, Franco OH, de Jongste JC, Klaver CCW, et al. The Generation R Study: Biobank update 2015. European Journal of Epidemiology 2014; 29:911-27.

Chapter 2

Early growth, childhood lung function and asthma

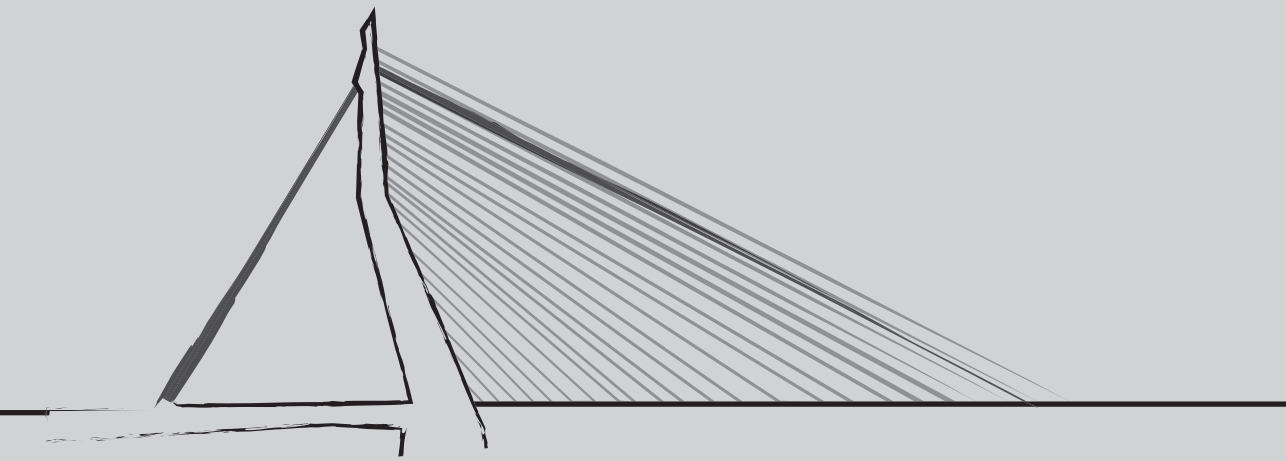


Chapter 2.1

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

HT den Dekker, AMM Sonnenschein-van der Voort, JC de Jongste, I Anessi–Maesano, S Hasan Arshad, H Barros, CS Beardsmore, H Bisgaard, S Correia, L Craig, G Devereux, CK van der Ent, A Esplugues, MP Fantini, C Flexeder, U Frey, F Forastiere, U Gehring, D Gori, AC van der Gugten, AJ Henderson, B Heude, J Ibarluzea, HM Inskip, T Keil, M Kogevinas, E Kreiner-Møller, CE Kuehni, S Lau, E Mélen, M Mommers, E Morales, J Penders, KC Pike, D Porta, IK Reiss, G Roberts, A Schmidt, ES Schultz, H Schulz, J Sunyer, M Torrent, M Vassilaki, AH Wijga, C Zabaleta, VWV Jaddoe, L Duijts

J Allergy Clin Immunol 2016;137(4):1026-35



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).

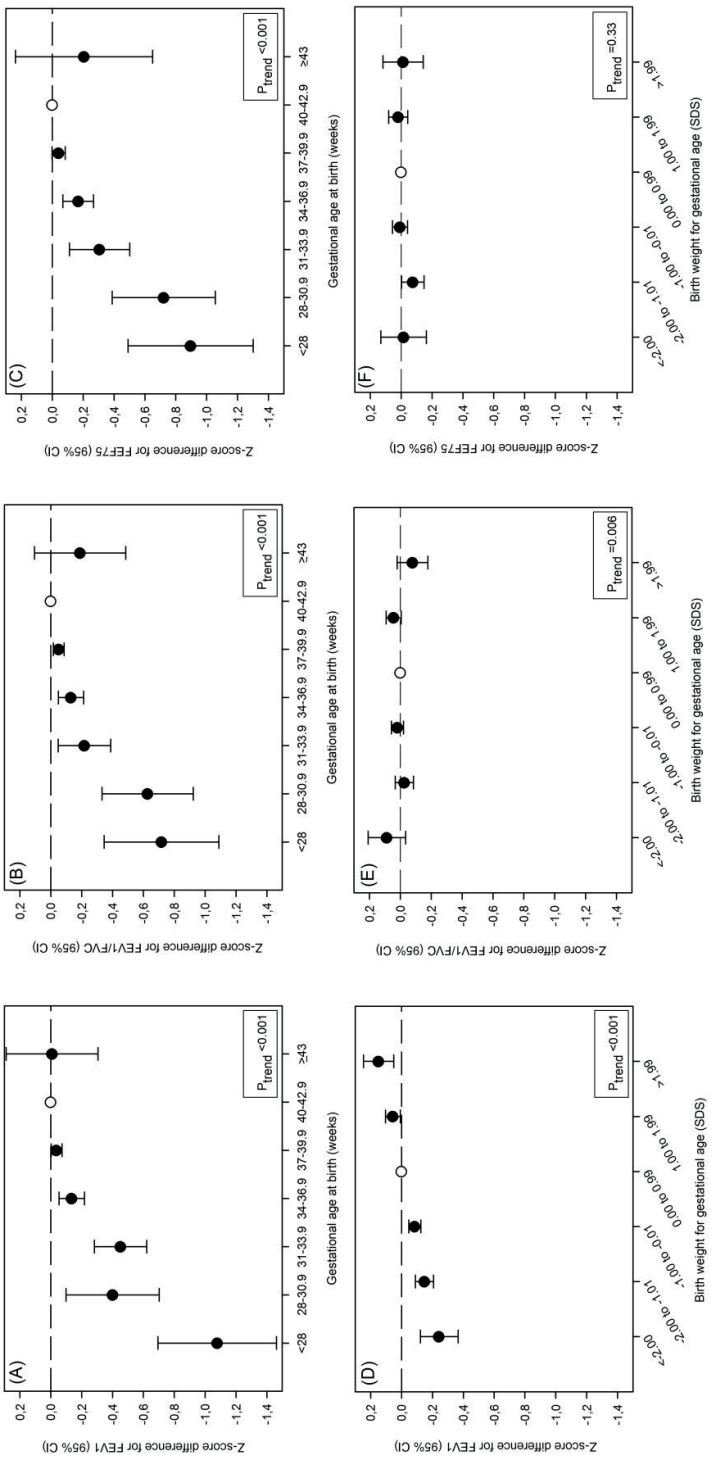


Figure 2.1.1. Associations of Gestational Age, Birth Weight and Infant Weight Gain with FEV_1, FEV_1/FVC and FEF_{75}.

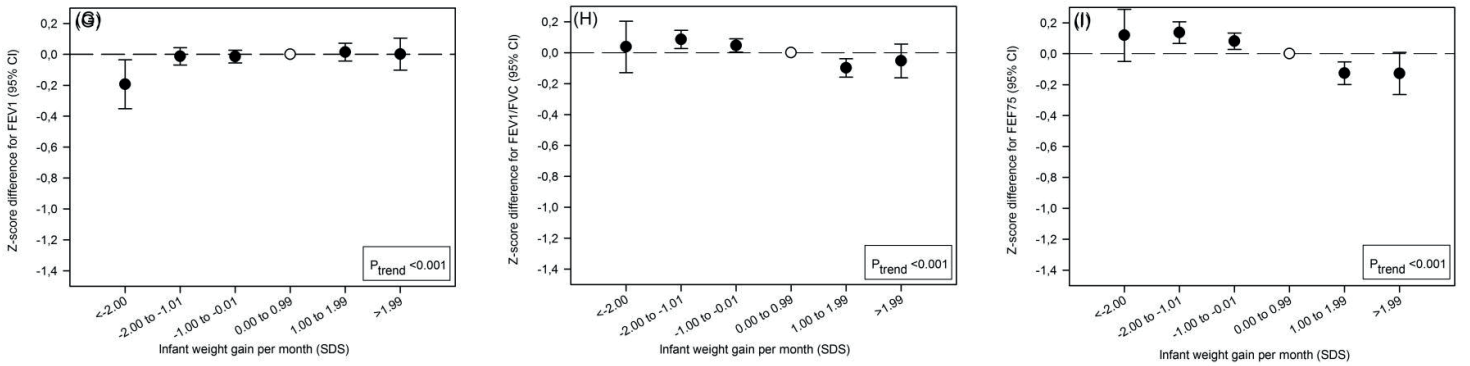


Figure 2.1.1. Associations of Gestational Age, Birth Weight and Infant Weight Gain with FEV_1, FEV_1/FVC and FEF_{75}. (continued)
Values represent Z-scores differences (95% confidence interval) from multi-level random effect models for the associations of gestational age at birth (A, B, C), gestational 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.

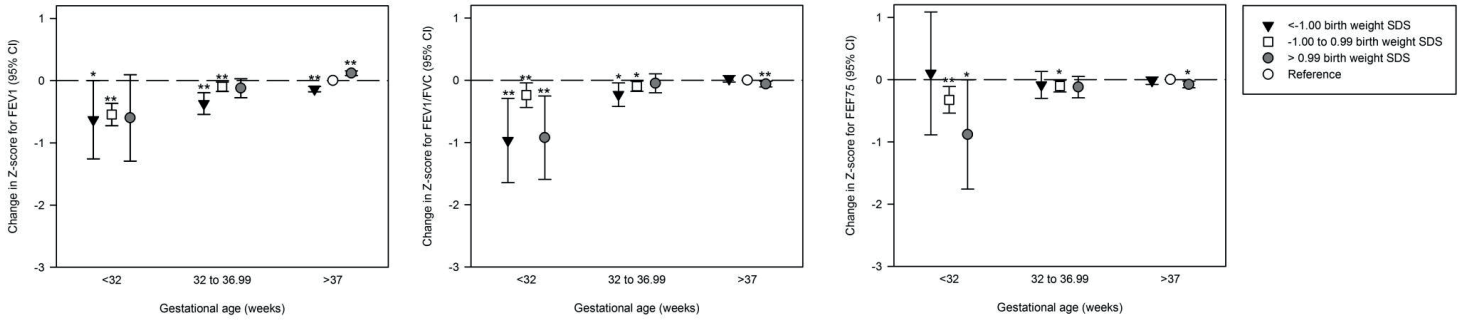


Figure 2.1.2. Combined Associations of Gestational Age and Birth Weight with FEV_1, FEV_1/FVC and FEF_{75}.

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.

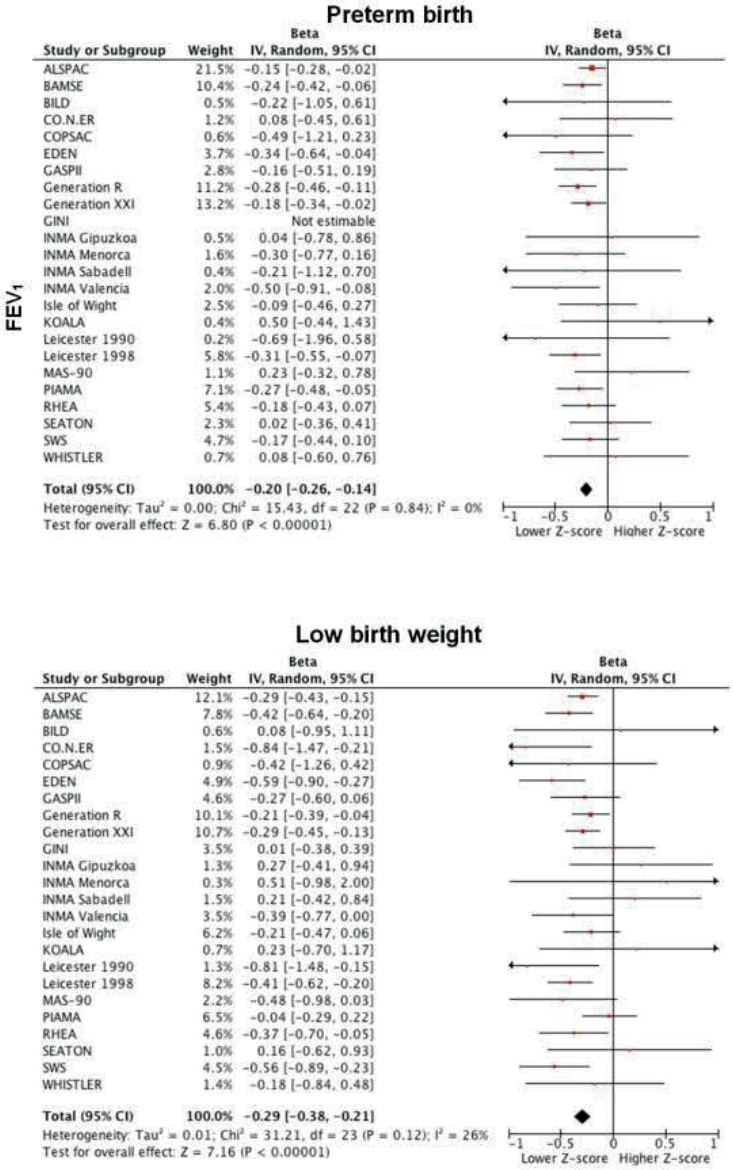


Figure 2.1.3. Forest Plots of the Associations between Preterm Birth and Low Birth Weight with FEV_1, FEV_1/FVC and FEF_{75}.

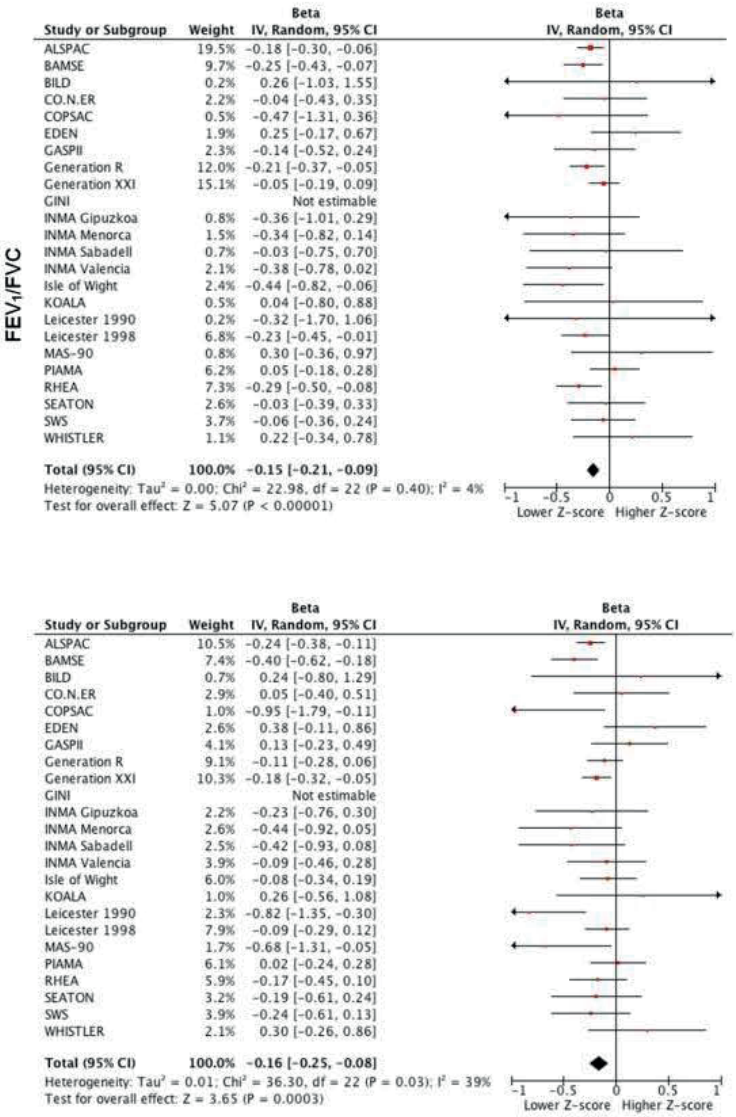
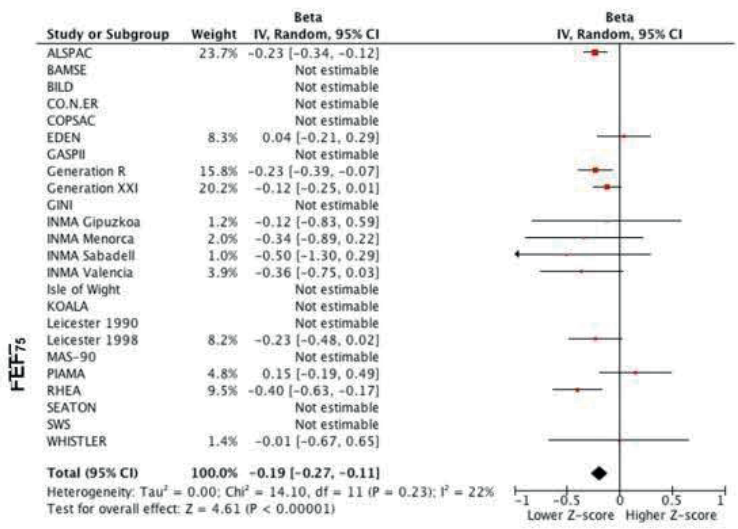


Figure 2.1.3. Forest Plots of the Associations between Preterm Birth and Low Birth Weight with FEV_1, FEV_1/FVC and FEF_{75}. (continued)



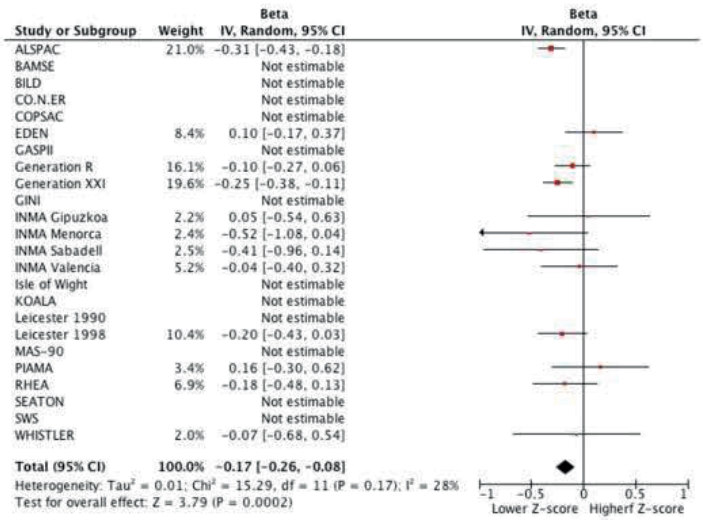


Figure 2.1.3. Forest Plots of the Associations between Preterm Birth and Low Birth Weight with FEV_1, FEV_1/FVC and FEF_{75}. (continued)

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

REFERENCES

1. Costeloe KL, Hennessy EM, Haider S, Stacey F, Marlow N, Draper ES. Short term outcomes after extreme preterm birth in England: comparison of two birth cohorts in 1995 and 2006 (the EPICure studies). BMJ 2012; 345:e7976.
2. Brostrom EB, Akre O, Katz-Salamon M, Jaraj D, Kaijser M. Obstructive pulmonary disease in old age among individuals born preterm. Eur J Epidemiol 2013; 28:79-85.
3. Been JV, Lugtenberg MJ, Smets E, van Schayck CP, Kramer BW, Mommers M, et al. Preterm birth and childhood wheezing disorders: a systematic review and meta-analysis. PLoS Med 2014; 11:e1001596.
4. Sonnenschein-van der Voort AM, Arends LR, de Jongste JC, Annesi-Maesano I, Arshad SH, Barros H, et al. Preterm birth, infant weight gain, and childhood asthma risk: A meta-analysis of 147,000 European children. J Allergy Clin Immunol 2014; 133:1317-29.
5. van der Gugten A, Korte K, van der Ent K, Uiterwaal C, Verheij T. Small airway caliber is the most important contributor of wheezing in healthy unselected newborns. Am J Respir Crit Care Med 2011; 183:553; author reply -4.
6. Rasmussen F, Taylor DR, Flannery EM, Cowan JO, Greene JM, Herbison GP, et al. Risk factors for hospital admission for asthma from childhood to young adulthood: a longitudinal population study. J Allergy Clin Immunol 2002; 110:220-7.
7. Hogg JC. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. Lancet 2004; 364:709-21.
8. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 2008; 359:61-73.
9. Canoy D, Pekkanen J, Elliott P, Pouta A, Laitinen J, Hartikainen AL, et al. Early growth and adult respiratory function in men and women followed from the fetal period to adulthood. Thorax 2007; 62:396-402.
10. Lawlor DA, Ebrahim S, Davey Smith G. Association of birth weight with adult lung function: findings from the British Women's Heart and Health Study and a meta-analysis. Thorax 2005; 60:851-8.
11. Suresh S, Mamun AA, O'Callaghan M, Sly PD. The impact of birth weight on peak lung function in young adults. Chest 2012; 142:1603-10.
12. Lima Rda C, Victora CG, Menezes AM, Barros FC. Respiratory function in adolescence in relation to low birth weight, preterm delivery, and intrauterine growth restriction. Chest 2005; 128:2400-7.
13. Lum S, Hoo AF, Dezateux C, Goetz I, Wade A, DeRooy L, et al. The association between birthweight, sex, and airway function in infants of nonsmoking mothers. Am J Respir Crit Care Med 2001; 164:2078-84.
14. Hancox RJ, Poulton R, Greene JM, McLachlan CR, Pearce MS, Sears MR. Associations between birth weight, early childhood weight gain and adult lung function. Thorax 2009; 64:228-32.
15. Sherrill DL, Guerra S, Wright AL, Morgan WJ, Martinez FD. Relation of early childhood growth and wheezing phenotypes to adult lung function. Pediatr Pulmonol 2011; 46:956-63.
16. van der Gugten AC, Koopman M, Evelein AM, Verheij TJ, Uiterwaal CS, van der Ent CK. Rapid early weight gain is associated with wheeze and reduced lung function in childhood. Eur Respir J 2012; 39:403-10.
17. Niklasson A, Ericson A, Fryer JG, Karlberg J, Lawrence C, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). Acta Paediatr Scand 1991; 80:756-62.
18. Bland JM, Altman DG. Measurement error. BMJ 1996; 312:1654.

19. Miller MR, Crapo R, Hankinson J, Brusasco V, Burgos F, Casaburi R, et al. General considerations for lung function testing. Eur Respir J 2005; 26:153-61.
20. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J 2005; 26:319-38.
21. Wanger J, Clausen JL, Coates A, Pedersen OF, Brusasco V, Burgos F, et al. Standardisation of the measurement of lung volumes. Eur Respir J 2005; 26:511-22.
22. Standardization of Spirometry, 1994 Update. American Thoracic Society. Am J Respir Crit Care Med 1995; 152:1107-36.
23. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. Eur Respir J Suppl 1993; 16:5-40.
24. Bjermer L. The role of small airway disease in asthma. Curr Opin Pulm Med 2014; 20:23-30.
25. Lipworth B, Manoharan A, Anderson W. Unlocking the quiet zone: the small airway asthma phenotype. Lancet Respir Med 2014; 2:497-506.
26. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. Eur Respir J 2012; 40:1324-43.
27. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J 1995; 8:483-91.
28. Boezen HM, Vonk JM, van Aalderen WM, Brand PL, Gerritsen J, Schouten JP, et al. Perinatal predictors of respiratory symptoms and lung function at a young adult age. Eur Respir J 2002; 20:383-90.
29. Kotecha SJ, Watkins WJ, Paranjothy S, Dunstan FD, Henderson AJ, Kotecha S. Effect of late preterm birth on longitudinal lung spirometry in school age children and adolescents. Thorax 2012; 67:54-61.
30. Debray TP, Moons KG, Abo-Zaid GM, Koffijberg H, Riley RD. Individual participant data meta-analysis for a binary outcome: one-stage or two-stage? PLoS One 2013; 8:e60650.
31. Normand SL. Meta-analysis: formulating, evaluating, combining, and reporting. Stat Med 1999; 18:321-59.
32. van Houwelingen HC, Arends LR, Stijnen T. Advanced methods in meta-analysis: multivariate approach and meta-regression. Stat Med 2002; 21:589-624.
33. Quanjer PH, Stanojevic S, Stocks J, Hall GL, Prasad KV, Cole TJ, et al. Changes in the FEV(1)/FVC ratio during childhood and adolescence: an intercontinental study. Eur Respir J 2010; 36:1391-9.
34. Baron RM, Kenny DA. The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. J Pers Soc Psychol 1986; 51:1173-82.
35. Cerin E, MacKinnon DP. A commentary on current practice in mediating variable analyses in behavioural nutrition and physical activity. Public Health Nutrition 2009; 12:1182-8.
36. MacKinnon DP, Fairchild AJ. Current Directions in Mediation Analysis. Current Directions in Psychological Science 2009; 18:16-20.
37. Narayanan M, Owers-Bradley J, Beardsmore CS, Mada M, Ball I, Garipov R, et al. Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. Am J Respir Crit Care Med 2012; 185:186-91.
38. Barker DJ, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO. Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. BMJ 1991; 303:671-5.

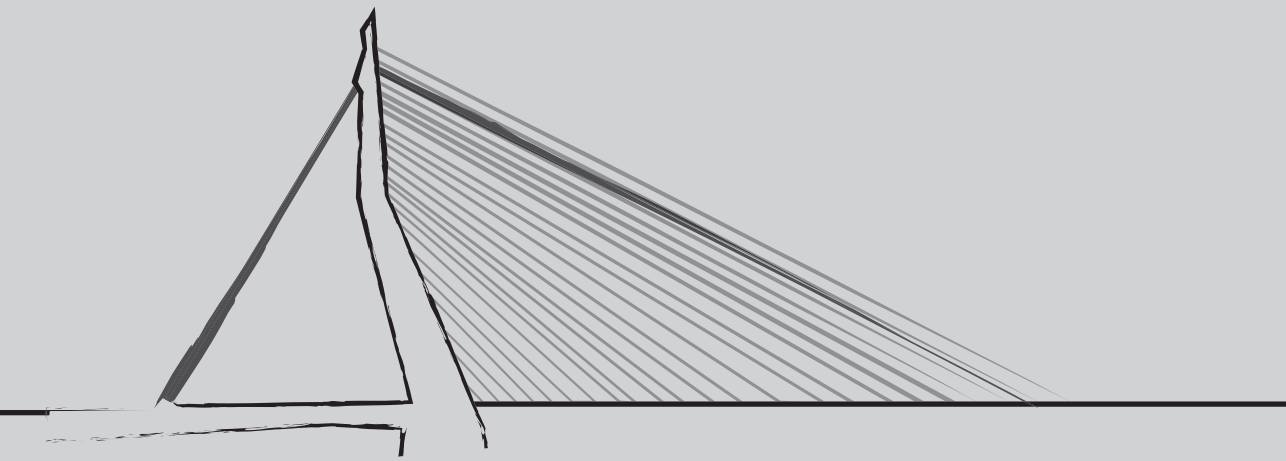
39. Bisgaard H, Jensen SM, Bonnelykke K. Interaction between asthma and lung function growth in early life. Am J Respir Crit Care Med 2012; 185:1183-9.
40. Haland G, Carlsen KC, Sandvik L, Devulapalli CS, Munthe-Kaas MC, Pettersen M, et al. Reduced lung function at birth and the risk of asthma at 10 years of age. N Engl J Med 2006; 355:1682-9.
41. Stocks J, Sonnappa S. Early life influences on the development of chronic obstructive pulmonary disease. Ther Adv Respir Dis 2013; 7:161-73.
42. Kotecha SJ, Edwards MO, Watkins WJ, Henderson AJ, Paranjothy S, Dunstan FD, et al. Effect of preterm birth on later FEV1: a systematic review and meta-analysis. Thorax 2013; 68:760-6.
43. Narang I, Rosenthal M, Cremonesini D, Silverman M, Bush A. Longitudinal evaluation of airway function 21 years after preterm birth. Am J Respir Crit Care Med 2008; 178:74-80.
44. Vollsaeter M, Roksund OD, Eide GE, Markestad T, Halvorsen T. Lung function after preterm birth: development from mid-childhood to adulthood. Thorax 2013; 68:767-76.
45. Miller MR, Pincock AC. Predicted values: how should we use them? Thorax 1988; 43:265-7.
46. ad hoc Statement Committee ATS. Mechanisms and limits of induced postnatal lung growth. Am J Respir Crit Care Med 2004; 170:319-43.
47. Scholtens S, Wijga AH, Seidell JC, Brunekreef B, de Jongste JC, Gehring U, et al. Overweight and changes in weight status during childhood in relation to asthma symptoms at 8 years of age. J Allergy Clin Immunol 2009; 123:1312-8 e2.
48. Rzehak P, Wijga AH, Keil T, Eller E, Bindslev-Jensen C, Smit HA, et al. Body mass index trajectory classes and incident asthma in childhood: results from 8 European Birth Cohorts--a Global Allergy and Asthma European Network initiative. J Allergy Clin Immunol 2013; 131:1528-36.
49. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 2004; 89:2548-56.
50. Dixon AE, Holguin F, Sood A, Salome CM, Pratley RE, Beuther DA, et al. An official American Thoracic Society Workshop report: obesity and asthma. Proc Am Thorac Soc 2010; 7:325-35.
51. Kotecha S. Lung growth for beginners. Paediatr Respir Rev 2000; 1:308-13.
52. Cole DA, Preacher KJ. Manifest variable path analysis: potentially serious and misleading consequences due to uncorrected measurement error. Psychol Methods 2014; 19:300-15.

Chapter 2.2

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

HT den Dekker, VWV Jaddoe, IK Reiss, JC de Jongste, L Duijts

Submitted



ABSTRACT

Background Children with lower birth weight are at increased risk of asthma-symptoms. We examined associations of fetal and infant growth with childhood lung function and asthma.

Methods This study was embedded in a population-based prospective cohort study among 5,536 children. Growth was estimated by repeated ultrasounds in 2nd and 3th trimester, and measured at birth and at 3, 6 and 12 months. At age 10 years, spirometry was performed and asthma was assessed by parental questionnaire. We applied linear and logistic regression analyses, and conditional regression taking into account correlations between repeated growth measures.

Results Restricted fetal weight growth between 2nd trimester and birth was independent of infant weight growth associated with a lower FEV_1 (range z-scores: -0.25 to -0.13) whereas restricted fetal weight growth in the same period followed by normal or accelerated weight growth during the first three postnatal months was associated with a lower FEV_1/FVC (z-score differences (95% CI): -0.18 (-0.32, -0.04) and -0.13 (-0.25, -0.00), respectively). Restricted fetal weight growth between 2nd trimester and birth followed by restricted or normal weight growth between birth and 3 months was associated with lower FEF_{25-75} (-0.29 (-0.56, -0.01) and FEF_{75} (-0.17 (-0.31, -0.03), respectively. Accelerated fetal weight growth followed by accelerated infant weight growth was associated with higher FVC (0.23 (0.07, 0.38)) and lower FEV_1/FVC (-0.23 (-0.39, -0.07)).

Conclusion Both restricted fetal weight growth, partly dependent of infant weight growth, and accelerated fetal and infant weight growth predispose children for lower lung function and an increased risk of asthma.

INTRODUCTION

Early growth characteristics have been associated with increased risks of respiratory morbidity in later life.[1,2] Altered fetal and infant growth may result in developmental adaptations with smaller airway dimensions, leading to a lower lung function and increased asthma risk.[3] Previous studies examining individual growth characteristics showed that higher fetal crown–rump length in first trimester, a greater abdominal circumference or a higher femur length in second pregnancy were associated with a lower risk of wheezing, atopic wheezing, asthma, or higher forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) and forced expiratory flow between 25-75% of FVC (FEF_{25-75}) at age 5-10 years.[4,5] Also, we previously showed that fetal growth restriction is associated with higher respiratory resistance in children aged 6 years [6], and that weight gain acceleration in early infancy is associated with an increased risk of asthma symptoms in preschool children.[7] Studies focused on combined fetal and infant growth patterns with respiratory morbidity in late childhood are scarce [6-8], and specific critical periods of growth have not yet been fully identified. Additionally, children's current body mass index (BMI) and atopy might affect associations of early growth with childhood lung function and asthma.[9]

Therefore, we aimed to identify critical periods for the effects of adverse growth in Fetal life and early infancy on respiratory morbidity using a population-based prospective cohort study. We examined the associations of fetal and infant individual growth measures, and combined growth patterns throughout fetal life and early infancy, with lung function and asthma among 5,635 children aged 9-10 years. Additionally, we examined whether these associations were modified by child's current BMI or inhalant allergic sensitization.

MATERIALS AND METHODS

General design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in Rotterdam, The Netherlands.[10] The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam (MEC 40020.078.12/2012/165). Written informed consent was obtained from parents or legal representatives (n = 7,393). Twins and children with missing data on all fetal and infant growth measures (n = 213), lung function or current asthma (n=1,545) were excluded, resulting in a total of 5,635 children for current analyses (S-Figure 2.2.1).

Fetal and infant growth

Gestational age was established by ultrasound in the first trimester.[7] We used crown-rump length to assess fetal growth only in mothers with a known and reliable first day of the last menstrual period and a regular cycle of 28 (range 24 - 32) days.[11] In the second and third trimester, head circumference, abdominal circumference and femur length were measured using standardized ultrasound procedures. Femur length was used as proxy for fetal length. Estimated fetal weight was calculated using the Hadlock formula.[12] Fetal growth characteristics were converted into standard deviation scores (SDS) according to reference growth charts.[13] Length and weight at birth were obtained from midwifes, general practitioners and hospital registries. Gestational age-adjusted SDS for length and weight at birth were constructed using reference growth charts.[14] Infant growth characteristics were measured at community health centers according to standard procedures at the ages of 3 months (range 3.0 - 4.0 months), 6 months (range 5.0 - 9.9 months) and 12 months (range 10.0 - 13.0 months). SDS for postnatal length and weight were obtained using reference growth charts (Growth Analyzer 3.0, Dutch Growth Research Foundation).[15] We used both fetal and infant length and weight measures to explore the role growth in relation to childhood asthma.

Childhood lung function and asthma

Spirometry was performed at a median age of 9.7 years (range 8.5 – 12.0 years) according to the American Thoracic Society and European Respiratory Society (ATS/ERS) recommendations.[16] Additionally, 122 children were included with individual spirometry curves with >5% deviation, but with at least one blow according to ATS/ERS criteria with adequate reach and duration of plateau. We observed no difference in lung function values between the group children with and without stringent criteria and no difference in size or direction of the effect estimates in our analyses when we included or excluded these children. All lung function variables were converted into sex-, age-, height- and ethnicity-adjusted z-scores according to the Global Lung Initiative (GLI) reference data.[17] Ever physician diagnosed asthma, wheezing and use of inhalant medication (bronchodilators, corticosteroids) in the previous 12 months at age 10 years were reported by a parental questionnaire. Response rate was 75%. Current asthma (no; yes) was defined as ever physician diagnosed asthma at age 10 years with either wheezing or the use of inhalant medication in the previous 12 months.

Covariates

Information about maternal age, pre-pregnancy BMI, highest level of education (primary or secondary; higher), history of asthma and atopy (no; yes), psychological distress during pregnancy (no; yes) and parity (nulli-; multiparous) was obtained by a maternal questionnaire completed at enrolment. The Global Severity Index was used to measure

psychological distress during pregnancy.[18] Maternal smoking during pregnancy was assessed by questionnaires throughout pregnancy and combined into smoking during pregnancy (never; until pregnancy known; continued throughout pregnancy). Child's sex and gestational age were obtained from midwife and hospital registries at birth. Child's ethnicity was based on parental countries of birth and classified according to the GLI definitions.[17] Breastfeeding (never; ever) was collected using questionnaires administered at 2, 6 and 12 months after birth. Child's length and weight were measured at the moment of spirometry. Inhalant allergic sensitization (no; yes) for *Dermatophagoides pteronyssinus*, 5-grass mixture (*Dactylis glomerata, Festuca pratensis, Lolium perenne, Phleum pratense, Poa pratensis*), birch (*Betula verrucosa*), cat (*Felis catus*) and dog (*Canis familiaris*) (ALK-Abelló B.V., Almere, The Netherlands) was measured by skin prick test using the 'scanned area method' at similar age of spirometry measurement.[19]

Statistical analysis

First, we compared characteristics of children included and not included in the study using ANOVA, Mann-Whitney tests, and Chi-square tests. Second, we used linear and logistic regression models to assess the associations of individual growth characteristics throughout early life with lung function and asthma. Third, we applied conditional regression analyses to identify independent critical early life growth periods associated with respiratory outcomes.[20] For these analyses, we constructed length and weight variables using standardized residuals resulting from the linear regression model of length and weight regressed on the prior corresponding growth measurements. These variables are statistically independent from each other and thereby allow simultaneous inclusion in multiple regression models.[20] Fourth, we explored growth patterns throughout early life. Restricted and accelerated weight growth were defined as weight growth percentile change between the time periods of less than -0.67 SDS or more than 0.67 SDS, respectively. These values represent the width of each percentile band on standard weight growth charts.[7,21] We assessed the associations of restricted and accelerated weight growth between the second trimester and birth, between birth and 3 months, and between 3 and 12 months using linear and logistic regression models. Last, we combined restricted and accelerated weight growth patterns in fetal life (second trimester to birth) and infancy (birth to 3 months) based on previous studies [22, 23] and our current observations with normal fetal and infant weight growth patterns as reference category. Selection of covariates was based on literature, if the effect estimate of the unadjusted analyses changed ≥10% after adjustment for a covariate, or if covariates were strongly related to the determinant and outcome and not in the causal pathway. The percentage of missing data for covariates was <20%, except for maternal psychological distress during pregnancy (24.5%). Missing data in covariates was imputed to reduce bias and improve efficiency using the Markov Chain Monte Carlo method to select the most likely value for a missing response.[24] Ten new datasets were

constructed. No differences in results were observed between analyses with and without imputed data. We only present results based on imputed data. The modifying effects of child's current BMI and inhalant allergic sensitization were tested by adding them as product terms with the growth characteristics in the models. Analyses were performed using SPSS version 21.0 for Windows (IBM, Chicago, Ill, USA).

RESULTS

Subject characteristics

Maternal and child characteristics are presented in Tables 2.2.1 and 2.2.2. Children were born after a median pregnancy duration of 40.1 (2.5-97.5% range: 35.8 - 42.3) weeks with a mean birth weight of 3,437 (SD 555) gram. Current asthma was reported in 5.7% (n=269). Mothers lost to follow-up were younger, lower educated and smoked more during pregnancy, and their children were more often of non-Caucasian ethnicity (S-Table 2.2.1).

Fetal and infant growth related to childhood respiratory outcomes

The associations of each individual Fetal, birth and infant growth characteristic with childhood lung function and asthma are shown in S-Tables 2.2.2 and 2.2.3. We observed in conditional analyses that fetal and infant length were not independently associated with lung function measures or current asthma (S-Table 2.2.4). In conditional analyses, we observed that a greater weight in the second and third trimester, at birth and at 12 months was independently of other time periods associated with higher FEV_1 and FVC (range z-score difference: 0.04 to 0.08, per SDS increase in weight), while only a greater weight at birth was associated with an increased risk of childhood asthma (OR (95% CI): 1.34 (1.09, 1.66) per SDS increase in weight) (Table 2.2.3). A greater weight at 3 months was associated with a higher FVC (z-score (95% CI): 0.06 (0.02, 0.10), and a lower FEV_1/FVC and FEF_{75} (-0.09 (-0.14, -0.05) and -0.09 (-0.13, -0.05), respectively).

Fetal and infant growth patterns related to childhood respiratory outcomes

When assessed per time period, we observed that restricted weight growth between 2nd trimester and birth was not associated with lung function measures or asthma, while accelerated growth was with higher FVC (0.10 (0.03, 0.16) and lower FEV_1/FVC (-0.08 (-0.15, -0.01)) compared with normal weight growth in that period (Figure 2.2.1, S-Table 5). Between birth and age 3 months, restricted weight growth was associated with an increased risk of childhood asthma (OR (95% CI): 1.57 (1.01, 2.45)), and accelerated weight growth with a lower FEV_1, FEV_1/FVC and FEF_{75} (-0.10 (-0.18, -0.02), -0.08 (-0.16, -0.00) and -0.07 (-0.14, -0.00), respectively), compared to normal weight growth. Weight growth patterns between age 3 and 12 months were not associated with lung function measures or asthma.

Table 2.2.1. Characteristics of Parents and Their Children.

	Study population n = 5,635
Maternal characteristics	
Age (years)	31.0 (4.9)
Pre-pregnancy body mass index (kg/m^2)$^{\#}$	23.7 (18.8 – 35.6)
Education (%)	
No, primary or secondary	51.6 (2,909)
Higher	48.4 (2,726)
History of asthma or atopy, yes (%)	39.6 (2,234)
Psychological distress, yes (%)	8.9 (503)
Nulli parity (%)	57.3 (3,229)
Smoking during pregnancy (%)	
Never	75.6 (4,262)
Until pregnancy known	9.0 (508)
Yes, continued	15.4 (865)
Child characteristics	
Female sex (%)	50.3 (2,829)
Gestational age at birth (weeks)$^{\#}$	40.1 (35.8-42.3)
Birth weight (grams)	3,437 (555)
Ethnicity (%)	
Caucasian	81.4 (4,588)
Black	14.6 (822)
Asian	2.6 (144)
Other/mixed	1.4 (81)
Breastfeeding, ever (%)	92.5 (5,212)
Inhalant allergic sensitization (yes)	33.1 (1,397)
Body mass index (kg/m^2)$^{\#}$	17.0 (14.0 – 24.8)
FEV_1 (L)	2.01 (0.30)
FVC (L)	2.32 (0.37)
FEV_1/FVC	0.87 (0.06)
FEF_{25-75} (L/s)	2.69 (0.65)
FEF_{75} (L/s)	1.14 (0.35)
Current asthma (yes / total)	5.7 (269)

Values are means (standard deviation), $^{\#}$medians (2.5-97.5 percentile) or percentages (absolute numbers) based on imputed data. Data on growth characteristics, lung function and current asthma were not imputed, and missing data ranged from 0 subjects (maternal age) tot 1,359 subjects (maternal psychological distress).

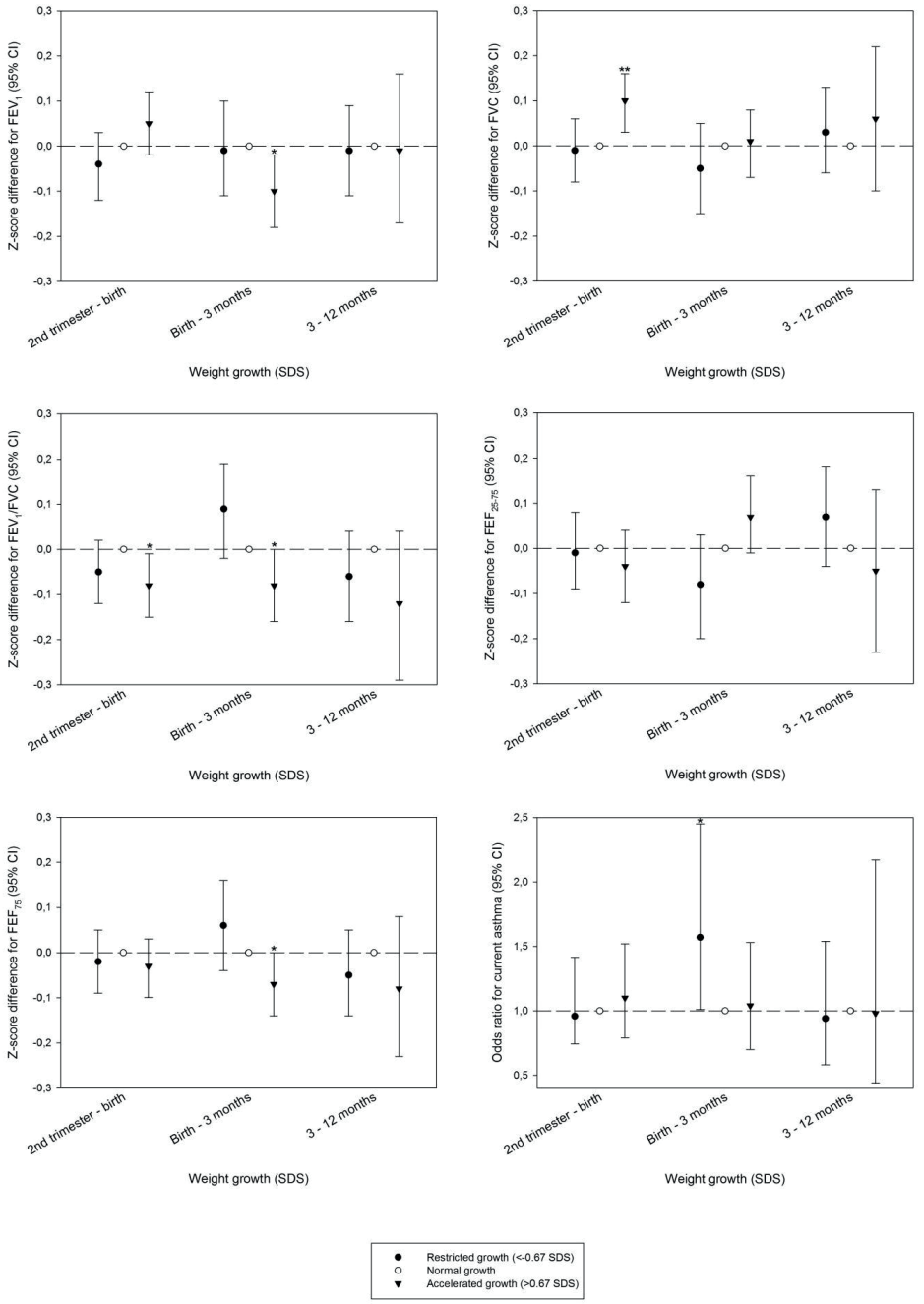


Figure 2.2.1. Associations of Weight Growth Patterns with Lung Function and Current Asthma.

Values are Z-scores (95% confidence interval) of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75} and odds ratio of current asthma per 1 SDS change in growth characteristic from linear and logistic regression models. Restricted fetal and infant growth was defined as <-0.67 SDS increase between second trimester and birth, between birth and 3 months, and between 3 and 12 months, respectively. Accelerated fetal and infant growth was defined as >0.67 SDS increase between second trimester and birth, between birth and 3 months, and between 3 and 12 months, respectively. Children with normal fetal and infant weight growth were used as reference. *P < 0.05, **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, smoking during pregnancy and child's sex, gestational age, ethnicity and breastfeeding.

Table 2.2.2. Descriptives of Growth Characteristics.

	Number of individuals	Mean / median
Growth characteristics		
First trimester		
Crown-rump length (mm)	1,096	60.9 (11.7)
Second trimester		
Abdominal circumference (mm)	4,870	157.1 (14.8)
Femur length (mm)	4,872	33.5 (3.5)
Estimated fetal weight[#] (g)	4,846	363.9 (246 – 624)
Third trimester		
Abdominal circumference (mm)	4,976	264.5 (16.7)
Femur length (mm)	4,987	57.5 (3.0)
Estimated fetal weight (g)[#]	4,968	1,626 (263)
Birth		
Weight (g)	5,635	3,436 (556)
Length (cm)	3,532	50.3 (2.4)
3 months		
Weight (g)	3,410	6,281 (758)
Length (cm)	2,905	61.4 (2.5)
6 months		
Weight (g)	4,261	7,858 (909)
Length (cm)	3,813	67.6 (2.7)
12 months		
Weight (g)	3,951	9,625 (1,060)
Length (cm)	3,947	74.3 (2.6)

Values are means (standard deviation) or [#]medians (2.5-97.5 percentile) based on observed data. "n =" represents the number of the total group. Crown-rump length was largely missing due to assessment only in mothers with a known and reliable first day of the last menstrual period and a regular cycle of 28 (range 24-32) days.

Table 2.2.3. Associations of Fetal and Infant Growth Characteristics with Lung Function and Current Asthma.

	FEV_1	FVC	FEV_1/FVC	FEF_{25-75}	FEF_{75}	Current asthma
	Z-score (95% CI)	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Odds ratio (95% CI)**
Weight (SDS)						
Second trimester	**0.05 (0.01, 0.10)***	**0.05 (0.01, 0.09)***	0.01 (-0.03, 0.05)	-0.01 (-0.05, 0.04)	0.03 (-0.01, 0.07)	1.18 (0.95, 1.46)
Third trimester	**0.08 (0.04, 0.13)****	**0.07 (0.03, 0.11)****	0.01 (-0.03, 0.05)	0.04 (-0.01, 0.08)	0.02 (-0.02, 0.06)	1.05 (0.85, 1.30)
Birth	**0.07 (0.03, 0.11)****	**0.07 (0.03, 0.11)****	-0.01 (-0.05, 0.04)	0.01 (-0.04, 0.05)	-0.00 (-0.04, 0.04)	**1.34 (1.09, 1.66)****
3 months	0.00 (-0.04, 0.05)	**0.06 (0.02, 0.10)****	**-0.09 (-0.14, -0.05)****	0.03 (-0.02, 0.07)	**-0.09 (-0.13, -0.05)****	1.09 (0.89, 1.35)
6 months	0.02 (-0.02, 0.06)	0.04 (-0.00, 0.07)	-0.04 (-0.08, 0.01)	0.02 (-0.03, 0.06)	-0.03 (-0.07, 0.01)	0.96 (0.78, 1.18)
12 months	**0.04 (0.00, 0.08)***	**0.05 (0.01, 0.09)***	-0.02 (-0.06, 0.02)	-0.03 (-0.07, 0.02)	-0.00 (-0.04, 0.04)	1.02 (0.83, 1.25)

Values are Z-scores (95% confidence interval) of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75} and odds ratio of current asthma per 1 SDS change in growth characteristic from conditional linear and logistic regression models taking correlation between Fetal and infant growth characteristics and measurements at multiple time points into account. *P < 0.05, **P < 0.01 and ***P < 0.001. Models were adjusted for maternal age, pre-pregnancy body mass index, educational level, history of asthma or atopy, psychological distress during pregnancy, parity, smoking during pregnancy and child's sex, gestational age, ethnicity and breastfeeding.

When we combined restricted and accelerated weight growth patterns in fetal and infant life, we observed that restricted fetal weight growth was associated with a lower FEV_1, independent of the infant weight growth pattern, compared with normal weight growth in fetal and infant life (range in z-score: -0.25 to -0.13) (Figure 2.2.2, S-Table 2.2.6). Restricted fetal weight growth was associated with a lower FVC and FEF_{25-75} (-0.30 (-0.53, -0.06) and -0.29 (-0.56, -0.01), respectively) if followed by restricted infant weight growth, with lower FEV_1/FVC (-0.18 (-0.32, -0.04) and -0.13 (-0.25, -0.00), respectively) if followed by normal or accelerated infant weight growth, and with lower FEF_{75} (-0.17 (-0.31, -0.03)) if followed by normal infant growth. Normal fetal weight growth followed by accelerated infant weight growth was associated with a higher FEF_{25-75} (0.14 (0.01, 0.27)). Accelerated fetal weight growth followed by accelerated infant weight growth was not associated with FEV_1, but was associated with a higher FVC (0.23 (0.07, 0.38)) and lower FEV_1/FVC (-0.23 (-0.39, -0.07)).

Associations between fetal and infant weight growth and lung function and asthma were not modified by child's current BMI or inhalant allergic sensitization (p-values for interaction >0.05).

DISCUSSION

We observed that in the full group overall greater weight in second and third trimester, at birth and at 12 months was associated with higher FEV_1 and FVC, while greater weight at 3 months was associated with a lower FEV_1/FVC and FEF_{75}, independent of weight in other periods. When using stratified analyses, restricted fetal weight growth was associated with a lower childhood FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and $FEF7_5$, partly dependent of weight growth patterns in infant life. Accelerated fetal weight growth was associated with higher FVC and lower FEV_1/FVC, but only if followed by accelerated infant weight growth. Length throughout fetal life and infancy was not associated with lung function and asthma in childhood. Results were not modified by child's current BMI or inhalant allergic sensitization.

Comparison with previous studies

A limited number of studies examined the associations of fetal growth with lung function and asthma.[4, 5, 7, 8] A prospective birth cohort study among 927 children showed that a higher crown–rump length in first trimester was associated with a higher FEV_1, FVC and FEF_{25-75}, and a lower risk of wheezing and asthma until age 10 years.[4,5] Also, a higher femur length in second trimester was associated with lower risk of asthma.[4] Compared with persistent greater growth in first and second trimester, defined as a greater CRL in first trimester and a greater BPD in second trimester, persistent smaller growth was

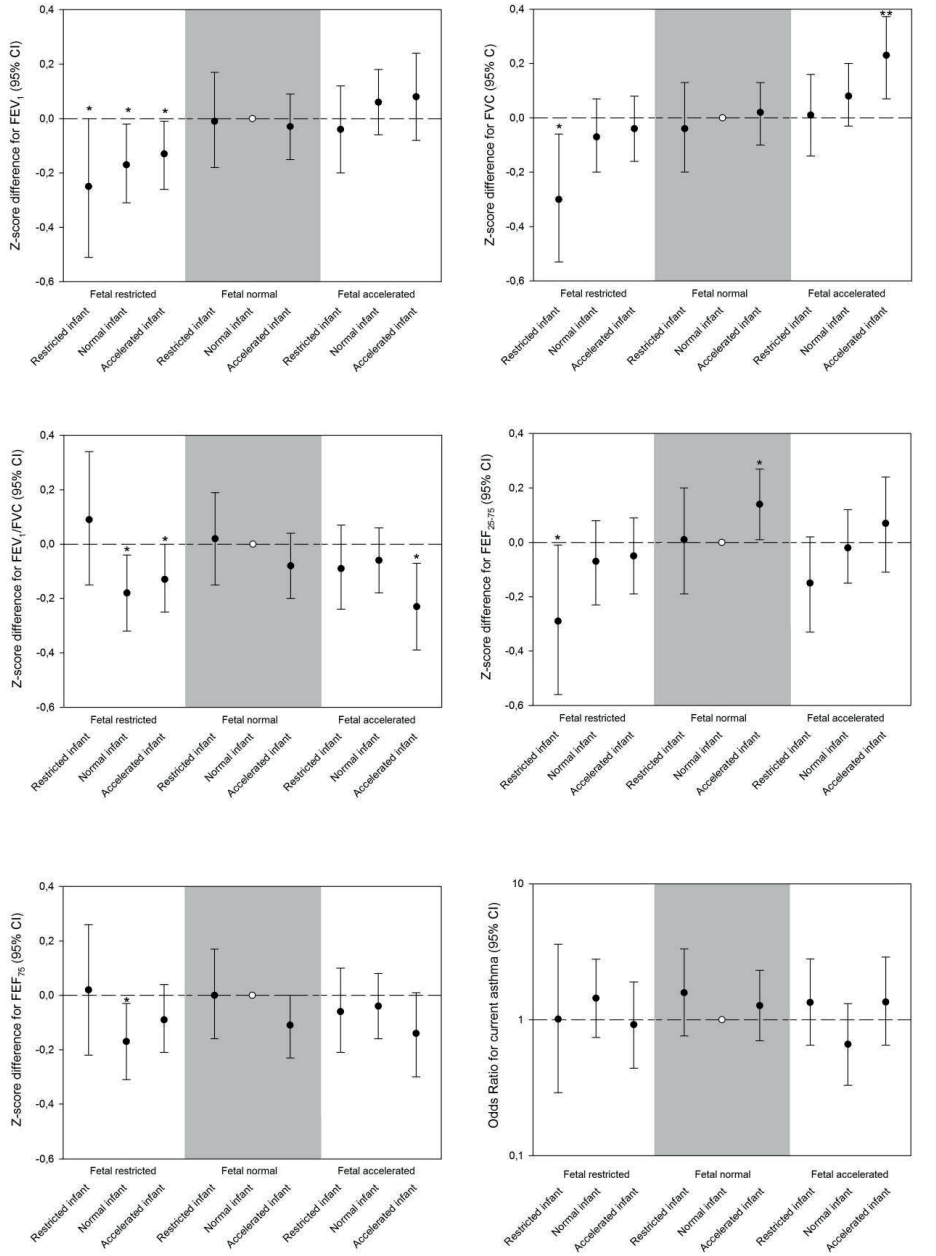


Figure 2.2.2. Associations of Weight Growth Patterns Combined from Fetal and Infant Life with Lung Function and Current Asthma.

Values are Z-scores (95% confidence interval) of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75} and odds ratio of current asthma per 1 SDS change in growth characteristic from linear and logistic regression models. Restricted fetal and infant growth was defined as <-0.67 SDS increase between second trimester and birth, and between birth and 3 months, respectively. Accelerated fetal and infant growth was defined as >0.67

SDS increase between second trimester and birth, and between birth and 3 months, respectively. Children with normal fetal and infant weight growth were used as reference and represented by the white dot. *P < 0.05, **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, smoking during pregnancy and child's sex, gestational age, ethnicity and breastfeeding.

associated with lower FEV_1 and increased risk of asthma.[5] In a population based birth cohort among 1,548 children, a greater abdominal circumference between 19 and 34 weeks of gestation was associated with a lower risk of atopic wheezing at age 3 years.[8] Smaller fetal head circumference growth between 11 and 19 weeks of gestation was associated with an increased risk of non-atopic wheeze.[8] We did observe similar associations between fetal abdominal circumference and femur length with childhood lung function. However, in conditional analyses we observed that femur length, as proxy for fetal length, and infant length were not independently associated with lung function or current asthma in childhood. Fetal weight was associated with childhood lung function. This suggests that the associations between fetal length characteristics are depending on length growth throughout fetal life and early pregnancy. Therefore, length measures during pregnancy are not solitary predictive for lung function and asthma in later childhood, whereas weight measures are.

Previous studies report inconsistent associations of estimated fetal weight growth with childhood wheezing or asthma.[4,6,7] Restricted fetal weight growth has been associated with increased respiratory resistance [6] and lower FEV_1 in childhood.[4] We did not observe associations of restricted fetal weight growth with lung function or asthma, when not taking into account infant weight growth. In contrast, we observed that accelerated weight growth between second trimester and birth was associated with a higher FVC and lower FEV_1/FVC.

However, when taking infant weight growth patterns into account, the associations of restricted and accelerated fetal weight growth structurally changed, suggesting a strong influence of weight growth in early infancy.

Studies on the associations of infant length with childhood lung function and asthma show conflicting results.[6-8, 22] Two studies reported that length growth between birth and 12 months was not associated with wheezing at ages 3 and 4 years.[7,8] More rapid height gain in early childhood and mid-childhood was associated with lower FEV_1 and FVC at age 15 years, but not with other lung function measures or with lung function at age 8 years.[22] In our cohort, lower infant length growth was previously associated with a higher airway resistance (Rint).[6] We observed that infant length growth was not associated with childhood lung function or the risk of asthma, which suggests that length measures in early infancy are not independently associated with childhood lung function and asthma. Differences in results with previous studies could be explained by the transient character of wheezing in early childhood and the use of conditional modelling.

Previous studies reported that term-born children with a greater infant weight growth had lower $FEV_{0.4}$-values in the first 3 months of life [25], and that increased growth between 1 and 12 months of age was associated with lower maximal flow at functional residual capacity during the same period.[26] In a population-based cohort study amongst 4,492 term born children followed up for 18 months, infant weight and infant weight gain velocity were associated with wheezing (ORs 1.28 and 1.30, respectively).[27] In a cohort study comprising 9,723 children, greater weight gain between birth and 3 months was associated with lower FEV_1/FVC and FEF_{25-75} at age 8 years.[22] We observed that accelerated weight growth between birth and 3 months, but not between 3 and 12 months, was associated with lower FEV_1, FEV_1/FVC and FEF_{75}, when not taking fetal growth into account. This is in line with a meta-analysis on BMI-gain in early childhood and mid-childhood, reporting that more greater BMI-gain in the first 2 years of life, but not thereafter, was associated with an increased incidence of asthma at age 6 years.[28] Similarly, in a meta-analyses amongst 25,000 European children, greater weight gain in the first year of infancy was associated with higher FEV_1, but lower FEV_1/FVC and FEF_{75}, and an increased risk of childhood asthma.[1] Additionally, although we observed similar associations of weight growth in infancy with lung function and asthma in childhood, we now observed that these associations are partly depending on fetal weight growth.

In summary, our results suggest that fetal and infant length are not independently associated with childhood lung function and asthma, whereas fetal and infant weight growth is. The associations of fetal weight growth are partly dependent of weight growth in early infancy and vice versa, which suggests that it is the combined weight growth in fetal life and the first 3 months of infancy most strongly is associated with childhood respiratory health.

Interpretation of results

We observed that both restricted and accelerated fetal weight growth were associated with lower childhood lung function and asthma. Different pathways might be responsible for this similar direction in association. The highest rates of airway and alveoli development occurs in fetal life, although both airways and alveoli development continues until the age of 21 years.[29] According to animal studies, fetal growth restriction might affect airway compliance.[30] Restricted fetal growth might lead to impaired growth of bronchial walls, alterations in mucus producing tissues, a decrease in number of alveoli, thicker inter-alveolar septa and a greater volume density of lung tissue.[31] This in in line with our observations that restricted fetal weight growth was associated with lower FEV_1, independent of weight growth in infancy, and that restricted fetal weight growth followed by restricted, normal or accelerated weight growth was associated with lower FVC and FEF_{25-75}, with lower FEV_1/FVC and FEF_{75}, and with lower FEV_1/FVC, respectively.

Furthermore, the associations of greater weight growth with increased risks of respiratory morbidity may be confounded by catch-up growth in infancy. Recent studies suggested that catch-up growth is associated with a lower pulmonary function, and increased risks of childhood asthma.[1,2] This is in line with our observations, as we observed that in children with restricted fetal weight growth, accelerated infant weight growth was associated with a lower FEV_1 and FEV_1/FVC, but not with asthma. Also, accelerated fetal weight growth followed by accelerated weight growth between birth and 3 months was not associated with FEV_1, but was associated with an increased FVC, resulting in a lower FEV_1/FVC. This suggests dysanapsis, a determinant of expiratory flow limitation, in which a disproportional growth between airway size and lung volume occurs.[32]

Several potential mechanisms may underlie the link between early growth and respiratory diseases in children. An increased weight could lead to increased intrathoracic and abdominal fat deposition, which reduces the pulmonary vital capacity and increase obstruction-related respiratory resistance and the risk of asthma symptoms.[33] Also, adiposity-related inflammation and an effect of energy-regulating hormones such as leptin and adiponectin might cause tissue-specific immunological and inflammatory effects with lung and airway remodelling.[9] Future mechanistic studies are needed to explore possible underlying mechanisms of the effect of fetal and infant growth patterns on lung development and obstructive respiratory diseases.

Strengths and limitations

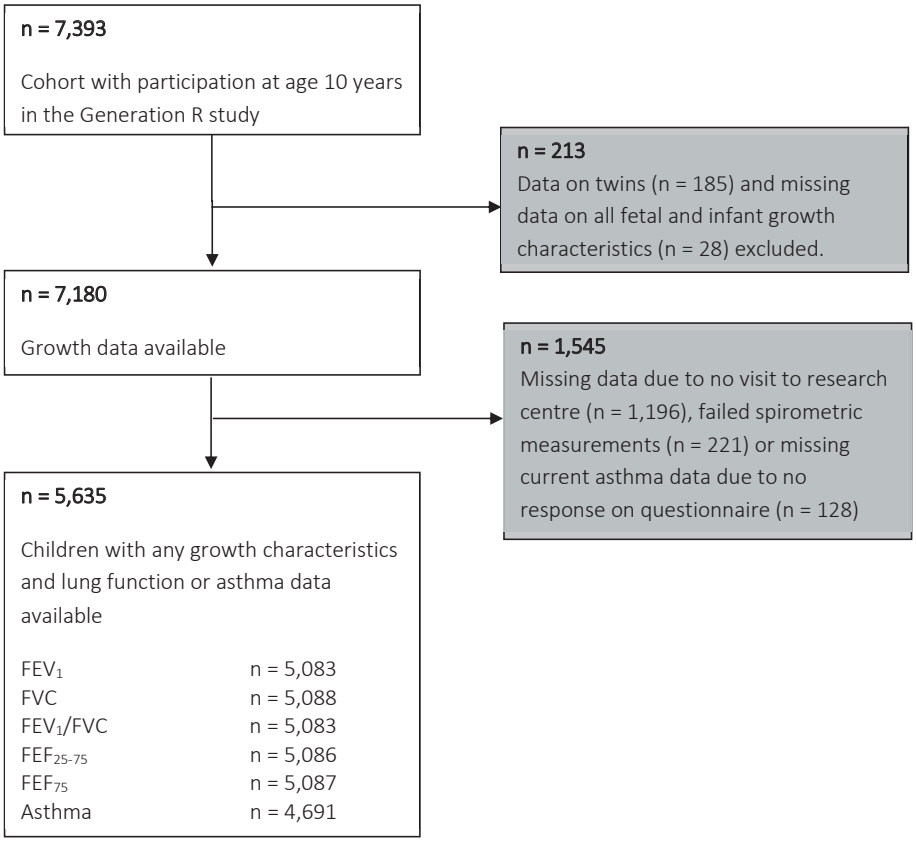
This study was embedded in a population-based prospective cohort study with a large number of subjects being studied from early fetal life onwards. Detailed and frequent measurements of head circumferences, length and weight in fetal and infant life were performed prospectively. Some limitations do apply. As in any prospective cohort study, our population was subject to loss to follow up. We did observe differences with individuals not included for analyses, which might have led to selection bias and limits generalizability of our results. Although the Hadlock formula is a validated tool to estimate fetal weight and intra- and inter-observer reproducibility for measurements of fetal growth in early pregnancy were high (34), we cannot exclude random measurement errors, which might have led to under- or overestimation of the true effect estimates. We adjusted for a large number of confounders, based on literature and univariate analyses. However, as in any observational study, we are not able to rule out residual confounding.

In conclusion, our results suggest that restricted weight growth between 2nd trimester and birth, partly dependent of infant weight growth, and accelerated weight growth between 2nd trimester and birth combined with accelerated infant growth between birth and 3 months, predisposes children for lower lung function and an increased risk of chronic obstructive respiratory diseases in later life. Further research for biological mechanisms and potential interventions is warranted.

REFERENCES

1. den Dekker HT, Sonnenschein-van der Voort AM, de Jongste JC, Anessi-Maesano I, Arshad SH, Barros H, et al. Early growth characteristics and the risk of reduced lung function and asthma: A meta-analysis of 25,000 children. J Allergy Clin Immunol. 2015.
2. Sonnenschein-van der Voort AM, Arends LR, de Jongste JC, Annesi-Maesano I, Arshad SH, Barros H, et al. Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. J Allergy Clin Immunol. 2014;133(5):1317-29.
3. Duijts L, Reiss IK, Brusselle G, de Jongste JC. Early origins of chronic obstructive lung diseases across the life course. Eur J Epidemiol. 2014;29(12):871-85.
4. Turner SW, Campbell D, Smith N, Craig LC, McNeill G, Forbes SH, et al. Associations between fetal size, maternal {alpha}-tocopherol and childhood asthma. Thorax. 2010;65(5):391-7.
5. Turner S, Prabhu N, Danielan P, McNeill G, Craig L, Allan K, et al. First- and second-trimester fetal size and asthma outcomes at age 10 years. Am J Respir Crit Care Med. 2011;184(4):407-13.
6. Sonnenschein-van der Voort AM, Gaillard R, de Jongste JC, Hofman A, Jaddoe VW, Duijts L. Foetal and infant growth patterns, airway resistance and school-age asthma. Respirology. 2016;21(4):674-82.
7. Sonnenschein-van der Voort AM, Jaddoe VW, Raat H, Moll HA, Hofman A, de Jongste JC, et al. Fetal and infant growth and asthma symptoms in preschool children: the Generation R Study. Am J Respir Crit Care Med. 2012;185(7):731-7.
8. Pike KC, Crozier SR, Lucas JS, Inskip HM, Robinson S, Southampton Women's Survey Study G, et al. Patterns of fetal and infant growth are related to atopy and wheezing disorders at age 3 years. Thorax. 2010;65(12):1099-106.
9. den Dekker HT, Ros KP, de Jongste JC, Reiss IK, Jaddoe VW, Duijts L. Body fat mass distribution and interrupter resistance, fractional exhaled nitric oxide, and asthma at school-age. J Allergy Clin Immunol. 2016.
10. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. Eur J Epidemiol. 2012;27(9):739-56.
11. Mook-Kanamori DO, Steegers EA, Eilers PH, Raat H, Hofman A, Jaddoe VW. Risk factors and outcomes associated with first-trimester fetal growth restriction. JAMA. 2010;303(6):527-34.
12. Hadlock FP, Harrist RB, Sharman RS, Deter RL, Park SK. Estimation of Fetal Weight with the Use of Head, Body, and Femur Measurements - a Prospective-Study. Am J Obstet Gynecol. 1985;151(3):333-7.
13. Verburg BO, Steegers EA, De Ridder M, Snijders RJ, Smith E, Hofman A, et al. New charts for ultrasound dating of pregnancy and assessment of fetal growth: longitudinal data from a population-based cohort study. Ultrasound Obstet Gynecol. 2008;31(4):388-96.
14. Niklasson A, Ericson A Fau - Fryer JG, Fryer Jg Fau - Karlberg J, Karlberg J Fau - Lawrence C, Lawrence C Fau - Karlberg P, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). (0001-656X (Print)).
15. Fredriks AM, van Buuren S, Burgmeijer RJ, Meulmeester JF, Beuker RJ, Brugman E, et al. Continuing positive secular growth change in The Netherlands 1955-1997. Pediatr Res. 2000;47(3):316-23.
16. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J. 2005;26(2):319-38.
17. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. Eur Respir J. 2012;40(6):1324-43.

18. Derogatis LR. Brief Symptom Inventory (BSI): Administration, Scoring and Procedures. Minneapolis, Minnesota: National Computer Systems; 1993.
19. van der Valk JP, Gerth van Wijk R, Hoorn E, Groenendijk L, Groenendijk IM, de Jong NW. Measurement and interpretation of skin prick test results. Clin Transl Allergy. 2015;6:8.
20. Keijzer-Veen MG, Euser AM, van Montfoort N, Dekker FW, Vandenbroucke JP, Van Houwelingen HC. A regression model with unexplained residuals was preferred in the analysis of the fetal origins of adult diseases hypothesis. J Clin Epidemiol. 2005;58(12):1320-4.
21. Ong KK, Ahmed ML, Emmett PM, Preece MA, Dunger DB. Association between postnatal catch-up growth and obesity in childhood: prospective cohort study. Bmj. 2000;320(7240):967-71.
22. Sonnenschein-van der Voort AM, Howe LD, Granell R, Duijts L, Sterne JA, Tilling K, et al. Influence of childhood growth on asthma and lung function in adolescence. J Allergy Clin Immunol. 2014.
23. van der Gugten AC, Koopman M, Evelein AM, Verheij TJ, Uiterwaal CS, van der Ent CK. Rapid early weight gain is associated with wheeze and reduced lung function in childhood. Eur Respir J. 2012;39(2):403-10.
24. Yuan YC. Multiple imputation for missing data: concepts and new development. Proceedings of the Twenty-Fifth Annual SAS Users Group International Conference. Paper No. 267. Cary, N.C.: SAS Institute; 2000.
25. Lucas JS, Inskip HM, Godfrey KM, Foreman CT, Warner JO, Gregson RK, et al. Small size at birth and greater postnatal weight gain: relationships to diminished infant lung function. Am J Respir Crit Care Med. 2004;170(5):534-40.
26. Turner S, Zhang G, Young S, Cox M, Goldblatt J, Landau L, et al. Associations between postnatal weight gain, change in postnatal pulmonary function, formula feeding and early asthma. Thorax. 2008;63(3):234-9.
27. Popovic M, Pizzi C, Rusconi F, Galassi C, Gagliardi L, De Marco L, et al. Infant weight trajectories and early childhood wheezing: the NINFEA birth cohort study. Thorax. 2016.
28. Rzehak P, Wijga AH, Keil T, Eller E, Bindslev-Jensen C, Smit HA, et al. Body mass index trajectory classes and incident asthma in childhood: Results from 8 European Birth Cohorts-a Global Allergy and Asthma European Network initiative. J Allergy Clin Immun. 2013;131(6):1528-+.
29. Narayanan M, Owers-Bradley J, Beardsmore CS, Mada M, Ball I, Garipov R, et al. Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. Am J Respir Crit Care Med. 2012;185(2):186-91.
30. Cock M, Hanna M, Sozo F, Wallace M, Yawno T, Suzuki K, et al. Pulmonary function and structure following mild preterm birth in lambs. Pediatr Pulmonol. 2005;40(4):336-48.
31. Harding R, Snibson K, O'Reilly M, Maritz GS. Early Life Origins of Human Health and Disease. Basel: Karger; 2009. p. 77-88.
32. Green M, Mead J, Turner JM. Variability of maximum expiratory flow-volume curves. J Appl Physiol. 1974;37(1):67-74.
33. Permaul P, Kanchongkittiphon W, Phipatanakul W. Childhood asthma and obesity-what is the true link? Ann Allerg Asthma Im. 2014;113(3):244-6.
34. Verburg BO, Mulder PG, Hofman A, Jaddoe VW, Witteman JC, Steegers EA. Intra- and interobserver reproducibility study of early fetal growth parameters. Prenat Diagn. 2008;28(4):323-31.



S-Figure 2.2.1. Flowchart of Participants.

S-Table 2.2.1. Comparison of the Study Population with Individuals Lost to Follow-up.

	Study population (n= 5,635)	Lost to follow-up (n = 1,545)	P-value for difference
Maternal characteristics			
Age (years)	31.0 (4.9)	29.4 (5.4)	<0.01
Pre-pregnancy body mass index[#] (kg/m^2)[#]	23.7 (18.8 – 35.6)	24.3 (18.5 – 36.7)	0.25
Education (%)			
No, primary or secondary	49.1 (2,568)	66.4 (881)	<0.01
Higher	50.9 (2,657)	33.6 (445)	
History of asthma or atopy, yes (%)	39.5 (1,811)	37.3 (431)	0.09
Psychological distress, yes (%)	8.1 (344)	13.3 (132)	<0.01
Nulli-parity (%)	57.3 (3,136)	51.1 (767)	<0.01
Smoking during pregnancy (%)			
Never	76.6 (3,800)	70.7 (915)	<0.01
Until pregnancy known	8.7 (430)	6.9 (89)	
Yes, continued	14.7 (728)	22.4 (290)	
Child characteristics			
Female sex (%)	50.2 (2,829)	48.3 (747)	0.20
Gestational age at birth (weeks)[#]	40.1 (35.7 – 42.3)	40.0 (35.9 – 42.3)	0.41
Birth weight (grams)	3,436 (553)	3,419 (556)	0.41
Ethnicity (%)			
Caucasian	82.0 (4,518)	78.6 (1,130)	<0.01
Black	14.7 (810)	18.0 (258)	
Asian	2.6 (144)	3.1 (44)	
Other/mixed	0.7 (38)	0.3 (5)	

Values are means (standard deviation), [#]medians (2.5-97.5 percentile) or percentages (absolute numbers) based on observed and imputed data. P-values for differences between the study population and individuals lost to follow-up were assessed by ANOVA, Mann-Whitney and Chi-square tests.

S-Table 2. Associations of Birth Characteristics with Lung Function and Current Asthma.

	FEV_1	FVC	FEV_1/FVC	FEF_{25-75}	FEF_{75}	Current asthma
	Z-score (95% CI)	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Odds ratio (95% CI)**
Gestational age						
Gestational age (weeks)	0.01 (-0.01, 0.02)	**-0.02 (-0.04, -0.00)***	**0.05 (0.03, 0.07)****	0.02 (0.00, 0.04)	**0.03 (0.02, 0.05)****	**0.88 (0.81, 0.96)****
Preterm birth (<37 weeks)	-0.10 (-0.24, 0.04)	0.06 (-0.08, 0.19)	**-0.29 (-0.43, -0.15)****	**-0.16 (-0.32, -0.00)***	**-0.25 (-0.38, -0.11)****	1.50 (0.83, 2.73)
Weight						
Birth weight (500 g)	**0.11 (0.08, 0.13)****	**0.08 (0.06, 0.11)****	**0.03 (0.00, 0.05)***	0.02 (-0.01, 0.05)	**0.05 (0.02, 0.07)****	1.03 (0.92, 1.16)
Gestational age adjusted birth weight (500 g)	**0.10 (0.07, 0.13)****	**0.11 (0.08, 0.14)****	-0.03 (-0.06, 0.01)	-0.00 (-0.04, 0.03)	0.01 (-0.02, 0.04)	1.12 (0.76, 1.65)
Low birth weight (<2,500 g)	**-0.31 (-0.44, -0.18)****	**-0.17 (-0.29, -0.04)****	**-0.20 (-0.34, -0.07)****	**-0.22 (-0.36, -0.07)****	**-0.19 (-0.32, -0.07)****	1.24 (0.77, 2.01)
Gestational age adjusted low birth weight (<2,500 g)	**-0.17 (-0.32, -0.02)***	-0.12 (-0.27, 0.03)	-0.03 (-0.18, 0.13)	**-0.17 (-0.34, -0.01)***	-0.03 (-0.17, 0.12)	0.82 (0.45, 1.50)

Values are Z-scores (95% confidence interval) of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75} and odds ratio of current asthma and reflect the change in Z-score or odds ratio per 500 grams or per week increase of gestational age. *P < 0.05, **P < 0.01, ***P < 0.001. Models were adjusted for maternal age, pre-pregnancy body mass index, educational level, history of asthma or atopy, psychological distress during pregnancy, parity, smoking during pregnancy and child's sex, ethnicity and breastfeeding.

Table S3. Associations of Fetal and Infant Growth Characteristics with Lung Function and Current Asthma.

	FEV_1	FVC	FEV_1/FVC	FEF_{25-75}	FEF_{75}	Current asthma
	Z-score (95% CI)	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Odds ratio (95% CI)**
First trimester						
Crown-rump length (SDS) n = 1,075	0.00 (-0.07, 0.08)	-0.01 (-0.08, 0.06)	0.02 (-0.06, 0.10)	-0.00 (-0.09, 0.08)	-0.01 (-0.08, 0.06)	1.29 (0.93, 1.81)
Second trimester						
Abdominal circumference (SDS) n = 4,785	**0.08 (0.05, 0.11)****	**0.08 (0.05, 0.11)****	-0.00 (-0.03, 0.03)	-0.01 (-0.04, 0.03)	**0.03 (0.00, 0.06)***	1.01 (0.88, 1.16)
Femur length (SDS) n = 4,787	-0.03 (-0.06, 0.00)	-0.02 (-0.05, 0.01)	-0.02 (-0.05, 0.01)	0.00 (-0.03, 0.04)	-0.02 (-0.04, 0.01)	**1.20 (1.05, 1.38)***
Estimated fetal weight[#] (SDS) n = 4,761	**0.04 (0.01, 0.07)***	**0.04 (0.02, 0.07)****	-0.01 (-0.04, 0.02)	-0.00 (-0.04, 0.03)	0.01 (-0.02, 0.04)	1.11 (0.97, 1.27)
Third trimester						
Abdominal circumference (SDS) n = 4,892	**0.11 (0.08, 0.14)****	**0.11 (0.08, 0.13)****	-0.01 (-0.04, 0.02)	-0.01 (-0.04, 0.02)	**0.04 (0.01, 0.07)****	0.98 (0.86, 1.13)
Femur length (SDS) n = 4,902	**-0.04 (-0.07, -0.01)***	-0.02 (-0.05, 0.01)	**-0.04 (-0.07, -0.02)****	0.01 (-0.02, 0.04)	**-0.05 (-0.08, -0.02)****	**1.15 (1.00, 1.32)***
Estimated fetal weight (SDS)[#] n = 4,884	**0.07 (0.04, 0.10)****	**0.08 (0.05, 0.10)****	-0.03 (-0.06, 0.00)	-0.01 (-0.04, 0.03)	0.01 (-0.02, 0.04)	1.03 (0.90, 1.18)
Birth						
Length (SDS) n = 3,468	**0.03 (0.00, 0.06)***	**0.04 (0.01, 0.07)****	-0.01 (-0.04, 0.02)	-0.01 (-0.04, 0.02)	0.00 (-0.03, 0.03)	1.12 (0.97, 1.28)
Weight (SDS) n = 5,536	**0.09 (0.06, 0.11)****	**0.09 (0.06, 0.12)****	-0.02 (-0.05, 0.00)	-0.01 (-0.04, 0.02)	0.01 (-0.02, 0.03)	1.27 (0.82, 1.94)
3 months						

Table S3. Associations of Fetal and Infant Growth Characteristics with Lung Function and Current Asthma. (continued)

	FEV_1	FVC	FEV_1/FVC	FEF_{25-75}	FEF_{75}	Current asthma
	Z-score (95% CI)	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Odds ratio (95% CI)**
Length (SDS) n = 2,854	-0.02 (-0.07, 0.03)	-0.01 (-0.06, 0.03)	-0.01 (-0.06, 0.04)	0.03 (-0.02, 0.09)	-0.03 (-0.08, 0.01)	0.98 (0.77, 1.26)
Weight (SDS) n = 3,345	0.00 (-0.04, 0.04)	**0.07 (0.03, 0.11)****	**-0.11 (-0.15, -0.07)****	0.04 (-0.01, 0.09)	**-0.10 (-0.14, -0.06)****	1.03 (0.83, 1.95)
6 months						
Length (SDS) n = 3,742	-0.01 (-0.05, 0.03)	-0.00 (-0.04, 0.03)	-0.02 (-0.06, 0.02)	0.02 (-0.02, 0.07)	**-0.05 (-0.09, -0.01)***	0.99 (0.82, 1.21)
Weight (SDS) n = 4,181	0.01 (-0.03, 0.05)	**0.07 (0.04, 0.11)****	**-0.10 (-0.14, -0.07)****	0.02 (-0.02, 0.06)	**-0.09 (-0.13, -0.06)****	0.99 (0.83, 1.19)
12 months						
Length (SDS) n = 3,875	-0.00 (-0.04, 0.04)	-0.01 (-0.04, 0.03)	-0.00 (-0.04, 0.04)	0.01 (-0.04, 0.05)	-0.03 (-0.07, 0.01)	0.90 (0.75, 1.08)
Weight (SDS) n = 3,878	**0.04 (0.00, 0.08)***	**0.09 (0.06, 0.13)****	**-0.10 (-0.14, -0.06)****	0.02 (-0.03, 0.06)	**-0.07 (-0.11, -0.04)****	0.99 (0.83, 1.19)

Values are Z-scores and Odds Ratios (95% confidence interval) and reflect the change in Z-score of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75} and odds ratio for current asthma from linear and logistic regression models, respectively. "n =" represents the numbers of the total groups. *P < 0.05, **P < 0.01 and ***P < 0.001. Models were adjusted for maternal age, pre-pregnancy body mass index, educational level, history of asthma or atopy, psychological distress during pregnancy, parity, smoking during pregnancy and child's sex, gestational age, ethnicity and breastfeeding.

S-Table 2.2.4. Associations of Fetal and Infant Length with Lung Function and Current Asthma.

	FEV_1	FVC	FEV_1/FVC	FEF_{25-75}	FEF_{75}	Current asthma
	Z-score (95% CI)	Z-score (95% CI)	Z-score (95% CI)	Z-score (95% CI)	Z-score (95% CI)	Odds ratio (95% CI)
Length (SDS)						
Second trimester	0.01 (-0.05, 0.07)	-0.01 (-0.07, 0.05)	0.03 (-0.03, 0.09)	0.03 (-0.03, 0.10)	0.02 (-0.04, 0.08)	1.33 (0.98, 1.81)
Third trimester	-0.02 (-0.08, 0.04)	-0.05 (-0.10, 0.01)	0.03 (-0.03, 0.09)	0.05 (-0.02, 0.11)	-0.02 (-0.08, 0.03)	0.79 (0.59, 1.06)
Birth	0.03 (-0.03, 0.08)	0.03 (-0.03, 0.08)	-0.00 (-0.06, 0.05)	-0.04 (-0.10, 0.02)	0.01 (-0.04, 0.06)	1.07 (0.84, 1.36)
3 months	0.01 (-0.06, 0.07)	0.01 (-0.05, 0.07)	-0.02 (-0.08, 0.04)	-0.01 (-0.08, 0.06)	-0.04 (-0.10, 0.02)	0.97 (0.72, 1.30)
6 months	0.00 (-0.05, 0.06)	0.01 (-0.05, 0.06)	-0.01 (-0.07, 0.04)	0.05 (-0.01, 0.11)	-0.04 (-0.09, 0.02)	1.03 (0.78, 1.37)
12 months	0.02 (-0.04, 0.08)	0.01 (-0.04, 0.07)	0.01 (-0.04, 0.07)	0.01 (-0.05, 0.07)	0.01 (-0.05, 0.06)	1.00 (0.74, 1.35)

Values are Z-scores (95% confidence interval) of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75} and odds ratio of current asthma per 1 SDS change in length from linear and logistic regression models. *P < 0.05, **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, smoking during pregnancy and child's sex, gestational age, ethnicity and breastfeeding.

S-Table 2.2.5. Associations of Fetal and Infant Weight Growth Patterns with Lung Function and Current Asthma.

	FEV_1 Z-score (95% CI)	**FVC Z-score (95% CI)**	**FEV_1/FVC Z-score (95% CI)**	**FEF_{25-75} Z-score (95% CI)**	**FEF_{75} Z-score (95% CI)**	**Current asthma Odds ratio (95% CI)**
Restricted growth in weight (vs normal) (SDS)						
2nd trimester – birth (n = 2,209)	-0.04 (-0.12, 0.03)	-0.01 (-0.08, 0.06)	-0.05 (-0.12, 0.02)	-0.01 (-0.09, 0.08)	-0.02 (-0.09, 0.05)	0.98 (0.70, 1.39)
Birth – 3 months (n = 782)	-0.01 (-0.11, 0.10)	-0.05 (-0.15, 0.05)	0.09 (-0.02, 0.19)	-0.08 (-0.20, 0.03)	0.06 (-0.04, 0.16)	**1.57 (1.01, 2.45)***
3 months – 12 months (n = 674)	-0.01 (-0.11, 0.09)	0.03 (-0.06, 0.13)	-0.06 (-0.16, 0.04)	0.07 (-0.04, 0.18)	-0.05 (-0.14, 0.05)	0.94 (0.58, 1.54)
Accelerated growth in weight (vs normal) (SDS)						
2nd trimester – birth (n = 2,255)	0.05 (-0.02, 0.12)	**0.10 (0.03, 0.16)****	**-0.08 (-0.15, -0.01)***	-0.04 (-0.12, 0.04)	-0.03 (-0.10, 0.03)	1.10 (0.79, 1.52)
Birth – 3 months (n = 1,987)	**-0.10 (-0.18, -0.02)***	0.01 (-0.07, 0.08)	**-0.08 (-0.16, -0.00)***	0.07 (-0.01, 0.16)	**-0.07 (-0.14, -0.00)***	1.04 (0.70, 1.53)
3 months – 12 months (n = 294)	-0.01 (-0.17, 0.16)	0.06 (-0.10, 0.22)	-0.12 (-0.29, 0.04)	-0.05 (-0.23, 0.13)	-0.08 (-0.23, 0.08)	0.98 (0.44,2.17)

Values are Z-scores (95% confidence interval) of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75} and odds ratio of current asthma per 1 SDS change in growth characteristic from linear and logistic regression models. Restricted Fetal and infant growth was defined as <-0.67 SDS increase between second trimester and birth, and between birth and 3 months, respectively. Accelerated Fetal and infant growth was defined as >0.67 SDS increase between second trimester and birth, and between birth and 3 months, respectively. *P < 0.05, **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, smoking during pregnancy and child's sex, gestational age, ethnicity and breastfeeding.

S-Table 2.2.6. Associations of Fetal and Infant Weight Growth Patterns Combined from Fetal and Infant Life with Lung Function and Current Asthma.

	FEV_1	FVC	FEV_1/FVC	FEF_{25-75}	FEF_{75}	Current asthma
	Z-score (95% CI)	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Z-score (95% CI)**	**Odds ratio (95% CI)**
Restricted fetal growth in weight (SDS)						
Restricted infant weight gain (n = 87)	**-0.25 (-0.51, -0.00)***	**-0.30 (-0.53, -0.06)***	0.09 (-0.15, 0.34)	**-0.29 (-0.56, -0.01)***	0.02 (-0.22, 0.26)	1.01 (0.29, 3.59)
Normal infant weight gain (n = 439)	**-0.17 (-0.31, -0.02)***	-0.07 (-0.20, 0.07)	**-0.18 (-0.32, -0.04)***	-0.07 (-0.23, 0.08)	**-0.17 (-0.31, -0.03)***	1.44 (0.74, 2.79)
Accelerated infant weight gain (n = 638)	**-0.13 (-0.26, -0.01)***	-0.04 (-0.16, 0.08)	**-0.13 (-0.25, -0.00)***	-0.05 (-0.19, 0.09)	-0.09 (-0.21, 0.04)	0.92 (0.44, 1.90)
Normal fetal growth in weight (SDS)						
Restricted infant weight gain (n = 228)	-0.01 (-0.18, 0.17)	-0.04 (-0.20, 0.13)	0.02 (-0.15, 0.19)	0.01 (-0.19, 0.20)	0.00 (-0.16, 0.17)	1.58 (0.76, 3.32)
Normal infant weight gain (n = 923)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Accelerated infant weight gain (n = 798)	-0.03 (-0.15, 0.09)	0.02 (-0.10, 0.13)	-0.08 (-0.20, 0.04)	**0.14 (0.01, 0.27)***	-0.11 (-0.23, 0.00)	1.27 (0.70, 2.30)
Accelerated fetal growth in weight (SDS)						
Restricted infant weight gain (n = 306)	-0.04 (-0.20, 0.12)	0.01 (-0.14, 0.16)	-0.09 (-0.24, 0.07)	-0.15 (-0.33, 0.02)	-0.06 (-0.21, 0.10)	1.34 (0.65, 2.80)
Normal infant weight gain (n = 644)	0.06 (-0.06, 0.18)	0.08 (-0.03, 0.20)	-0.06 (-0.18, 0.06)	-0.02 (-0.15, 0.12)	-0.04 (-0.16, 0.08)	0.66 (0.33, 1.31)
Accelerated infant weight gain (n = 301)	0.08 (-0.08, 0.24)	**0.23 (0.07, 0.38)****	**-0.23 (-0.39, -0.07)***	0.07 (-0.11, 0.24)	-0.14 (-0.30, 0.01)	1.35 (0.65, 2.89)

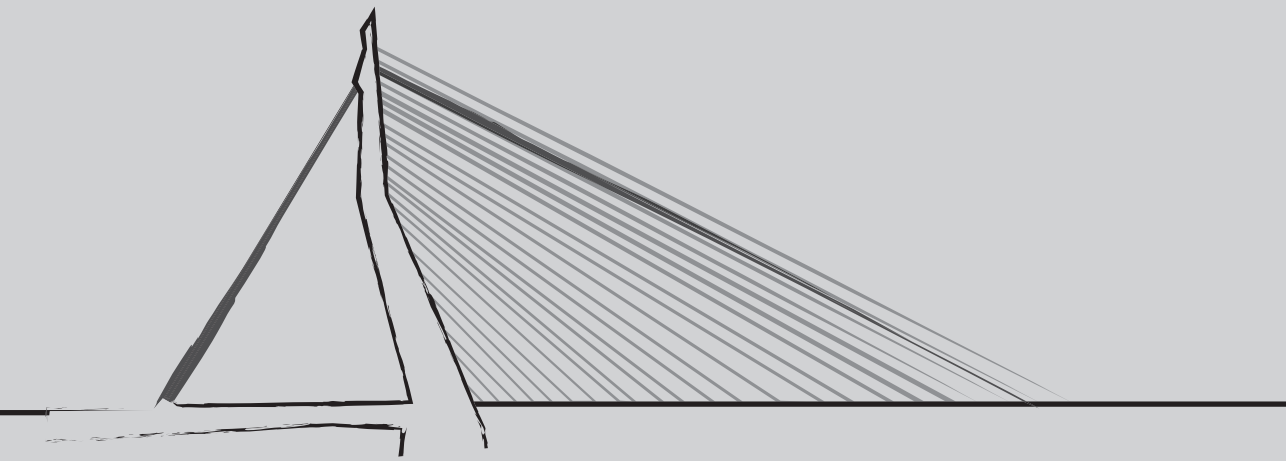
Values are Z-scores and Odds Ratios (95% confidence interval) and reflect the change in Z-score of lung function and odds ratio for current asthma from linear and logistic regression models. "n =" represents the numbers of the total groups. *P < 0.05, **P < 0.01 and ***P < 0.001. Models were adjusted for maternal age, pre-pregnancy body mass index, educational level, history of asthma or atopy, psychological distress during pregnancy, parity, smoking during pregnancy and child's sex, gestational age, ethnicity and breastfeeding.

Chapter 2.3

Body fat mass distribution and asthma at school-age

HT den Dekker, KPI Ros, JC de Jongste, IK Reiss, VWV Jaddoe, L Duijts

J Allergy Clin Immunol. 2016 Jul 16



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).

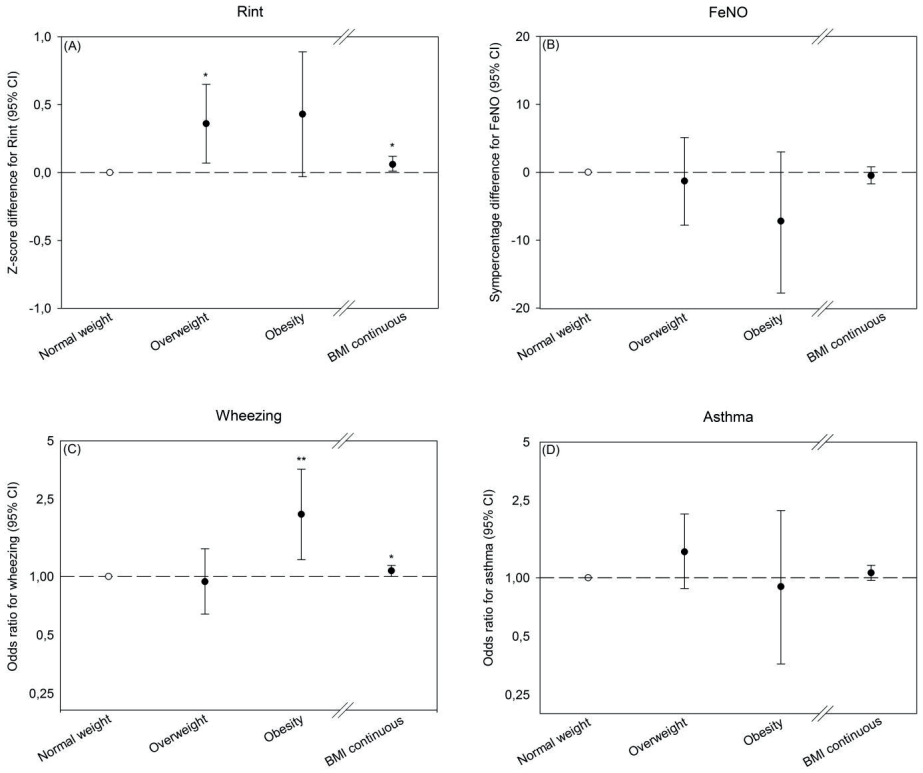


Figure 2.3.1. Association of Body Mass Index in Categories and Continuously with Rint, FeNO, Wheezing and Asthma in Children at Age 6 Years.
Values are changes in Z-score for Rint (A) or in sympercent for FeNO (B), and odds ratios for wheezing (C) and asthma (D) (95% confidence interval presented as 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.

Detailed acknowledgements and online resources can be found in the published article online: http://www.sciencedirect.com/science/article/pii/S009167491630625X

REFERENCES

1. Lai CKW, Beasley R, Crane J, Foliaki S, Shah J, Weiland S, et al. Global variation in the prevalence and severity of asthma symptoms: Phase Three of the International Study of Asthma and Allergies in Childhood (ISAAC). Thorax 2009; 64:476-83.
2. Kipping RR, Jago R, Lawlor DA. Obesity in children. Part 2: Prevention and management. British Medical Journal 2008; 337.
3. Permaul P, Kanchongkittiphon W, Phipatanakul W. Childhood asthma and obesity--what is the true link? Ann Allergy Asthma Immunol 2014; 113:244-6.
4. Guibas GV, Manios Y, Xepapadaki P, Moschonis G, Douladiris N, Mavrogianni C, et al. The obesity-asthma link in different ages and the role of body mass index in its investigation: findings from the Genesis and Healthy Growth Studies. Allergy 2013; 68:1298-305.
5. Ashwell M, Hsieh SD. Six reasons why the waist-to-height ratio is a rapid and effective global indicator for health risks of obesity and how its use could simplify the international public health message on obesity. Int J Food Sci Nutr 2005; 56:303-7.
6. Gaggini M, Saponaro C, Gastaldelli A. Not all fats are created equal: adipose vs. ectopic fat, implication in cardiometabolic diseases. Horm Mol Biol Clin Investig 2015.
7. Mook-Kanamori DO, Holzhauer S, Hollestein LM, Durmus B, Manniesing R, Koek M, et al. Abdominal fat in children measured by ultrasound and computed tomography. Ultrasound Med Biol 2009; 35:1938-46.
8. Holzhauer S, Zwijsen RM, Jaddoe VW, Boehm G, Moll HA, Mulder PG, et al. Sonographic assessment of abdominal fat distribution in infancy. Eur J Epidemiol 2009; 24:521-9.
9. Kruithof CJ, Kooijman MN, van Duijn CM, Franco OH, de Jongste JC, Klaver CC, et al. The Generation R Study: Biobank update 2015. Eur J Epidemiol 2014; 29:911-27.
10. Kaul S, Rothney MP, Peters DM, Wacker WK, Davis CE, Shapiro MD, et al. Dual-energy X-ray absorptiometry for quantification of visceral fat. Obesity (Silver Spring) 2012; 20:1313-8.
11. Helba M, Binkovitz LA. Pediatric body composition analysis with dual-energy X-ray absorptiometry. Pediatr Radiol 2009; 39:647-56.
12. Nutrition, Physical activity and Obesity. 2013.] Available from http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/country-work/netherlands.
13. American Thoracic S, European Respiratory S. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med 2005; 171:912-30.
14. Beydon N, Davis SD, Lombardi E, Allen JL, Arets HG, Aurora P, et al. An official American Thoracic Society/European Respiratory Society statement: pulmonary function testing in preschool children. Am J Respir Crit Care Med 2007; 175:1304-45.
15. Merkus PJ, Stocks J, Beydon N, Lombardi E, Jones M, McKenzie SA, et al. Reference ranges for interrupter resistance technique: the Asthma UK Initiative. Eur Respir J 2010; 36:157-63.
16. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J 1995; 8:483-91.
17. Beurs E. Brief Symptom Inventory, handleiding addendum 2009.
18. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 2000; 320:1240-3.
19. Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, et al. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. BMJ 2009; 338:b2393.

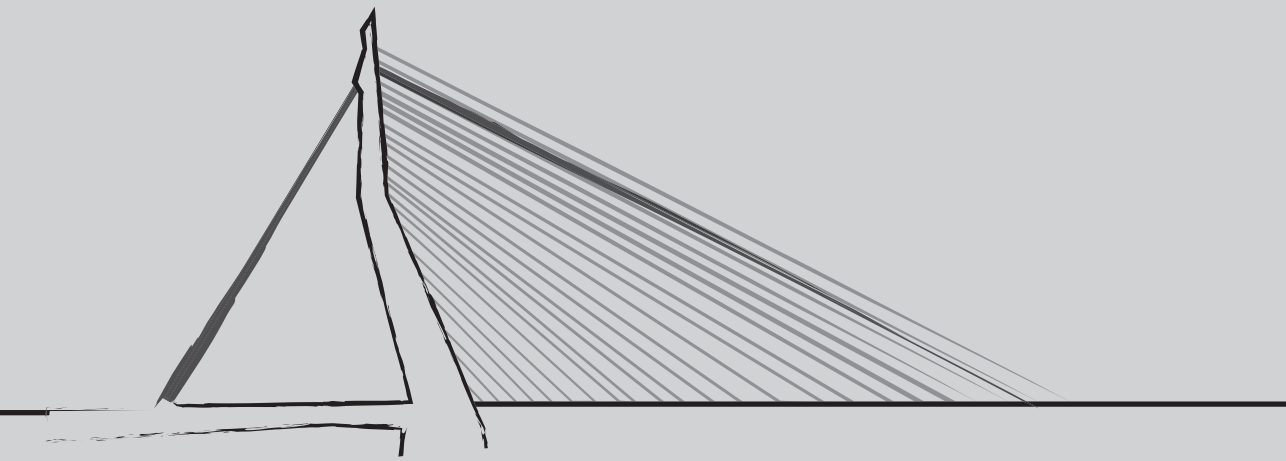
20. Cole TJ. Sympercents: symmetric percentage differences on the 100 log(e) scale simplify the presentation of log transformed data. Stat Med 2000; 19:3109-25.
21. Egan KB, Ettinger AS, Bracken MB. Childhood body mass index and subsequent physician-diagnosed asthma: a systematic review and meta-analysis of prospective cohort studies. BMC Pediatr 2013; 13:121.
22. Weinmayr G, Forastiere F, Buchele G, Jaensch A, Strachan DP, Nagel G, et al. Overweight/obesity and respiratory and allergic disease in children: international study of asthma and allergies in childhood (ISAAC) phase two. PLoS One 2014; 9:e113996.
23. Bekkers MBM, Elstgeest LEM, Soholtens S, Haveman-Nies A, de Jongste JC, Kerkhof M, et al. Maternal use of folic acid supplements during pregnancy, and childhood respiratory health and atopy. European Respiratory Journal 2012; 39:1468-74.
24. Consilvio NP, Di Pillo S, Verini M, de Giorgis T, Cingolani A, Chiavaroli V, et al. The reciprocal influences of asthma and obesity on lung function testing, AHR, and airway inflammation in prepubertal children. Pediatr Pulmonol 2010; 45:1103-10.
25. Tantisira KG, Litonjua AA, Weiss ST, Fuhlbrigge AL, Childhood Asthma Management Program Research G. Association of body mass with pulmonary function in the Childhood Asthma Management Program (CAMP). Thorax 2003; 58:1036-41.
26. van der Valk RJ, Duijts L, Timpson NJ, Salam MT, Standl M, Curtin JA, et al. Fraction of exhaled nitric oxide values in childhood are associated with 17q11.2-q12 and 17q12-q21 variants. J Allergy Clin Immunol 2014; 134:46-55.
27. Singleton MD, Sanderson WT, Mannino DM. Body mass index, asthma and exhaled nitric oxide in U.S. adults, 2007-2010. J Asthma 2014; 51:756-61.
28. Fenger RV, Gonzalez-Quintela A, Vidal C, Gude F, Husemoen LL, Aadahl M, et al. Exploring the obesity-asthma link: do all types of adiposity increase the risk of asthma? Clinical and Experimental Allergy 2012; 42:1237-45.
29. Forno E, Acosta-Perez E, Brehm JM, Han YY, Alvarez M, Colon-Semidey A, et al. Obesity and adiposity indicators, asthma, and atopy in Puerto Rican children. J Allergy Clin Immunol 2014; 133:1308-14, 14 e1-5.
30. Chen YC, Tu YK, Huang KC, Chen PC, Chu DC, Lee YL. Pathway from central obesity to childhood asthma. Physical fitness and sedentary time are leading factors. Am J Respir Crit Care Med 2014; 189:1194-203.
31. Sideleva O, Suratt BT, Black KE, Tharp WG, Pratley RE, Forgione P, et al. Obesity and Asthma An Inflammatory Disease of Adipose Tissue Not the Airway. American Journal of Respiratory and Critical Care Medicine 2012; 186:598-605.
32. Lu FL, Johnston RA, Flynt L, Theman TA, Terry RD, Schwartzman IN, et al. Increased pulmonary responses to acute ozone exposure in obese db/db mice. American Journal of Physiology-Lung Cellular and Molecular Physiology 2006; 290:L856-L65.
33. Shore SA, Terry RD, Flynt L, Xu AM, Hug C. Adiponectin attenuates allergen-induced airway inflammation and hyperresponsiveness in mice. Journal of Allergy and Clinical Immunology 2006; 118:389-95.
34. Salvi S, Semper A, Blomberg A, Holloway J, Jaffar Z, Papi A, et al. Interleukin-5 production by human airway epithelial cells. American Journal of Respiratory Cell and Molecular Biology 1999; 20:984-91.
35. Yokoyama A, Kohno N, Fujino S, Hamada H, Inoue Y, Fujioka S, et al. Circulating Interleukin-6 Levels in Patients with Bronchial-Asthma. American Journal of Respiratory and Critical Care Medicine 1995; 151:1354-8.

36. Arteaga-Solis E, Zee T, Emala CW, Vinson C, Wess J, Karsenty G. Inhibition of Leptin Regulation of Parasympathetic Signaling as a Cause of Extreme Body Weight-Associated Asthma. Cell Metabolism 2013; 17:35-48.
37. Altomonte J, Harbaran S, Richter A, Dong HJ. Fat depot-specific expression of adiponectin is impaired in Zucker fatty rats. Metabolism-Clinical and Experimental 2003; 52:958-63.
38. Makki K, Froguel P, Wolowczuk I. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. ISRN Inflamm 2013; 2013:139239.
39. Calixto MC, Lintomen L, Schenka A, Saad MJ, Zanesco A, Antunes E. Obesity enhances eosinophilic inflammation in a murine model of allergic asthma. Br J Pharmacol 2010; 159:617-25.
40. Eising JB, Uiterwaal CSPM, Evelein AMV, Visseren FLJ, van der Ent CK. Relationship between leptin and lung function in young healthy children. European Respiratory Journal 2014; 43:1189-92.
41. Lara A, Khatri SB, Wang Z, Comhair SA, Xu W, Dweik RA, et al. Alterations of the arginine metabolome in asthma. Am J Respir Crit Care Med 2008; 178:673-81.
42. Dweik RA, Boggs PB, Erzurum SC, Irvin CG, Leigh MW, Lundberg JO, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. Am J Respir Crit Care Med 2011; 184:602-15.
43. Komakula S, Khatri S, Mermis J, Savill S, Haque S, Rojas M, et al. Body mass index is associated with reduced exhaled nitric oxide and higher exhaled 8-isoprostanes in asthmatics. Respir Res 2007; 8:32.
44. Berg CM, Thelle DS, Rosengren A, Lissner L, Toren K, Olin AC. Decreased fraction of exhaled nitric oxide in obese subjects with asthma symptoms: data from the population study INTERGENE/ADONIX. Chest 2011; 139:1109-16.
45. Benson RC, Hardy KA, Morris CR. Arginase and arginine dysregulation in asthma. J Allergy (Cairo) 2011; 2011:736319.
46. Ashwell M, Hsieh SD. Six reasons why the waist-to-height ratio is a rapid and effective global indicator for health risks of obesity and how its use could simplify the international public health message on obesity. International Journal of Food Sciences and Nutrition 2005; 56:303-7.
47. Kouda K, Nakamura H, Ohara K, Fujita Y, Iki M. Increased Ratio of Trunk-to-Appendicular Fat and Decreased Adiponectin: A Population-Based Study of School Children in Hamamatsu, Japan. J Clin Densitom 2015.
48. Staiger H, Tschritter O, Machann J, Thamer C, Fritsche A, Maerker E, et al. Relationship of serum adiponectin and leptin concentrations with body fat distribution in humans. Obes Res 2003; 11:368-72.
49. Farb MG, Ganley-Leal L, Mott M, Liang Y, Ercan B, Widlansky ME, et al. Arteriolar function in visceral adipose tissue is impaired in human obesity. Arterioscler Thromb Vasc Biol 2012; 32:467-73.
50. Kujawska-Luczak M, Sulibursk a J, Markuszewski L, Pupek-Musialik D, Jablecka A, Bogdanski P. The effect of L-arginine and ascorbic acid on the visceral fat and the concentrations of metalloproteinases 2 and 9 in high-fat-diet rats. Endokrynol Pol 2015; 66:526-32.
51. Miczke A, Sulibursk a J, Pupek-Musialik D, Ostrowska L, Jablecka A, Krejpcio Z, et al. Effect of L-arginine supplementation on insulin resistance and serum adiponectin concentration in rats with fat diet. Int J Clin Exp Med 2015; 8:10358-66.
52. Savgan-Gurol E, Bredella M, Russell M, Mendes N, Klibanski A, Misra M. Waist to hip ratio and trunk to extremity fat (DXA) are better surrogates for IMCL and for visceral fat respectively than for subcutaneous fat in adolescent girls. Nutr Metab (Lond) 2010; 7:86.
53. Chan DC, Watts GF, Barrett PH, Burke V. Waist circumference, waist-to-hip ratio and body mass index as predictors of adipose tissue compartments in men. QJM 2003; 96:441-7.

54. Pouliot MC, Despres JP, Lemieux S, Moorjani S, Bouchard C, Tremblay A, et al. Waist circumference and abdominal sagittal diameter: best simple anthropometric indexes of abdominal visceral adipose tissue accumulation and related cardiovascular risk in men and women. Am J Cardiol 1994; 73:460-8.
55. Permaul P, Kanchongkittiphon W, Phipatanakul W. Childhood asthma and obesity-what is the true link? Annals of Allergy Asthma & Immunology 2014; 113:244-6.
56. Delacourt C, Lorino H, Fuhrman C, Herve-Guillot M, Reinert P, Harf A, et al. Comparison of the forced oscillation technique and the interrupter technique for assessing airway obstruction and its reversibility in children. Am J Respir Crit Care Med 2001; 164:965-72.
57. Beydon N, M'Buila C, Bados A, Peiffer C, Bernard A, Zaccaria I, et al. Interrupter resistance short-term repeatability and bronchodilator response in preschool children. Respir Med 2007; 101:2482-7.
58. Merkus PJ, Mijnsbergen JY, Hop WC, de Jongste JC. Interrupter resistance in preschool children: measurement characteristics and reference values. Am J Respir Crit Care Med 2001; 163:1350-5.
59. Klug B, Bisgaard H. Specific airway resistance, interrupter resistance, and respiratory impedance in healthy children aged 2-7 years. Pediatr Pulmonol 1998; 25:322-31.
60. Turner EL, Dobson JE, Pocock SJ. Categorisation of continuous risk factors in epidemiological publications: a survey of current practice. Epidemiol Perspect Innov 2010; 7:9.

Chapter 3

Early environmental exposures, childhood lung function and asthma

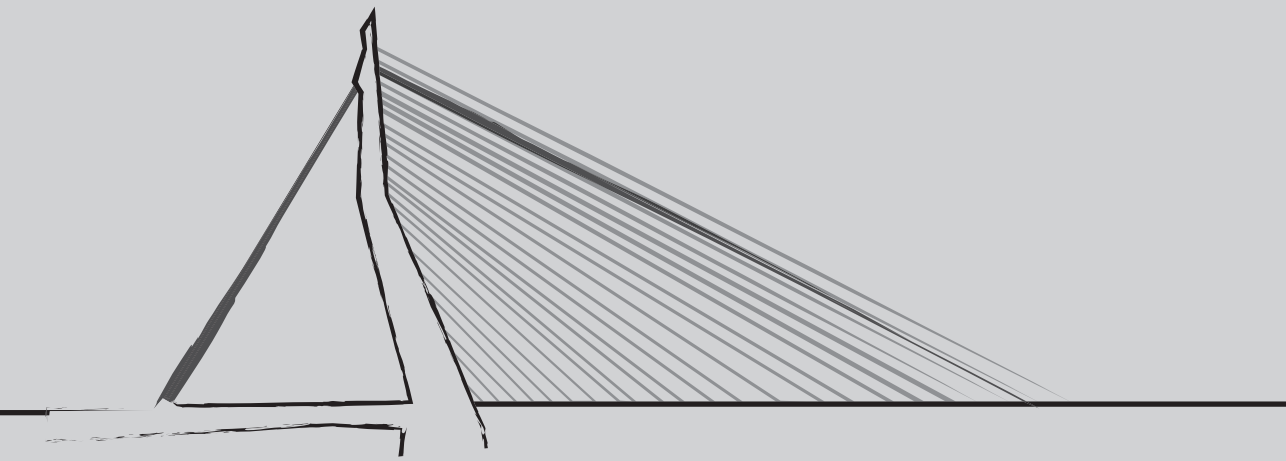


Chapter 3.1

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

HT den Dekker, AMM Sonnenschein – van der Voort, JC de Jongste, IK Reiss, A Hofman, VWV Jaddoe, L Duijts

Chest 2015;148(3):607-17



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).

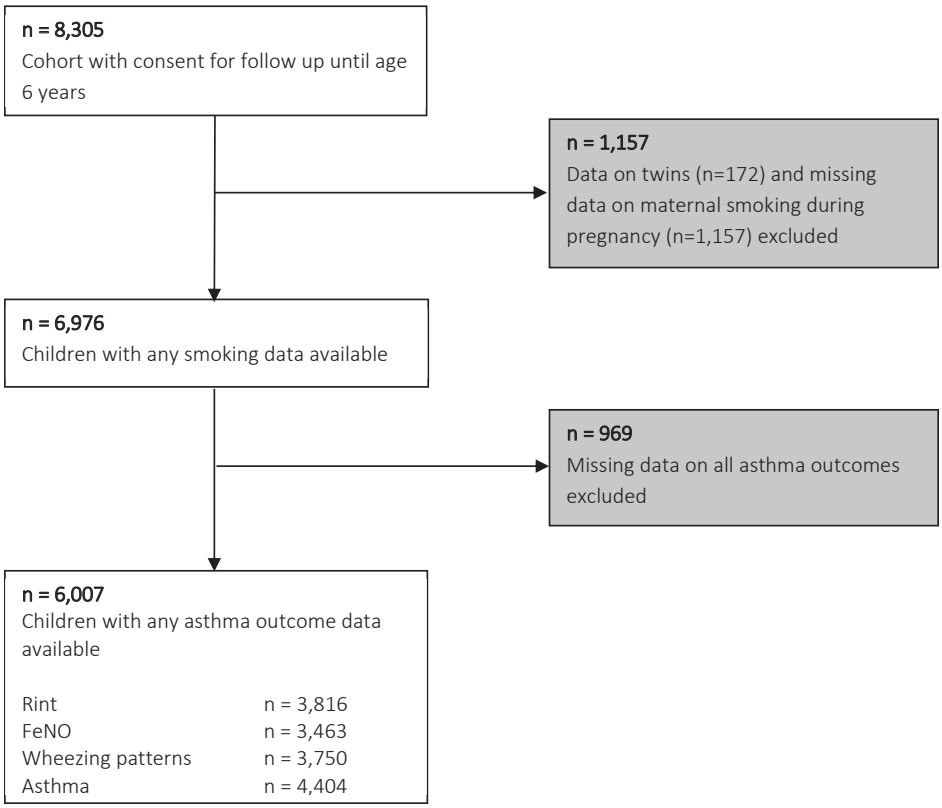


Figure 1. Flowchart of Participants.

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

REFERENCES

1. Burke H, Leonardi-Bee J, Hashim A, Pine-Abata H, Chen Y, Cook DG, et al. Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. Pediatrics 2012; 129:735-44.
2. Neuman A, Hohmann C, Orsini N, Pershagen G, Eller E, Kjaer HF, et al. Maternal smoking in pregnancy and asthma in preschool children: a pooled analysis of eight birth cohorts. Am J Respir Crit Care Med 2012; 186:1037-43.
3. Haberg SE, Stigum H, Nystad W, Nafstad P. Effects of pre- and postnatal exposure to parental smoking on early childhood respiratory health. Am J Epidemiol 2007; 166:679-86.
4. Hoppin JA, Umbach DM, London SJ, Alavanja MC, Sandler DP. Diesel exhaust, solvents, and other occupational exposures as risk factors for wheeze among farmers. Am J Respir Crit Care Med 2004; 169:1308-13.
5. Anderson HR, Ruggles R, Pandey KD, Kapetanakis V, Brunekreef B, Lai CK, et al. Ambient particulate pollution and the world-wide prevalence of asthma, rhinoconjunctivitis and eczema in children: Phase One of the International Study of Asthma and Allergies in Childhood (ISAAC). Occup Environ Med 2010; 67:293-300.
6. Gilliland FD, Berhane K, Li YF, Rappaport EB, Peters JM. Effects of early onset asthma and in utero exposure to maternal smoking on childhood lung function. Am J Respir Crit Care Med 2003; 167:917-24.
7. Duijts L. Fetal and infant origins of asthma. Eur J Epidemiol 2012; 27:5-14.
8. Rehan VK, Asotra K, Torday JS. The effects of smoking on the developing lung: insights from a biologic model for lung development, homeostasis, and repair. Lung 2009; 187:281-9.
9. Maritz GS. Perinatal exposure to nicotine and implications for subsequent obstructive lung disease. Paediatr Respir Rev 2013; 14:3-8.
10. Hollams EM, de Klerk NH, Holt PG, Sly PD. Persistent Effects of Maternal Smoking during Pregnancy on Lung Function and Asthma in Adolescents. Am J Respir Crit Care Med 2013.
11. Miyake Y, Tanaka K. Lack of relationship between birth conditions and allergic disorders in Japanese children aged 3 years. J Asthma 2013; 50:555-9.
12. Alati R, Al Mamun A, O'Callaghan M, Najman JM, Williams GM. In utero and postnatal maternal smoking and asthma in adolescence. Epidemiology 2006; 17:138-44.
13. Stein RT, Holberg CJ, Sherrill D, Wright AL, Morgan WJ, Taussig L, et al. Influence of parental smoking on respiratory symptoms during the first decade of life: the Tucson Children's Respiratory Study. Am J Epidemiol 1999; 149:1030-7.
14. Sonnenschein-van der Voort AM, Arends LR, de Jongste JC, Annesi-Maesano I, Arshad SH, Barros H, et al. Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. J Allergy Clin Immunol 2014; 133:1317-29.
15. G. Davey Smith SL, A. Ness In: Berthold Koletzko TD, Dees Molnar, Anne de la Hunty, editor. Early Nutrition Programming and Health Outcomes in Later Life. Amsterdam: Springer Netherlands; 2009. p. 1-14.
16. Raherison C, Penard-Morand C, Moreau D, Caillaud D, Charpin D, Kopfersmitt C, et al. In utero and childhood exposure to parental tobacco smoke, and allergies in schoolchildren. Respir Med 2007; 101:107-17.
17. Xepapadaki P, Manios Y, Liarigkovinos T, Grammatikaki E, Douladiris N, Kortsalioudaki C, et al. Association of passive exposure of pregnant women to environmental tobacco smoke with asthma symptoms in children. Pediatr Allergy Immunol 2009; 20:423-9.

18. Jaakkola JJ, Ahmed P, Ieromnimon A, Goepfert P, Laiou E, Quansah R, et al. Preterm delivery and asthma: a systematic review and meta-analysis. J Allergy Clin Immunol 2006; 118:823-30.
19. Kotecha SJ, Watkins WJ, Heron J, Henderson J, Dunstan FD, Kotecha S. Spirometric lung function in school-age children: effect of intrauterine growth retardation and catch-up growth. Am J Respir Crit Care Med 2010; 181:969-74.
20. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. Eur J Epidemiol 2012; 27:739-56.
21. Jaddoe VW, Bakker R, van Duijn CM, van der Heijden AJ, Lindemans J, Mackenbach JP, et al. The Generation R Study Biobank: a resource for epidemiological studies in children and their parents. Eur J Epidemiol 2007; 22:917-23.
22. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med 1995; 332:133-8.
23. Brussee JE, Smit HA, Koopman LP, Wijga AH, Kerkhof M, Corver K, et al. Interrupter resistance and wheezing phenotypes at 4 years of age. Am J Respir Crit Care Med 2004; 169:209-13.
24. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J 1995; 8:483-91.
25. Cole TJ. Sympercents: symmetric percentage differences on the 100 log(e) scale simplify the presentation of log transformed data. Stat Med 2000; 19:3109-25.
26. Silvestri M, Franchi S, Pistorio A, Petecchia L, Rusconi F. Smoke exposure, wheezing, and asthma development: A systematic review and meta-analysis in unselected birth cohorts. Pediatr Pulmonol 2014.
27. Pattenden S, Antova T, Neuberger M, Nikiforov B, De Sario M, Grize L, et al. Parental smoking and children's respiratory health: independent effects of prenatal and postnatal exposure. Tob Control 2006; 15:294-301.
28. Arshad SH, Karmaus W, Raza A, Kurukulaaratchy RJ, Matthews SM, Holloway JW, et al. The effect of parental allergy on childhood allergic diseases depends on the sex of the child. J Allergy Clin Immunol 2012; 130:427-34 e6.
29. Shaheen S, Barker DJ. Early lung growth and chronic airflow obstruction. Thorax 1994; 49:533-6.
30. Kotecha SJ, Edwards MO, Watkins WJ, Henderson AJ, Paranjothy S, Dunstan FD, et al. Effect of preterm birth on later FEV1: a systematic review and meta-analysis. Thorax 2013; 68:760-6.
31. Canoy D, Pekkanen J, Elliott P, Pouta A, Laitinen J, Hartikainen AL, et al. Early growth and adult respiratory function in men and women followed from the fetal period to adulthood. Thorax 2007; 62:396-402.
32. Kooi EM, Vrijlandt EJ, Boezen HM, Duiverman EJ. Children with smoking parents have a higher airway resistance measured by the interruption technique. Pediatr Pulmonol 2004; 38:419-24.
33. Kalliola S, Pelkonen AS, Malmberg LP, Sarna S, Hamalainen M, Mononen I, et al. Maternal smoking affects lung function and airway inflammation in young children with multiple-trigger wheeze. J Allergy Clin Immunol 2013; 131:730-5.
34. Blacquiere MJ, Timens W, Melgert BN, Geerlings M, Postma DS, Hylkema MN. Maternal smoking during pregnancy induces airway remodelling in mice offspring. Eur Respir J 2009; 33:1133-40.
35. Lambers DS, Clark KE. The maternal and fetal physiologic effects of nicotine. Semin Perinatol 1996; 20:115-26.
36. Manning FA, Feyerabend C. Cigarette smoking and fetal breathing movements. Br J Obstet Gynaecol 1976; 83:262-70.
37. Maritz GS, Dennis H. Maternal nicotine exposure during gestation and lactation interferes with alveolar development in the neonatal lung. Reprod Fertil Dev 1998; 10:255-61.

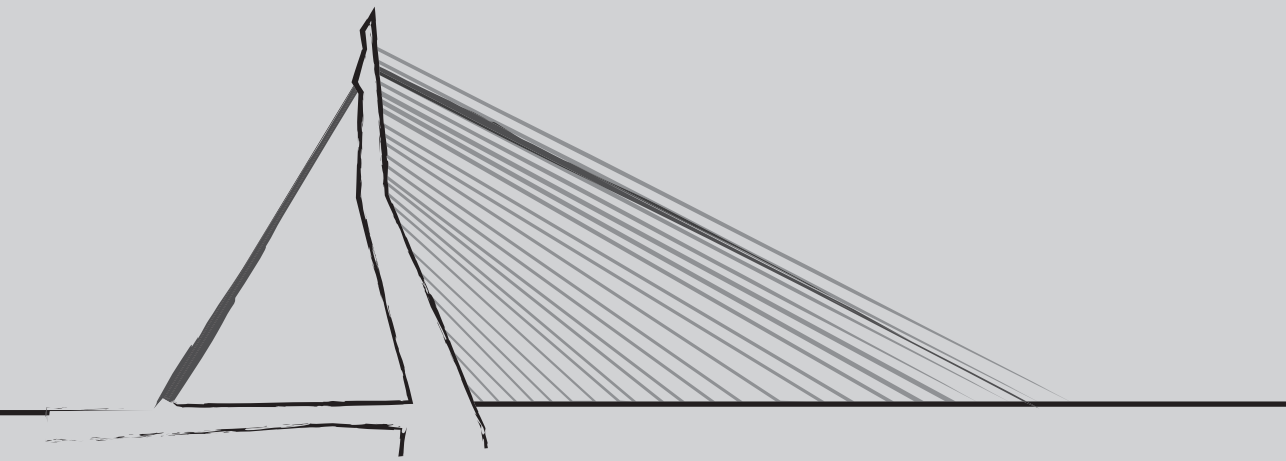
38. Bouzigon E, Corda E, Aschard H, Dizier MH, Boland A, Bousquet J, et al. Effect of 17q21 variants and smoking exposure in early-onset asthma. N Engl J Med 2008; 359:1985-94.
39. Martino D, Prescott S. Epigenetics and prenatal influences on asthma and allergic airways disease. Chest 2011; 139:640-7.
40. Breton CV, Byun HM, Wenten M, Pan F, Yang A, Gilliland FD. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. Am J Respir Crit Care Med 2009; 180:462-7.
41. Shipton D, Tappin DM, Vadiveloo T, Crossley JA, Aitken DA, Chalmers J. Reliability of self reported smoking status by pregnant women for estimating smoking prevalence: a retrospective, cross sectional study. BMJ 2009; 339:b4347.
42. Carlsten C, Dimich-Ward H, DyBuncio A, Becker AB, Chan-Yeung M. Cotinine versus questionnaire: early-life environmental tobacco smoke exposure and incident asthma. BMC Pediatr 2012; 12:187.
43. Gaffin JM, Sheehan WJ, Morrill J, Cinar M, Borras Coughlin IM, Sawicki GS, et al. Tree nut allergy, egg allergy, and asthma in children. Clin Pediatr (Phila) 2011; 50:133-9.
44. Beydon N, Mahut B, Maingot L, Guillo H, La Rocca MC, Medjahdi N, et al. Baseline and post-bronchodilator interrupter resistance and spirometry in asthmatic children. Pediatr Pulmonol 2012; 47:987-93.
45. Black J, Baxter-Jones AD, Gordon J, Findlay AL, Helms PJ. Assessment of airway function in young children with asthma: comparison of spirometry, interrupter technique, and tidal flow by inductance plethsmography. Pediatr Pulmonol 2004; 37:548-53.
46. Kaminsky DA. What does airway resistance tell us about lung function? Respir Care 2012; 57:85-96; discussion -9.

Chapter 3.2

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

HT den Dekker, VWV Jaddoe, IK Reiss, JC de Jongste, L Duijts

Submitted



ABSTRACT

Background: Folic acid supplement use during pregnancy might affect childhood respiratory health, potentially mediated by methylenetetrahydrofolate-reductase polymorphism C677T (*MTHFR-C677T*) carriership.

Objectives We examined the associations of maternal folic acid supplement use and folate, vitamin B12 and homocysteine concentrations during pregnancy with childhood lung function and asthma.

Methods: This study was embedded in a population-based prospective cohort study among 5,653 children. Folic acid supplement use was assessed by questionnaires. Folate, vitamin B_{12} and homocysteine plasma concentrations were measured in early pregnancy and at birth. At age 10 years, forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC, forced expiratory flow between 25% and 75% (FEF_{25-75}), at 75% of FVC (FEF_{75}), and asthma were examined.

Results: Among mothers carrying MTHFR-C677T variants, preconceptional start of folic acid supplement use was associated with lower FEV_1/FVC (-0.17 (-0.32, -0.02)) and FEF_{25-75} (-0.24 (-0.40, -0.07)), but not with asthma, compared with no folic acid supplement use. Among children carrying MTHFR-C677T wildtype, a higher vitamin B_{12} level at birth was associated with a lower FEV_1 (-0.07 (-0.12, -0.01)) and FVC (-0.09 (-0.15, -0.04). Folate and homocysteine concentrations were not consistently associated with lower childhood lung function or asthma.

Conclusions: Preconceptional start of maternal folic acid supplement use and higher vitamin B_{12} concentrations at birth might adversely affect childhood lung function depending on MTHFR-C677T carriership. The clinical implications need to be evaluated.

INTRODUCTION

Folate is vital for fetal development. Folic acid supplement use during pregnancy is recommended to prevent neural tube defects[1], but high dosages have been associated with increased risks of asthma and allergic diseases in animal studies.[2] Human studies that assessed associations of maternal folic acid supplement use or related folate blood concentrations during pregnancy with childhood lung function and asthma show conflicting results.[3-12] This could be due to different methods used to assess folic acid supplement use, including food frequency questionnaires, diet records and self-administered questionnaires, and differences in techniques and ages at which folate concentrations were measured. Besides folic acid use and related folate blood concentrations, vitamin B_{12} and homocysteine are involved in the one-carbon metabolism, which is important for DNA-methylation.[13] The one-carbon metabolism is influenced by polymorphisms in the methylenetetrahydrofolate reductase gene (*MTHFR*).[14,15] The variant *C677T* is known to affect the activity of the *MTHFR* enzyme, leading to lower circulating folate and higher homocysteine concentrations.[16] We previously observed that higher circulating maternal folate concentrations during pregnancy and not at birth were associated with increased risk of atopic dermatitis[10], but not with wheezing, higher respiratory resistance or fractional exhaled nitric oxide.[4] 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 less clear.[4,9,10]

Therefore, we aimed to assess in a population-based prospective cohort study the associations of folic acid supplement use and folate, vitamin B_{12} and homocysteine concentrations in early pregnancy and at birth with lung function and asthma at age 10 years. Second, we assessed whether these associations were modified by *MTHFR-C677T* polymorphisms.[17]

METHODS

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards situated in Rotterdam, The Netherlands.[18] This study was approved by the Medical Ethical Committee of Erasmus MC, University Medical Center Rotterdam, The Netherlands (MEC_2012-165 NL40020.078.12). Written informed consent was obtained from parents or legal guardians. Twins, children with missing data on maternal folic acid supplement use and folate, vitamin B_{12} and homocysteine concentrations in early pregnancy and at birth (n=186), and with missing data on lung function and current asthma at age 10 years (n=1,554) were excluded, resulting in a total of 5,653 children and their mothers for analyses (S-Figure 3.2.1).

Maternal folic acid supplement use

In the Netherlands, 400–500 µg folic acid solely or within multivitamin supplements are recommended during pregnancy, and mandatory food fortification with folic acid is not present. Mothers reported their folic acid or multivitamin use and when they started these supplements by questionnaires before 18 weeks of gestation (median 13.2 weeks; 90%-range 10.5–17.2 weeks). We categorized folic acid supplement use into "no use" (n=855 (18.9%)) and "use" (n=3,670 (81.3%)), and further classified use of folic acid supplements into duration of use: 1) start ≥10 weeks of pregnancy (n=575 (15.7%)), 2) start <10 weeks of pregnancy (n=1,265 (34.5%)) and 3) start preconceptional (n=1,830 (49.9%)). Self-reported folic acid use was validated by serum folate and homocysteine concentrations before 12 weeks of gestation in a small random subsample of this study (*n* = 276), which provided sensitivity and specificity scores of respectively 97% and 56%.[19]

Folate, homocysteine and vitamin B_{12} concentrations

Non-fasting maternal blood samples in early pregnancy (median 13.4 weeks of gestation, 95%-range 9.8-17.5), and cord blood samples were collected at birth (median 40.1 weeks of gestation, 95%-range 35.8 – 42.3). Folate, vitamin B_{12} and homocysteine concentrations were analysed using an immunoelectrochemoluminescence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands).[20] The between-run coefficient of variation ranged from 1.5% to 8.9%. Detailed information is provided in the Supplementary Material.

casian ethnicity was defined as having principal components within 4 SD values of the CEU cluster of HapMap (18). Genotype data were extracted from an imputed genome-wide association scan (HapMap phase II release 22). The imputation quality of the two SNPs was good (rs1801133: RSQ = 0.985, rs1801131: RSQ = 0.997). The genotype frequencies of *MTHFR-C677T* were 46.0% [CC], 43.6% (TC) and 10.4% [TT] (Hardy–Weinberg P = 1.00), and A1298C were 45.6% (AA), 43.7% (CA) and 10.7% [CC] (Hardy–Weinberg P = 0.79). The two MTHFR SNPs are in LD, but do not tag the same genetic variation (HapMap pairwise LD (phase II release 22 CEU); D' = 1.000, r^2 = 0.178).

Childhood lung function and asthma

Lung function measures at a median age of 9.7 years (range 8.5 – 12.0 years) comprised FEV_1, FEV_1/FVC and FEF_{75}[21], which were converted into sex-, age-, height- and ethnicity-adjusted z-scores.[22] Quality criteria required 3 reproducible curves and no use of inhalant bronchodilators or corticosteroids within 48 hours before spirometry, which was met for 4,583 (87%) individuals. Additionally, 521 children were included with individual spirometry curves with >5% deviation, but with at least one blow according to ATS/ERS criteria with adequate reach and duration of plateau. Ever physician diagnosed asthma, wheezing and use of bronchodilators or inhalant corticosteroids was reported by a

parental questionnaire at age 10 years. Current asthma (no; yes) was defined as ever physician diagnosed asthma, with either wheezing or the use of inhalant medication in the past 12 months.

MTHFR-C677T

Maternal DNA was extracted from white blood cells obtained in early pregnancy. Genotyping of *MTHFR-C677T* was performed using TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg, Germany).[18] Child's DNA was extracted from white cells in cord blood. Samples were genotyped using Illumina Infinium II HumanHap610-660 Quad Arrays following standard manufacturer's protocols. Genotype data were extracted from an imputed genome-wide association scan (1000G phase Iv3).[18] The imputation quality of *MTHFR-C677T* was high ($r^2 = 0.99$).

Covariates

We collected information about maternal age, parity (nulli-; multiparous), history of asthma, eczema or allergy (no; yes), and educational level (low: secondary school or lower; and high: higher than secondary school as highest finished education) from maternal questionnaires at enrollment. Maternal weight and height were measured at enrollment. We obtained information on maternal smoking (no; first trimester only; continued during pregnancy) and alcohol use (no; first trimester only, continued during pregnancy) by multiple questionnaires during pregnancy. Child's sex, gestational age at birth and birthweight were obtained from midwifes and hospital records. The country of birth of parents was used to define the child's ethnicity.[22] We measured inhalant allergic sensitization by skin prick test using the 'scanned area method[23], for which detailed information is provided in the Supplementary Material.

Statistical analyses

We used linear and logistic regression models to examine the associations of folic acid supplement use and folate, vitamin B_{12} and homocysteine blood concentrations in early pregnancy and at birth with lung function and asthma. We created standard deviations scores (SDS) for folate, vitamin B_{12} and homocysteine to enable comparison between effect estimates. For clinical interpretation, we categorized folate, vitamin B_{12} and homocysteine concentrations into three groups: "low" (SDS < -1.00), "normal" (-1.00 ≤ SDS ≥ 1.00) and "high" (SDS > 1.00). These groups reflect the 16th and 84th percentile, respectively. The "normal" group was used as reference category. The modifying effects of inhalant allergic sensitization and *MTHFR-C677T* polymorphisms were tested by adding them as product terms with the exposures in the models. Models with p-values for interaction <0.05 were stratified. The most common *MTHFR* genotype [CC] was used as

reference genotype (wildtype). Because of low prevalences, the variant genotypes [CT] and [TT] were combined and considered as "variant" genotype. All models were adjusted for covariates based on literature, if the covariate was associated with the exposure and outcome, or if it changed the effect estimates of univariate associations with ≥10%. For all covariates, the percentage of missing values was <20% (S-Table 3.2.1). Missing data on covariates was imputed using the Markov Chain Monte Carlo method to select the most likely value for a missing response. Ten imputed data sets were created and analysed together. No major differences in the magnitude or direction of the effect estimates were observed between imputed data and complete cases only. To further strengthen observed associations and to minimalize reporting false-positive findings, we additionally performed canonical correlations analyses [24], of which detailed information is provided in the Supplementary Material. All measures of association are presented with their 95% Confidence Interval (95% CI). Statistical analyses were performed using SPSS 21.0 (IBM, Chicago, Ill, USA).

RESULTS

Participant characteristics

An overview of the study population is given in Table 3.2.1. Prevalence of *MTHFR-C677T* variant genotype in mothers was 49.1%, and in children 50.6%. Mean (SD) FEV_1 was 2.02 (0.98) L/s, for FVC 2.34 (0.36) L, for FEV_1/FVC 0.87 (0.06), for FEF_{25-75} 2.71 (0.63) L/s and for FEF_{75} 1.14 (0.34) L/s. Current asthma was reported for 269 children (5.7%). Children lost to follow-up had younger, lower educated mothers who smoked more and used less alcohol, and were more often of non-European origin, compared to children included (S-Table 3.2.1).

Folic acid supplement use and respiratory outcomes

Maternal folic acid supplement use during pregnancy was associated with higher FEV_1 and FVC (z-score difference (95% CI): 0.14 (0.05, 0.22) and 0.17 (0.09, 0.25), respectively), but not with FEV_1/FVC, FEF_{25-75}, FEF_{75} or current asthma, compared to mothers who did not use folic acid supplements (Table 3.2.2). Preconceptional start of folic acid supplement use was associated with a lower FEV_1/FVC (Z-score difference (95% CI): -0.11 (-0.21, -0.02)). A longer duration of folic acid supplement use was associated with a lower FEF_{25-75} (p-value for trend 0.04). When stratified for maternal *MTHFR-C677T*, folic acid supplement use was associated with a high FEV_1, FVC, and FEF_{25-75}, compared with no folic acid supplement use, among mothers carrying wildtype (p-values for interaction: <0.01, <0.01 and 0.05, respectively) (Figure 3.2.1, S-Table 3.2.3). Preconceptional start of folic acid supplement use was associated with lower FEV_1/FVC and FEF_{25-75} among mothers carrying variant alleles (z-score difference (95% CI): -0.17 (-0.32, -0.02) and -0.24

Table 3.2.1. Characteristics of Children and Their Mothers.

	n = 5,653
Maternal characteristics	
Age (years)	31.0 (4.9)
Body mass index at intake (kg/m^2)[#]	23.9 (18.9 – 35.4)
Nullipara (%)	57.0 (3,215)
History of asthma or atopy, yes (%)	38.7 (2,190)
Higher education (%)	49.5 (2,793)
Smoking during pregnancy (%)	24.1 (1,363)
Alcohol consumption during pregnancy	56.0 (3,200)
Folic acid supplement use (%)[¶]	
No use	15.1 (855)
Start after first 10 weeks of pregnancy	10.2 (575)
Start within first 10 weeks of pregnancy	22.4 (1,265)
Preconceptional start	32.4 (1,830)
Folate in early pregnancy (nmol/l)[#¶]	17.5 (6.0 – 37.9)
Vitamin B_{12} in early pregnancy (pmol/l)[#¶]	172.0 (77.0 – 406.0)
Homocysteine in early pregnancy (µmol/l)[#¶]	9.1 (5.2 – 16.6)
MTHFR-C677T variant, [CT or TT] (%)[¶]	49.1 (2,127)
Child's characteristics	
Female sex (%)	50.6 (2,860)
Gestational age at birth (weeks)[#]	40.1 (35.8 – 42.3)
Birth weight (grams)	3,440 (554)
European ethnicity (%)	68.7 (3,674)
Folate at birth (nmol/l)[#¶]	20.9 (10.7 – 38.4)
Vitamin B_{12} at birth (nmol/l)[#¶]	303.0 (121.0 – 902.2)
Homocysteine at birth (µmol/l) [#¶]	6.9 (4.6 – 12.0)
MTHFR-C677T variant, [CT or TT] (%)[¶]	50.6 (1,850)
Age at spirometry and asthma-assessment	9.8 (0.3)
FEV_1 (L/s) [¶]	2.02 (0.30)
FVC (L) [¶]	2.34 (0.36)
FEV_1/FVC[¶]	0.87 (0.06)
FEF_{25-75} (L/s) [¶]	2.71 (0.63)
FEF_{75} (L/s) [¶]	1.14 (0.34)
Current asthma, yes (%)[¶]	5.7 (269)

Values are means (SD), valid percentages (absolute numbers) or [#]medians (95% range). FEV_1: forced expiratory volume in the 1st second, FEV_1: forced expiratory volume in the 1st 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 exhalation. [¶]not imputed.

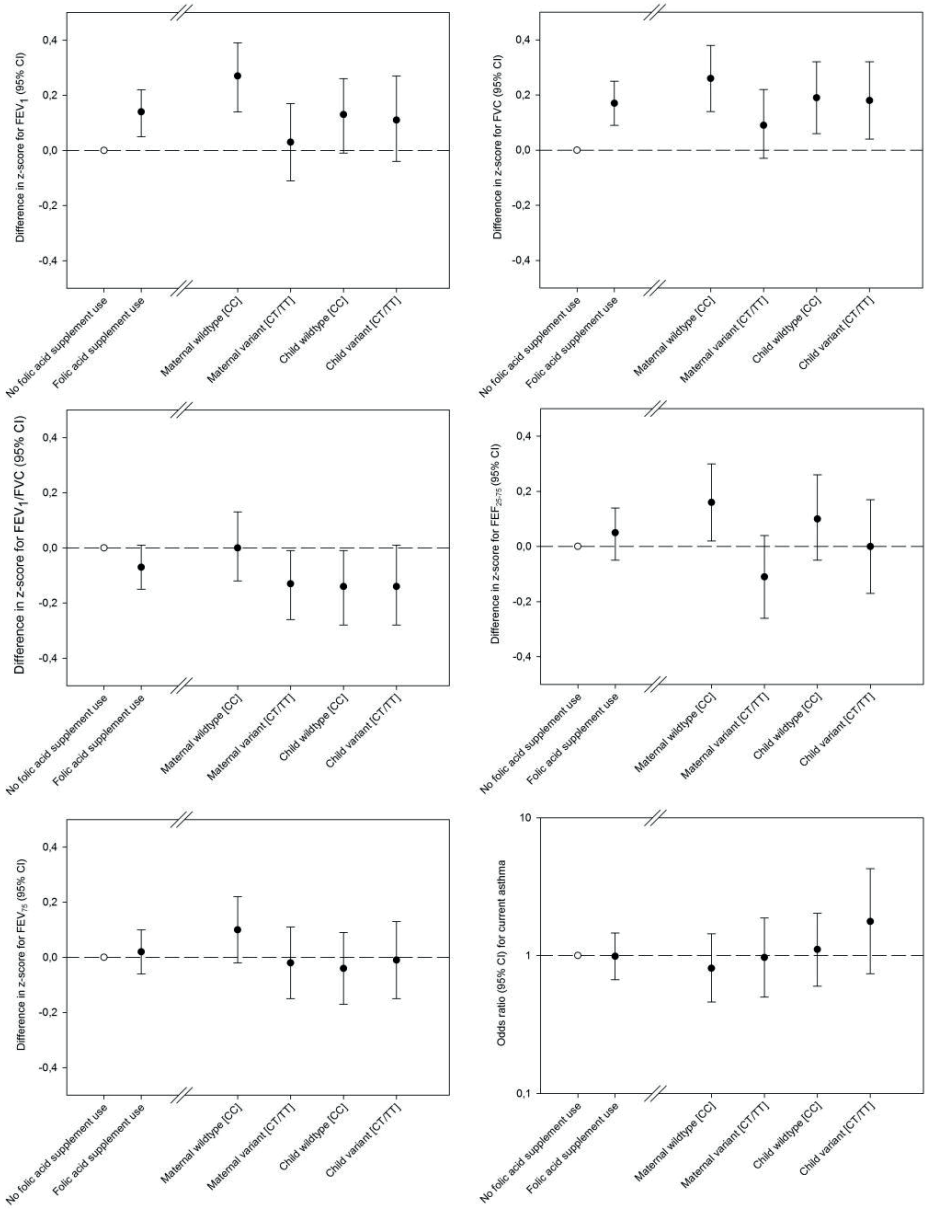


Figure 3.2.1. Associations of Maternal Folic Acid Supplement Use during Pregnancy with A) FEV_1, B) FVC, C) FEV_1/FVC, D) FEF_{25-75}, E) FEF_{75} and F) Current Asthma, for the Total Population and Stratified by Maternal and Child *MTHFR-C677T* Genotype.

Values are z-score mean differences or odds ratios (95% confidence intervals) with "never used folic acid supplements during pregnancy" as reference group. Associations for the entire population, and stratification by maternal and child *MTHFR-C677T* genotype are shown. Lung function variables were converted into sex-, height-, age- and ethnicity-adjusted z-scores. FEV_1: forced expiratory volume in the 1st 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 exhalation. Models were adjusted for maternal age and BMI at intake, parity, history of asthma or atopy, educational level, smoking or alcohol use during pregnancy, child's gestational age at birth, birthweight and ethnicity. *p < 0.05, ** p < 0.01.

Table 3.2.2. Associations of Folic Acid Supplement Use during Pregnancy with Childhood Lung Function and Asthma.

	FEV_1 Z-score (95% CI) n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
Folic acid supplement use						
Never (n = 855)	*Reference n = 794*	*Reference n = 795*	*Reference n = 794*	*Reference n =794*	*Reference n = 795*	*Reference n = 43 / 597*
Any use of folic acid (n = 3,670)	**0.14 (0.05, 0.22)**** n = 3,305	**0.17 (0.09, 0.25)**** n = 3,309	-0.07 (-0.15, 0.01) n = 3,305	0.05 (-0.05, 0.14) n = 3,308	0.02 (-0.06, 0.10) n = 3,308	0.99 (0.67, 1.46) n = 180 / 3,213
Start > 10 weeks (n = 575)	**0.13 (0.02, 0.24)*** n = 512	**0.14 (0.04, 0.25)**** n = 512	-0.05 (-0.16, 0.06) n = 512	**0.12 (0.00, 0.25)*** n = 512	0.04 (-0.07, 0.14) n = 512	0.85 (0.60, 1.43) n = 26 / 473
Start 0-10 weeks (n = 1,265)	**0.17 (0.07, 0.26)**** n = 1,145	**0.18 (0.09, 0.27)**** n = 1,148	-0.04 (-0.13, 0.05) n = 1,148	0.05 (-0.05, 0.15) n = 1,148	0.06 (-0.03, 0.15) n = 1,148	1.33 (0.74, 1.73) n = 74 / 1,105
Start preconceptional (n = 1,830)	**0.11 (0.02, 0.21)*** n = 1,648	**0.18 (0.10, 0.27)**** n = 1,649	**-0.11 (-0.21, -0.02)**** n = 1,648	0.02 (-0.09, 0.12) n = 1,649	-0.02 (-0.11, 0.06) n = 1,649	0.92 (0.50, 1.44) n = 80 / 1,635
P-value for trend	*0.68*	*0.39*	*0.09*	***0.04***	*0.11*	*0.56*

Values are z-score mean differences or odds ratios (95% confidence intervals) with "never folic acid supplement use during pregnancy" as reference group. "n =": number of individuals assessed (lung function) and number of cases / number of individuals assessed (current asthma). Any use of folic acid supplements was categorized into start after the first 10 weeks of pregnancy, start within the first 10 weeks of pregnancy and preconceptional start. P-value for trend depicts line for increased duration of folic acid supplements use. Lung function variables were converted into sex-, height-, age- and ethnicity-adjusted z-scores. FEV_1: forced expiratory volume in the 1st 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 exhalation. Models were adjusted for maternal age and body mass index at intake, parity, history of asthma or atopy, educational level, smoking or alcohol use during pregnancy, child's gestational age at birth, birthweight and ethnicity. *$p < 0.05$, ** $p < 0.01$.

(-0.40, -0.07), respectively). Effect estimates of preconceptional folic acid supplement use and lower FEV_1/FVC and FEF_{25-75} were similar in size and direction among *MTHFR-C677T* genotypes in children (p-values for interaction<0.05) (S-Table 3.2.4).

Folate, vitamin B_{12} and homocysteine concentrations and respiratory outcomes

In early pregnancy, higher maternal folate concentrations were associated with higher FEF_{75} among mothers carrying wildtype only (z-score (95% CI): 0.06 (0.00, 0.11), per 1 SDS increase), but not with other lung function measures (Table 3.2.3). We observed a borderline significant association of higher maternal folate concentrations with childhood current asthma among mothers carrying wildtype only (OR (95% CI): 0.78 (0.61, 1.00)). Vitamin B_{12} concentrations were not associated with lung function or current asthma. Higher homocysteine concentrations were associated with higher FVC (z-score difference (95% CI): 0.04 (0.01, 0.07) per 1 SDS increase), but not among the different maternal *MTHFR-C677T* genotype groups (p-value for interaction >0.05). When categorized, folate, vitamin B_{12} and homocysteine concentrations were not associated with lung function or current asthma (S-Table 3.2.5).

At birth, folate concentrations were not associated with lung function or current asthma (Table 3.2.4). When categorized, a low folate concentration at birth was associated with a higher FEV_1, FEV_1/FVC and FEF_{75} (0.11 (0.01, 0.21), 0.14 (0.04, 0.23) and 0.10 (0.01, 0.19), respectively) (S-Table 3.2.6). Higher vitamin B_{12} at birth was associated with lower FVC and higher FEV_1/FVC (z-score difference (95% CI): -0.05 (-0.08, -0.01) and 0.04, (0.00, 0.07), respectively, per 1 SDS increase). Effect estimates were more prominent among children carrying wildtype showing that a higher vitamin B_{12} concentration at birth was associated with a lower FEV_1 and FVC and higher FEV_1/FVC and FEF_{75} (p-values for interaction: 0.15, 0.01, 0.06 and 0.14, respectively) (Table 3.2.4). When categorized, a high vitamin B_{12} concentration was associated with a low FVC (-0.18 (-0.33, -0.03) in children carrying wildtype. A low vitamin B_{12} concentration was associated with a low FEF_{25-75} (-0.23 (-0.40, -0.06), compared with a normal vitamin B_{12} concentration, only among children carrying (S-Table 3.2.6). Homocysteine concentrations at birth were not associated with lung function and current asthma. Canonical correlation analyses showed that folate, vitamin B_{12} and homocysteine in early pregnancy and at birth explained 3.1% of the variance of the combined lung function measures (S-Tables 3.2.7). When stratified for *MTHFR-C677T* status, the explained variance was 5.7% (p-value 0.02) among mothers carrying variants, and 5.4% (p-value 0.04) among children carrying wildtype. No explained variance was observed among mothers carrying wildtype and children carrying variants (p-values 0.71 and 0.35, respectively).

We did not observe interactions between inhalant allergic sensitization and folic acid supplement use, folate, vitamin B_{12} or homocysteine for their association with lung function and current asthma.

Table 3.2.3. Associations of Folate, Vitamin B_{12} and Homocysteine Concentrations in Early Pregnancy with Childhood Lung Function and Asthma.

	FEV_1 Z-score (95% CI) n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
Folate concentration early pregnancy (SDS)						
Total population (n = 3,719)	0.01 (-0.02, 0.05)	0.02 (-0.02, 0.05)	-0.01 (-0.04, 0.03)	-0.01 (-0.05, 0.03)	0.00 (-0.03, 0.04)	0.93 (0.79, 1.09)
Maternal wildtype [CC] (n = 1,666)	0.04 (-0.01, 0.10)	0.02 (-0.03, 0.08)	0.04 (-0.02, 0.09)	0.01 (-0.05, 0.08)	**0.06 (0.00, 0.11)***	0.78 (0.61, 1.00)
Maternal variant [CT/TT] (n = 1,656)	-0.01 (-0.07, 0.04)	-0.01 (-0.06, 0.04)	-0.01 (-0.06, 0.05)	-0.05 (-0.11, 0.01)	-0.01 (-0.06, 0.04)	1.01 (0.78, 1.31)
Vitamin B_{12} concentration early pregnancy (SDS)						
Total population (n = 3,559)	-0.00 (-0.04, 0.03)	-0.00 (-0.03, 0.03)	0.01 (-0.03, 0.05)	0.00 (-0.04, 0.04)	0.01 (-0.02, 0.04)	0.95 (0.81, 1.11)
Maternal wildtype [CC] (n = 1,599)	-0.01 (-0.06, 0.05)	0.00 (-0.05, 0.05)	0.01 (-0.04, 0.06)	0.02 (-0.03, 0.08)	0.00 (-0.05, 0.05)	1.00 (0.79, 1.27)
Maternal variant [CT/TT] (n = 1,578)	-0.01 (-0.06, 0.04)	0.00 (-0.05, 0.05)	-0.03 (-0.08, 0.02)	-0.01 (-0.07, 0.05)	-0.00 (-0.05, 0.05)	0.89 (0.69, 1.15)
Homocysteine concentration early pregnancy (SDS)						
Total population (n = 3,683)	0.03 (-0.01, 0.06)	**0.04 (0.01, 0.07)***	-0.02 (-0.06, 0.01)	-0.02 (-0.06, 0.02)	0.00 (-0.03, 0.03)	1.05 (0.90, 1.22)
Maternal wildtype [CC] (n = 1,654)	0.00 (-0.06, 0.06)	0.02 (-0.04, 0.07)	-0.03 (-0.09, 0.03)	-0.01 (-0.08, 0.05)	-0.01 (-0.06, 0.04)	1.18 (0.95, 1.47)
Maternal variant [CT/TT] (n = 1,635)	0.01 (-0.04, 0.06)	0.03 (-0.02, 0.08)	-0.02 (-0.07, 0.03)	-0.02 (-0.08, 0.04)	-0.01 (-0.05, 0.04)	0.93 (0.71, 1.22)

Values are z-score mean differences or odds ratios (95% confidence intervals) with "never folic acid supplement use during pregnancy" as reference group. "n =": number of individuals assessed (lung function at age 10 years) and number of cases / number of individuals assessed (current asthma at age 10 years). Lung function variables were converted into sex-, height-, age- and ethnicity-adjusted z-scores. FEV_1: forced expiratory volume in the 1st 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 exhalation. Models were adjusted for maternal age and BMI at intake, parity, history of asthma or atopy, educational level, smoking or alcohol use during pregnancy, child's gestational age at birth, birthweight and ethnicity. Models were stratified for maternal or child *MTHFR-C677T* carriership when interaction (p-value < 0.05) was present. No significant interactions were observed between folate, vitamin B_{12} and homocysteine concentrations at birth with child *MTHFR-C677T* carriership. *p < 0.05, ** p < 0.01.

Table 3.2.4. Associations of Folate, Vitamin B_{12} and Homocysteine Concentrations at Birth with Childhood Lung Function and Asthma.

	FEV_1 Z-score (95% CI) n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
Folate concentration birth (SDS)						
Total population (n = 3,095)	-0.02 (-0.06, 0.02)	-0.00 (-0.04, 0.04)	-0.03 (-0.06, 0.01)	-0.03 (-0.07, 0.02)	-0.01 (-0.05, 0.02)	1.05 (0.88, 1.24)
Child wildtype [CC] (n = 1,430)	-0.03 (-0.09, 0.02)	-0.03 (-0.08, 0.03)	-0.00 (-0.06, 0.05)	-0.05 (-0.11, 0.01)	0.01 (-0.04, 0.07)	1.12 (0.98, 1.43)
Child variant [CT/TT] (n = 1,464)	-0.00 (-0.06, 0.05)	0.03 (-0.02, 0.08)	-0.04 (-0.10, 0.01)	0.00 (-0.06, 0.06)	-0.03 (-0.08, 0.02)	0.97 (0.74, 1.26)
Vitamin B_{12} concentration birth (SDS)						
Total population (n = 3,135)	-0.03 (-0.07, 0.00)	**-0.05 (-0.08, -0.01)***	**0.04 (0.00, 0.07)***	0.02 (-0.02, 0.06)	0.02 (-0.01, 0.06)	0.99 (0.84, 1.18)
Child wildtype [CC] (n = 1,437)	**-0.07 (-0.12, -0.01)***	**-0.09 (-0.15, -0.04)***	**0.07 (0.01, 0.12)***	-0.04 (-0.10, 0.03)	**0.05 (0.00, 0.10)***	1.15 (0.91, 1.46)
Child variant [CT/TT] (n = 1,489)	-0.02 (-0.08, 0.03)	-0.02 (-0.07, 0.03)	0.01 (-0.05, 0.06)	0.04 (-0.02, 0.10)	-0.00 (-0.05, 0.05)	0.81 (0.61, 1.07)
Homocysteine concentration birth (SDS)						
Total population (n = 3,003)	0.02 (-0.01, 0.06)	0.01 (-0.02, 0.05)	0.01 (-0.02, 0.05)	0.02 (-0.03, 0.06)	0.01 (-0.02, 0.05)	1.04 (0.88, 1.24)
Child wildtype [CC] (n = 1,389)	0.04 (-0.02, 0.09)	0.04 (-0.02, 0.09)	-0.02 (-0.07, 0.04)	0.04 (-0.03, 0.10)	-0.01 (-0.06, 0.05)	0.89 (0.68, 1.17)
Child variant [CT/TT] (n = 1,420)	0.02 (-0.03, 0.07)	-0.00 (-0.05, 0.05)	0.04 (-0.01, 0.09)	0.01 (-0.05, 0.07)	0.04 (-0.01, 0.08)	1.13 (0.89, 1.42)

Values are z-score mean differences or odds ratios (95% confidence intervals) with "never folic acid supplement use during pregnancy" as reference group. "n =": number of individuals assessed (lung function at age 10 years) and number of cases / number of individuals assessed (current asthma at age 10 years). Lung function variables were converted into sex-, height-, age- and ethnicity-adjusted z-scores. FEV_1: forced expiratory volume in the 1st 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 exhalation. Models were adjusted for maternal age and BMI at intake, parity, history of asthma or atopy, educational level, smoking or alcohol use during pregnancy, child's gestational age at birth, birthweight and ethnicity. Models were stratified for maternal or child *MTHFR-C677T* carriership when interaction (p-value < 0.05) was present. No significant interactions were observed between folate, vitamin B_{12} and homocysteine concentrations at birth with maternal *MTHFR-C677T* carriership. *p < 0.05, ** p < 0.01.

DISCUSSION

We observed that folic acid supplement use during pregnancy was associated with higher childhood FEV_1, FVC and FEF_{25-75} among mothers carrying *MTHFR*-C677*T* wildtype and with lower FEV_1/FVC and FEF_{25-75} among mothers carrying*MTHFR-C677T* variants, but not with childhood asthma. A higher vitamin B_{12} concentration at birth was associated with a lower FEV_1 and FVC among children carrying *MTHFR-C677T* wildtype and no associations were observed when carrying *MTHFR-C677T* variants. Folate, vitamin B_{12} and homocysteine concentrations in early pregnancy, or folate and homocysteine concentrations at birth, were not consistently associated with lower childhood lung function and asthma.

Comparison with previous studies

Previous studies present conflicting epidemiologic evidence of the effects of maternal folic acid supplement use and folate concentrations during pregnancy on risk of childhood asthma.[3-11] Two studies among 32,077 and >100,000 children showed that folic acid supplement use or folic acid prescription during pregnancy was associated with an up to 1.3-fold increased risks of wheeze and respiratory tract infections up to 18 months of age[6], and of asthma at ages 4-6 years[11], respectively. Similarly, in a population-based cohort study amongst 39,846 children, the higher quintile of maternal total folate intake was associated with an 1.2 increased relative risk of asthma at age 7 years, compared with the lowest quintile.[12] In contrast, a randomized controlled trial among 793 Nepalese children aged 8 years examined the effects of maternal micronutrient supplementation, including folic acid, and observed no differences in childhood FEV_1, FVC and FEV_1/FVC between the group with and without supplementation.[25] Results are difficult to generalize to western populations because the Nepalese study assessed multiple micronutrients in a population with depleted micronutrient sources. Folic acid supplement use during pregnancy was not associated with lung function, asthma or atopic outcomes among 1,902 Dutch children aged 6-7 years.[3] Higher intracellular folate concentrations in late pregnancy were associated with a lower risk of asthma in a dose-dependent manner. Results were limited by a small sample size with a low number of asthma-cases and a young study population.[26,27]

Studies on the associations of folate, vitamin B_{12} and homocysteine concentrations during pregnancy with lung function and asthma are scarce and conflicting.[3,8,10,28] Most studies were limited to an age of asthma diagnosis up to 4 years, which partly is biased by transient wheezing phenotypes. Only folate concentrations in late pregnancy seem to be consistently associated with an increased risk of wheezing and asthma in childhood.[26]

A study among 5,364 children aged 7-8 years[9] observed no modifying effect of maternal *MTHFR-C677T* on the associations between folic acid supplement use at 18 and 32 weeks of gestation and risk of asthma and atopic disease. We also observed no associations between *MTHFR-C677T* genotypes and the risk of childhood asthma. We did observe that associations of folic acid supplement use in early pregnancy with childhood lung function were affected by maternal *MTHFR-C677T* variant carriership, and of vitamin B_{12} concentrations at birth with FVC and FEF_{25-75} by type of child's *MTHFR-C677T* carriership. Canonical correlation analyses taking simultaneous comparison of correlated variables into account supported the observed associations. This suggests a role of genetic predisposition affecting the associations of folic supplement use and vitamin B_{12} blood concentrations with childhood lung function. The difference in findings with lung function and physician-diagnosed asthma in the current study could be due to the low number of asthma-cases, as an increased risk between high folic acid supplement use during pregnancy and childhood asthma was observed in large study populations.[6, 11, 12]

Interpretation of results

Folic acid supplement use in pregnancy was associated with higher childhood FEV_1 and FVC, but only among mothers carrying *MTHFR-C677T* wildtype. For these women, folic acid supplement use seems beneficial for their child's respiratory health in later life. Among mothers carrying *MTHFR-C677T* variants, we observed that folic acid supplement use was associated with lower FEV_1/FVC and FEF_{25-75}. *MTHFR* produces an enzyme that converts dietary folic acid to folate.[29] Homozygous *C677T* [TT] individuals have ~30% of the expected *MTHFR* enzyme activity, and heterozygotes [CT] have ~65% activity, compared to wildtype [CC].[16] Therefore, less conversion of folic acid occurs in *MTHFR-C677T* variant carriership with potential consequences for child's respiratory health. Folate, vitamin B_{12} and homocysteine are essential components in the one-carbon metabolism, which is responsible for the synthesis of purines and pyrimidines, numerous amino acids and S-adenosylmethionine (SAM). SAM is a key methyl donor in DNA-methylation. High concentrations of folic acid could inhibit the formation of folate and lead to a decrease in methionine synthesis and DNA hypomethylation. Similarly, vitamin B_{12} deficiency results in an excessive incorporation of uracil into DNA and DNA hypomethylation.[30] Experimental studies support the role of folate in cell division, nucleotide synthesis and DNA-methylation during fetal development [26]. Elevated folic acid exposure during pregnancy has been linked to cellular modifications associated with asthma and allergic diseases.[31] In animal models, enriched maternal diet with amongst others folic acid and vitamin B_{12} during pregnancy was associated with epigenetic changes and phenotypic differences in offspring, including immune functioning and insulin resistance, compared with offspring of mothers fed a low methyl donor diet.[31, 32] Human studies are scarce. We previously observed that folate concentrations in early pregnancy were associated

with differential DNA-methylation of 443 CpGs linked to 320 genes at birth.[33] These findings suggest that insufficient or excess intake of methyl donors leads to altered folate, vitamin B_{12} and homocysteine concentrations, which could affect DNA-methylation. Whether this mechanism affects lung development, lung function and risk of asthma remains to be studied.

Strengths and limitations

We used a prospective population-based design with a large number of participants being studied from early life with detailed and repeatedly measured exposures and multiple respiratory outcomes. An association of folic acid supplement use with lung function is supported by our stratified analyses on *MTHFR-C677T*. This genetic variant has an independent distribution in the population. Therefore, it is unlikely that the differences in associations between folic acid supplement use amongst mothers with *MTHFR-C677T* variants and lung function were biased by residual socio-economic and lifestyle-related factors. Still, some limitations do apply. The major source of selection bias in cohort studies usually arises from loss to follow-up[34], which was 25.3% in our study. We observed differences in characteristics between included participants and those lost to follow-up, and can therefore not fully exclude selection bias. Plasma folate does not reflect self-reported folic acid supplementation use in early pregnancy.[35] Plasma folate concentrations are sensitive to dietary changes and reflect short-term balance of folate. Therefore, the associations of folate concentrations with lung function and asthma do not reflect folate concentrations throughout pregnancy. Last, analyses stratified on genotype were limited by numbers of asthma cases, which might have led to under- or overestimation of the true effect sizes.

In conclusion, preconceptional start of maternal folic acid supplement use and higher vitamin B_{12} concentration at birth seem associated with lower childhood lung function depending on maternal or child *MTHFR-C677T* carriership. Further understanding of the interactions between folic acid exposure, blood methyl donor concentrations and genetic vulnerability is needed to provide insight into mechanisms for the development of chronic obstructive respiratory diseases in later life. Given the importance of folic acid during pregnancy to prevent congenital abnormalities, potential adverse consequences of supplement use needs to be carefully evaluated before modifications in current recommendations are considered.

REFERENCES

1. Wolff T, Witkop CT, Miller T, Syed SB, Force USPST. Folic acid supplementation for the prevention of neural tube defects: an update of the evidence for the U.S. Preventive Services Task Force. Ann Intern Med 2009; 150:632-9.
2. Sharma S, Litonjua A. Asthma, allergy, and responses to methyl donor supplements and nutrients. J Allergy Clin Immunol 2014; 133:1246-54.
3. Magdelijns FJ, Mommers M, Penders J, Smits L, Thijs C. Folic acid use in pregnancy and the development of atopy, asthma, and lung function in childhood. Pediatrics 2011; 128:e135-44.
4. van der Valk RJ, Kiefte-de Jong JC, Sonnenschein-van der Voort AM, Duijts L, Hafkamp-de Groen E, Moll HA, et al. Neonatal folate, homocysteine, vitamin B12 levels and methylenetetrahydrofolate reductase variants in childhood asthma and eczema. Allergy 2013; 68:788-95.
5. Bekkers MB, Elstgeest LE, Scholtens S, Haveman-Nies A, de Jongste JC, Kerkhof M, et al. Maternal use of folic acid supplements during pregnancy, and childhood respiratory health and atopy. Eur Respir J 2012; 39:1468-74.
6. Haberg SE, London SJ, Stigum H, Nafstad P, Nystad W. Folic acid supplements in pregnancy and early childhood respiratory health. Archives of Disease in Childhood 2009; 94:180-4.
7. Whitrow MJ, Moore VM, Rumbold AR, Davies MJ. Effect of supplemental folic acid in pregnancy on childhood asthma: a prospective birth cohort study. Am J Epidemiol 2009; 170:1486-93.
8. Haberg SE, London SJ, Nafstad P, Nilsen RM, Ueland PM, Vollset SE, et al. Maternal folate levels in pregnancy and asthma in children at age 3 years. J Allergy Clin Immunol 2011; 127:262-4, 4 e1.
9. Granell R, Heron J, Lewis S, Davey Smith G, Sterne JA, Henderson J. The association between mother and child MTHFR C677T polymorphisms, dietary folate intake and childhood atopy in a population-based, longitudinal birth cohort. Clin Exp Allergy 2008; 38:320-8.
10. Kiefte-de Jong JC, Timmermans S, Jaddoe VW, Hofman A, Tiemeier H, Steegers EA, et al. High circulating folate and vitamin B-12 concentrations in women during pregnancy are associated with increased prevalence of atopic dermatitis in their offspring. J Nutr 2012; 142:731-8.
11. Veeranki SP, Gebretsadik T, Mitchel EF, Tylavsky FA, Hartert TV, Cooper WO, et al. Maternal Folic Acid Supplementation During Pregnancy and Early Childhood Asthma. Epidemiology 2015; 26:934-41.
12. Parr CL, Magnus MC, Karlstad O, Haugen M, Refsum H, Ueland PM, et al. Maternal Folate Intake During Pregnancy and Childhood Asthma in a Population Based Cohort. Am J Respir Crit Care Med 2016.
13. Sonnenschein-van der Voort AM, Howe LD, Granell R, Duijts L, Sterne JA, Tilling K, et al. Influence of childhood growth on asthma and lung function in adolescence. J Allergy Clin Immunol 2014.
14. Tanaka T, Scheet P, Giusti B, Bandinelli S, Piras MG, Usala G, et al. Genome-wide association study of vitamin B6, vitamin B12, folate, and homocysteine blood concentrations. Am J Hum Genet 2009; 84:477-82.
15. Hazra A, Kraft P, Lazarus R, Chen C, Chanock SJ, Jacques P, et al. Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway. Hum Mol Genet 2009; 18:4677-87.
16. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995; 10:111-3.
17. Thuesen BH, Husemoen LL, Ovesen L, Jorgensen T, Fenger M, Gilderson G, et al. Atopy, asthma, and lung function in relation to folate and vitamin B(12) in adults. Allergy 2010; 65:1446-54.
18. Kruithof CJ, Kooijman MN, van Duijn CM, Franco OH, de Jongste JC, Klaver CC, et al. The Generation R Study: Biobank update 2015. Eur J Epidemiol 2014; 29:911-27.

19. Timmermans S, Jaddoe VW, Silva LM, Hofman A, Raat H, Steegers-Theunissen RP, et al. Folic acid is positively associated with uteroplacental vascular resistance: the Generation R study. Nutr Metab Cardiovasc Dis 2011; 21:54-61.
20. Bergen NE, Jaddoe VW, Timmermans S, Hofman A, Lindemans J, Russcher H, et al. Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. Bjog 2012; 119:739-51.
21. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J 2005; 26:319-38.
22. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. Eur Respir J 2012; 40:1324-43.
23. van der Valk JP, Gerth van Wijk R, Hoorn E, Groenendijk L, Groenendijk IM, de Jong NW. Measurement and interpretation of skin prick test results. Clin Transl Allergy 2015; 6:8.
24. Sherry A, Henson RK. Conducting and interpreting canonical correlation analysis in personality research: a user-friendly primer. J Pers Assess 2005; 84:37-48.
25. Devakumar D, Stocks J, Ayres JG, Kirkby J, Yadav SK, Saville NM, et al. Effects of antenatal multiple micronutrient supplementation on lung function in mid-childhood: follow-up of a double-blind randomised controlled trial in Nepal. Eur Respir J 2015; 45:1566-75.
26. Brown SB, Reeves KW, Bertone-Johnson ER. Maternal folate exposure in pregnancy and childhood asthma and allergy: a systematic review. Nutr Rev 2014; 72:55-64.
27. Blatter J, Han YY, Forno E, Brehm J, Bodnar L, Celedon JC. Folate and asthma. Am J Respir Crit Care Med 2013; 188:12-7.
28. Miyake Y, Sasaki S, Tanaka K, Hirota Y. Maternal B vitamin intake during pregnancy and wheeze and eczema in Japanese infants aged 16-24 months: the Osaka Maternal and Child Health Study. Pediatr Allergy Immunol 2011; 22:69-74.
29. Kim MW, Hong SC, Choi JS, Han JY, Oh MJ, Kim HJ, et al. Homocysteine, folate and pregnancy outcomes. J Obstet Gynaecol 2012; 32:520-4.
30. Okun JG, Horster F, Farkas LM, Feyh P, Hinz A, Sauer S, et al. Neurodegeneration in methylmalonic aciduria involves inhibition of complex II and the tricarboxylic acid cycle, and synergistically acting excitotoxicity. J Biol Chem 2002; 277:14674-80.
31. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A 2007; 104:19351-6.
32. Hollingsworth JW, Maruoka S, Boon K, Garantziotis S, Li Z, Tomfohr J, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. J Clin Invest 2008; 118:3462-9.
33. Joubert BR, den Dekker HT, Felix JF, Bohlin J, Ligthart S, Beckett E, et al. Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. Nat Commun 2016; 7:10577.
34. Nohr EA, Frydenberg M, Henriksen TB, Olsen J. Does low participation in cohort studies induce bias? Epidemiology 2006; 17:413-8.
35. Roth C, Bjorke-Monsen AL, Reichborn-Kjennerud T, Nilsen RM, Smith GD, Stoltenberg C, et al. Use of folic acid supplements in early pregnancy in relation to maternal plasma levels in week 18 of pregnancy. Mol Nutr Food Res 2013; 57:653-60.

SUPPLEMENTAL MATERIAL

METHODS

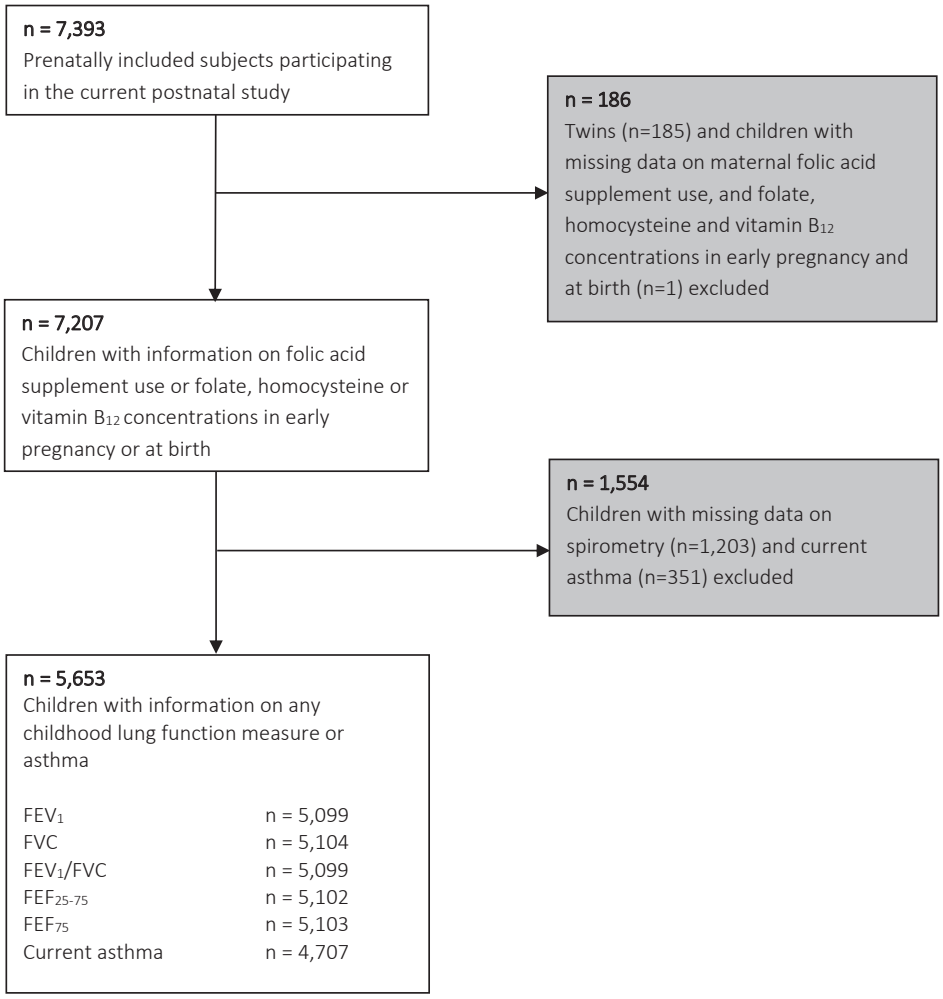
Folate, homocysteine and vitamin B_{12} concentrations Samples were transported to a dedicated laboratory facility (STAR-MDC) (1). In 2008, plasma samples (folate and homocysteine) and serum samples (vitamin B_{12}) were analysed at the Department of Clinical Chemistry at the Erasmus MC, University Medical Center Rotterdam (2). After thawing, folate, vitamin B_{12} and homocysteine concentrations were analysed using an immunoelectrochemoluminescence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands) (2). The between-run coefficient of variation for plasma folate was 8.9% at 5.6 nmol/l, 2.5% at 16.6 nmol/l and 1.5% at 33.6 nmol/l, with an analytical range of 1.8-45.3 nmol/l. The between-run coefficient of variation for serum vitamin B_{12} was 3.6% at 142 pmol/l, 7.5% at 308 pmol/l and 3.1% at 633 pmol/l, with an analytical range of 44-1476 pmol/l (2). The between-run coefficient of variation for plasma homocysteine was 3.1% at 7.2 µmol/l, 3.1% at 12.9 µmol/l and 2.1% at 26.1 µmol/l, with an analytic range of 1-50 µmol/l. Folate, vitamin B_{12} and homocysteine concentrations were available for 80.8%, 76.5% and 79.9% mothers and 71.2%, 71.2% and 68.4% children (1).

Inhalant allergic sensitization Allergic sensitization was measured by skin prick tests on the volar surface of the left forearm with single-use sterile 1 mm-tip metal lancets (ALK-Abelló A/S, Hørsholm, Denmark). Histamine dihydrochloride (10 mg/mL) and a saline solution (NaCl 0.9%) served as 2 positive controls and 1 negative control, respectively. Inhalant allergens tested were *Dermatophagoides pteronyssinus*, 5-grass mixture (*Dactylis glomerata, Festuca pratensis, Lolium perenne, Phleum pratense, Poa pratensis*), birch (*Betula verrucosa*), cat (*Felis catus*) and dog (*Canis familiaris*) (ALK-Abelló B.V., Almere, The Netherlands). Skin responses were recorded 15 minutes after applying allergens to the skin by measuring the area of the wheal (in mm^2) using the Precise Automated Area Measurement of Skin Test (PAAMOST) software and considered positive if the area of the wheal was ≥40% of the histamine response (i.e., histamine equivalent prick (HEP) index area ≥0.40) (3). A skin prick test was not performed if children could not omit antihistamine intake or corticosteroid ointment ≤72 h and ≤48 h prior to the test, respectively, used oral prednisone ≥10 mg daily or had eczema on the volar surface of the left forearm (n=51).

Canonical correlation analysis Canonical correlation analysis represents the highest level of the general linear modeling and can be interpreted as a method similar to the Pearson correlation coefficient (4). Canonical correlation analysis allows for simultaneous comparison among correlated variables. We tested whether 6 variables (folic acid, vitamin B_{12} and homocysteine during pregnancy and at birth) could predict 5 lung function variables. In that case, 30 multiple regressions are needed to examine the

associations between each exposure and outcome separately. This will result in and increased experiment-wise (EW) Type I error rate. At a test-wise (TW) error rate of 0.05, the experiment-wise error rate could be estimated as αEW = 1 – (1 – αTW)k = 1 – (1 – .05)^30 = 0.79, which is substantial. Also, we cannot identify which of the statistically significant results are true or false. Multivariate techniques such as canonical correlation analysis enables simultaneous assessment of the relationships between the 6 exposure variables and the 5 lung function variables. Because only one test is performed, the risk of committing a Type I error is minimized (4). Therefore, we conducted a canonical correlation analysis using folate, vitamin B_{12} and homocysteine levels in early pregnancy and at birth as predictors of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75} to evaluate the multivariate shared relationship between the two variable sets.

In the full study population (n = 5,653), the analysis yielded 5 functions with squared canonical correlations of 0.02452, 0.00483, 0.00176, 0.00040 and 0.00006 for each successive function. Collectively, the full model across all functions was statistically significant (Wilks's λ = .96861, p=.002). Because Wilks's λ represents the unexplained variance, 1 – λ shows the full model effect size as an r^2 value. Thus, for the set of 5 canonical functions, the r^2 type effect size was 0.03139, which indicates that the full model explained about 3.1% of the variance shared between the variable sets. Only function 1 was significant in all models.



S-Figure 3.2.1. Flowchart of Inclusion of Participants.

S-Table 3.2.1. Characteristics of Children and Their Mothers (n = 5,653)

	Original Data	Data after multiple imputation
Maternal characteristics		
Age (years)	31.0 (4.9)	31.0 (4.9)
Missing	-	-
Body mass index at intake (kg/m^2)[#]	23.7 (18.8 – 35.6)	23.9 (18.9 – 35.4)
Missing	9.8 (554)	-
Parity (%)		
Nullipara	57.4 (3,141)	57.0 (3,215)
Multipara	42.6 (2,331)	43.0 (2,438)
Missing	3.2 (181)	-
History of asthma or atopy (%)		
No	60.4 (2,772)	61.3 (3,463)
Yes	39.6 (1,818)	38.7 (2,190)
Missing	18.8 (1,063)	-
Education (%)		
Low (no, primary, secondary education)	49.0 (2,557)	50.5 (2,860)
High (higher education)	51.0 (2,661)	49.5 (2,793)
Missing	7.7 (435)	-
Smoking during pregnancy (%)		
No smoking throughout pregnancy	76.6 (3,802)	75.9 (4,288)
Smoking during first trimester	8.7 (432)	8.6 (485)
Smoking throughout pregnancy	14.7 (729)	15.6 (880)
Missing	12.2 (690)	-
Alcohol consumption during pregnancy (%)		
No alcohol throughout pregnancy	42.7 (1,943)	43.4 (2,453)
Alcohol during first trimester	14.1 (642)	14.2 (803)
Alcohol throughout pregnancy	43.2 (1,966)	42.4 (2,397)
Missing	19.5 (1,102)	-
Folic acid supplement use (%)		NI
No use	15.1 (855)	
Start after first 10 weeks of pregnancy	10.2 (575)	
Start within first 10 weeks of pregnancy	22.4 (1,265)	
Preconceptional start	32.4 (1,830)	
Missing	19.9 (1,128)	
Folate in early pregnancy (nmol/l)[#]	17.5 (6.0 – 37.9)	NI
Missing	34.2 (1,933)	
Vitamin B_{12} in early pregnancy (pmol/l)[#]	172.0 (77.0 – 406.0)	NI
Missing	37.1 (2,097)	
Homocysteine in early pregnancy (µmol/l)[#]	9.1 (5.2 – 16.6)	NI
Missing	34.6 (1,956)	
MTHFR-C677T (%)		NI
CC	51.0 (2,214)	

S-Table 3.2.1. Characteristics of Children and Their Mothers (n = 5,653) (continued)

	Original Data	Data after multiple imputation
CT	40.0 (1,738)	
TT	9.0 (391)	
Missing	23.2 (1,310)	
Child's characteristics		
Sex (%)		
Female	50.6 (2,860)	50.6 (2,860)
Male	49.4 (2,793)	49.4 (2,793)
Missing	-	-
Gestational age at birth (weeks)[#]	40.1 (35.8 – 42.3)	40.1 (35.8 – 42.3)
Missing	0.5 (33)	
Birth weight (grams)	3440 (554)	3440 (554)
Missing	0.1 (7)	-
Ethnicity (%)		
European	68.7 (3,674)	68.9 (3,895)
Non-European	31.3 (1,674)	31.1 (1,758)
Missing	5.4 (305)	
Folate at birth (nmol/l)[#]	20.9 (10.7 – 38.4)	NI
Missing	45.2 (2,555)	
Vitamin B_{12} at birth (nmol/l)[#]	303.0 (121.0 – 902.2)	NI
Missing	44.6 (2,521)	
Homocysteine at birth (µmol/l) [#]	6.9 (4.6 – 12.0)	NI
Missing	46.9 (2,651)	
MTHFR-C677T (%)		NI
CC	49.3 (1,773)	
CT	41.7 (1,500)	
TT	8.9 (320)	
Missing	35.1 (1,660)	
FEV_1 (L/s)	2.02 (0.30)	NI
FVC (L)	2.34 (0.36)	NI
FEV_1/FVC	0.87 (0.06)	NI
FEF_{25-75} (L/s)	2.71 (0.63)	NI
FEF_{75} (L/s)	1.14 (0.34)	NI
Current asthma (%)		NI
No	94.3 (4,438)	
Yes	5.7 (269)	
Missing	16.7 (946)	

Values are means (SD), valid percentages (absolute numbers) or [#]medians (95% range). FEV_1: forced expiratory volume in the 1st second, FEV_1: forced expiratory volume in the 1st 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 exhalation. NI: not imputed.

S-Table 3.2.2. Characteristics of Included (Population for Analysis) and Excluded (Lost to Follow-up) Participants.

	Included (n = 5,653)	**Lost to follow-up (n = 1,554)**	**P-value**
Maternal characteristics			
Age (years)	31.0 (4.9)	30.9 (4.9)	
Body mass index at intake (kg/m^2)#	23.9 (18.9 – 35.3)	24.4 (19.1 – 35.3)	0.18
Nulli-parity	57.4 (3,141)	52.3 (811)	<0.01
History of asthma or atopy, yes (%)	39.3 (2,180)	40.3 (626)	0.10
Lower education (%)	50.5 (2,860)	50.6 (786)	<0.01
Smoking during pregnancy (%)			
No smoking throughout pregnancy	75.9 (4,288)	70.4 (1,094)	0.46
Smoking during first trimester	8.6 (485)	8.4 (131)	
Smoking throughout pregnancy	15.6 (880)	21.2 (329)	
Alcohol consumption during pregnancy			
No alcohol throughout pregnancy	43.4 (2,453)	55.2 (858)	<0.01
Alcohol during first trimester	14.2 (803)	12.7 (197)	
Alcohol throughout pregnancy	42.4 (2,397)	32.1 (499)	
Folic acid supplement use	84.6 (3,616)	79.0 (1,228)	<0.01
Folate in early pregnancy (nmol/l)#	17.4 (6.0 – 37.9)	14.8 (5.3, 37.4)	<0.01
Vitamin B_{12} in early pregnancy (pmol/l)#	172 (77 – 406)	165 (68 – 411)	0.06
Homocysteine in early pregnancy (µmol/l)	7.2 (2.0)	7.3 (2.3)	0.08
Child's characteristics			
Female sex (%)	50.6 (2,860)	49.2 (765)	0.30
Gestational age at birth (weeks)#	40.1 (35.8 – 42.3)	39.7 (32.5 – 42.1)	0.05
Birth weight (grams)	3440 (554)	3213 (690)	<0.01
European ethnicity (%)	68.9 (3,895)	67.8 (1,054)	0.62
Folate at birth (nmol/l)#	20.9 (10.7 – 38.4)	20.0 (10.0, 38.5)	<0.01
Vitamin B_{12} at birth (nmol/l)#	313 (117 – 929)	294 (118 – 919)	0.19
Homocysteine at birth (µmol/l)	9.7 (3.2)	9.7 (3.1)	0.51

Values are means (SD), valid percentages (absolute numbers) or #medians (95% range). Differences between the included population and loss to follow-up were assessed with T-tests, Mann-Whitney U tests and chi-square tests.

S-Table 3.2.3. Associations of Folic Acid Supplement Use during Pregnancy with Childhood Lung Function and Asthma, Stratified for Maternal rs1801133.

		FEV_1 Z-score (95% CI) n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
Maternal genotype	**Folic acid use**						
Wildtype [CC]	No use n = 388	*Reference* *n = 362*	*Reference* *n = 363*	*Reference* *n = 363*	*Reference* *n = 363*	*Reference* *n = 363*	*Reference* *n = 24 / 257*
	Any folic acid supplementation n = 1,533	**0.27 (0.14, 0.39)**** n = 1,389	**0.26 (0.14, 0.38)**** n = 1,391	0.00 (-0.12, 0.13) n = 1,389	**0.16 (0.02, 0.30)*** n = 1,391	0.10 (-0.02, 0.22) n = 1,391	0.81 (0.46, 1.44) n = 82 / 1,386
	Start > 10 weeks n = 268	**0.24 (0.08, 0.41)**** n = 240	**0.23 (0.07, 0.37)**** n = 240	0.03 (-0.13, 0.19) n = 240	0.13 (-0.05, 0.10) n = 240	0.14 (-0.01, 0.29) n = 240	0.71 (0.33, 1.49) n = 13 / 224
	Start ≤ 10 weeks n = 537	**0.29 (0.15, 0.43)**** n = 491	**0.25 (0.11, 0.38)**** n = 491	0.05 (-0.09, 0.19) n = 491	0.14 (-0.02, 0.30) n = 491	**0.17 (0.04, 0.31)*** n = 491	0.93 (0.49, 1.75) n = 29 / 461
	Start preconceptional n = 749	**0.26 (0.12, 0.40)**** n = 660	**0.30 (0.16, 0.43)**** n = 660	-0.07 (-0.21, 0.07) n = 660	**0.19 (0.03, 0.35)*** n = 660	0.02 (-0.11, 0.15) n = 660	0.78 (0.40, 1.52) n = 29 / 668
	P-value for trend	*0.99*	*0.26*	*0.07*	*0.47*	*0.38*	*0.66*

S-Table 3.2.3. Associations of Folic Acid Supplement Use during Pregnancy with Childhood Lung Function and Asthma, Stratified for Maternal rs1801133. (continued)

		FEV_1 Z-score (95% CI) n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
Variant [CT/TT]	No use n = 313	*Reference n = 290*	*Reference n = 290*	*Reference n = 290*	*Reference n = 290*	*Reference n = 290*	*Reference n = 14 / 228*
	Any folic acid supplementation n = 1,541	0.03 (-0.11, 0.17) n = 1,420	0.09 (-0.03, 0.22) n = 1,421	**-0.13 (-0.26, -0.00)*** n = 1,419	-0.11 (-0.26, 0.04) n = 1,421	-0.02 (-0.15, 0.11) n = 1,421	0.97 (0.50, 1.88) n = 77 / 1,424
	Start > 10 weeks n = 225	0.04 (-0.14, 0.22) n = 198	0.08 (-0.09, 0.25) n = 198	-0.010 (-0.28, 0.08) n = 198	0.10 (-0.10, 0.30) n = 198	0.01 (-0.16, 0.18) n = 198	1.05 (0.45, 2.45) n = 11 / 182
	Start ≤ 10 weeks n = 530	0.08 (-0.07, 0.23) n = 477	0.12 (-0.02, 0.26) n = 477	-0.09 (-0.24, 0.06) n = 477	-0.09 (-0.26, 0.08) n = 477	0.02 (-0.12, 0.16) n = 477	1.07 (0.52, 2.19) n = 28 / 471
	Start preconceptional n = 811	-0.02 (-0.17, 0.13) n = 746	0.08 (-0.06, 0.22) n = 746	**-0.17 (-0.32, -0.02)*** n = 746	**-0.24 (-0.40, -0.07)*** n = 746	-0.04 (-0.18, 0.10) n = 746	0.85 (0.41, 1.76) n = 35 / 725
	P-value for trend	*0.24*	*0.96*	*0.16*	***<0.01***	*0.31*	*0.74*

Values are z-score differences or odds ratios (95% confidence intervals) with "never uses folic acid supplements during pregnancy" as reference group. Any use of folic acid supplements was categorized into start after the first 10 weeks of pregnancy, start within the first 10 weeks of pregnancy and preconceptional start. P-value for trend depicts line for increased duration of folic acid supplements use. Lung function variables were converted into sex-, height-, age- and ethnicity-adjusted z-scores. FEV_1: forced expiratory volume in the 1st 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 exhalation. Models were adjusted for maternal age and BMI at intake, parity, history of asthma or atopy, educational concentration, smoking or alcohol use during pregnancy, child's gestational age at birth, birthweight and ethnicity. *p < 0.05, ** p < 0.01.

S-Table 3.2.4. Associations of Folic Acid Supplement Use during Pregnancy with Childhood Lung Function and Asthma, Stratified for Child's rs1801133.

		FEV_1 Z-score (95% CI) n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
Childs genotype	**Folic acid use**						
Wildtype [CC]	No use n = 318	*Reference n = 295*	*Reference n = 295*	*Reference n = 295*	*Reference n = 295*	*Reference n = 295*	*Reference n = 18 / 228*
	Any folic acid supplementation n = 1,250	0.13 (-0.01, 0.26) n = 1,020	**0.19 (0.06, 0.32)*** n = 1,020	**-0.14 (-0.28, -0.00)*** n = 1,020	0.10 (-0.05, 0.26) n = 1,020	-0.04 (-0.17, 0.09) n = 1,020	1.11 (0.60, 2.03) n = 68 / 1,101
	Start > 10 weeks n = 202	0.08 (-0.11, 0.26) n = 178	0.15 (-0.03, 0.33) n = 178	-0.14 (-0.33, 0.04) n = 178	0.08 (-0.13, 0.29) n = 178	-0.03 (-0.20, 0.14) n = 178	0.98 (0.43, 1.99) n = 11 / 170
	Start ≤ 10 weeks n = 449	**0.16 (0.01, 0.32)*** n = 408	**0.21 (0.06, 0.36)**** n = 408	-0.12 (-0.28, 0.03) n = 408	0.08 (-0.10, 0.25) n = 408	0.00 (-0.14, 0.14) n = 408	1.27 (0.66, 2.46) n = 31 / 386
	Start preconceptional n = 599	0.11 (-0.04, 0.27) n = 539	**0.19 (0.04, 0.34)**** n = 539	**-0.16 (-0.32, -0.01)*** n = 539	0.15 (-0.03, 0.33) n = 539	-0.09 (-0.24, 0.05) n = 539	0.98 (0.48, 1.99) n = 26 / 545
	P-value for trend	*0.94*	*0.77*	*0.76*	*0.35*	*0.25*	*0.80*

S-Table 3.2.4. Associations of Folic Acid Supplement Use during Pregnancy with Childhood Lung Function and Asthma, Stratified for Child's rs1801133. (continued)

		FEV_1 Z-score (95% CI) n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
Variant [CT/TT]	No use n = 244	*Reference n = 222*	*Reference n = 222*	*Reference n = 222*	*Reference n = 222*	*Reference n = 222*	*Reference n = 8 / 174*
	Any folic acid supplementation n = 1,398	0.11 (-0.04, 0.27) n = 1,256	**0.18 (0.04, 0.32)*** n = 1,256	-0.14 (-0.28, 0.01) n = 1,256	0.00 (-0.17, 0.17) n = 1,256	-0.01 (-0.15, 0.13) n = 1,256	1.77 (0.74, 4.28) n = 64 / 1,235
	Start > 10 weeks n = 196	0.18 (-0.01, 0.37) n = 177	**0.20 (0.02, 0.38)*** n = 177	-0.07 (-0.26, 0.12) n = 177	0.19 (-0.03, 0.41) n = 177	0.04 (-0.15, 0.22) n = 177	1.70 (0.57, 4.09) n = 8 / 158
	Start ≤ 10 weeks n = 469	0.11 (-0.06, 0.27) n = 426	**0.16 (0.01, 0.32)*** n = 426	-0.13 (-0.29, 0.04) n = 426	-0.01 (-0.20, 0.17) n = 426	-0.01 (-0.16, 0.15) n = 426	1.94 (0.77, 4.88) n = 25 / 418
	Start preconceptional n = 733	0.09 (-0.08, 0.26) n = 653	**0.19 (0.04, 0.34)*** n = 653	**-0.18 (-0.34, -0.01)*** n = 653	-0.07 (-0.26, 0.11) n = 653	-0.04 (-0.20, 0.12) n = 653	1.63 (0.64, 4.17) n = 31 / 659
	P-value for trend	*0.51*	*0.41*	*0.22*	***0.01***	*0.43*	*0.80*

Values are z-score differences or odds ratios (95% confidence intervals) with "never uses folic acid supplements during pregnancy" as reference group. Any use of folic acid supplements was categorized into start after the first 10 weeks of pregnancy, start within the first 10 weeks of pregnancy and preconceptional start. P-value for trend depicts line for increased duration of folic acid supplements use. Lung function variables were converted into sex-, height-, age- and ethnicity-adjusted z-scores. FEV_1: forced expiratory volume in the 1st 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 exhalation. Models were adjusted for maternal age and BMI at intake, parity, history of asthma or atopy, educational concentration, smoking or alcohol use during pregnancy, child's gestational age at birth, birthweight and ethnicity. *p < 0.05, ** p < 0.01.

S-Table 3.2.5. Associations of Folate, Vitamin B_{12}, and Homocysteine Concentrations in Early Pregnancy with Spirometry Measures and Childhood Asthma, additionally Stratified by Maternal Genotype.

Genotype		**FEV_1 Z-score (95% CI)** n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
	Folate concentration early pregnancy (SDS)						
Total population	Low (n = 702)	-0.04 (-0.14, 0.05)	-0.06 (-0.15, 0.03)	0.03 (-0.06, 0.13)	-0.02 (-0.12, 0.09)	0.01 (-0.08, 0.09)	1.35 (0.91, 2.01)
	Normal (n = 2,324)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 693)	-0.01 (-0.10, 0.07)	-0.01 (-0.10, 0.07)	-0.01 (-0.09, 0.08)	-0.03 (-0.13, 0.07)	-0.01 (-0.10, 0.07)	1.11 (0.74, 1.66)
Maternal wildtype [CC]	Low (n = 314)	-0.07 (-0.21, 0.07)	-0.06 (-0.19, 0.08)	-0.02 (-0.16, 0.12)	0.00 (-0.15, 0.16)	-0.06 (-0.19, 0.07)	1.80 (0.97, 3.21)
	Normal (n = 1,579)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 315)	-0.02 (-0.15, 0.12)	-0.03 (-0.16, 0.10)	0.03 (-0.10, 0.16)	-0.05 (-0.19, 0.10)	0.03 (-0.10, 0.15)	1.05 (0.55, 1.99)
Maternal variant [CT/TT]	Low (n = 298)	0.02 (-0.12, 0.16)	0.01 (-0.12, 0.15)	0.01 (-0.13, 0.15)	-0.04 (-0.19, 0.12)	0.06 (-0.07, 0.19)	1.17 (0.60, 2.28)
	Normal (n = 1,607)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 303)	0.05 (-0.09, 0.18)	0.04 (-0.09, 0.16)	-0.01 (-0.14, 0.13)	-0.05 (-0.20, 0.10)	0.02 (-0.10, 0.15)	1.05 (0.58, 1.92)
	Vitamin B_{12} concentration early pregnancy (SDS)						
Total population	Low (n = 524)	0.03 (-0.06, 0.13)	-0.01 (-0.10, 0.09)	0.05 (-0.05, 0.14)	-0.05 (-0.16, 0.06)	0.05 (-0.04, 0.14)	0.98 (0.63, 1.53)
	Normal (n = 2,485)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 550)	0.00 (-0.09, 0.10)	-0.04 (-0.13, 0.05)	0.07 (-0.03, 0.16)	0.01 (-0.09, 0.12)	0.08 (-0.01, 0.17)	0.82 (0.51, 1.31)
	Homocysteine concentration early pregnancy (SDS)						
Total population	Low (n = 275)	-0.01 (-0.13, 0.12)	-0.18 (-0.20, 0.04)	0.11 (-0.02, 0.24)	-0.05 (-0.19, 0.09)	0.11 (-0.01, 0.23)	0.80 (0.41, 1.55)
	Normal (n = 3,047)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 361)	0.02 (-0.09, 0.13)	0.04 (-0.07, 0.14)	-0.03 (-0.14, 0.08)	-0.01 (-0.14, 0.11)	0.02 (-0.08 0.13)	1.07 (0.64, 1.77)

S-Table 3.2.5. Associations of Folate, Vitamin B_{12}, and Homocysteine Concentrations in Early Pregnancy with Spirometry Measures and Childhood Asthma, additionally Stratified by Maternal Genotype. (continued)

Genotype		**FEV_1 Z-score (95% CI)** n = 5,099	**FVC Z-score (95% CI)** n = 5,104	**FEV_1/FVC Z-score (95% CI)** n = 5,099	**FEF_{25-75} Z-score (95% CI)** n = 5,102	**FEF_{75} Z-score (95% CI)** n = 5,103	**Current asthma Odds Ratio (95% CI)** n = 269 / 4,707
Maternal wildtype [CC]	Low (n = 131)	0.06 (-0.13, 0.24)	-0.01 (-0.19, 0.17)	0.16 (-0.03, 0.34)	-0.07 (-0.28, 0.15)	0.15 (-0.02, 0.33)	0.86 (0.33, 2.28)
	Normal (n = 1,392)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 131)	-0.01 (-0.19, 0.17)	0.02 (-0.16, 0.19)	-0.07 (-0.25, 0.11)	-0.04 (-0.25, 0.17)	0.03 (-0.14, 0.23)	1.20 (0.55, 2.62)
Maternal variant [CT/TT]	Low (n = 112)	-0.02 (-0.22, 0.17)	-0.11 (-0.30, 0.07)	0.10 (-0.10, 0.30)	0.00 (-0.22, 0.22)	0.10 (-0.08, 0.29)	0.56 (0.17, 1.85)
	Normal (n = 1,347)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 176)	-0.08 (-0.24, 0.08)	-0.06 (-0.21, 0.09)	-0.02 (-0.17, 0.15)	-0.01 (-0.19, 0.17)	-0.02 (-0.17, 0.13)	0.94 (0.42, 2.14)

Values are z-score differences or odds ratios (95% confidence intervals) with "never uses folic acid supplements during pregnancy" as reference group. Any use of folic acid supplements was categorized into start after the first 10 weeks of pregnancy, start within the first 10 weeks of pregnancy and preconceptional start. Lung function variables were converted into sex-, height-, age- and ethnicity-adjusted z-scores. FEV_1: forced expiratory volume in the 1st 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 exhalation. Models were adjusted for maternal age and BMI at intake, parity, history of asthma or atopy, educational concentration, smoking or alcohol use during pregnancy, child's gestational age at birth, birthweight and ethnicity. Models were stratified for maternal or child *MTHFR-C677T* carriership when interaction (p-value < 0.05) was present. *p < 0.05, ** p < 0.01.

S-Table 3.2.6. Associations of Folate, Vitamin B_{12}, and Homocysteine Concentrations at Birth with Spirometry Measures and Childhood Asthma, Stratified by Child Genotype.

Genotype		FEV_1 Z-score (95% CI) n = 5,099	FVC Z-score (95% CI) n = 5,104	FEV_1/FVC Z-score (95% CI) n = 5,099	FEF_{25-75} Z-score (95% CI) n = 5,102	FEF_{75} Z-score (95% CI) n = 5,103	Current asthma Odds Ratio (95% CI) n = 269 / 4,707
	Folate concentration birth (SDS)						
Total Population	Low (n = 548)	**0.11 (0.01, 0.21)***	0.02 (-0.07, 0.12)	**0.14 (0.04, 0.23)****	0.07 (-0.04, 0.18)	**0.10 (0.01, 0.19)***	0.75 (0.46, 1.22)
	Normal (n = 1,981)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 566)	0.02 (-0.07, 0.12)	0.01 (-0.09, 0.10)	0.04 (-0.05, 0.14)	0.02 (-0.08, 0.13)	0.04 (-0.05, 0.13)	1.11 (0.72, 1.70)
	Vitamin B_{12} concentration birth (SDS)						
Total Population	Low (n = 475)	0.03 (-0.08, 0.13)	0.08 (-0.02, 0.18)	-0.07 (-0.18, 0.03)	-0.11 (-0.22, 0.01)	-0.05 (-0.14, 0.05)	0.94 (0.58, 1.53)
	Normal (n = 2,174)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 486)	-0.09 (-0.19, 0.02)	-0.09 (-0.19, 0.01)	0.04 (-0.06, 0.15)	-0.05 (-0.16, 0.07)	0.05 (-0.05, 0.14)	1.04 (0.65, 1.66)
Child wildtype [CC]	Low (n = 218)	0.09 (-0.07, 0.24)	0.14 (-0.01, 0.29)	-0.10 (-0.25, 0.05)	0.05 (-0.12, 0.22)	-0.11 (-0.25, 0.03)	0.70 (0.33, 1.48)
	Normal (n = 995)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 224)	-0.14 (-0.29, 0.02)	**-0.18 (-0.33, -0.03)***	0.11 (-0.04, 0.26)	-0.02 (-0.19, 0.15)	0.06 (-0.08, 0.19)	1.43 (0.78, 2.63)

Child variant [CT/TT]	Low (n = 225)	-0.02 (-0.17, 0.13)	0.02 (-0.12, 0.16)	-0.05 (-0.20, 0.10)	**-0.23 (-0.40, -0.06)****	-0.00 (-0.14, 0.14)	1.21 (0.60, 2.47)
	Normal (n = 1,036)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 228)	-0.08 (-0.23, 0.07)	0.03 (-0.16, 0.14)	-0.05 (-0.20, 0.10)	-0.14 (-0.31, 0.03)	0.01 (-0.13, 0.15)	0.63 (0.26, 1.51)
	Homocysteine concentration birth (SDS)						
Total population	Low (n = 304)	0.04 (-0.09, 0.16)	-0.01 (-0.13, 0.11)	0.08 (-0.04, 0.20)	-0.04 (-0.18, 0.09)	0.08 (-0.03, 0.19)	0.72 (0.37, 1.41)
	Normal (n = 2,315)	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
	High (n = 384)	0.11 (-0.14, 0.22)	0.08 (-0.03, 0.19)	-0.03 (-0.08, 0.14)	0.08 (-0.04, 0.21)	0.05 (-0.05, 0.15)	0.97 (0.58, 1.63)

Values are z-score differences or odds ratios (95% confidence intervals) with "never uses folic acid supplements during pregnancy" as reference group. Any use of folic acid supplements was categorized into start after the first 10 weeks of pregnancy, start within the first 10 weeks of pregnancy and preconceptional start. Lung function variables were converted into sex-, height-, age- and ethnicity-adjusted z-scores. FEV_1: forced expiratory volume in the 1st 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 exhalation. Models were adjusted for maternal age and BMI at intake, parity, history of asthma or atopy, educational concentration, smoking or alcohol use during pregnancy, child's gestational age at birth, birthweight and ethnicity. Models were stratified for maternal or child *MTHFR-C677T* carriership when interaction (p-value < 0.05) was present. *p < 0.05, ** p < 0.01.

S-Table 3.2.7a. Canonical Correlation Analyses of Folate, Vitamin B_{12} and Homocysteine Levels in Early Pregnancy and at Birth with Lung Function in Childhood in the Full Cohort (n = 5,554).

	Coef	R_s	R^2_s
Folic acid in early pregnancy	0.81521	**-0.68265**	46.6011
Vitamin B_{12} in early pregnancy	0.19843	-0.10116	1.023335
Homocysteine in early pregnancy	0.31683	0.12982	1.685323
Folic acid at birth	-0.09881	0.13971	1.951888
Vitamin B_{12} at birth	-0.73317	**0.58409**	34.11553
Homocysteine at birth	-0.17574	-0.04560	0.207936
$zFEV_1$	5.25966	-0.06397	0.409216
zFVC	-4.20692	0.11063	1.2239
$zFEV_1$/FVC	-1.75401	-0.43037	18.52183
$zFEF_{25-75}$	-0.79941	-0.39034	15.23653
$zFEF_{75}$	-1.5503	**-0.47407**	22.47424

Coef = standardized canonical function coefficient; R_s = structure coefficient; R^2_s= squared structure coefficient. The analysis yielded 5 functions with squared canonical correlations of 0.02452, 0.00483, 0.00176, 0.00040 and 0.00006 for each successive function. Collectively, the full model across all functions was statistically significant using the Wilks's λ = .96861 criterion, F(30, 7138.00) = 1.904783, p=.002. The r^2 type effect size was 0.03139, which indicates that the full model explained about 3.1%, of the variance shared between the variable sets. For emphasis, structure coefficients <-0.45 or >0.45 are presented bold, indicating a "fair" factor loading, following Comrey et al. (Comrey A.L., Lee H.B.; A First encounter in factor analysis; Psychology Press; 2nd edition (1992)).

S-Table 3.2.7b. Canonical Correlation Analyses of Folate, Vitamin B_{12} and Homocysteine Levels in Early Pregnancy and at Birth with Lung Function in Childhood, Stratified by Maternal *MTHFR*-genotype.

	Maternal wildtype [CC]			**Maternal variant [CT/TT]**		
	Coef	**R_s**	**R^2_s**	**Coef**	**R_s**	**R^2_s**
Folic acid in early pregnancy	-0.87894	**-0.76565**	58.62199	-0.698	**-0.62437**	38.98379
Vitamin B_{12} in early pregnancy	-0.0917	0.14038	1.970654	-0.40151	-0.05235	0.274052
Homocysteine in early pregnancy	-0.06106	0.01838	0.033782	-0.23213	-0.01964	0.038573
Folic acid at birth	0.24131	-0.06156	0.378963	-0.08728	-0.24184	5.848659
Vitamin B_{12} at birth	0.60445	**0.58503**	34.22601	0.90281	**0.56253**	31.644
Homocysteine at birth	-0.02777	-0.08183	0.669615	0.17493	0.05519	0.304594
$zFEV_1$	-5.53056	-0.4227	17.86753	-4.94953	0.24971	6.235508
zFVC	4.05997	-0.3622	13.12178	4.14401	0.07658	0.58645
$zFEV_1$/FVC	1.31866	0.01057	0.011172	1.74442	0.40981	16.79442
$zFEF_{25-75}$	0.59887	0.05505	0.30305	0.96257	**0.64068**	41.04709
$zFEF_{75}$	1.65360	0.05200	0.27040	1.33863	0.43852	19.22998

Coef = standardized canonical function coefficient; R_s = structure coefficient; R^2_s= squared structure coefficient. For maternal wildtype genotype [CC], the full model across all functions was not statistically significant using the Wilks's $\lambda = 0.95641$ criterion, $F(30, 3122.00) = 1.16591$, $p=0.245$. For maternal variant genotype [CT/TT], the full model across all functions was statistically significant using the Wilks's $\lambda = 0.94309$ criterion, $F(30, 3218.00) = 1.58296$, $p=0.023$. The r^2 type effect size was 0.05691, which indicates that the full model explained about 5.7% of the variance shared between the variable sets. For emphasis, structure coefficients <-0.45 or >0.45 are presented bold, indicating a "fair" factor loading, following Comrey et al. (Comrey A.L., Lee H.B.; A First encounter in factor analysis; Psychology Press; 2nd edition (1992)).

S-Table 3.2.7c. Canonical Correlation Analyses of Folate, Vitamin B_{12} and Homocysteine Levels in Early Pregnancy and at Birth with Lung Function in Childhood, Stratified by Child's *MTHFR*-genotype.

	Child wildtype [CC]			**Child [CT/TT]**		
	Coef	**R_s**	**R^2_s**	**Coef**	**R_s**	**R^2_s**
Folic acid in early pregnancy	-0.80174	**-0.68318**	46.67349	-0.5916	-0.37101	13.76484
Vitamin B_{12} in early pregnancy	0.01434	0.32690	10.68636	-0.31106	0.0171	0.029241
Homocysteine in early pregnancy	-0.11453	-0.02947	0.086848	-0.4014	-0.20952	4.389863
Folic acid at birth	0.15967	-0.02351	0.055272	0.25349	0.06207	0.385268
Vitamin B_{12} at birth	0.65312	**0.67500**	45.5625	0.90781	**0.63458**	40.26918
Homocysteine at birth	-0.04658	-0.15288	2.337229	0.47162	0.23306	5.431696
$zFEV_1$	-4.78868	-0.22450	5.040025	-1.74626	0.75850	57.53223
zFVC	3.43064	-0.39679	15.74423	2.29663	**0.54205**	29.38182
$zFEV_1$/FVC	1.07971	0.43914	19.28439	1.45735	0.35409	12.53797
$zFEF_{25-75}$	0.49417	-0.00925	0.008556	0.66005	**0.82766**	68.50211
$zFEF_{75}$	1.82369	0.44780	20.05248	0.03688	**0.46946**	22.03927

Coef = standardized canonical function coefficient; R_s = structure coefficient; R^2_s= squared structure coefficient. For children with child wildtype genotype, the full model across all functions was statistically significant using the Wilks's $\lambda = 0.94639$ criterion, $F(30, 3250.00) = 1.50257$, $p=0.039$. The r^2 type effect size was 0.05361, which indicates that the full model explained about 5.4% of the variance shared between the variable sets. For children with variant genotype [CT/TT], the full model across all functions was not statistically significant using the Wilks's $\lambda = 0.96266$ criterion, $F(30, 3382.00) = 1.07760$, $p=0.353$. For emphasis, structure coefficients <-0.45 or >0.45 are presented bold, indicating a "fair" factor loading, following Comrey et al. (Comrey A.L., Lee H.B.; A First encounter in factor analysis; Psychology Press; 2nd edition (1992)).

REFERENCES

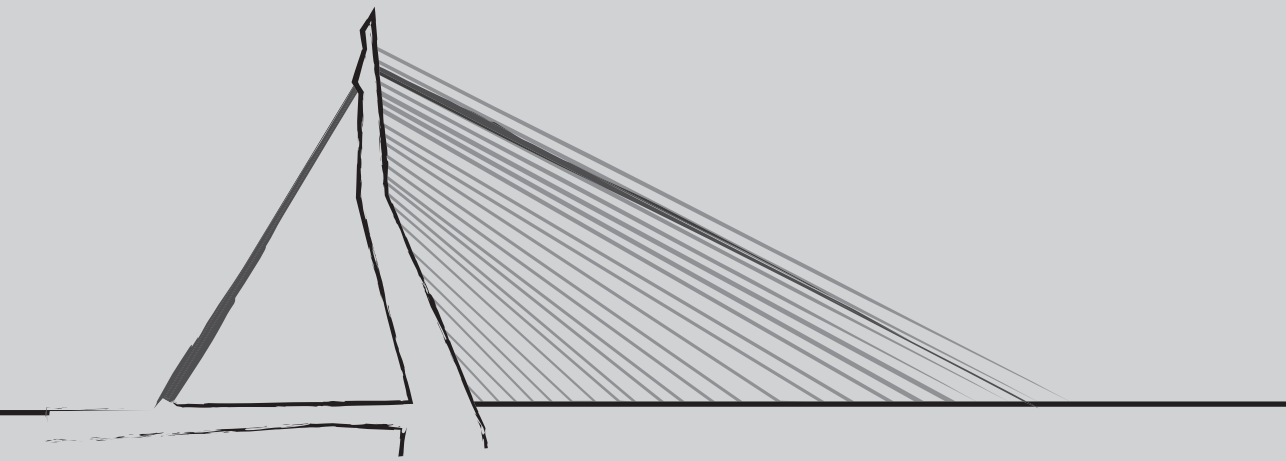
1. Kruithof CJ, Kooijman MN, van Duijn CM, Franco OH, de Jongste JC, Klaver CC, et al. The Generation R Study: Biobank update 2015. Eur J Epidemiol. 2014;29(12):911-27.
2. Bergen NE, Jaddoe VW, Timmermans S, Hofman A, Lindemans J, Russcher H, et al. Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. Bjog. 2012;119(6):739-51.
3. van der Valk JP, Gerth van Wijk R, Hoorn E, Groenendijk L, Groenendijk IM, de Jong NW. Measurement and interpretation of skin prick test results. Clin Transl Allergy. 2015;6:8.
4. Sherry A, Henson RK. Conducting and interpreting canonical correlation analysis in personality research: a user-friendly primer. J Pers Assess. 2005;84(1):37-48.

Chapter 3.3

Duration and exclusiveness of breastfeeding and outcome in asthma

HT den Dekker, AMM Sonnenschein – van der Voort, VWV Jaddoe, IK Reiss, JC de Jongste, L Duijts

Pediatr Allergy Immunol 2016;27(5):486-92



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.

REFERENCES

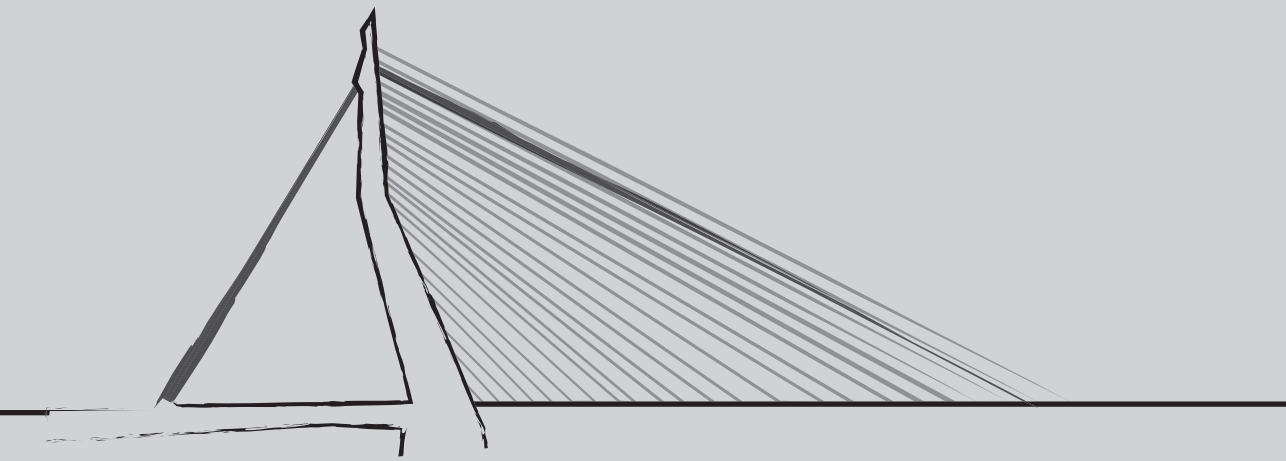
1. Duijts L, Reiss IK, Brusselle G, de Jongste JC. Early origins of chronic obstructive lung diseases across the life course. Eur J Epidemiol 2014; 29:871-85.
2. Elliott L, Henderson J, Northstone K, Chiu GY, Dunson D, London SJ. Prospective study of breast-feeding in relation to wheeze, atopy, and bronchial hyperresponsiveness in the Avon Longitudinal Study of Parents and Children (ALSPAC). J Allergy Clin Immunol 2008; 122:49-54, e1-3.
3. Kramer MS, Matush L, Vanilovich I, Platt R, Bogdanovich N, Sevkovskaya Z, et al. Effect of prolonged and exclusive breast feeding on risk of allergy and asthma: cluster randomised trial. BMJ 2007; 335:815.
4. Sonnenschein-van der Voort AM, Jaddoe VW, van der Valk RJ, Willemsen SP, Hofman A, Moll HA, et al. Duration and exclusiveness of breastfeeding and childhood asthma-related symptoms. Eur Respir J 2012; 39:81-9.
5. Dogaru CM, Nyffenegger D, Pescatore AM, Spycher BD, Kuehni CE. Breastfeeding and childhood asthma: systematic review and meta-analysis. Am J Epidemiol 2014; 179:1153-67.
6. Friedman NJ, Zeiger RS. The role of breast-feeding in the development of allergies and asthma. J Allergy Clin Immunol 2005; 115:1238-48.
7. Hoppu U, Kalliomaki M, Laiho K, Isolauri E. Breast milk--immunomodulatory signals against allergic diseases. Allergy 2001; 56 Suppl 67:23-6.
8. Oddy WH. A review of the effects of breastfeeding on respiratory infections, atopy, and childhood asthma. J Asthma 2004; 41:605-21.
9. Kramer MS. Invited commentary: Does breastfeeding protect against "asthma"? Am J Epidemiol 2014; 179:1168-70.
10. Kruithof CJ, Kooijman MN, van Duijn CM, Franco OH, de Jongste JC, Klaver CC, et al. The Generation R Study: Biobank update 2015. Eur J Epidemiol 2014; 29:911-27.
11. American Thoracic S, European Respiratory S. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med 2005; 171:912-30.
12. Merkus PJ, Stocks J, Beydon N, Lombardi E, Jones M, McKenzie SA, et al. Reference ranges for interrupter resistance technique: the Asthma UK Initiative. Eur Respir J 2010; 36:157-63.
13. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J 1995; 8:483-91.
14. Beurs E. Brief Symptom Inventory, handleiding Addendum. Leiden, the Netherlands. PITS BV. 2009.
15. Guxens M, Sonnenschein-van der Voort AM, Tiemeier H, Hofman A, Sunyer J, de Jongste JC, et al. Parental psychological distress during pregnancy and wheezing in preschool children: the Generation R Study. J Allergy Clin Immunol 2014; 133:59-67 e1-12.
16. Mackinnon DP, Fairchild AJ. Current Directions in Mediation Analysis. Curr Dir Psychol Sci 2009; 18:16.
17. Cole TJ. Sympercents: symmetric percentage differences on the 100 log(e) scale simplify the presentation of log transformed data. Stat Med 2000; 19:3109-25.
18. Dogaru CM, Strippoli MP, Spycher BD, Frey U, Beardsmore CS, Silverman M, et al. Breastfeeding and lung function at school age: does maternal asthma modify the effect? Am J Respir Crit Care Med 2012; 185:874-80.
19. Nagel G, Buchele G, Weinmayr G, Bjorksten B, Chen YZ, Wang H, et al. Effect of breastfeeding on asthma, lung function and bronchial hyperreactivity in ISAAC Phase II. Eur Respir J 2009; 33:993-1002.

20. Ogbuanu IU, Karmaus W, Arshad SH, Kurukulaaratchy RJ, Ewart S. Effect of breastfeeding duration on lung function at age 10 years: a prospective birth cohort study. Thorax 2009; 64:62-6.
21. Soto-Ramirez N, Alexander M, Karmaus W, Yousefi M, Zhang H, Kurukulaaratchy RJ, et al. Breast-feeding is associated with increased lung function at 18 years of age: a cohort study. Eur Respir J 2012; 39:985-91.
22. Shaukat A, Freudenheim JL, Grant BJ, Muti P, Ochs-Balcom HM, McCann SE, et al. Is being breastfed as an infant associated with adult pulmonary function? J Am Coll Nutr 2005; 24:327-33.
23. Kim HS, Kim YH, Kim MJ, Lee HS, Han YK, Kim KW, et al. Effect of breastfeeding on lung function in asthmatic children. Allergy Asthma Proc 2015; 36:116-22.
24. Kull I, Melen E, Alm J, Hallberg J, Svartengren M, van Hage M, et al. Breast-feeding in relation to asthma, lung function, and sensitization in young schoolchildren. J Allergy Clin Immunol 2010; 125:1013-9.
25. Guilbert T, Stern D, Morgan W, Martinez F, Wright A. Effect of Breastfeeding on Lung Function in Childhood and Modulation by Maternal Asthma and Atopy. Am J Respir Crit Care Med. 2007; 176:843-8.
26. Wright AL, Holberg CJ, Taussig LM, Martinez FD. Factors influencing the relation of infant feeding to asthma and recurrent wheeze in childhood. Thorax 2001; 56:192-7.
27. Walker WA, Iyengar RS. Breast milk, microbiota, and intestinal immune homeostasis. Pediatr Res 2015; 77:220-8.
28. Morales E, Garcia-Esteban R, Guxens M, Guerra S, Mendez M, Molto-Puigmarti C, et al. Effects of prolonged breastfeeding and colostrum fatty acids on allergic manifestations and infections in infancy. Clin Exp Allergy 2012; 42:918-28.
29. Tarrant M, Kwok MK, Lam TH, Leung GM, Schooling CM. Breast-feeding and childhood hospitalizations for infections. Epidemiology 2010; 21:847-54.
30. Wurzel DF, Marchant JM, Clark JE, Masters IB, Yerkovich ST, Upham JW, et al. Wet cough in children: Infective and inflammatory characteristics in broncho-alveolar lavage fluid. Pediatr Pulmonol 2013.
31. Snijders D, Agostini S, Bertuola F, Panizzolo C, Baraldo S, Turato G, et al. Markers of eosinophilic and neutrophilic inflammation in bronchoalveolar lavage of asthmatic and atopic children. Allergy 2010; 65:978-85.
32. Hancox RJ, Cowan DC, Aldridge RE, Cowan JO, Palmay R, Williamson A, et al. Asthma phenotypes: consistency of classification using induced sputum. Respirology 2012; 17:461-6.
33. Sonnenschein-van der Voort AM, Jaddoe VW, Raat H, Moll HA, Hofman A, de Jongste JC, et al. Fetal and infant growth and asthma symptoms in preschool children: the Generation R Study. Am J Respir Crit Care Med 2012; 185:731-7.
34. Brew B, Allen C, Toelle B, Marks G. Systematic review and meta-analysis investigating breast feeding and childhood wheezing illness. Paediatr Perinat Epidemiol 2011; 25:507-18.
35. Sears M, Greene J, Willan A, Taylor D, Flannery E, Cowan J, et al. Long-term relation between breast-feeding and development of atopy and asthma in children and young adults: a longitudinal study. Lancet 2002; 360:901-7.
36. Gaffin JM, Shotola NL, Martin TR, Phipatanakul W. Clinically useful spirometry in preschool-aged children: evaluation of the 2007 American Thoracic Society Guidelines. J Asthma 2010; 47:762-7.
37. Koopman M, Brackel HJ, Vaessen-Verberne AA, Hop WC, van der Ent CK, Group CO-RR. Evaluation of interrupter resistance in methacholine challenge testing in children. Pediatr Pulmonol 2011; 46:266-71.
38. Merkus PJ, Mijnsbergen JY, Hop WC, de Jongste JC. Interrupter resistance in preschool children: measurement characteristics and reference values. Am J Respir Crit Care Med 2001; 163:1350-5.

39. Richiardi L, Bellocco R, Zugna D. Mediation analysis in epidemiology: methods, interpretation and bias. Int J Epidemiol 2013; 42:1511-9.
40. Chu L, Rennie D, Cockcroft D, Pahwa P, Dosman J, Hagel L, et al. Agreement between questionnaire report of allergy-related outcomes in school-age children and objective measures of atopy: the Saskatchewan rural health study. Clin Exp Allergy 2015; 45:1337-45.
41. Braun-Fahrlander C, Wuthrich B, Gassner M, Grize L, Sennhauser FH, Varonier HS, et al. Validation of a rhinitis symptom questionnaire (ISAAC core questions) in a population of Swiss school children visiting the school health services. SCARPOL-team. Swiss Study on Childhood Allergy and Respiratory Symptom with respect to Air Pollution and Climate. International Study of Asthma and Allergies in Childhood. Pediatr Allergy Immunol 1997; 8:75-82.

Chapter 4

Genetics and epigenetics of childhood lung function and asthma

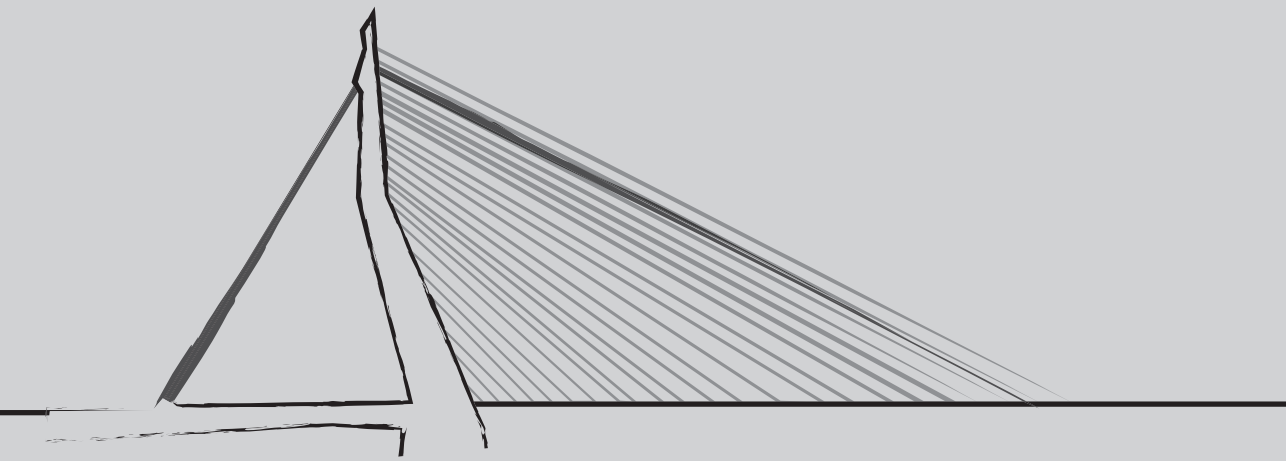


Chapter 4.1

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

K Bønnelykke, P Sleiman, K Nielsen, E Kreiner-Møller, JM Mercader, D Belgrave, HT den Dekker, A Husby, A Sevelsted, G Faura- Tellez, LJ Mortensen, L Paternoster, R Flaaten, A Mølgaard, DE Smart, PF Thomsen, MA Rasmussen, S Bonàs-Guarch, C Holst, EA Nohr, R Yadav, ME March, T Blicher, PM Lackie, VWV Jaddoe, A Simpson, JW Holloway, L Duijts, A Custovic, DE Davies, D Torrents, R Gupta, MV Hollegaard, DM Hougaard, H Hakonarson, H Bisgaard

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.

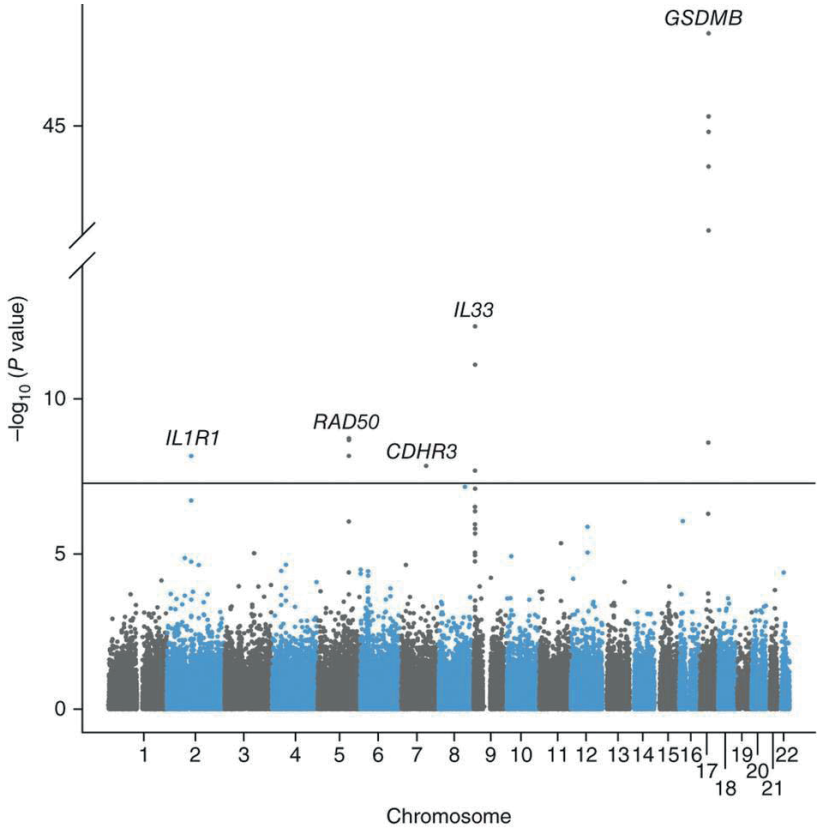


Figure 4.1.1. Manhattan Plot for the Discovery Genome-wide Association Analysis. The Horizontal Line indicates the Genome-wide Significance Threshold ($P < 5 \times 10^{-8}$).

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).

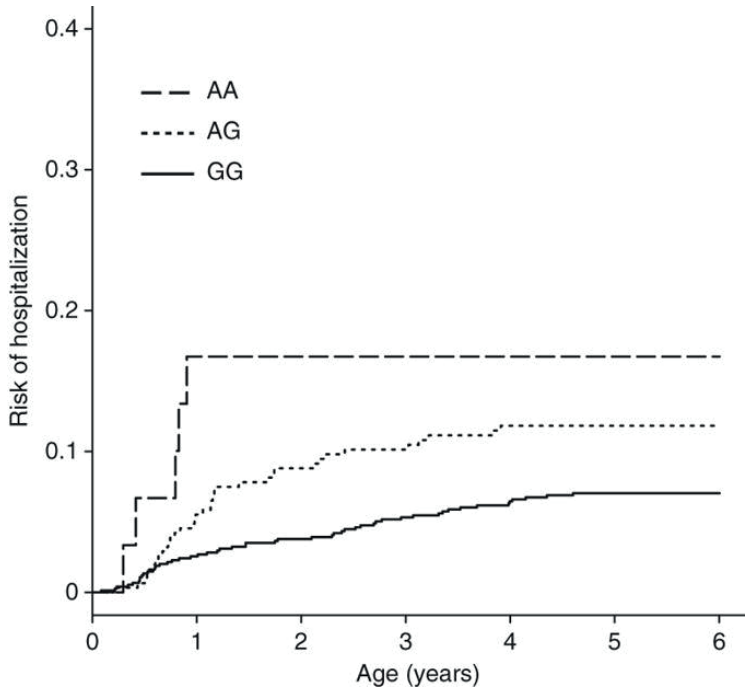


Figure 4.1.2. Cumulative Risk of Asthma Hospitalization during the First 6 Years of Life Stratified on *CDHR3* (rs6967330) Genotype.
Data are from combined analysis of the $COPSAC_{2000}$ and MAAS birth cohorts (replication), including a total of 1,091 children, of whom 92 were hospitalized for asthma. 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]

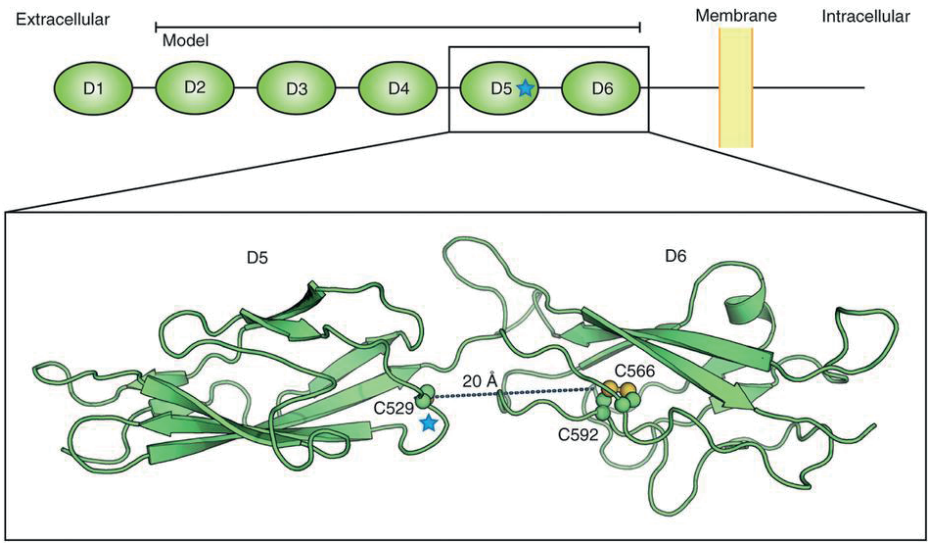


Figure 4.1.3. Overview of the *CDHR3* Protein Model.
The model covers cadherin domains 2–6 (D2–D6) and is based on the structure of the entire mouse N-cadherin ectodomain (Protein Data Bank (PDB) 3Q2W; domains 1–5). The location of the alteration at position 529 is indicated with a blue star. The distance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

REFERENCES

1. Kocevar VS, Bisgaard H, Jonsson L, Valovirta E, Kristensen F, Yin DD, et al. Variations in pediatric asthma hospitalization rates and costs between and within Nordic countries. Chest 2004; 125:1680-4.
2. Lozano P, Sullivan SD, Smith DH, Weiss KB. The economic burden of asthma in US children: estimates from the National Medical Expenditure Survey. J Allergy Clin Immunol 1999; 104:957-63.
3. Matterne U, Schmitt J, Diepgen TL, Apfelbacher C. Children and adolescents' health-related quality of life in relation to eczema, asthma and hay fever: results from a population-based cross-sectional study. Qual Life Res 2011; 20:1295-305.
4. Smith DH, Malone DC, Lawson KA, Okamoto LJ, Battista C, Saunders WB. A national estimate of the economic costs of asthma. Am J Respir Crit Care Med 1997; 156:787-93.
5. Bush A. Practice imperfect--treatment for wheezing in preschoolers. N Engl J Med 2009; 360:409-10.
6. Duffy DL, Martin NG, Battistutta D, Hopper JL, Mathews JD. Genetics of asthma and hay fever in Australian twins. Am Rev Respir Dis 1990; 142:1351-8.
7. van Beijsterveldt CE, Boomsma DI. Genetics of parentally reported asthma, eczema and rhinitis in 5-yr-old twins. Eur Respir J 2007; 29:516-21.
8. Ferreira MAR, Matheson MC, Duffy DL, Marks GB, Hui JN, Le Souef P, et al. Identification of IL6R and chromosome 11q13.5 as risk loci for asthma. Lancet 2011; 378:1006-14.
9. Gudbjartsson DF, Bjornsdottir US, Halapi E, Helgadottir A, Sulem P, Jonsdottir GM, et al. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. Nat Genet 2009; 41:342-7.
10. Himes BE, Hunninghake GM, Baurley JW, Rafaels NM, Sleiman P, Strachan DP, et al. Genome-wide association analysis identifies PDE4D as an asthma-susceptibility gene. Am J Hum Genet 2009; 84:581-93.
11. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. N Engl J Med 2010; 363:1211-21.
12. Sleiman PM, Flory J, Imielinski M, Bradfield JP, Annaiah K, Willis-Owen SA, et al. Variants of DENND1B associated with asthma in children. N Engl J Med 2010; 362:36-44.
13. Torgerson DG, Ampleford EJ, Chiu GY, Gauderman WJ, Gignoux CR, Graves PE, et al. Meta-analysis of genome-wide association studies of asthma in ethnically diverse North American populations. Nat Genet 2011; 43:887-92.
14. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. Lancet 2008; 372:1107-19.
15. Hollegaard MV, Grauholm J, Borglum A, Nyegaard M, Norgaard-Pedersen B, Orntoft T, et al. Genome-wide scans using archived neonatal dried blood spot samples. BMC Genomics 2009; 10:297.
16. Hollegaard MV, Grove J, Grauholm J, Kreiner-Moller E, Bonnelykke K, Norgaard M, et al. Robustness of genome-wide scanning using archived dried blood spot samples as a DNA source. BMC Genet 2011; 12:58.
17. Hao K, Bosse Y, Nickle DC, Pare PD, Postma DS, Laviolette M, et al. Lung eQTLs to help reveal the molecular underpinnings of asthma. PLoS Genet 2012; 8:e1003029.
18. Hulpiau P, van Roy F. Molecular evolution of the cadherin superfamily. Int J Biochem Cell Biol 2009; 41:349-69.
19. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJ, Heijink IH. E-cadherin: gatekeeper of airway mucosa and allergic sensitization. Trends Immunol 2011; 32:248-55.

20. Koppelman GH, Meyers DA, Howard TD, Zheng SL, Hawkins GA, Ampleford EJ, et al. Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. Am J Respir Crit Care Med 2009; 180:929-35.
21. Yanai I, Benjamin H, Shmoish M, Chalifa-Caspi V, Shklar M, Ophir R, et al. Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics 2005; 21:650-9.
22. McCall MN, Uppal K, Jaffee HA, Zilliox MJ, Irizarry RA. The Gene Expression Barcode: leveraging public data repositories to begin cataloging the human and murine transcriptomes. Nucleic Acids Res 2011; 39:D1011-5.
23. Ross AJ, Dailey LA, Brighton LE, Devlin RB. Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. Am J Respir Cell Mol Biol 2007; 37:169-85.
24. Kho AT, Bhattacharya S, Tantisira KG, Carey VJ, Gaedigk R, Leeder JS, et al. Transcriptomic analysis of human lung development. Am J Respir Crit Care Med 2010; 181:54-63.
25. Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. Immunol Rev 2011; 242:205-19.
26. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, et al. Defective epithelial barrier function in asthma. Journal of Allergy and Clinical Immunology 2011; 128:549-U177.
27. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. Can J Physiol Pharmacol 2008; 86:105-12.
28. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et al. Community Study of Role of Viral-Infections in Exacerbations of Asthma in 9-11 Year-Old Children. British Medical Journal 1995; 310:1225-9.
29. Bisgaard H, Hermansen MN, Bonnelykke K, Stokholm J, Baty F, Skytt NL, et al. Association of bacteria and viruses with wheezy episodes in young children: prospective birth cohort study. BMJ 2010; 341:c4978.
30. Iskandar A, Andersen ZJ, Bonnelykke K, Ellermann T, Andersen KK, Bisgaard H. Coarse and fine particles but not ultrafine particles in urban air trigger hospital admission for asthma in children. Thorax 2012; 67:252-7.
31. Di Rienzo A, Hudson RR. An evolutionary framework for common diseases: the ancestral-susceptibility model. Trends in Genetics 2005; 21:596-601.
32. Li X, Howard TD, Zheng SL, Haselkorn T, Peters SP, Meyers DA, et al. Genome-wide association study of asthma identifies RAD50-IL13 and HLA-DR/DQ regions. J Allergy Clin Immunol 2010; 125:328-35 e11.
33. Thomsen SF, Duffy DL, Kyvik KO, Backer V. Genetic influence on the age at onset of asthma: A twin study. Journal of Allergy and Clinical Immunology 2010; 126:626-30.
34. Lynge E, Sandegaard JL, Rebolj M. The Danish National Patient Register. Scand J Public Health 2011; 39:30-3.
35. Norgaard-Pedersen B, Hougaard DM. Storage policies and use of the Danish Newborn Screening Biobank. Journal of Inherited Metabolic Disease 2007; 30:530-6.
36. Paternoster L, Evans DM, Nohr EA, Holst C, Gaborieau V, Brennan P, et al. Genome-Wide Population-Based Association Study of Extremely Overweight Young Adults - The GOYA Study. Plos One 2011; 6.
37. Bisgaard H. The Copenhagen Prospective Study on Asthma in Childhood (COPSAC): design, rationale, and baseline data from a longitudinal birth cohort study. Annals of Allergy Asthma & Immunology 2004; 93:381-9.

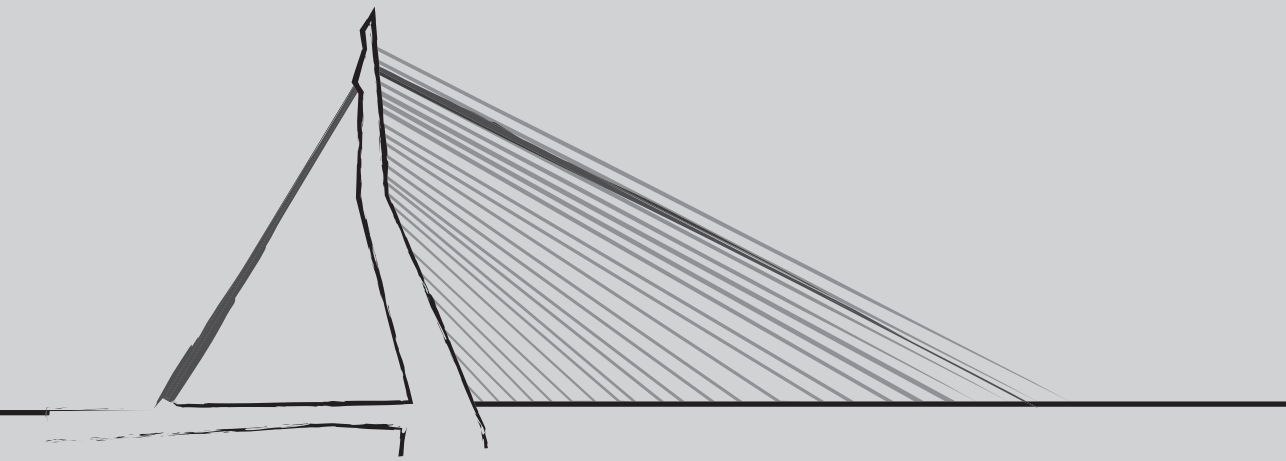
38. Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F. Intermittent inhaled corticosteroids in infants with episodic wheezing. New England Journal of Medicine 2006; 354:1998-2005.
39. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, et al. Childhood asthma after bacterial colonization of the airway in neonates. New England Journal of Medicine 2007; 357:1487-95.
40. Bisgaard H, Pipper CB, Bonnelykke K. Endotyping early childhood asthma by quantitative symptom assessment. Journal of Allergy and Clinical Immunology 2011; 127:1155-U428.
41. Bisgaard H, Bonnelykke K, Sleiman PMA, Brasholt M, Chawes B, Kreiner-Moller E, et al. Chromosome 17q21 Gene Variants Are Associated with Asthma and Exacerbations but Not Atopy in Early Childhood. American Journal of Respiratory and Critical Care Medicine 2009; 179:179-85.
42. Hanifin JM, Rajka G. Diagnostic Features of Atopic-Dermatitis. Acta Dermato-Venereologica 1980:44-7.
43. Lowe L, Murray CS, Custovic A, Simpson BM, Kissen PM, Woodcock A, et al. Specific airway resistance in 3-year-old children: a prospective cohort study. Lancet 2002; 359:1904-8.
44. Reddel HK, Taylor DR, Bateman ED, Boulet LP, Boushey HA, Busse WW, et al. An Official American Thoracic Society/European Respiratory Society Statement: Asthma Control and Exacerbations Standardising Endpoints for Clinical Asthma Trials and Clinical Practice. American Journal of Respiratory and Critical Care Medicine 2009; 180:59-99.
45. Jaddoe VWV, Bakker R, van Duijn CM, van der Heijden AJ, Lindemans J, Mackenbach JP, et al. The Generation R Study Biobank: a resource for epidemiological studies in children and their parents. European Journal of Epidemiology 2007; 22:917-23.
46. Jaddoe VWV, van Duijn CM, Franco OH, van der Heijden AJ, van IIzendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. European Journal of Epidemiology 2012; 27:739-56.
47. Ward LD, Kellis M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. Nucleic Acids Research 2012; 40:D930-D4.
48. Howie B, Fuchsberger C, Stephens M, Marchini J, Abecasis GR. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. Nature Genetics 2012; 44:955-+.
49. Delaneau O, Marchini J, Zagury JF. A linear complexity phasing method for thousands of genomes. Nature Methods 2012; 9:179-81.
50. Howie B, Marchini J, Stephens M. Genotype Imputation with Thousands of Genomes. G3-Genes Genomes Genetics 2011; 1:457-69.
51. Altshuler DM, Durbin RM, Abecasis GR, Bentley DR, Chakravarti A, Clark AG, et al. An integrated map of genetic variation from 1,092 human genomes. Nature 2012; 491:56-65.
52. Auer PL, Johnsen JM, Johnson AD, Logsdon BA, Lange LA, Nalls MA, et al. Imputation of Exome Sequence Variants into Population-Based Samples and Blood-Cell-Trait-Associated Loci in African Americans: NHLBI GO Exome Sequencing Project. American Journal of Human Genetics 2012; 91:794-808.
53. Marchini J, Howie B. Genotype imputation for genome-wide association studies. Nature Reviews Genetics 2010; 11:499-511.
54. Soding J, Biegert A, Lupas AN. The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Research 2005; 33:W244-W8.

Chapter 4.2

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

S Shagiwal, HT den Dekker, JC de Jongste, GG Brusselle, VWV Jaddoe, JF Felix, L Duijts

Submitted



ABSTRACT

Background Genetic variants associated with adult lung function could already exert effects in childhood lung function.

Objective To examine the associations of genetic variants identified for adult lung function with childhood lung function and asthma, and whether these associations were modified by atopic predisposition, tobacco smoke exposure, or early growth characteristics.

Methods In a population-based prospective cohort study among 3,347 children, we selected 7 and 20 SNPs associated with adult forced expiratory volume in 1 second (FEV_1) and FEV_1/forced vital capacity (FEV_1/FVC), respectively. Weighted genetic risk scores (GRSs) for FEV_1 and FEV_1/FVC were constructed. At age 10 years, FEV_1, FVC, FEV_1/FVC, forced expiratory flow between 25-75% (FEF_{25-75}) and at 75% (FEF_{75}) of FVC were measured, and asthma-diagnosis was obtained by questionnaire.

Results The FEV1-GRS was associated with lower childhood FEV_1, FEV_1/FVC and FEF_{75} (Z-score (95% CI): -0.03 (-0.05,-0.01), -0.03 (-0.05,-0.01) and -0.04 (-0.05, -0.01) respectively, per additional risk allele). The FEV_1/FVC-GRS was associated with lower childhood FEV_1/FVC and FEF_{75} (Z-score (95% CI): -0.04 (-0.05, -0.03) and -0.03 (-0.05, -0.02), respectively, per additional risk allele). Effect estimates of adult FEV_1-GRS with FEF_{75}, and adult FEV_1/FVC-GRS with FEV_1/FVC or FEF_{75} were stronger among children exposed to maternal smoking during childhood, maternal smoking during pregnancy or born at term (p-values for interaction < 0.05). Atopic predisposition did not modify the associations.

Conclusion Adult lung function-related genetic variants were associated with lung function but not with asthma at age 10 years. Exposure to maternal smoking and gestational age at birth modified the observed effects.

INTRODUCTION

Chronic obstructive respiratory diseases, including asthma and chronic obstructive pulmonary disease (COPD), have their origins partly in early life.[1] Chronic obstructive respiratory diseases are characterized by lower lung function measures of airway patency, such as Forced Expiratory Volume in 1 second (FEV_1) and a lower FEV_1/Forced Vital Capacity (FEV_1/FVC). Genetic factors are suggested to contribute to lung function in later life.[2] Familial aggregation and twin studies suggest heritability estimates of lung function of more than 40%.[3-6] Three meta-analyses of genome-wide association (GWA) studies from population-based studies showed that 35 genetic variants are associated with lung function in European adults.[7-10] Most of the genes closest to these variants are expressed in lung tissue and are involved in several biological processes including airway inflammation, morphogenesis, growth-related processes, repair responses to injury and cell signaling.[9] Previous studies have aimed to replicate adult lung function-related genetic variants, either individually or combined into genetic risk scores (GRSs), in European children aged 7-9 years.[9,11,12] Results across these studies were inconsistent, which might be explained by differences in the included study populations, definition and time of lung function measurements and adjustment for confounders. Furthermore, whether the associations of adult lung function-related genetic variants with childhood lung function and asthma are modified by known early life risk factors such as atopic predisposition, maternal smoking during pregnancy or in childhood, or mediated by infant characteristics such as gestational age and weight at birth is unknown.[13,14]

Therefore, we aimed to examine among 3,347 children the associations of adult lung function-related genetic variants, individually and combined into genetic risk scores, with lung function measures and current asthma at the age of 10 years in a population-based prospective cohort study. Second, we examined whether these associations were influenced by atopic predisposition, maternal smoking during pregnancy or in childhood, or early growth characteristics.

MATERIALS AND METHODS

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life onwards, in Rotterdam, The Netherlands.[15] The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands (16). Written informed consent was obtained from parents or legal guardians. A total of 3,347 children were included

in the current analyses after excluding twins, siblings and children with missing data on lung function measures or asthma (S-Figure 4.2.1).

Genetic variants and risk scores

Previous GWA studies of general population cohorts identified 35 single nucleotide polymorphisms (SNPs) associated with adult FEV_1 and FEV_1/FVC.[7-9,17] In the current study, we extracted those SNPs from a previously run GWA scan performed on DNA isolated from cord blood samples using the Illumina 610 Quad and 660 W platforms.[16] If a cord blood sample was unavailable, DNA was isolated from blood samples taken at the 6-year visit. For this GWA screen, quality control comprised exclusion of duplicates, sex mismatches and low sample call rates. Further, SNPs with call rates <98%, low minor allele frequencies (<0.1%), and significant deviations from Hardy-Weinberg equilibrium ($P < 1x10^{-6}$) were excluded. Genotypes were imputed for all polymorphic SNPs to the 1000 Genomes GIANTv3 panel. Information on 33 previously identified SNPs was directly available from the GWAS dataset (S-Table 1). Of these, 7 SNP pairs were in strong linkage disequilibrium ($r^2 \geq 0.8$).[18] Of each pair, we included the SNP with the strongest association with either FEV_1 or FEV_1/FVC, based on the p-value from the adult study. One SNP (rs2045517) was monomorphic in our study population, and therefore not included in analyses. Two SNPs (rs2865531 and rs9978142) were not directly available in our genetic data and therefore proxies (rs8050059 and rs10470171, respectively), which were in perfect linkage disequilibrium (r^2=1.00) were selected. In total, 26 SNPs were used for analyses.

We constructed genetic risk scores (GRSs) for FEV_1 and FEV_1/FVC ratio based on known SNPs related to adult FEV_1 and FEV_1/FVC, respectively. Risk alleles were defined as the alleles that previously have been associated with a lower lung function. Unweighted GRSs were calculated by summation of the number of risk alleles across all SNPs in the score, using the dosage data from the GWAs dataset.[9] Weighted GRSs were calculated the same way, but weighting the dosage information of each SNP by the previously reported effect estimates in adults.[9]

Childhood lung function and asthma

At a mean age of 10 years (standard deviation (SD): 0.3), lung function was measured by spirometry and performed according to American Thoracic Society (ATS) and European Respiratory Society (ERS) recommendations[19] Lung function measures of interest were FEV_1, FVC, FEV_1/FVC, forced expiratory flow at 25-75% (FEF_{25-75}) and forced expiratory flow at 75% (FEF_{75}) of FVC, and converted into sex-, height-, age-, and ethnicity-adjusted Z-scores according to the Global Lung Initiative reference values.[20] Ever asthma, current wheezing and use of inhaled medication in the past 12 months (no, yes) were reported by parents using questions adapted from the International Study on Asthma and Allergy in Childhood (ISAAC).[21] Current asthma was defined as ever physician diagnosed asthma

ever at age 10 years with parental reported wheezing and/or the use of inhaled medication in the past 12 months.

Covariates

Information on maternal history of asthma, eczema or allergy was obtained by a questionnaire at enrollment, and on smoking during pregnancy by multiple questionnaires in early, mid- and late pregnancy. Maternal smoking in childhood was assessed by questionnaire at the age of 6 years. Child's sex, gestational age at birth and birth weight were retrieved from hospital and midwife registries. Current height was measured at age 10 years using a stadiometer (Holtain Limited, Crosswell, Crymych, UK).

Statistical analysis

First, we examined associations of individual SNPs related to lung function in adults with childhood lung function and current asthma using multivariable linear and logistic regression analyses, respectively. Second, we examined the associations of the unweighted and weighted GRSs based on adult lung function with childhood lung function measures and current asthma using similar models. The risk scores were divided into categories with a minimum of 30 individuals per category. The difference in the explained variance in the null (without the GRS) and alternative model (with the GRS) was considered as the variance in the outcome explained by the GRS. All analyses were first performed in the full group. As a sensitivity analysis, we subsequently repeated all analyses in Europeans as this was the largest ethnic subgroup. A child was classified as European if he or she was within four standard deviations from the HapMap CEU panel for all first four principal components, based on the genetic data. Models were adjusted for child's age, age^2, sex, current height and the first four ancestry principal components for either the full group or Europeans only. We considered maternal asthma, allergy or eczema status, smoking during pregnancy and in childhood, and child's gestational age and weight at birth as potential effect modifiers reflecting atopic predisposition, adverse environmental exposures and infant characteristics, respectively. These were evaluated by adding the product term of the weighted GRSs and potential effect modifier as an independent variable into the model. We stratified the analyses for the potential effect modifier for the models in which a significant interaction (p-value <0.05) was observed. Furthermore, we assessed whether gestational age at birth and birth weight explained associations between the GRS and childhood lung function and asthma by additional adjustment for these variables in the models. To adjust for multiple testing, we applied Bonferroni correction for the number of individual SNPs per outcome and for the number of tested outcomes in the analyses of weighted GRSs. All measures of association are presented as change in Z-scores or odds ratios with corresponding 95% confidence Intervals (95% CI). Statistical analyses were performed using SPSS version 21.0 for Windows software (IBM, Chicago, Ill, USA).

RESULTS

Table 4.2.1 shows the characteristics for all children and for the European children separately. In the full group, 50.9% were girls. Mean FEV_1 was 2.02 (SD 0.30) L, mean FVC 2.34 (SD (0.36) L, mean FEV_1/FVC 86.7% (SD 5.67) %, mean $FEF_{25-75\%}$ 2.70 (SD 0.64) L/sec and mean $FEF_{75\%}$ 1.15 (SD 0.34) L/sec.

Table 4.2.1. Characteristics of Children and Their Mothers.

	Full group n = 3,347	**Europeans n = 1,924**
Maternal characteristics		
Age (years)	31.0 (4.8)	32.0 (4.0)
History of asthma, eczema or allergy, yes (%)	37.2 (1,111)	37.8 (663)
Smoking during pregnancy, yes (%)	24.4 (743)	22.5 (403)
Smoking in childhood, yes (%)	6.6 (173)	5.5 (92)
Child characteristics		
Female sex (%)	50.9 (1,705)	50.8 (977)
Gestational age at birth (weeks)	40.1 (36.3-42.4)	40.2 (36.6-42.4)
Birth weight (kilogram)	3,459 (514)	3,536 (511)
Age (years)	9.8 (0.3)	9.8 (0.3)
Height (cm)	141.6 (6.5)	142.1 (6.4)
Lung function measures		
FEV_1 (L)	2.02 (0.30)	2.05 (0.30)
FVC (L)	2.34 (0.36)	2.39 (0.35)
FEV_1/FVC (%)	86.7 (5.69)	86.1 (5.68)
FEF_{25-75} (L/s)	2.70 (0.64)	2.67 (0.63)
FEF_{75} (L/s)	1.15 (0.34)	1.15 (0.34)
Current asthma, yes (%)	7.6 (214)	6.1 (108)

Values are means (SD), medians (2.5-97.5 percentile) or percentages (absolute numbers).
Abbreviations: FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; FEF_{25-75}, forced expiratory flow between 25–75% of FVC; FEF_{75}, forced expiratory flow at 75% of FVC.

Individual SNPs related with adult lung function and childhood lung function and asthma

Of the seven adult-related FEV_1 SNPs, rs10516526 was associated with childhood FEV_1, FEV_1/FVC and FEF_{75} (p-values < 0.007) and none with current asthma (S-Table 4.2.2). Of the 20 adult-related FEV_1/FVC SNPs, rs262129 was associated with childhood FEF_{75} (p-values < 0.0025) and none with FEV_1, FVC, FEV_1/FVC, FEF_{25-75} or current asthma (S-Table 4.2.3). The direction and magnitude of effect sizes did not materially differ among European children only (S-Table 4.2.3).

Genetic risk scores and childhood lung function and current asthma

The weighted adult FEV_1-GRS was associated with lower childhood FEV_1, FEV_1/FVC and FEF_{75} (Z-score (95% CI): -0.03 (-0.05, -0.01), -0.03 (-0.05,-0.01), -0.04 (-0.06,-0.01) respectively, per additional adult FEV_1 lowering allele) (Table 4.2.2). The weighted adult FEV_1-GRS explained 0.2% and 2.5% of the variation in childhood FEV_1 and FEF_{75}, respectively. The weighted adult FEV_1/FVC-GRS was associated with lower childhood FEV_1/FVC and FEF_{75} (Z-score (95% CI): -0.04 (-0.05, -0.0) and -0.03 (-0.05, -0.02) respectively, per additional FEV_1/FVC lowering allele), and explained 1.2% and 3.6% of the variation in childhood FEV_1/FVC and $FEF_{75,}$ respectively. The direction and magnitude of effect sizes did not materially differ in European children only (Table 4.2.2). The difference in mean FEV_1 between the two extreme groups (≤5 and ≥12 risk alleles) was 0.3 SDS (Figure 4.2.1A). The difference in mean FEV_1/FVC between the two extreme categories (≤14 and ≥27 risk alleles) was 0.8 SDS (Figure 4.2.2C). The weighted adult FEV_1-GRS and FEV_1/FVC-GR were not associated with current asthma. The results of the unweighted GRSs with childhood lung function were largely in line with the findings of the weighted GRSs, although the FEV_1-GRS was not associated with childhood FEV_1 in European children only (S-Table 4.2.4).

Table 4.2.2. Association of Weighted Genetic Risk Scores with Childhood Lung Function and Current Asthma.

	FEV_1 Z-score (95% CI)	**FVC Z-score (95% CI)**	**FEV_1/FVC Z-score (95% CI)**	**FEF_{25-75} Z-score (95% CI)**	**FEF_{75} Z-score (95% CI)**	**Current asthma Odds ratio (95% CI)**
FEV_1 genetic risk score						
Full cohort (n=3,347)	**-0.03 (-0.04,-0.01)***	-0.01 (-0.03,0.01)	**-0.03 (-0.05,-0.01)***	-0.01 (-0.03,0.02)	**-0.04 (-0.06,-0.01)***	1.02 (0.92,1.13)
Europeans (n=1,924)	**-0.04 (-0.07,-0.01)***	-0.02 (-0.05,0.01)	-0.03 (-0.06,0.00)	-0.01 (-0.04,0.02)	-0.03 (-0.06,-0.01)	1.04 (0.89,1.21)
FEV_1/FVC genetic risk score						
Full cohort (n=3,347)	-0.01 (-0.03,-0.00)	0.01 (-0.00,0.02)	**-0.04 (-0.05,-0.03)***	-0.02 (-0.03,-0.00)	**-0.03 (-0.05,-0.02)***	1.04 (0.97,1.11)
Europeans (n=1,924)	-0.01 (-0.03,0.00)	0.01 (-0.00,0.03)	**-0.04 (-0.06,-0.03)***	-0.02 (-0.04,0.00)	**-0.04 (-0.05,-0.02)***	1.02 (0.93,1.11)

Values represent change in Z-score or odds ratio with their corresponding 95% confidence interval (CI) of forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEF_{25-75}, forced expiratory flow between 25–75% of FVC; FEF_{75}, forced expiratory flow at 75% of FVC or current asthma, per additional lowering allele of the genetic risk score. Models were adjusted for child's age, age^2, sex, height and the first four ancestry principal components. *p-value < 0.01. Because of Bonferroni-correction, a p-value < 0.01 was considered significant.

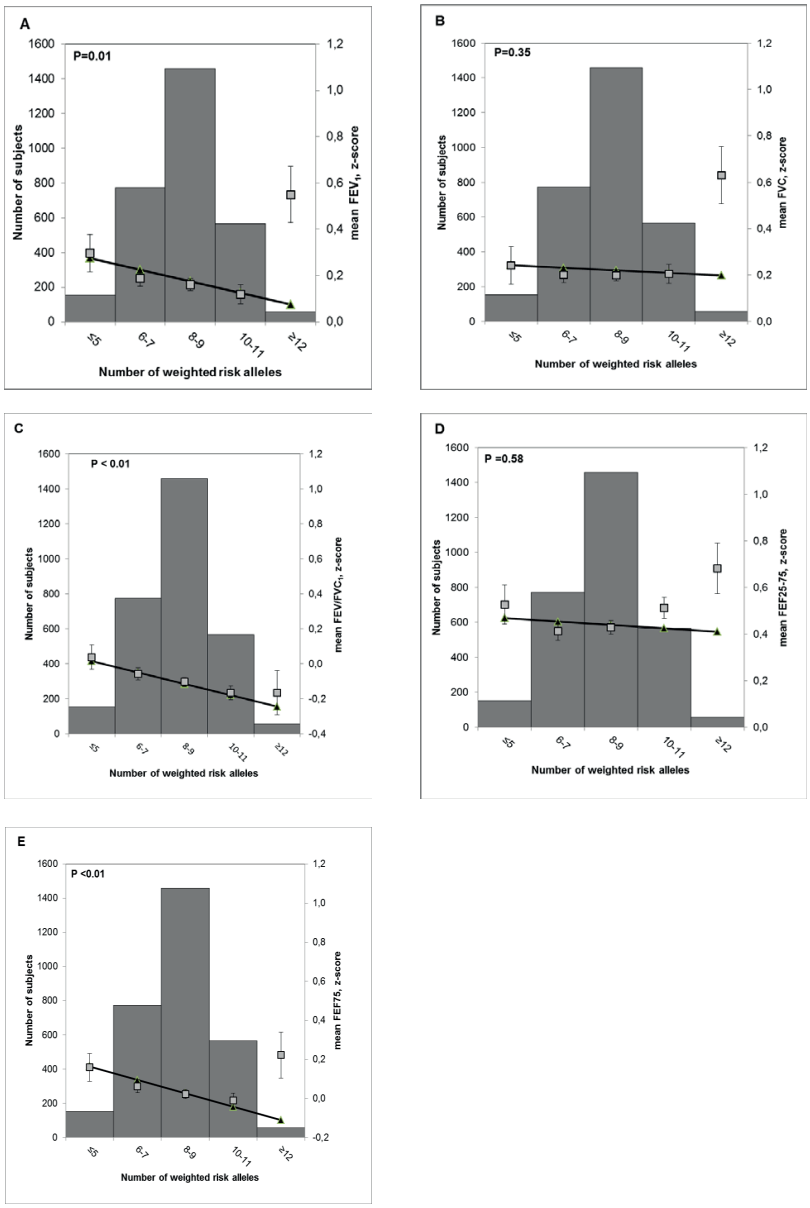


Figure 4.2.1. Associations of Weighted FEV_1-GRS Categorized by Number of Risk Alleles with (A) Forced Expiratory Volume in One Second (FEV_1), (B) Forced Vital Capacity (FVC), (C) FEV_1/FVC, (D) Forced Expiratory Flow between 25-75% (FEF_{25-75}) and (E) Forced Expiratory Flow at 75% (FEF_{75}) in Childhood.
The x-axis represents the distribution of the number of weighted risk alleles. The left y-axis represents the number of subjects in each risk score category shown as bars and the right y-axis the mean (SD) of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} or FEF_{75} z-scores in each category of weighted risk alleles shown as boxes. The line represents the regression of the mean FEV_1, FVC, FEV_1/FVC, FEF_{25-75} or FEF_{75} values per category of the weighted risk allele score shown as triangles. Regression models were adjusted for child's age, age^2, sex, height and the first four principal components.

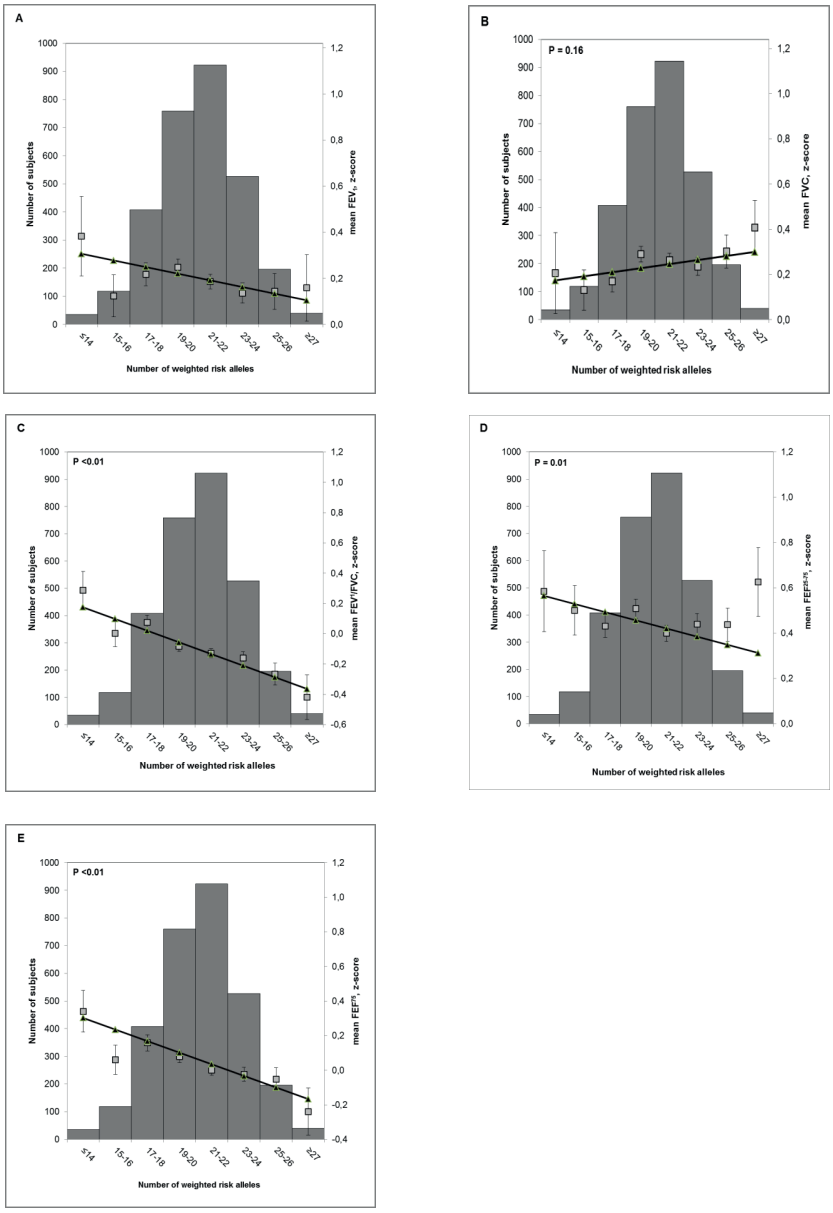


Figure 4.2.2. Associations of Weighted FEV_1/FVC-GRS Categorized by Number of Risk Alleles with (A) Forced Expiratory Volume in One Second (FEV_1), (B) Forced Vital Capacity (FVC), (C) FEV_1/FVC, (D) Forced Expiratory Flow between 25-75% (FEF_{25-75}) and (E) Forced Expiratory Flow at 75% (FEF_{75}) in Childhood .
The x-axis represents the distribution of the number of FEV_1/FVC weighted risk alleles. The left y-axis represents the number of subjects in each risk score category shown as bars and the right y-axis the mean (SD) of FEV_1, FVC, FEV_1/FVC, FEF_{25-75} or FEF_{75} z-scores in each category of weighted risk alleles shown as boxes. The line represents the regression of the mean FEV_1, FVC, FEV_1/FVC, FEF_{25-75} or FEF_{75} values per category of the weighted risk allele score shown as triangles. Regression models were adjusted for child's age, age^2, sex, height and the first four principal components.

Modifying and mediating factors

Maternal asthma, allergy or eczema status and child's weight at birth did not modify the associations of adult FEV_1-GRS or FEV1/FVC GRS with childhood lung function measures (p-values for interaction > 0.05). The weighted adult FEV_1-GRS was associated with a lower FEF_{75}, among children exposed to maternal smoking during childhood than those who were not (-0.12 (-0.21, -0.04) vs. -0.03 (-0.05, -0.00), per additional FEV_1 lowering allele, p-value for interaction 0.02). The weighted adult FEV_1/FVC-GRS was associated with a lower FEV_1/FVC among children exposed to maternal smoking during pregnancy compared to mothers who were not (-0.07 (-0.10, -0.04) vs. -0.04 (-0.05, -0.02), per additional FEV_1/FVC lowering allele, p-value for interaction 0.03). The weighted adult FEV_1/FVC-GRS was associated with a lower FEV_1/FVC and FEF_{75} among children born at term than those who were not (-0.04 (-0.05,-0.03) vs. -0.02 (-0.10, 0.07), and -0.04 (-0.05, -0.03) vs. -0.01 (-0.09, 0.07), respectively, per additional FEV_1/FVC lowering allele). The effect estimates for associations of weighted GRSs with childhood lung function did not change when models were additionally adjusted for gestational age or birth weight.

DISCUSSION

In this large population-based prospective cohort study, each additional FEV_1 and FEV_1/FVC lowering allele in a weighted GRS based on 26 individual SNPs that have been shown to be related to adult lung function previously, was associated with lower childhood FEV_1, FEV_1/FVC, FEF_{75}, FEV_1/FVC or FEF_{75}. Similar results were observed among European children only. Effect estimates of adult FEV_1-GRS with FEF_{75}, and adult FEV_1/FVC-GRS with FEV_1/FVC or FEF_{75} were stronger among children exposed to maternal smoking during childhood, and maternal smoking during pregnancy or who were born at term.

Comparison with previous studies

To date, three meta-analyses of GWAS studies have identified 35 genetic variants in or near 26 genes associated with lung function measures in healthy adults of European ancestry.[7-9] These genetic variants, either individually or combined into GRSs were partly replicated with lung function in children aged 7-9 years on a population-based level.[9,11,12] The magnitude and direction of effect estimates were comparable to the adult GWAS in or near 23 genes.[9] A study among 411 high-risk asthmatic children showed that a GRS based on variants related with lower adult FEV_1/FVC-GRS was not associated with neonatal lung function or bronchial responsiveness, but was associated with lower forced expiratory flow at 50% (FEF_{50}) and higher bronchial responsiveness (PD_{20}) at age 7 years, or a change of these parameters between birth and age 7 years.[11] In contrast to the present findings, these authors did not observe an association of weighted FEV_1-GRS

with lung function measures while we did with FEV_1/FVC.[11] Differences in results are most probably due to our non-high risk population and larger sample size. In addition to previous studies, we examined the associations between the GRSs and peripheral airway function including FEF_{25-75} and FEF_{75}, or asthma, and also explored the modifying effects of atopic predisposition, adverse environmental exposures and characteristics. In the current study, maternal smoking in childhood modified the association of weighted FEV_1-GRS with childhood FEF_{75}. Maternal smoking during pregnancy and child's gestational age at birth modified the associations of FEV_1/FVC-GRS with childhood FEV_1/FVC and FEF_{75}, respectively. However, these findings should be interpreted cautiously due to the relatively small number of children included in these analyses.

Interpretation of results

We observed that weighted GRSs based on SNPs associated with FEV_1 and FEV_1/FVC in adults were associated with childhood lung function measures. These findings indicate that there is a polygenic effect on lung function in children, and also suggest that there is overlap in genetic background of lung function in children and adults. However, the biological functions of the genes associated with adult lung function remain poorly understood. In addition, to what extent gene-gene and gene-environment interactions play a role remains unclear. Therefore, future studies are needed to determine whether the nearest genes are indeed those with causal effects. Furthermore, previous GWA studies have focused on cross-sectional measured lung function phenotypes.[7-9] Additional studies in large pediatric non high-risk populations with longitudinal data will be necessary to explore the effects of the SNPs related to adult lung function on lung function development across childhood into adulthood.

Measures of lung function have been shown to be correlated with each other and have been found to be heritable, as estimated from twin and family studies.[3-6] Yet the percentage of variance explained by the GRSs and the individual SNPs was low in the current study. This could be attributable to the small-to-moderate effect estimates of the previously identified SNPs related to adult lung. In addition, rare genetic variants, epigenetic factors and gene-gene/gene-environment interactions may account for this difference. Therefore further large meta-analyses of genetic and epigenetic data are required.

We observed that the variance explained by the weighted GRSs was higher for FEF_{75} than for FEV and FEV_1/FVC. The GWA studies that identified SNPs associated with adult lung function were restricted to FEV_1 and FEV_1/FVC. Therefore, our results suggest that the SNPs associated with adult lung function might be associated even stronger with peripheral flow measures.

The adult FEV_1-GRS was based on seven SNPs, one of which showed an individual association with childhood FEV_1. This SNP, rs10516526, is located in intron 5 of glutathione

S-transferase C-terminal domain containing protein (*GSTCD*). The potential function of *GSTCD* remains unclear, but studies have reported that the encoded protein could influence lung function via mechanisms involving the detoxification by glutathione S-transferases of xenobiotics that might damage the lungs.[7] In addition, it has been shown to be expressed across fetal lung developmental stages.[22] The adult FEV_1/FVC-GRS was based on 20 SNPs, of which one showed an individual association with childhood FEF_{75} but not with FEV_1/FVC. This SNP, rs262129, is located in the gene *LOC153910* of which the potential function remains unclear to date.[7-9,11,12] Furthermore, in previous population-based studies, SNPs in or near *GSTCD* and *LOC153910* have been suggested to be associated with airflow obstruction and risk of COPD.[23-26] Although both reduced growth and accelerated lung function decline lead to lower lung function in adults, we believe that these SNPs may to some degree be involved in altering lung growth or development rather than in the accelerated decline of lung function, given that the majority of SNPs were not associated with childhood lung function measures. Associations of single SNPs with childhood lung were limited by our sample size. Lastly, we did not observe any associations between the GRSs and childhood asthma. This could be due to the small number of subjects in the asthma subgroup. Alternatively, other SNPs than those associated with adult lung function may be involved in the development of asthma. No associations between SNPs related to adult lung function and asthma have been reported before.[9,27]

Strengths and limitations

This study was embedded in a large population-based prospective cohort study with detailed childhood lung function measures. Some limitations should be acknowledged. First, the study population is from a prospective cohort study and is subject to loss to follow-up. Of all children with genetic information, information on lung function measures was available in 72%. Compared to children with genetic information, children without genetic information had lower birth weight and were born at a younger gestational age (data not shown). This selective loss to follow-up could be of concern if the examined associations would be different in those included and not included in the analysis, but this seems unlikely. Second, the previously identified SNPs related to adult lung function explain only a small proportion of the variance in the phenotype.[9] We only selected SNPs that reached genome-wide significance levels and might have missed SNPs with true effects that failed to reach the stringent GWA threshold levels in previous studies. Future studies should explore if the addition of further variants increase the explained variation in lung function measures. Third, the individual SNPs for lung function may have varying effect sizes across the life course. Therefore, weighing based on effect estimates from adult studies may not reflect the true effect sizes in children. However, in the current study, similar findings were observed for the unweighted and weighted GRSs, suggest-

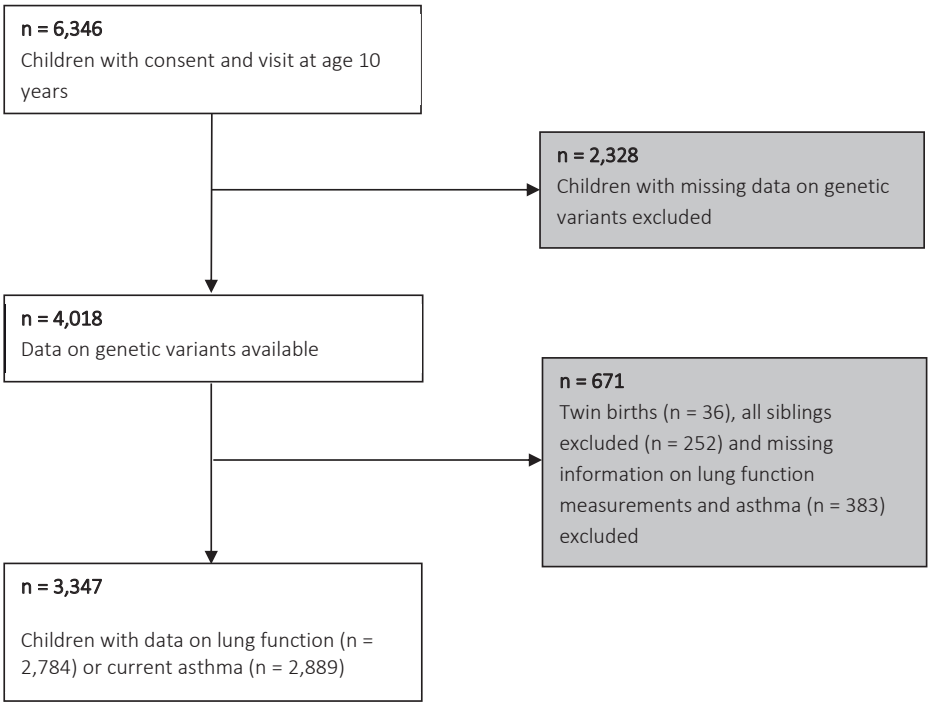
ing that the adult weights used in the GRSs did not strongly influence the observed associations. Last, ethnic differences in lung function measures have been observed.[28] We aimed to address this issue by using GLI reference data, a method that uses updated and accurate reference values for lung function measures of a wide age range with different ethnicities and has been adopted by the ATS and ERS.[20] Furthermore, we adjusted for ancestry using principal components from the GWAs data and observed only minor differences between the full population and individuals with European ancestry only.

In conclusion, our findings support the hypothesis that genetic variants associated with adult lung function affect lung function already in childhood. Maternal smoking during pregnancy, in childhood and gestational age at birth partly modified the associations of weighted FEV_1 and FEV_1/FVC-GRSs with lung function measures in children. Future studies are needed to explore the role of genetic variants in lung development, lung function impairment and chronic obstructive respiratory diseases throughout life.

REFERENCES

1. Duijts L, Reiss IK, Brusselle G, de Jongste JC. Early origins of chronic obstructive lung diseases across the life course. Eur J Epidemiol. 2014;29(12):871-85.
2. Eisner MD, Anthonisen N, Coultas D, Kuenzli N, Perez-Padilla R, Postma D, et al. An official American Thoracic Society public policy statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2010;182(5):693-718.
3. DeMeo DL, Silverman EK. Genetics of chronic obstructive pulmonary disease. Semin Respir Crit Care Med. 2003;24(2):151-60.
4. Pauwels RA, Buist AS, Ma P, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: National Heart, Lung, and Blood Institute and World Health Organization Global Initiative for Chronic Obstructive Lung Disease (GOLD): executive summary. Respir Care. 2001;46(8):798-825.
5. Hubert HB, Fabsitz RR, Feinleib M, Gwinn C. Genetic and environmental influences on pulmonary function in adult twins. Am Rev Respir Dis. 1982;125(4):409-15.
6. McClearn GE, Svartengren M, Pedersen NL, Heller DA, Plomin R. Genetic and environmental influences on pulmonary function in aging Swedish twins. J Gerontol. 1994;49(6):264-8.
7. Hancock DB, Eijgelsheim M, Wilk JB, Gharib SA, Loehr LR, Marciante KD, et al. Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. Nat Genet. 2010;42(1):45-52.
8. Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat M, et al. Genome-wide association study identifies five loci associated with lung function. Nat Genet. 2010;42(1):36-44.
9. Artigas M, Loth DW, Wain LV, Gharib SA, Obeidat M, Tang W, et al. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. Nat Genet. 2011;43(11):1082-90.
10. Wilk JB, Chen TH, Gottlieb DJ, Walter RE, Nagle MW, Brandler BJ, et al. A genome-wide association study of pulmonary function measures in the Framingham Heart Study. PLoS Genet. 2009;5(3):e1000429.
11. Kreiner-Moller E, Bisgaard H, Bonnelykke K. Prenatal and postnatal genetic influence on lung function development. J Allergy Clin Immunol. 2014;134(5):1036-42 e15.
12. Panasevich S, Melen E, Hallberg J, Bergstrom A, Svartengren M, Pershagen G, et al. Investigation of novel genes for lung function in children and their interaction with tobacco smoke exposure: a preliminary report. Acta Paediatr. 2013;102(5):498-503.
13. Sonnenschein-van der Voort AM, Howe LD, Granell R, Duijts L, Sterne JA, Tilling K, et al. Influence of childhood growth on asthma and lung function in adolescence. J Allergy Clin Immunol. 2015;135(6):1435-43 e7.
14. den Dekker HT, Sonnenschein-van der Voort AM, de Jongste JC, Reiss IK, Hofman A, Jaddoe VW, et al. Tobacco Smoke Exposure, Airway Resistance, and Asthma in School-age Children: The Generation R Study. Chest. 2015;148(3):607-17.
15. Kruithof CJ, Kooijman MN, van Duijn CM, Franco OH, de Jongste JC, Klaver CC, et al. The Generation R Study: Biobank update 2015. Eur J Epidemiol. 2014;29(12):911-27.
16. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. Eur J Epidemiol. 2012;27(9):739-56.
17. Loth DW, Ittermann T, Lahousse L, Hofman A, Leufkens HG, Brusselle GG, et al. Normal spirometry values in healthy elderly: the Rotterdam Study. Eur J Epidemiol. 2013;28(4):329-34.

18. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI. SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. Bioinformatics. 2008;24(24):2938-9.
19. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J. 2005;26(2):319-38.
20. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. Eur Respir J. 2012;40(6):1324-43.
21. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J. 1995;8(3):483-91.
22. Obeidat M, Miller S, Probert K, Billington CK, Henry AP, Hodge E, et al. GSTCD and INTS12 regulation and expression in the human lung. PLoS One. 2013;8(9):e74630.
23. Soler Artigas M, Wain LV, Repapi E, Obeidat M, Sayers I, Burton PR, et al. Effect of five genetic variants associated with lung function on the risk of chronic obstructive lung disease, and their joint effects on lung function. Am J Respir Crit Care Med. 2011;184(7):786-95.
24. Castaldi PJ, Cho MH, Litonjua AA, Bakke P, Gulsvik A, Lomas DA, et al. The association of genome-wide significant spirometric loci with chronic obstructive pulmonary disease susceptibility. Am J Respir Cell Mol Biol. 2011;45(6):1147-53.
25. Pillai SG, Ge D, Zhu G, Kong X, Shianna KV, Need AC, et al. A genome-wide association study in chronic obstructive pulmonary disease (COPD): identification of two major susceptibility loci. PLoS Genet. 2009;5(3):e1000421.
26. Cho MH, Boutaoui N, Klanderman BJ, Sylvia JS, Ziniti JP, Hersh CP, et al. Variants in FAM13A are associated with chronic obstructive pulmonary disease. Nat Genet. 2010;42(3):200-2.
27. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. N Engl J Med. 2010;363(13):1211-21.
28. Strippoli MP, Kuehni CE, Dogaru CM, Spycher BD, McNally T, Silverman M, et al. Etiology of ethnic differences in childhood spirometry. Pediatrics. 2013;131(6):e1842-9.



S-Figure 4.2.1. Flow Chart of Participants Included for Analyses.

S-Table 4.2.1. List of Identified Genetic Variants Associated with Adult Lung Function.

Phenotype	Chromosome	Position	SNP ID	Locus	EA*	Effect in original study*
FEV_1	2	218391399	rs2571445	*TNS1*	A	0.047
FEV_1	3	170782913	rs1344555	*MECOM*	T	0.025
FEV_1	4	106908353	rs10516526	*GSTCD*	A	0.108
FEV_1	5	147827981	rs1985524	*HTR4*	G	0.048
FEV_1	6	28430275	rs6903823	*ZKSCAN3/ ZNF323*	G	0.029
FEV_1	10	12317998	rs7068966	*CDC123*	C	0.022
FEV_1	10	77985230	rs11001819	*C10orf11*	G	0.022
FEV_1/FVC	1	17179262	rs2284746	*MFAP2*	G	0.038
FEV_1/FVC	1	216926691	rs993925	*TGFB2*	C	0.023
FEV_1/FVC	2	239542085	rs12477314	*HDAC4*	C	0.031
FEV_1/FVC	3	25495586	rs1529672	*RARB*	C	0.038
FEV_1/FVC	4	145698589	rs11100860	*HHIP*	A	0.064
FEV_1/FVC	5	147822546	rs11168048	*HTR4*	T	0.047
FEV_1/FVC	5	156869344	rs11134779	*ADAM19*	G	0.042
FEV_1/FVC	5	95062456	rs153916	*SPATA9*	T	0.025
FEV_1/FVC	6	31676448	rs2857595	*NCR3*	A	0.028
FEV_1/FVC	6	109374743	rs2798641	*ARMC2*	T	0.03
FEV_1/FVC	6	32259421	rs2070600	*AGER*	C	0.126
FEV_1/FVC	6	142894837	rs262129	*LOC153910*	A	0.056
FEV_1/FVC	9	97244613	rs16909859	*PTCH1*	A	0.08
FEV_1/FVC	10	12317998	rs7068966	*CDC123*	C	0.023
FEV_1/FVC	12	55813550	rs11172113	*LRP1*	T	0.026
FEV_1/FVC	12	94795559	rs1036429	*CCDC38*	C	0.028
FEV_1/FVC	15	69467134	rs8033889	*THSD4*	T	0.072
FEV_1/FVC	16	56632783	rs12447804	*MMP15*	T	0.021
FEV_1/FVC	16	73947817	rs2865531	*CFDP1*	A	0.024
FEV_1/FVC	21	34574109	rs9978142	*KCNE2*	C	0.031

*Reported effect estimates, standard errors and p-values for the associations between SNPs and corresponding lung function measures by Artigas et al. EA, effect allele; SE, standard error; P; p-value.

S-Table 4.2.2. Associations of Individual SNPs Related to Adult FEV_1 with Childhood Lung Function and Current Asthma.

SNP ID	Chr.	Position	EA/ OA	EAF	FEV_1 Z-score (95% CI)	FVC Z-score (95% CI)	FEV_1/FVC Z-score (95% CI)	FEF_{25-75} Z-score (95% CI)	FEF_{75} Z-score (95% CI)	Current asthma Odds ratio (95% CI)
Full cohort (n=3,347)										
rs2571445	2	218391399	A/G	0.35	0.02 (-0.03,0.07)	0.01 (-0.04,0.06)	0.02 (-0.03,0.07)	-0.01 (-0.07,0.04)	0.02 (-0.03,0.06)	1.00 (0.78,1.28)
rs1344555	3	170782913	C/T	0.22	-0.01 (-0.08,0.05)	-0.00 (-0.06,0.06)	-0.03 (-0.09,-0.03)	-0.02 (-0.09,0.05)	-0.03 (-0.08,0.03)	1.03 (0.76,1.39)
rs10516526	4	106908353	A/G	0.94	**-0.17 (-0.27,-0.06)****	-0.03 (-0.13,0.07)	**-0.22 (-0.32,-0.12)****	-0.01 (-0.12,0.11)	**-0.24 (-0.34,-0.14)****	1.24 (0.72,2.16)
rs1985524	5	147827981	C/G	0.48	0.01 (-0.04,0.06)	-0.00 (-0.05,0.04)	0.02 (-0.04,0.06)	-0.02 (-0.08,0.03)	0.02 (-0.03,0.06)	0.99 (0.78,1.27)
rs6903823	6	28430275	A/G	0.24	0.07 (0.01,0.14)	0.07 (0.01,0.13)	-0.00 (-0.06,0.05)	0.01 (-0.06,0.07)	0.05 (-0.00,0.11)	0.91 (0.68,1.21)
rs7068966	10	12317998	C/T	0.52	0.02 (-0.03,0.07)	0.05 (0.00,0.10)	-0.05 (-0.10,0.00)*	-0.00 (-0.06,0.05)	-0.02 (-0.07,0.02)	0.93 (0.72,1.19)
rs11001819	10	77985230	A/G	0.58	-0.03 (-0.08,0.02)	-0.03 (-0.08,0.02)	-0.01 (-0.06,0.04)	-0.02 (-0.07,0.03)	-0.02 (-0.06,0.03)	1.10 (0.87,1.41)
Europeans (n=1,924)										
rs2571445	2	218391399	A/G	0.37	0.03 (-0.04,0.09)	0.02 (-0.03,0.08)	0.00 (-0.06,0.07)	-0.01 (-0.09,0.06)	0.01 (-0.05,0.07)	1.00 (0.70,1.41)
rs1344555	3	170782913	C/T	0.20	0.04 (-0.04,0.12)	0.04 (-0.03,0.11)	0.00 (-0.08,0.08)	0.00 (-0,09,0.09)	0.00 (-0.07,0.08)	1.31 (0.81,2.10)
rs10516526	4	106908353	A/G	0.94	**-0.21 (-0.34,-0.08)****	-0.08 (-0.20,0.04)	**-0.21 (-0.34,-0.08)****	-0.04(-0.19,0.11)	**-0.24 (-0.37,-0.12)****	1.12 (0.54,2.33)
rs1985524	5	147827981	C/G	0.46	0.02 (-0.04,0.08)	-0.00 (-0.06,0.05)	0.04 (-0.02,0.11)	-0.01 (-0.08,0.07)	0.03 (-0.03,0.09)	1.14 (0.80,1.61)
rs6903823	6	28430275	A/G	0.21	0.05 (-0.03,0.12)	0.02 (-0.05,0.09)	0.04 (-0.04,0.12)	0.04 (-0.05,0.12)	0.06 (-0.02,0.13)	0.91 (0.61,1.36)
rs7068966	10	12317998	C/T	0.48	0.02 (-0.04,0.08)	0.03 (-0.02,0.09)	-0.03 (-0.09,0.04)	-0.02 (-0.09,0.05)	0.01 (-0.05,0.07)	0.79 (0.56,1.12)
rs11001819	10	77985230	A/G	0.53	-0.03 (-0.09,0.03)	-0.02 (-0.08,0.03)	-0.01 (-0.08,0.05)	-0.02 (-0.09,0.05)	-0.01 (-0.07,0.04)	1.18 (0.85,1.65)

Values represent differences in Z-score or odds ratio with their corresponding 95% confidence interval (CI) for the effect allele (EA) compared to the other allele (OA). Models were adjusted for child's age, age^2, sex, height and the first four ancestry principal components. Chr. Chromosome; EA, effect allele; OA, other allele; EAF, effect allele frequency; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; FEF_{25-75}, forced expiratory flow between 25–75% of FVC; FEF_{75}, forced expiratory flow at 75% of FVC. ** p-value < 0.01. Because of Bonferroni-correction, a p-value < 0.01 was considered significant.

S-Table 4.2.3. Associations of Individual SNPs Related to Adult FEV_1/FVC with Childhood Lung Function and Current Asthma.

SNP ID	Chr.	Position	EA/ OA	EAF	FEV_1 Z-score (95% CI)	FVC Z-score (95% CI)	FEV_1/FVC Z-score (95% CI)	FEF_{25-75} Z-score (95% CI)	FEF_{75} Z-score (95% CI)	Current asthma Odds Ratio (95% CI)
Full cohort (n=3,347)										
rs993925	1	216926691	C/T	0.67	0.05 (0.00,0.11)	0.05 (0.00,0.11)	0.01 (-0.05,0.05)	0.01 (-0.05,0.07)	0.02 (-0.03,0.07)	1.08 (0.83,1.40)
rs2284746	1	17179262	C/G	0.53	-0.03 (-0.08,0.02)	-0.01 (-0.07,0.04)	-0.03 (-0.08,0.02)	-0.08 (-0.14,-0.02)	-0.02 (-0.06,0.03)	1.05 (0.81,1.36)
rs12477314	2	239542085	C/T	0.82	-0.05 (-0.11,0.02)	-0.02 (-0.08,0.04)	-0.05 (-0.12,0.01)	-0.01 (-0.08,0.06)	-0.07 (-0.12,-0.01)	1.19 (0.86,1.65)
rs1529672	3	25495586	A/C	0.82	-0.05 (-0.12,0.02)	-0.00 (-0.07,0.06)	-0.07 (-0.13,0.00)	0.00 (-0.07,0.08)	-0.08 (-0.14,-0.02)	1.11 (0.80,1.56)
rs11100860	4	145698589	A/G	0.46	0.04 (-0.02,0.10)	0.06 (-0.00,0.11)	-0.03 (-0.09,0.03)	0.01 (-0.06,0.08)	-0.02 (-0.07,0.04)	1.04 (0.77,1.41)
rs11168048	5	147822546	C/T	0.59	-0.03 (-0.08,0.02)	-0.02 (-0.07,0.03)	-0.00 (-0.05,0.05)	-0.01 (-0.07,0.05)	-0.01 (-0.06,0.04)	0.99 (0.77,1.27)
rs11134779	5	156869344	A/G	0.39	0.04 (-0.01,0.09)	0.02 (-0.03,0.07)	0.04 (-0.01,0.09)	0.03 (-0.03,0.09)	0.05 (0.01,0.10)	1.28 (0.98,1.65)
rs153916	5	95062456	C/T	0.55	0.02 (-0.03,0.07)	0.04 (-0.00,0.09)	-0.04 (-0.09,0.01)	-0.02 (-0.08,0.03)	-0.02 (-0.07,0.02)	1.10 (0.87,1.41)
rs2857595	6	31676448	A/G	0.22	0.03 (-0.03,0.10)	-0.01 (-0.07,0.05)	0.08 (0.01,0.14)	-0.01 (-0.07,0.06)	0.06 (0.01,0.12)	0.91 (0.68,1.21)
rs2798641	6	109374743	C/T	0.17	0.06 (-0.01,0.13)	0.01 (-0.05,0.08)	0.08 (0.00,0.14)	0.08 (0.01,0.15)	0.08 (0.02,0.14)	1.02 (0.74,1.41)
rs2070600	6	32259421	C/T	0.97	0.04 (-0.11,0.19)	0.15 (0.01,0.29)	**-**0.21 (-0.36,-0.07)	-0.05 (-0.21,0.12)	-0.13 (-0.26,0.00)	1.82 (0.73,4.53)
rs262129	6	142894837	A/G	0.62	0.05 (-0.01,0.10)	-0.01 (-0.06,0.04)	0.08 (0.03,0.14)	0.02 (-0.04,0.09)	**0.10 (0.05,0.15)****	1.02 (0.78,1.34)
rs16909859	9	97244613	A/G	0.91	0.05 (-0.05,0.16)	0.03 (-0.07,0.14)	0.01 (-0.10,0.11)	-0.03 (-0.14,0.09)	0.04 (-0.05,0.14)	1.35 (0.77,2.36)
rs7068966	10	12317998	C/T	0.52	0.02 (-0.03,0.07)	0.05 (-0.00,0.10)	-0.05 (-0.10,0.00)	-0.01 (-0.07,0.04)	-0.02 (-0.07,0.02)	0.93 (0.72,1.19)
rs11172113	12	55813550	C/T	0.60	0.03 (-0.02,0.09)	0.04 (-0.01,0.09)	-0.02 (-0.07,0.02)	-0.00 (-0.06,0.06)	-0.00 (-0.05,0.05)	1.18 (0.92,1.51)
rs1036429	12	94795559	C/T	0.79	0.01 (-0.06,0.07)	0.00 (-0.06,0.06)	0.02 (-0.04,0.08)	0.01 (-0.06,0.07)	0.01 (-0.05,0.06)	1.17 (0.85,1.61)
rs8033889	15	69467134	G/T	0.23	0.02 (-0.04,0.08)	-0.01 (-0.06,0.05)	0.06 (-0.00,0.11)	0.03 (-0.03,0.10)	0.05 (-0.00,0.10)	0.81 (0.62,1.06)
rs12447804	16	56632783	C/T	0.17	0.10 (0.03,0.16)	0.06 (-0.00,0.13)	0.06 (-0.01,0.12)	0.02 (-0.05,0.09)	0.09 (0.03,0.15)	0.79 (0.61,1.02)
rs8050059[$]	16	73947817	A/T	0.55	0.06 (0.01,0.11)	0.02 (-0.03,0.07)	0.07 (0.02,0.12)	0.04 (-0.01,0.10)	0.06 (0.02,0.11)	1.15 (0.90,1.47)
rs10470171[#]	21	34574109	C/T	0.14	0.02 (-0.06,0.09)	0.00 (-0.07,0.07)	0.02 (-0.05,0.09)	0.01 (-0.07,0.09)	-0.01 (-0.08,0.05)	1.01 (0.71,1.45)

S-Table 4.2.3. Associations of Individual SNPs Related to Adult FEV_1/FVC with Childhood Lung Function and Current Asthma. (continued)

SNP ID	Chr.	Position	EA/ OA	EAF	FEV_1 Z-score (95% CI)	FVC Z-score (95% CI)	FEV_1/FVC Z-score (95% CI)	FEF_{25-75} Z-score (95% CI)	FEF_{75} Z-score (95% CI)	Current asthma Odds Ratio (95% CI)
Europeans (n=1,924)										
rs993925	1	216926691	C/T	0.66	0.06 (-0.00,0.13)	0.05 (-0.01,0.11)	0.02 (-0.04,0.08)	0.02 (-0.06,0.10)	0.03 (-0.03,0.09)	1.27 (0.87,1.84)
rs2284746	1	17179262	C/G	0.47	-0.02 (-0.08,0.04)	-0.00 (-0.06,0.06)	-0.03 (-0.09,0.04)	-0.10 (-0.17,-0.02)	-0.01 (-0.07,0.05)	1.04 (0.73, 1.47)
rs12477314	2	239542085	C/T	0.79	-0.01 (-0.08,0.07)	0.00 (-0.07,0.07)	-0.03 (-0.11,0.04)	-0.03 (-0.11,0.06)	-0.03 (-0.10,0.04)	0.94 (0.63,1.41)
rs1529672	3	25495586	A/C	0.84	-0.05 (-0.14,0.04)	-0.03 (-011,0.05)	-0.04 (-0.12,0.04)	-0.00 (-0.10,0.10)	-0.06 (-0.14,0.01)	0.98 (0.61,1.55)
rs11100860	4	145698589	A/G	0.43	0.07 (-0.01,0.14)	0.09 (0.02,0.16)	-0.04 (-0.11,0.04)	0.03 (-0.06,0.12)	-0.01 (-0.08,0.06)	1.15 (0.76, 1.73)
rs11168048	5	147822546	C/T	0.57	-0.02 (-0.09,0.04)	-0.00 (-0.06,0.06)	-0.03 (-0.09,0.04)	0.00 (-0.07,0.08)	-0.02 (-0.08,0.03)	0.93 (0.66,1.31)
rs11134779	5	156869344	A/G	0.33	0.05 (-0.02,0.11)	0.01 (-0.06,0.07)	0.08 (0.01,0.14)	0.03 (-0.05,0.10)	0.08 (0.01,0.14)	1.10 (0.76,1.59)
rs153916	5	95062456	C/T	0.54	0.01 (-0.05,0.08)	0.05 (-0.01,0.11)	-0.05 (-0.12,0.01)	-0.01 (-0.09,0.06)	-0.04 (-0.10,0.02)	1.05 (0.75,1.46)
rs2857595	6	31676448	A/G	0.21	0.02 (-0.06,0.10)	-0.02 (-0.09,0.05)	0.06 (-0.02,0.14)	0.02 (-0.07,0.11)	0.04 (-0.03,0.12)	1.01 (0.66,1.54)
rs2798641	6	109374743	C/T	0.18	0.10 (0.01,0.18)	0.05 (-0.03,0.12)	0.08 (-0.00,0.16)	0.07 (-0.02,0.17)	0.10 (0.03,0.18)	1.19 (0.75,1.90)
rs2070600	6	32259421	C/T	0.97	0.08 (-0.10,0.27)	0.12 (-0.04,0.29)	-0.09 (-0.27,0.09)	0.07 (-0.14,0.27)	-0.06 (-0.23,0.10)	2.34 (0.57,9.69)
rs262129	6	142894837	A/G	0.71	0.06 (-0.01,0.13)	0.00 (-0.06,0.07)	0.08 (0.00,0.15)	0.05 (-0.03,0.13)	0.09 (0.02,0.15)	0.93 (0.63,1.37)
rs16909859	9	97244613	A/G	0.91	0.02 (-0.11,0.15)	-0.02 (-0.14,0.10)	0.10 (-0.03,0.23)	-0.05 (-0.20,0.10)	0.08 (-0.04,0.21)	1.21 (0.58,2.56)
rs7068966	10	12317998	C/T	0.48	0.02 (-0.04,0.08)	0.03 (-0.02,0.09)	-0.02 (-0.08,0.04)	-0.02 (-0.09,0.05)	0.01 (-0.05,0.07)	0.79 (0.56,1.12)
rs11172113	12	55813550	C/T	0.58	0.04 (-0.03,0.10)	0.05 (-0.01,0.11)	-0.03 (-0.10,0.03)	0.00 (-0.07,0.07)	-0.01 (-0.07,0.05)	1.33 (0.93,1.89)
rs1036429	12	94795559	C/T	0.80	-0.02 (-0.10,0.06)	0.00 (-0.07,0.07)	-0.02 (-0.09,0.06)	-0.00 (-0.10,0.08)	-0.04 (-0.11,0.03)	0.89 (0.58,1.35)
rs8033889	15	69467134	G/T	0.24	0.05 (-0.03,0.12)	0.01 (-0.06,0.07)	0.07 (-0.00,0.14)	0.03 (-0.05,0.11)	0.07 (-0.00,0.14)	0.88 (0.60,1.29)
rs12447804	16	56632783	C/T	0.20	0.09 (0.01,0.17)	0.07 (-0.00,0.14)	0.03 (-0.05,0.10)	0.03 (-0.06,0.11)	0.06 (-0.01,0.13)	0.97 (0.64,1.48)
rs8050059[$]	16	73947817	A/T	0.59	0.07 (0.00,0.13)	0.02 (-0.04,0.07)	0.08 (0.02,0.15)	0.04 (-0.03,0.11)	0.08 (0.02,0.14)	0.95 (0.67,1.35)
rs10470171[#]	21	34574109	C/T	0.15	-0.01 (-0.09,0.08)	-0.01 (-0.09,0.07)	-0.01 (-0.10,0.07)	-0.02 (-0.12,0.08)	-0.01 (-0.10,0.07)	1.37 (0.82,2.29)

Values represent differences in Z-score or odds ratio with their corresponding 95% confidence interval (CI) for the effect allele (EA) compared to the other allele (OA). Models were adjusted for child's age, age^2, sex, height and the first four ancestry principal components. Chr. chromosome; EA, effect allele; OA, other allele; EAF, effect allele frequency; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; FEF_{25-75}, forced expiratory flow between 25–75% of FVC; FEF_{75}, forced expiratory flow at 75% of FVC. [$]proxy for rs2865531, [#]proxy for rs9978142. ** p-value < 0.0025. Because of Bonferroni-correction, a p-value of < 0.0025 was considered significant.

S-Table 4.2.4. Association of Unweighted Genetic Risk Scores with Childhood Lung Function and Current Asthma.

	FEV_1, Z-score (95% CI)	**FVC Z-score (95% CI)**	**FEV_1/FVC, Z-score (95% CI)**	**FEF_{25-75} Z-score (95% CI)**	**FEF_{75}, Z-score (95% CI)**	**Current asthma Odds ratio (95% CI)**
FEV_1 Genetic risk scores						
Full cohort (n=3,347)	-0.02 (-0.04,0.00)	-0.01 (-0.03,0.01)	-0.02 (-0.04,0.00)	-0.01 (-0.03,0.01)	-0.02 (-0.04,-0.00)	1.02 (0.92,1.13)
Europeans (n=1,924)	-0.02 (-0.05,0.00)	-0.01 (-0.04,0.01)	-0.01 (-0.04,0.01)	-0.01 (-0.04,0.02)	-0.01 (-0.04, 0.01)	1.00 (0.87,1.16)
FEV_1/FVC Genetic risk scores						
Full cohort (n=3,347)	-0.02 (-0.03,-0.00)	0.01 (-0.00,0.02)	**-0.04 (-0.05,-0.03)****	-0.02 (-0.03,-0.00)	**-0.03 (-0.05,-0.02)****	1.04 (0.97,1.11)
Europeans (n=1,924)	-0.01 (-0.03,-0.00)	0.01 (-0.00,0.03)	**-0.04 (-0.06,-0.03)****	-0.02 (-0.04,0.00)	**-0.04 (-0.05,-0.02)****	1.01 (0.92,1.10)

Values represent change in Z-score or odds ratio with their corresponding 95% confidence interval (CI) of forced expiratory volume in one second (FEV_1), FEV_1/ to forced vital capacity (FEV_1/FVC), per additional lowering allele of the genetic risk score using linear and logistic regression models. Regression models were adjusted for child's age, age^2, sex, height and the first four ancestry principal components. Chr. chromosome; EA, effect allele; OA, other allele; EAF, effect allele frequency; FEF_{25-75}, forced expiratory flow between 25–75% of FVC; FEF_{75}, forced expiratory flow at 75% of FVC. ** p-value < 0.01. Because of Bonferroni-correction, a p-value of < 0.01 was considered significant.

Chapter 4.3

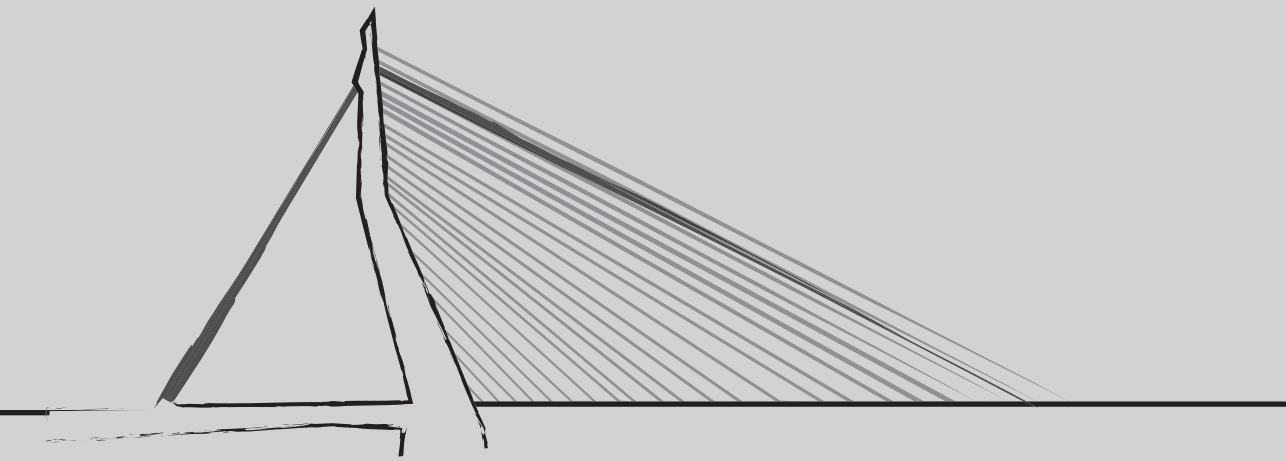
Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns

BR Joubert*, HT den Dekker*, JF Felix, J Bohlin, S Ligthart, E Beckett, H Tiemeier, JB van Meurs, AG Uitterlinden, A Hofman, SE Håberg, SE Reese, MJ Peters, B Kulle Andreassen, EA Steegers, RM Nilsen, SE Vollset, Ø Midttun, PM Ueland, OH Franco, A Dehghan, JC de Jongste, MC Wu, T Wang, SD Peddada, VWV Jaddoe, W Nystad, L Duijts#, SJ London#

** Both authors contributed equally*

 # Both authors contributed equally

Nat Commun. 2016;10;7:10577



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

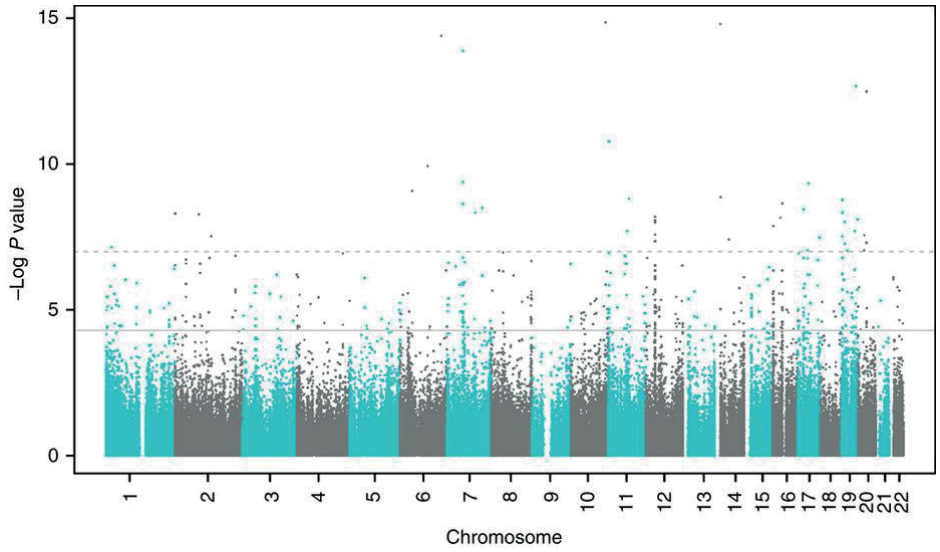


Figure 4.3.1. Association between Maternal Plasma Folate and DNA-methylation in Newborn Cord Blood: Meta-analysis Results for MoBa (n = 1,275) and Generation R (n = 713) Cohorts.
The uncorrected –log10(P values) are plotted by CpG genomic position. Multiple testing was accounted for using the false discovery 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.

Detailed acknowledgements and Supplementary Data can be found in the published article online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4749955/

REFERENCES

1. Miller JW, Ulrich CM. Folic acid and cancer--where are we today? Lancet. 2013;381(9871):974-6.
2. De-Regil LM, Fernandez-Gaxiola AC, Dowswell T, Pena-Rosas JP. Effects and safety of periconceptional folate supplementation for preventing birth defects. Cochrane Database Syst Rev. 2010(10):CD007950.
3. Bortolus R, Blom F, Filippini F, van Poppel MN, Leoncini E, de Smit DJ, et al. Prevention of congenital malformations and other adverse pregnancy outcomes with 4.0 mg of folic acid: community-based randomized clinical trial in Italy and the Netherlands. BMC Pregnancy Childbirth. 2014;14:166.
4. Barua S, Kuizon S, Junaid MA. Folic acid supplementation in pregnancy and implications in health and disease. J Biomed Sci. 2014;21:77.
5. O'Neill RJ, Vrana PB, Rosenfeld CS. Maternal methyl supplemented diets and effects on offspring health. Front Genet. 2014;5:289.
6. Choumenkovitch SF, Selhub J, Wilson PW, Rader JI, Rosenberg IH, Jacques PF. Folic acid intake from fortification in United States exceeds predictions. J Nutr. 2002;132(9):2792-8.
7. Nakouzi GA, Nadeau JH. Does dietary folic acid supplementation in mouse NTD models affect neural tube development or gamete preference at fertilization? Bmc Genet. 2014;15.
8. Fox JT, Stover PJ. Folate-Mediated One-Carbon Metabolism. Vitam Horm. 2008;79:1-44.
9. Begin P, Nadeau KC. Epigenetic regulation of asthma and allergic disease. Allergy Asthma Cl Im. 2014;10.
10. Chang HB, Zhang T, Zhang ZP, Bao R, Fu CB, Wang ZG, et al. Tissue-specific distribution of aberrant DNA methylation associated with maternal low-folate status in human neural tube defects. J Nutr Biochem. 2011;22(12):1172-7.
11. Loke YJ, Galati JC, Morley R, Joo EJH, Novakovic B, Li X, et al. Association of maternal and nutrient supply line factors with DNA methylation at the imprinted IGF2/H19 locus in multiple tissues of newborn twins. Epigenetics-Us. 2013;8(10):1069-79.
12. Amarasekera M, Martino D, Ashley S, Harb H, Kesper D, Strickland D, et al. Genome-wide DNA methylation profiling identifies a folate-sensitive region of differential methylation upstream of ZFP57-imprinting regulator in humans. Faseb J. 2014;28(9):4068-76.
13. Magnus P, Irgens LM, Haug K, Nystad W, Skjaerven R, Stoltenberg C, et al. Cohort profile: the Norwegian Mother and Child Cohort Study (MoBa). Int J Epidemiol. 2006;35(5):1146-50.
14. Ronningen KS, Paltiel L, Meltzer HM, Nordhagen R, Lie KK, Hovengen R, et al. The biobank of the Norwegian Mother and Child Cohort Study: a resource for the next 100 years. Eur J Epidemiol. 2006;21(8):619-25.
15. Haberg SE, London SJ, Nafstad P, Nilsen RM, Ueland PM, Vollset SE, et al. Maternal folate levels in pregnancy and asthma in children at age 3 years. J Allergy Clin Immunol. 2011;127(1):262-4, 4 e1.
16. Jaddoe VW, van Duijn CM, Franco OH, van der Heijden AJ, van Iizendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. Eur J Epidemiol. 2012;27(9):739-56.
17. Kruithof CJ, Kooijman MN, van Duijn CM, Franco OH, de Jongste JC, Klaver CC, et al. The Generation R Study: Biobank update 2015. Eur J Epidemiol. 2014;29(12):911-27.
18. O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. J Clin Pathol. 1992;45(4):344-7.
19. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics. 2007;8(1):118-27.

20. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics. 2013;29(2):189-96.
21. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13:86.
22. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30(10):1363-9.
23. Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlen SE, Greco D, et al. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. PLoS One. 2012;7(7):e41361.
24. Team RDC. R: A language and environment for statistical computing. . Vienna, Austria: R Foundation for Statistical Computing; 2010.
25. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010;26(17):2190-1.
26. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. J Roy Stat Soc B Met. 1995;57(1):289-300.
27. Tsang BL, Devine OJ, Cordero AM, Marchetta CM, Mulinare J, Mersereau P, et al. Assessing the association between the methylenetetrahydrofolate reductase (MTHFR) 677C > T polymorphism and blood folate concentrations: a systematic review and meta-analysis of trials and observational studies. Am J Clin Nutr. 2015;101(6):1286-94.
28. van der Valk RJP, Kiefte-de Jong JC, Sonnenschein-van der Voort AMM, Duijts L, Hafkamp-de Groen E, Moll HA, et al. Neonatal folate, homocysteine, vitamin B12 levels and methylenetetrahydrofolate reductase variants in childhood asthma and eczema. Allergy. 2013;68(6):788-95.
29. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37(1):1-13.
30. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. Bmc Bioinformatics. 2009;10.
31. Devlin B, Roeder K. Genomic control for association studies. Biometrics. 1999;55(4):997-1004.
32. Slattery ML, Lundgreen A, John EM, Torres-Mejia G, Hines L, Giuliano AR, et al. MAPK Genes Interact with Diet and Lifestyle Factors to Alter Risk of Breast Cancer: The Breast Cancer Health Disparities Study. Nutr Cancer. 2015;67(2):292-304.
33. Jarrett CR, Blancato J, Cao T, Bressette DS, Cepeda M, Young PE, et al. Human APC2 localization and allelic imbalance. Cancer Res. 2001;61(21):7978-84.
34. Shintani T, Ihara M, Tani S, Sakuraba J, Sakuta H, Noda M. APC2 Plays an Essential Role in Axonal Projections through the Regulation of Microtubule Stability. J Neurosci. 2009;29(37):11628-40.
35. van Es JH, Kirkpatrick C, van de Wetering M, Molenaar M, Miles A, Kuipers J, et al. Identification of APC2, a homologue of the adenomatous polyposis coli tumour suppressor. Curr Biol. 1999;9(2):105-8.
36. Sie KKY, Li J, Ly A, Sohn KJ, Croxford R, Kim YI. Effect of maternal and postweaning folic acid supplementation on global and gene-specific DNA methylation in the liver of the rat offspring. Mol Nutr Food Res. 2013;57(4):677-85.
37. Lubecka-Pietruszewska K, Kaufman-Szymczyk A, Stefanska B, Fabianowska-Majewska K. Folic acid enforces DNA methylation-mediated transcriptional silencing of PTEN, APC and RARbeta2 tumour suppressor genes in breast cancer. Biochem Bioph Res Co. 2013;430(2):623-8.

38. Mukherjee S, Manahan-Vaughan D. Role of metabotropic glutamate receptors in persistent forms of hippocampal plasticity and learning. Neuropharmacology. 2013;66:65-81.
39. Elia J, Glessner JT, Wang K, Takahashi N, Shtir CJ, Hadley D, et al. Genome-wide copy number variation study associates metabotropic glutamate receptor gene networks with attention deficit hyperactivity disorder. Nat Genet. 2012;44(1):78-U113.
40. Prasad A, Merico D, Thiruvahindrapuram B, Wei J, Lionel AC, Sato D, et al. A Discovery Resource of Rare Copy Number Variations in Individuals with Autism Spectrum Disorder. G3-Genes Genom Genet. 2012;2(12):1665-85.
41. Kloeckener-Gruissem B, Vandekerckhove K, Nurnberg G, Neidhardt J, Zeitz C, Nuernberg P, et al. Mutation of solute carrier SLC16A12 associates with a syndrome combining juvenile cataract with microcornea and renal glucosuria. Am J Hum Genet. 2008;82(3):772-9.
42. Wang SK, Hu Y, Simmer JP, Seymen F, Estrella NMRP, Pal S, et al. Novel KLK4 and MMP20 Mutations Discovered by Whole-exome Sequencing. J Dent Res. 2013;92(3):266-71.
43. Sandbacka M, Laivuori H, Freitas E, Halttunen M, Jokimaa V, Morin-Papunen L, et al. TBX6, LHX1 and copy number variations in the complex genetics of Mullerian aplasia. Orphanet J Rare Dis. 2013;8.
44. Bedont JL, LeGates TA, Slat EA, Byerly MS, Wang H, Hu JF, et al. Lhx1 Controls Terminal Differentiation and Circadian Function of the Suprachiasmatic Nucleus. Cell Rep. 2014;7(3):609-22.
45. Corrado L, Caromagno Y, Falasco L, Mellone S, Godi M, Cova E, et al. A novel peripherin gene (PRPH) mutation identified in one sporadic amyotrophic lateral sclerosis patient. Neurobiol Aging. 2011;32(3).
46. Xie T, Deng LB, Mei PM, Zhou YY, Wang B, Zhang J, et al. A genome-wide association study combining pathway analysis for typical sporadic amyotrophic lateral sclerosis in Chinese Han populations. Neurobiol Aging. 2014;35(7).
47. Hindorff LA MJEBI, Morales J (European Bioinformatics Institute), Junkins HA, Hall PN, Klemm AK, Manolio TA. A Catalog of Published Genome-Wide Association Studies [cited 2015 15 May]. Available from: http://www.genome.gov/gwastudies
48. Kempkensteffen C, Christoph F, Weikert S, Krause H, Kollermann J, Schostak M, et al. Epigenetic silencing of the putative tumor suppressor gene testisin in testicular germ cell tumors. J Cancer Res Clin. 2006;132(12):765-70.
49. Kristensen DG, Skakkebaek NE, Meyts ERD, Almstrup K. Epigenetic features of testicular germ cell tumours in relation to epigenetic characteristics of foetal germ cells. Int J Dev Biol. 2013;57(2-4):309-17.
50. Fetahu IS, Hobaus J, Kallay E. Vitamin D and the epigenome. Front Physiol. 2014;5.
51. Smith DE, Hornstra JM, Kok RM, Blom HJ, Smulders YM. Folic acid supplementation does not reduce intracellular homocysteine, and may disturb intracellular one-carbon metabolism. Clin Chem Lab Med. 2013;51(8):1643-50.
52. Christensen KE, Mikael LG, Leung KY, Levesque N, Deng L, Wu Q, et al. High folic acid consumption leads to pseudo-MTHFR deficiency, altered lipid metabolism, and liver injury in mice. Am J Clin Nutr. 2015;101(3):646-58.
53. Hoyo C, Murtha AP, Schildkraut JM, Jirtle R, Demark-Wahnefried W, Forman MR, et al. Methylation variation at IGF2 differentially methylated regions and maternal folic acid use before and during pregnancy. Epigenetics-Us. 2011;6(7):928-36.
54. Ally MS, Al-Ghnaniem R, Pufulete M. The relationship between gene-specific DNA methylation in leukocytes and normal colorectal mucosa in subjects with and without colorectal tumors. Cancer Epidemiol Biomarkers Prev. 2009;18(3):922-8.

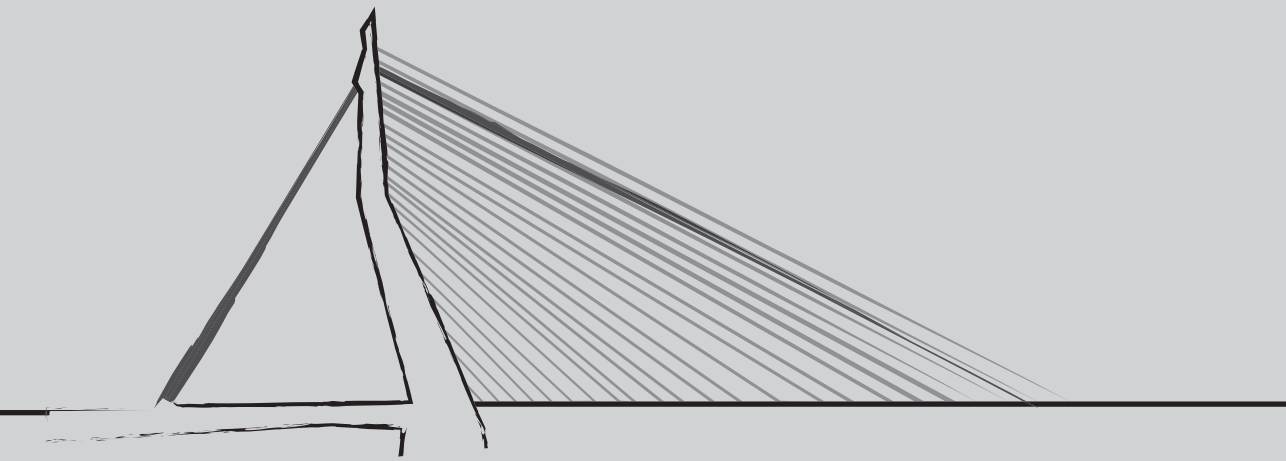
55. Byun HM, Siegmund KD, Pan F, Weisenberger DJ, Kanel G, Laird PW, et al. Epigenetic profiling of somatic tissues from human autopsy specimens identifies tissue- and individual-specific DNA methylation patterns. Hum Mol Genet. 2009;18(24):4808-17.
56. Talens RP, Boomsma DI, Tobi EW, Kremer D, Jukema JW, Willemsen G, et al. Variation, patterns, and temporal stability of DNA methylation: considerations for epigenetic epidemiology. Faseb J. 2010;24(9):3135-44.

Chapter 4.4

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

HT den Dekker, K Burrows, JF Felix, LA Salas, I Nedeljkovic, J Yao, SL Rifas-Shiman, C Ruiz-Arenas, N Amin, M Bustamante, DL DeMeo, AJ Henderson, CG Howe, MF Hivert, MA Ikram, JC de Jongste, L Lahousse, PR Mandaviya, JB van Meurs, M Pinart, L Stolk, AG Uitterlinden, JM Anto, AA Litonjua, CV Breton, GG Brusselle, J Sunyer, GD Smith, CL Relton, VWV Jaddoe, L Duijts

Submitted



ABSTRACT

Background The observed associations of fetal exposures with respiratory diseases may be explained by DNA-methylation. We aimed to identify differentially methylated regions (DMRs) in neonatal cord blood DNA associated with childhood lung function and the risks of asthma and chronic obstructive pulmonary disease (COPD) across the life course.

Methods We meta-analyzed epigenome-wide data of 1688 children from five cohorts to identify cord blood DMRs (Illumina HumanMethyl450 Beadchip) and their annotated genes, in relation to Forced Expiratory Volume in 1 second (FEV_1), FEV_1/Forced Vital Capacity (FVC), and Forced Expiratory Flow at 75% of FVC (FEF_{75}) at ages 7 to 13 years. Identified DMRs were explored for associations with childhood asthma, adult lung function and COPD, and gene expression and involvement in biological processes.

Findings We identified 59 DMRs associated with childhood lung function, of which 18 were associated with childhood asthma and 9 with COPD in adulthood. Genes annotated to the top ten identified DMRs were *HOXA5*, *PAOX*, *LINC00602*, *ABCA7*, *PER3*, *CLCA1*, *VENTX*, *NUDT12*, *PTPRN2* and *TCL1A*. Differential gene expression in blood was observed for 32 DMRs in childhood and 18 in adulthood, and genes related to 28 DMRs were expressed in adult lung tissue. Genes related with 16 identified DMRs were associated with respiratory developmental or pathogenic pathways. Specifically, the DMR annotated to *HOXA5* was associated with childhood FEV_1, risk of COPD and differential expression of *HOXA1*, *HOXA4* and *HOXA7*.

Interpretation Our findings suggest that the epigenetic status of the newborn affects respiratory health and disease across the life course.

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are major global health problems.[1] Both diseases are characterized by airway obstruction, as indicated by a reduced Forced Expiratory Volume in 1 second (FEV_1), FEV_1 to Forced Vital Capacity (FVC) ratio, and Forced Expiratory Flow at 75% of FVC (FEF_{75}).[2] Childhood lung function predicts lung function and risks of asthma and COPD in later life.[3] An accumulating body of evidence suggests that asthma and COPD have at least part of their origins in fetal life.[4,5] Adverse fetal exposures, such as maternal smoking and suboptimal diet, increase the risk of asthma and COPD.[5] The pathways linking fetal life with life course respiratory disease may include epigenetic changes, including DNA-methylation.[5] Fetal development is characterized by high rates of DNA-methylation changes and rapid organ development.[5] We hypothesized that fetal differential DNA-methylation reflected in cord blood DNA of newborns affect gene expression and subsequent respiratory tract development, and predispose individuals for obstructive airway diseases in later life.[6,7]

We meta-analyzed five epigenome-wide association studies using data from 1,688 children participating in prospective cohort studies to identify differential DNA-methylated regions (DMRs) of newborns associated with childhood FEV_1, FEV_1/FVC and FEF_{75}. Identified top DMRs were subsequently explored for their associations with childhood asthma, lung function in adolescence and adulthood, and COPD in adulthood, and explored for association with gene expression and involvement in biological processes.

METHODS

Study Design and Data Sources

We included population-based cohort studies participating in PACE consortium with data on epigenome-wide DNA-methylation at birth and lung function in childhood.[8] We used data from 1,688 Caucasian children aged 7 to 13 years participating in the Avon Longitudinal Study of Parents and Children (ALSPAC) (United Kingdom), Generation R (Netherlands), INfancia y Medio Ambiente Study (INMA) (Spain), Children's Health Study (CHS) and Project Viva (both from the U.S.A.). These data were used for the primary discovery epigenome-wide meta-analysis to identify DMRs of newborns related to childhood lung function. We focused on identification of DMRs because regional methylation of CpGs is likely to control gene transcription.[9,10]

We used several resources for the secondary analyses. For clinical outcomes, we used childhood asthma data (Generation R, mean age 6 years), lung function data from adolescents (ALSPAC, mean age 15 years) and adults (Rotterdam Study, mean age 66 years, The Netherlands), and COPD data in adults (Rotterdam Study) (Figure 4.4.1). For gene

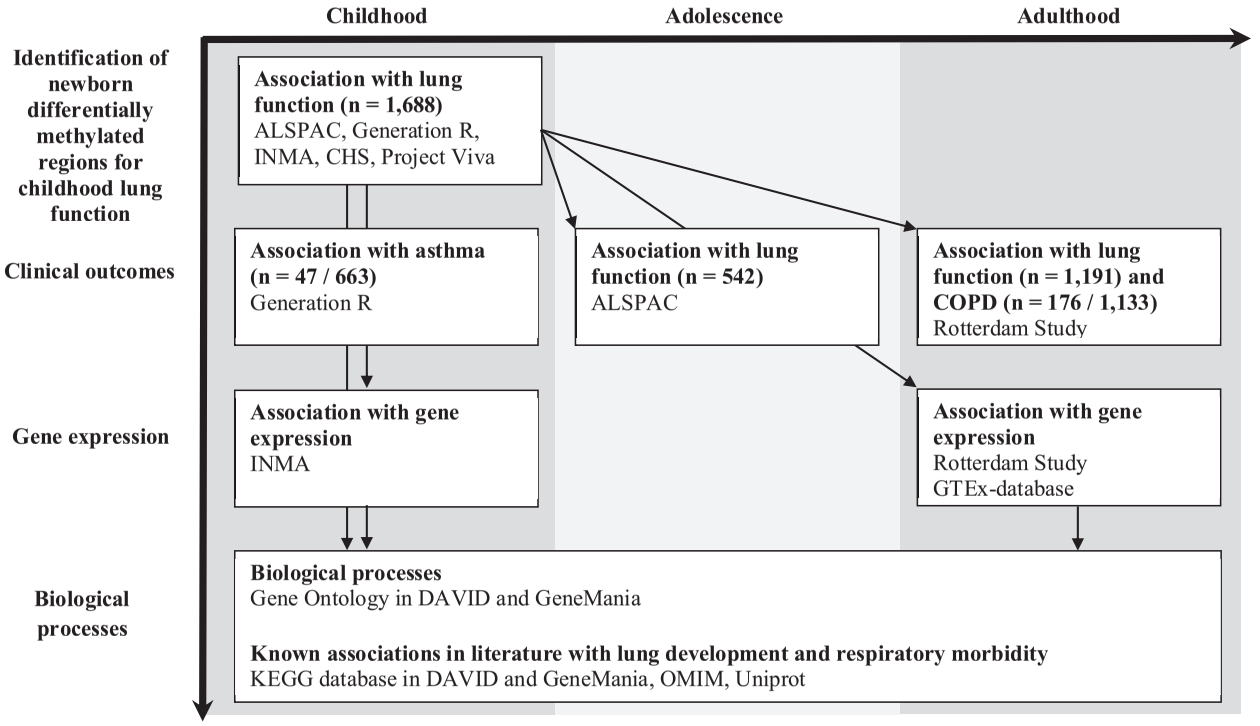


Figure 4.4.1. Overall Study Design.

Epigenome-wide meta-analyses were performed to identify methylated CpGs associated with lung function in children using data from 1,689 children participating in ALSPAC, Generation R, INMA, CHS and Project Viva. Identified differentially methylated regions (DMRs) were annotated to their nearest gene using PAVIS. Next, we examined if identified DMRs were associated with asthma in children participating in Generation R, lung function in adolescents and adults participating in the ALSPAC or Rotterdam Study, or COPD in adults participating in Rotterdam Study, and with gene expression levels in children participating in INMA, adults in Rotterdam Study, and the GTEx-database. We further explored biological processes and associations with lung development and respiratory morbidity using publicly available resources (DAVID, GeneMania, OMIM and UniProt). N = x: number of participants included for the analysis. N = x/x: number of cases / total number of participants included in the analysis.

expression, we used blood samples from children (INMA, at birth and mean age 4 years) and adults (Rotterdam Study). Last, we used publicly available resources to relate identified DMRs with biological processes.[11-13] Parents, legal representatives or participants provided informed consent in accordance with local ethics policies. Detailed information about the study design and cohorts is provided in the Supplementary material.

DNA-methylation

All cohorts extracted DNA from blood samples and used the EZ-96 DNA Methylation kit (Zymo Research Corporation, Irvine, USA) for bisulfite conversion. Samples were processed with the Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, USA) followed by cohort-specific quality control, probe exclusion and data normalization. Detailed information on cohort-specific data acquisition and quality control is provided in the Supplementary material.

Respiratory Outcome Assessment

Lung function measures comprised pre-bronchodilator FEV_1, FEV_1/FVC and FEF_{75}, which were converted into sex-, age-, height- and ethnicity-adjusted z-scores.[14] Physician-diagnosed asthma was obtained by questions adapted from the International Study on Asthma and Allergy in Childhood.[15] COPD was defined as pre-bronchodilator FEV_1/FVC <0·70 in the absence of asthma, or a doctor diagnosis.[16]

Statistical Analyses

Primary Meta-analysis on Childhood Lung Function

A detailed description of applied methods is presented in the Supplementary material. Individual cohorts used robust linear regression models to examine the associations of DNA-methylation levels of CpGs with childhood FEV_1, FEV_1/FVC and FEF_{75}. Analyses were adjusted for maternal age, socio-economic status, smoking during pregnancy, parity, asthma or atopy, technical covariates and estimated cell counts.[17] Results were combined using inverse variance-weighted fixed-effect meta-analyses. Results from unadjusted models were similar to fully adjusted models (S-Table 1). We identified DMRs using the software-tool Comb-p, which is the most robust tool to identify DMRs with small effect sizes.[18,19] We used a maximum window of 500 bases between neighboring CpGs, an FDR-threshold <0·05 after correlation-correction between neighboring CpGs, and a minimum of 2 CpGs to start identification of a DMR. DMR p-values were adjusted for multiple testing by Šidák-correction, which is comparable to Bonferroni-correction. We considered Šidák-corrected p-values <0·05 statistically significant. DMRs were annotated to the nearest gene with PAVIS, using the human GRCh37/hg19 assembly as reference genome.[20] We performed a sensitivity analysis restricted to probes providing unbiased measures of DNA-methylation because genomic variants in the Illumina-probes may compromise methylation profiling.[21]

Table 4.4.1. Characteristics of Cohorts and Their Participants.

	No. of participants	Birth years	Type of blood sample for DNA-methylation	No. of available CpGs	Age in years (SD) at lung function measurement	FEV_1 z-score (SD)	FEV_1/FVC z-score (SD)	FEF_{75} z-score (SD)
Childhood								
ALSPAC (UK)	654	1991-1992	Cord blood	471,193	8.6 (0.2)	-0.07 (0.97)	0.03 (1.03)	0.14 (0.97)
Generation R (NL)	643	2002-2006	Cord blood	436,013	9.8 (0.3)	0.05 (0.88)	-0.12 (0.95)	-0.07 (0.85)
INMA (Spain)	140	2004-2007	Cord blood	439,306	6.9 (0.3)	1.37 (0.93)	-0.55 (0.85)	-0.80 (0.74)
CHS (USA)	75	2002	Cord blood	383,857	13.3 (0.6)	0.41 (1.15)	-0.08 (1.05)	0.37 (1.01)
Project Viva (USA)	176	1999 - 2002	Cord blood	470,870	7.9 (0.7)	-0.36 (1.07)	-0.88 (0.84)	-0.40 (1.10)
Adolescence / Adulthood								
ALSPAC (UK)	542	1991-1992	Cord blood	n.a.	15.4 (0.2)	-0.69 (1.24)	0.52 (1.16)	0.20 (1.00)
Rotterdam Study – I (NL)	488	1921-1960	Peripheral blood	n.a.	64.0 (6.3)	-0.09 (1.15)	-0.26 (0.97)	0.08 (0.76)
Rotterdam Study – II (NL)	703	1930-1960	Peripheral blood	n.a.	67.5 (5.9)	-0.16 (1.07)	-0.12 (0.94)	0.04 (0.71)

Lung function was obtained by spirometry and sex-, age-, height- and ethnicity-adjusted Z-scores were calculated. FEV_1: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; FEF_{75}: Forced Expiratory Flow at 75% of FVC; n.a.: not applicable. UK: United Kingdom. NL: the Netherlands. USA: United States of America.

Secondary Analyses on Later Life Lung Function and Respiratory Diseases

We used linear and logistic regression models to examine the associations of CpGs within identified DMRs with asthma in childhood, FEV_1, FEV_1/FVC and FEF_{75} in adolescence and adulthood, and COPD in adulthood. Single CpG p-values were used to reconstruct the identified DMRs with Comb-p, applying identical parameter settings as in the discovery meta-analyses including false discovery rate (FDR)-correction.[18,22] We did not apply Šidák-correction because analyses were limited to the identified DMRs.

Gene Expression Analyses

We assessed the associations of CpGs within identified DMRs with gene expression in a region of ±250kb in blood samples from children and adults. P-values of CpGs associated with gene expression were combined for each DMR using a modified generalized Fisher method and FDR-correction.[22,23] Additionally, we explored whether the annotated and differentially expressed genes were expressed in human lung specimens of the Genotype-Tissue Expression (GTEx) database.[11]

Exploration biological processes

The Gene Ontology database implemented in DAVID and Genemania was used to examine gene function in biological processes.[12,13] We examined pathways for all genes annotated to the DMRs and for genes with differential expression in association with the identified DMRs. We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database in DAVID and Genemania, the OMIM database and the Universal Protein Resource (UniProt) to explore whether annotated or expressed genes have been related to respiratory development or diseases.[24] We used the Ensemble Genome browser to visualize the genomic structure of the identified DMRs.

RESULTS

Meta-analysis of Epigenome-wide Association Studies on Childhood Lung Function

Characteristics of the participating cohorts are given in Table 4.4.1 and S-Table 4.4.2.

We identified 22, 15 and 22 DMRs associated with FEV_1, FEV_1/FVC and FEF_{75}, respectively (Figure 4.4.2, S-Tables 4.4.3 and 4.4.4). One DMR was associated with both FEV_1 and FEF_{75}, and four DMRs with both FEV_1/FVC and FEF_{75}. A higher mean methylation of CpGs located within 37 (63%) of the identified DMRs was associated with higher lung function measures, and within 22 (37%) of identified DMRs with lower lung function measures.

Of the top ten significant DMRs associated with childhood lung function, the 5 DMRs and their annotated genes for FEV_1 were located at chr7:27,183,133-27,184,854

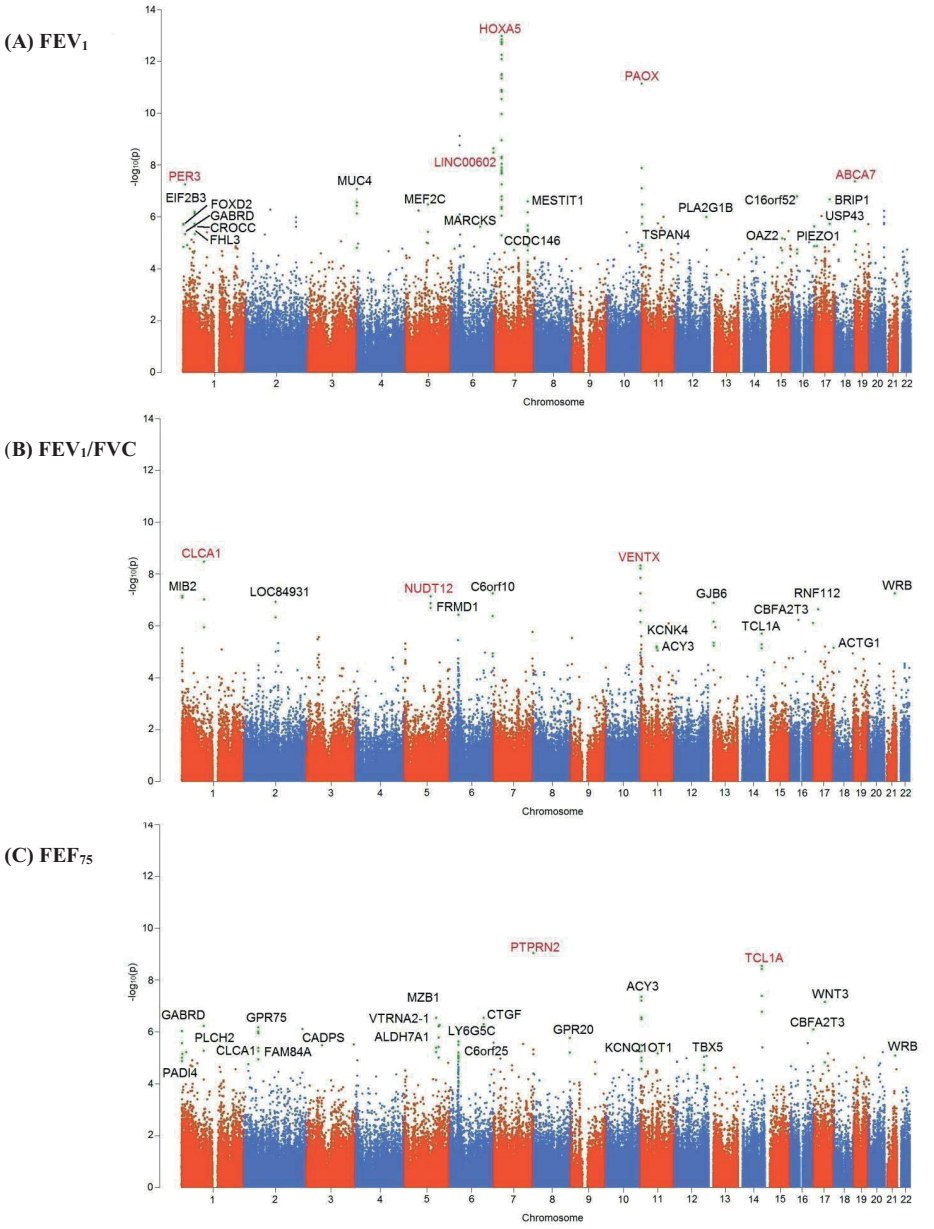


Figure 4.4.2. Manhattan Plots of Associations of CpGs located in Differentially Methylated Regions with Childhood Lung Function Outcomes.

Green dots represent p-values from associations of CpGs located in differentially methylated regions (DMRs) at birth with childhood Forced Expiratory Volume in 1 second (FEV_1), FEV_1/ Forced Vital Capacity (FVC) and Forced Expiratory Flow at 75% of FVC (FEF_{75}). P-values of DMRs ranged from 3.05E-14 to 0.031, and details are provided in Supplementary Appendix Table 3. Nearest annotated genes of DMRs are provided. The genes annotated to the top ten significant DMRs associated with childhood lung function are written in red. Single CpGs are presented as red and blue dots, corrected for correlations with neigboring CpGs.

(*HOXA5),* chr10:135,202,522-135,203,201 (*PAOX),* chr6:166,418,799-166,419,139 *(LINC00602)*, chr19:1,063,624-1,064,219 (*ABCA7)* and chr1:7,887,199-7,887,561 (*PER3*). Three DMRs and their annotated genes for FEV_1/FVC were located at chr1:86,968,087-86,968,544 *(CLCA1)*, chr10:135,051,149-135,051,582 (*VENTX),* and chr5:102,898,223-102,898,734 (*NUDT12).* Two DMRs for FEF_{75} and their annotated genes were located at chr7:158,045,980-158,046,359 *(PTPRN2)* and chr14:96,180,406-96,181,045 (*TCL1A)*. After exclusion of probes containing genomic variants, 22 (54%) of the identified DMRs with ≥2 CpGs (n=41) replicated (S-Table 4.4.5).

Identified DMRs and Lung Function and Respiratory Diseases Across the Life Course

Of all 59 identified DMRs related with childhood lung function, 18 (31%) were associated with childhood asthma (Figure 4.4.3, S-Table 4.4.6). Furthermore, 11 (19%) and 9 (15%) DMRs were associated with lung function in adolescence and adulthood, respectively, and 9 (15%) were associated with COPD. The DMRs annotated to *HOXA5* and *PAOX* were associated with childhood and adolescence FEV_1 and COPD, but not with childhood asthma or adult lung function. The DMRs annotated to *PER3* and *VENTX* were associated with childhood and adolescence FEV_1 and FEV_1/FVC, respectively. The DMR annotated to *NUDT12* was associated with childhood FEV_1/FVC and COPD. The DMRs annotated to *PTPRN2* and *TCL1A* were associated with childhood FEF_{75} and asthma. The DMRs annotated to *LINC00602*, *ABCA7* and *CLCA1* were associated with childhood lung function but not with other outcomes.

Identified DMRs and Gene Expression

Of the 59 identified DMRs, 32 (54%) DMRs at birth were associated with gene expression at age 4 years, and 18 (31%) DMRs with gene expression in adulthood (S-Table 4.4.7). The DMR annotated to *HOXA5* was associated with differential expression of several genes of the *HOX*-family (Table 4.4.2). The DMRs annotated to *PER3*, *VENTX, NUDT12* and *TCL1A* were associated with differential expression of their respective genes. The DMRs annotated to *PAOX*, *LINC00602*, *ABCA7*, *CLCA1* and *PTPRN2* were not associated with expression of their corresponding genes. Genes annotated to 28 (47%) of all identified DMRs were expressed in adult lung tissue, including the top significant DMRs annotated to *PAOX, ABCA7,CLCA1*, *VENTX* and *NUDT12* (S-Table 4.4.8).

Identified DMRs and related biological processes

Of all 59 identified DMRs, 43 were annotated to genes not previously associated with lung function or respiratory morbidity (S- Table 4.4.7). Of the genes annotated to the top ten significant DMRs, *HOXA5*, *CLCA1*, *TCL1A* and *NUDT12* were previously associated with respiratory development including alveogenesis, respiratory diseases and cellular immunity (Table 4.4.2). Genes related to the identified DMRs, including *HOXA5*,


Molecular location of the differentially methylated region (Chromosome: start – end)
Childhood
Adole-scence
Adulthood
Lung function
Asthma
Lung function
Lung function
COPD
FEV1
1:1,957,385-1,957,806
1:7,887,199-7,887,561
1:17,222,405-17,222,716
1:8,461,540-38,461,897
1:45,452,166-45,452,606
1:47,915,553-47,915,952
3:195,489,708-195,490,310
5:88,179,235-88,179,540
6:114,181,482-114,181,799
6:166,418,799-166,419,139
7:27,183,133-27,184,854
7:76,828,885-76,829,169
7:130,131,480-130,132,162
10:135,202,522-135,203,201
11:843,897-844,285
12:120,763,487-120,763,870
15:64,995,133-64,995,502
16:22,018,727-22,019,338
16:88,803,803-88,804,052
17:9,550,137-9,550,546
17:59,940,709-59,941,133
19:1,063,624-1,064,219
FEV1/FVC
1:1,564,422-1,564,921
1:186,968,087-86,968,544
2:121,223,534-121,223,965
5:102,898,223-102,898,734
6:32,305,068-32,305,146
6:168,533,375-168,533,690
10:135,051,149-135,051,582
11:64,064,947-64,065,372
11:67,417,958-67,418,406
13:20,805,380-20,805,896
14:96,180,406-96,181,045
16:89,023,389-89,023,634
17:19,314,299-19,314,619
17:79,485,529-79,485,710
21:40,759,534-40,759,695
FEF75
1:1,957,073-1,957,712
1:2,383,438-2,383,688
1:17,634,543-17,634,717
1:86,968,087-86,968,256
2:14,774,786-14,774,903
2:54,086,854-54,087,553
3:62,860,103-62,860,225
5:125,931,001-125,931,363
5:135,416,029-135,416,614
5:138,725,400-138,725,831
6:31,650,735-31,651,159
6:31,690,998-31,691,540
6:132,271,361-132,271,589
7:158,045,980-158,046,359
8:142,377,303-142,377,490
11:2,721,207-2,721,633
11:67,417,958-67,418,406
12:114,843,919-114,844,266
14:96,180,406-96,181,045
16:89,023,389-89,023,634
17:44,897,431-44,897,513
21:40,759,534-40,759,695

Figure 4.4.3.

Figure 4.4.3. Identified Differentially Methylated Regions and Their Location, and Direction of Associations with Childhood Lung Function, Childhood Asthma, Adolescent Lung Function, and Adult Lung Function and COPD.
Results present identified differentially methylated regions (DMRs) from association-analyses of DNA-methylation at birth with childhood Forced Expiratory Volume in 1 second (FEV_1), FEV_1/ Forced Vital Capacity (FVC) and Forced Expiratory Flow at 75% of FVC (FEF_{75}), their location, and their direction of association with childhood lung function, childhood asthma, adolescent lung function, and adult lung function and COPD. Molecular locations of the top ten significant DMRs are presented in bold. Identified DMRs associated with childhood lung function and other respiratory outcomes are marked in grey, and if not associated with respiratory outcomes in white. Directions of associations are marked ↓ if a higher mean methylation of the DMRs was associated with a lower z-score for lung function or lower risk of asthma or COPD, and marked ↑ if a higher mean methylation of the DMRs was associated with a higher z-score of lung function or higher risk of asthma or COPD. Red colored arrows represent disadvantageous effect estimates (lower lung function, increased risk of asthma or COPD), and green colored arrows beneficial effect estimates (higher lung function, lower risk of asthma or COPD).

PER3, *CLCA1*, *NUDT12* and *PTPRN2*, were located in pathways related to regionalization, DNA- and RNA-regulation and embryonic development (S-Tables 4.4.9). The genes *HLA-DRB4* and *HLA-DRB5* were enriched in processes including asthma. These genes were associated with the DMR located at chr6:32,305,068-32,305,146, which was related with childhood and adulthood FEV_1/FVC and COPD.

Of the top ten significant DMRs, the DMRs annotated to *HOXA5*, *CLCA1* and *TCL1A* contained *CTCF*-binding sites (S-Figure 4.4.2). The DMRs annotated to *HOXA5*, *PAOX*, *PER3* and *NUDT12* were located in promotor regions of their respective genes. The DMR annotated to *ABCA7* was located in a CpG-island.

DISCUSSION

We identified 59 DMRs in neonatal cord blood associated with childhood lung function. Eighteen (31%) of all identified DMRs were also associated with childhood asthma, 11 (19%) and 9 (15%) with adolescent and adult lung function, respectively, and 9 (15%) with COPD. Differential gene expression was observed for 32 (54%) DMRs in childhood and 18 (31%) DMRs in adulthood. Multiple genes related to the identified DMRs have previously been associated with respiratory development and morbidity, and many identified DMRs were located within known regulatory elements for gene expression.

Reduced lung function in childhood is associated with reduced lung function and increased risks of asthma and COPD many decades later.[6,25] Development of lung function, asthma and COPD partly originates in fetal life, in which differential DNA-methylation may be an underlying pathway.[5] 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. An epigenome-wide study among

Table 4.4.2. Associations of the Top Ten Significant Identified Differentially Methylated Regions with Gene Expression and Related Respiratory Outcomes.

Molecular location of the differentially methylated region (Chromosome: start – end)	Lung function	Annotated gene*	Expressed gene†	Gene expression in children‡	Gene expression in adults§	Previously associated with lung development or respiratory morbidity‖
chr1: 7,887,199 - 7,887,561	FEV_1	*PER3*	*PER3, RP3-467I1.4, RNA5SP23, RP4-726F1.1*	↓	-	
			RP11-431K24.1	-	↑	
chr1: 86,968,087 – 86,968,544	FEV_1/FVC	*CLCA1*	no expression	-	-	Lung development, asthma, COPD
chr5: 102,898,223 - 102,898,734	FEV_1/FVC	*NUDT12*	*NUDT12*	↓	-	Smoking behavior in COPD
			CMBL	↓	-	
chr6: 166,418,799 – 166,419,139	FEV_1	*LINC00602*	no expression	-	-	
chr7: 27,183,133 - 27,184,854	FEV_1	*HOXA5*	*HOXA1, HOTTIP*	↓	↓	Lung development, FEV_1, FEV_1/FVC
			EVX1, HOXA4, HOXA7	↓	-	Lung development, asthma, COPD
chr7: 158,045,980 – 158,046,359	FEF_{75}	*PTPRN2*	no expression	-	-	
chr10: 135,202,522 – 135,203,201	FEV_1	*PAOX*	no expression	-	-	
chr10: 135,051,149 – 135,051,582	FEV_1/FVC	*VENTX*	*TUBGCP2, RP11-122K13.12*	↓	-	
			VENTX, ECHS1	↑	-	
			SPRN	↑	↓	
			ZNF511	-	↑	
chr14: 96,180,406 – 96,181,045	FEF_{75}	*TCL1A*	*TCL1A,CCDC85C*	↓	-	Asthma
chr19: 1,063,624 – 1,064,219	FEV_1	*ABCA7*	no expression	-	-	

Results present identified differentially methylated regions (DMRs) from association-analyses of DNA-methylation at birth with childhood Forced Expiratory Volume in 1 second (FEV_1), FEV_1/ Forced Vital Capacity (FVC) and Forced Expiratory Flow at 75% of FVC (FEF_{75}). *DMRs were annotated to their nearest gene. †: Identified DMRs at birth were associated with gene expression in: ‡: childhood (INMA; mean age 4 years) and §: adulthood (the Rotterdam Study, mean age 66 years). Gene expressions levels were assessed limited to 250kb up- and downstream of the outer border of the DMR. Directions of associations are marked ↓ if a higher methylation of the DMR was associated with a decreased expression of the specific gene, ↑ if a higher methylation of the DMR was associated with an increased expression of the specific gene, and - if no direction of associations were observed. ‖: Associations of expressed genes with lung development and respiratory morbidity were explored in previous published studies the OMIM database and UniProt.

97 asthmatics and 97 healthy children aged 6-12 years identified 81 DMRs associated with asthma, of which 16 DMRs were also associated with FEV_1.[26] Of these 81 DMRs, 19 were located within 500kb of our identified DMRs and may affect the same genes. Another epigenome-wide study in 1,454 adults identified 349 CpGs associated with COPD.[27] Four annotated genes in this adult study (*CBFA2T3*, *PADI4*, *LST1*, *KCNQ1*) were replicated in our study of children. Multiple genes associated with the identified DMRs have previously been related with asthma and COPD in genome-wide association studies. *TCL1A* has been identified as asthma-susceptibility gene.[28] Nine (15%) of the 59 DMRs we identified were associated with adult lung function, and annotated to, or associated with differential expression of 11 genes. Nine of these genes were previously linked with pulmonary structures (*CROCC*, *CLCA1*), immunity (*MARCKS*, *FOXD2*, *MEF2C*, *CMBL*, *CLCA1*), asthma (*MARCKS*, *HCG23*, *CLCA1*), COPD (*MARCKS*, *TBX5*, *CLCA1*), and smoking behavior in COPD (*NUDT12*).[24] This suggests that genes associated with respiratory diseases could be influenced by differential DNA-methylation from early life onwards.

We explored the biological processes of the top significant DMRs for development of respiratory morbidity.[24] The DMR annotated to *HOXA5* was associated with childhood and adolescent FEV_1, COPD and differential expression of *HOXA1*, *HOXA4* and *HOXA7*. One DMR associated with childhood FEV_1/FVC was annotated to *VENTX*, which is a member of the *HOX*-gene family. The DMR annotated to *LINC00602* (Long Intergenic Non-Protein Coding RNA (lncRNA) 602) was linked to childhood FEV_1. LncRNAs influence gene-specific epigenetic regulation and interact amongst others with the transcription of *HOX*-genes. *HOX*-genes are critical for segmental fetal development, and especially *HOXA5* is required for embryonic respiratory tract morphogenesis.[29,30] The DMR annotated to *PAOX* was linked to childhood and adolescence FEV_1 and COPD. *PAOX* is involved in the regulation of intracellular polyamine, which is essential for protein synthesis. The DMR linked to *ABCA7* was associated with FEV_1 in childhood and adolescence. *ABCA7* is involved in the lipid homeostasis in the cellular immune system and is essential for phagocytosis of apoptotic cells by alveolar macrophages.[31]*PER3*, annotated to a DMR associated with FEV_1 in children and adolescents, is a key element in the endogenous circadian rhythm. The DMR linked to *CLCA1* was associated with childhood FEV_1/FVC and FEF_{75}, and expressed in adult lung tissue. *CLCA1* affects IL-13 driven mucus production in human airway epithelial cells and is associated with asthma and COPD.[32-34]*NUDT12*, annotated to a DMR associated with childhood FEV_1/FVC and COPD, is involved in intracellular biochemical reactions. *NUDT12* is associated with smoking behavior in COPD.[35]

PTPRN2, annotated to a DMR associated with childhood FEF_{75} and asthma is member of a gene family regulating cell growth and differentiation, and is involved in vesicle-mediated secretory processes. DNA-methylation of *PTPRN2* differentiates between lung cancer, pulmonary fibrosis and COPD.[36]*TCL1A*, annotated to a DMR associated with childhood FEV_1/FVC, FEF_{75} and asthma, is specific to developing lymphocytes when

expressed and is associated with asthma.[28] Thus, many of the genes annotated to the top significant DMRs are involved in respiratory development, cellular immunity and respiratory morbidity, which warrant further studies.

This is the largest study to date evaluating the associations of newborn epigenome-wide DNA-methylation with lung function and respiratory disease in children and adults, and it provides new insights into the epigenetic changes in fetal life that increase the risk of life-time respiratory morbidity. These results cannot currently be used as predictors of disease in individuals, but are important from an etiological perspective. Genes associated with 29 of the identified DMRs, including *HOXA5, PAOX, VENTX, PTPRN2* and *TCL1A*, have been reported to be differentially methylated in relation with maternal smoking during pregnancy.[8] Genes related with four identified DMRs associated with childhood lung function were differentially methylated in association with maternal folate levels during pregnancy.[37] This supports the hypothesis that adverse exposures in fetal life may impact DNA-methylation at birth, gene expression and subsequent respiratory development in the child, predisposing individuals for obstructive airway diseases. Further experimental or Mendelian randomization studies on the identified DMRs and associated genes might inform strategies in early life to improve lung function and lower the lifetime risk of obstructive respiratory diseases.

Some limitations should be discussed. We measured DNA-methylation in blood because it is easily accessible in large cohort studies. Blood DNA-methylation does not necessarily reflect lung epithelial DNA-methylation. However, asthma and COPD have systemic manifestations, characterized by increased inflammatory blood markers.[38,39] Although the analyses were adjusted for estimated cell counts, we cannot rule out residual confounding due to alterations in cell type distribution. Recently, two new reference sets for cell type adjustment in cord blood samples were published.[40,41] These reference sets are currently being validated, and future studies will shed light on the differences between reference panels. Some associations of DMRs with lung function and clinical outcomes differed in direction per age period. Expression of genes may differ depending on the developmental stage.[42] Identified DMRs could have been affected by genomic variants in Illumina-probes. Whether probes containing genomic variants should be retained in analyses, is currently under debate.[21,43] We aimed to reduce this possible effect by the stringent requirement of at least two CpGs to form a DMR. Further research is needed to determine the effect of genetic variants in probes on DNA-methylation analyses. Several identified DMRs were associated with gene expression other than the nearest and therefore annotated gene, which limits the potential biological effect of the annotated genes.

In conclusion, we identified 59 DMRs in cord blood that were associated with childhood lung function. Multiple DMRs were additionally related with childhood asthma, adolescent and adult lung function, or adult COPD. Also, multiple DMRs were associated

with differential gene expression of genes involved in embryonic and respiratory tract development, or were located in regulatory elements for gene expression. These findings suggest that epigenetic changes during fetal life might modify the risk of respiratory diseases across the life course.

REFERENCES

1. Chronic obstructive pulmonary disease (COPD). World Health Organisation, 2015. (Accessed June 16, 2016, at http://www.who.int/mediacentre/factsheets/fs315/en/.)
2. McDonough JE, Yuan R, Suzuki M, et al. Small-airway obstruction and emphysema in chronic obstructive pulmonary disease. N Engl J Med 2011;365:1567-75.
3. McGeachie MJ, Yates KP, Zhou X, et al. Patterns of Growth and Decline in Lung Function in Persistent Childhood Asthma. New Engl J Med 2016;374:1842-52.
4. Martinez FD. Early-Life Origins of Chronic Obstructive Pulmonary Disease. N Engl J Med 2016;375:871-8.
5. Duijts L, Reiss IK, Brusselle G, de Jongste JC. Early origins of chronic obstructive lung diseases across the life course. Eur J Epidemiol 2014;29:871-85.
6. Postma DS, Rabe KF. The Asthma-COPD Overlap Syndrome. N Engl J Med 2015;373:1241-9.
7. Fu JJ, McDonald VM, Baines KJ, Gibson PG. Airway IL-1 beta and Systemic Inflammation as Predictors of Future Exacerbation Risk in Asthma and COPD. Chest 2015;148:618-29.
8. Joubert BR, Felix JF, Yousefi P, et al. DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. Am J Hum Genet 2016;98:680-96.
9. Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 2009;462:315-22.
10. Bock C. Analysing and interpreting DNA methylation data. Nat Rev Genet 2012;13:705-19.
11. Carithers LJ, Ardlie K, Barcus M, et al. A Novel Approach to High-Quality Postmortem Tissue Procurement: The GTEx Project. Biopreserv Biobank 2015;13:311-9.
12. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research 2009;37:1-13.
13. Warde-Farley D, Donaldson SL, Comes O, et al. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic acids research 2010;38:W214-20.
14. Quanjer PH, Stanojevic S, Cole TJ, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. Eur Respir J 2012;40:1324-43.
15. Asher MI, Keil U, Anderson HR, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J 1995;8:483-91.
16. Terzikhan N, Verhamme KM, Hofman A, Stricker BH, Brusselle GG, Lahousse L. Prevalence and incidence of COPD in smokers and non-smokers: the Rotterdam Study. Eur J Epidemiol 2016.
17. Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC bioinformatics 2012;13:86.
18. Pedersen BS, Schwartz DA, Yang IV, Kechris KJ. Comb-p: software for combining, analyzing, grouping and correcting spatially correlated P-values. Bioinformatics 2012;28:2986-8.
19. Peters TJ, Buckley MJ, Statham AL, et al. De novo identification of differentially methylated regions in the human genome. Epigenet Chromatin 2015;8.
20. Huang W, Loganantharaj R, Schroeder B, Fargo D, Li L. PAVIS: a tool for Peak Annotation and Visualization. Bioinformatics 2013;29:3097-9.
21. Naeem H, Wong NC, Chatterton Z, et al. Reducing the risk of false discovery enabling identification of biologically significant genome-wide methylation status using the HumanMethylation450 array. BMC Genomics 2014;15:51.
22. Benjamini Y, Hochberg Y. Controlling for False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society, Series B 1995;57:289-300.

23. Dai H, Leeder JS, Cui Y. A modified generalized Fisher method for combining probabilities from dependent tests. Front Genet 2014;5:32.
24. UniProt C. UniProt: a hub for protein information. Nucleic acids research 2015;43:D204-12.
25. McGeachie MJ, Yates KP, Zhou X, et al. Patterns of Growth and Decline in Lung Function in Persistent Childhood Asthma. N Engl J Med 2016;374:1842-52.
26. Yang IV, Pedersen BS, Liu A, et al. DNA methylation and childhood asthma in the inner city. J Allergy Clin Immunol 2015;136:69-80.
27. Qiu W, Baccarelli A, Carey VJ, et al. Variable DNA methylation is associated with chronic obstructive pulmonary disease and lung function. Am J Respir Crit Care Med 2012;185:373-81.
28. George BJ, Reif DM, Gallagher JE, et al. Data-driven asthma endotypes defined from blood biomarker and gene expression data. PloS one 2015;10:e0117445.
29. Golpon HA, Geraci MW, Moore MD, et al. HOX genes in human lung: altered expression in primary pulmonary hypertension and emphysema. Am J Pathol 2001;158:955-66.
30. Mandeville I, Aubin J, LeBlanc M, et al. Impact of the loss of Hoxa5 function on lung alveogenesis. American Journal of Pathology 2006;169:1312-27.
31. Jehle AW, Gardai SJ, Li S, et al. ATP-binding cassette transporter A7 enhances phagocytosis of apoptotic cells and associated ERK signaling in macrophages. J Cell Biol 2006;174:547-56.
32. Poole A, Urbanek C, Eng C, et al. Dissecting childhood asthma with nasal transcriptomics distinguishes subphenotypes of disease. J Allergy Clin Immunol 2014;133:670-8 e12.
33. Hegab AE, Sakamoto T, Uchida Y, et al. CLCA1 gene polymorphisms in chronic obstructive pulmonary disease. J Med Genet 2004;41:e27.
34. Alevy YG, Patel AC, Romero AG, et al. IL-13-induced airway mucus production is attenuated by MAPK13 inhibition. Journal of Clinical Investigation 2012;122:4555-68.
35. Siedlinski M, Cho MH, Bakke P, et al. Genome-wide association study of smoking behaviours in patients with COPD. Thorax 2011;66:894-902.
36. Wielscher M, Vierlinger K, Kegler U, Ziesche R, Gsur A, Weinhausel A. Diagnostic Performance of Plasma DNA Methylation Profiles in Lung Cancer, Pulmonary Fibrosis and COPD. EBioMedicine 2015;2:929-36.
37. Joubert BR, den Dekker HT, Felix JF, et al. Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. Nat Commun 2016;7:10577.
38. Barnes PJ. Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. J Allergy Clin Immunol 2016;138:16-27.
39. Nadif R, Siroux V, Boudier A, et al. Blood granulocyte patterns as predictors of asthma phenotypes in adults from the EGEA study. Eur Respir J 2016.
40. Bakulski KM, Feinberg JI, Andrews SV, et al. DNA methylation of cord blood cell types: Applications for mixed cell birth studies. Epigenetics 2016;11:354-62.
41. Gervin K, Page CM, Aass HC, et al. Cell type specific DNA methylation in cord blood: a 450K-reference data set and cell count-based validation of estimated cell type composition. Epigenetics 2016:0.
42. Feng L, Wang J, Cao B, et al. Gene expression profiling in human lung development: an abundant resource for lung adenocarcinoma prognosis. PloS one 2014;9:e105639.
43. Lehne B, Drong AW, Loh M, et al. A coherent approach for analysis of the Illumina HumanMethylation450 BeadChip improves data quality and performance in epigenome-wide association studies (vol 16, 37, 2015). Genome Biol 2016;17.

SUPPLEMENTARY MATERIAL

S-Text

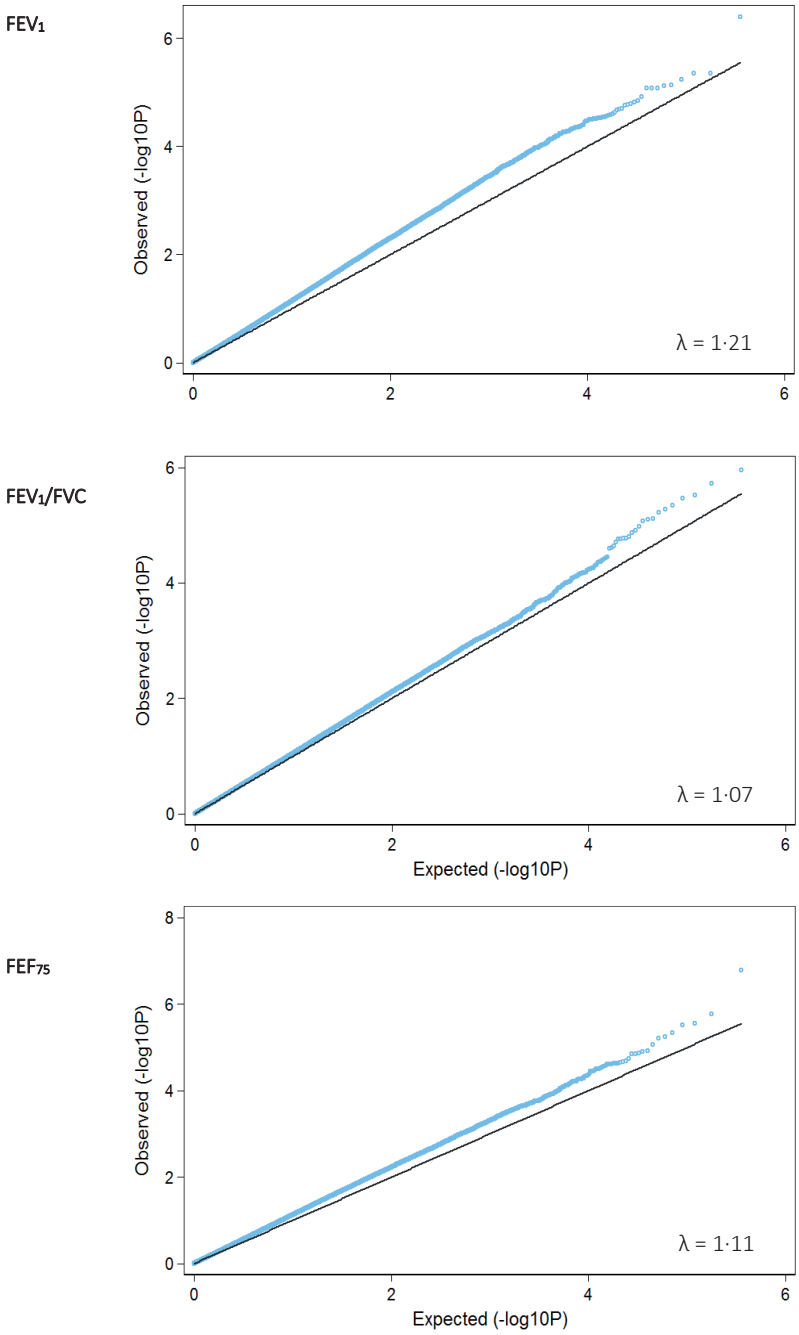
Statistical Analyses

Primary Meta-analysis on Childhood Lung Function

All cohorts performed spirometry according to the American Thoracic Society / European Respiratory Society recommendations.[1] The cohort-specific statistical models were run independently. For each cohort, robust linear regression models were used to evaluate the associations of cord blood DNA-methylation for each CpG-site with FEV1, FEV1/FVC and FEF75. All cohorts excluded DNA-methylation data outside the range ([25th percentile – 3* interquartile range) and (75th percentile + 3* interquartile range]). For the meta-analyses, we excluded probes that were missing in ≥3 cohorts (n=12,960), probes that mapped to X- or Y- chromosomes (n=11,448) and control probes (n = 65). A total of 457,748 CpGs were used for meta-analyses. First, models were adjusted for batch effects only. Second, models were additionally adjusted for maternal age, socio-economic status, smoking during pregnancy, parity, and asthma or atopy, and estimated cell type proportions using the Houseman method.[2] Results did not materially differ without adjustment for estimated cell type proportions. Inverse variance-weighted fixed-effects meta-analysis was completed using *METAL*.[3] The median I^2 value for all CpGs located in differentially methylated regions (DMRs) associated with FEV1 was 4 .9, with FEV1/FVC 0.0, and with FEF75 2 .4, indicating a strong homogeneity of the results across the cohorts. The genomic inflation factors (λ) for the associations with childhood FEV1, FEV1/FVC and FEF75 were 1 .21, 1 .07 and 1 .11, respectively (S-Figure 4.4.1).

The rationale to identify associations of DMRs instead of single CpGs is threefold. First, the vast majority of DMRs reported in the literature fall within a size range of a few hundred to a few thousand bases. This range coincides with typical sizes of gene-regulatory regions, and it is widely believed that DMRs can control cell-type-specific transcription of an associated gene. Second, while differences at any individual site may be small, if they are consistent across a region, statistical power to detect them may be greater. Third, the use of DMRs instead of single CpGs minimizes the effects of genetic variants in the methylation analysis. This is driven by the applied Stouffer-Liptak-Kechris (SLK)-correction, in which each *p*-value is adjusted according to adjacent *p*-values as weighted according to the correlation with neighboring CpGs. A given *p*-value will be pulled down if its neighbors also have low *p*-values (and little auto-correlation) and likely remain insignificant if the neighboring *p*-values are also high.

Using *p*-values obtained from the meta-analyses we identified DMRs with the software-tool Comb-p, using a window size of 500 bases and FDR-threshold <0 .05 with a minimum



S-Figure 4.4.1. QQ-plot of Associations of CpGs with Childhood FEV_1, FEV_1/FVC and FEF_{75}, respectively. QQ-plot of the associations of DNA-methylation of CpGs at birth with childhood FEV_1, FEV_1/FVC and FEF_{75}, respectively, adjusted for covariates and estimated cell counts. The–log10(p-values) are plotted by expected –log10(p-values). Λ indicates the genomic inflation factor (lambda) for the model.

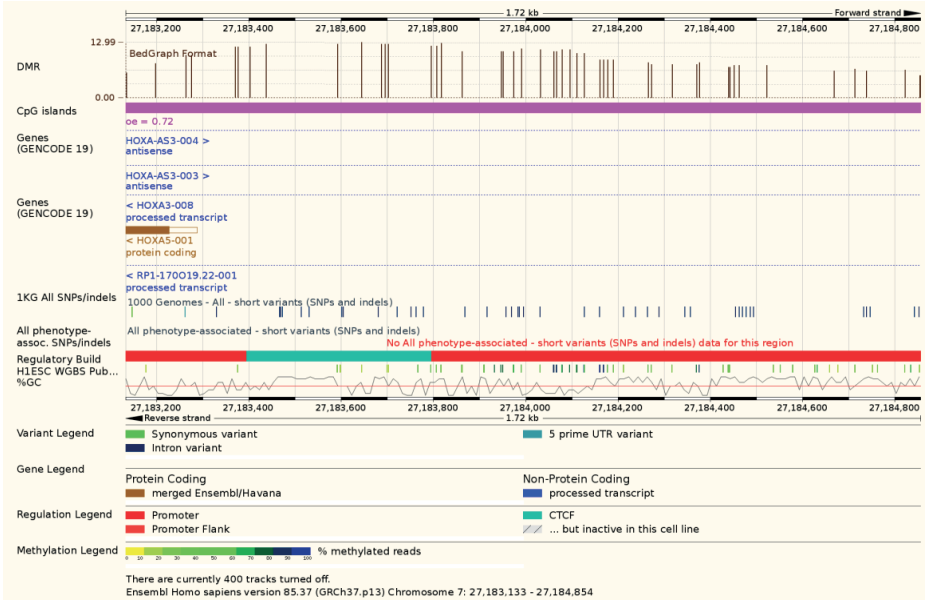
of 2 probes to start a region.[4] The window size of 500 bases was based on the strong correlation of CpGs within this distance, whereas at distances larger than 1,000 bases no correlation between CpG methylation and spatial distance is detectable.[5] Comb-p uses unadjusted p-values for each probe as input, and calculates adjusted p-values for each probe that accounts for the correlation with nearby CpGs (SLK-correction).[5] Next, the SLK p-values are adjusted for multiple testing and adjusted into q-values using the false-discovery rate (FDR) procedure of Benjamini-Hochberg. Comb-p finds DMRs based on the q-values and calculates p-values for these DMRs based on Stouffer-Liptak correction of the original p-values. Finally, the DMR p-values are adjusted for multiple testing using the Šidák-correction based on the size of the region and number of possible regions of that size. For a given region, the number of possible tests in the Šidák-correction is the total bases covered by all input probes divided by the size of the given region, such that larger regions undergo less stringent correction because there are fewer possible large regions.

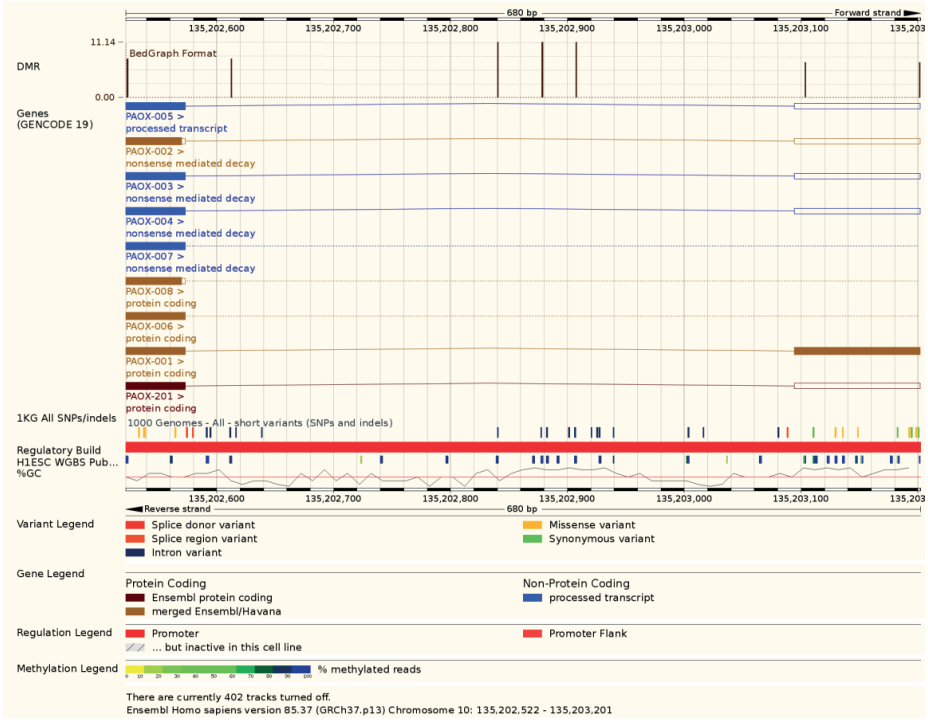
Annotation of the genes located nearest to the DMRs was performed using Peak Annotation and Visualization (PAVIS).[6] We limited annotation to a region of 500 kb (250kb upstream, 250kb downstream). All annotations were based on human GRCh37/hg19 assembly. Because genetic variants in Infinium probes could result in spurious methylation measurements, we performed a sensitivity analysis in a subset of high-quality probes (n=294,834) without SNPs, insertions or deletions, repeats and bisulfite induced reduced genomic complexity.[7]

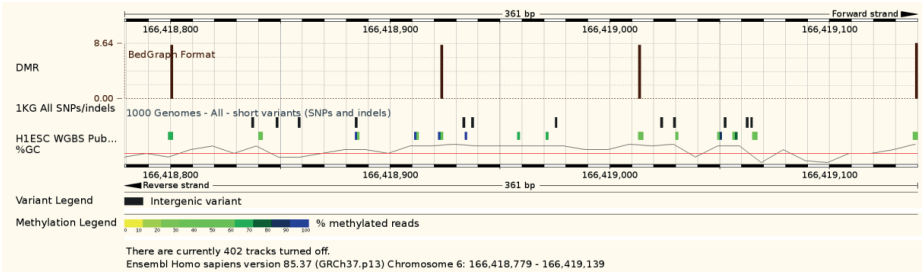
Secondary Analyses on Later Life Lung Function and Respiratory Diseases The associations of all CpGs present in the DMRs with lung function were replicated at older age in ALSPAC (DNA-methylation in cord blood) and the Rotterdam Study (DNA-methylation in peripheral blood samples). The statistical models in ALSPAC were adjusted for similar confounders used in the discovery analyses. The statistical models in the Rotterdam Study were adjusted for participants current age, sex, social class (low, middle, high), smoking status (never, ever), technical covariates and estimated cell counts.[2] We combined the p-values of the individual associations into the previous identified DMRs using Comb-p, applying correlation-correction based on the discovery meta-analysis using identical parameter settings. We considered SL p-values <0 .05 significant. We used logistic regression models to examine the associations of CpGs within identified DMRs with ever physician-diagnosed asthma in childhood. The statistical model was adjusted for maternal age, smoking during pregnancy, maternal asthma or atopy, gender, batch effects and estimated cell counts.[2] We did not additionally adjust for the correlation between asthma and lung function in children in Generation R, because most school-aged children have an FEV_1 >80% predicted, independent of their asthma severity when defined on the basis of symptoms.[8] In childhood, FEV_1 may not be sensitive and may have a different physiological meaning as compared with older ages.[9] We used linear

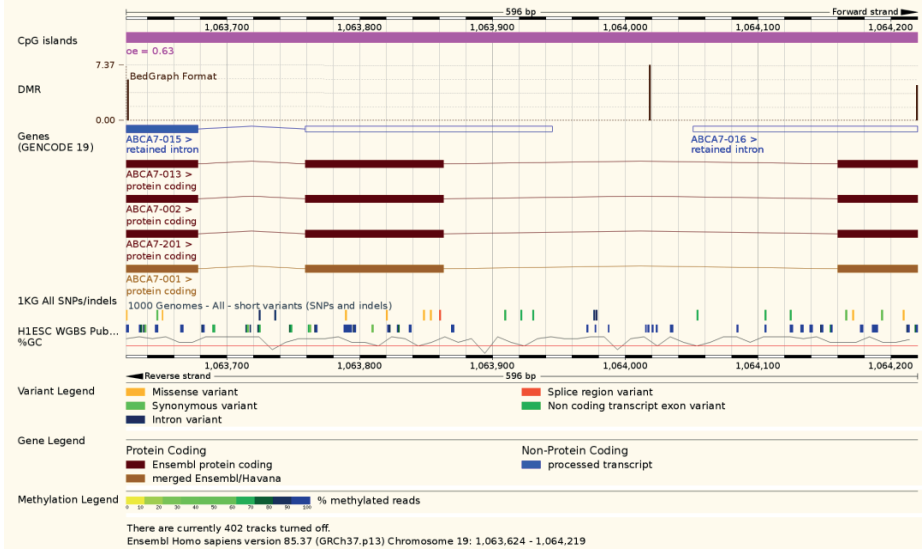
regression models to examine the associations of CpGs within identified DMRs with FEV_1, FEV_1/FVC and FEF_{75} in adolescence. Statistical models were adjusted for maternal age, socio-economic status, smoking during pregnancy, parity, asthma or atopy, batch effects and estimated cell counts. Last, we used linear and logistic regression models to examine the associations of CpGs within identified DMRs with FEV_1, FEV_1/FVC and FEF_{75} and COPD in adulthood. The statistical models were adjusted for participants age, sex, social class (low, middle, high), smoking status (never, ever), technical covariates and estimated cell counts.[2]

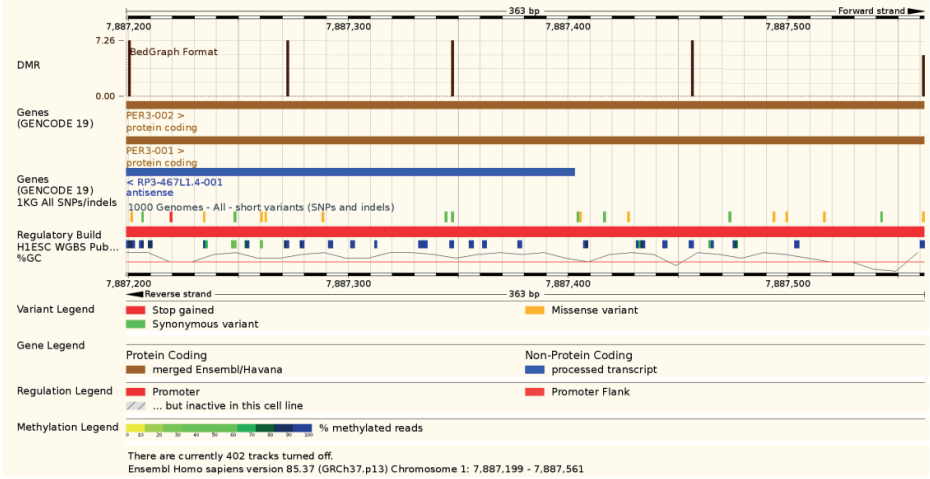
Gene Expression Analyses We examined the associations of methylation of individual CpGs with quantitative levels of gene expression for all significant CpGs located in the DMRs. We used 450K DNA-methylation data in cord blood and at age 4 years, and gene expression data at age 4 years obtained from whole blood from 107 individuals in INMA. Furthermore, we used gene expression and 450K methylation data both from white blood cells from 730 adults aged >45 years in the Rotterdam Study. Individual associations of CpGs with expression levels of all genes within a distance of 250 kb upstream and downstream (total region 500 kb) were assessed. Since correlations between CpGs for gene expression might differ from the correlations between CpGs for lung function parameters, we could not use Comb-p to reconstruct DMRs. Therefore, we combined the p-values of the individual associations between CpGs and gene expression into DMRs using a modified generalized Fisher method with Lancaster procedure, after which we applied multiple testing correction using the Benjamini-Hochberg procedure.[10] Last, we assessed whether expressed genes were also expressed in 278 human adult lung tissue specimen using the Genotype-Tissue Expression (GTEx) Version 6 database.[11]

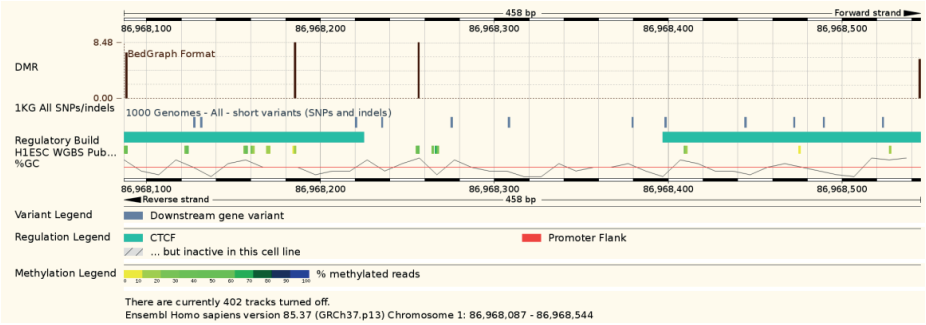

1.72 kb
Forward strand
27,183,200
27,183,400
27,183,600
27,183,800
27,184,000
27,184,200
27,184,400
27,184,600
27,184,800
12.99
DMR
BedGraph Format
0.00
CpG islands
oe = 0.72
Genes (GENCODE 19)
HOXA-AS3-004 > antisense
HOXA-AS3-003 > antisense
Genes (GENCODE 19)
< HOXA3-008 processed transcript
< HOXA5-001 protein coding
< RP1-170O19.22-001 processed transcript
1KG All SNPs/indels
1000 Genomes - All - short variants (SNPs and indels)
All phenotype-assoc. SNPs/indels
All phenotype-associated - short variants (SNPs and indels)
No All phenotype-associated - short variants (SNPs and indels) data for this region
Regulatory Build H1ESC WGBS Pub... %GC
Reverse strand
Variant Legend
Synonymous variant
Intron variant
5 prime UTR variant
Gene Legend
Protein Coding
merged Ensembl/Havana
Non-Protein Coding
processed transcript
Regulation Legend
Promoter
Promoter Flank
CTCF
... but inactive in this cell line
Methylation Legend
% methylated reads
There are currently 400 tracks turned off.
Ensembl Homo sapiens version 85.37 (GRCh37.p13) Chromosome 7: 27,183,133 - 27,184,854

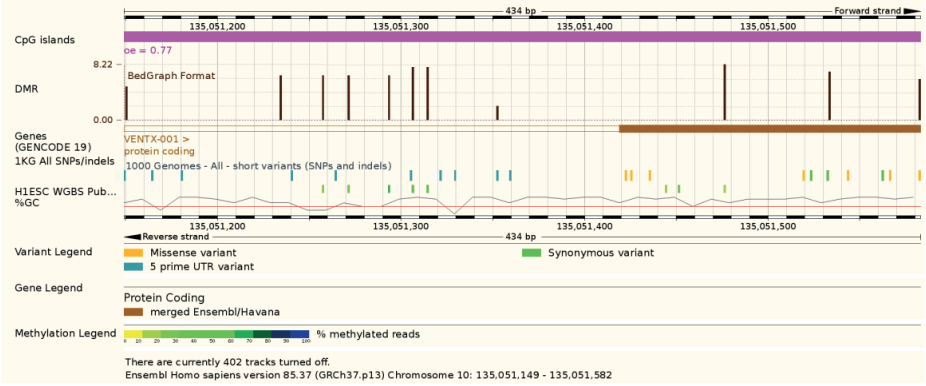

680 bp
Forward strand
135,202,600
135,202,700
135,202,800
135,202,900
135,203,000
135,203,100
135,203
11.14
DMR
BedGraph Format
0.00
Genes (GENCODE 19)
PAOX-005 > processed transcript
PAOX-002 > nonsense mediated decay
PAOX-003 > nonsense mediated decay
PAOX-004 > nonsense mediated decay
PAOX-007 > nonsense mediated decay
PAOX-008 > protein coding
PAOX-006 > protein coding
PAOX-001 > protein coding
PAOX-201 > protein coding
1KG All SNPs/indels
1000 Genomes - All - short variants (SNPs and indels)
Regulatory Build H1ESC WGBS Pub... %GC
Reverse strand
Variant Legend
Splice donor variant
Splice region variant
Intron variant
Missense variant
Synonymous variant
Gene Legend
Protein Coding
Ensembl protein coding
merged Ensembl/Havana
Non-Protein Coding
processed transcript
Regulation Legend
Promoter
... but inactive in this cell line
Promoter Flank
Methylation Legend
% methylated reads
There are currently 402 tracks turned off.
Ensembl Homo sapiens version 85.37 (GRCh37.p13) Chromosome 10: 135,202,522 - 135,203,201

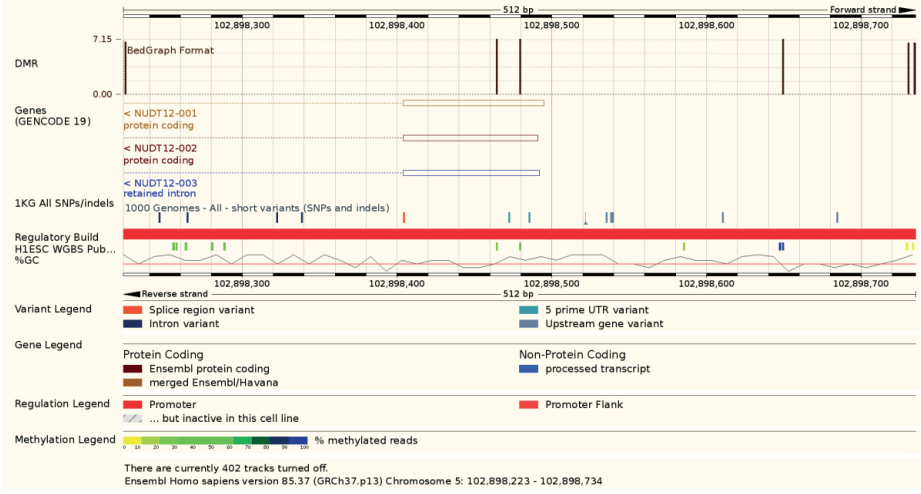

361 bp
Forward strand
166,418,800
166,418,900
166,419,000
166,419,100
8.64
DMR
BedGraph Format
0.00
1KG All SNPs/indels
1000 Genomes - All - short variants (SNPs and indels)
H1ESC WGBS Pub...
%GC
Reverse strand
Variant Legend
Intergenic variant
Methylation Legend
% methylated reads
There are currently 402 tracks turned off.
Ensembl Homo sapiens version 85.37 (GRCh37.p13) Chromosome 6: 166,418,779 - 166,419,139

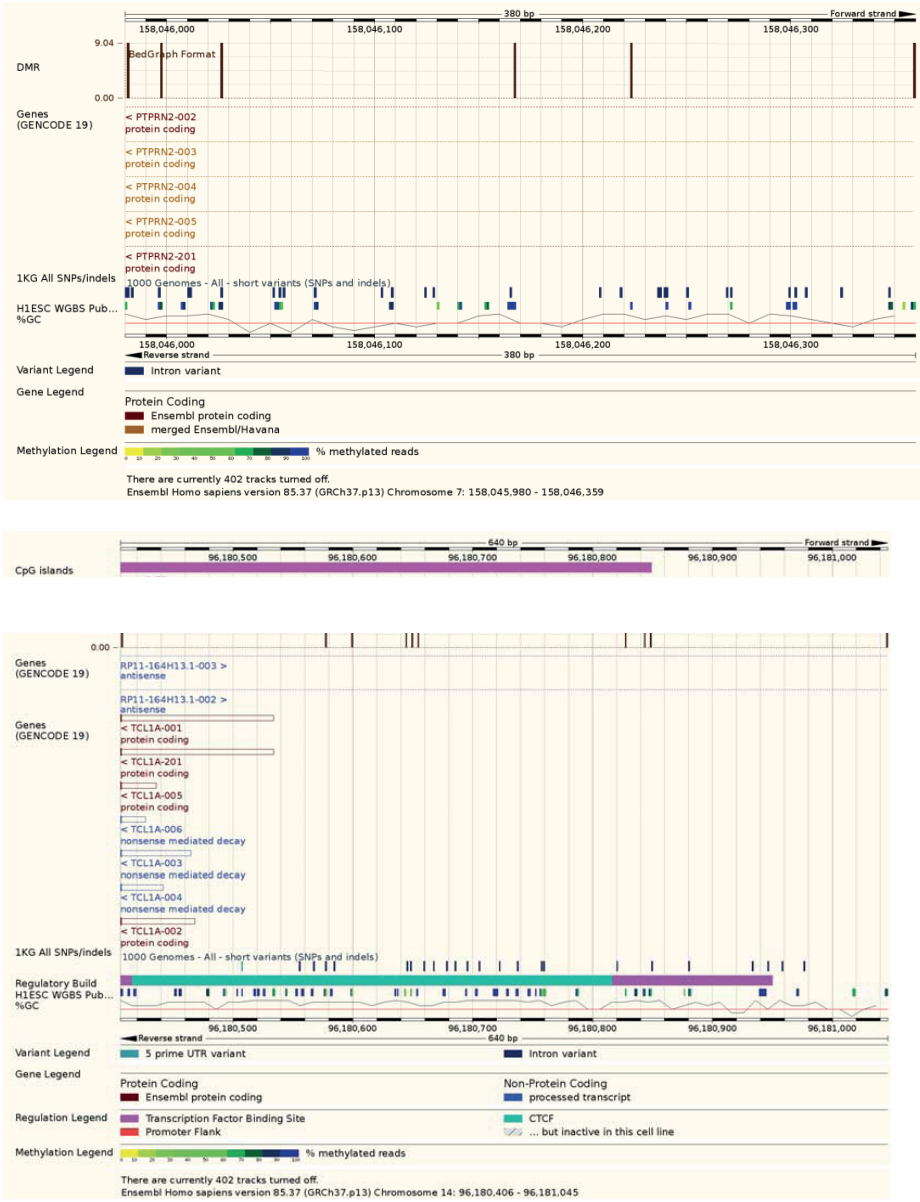

596 bp
Forward strand
1,063,700
1,063,800
1,063,900
1,064,000
1,064,100
1,064,200
CpG islands
oe = 0.63
7.37
DMR
BedGraph Format
0.00
Genes
(GENCODE 19)
ABCA7-015 >
retained intron
ABCA7-016 >
retained intron
ABCA7-013 >
protein coding
ABCA7-002 >
protein coding
ABCA7-201 >
protein coding
ABCA7-001 >
protein coding
1KG All SNPs/indels
1000 Genomes - All - short variants (SNPs and indels)
H1ESC WGBS Pub...
%GC
Reverse strand
Variant Legend
Missense variant
Synonymous variant
Intron variant
Splice region variant
Non coding transcript exon variant
Gene Legend
Protein Coding
Ensembl protein coding
merged Ensembl/Havana
Non-Protein Coding
processed transcript
Methylation Legend
% methylated reads
There are currently 402 tracks turned off.
Ensembl Homo sapiens version 85.37 (GRCh37.p13) Chromosome 19: 1,063,624 - 1,064,219


363 bp
Forward strand
7,887,200
7,887,300
7,887,400
7,887,500
7.26
DMR
BedGraph Format
0.00
Genes
(GENCODE 19)
PER3-002 >
protein coding
PER3-001 >
protein coding
Genes
(GENCODE 19)
1KG All SNPs/indels
< RP3-467L1.4-001
antisense
1000 Genomes - All - short variants (SNPs and indels)
Regulatory Build
H1ESC WGBS Pub...
%GC
Reverse strand
Variant Legend
Stop gained
Synonymous variant
Missense variant
Gene Legend
Protein Coding
merged Ensembl/Havana
Non-Protein Coding
processed transcript
Regulation Legend
Promoter
... but inactive in this cell line
Promoter Flank
Methylation Legend
% methylated reads
There are currently 402 tracks turned off.
Ensembl Homo sapiens version 85.37 (GRCh37.p13) Chromosome 1: 7,887,199 - 7,887,561


458 bp
Forward strand
86,968,100
86,968,200
86,968,300
86,968,400
86,968,500
8.48
DMR
BedGraph Format
0.00
1KG All SNPs/indels
1000 Genomes - All - short variants (SNPs and indels)
Regulatory Build
H1ESC WGBS Pub...
%GC
Reverse strand
Variant Legend
Downstream gene variant
Regulation Legend
CTCF
Promoter Flank
... but inactive in this cell line
Methylation Legend
% methylated reads
There are currently 402 tracks turned off.
Ensembl Homo sapiens version 85.37 (GRCh37.p13) Chromosome 1: 86,968,087 - 86,968,544


434 bp
Forward strand
135,051,200
135,051,300
135,051,400
135,051,500
CpG islands
oe = 0.77
8.22
DMR
BedGraph Format
0.00
Genes
(GENCODE 19)
VENTX-001 >
protein coding
1KG All SNPs/indels
1000 Genomes - All - short variants (SNPs and indels)
H1ESC WGBS Pub...
%GC
Reverse strand
Variant Legend
Missense variant
Synonymous variant
5 prime UTR variant
Gene Legend
Protein Coding
merged Ensembl/Havana
Methylation Legend
% methylated reads
There are currently 402 tracks turned off.
Ensembl Homo sapiens version 85.37 (GRCh37.p13) Chromosome 10: 135,051,149 - 135,051,582


512 bp
Forward strand
102,898,300
102,898,400
102,898,500
102,898,600
102,898,700
7.15
DMR
BedGraph Format
0.00
Genes
(GENCODE 19)
< NUDT12-001
protein coding
< NUDT12-002
protein coding
< NUDT12-003
retained intron
1KG All SNPs/indels
1000 Genomes - All - short variants (SNPs and indels)
Regulatory Build
H1ESC WGBS Pub...
%GC
Reverse strand
Variant Legend
Splice region variant
Intron variant
5 prime UTR variant
Upstream gene variant
Gene Legend
Protein Coding
Ensembl protein coding
merged Ensembl/Havana
Non-Protein Coding
processed transcript
Regulation Legend
Promoter
Promoter Flank
... but inactive in this cell line
Methylation Legend
% methylated reads
There are currently 402 tracks turned off.
Ensembl Homo sapiens version 85.37 (GRCh37.p13) Chromosome 5: 102,898,223 - 102,898,734



S-Figure 4.4.2. Visualization of the Underlying Genomic Structure of the Top 10 Identified Differentially Methylated Regions Associated With Childhood Lung Function Outcomes.

Data were visualized using the Encyclopedia of DNA Elements (ENCODE) Consortium embedded in the Ensembl Genome browser. Results present the underlying genomic structure of the top 10 identified differentially methylated regions (DMRs) with childhood lung function. $-Log_{10}$-transformed p-values of the CpGs located within the DMR are visualized. The presence of CpG Islands is shown, if applicable. Nearest genes and genomic variants (SNPs, Indels) are visualized. Regulatory elements are shown as defined in the specific figure legends. The DNA-methylation of a reference dataset of embryonic tissue (H1ESC WGBS) is shown.

S-Table 4.4.1. Unadjusted and Fully Adjusted Associations of Differentially Methylated Regions with Childhood Lung Function Outcomes.

				Unadjusted model		Full model	
	Chr	**Start**	**End**	**No. of probes**	**P-value**	**No. of probes**	**P-value**
FEV_1	1	1,957,620	1,957,712	2	0.99	4	0 .016
	1	7,887,199	7,887,456	4	0 .121	5	7 .27E-05
	1	17,222,405	17,222,716	-	-	2	0 .019
	1	38,461,540	38,461,897	-	-	4	0 .029
	1	45,452,166	45,452,606	7	3 .01E-04	7	6 .89E-04
	1	47,915,553	47,915,952	6	6 .61E-03	6	5 .50E-03
	3	195,489,708	195,490,310	8	1 .71E-04	8	7 .46E-05
	5	88,179,496	88,179,540	2	1	6	9 .96E-03
	6	114,181,482	114,181,799	3	0 .013	3	3 .59E-03
	6	166,418,799	166,419,139	4	2 .24E-05	4	4 .55E-06
	7	27,182,493	27,184,854	49	3 .48E-21	47	3 .05E-14
	7	76,828,885	76,829,169	-	-	2	0 .031
	7	130,131,480	130,132,162	-	-	18	1 .34E-03
	10	135,202,522	135,203,201	7	2 .19E-06	7	6 .65E-09
	11	843,897	844,285	-	-	6	0 .016
	12	120,763,487	120,763,870	5	1 .81E-03	5	1 .23E-03
	15	64,995,494	64,995,502	3	1	5	0 .025
	16	22,018,727	22,019,139	3	7 .19E-03	4	1 .42E-04
	16	88,803,803	88,804,052	4	7 .25E-03	4	4 .36E-03
	17	9,550,137	9,550,546	-	-	5	0 .015
	17	59,490,484	59,490,614	2	0 .072	8	2 .09E-03
	19	1,063,624	1,064,018	2	6 .21E-03	3	3 .83E-05
FEV_1/FVC	1	1,564,482	1,564,921	2	1 .17E-03	4	8 .51E-05
	1	86,968,087	86,968,256	3	4 .02E-03	4	3 .59E-06
	2	12,1223,534	121,223,965	-	-	6	1 .28E-04
	5	102,898,223	102,898,734	6	2 .56E-05	6	6 .95E-05
	6	32,305,068	32,305,146	-	-	3	2 .21E-03
	6	168,533,689	168,533,690	1	1	4	0 .017
	10	135,051,305	135,051,352	3	0 .99	11	4 .28E-05
	11	64,064,947	64,065,372	3	4 .23E-03	3	7 .71E-03
	11	67,417,958	67,418,406	-	-	13	9 .21E-03
	13	20,805,380	20,805,896	9	1 .02E-05	9	1 .22E-04
	14	96,180,406	96,181,045	-	-	10	1 .49E-03
	16	89,023,389	89,023,634	-	-	3	1 .45E-03
	17	19,314,299	19,314,619	-	-	6	3 .39E-04
	17	79,485,529	79,485,710	-	-	2	0 .029

S-Table 4.4.1. Unadjusted and Fully Adjusted Associations of Differentially Methylated Regions with Childhood Lung Function Outcomes. (continued)

				Unadjusted model		Full model	
	Chr	**Start**	**End**	**No. of probes**	**P-value**	**No. of probes**	**P-value**
	21	40,759,534	40,759,695	5	2 .29E-04	5	1 .66E-04
FEF_{75}	1	1,957,073	1,957,712	5	0 .012	5	7 .53E-03
	1	2,383,438	2,383,688	-	-	4	0 .013
	1	17,634,543	17,634,717	4	3 .24E-03	4	0 .016
	1	86,968,087	86,968,256	3	0 .075	3	0 .015
	2	14,774,786	14,774,903	-	-	3	0 .013
	2	54,086,854	54,087,518	6	0 .010	14	4 .08E-03
	3	62,860,103	62,860,225	2	3 .12E-03	2	0 .013
	5	125,930,870	125,931,363	8	7 .26E-04	7	7 .83E-03
	5	135,416,331	135,416,399	4	1	11	1 .34E-03
	5	138,725,400	138,725,831	5	8 .21E-03	5	6 .06E-04
	6	31,650,735	31,651,363	21	3 .61E-05	16	3 .45E-03
	6	31,690,998	31,691,540	-	-	10	0 .020
	6	132,271,361	132,271,589	-	-	3	5 .89E-04
	7	158,045,980	158,046,359	6	3 .29E-06	6	1 .14E-06
	8	142,377,303	142,377,768	6	3 .06E-03	5	4 .31E-03
	11	2,721,207	2,721,867	19	1 .98E-05	14	0 .029
	11	67,417,958	67,418,406	-	-	13	7 .07E-03
	12	114,843,583	114,844,266	7	3 .63E-04	3	0 .025
	14	96,180,406	96,181,045	10	8 .50E-07	10	2 .20E-06
	16	89,023,389	89,023,634	3	3 .00E-03	3	1 .55E-03
	17	44,897,512	44,897,513	1	1	2	3 .95E-04
	21	40,759,534	40,759,695	-	-	5	0 .024

Results present identified differentially methylated regions (DMRs) from association-analyses of DNA-methylation at birth with childhood Forced Expiratory Volume in 1 second (FEV_1), FEV_1/ Forced Vital Capacity (FVC) and Forced Expiratory Flow at 75% of FVC (FEF_{75}). Crude model: adjusted for batch effect. Full model: adjusted model, additionally adjusted for estimated cell counts. Start: Genetic location of the start of the DMR. End: Genetic location of the end of the DMR. No.: total number of CpGs located in the DMR. P-value: Šidák-corrected p-values.

S-Table 4.4.2. Characteristics of the Covariates of Cohorts.

	Maternal age in years mean (SD)	Maternal socio-economic status % (n)			Maternal smoking during pregnancy % (n)	Ever smoking % (n)	Maternal nulliparity % (n)	Maternal asthma or atopy % (n)
		Low	Middle	High				
Childhood								
ALSPAC (UK)	30 .3 (4 .2)	8 .3 (54)	43 .6 (285)	48 .2 (315)	11 .8 (77)	n.a.	48 .9 (320)	58 .7 (384)
Generation R (NL)	31 .6 (4 .2)	12 .4 (71)	49 .2 (282)	38 .4 (220)	19 .4 (111)	n.a.	63 .7 (365)	38 .7 (222)
INMA (Spain)	32 .1 (4 .1)	21 .4 (30)	30 .7 (43)	47 .9 (67)	24 .3 (34)	n.a.	51 .4 (72)	32 .1 (45)
Children's Health Study (CHS) (USA)	30 .7 (5 .4)	16 .0 (12)	37 .3 (28)	46 .7 (35)	8 .0 (6)	n.a.	41 .3 (31)	53 .3 (40)
Project Viva (USA)	33 .4 (4 .5)	20 .5 (36)[#]	n.a.[#]	79 .5 (140)[#]	6 .3 (11)	n.a.	47 .2 (83)	39 .8 (70)
Adolescents / adults								
ALSPAC (UK)	30 .2 (4 .2)	8 .9 (48)	43 .5 (236)	47 .6 (258)	11 .8 (64)	n.a.	50 .4 (269)	57 .2 (310)
Rotterdam Study – I (NL)*	n.a.	7 .6 (37)	59 .5 (290)	32 .9 (323)	n.a.	66 .2 (323)	n.a.	n.a.
Rotterdam Study – II (NL)*	n.a.	16 .2 (114)	58 .7 (412)	25 .1 (176)	n.a.	67 .7 (475)	n.a.	n.a.

[#]Project Viva: Maternal socio-economic status was defined as college graduate (no: lower educated; yes: higher educated). n.a.: not applicable.

S-Table 4.4.3. Associations of Differentially Methylated Regions (DMRs) with Childhood Lung Function Outcomes, Location of DMRs, and Direction of Association of DMRs with Childhood Lung Function Outcomes.

	Chromosome	Start	End	No. of probes	P-value	Nearest gene	Location	Distance to nearest transcription start site (bases)	Sequence location	Direction of association
FEV_1	1	1,957,385	1,957,806	4	0 .016	*GABRD*	S-Shore	6,828	Intron	↓
	1	7,887,199	7,887,561	5	7 .27E-05	*PER3*	Island	42,618	Exon	↓
	1	17,222,405	17,222,716	2	0 .019	*CROCC*	Island	-25,884	Upstream	↑
	1	38,461,540	38,461,897	4	0 .029	*FHL3*	Island	9,469	Downstream	↓
	1	45,452,166	45,452,606	7	6 .89E-04	*EIF2B3*	Island	8	5'UTR	↑
	1	47,915,553	47,915,952	6	5 .50E-03	*FOXD2*	Island	14,064	Downstream	↑
	3	195,489,708	195,490,310	8	7 .46E-05	*MUC4*	Island	48,835	Intron	↓
	5	88,179,235	88,179,540	5	9 .96E-03	*MEF2C*	Island	20,535	Intron	↑
	6	114,181,482	114,181,799	3	3 .59E-03	*MARCKS*	Island	3,114	Exon	↓
	6	166,418,799	166,419,139	4	4 .55E-06	*LINC00602*	N-Shelf	17,931	Downstream	↑
	7	27,183,133	27,184,854	47	3 .05E-14	*HOXA5*	Island	-706	Intron	↓
	7	76,828,885	76,829,169	2	0 .031	*CCDC146*	Open sea	77,094	Intron	↓
	7	130,131,480	130,132,162	18	1 .34E-03	*MEST*	Open sea	-808	Upstream	↓
	10	135,202,522	135,203,201	7	6 .65E-09	*PAOX*	Open sea	10,121	Intron	↑
	11	843,897	844,285	6	0 .016	*TSPAN4*	S-Shore	1,268	Intron	↑
	12	120,763,487	120,763,870	5	1 .23E-03	*PLA2G1B*	Island	1,914	Exon	↑
	15	64,995,133	64,995,502	5	0 .025	*OAZ2*	Island	145	5'UTR	↑
	16	22,018,727	22,019,338	4	1 .42E-04	*C16orf52*	Island	-423	Upstream	↑
	16	88,803,803	88,804,052	4	4 .36E-03	*PIEZO1*	Island	47,445	Intron	↓
	17	9,550,137	9,550,546	5	0 .015	*USP43*	N-Shore	1,488	Upstream	↑

S-Table 4.4.3. Associations of Differentially Methylated Regions (DMRs) with Childhood Lung Function Outcomes, Location of DMRs, and Direction of Association of DMRs with Childhood Lung Function Outcomes. (continued)

	Chromosome	Start	End	No. of probes	P-value	Nearest gene	Location	Distance to nearest transcription start site (bases)	Sequence location	Direction of association
	17	59,940,709	59,941,133	8	2 .09E-03	*BRIP1*	Island	-1	Upstream	↑
	19	1,063,624	1,064,219	3	3 .83E-05	*ABCA7*	Island	23,820	Intron	↓
FEV_1/FVC	1	1,564,422	1,564,921	4	8 .51E-05	*MIB2*	Island	13,877	Exon	↓
	1	86,968,087	86,968,544	4	3 .59E-06	*CLCA1*	Open sea	33,790	Downstream	↓
	2	121,223,534	121,223,965	6	1 .28E-04	*LOC84931*	Open sea	176	3'UTR	↑
	5	102,898,223	102,898,734	6	6 .95E-05	*NUDT12*	Open sea	12	5'UTR	↑
	6	32,305,068	32,305,146	3	2 .21E-03	*C6orf10*	Open sea	34,549	Intron	↓
	6	168,533,375	168,533,690	4	0 .017	*FRMD1*	S-Shelf	-53,693	Upstream	↑
	10	135,051,149	135,051,582	11	4 .28E-05	*VENTX*	Island	-42	Upstream	↑
	11	64,064,947	64,065,372	3	7 .71E-03	*KCNK4*	N-Shore	6,367	Intron	↑
	11	67,417,958	67,418,406	13	9 .21E-03	*ACY3*	Open sea	-52	Upstream	↓
	13	20,805,380	20,805,896	9	1 .22E-04	*GJB6*	N-Shore	896	Intron	↑
	14	96,180,406	96,181,045	10	1 .49E-03	*TCL1A*	Island	-192	Upstream	↑
	16	89,023,389	89,023,634	3	1 .45E-03	*CBFA2T3*	Open sea	19,993	Intron	↓
	17	19,314,299	19,314,619	6	3 .39E-04	*RNF112*	Open sea	-31	Upstream	↑
	17	79,485,529	79,485,710	2	0 .029	*ACTG1*	Island / N-Shore	-5,727	Upstream	↑
	21	40,759,534	40,759,695	5	1 .66E-04	*WRB*	N-Shore	7,402	Intron	↑
FEF_{75}	1	1,957,073	1,957,712	5	7 .53E-03	*GABRD*	Island	6,625	Intron	↓
	1	2,383,438	2,383,688	4	0 .013	*PLCH2*	N-Shelf	-24,190	Upstream	↑

S-Table 4.4.3. Associations of Differentially Methylated Regions (DMRs) with Childhood Lung Function Outcomes, Location of DMRs, and Direction of Association of DMRs with Childhood Lung Function Outcomes. (continued)

Chromosome	Start	End	No. of probes	P-value	Nearest gene	Location	Distance to nearest transcription start site (bases)	Sequence location	Direction of association
1	17,634,543	17,634,717	4	0 .016	*PADI4*	Open sea	-59	Upstream	↑
1	86,968,087	86,968,256	3	0 .015	*CLCA1*	Open sea	33,646	Downstream	↓
2	14,774,786	14,774,903	3	0 .013	*FAM84A*	Island	2,035	Exon	↑
2	54,086,854	54,087,553	14	4 .08E-03	*GPR75*	Island	-33	Upstream	↓
3	62,860,103	62,860,225	2	0 .013	*CADPS*	Island	900	Intron	↑
5	125,931,001	125,931,363	7	7 .83E-03	*ALDH7A1*	S-Shore	-100	Upstream	↑
5	135,416,029	135,416,614	11	1 .34E-03	*VTRNA2-1*	Island	-35	Upstream	↑
5	138,725,400	138,725,831	5	6 .06E-04	*MZB1*	N-Shore	-10	Upstream	↑
6	31,650,735	31,651,159	16	3 .45E-03	*LY6G5C*	Island	-2,797	Upstream	↑
6	31,690,998	31,691,540	10	0 .020	*C6orf25*	Island	149	Intron	↑
6	132,271,361	132,271,589	3	5 .89E-04	*CTGF*	Island	1,043	Exon	↑
7	158,045,980	158,046,359	6	1 .14E-06	*PTPRN2*	Open sea	334,313	Intron	↑
8	142,377,303	142,377,490	5	4 .31E-03	*GPR20*	Open sea	-31	Upstream	↑
11	2,721,207	2,721,633	14	0 .029	*KCNQ1OT1*	Island	-192	Upstream	↓
11	67,417,958	67,418,406	13	7 .07E-03	*ACY3*	Open sea	-52	Upstream	↓
12	114,843,919	114,844,266	3	0 .025	*TBX5*	N-Shore	2,155	Intron	↑
14	96,180,406	96,181,045	10	2 .20E-06	*TCL1A*	Island	-192	Upstream	↑
16	89,023,389	89,023,634	3	1 .55E-03	*CBFA2T3*	Open sea	19,993	Intron	↓
17	44,897,431	44,897,513	2	3 .95E-04	*WNT3*	S-Shore	-1,346	Upstream	↑
21	40,759,534	40,759,695	5	0 .024	*WRB*	N-Shore	7,402	Intron	↑

Results present identified differentially methylated regions (DMRs) from association-analyses of DNA-methylation at birth with Forced Expiratory Volume in 1 second (FEV_1), FEV_1/ Forced Vital Capacity (FVC) and Forced Expiratory Flow at 75% of FVC (FEF_{75}) observed in the discovery meta-analyses and shown in numeric order. Start: Genetic location of the start of the DMR. End: Genetic location of the end of the DMR. No. of probes: total number of CpGs located in the DMR. P-value: Šidák-corrected p-values. Nearest gene: gene located nearest to the DMR. Location: location of the DMR in the epigenome. N-shelf: North shelf. N-Shore: North shore. S-Shore: South shore. Direction of association: mean direction of beta-coefficient of associations of identified DMRs with lung function, marked ↓ if a higher mean methylation of the DMRs was associated with a lower z-score for lung function and ↑ if a higher mean methylation of the DMRs was associated with a higher z-score of lung function.

S-Table 4.4.4a/b/c. Associations of CpGs located in Differentially Methylated Regions with Childhood FEV_1, FEV_1/FVC and FEF_{75}.

This table is provided as a separate Microsoft Excel file, and is available upon request.

All CpGs located in differentially methylated regions (DMRs) associated with a) Forced Expiratory Volume in 1 second (FEV_1), b) FEV_1/Forced Vital Capacity (FVC), and c) Forced Expiratory Flow at 75% of FVC (FEF_{75}) observed in the discovery meta-analyses are shown, ordered by significance of the association. Chr: chromosome. Start: Genetic location of the start of the DMR. End: Genetic location of the end of the DMR. P-value: Šidák-corrected p-values. Effect: beta of the association between methylation of the specified CpG and FEV_1, FEV_1/FVC and FEF_{75}, respectively. StdErr: Standard Error of the beta of the association between methylation of the specified CpG and FEV_1 FEV_1/FVC and FEF_{75}, respectively. P.value: original p-value of the association between methylation of the specified CpG and FEV_1, FEV_1/FVC and FEF_{75}, respectively. Direction: direction of the effect of the association between methylation of the specified CpG and FEV_1 for ALSPAC, Generation R, INMA, CHS and Project Viva, respectively. +: positive effect estimate in the respective cohort, -: negative effect estimate in the respective cohort, ?: CpG not available for the respective cohort. HetIsq: I^2-statistic for heterogeneity between cohorts for the association between methylation of the specified CpG and FEV_1. HetPVal: p-value for the heterogeneity between cohorts for the association between methylation of the specified CpG and FEV_1, FEV_1/FVC and FEF_{75}, respectively. * CpG in which array probes contain SNPs, insertions, deletions, repeats or bisulfite induced reduced genomic complexity.[7]

S-Table 4.4.5. Associations of Differentially Methylated Regions Associated with Childhood Lung Function Outcomes in Quality Probes Only.

	Chromosome	Start	End	No. of probes	P-value
FEV_1	1	38,461,540	38,461,897	4	0 .018
	1	47,915,553	47,915,952	4	9 .90E-03
	3	195,489,708	195,490,095	5	2 .44E-03
	5	88,179,235	88,179,497	4	0 .023
	6	166,418,922	166,419,013	2	0 .030
	7	27,183,196	27,184,854	36	3 .73E-11
	7	130,131,826	130,131,932	8	0 .014
	10	135,202,522	135,203,201	4	5 .30E-07
	11	843,943	844,086	3	1 .62E-03
	12	120,763,487	120,763,870	5	7 .72E-04
	15	64,995,133	64,995,502	5	6 .49E-03
	16	22,019,138	22,019,338	2	1 .67E-03
	17	59,940,709	59,941,133	8	1 .37E-03
FEV_1/FVC	5	102,898,223	102,898,730	5	2 .14E-06
FEF_{75}	2	54,086,854	54,087,553	12	3 .14E-03
	3	62,860,103	62,860,225	2	7 .71E-03
	5	135,416,205	135,416,399	4	5 .28E-03
	5	138,725,400	138,725,831	5	3 .83E-04
	6	132,271,361	132,271,589	3	3 .92E-04
	11	2,721,480	2,721,867	7	7 .56E-06
	11	67,418,045	67,418,406	10	1 .71E-03
	14	96,505,869	96,505,875	2	3 .32E-04

Results present differentially methylated regions (DMRs) at birth associated with childhood Forced Expiratory Volume in 1 second (FEV_1), FEV_1/ Forced Vital Capacity (FVC) and Forced Expiratory Flow at 75% of FVC (FEF_{75}), excluding all probes potentially containing genomic variant. Start: Genetic location of the start of the differentially methylated region (DMRs). End: Genetic location of the end of the DMR. No. of probes: total number of CpGs located in the DMR. P-value: Šidák-corrected p-values.

S- 4.4.6. Associations of CpGs Located in Identified Differentially Methylated Regions Related with Childhood Asthma, Adolescent and Adult Lung Function, and Adult COPD.

This table is provided as a separate Microsoft Excel file, and is available upon request.

Results present CpGs located in identified differentially methylated regions (DMRs) at birth associated with childhood asthma, adolescent and adult lung function, and adult COPD. Start: Genetic location of the start of the differentially methylated region (DMR). End: Genetic location of the end of the DMR. CpG: CpG probes located in the DMR. Effect: effect estimate of the association of differential DNA-methylation if the CpG with the corresponding outcome. StdErr: standard error of the effect estimate. P.value: Stouffer-Liptak p-value from associations of CpGs with childhood asthma, adolescent and adult lung function and adult COPD. DMR: q-value derived from the DMRs identified with Comb-p. The p-values of neighboring CpGs in the discovery meta-analyses were adjusted for local correlation and adjusted for multiple testing using the False-Discovery-Rate correction. N.R.: Not reconstructed; some DMRs could not be reconstructed as the regions did not meet the criteria for identification (2 or more probes within 500 bases).

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood.

	Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
FEV_1	chr7: 27,183,133-27,184,854	*HOXA1*, HOTTIP*	↓	↓	↓	*Fetal lung development, FEV_1, FEV_1/FVC [12]
		EVX1, HOXA4**, HOXA7*	↓	↓		*COPD [13]; **Lung development [14], asthma [15], COPD[14]
		EVX1-AS, RP1-170O19.17	↑			
	chr10: 135,202,522-135,203,201	*SPRN*	↑	↑	↓	
		RP11-122K13.7	↓			
	chr1: 7,887,199-7,887,561	*PER3¶, RP3-467I1.4, RNA5SP23, RP4-726F1.1*	↓	↓		
		RNU6-1102P		↑		
		RP11-431K24.1			↑	
	chr3: 195,489,708-195,490,310	*MUC20**	↓	↓		*Ciliated epithelial lung cells [16]
		NCI_CGAP_Lu24			↓	
		*MIR570, SDHAP1, PPP1R2**	↑	↑		*Lung development[17]
	chr12: 120,763,487-120,763,870	*SIRT4*	↓			

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
	*LINC1089, TMEM142A**			↓	*Lung mast cell mediator release [18], bronchoconstriction [19]
	COQ5			↑	
chr16: 88,803,803-88,804,052	*APRT*	↓	↓		
	TRAPPC2L		↓		
	LOC100129697	↑			
chr1: 47,915,553-4,7915,952	*RP11-511I2.2*	↑			
chr1: 1,957,385-1,957,806	*MIR3120, RP5-1114G22.2, SNORA77, RP3-329E20.2, GAS5*, RP11-95P13.2*	↓	↓		* Asthma, COPD [20]
	CAMSAP2, RNU6-778P, TPR, RPS6KC1, RNU6-773P, RP3-467L1.4		↓		
	LOC100506801, IPO9-AS1, PADI4, CHI3L1, TMEM52, CDA, PPP1R11P1, PRDX6, RP11-15I11.2		↑		

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

	Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
		RNU6-307P, PM20D1, RP11-160H22.3, RP11-108M9.4, TNFSF4**, FMO3, LINC01036, LOC101929541*	↑			*Fetal lung development, FEV_1/FVC [12]; **asthma [21, 22]
	chr1: 38,461,540-38,461,897	*MIR3659, INPP5B*	↓	↓		
		YRDC	↑			
	chr11: 843,897–844,285	*AP2A2**		↑	↑	*COPD, chronic bronchitis [23]
FEV_1/FVC	chr10: 135,051,149-135,051,582	*RP11-122K13.12, TUBGCP2*	↓			
		TUBGCP2		↓		
		SPRN	↑		↓	
		VENTX¶, ECHS1	↑			
		ECHS1		↑		
		ZNF511			↑	
	chr5: 102,898,223-102,898,734	*NUDT12¶, CMBL*	↓			
		NUDT12		↓		
	chr2: 121,223,534-121,223,965	*AC012363.13*	↓	↓		

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
	INHBB, LINC01101, AC073257.2	↑	↑		
chr21: 40,759,534-40,759,695	*BRWD1-IT2,SH3BGR*	↓			
	BRWD1-IT2		↓		
	BRWD1,BRWD1-AS1,HMGN1, BRWD1-IT1*	↑			*Fetal lung development, FEV_1 [12]
	BRWD1-AS1		↑		
chr17: 19,314,299-19,314,619	*AC099684.1*	↓			
	SNORD3A, SNORD3C	↑			
chr14: 96,180,406-96,181,045	*CCDC85C,TCL1A¶*	↓			
	TCL1A¶		↓		
chr6: 32,305,068-32,305,146	*HCG23*, XXbac-BPG154L12.4, C6orf10¶, LOC285768, OFCC1*	↓			*Asthma [24], **adult asthma [25]
	*RNF5**			↓	*Cystic fibrosis [26]
	HLA-DRB4, HLA-DRB5***	↑			*Fetal lung development [12] ** FEV_1/FVC [12]
	HLA-DRB4		↑		
	TNXA			↑	

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

	Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
	chr11: 67,417,958-67,418,406	*NUDT8, CABP2*, CABP4, C11orf72, ACY3*[¶]	↓			*Asthma [27]
		NUDT8, CABP2		↓		
		AIP, NDUFV1, FAM86C2P, LOC729196	↑			
		AIP		↑		
	chr6: 168,533,375-168,533,690	*KIF25-AS1, DACT2, KIF25, FRMD1*[¶]	↑			
		KIF25-AS1, DACT2		↑		
	chr17: 79,485,529-79,485,710	*HGS*	↓			
		OXLD1	↑			
	chr13: 20,805,380 – 20,805,896	*CRYL1*			↓	
		MRPL57, MRP63			↑	
	chr11: 64,064,947 – 64,065,372	*PRDX5**			↓	*Lung development [28], COPD [29], FEV_1 and FEV_1/FVC [12, 30]
	chr1: 1,564,422 – 1,564,921	*LOC728661*			↑	
FEF_{75}	chr14: 96,180,406-96,181,045	*CCDC85C,TCL1A*[¶]	↓			
		TCL1A		↓		

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
chr6: 132,271,361-132,271,589	*RP11-69I8.2*	↑	↑		
chr16: 89,023,389-89,023,634	*APRT, TRAPPC2L*	↑	↑		
chr6: 31,650,735-31,651,159	*NEU1*, SNORA38, VWA7, LST1**	↓	↓		*FEV_1/FVC [12]
	CSNK2B, HSPA1L**,*			↓	*FEV_1 and FEV_1/FVC [12]; **FEV_1/FVC [12]
	ABHD16A, LSM2**, LY6G5C¶***,PRRC2A*****	↑	↑		*Steroid use in COPD [31]; ** FEV_1/FVC [12]; *** FEV_1 [12]; ****asthma [32]
	BAG6		↓		
	TSTD3		↑		
	C6orf25			↑	
chr2: 54,086,854-54,087,553	*ASB3*, PSME4*			↓	*Childhood asthma [33]
	ACYP2, ASB3	↑			
	ACYP2		↑		
chr8: 142,377,303-142,377,490	*RP11-10J21.3*	↓			
chr11: 67,417,958-67,418,406	*NUDT8, CABP2*, CABP4*, C11orf72**	↓			*Bronchopulmonary dysplasia [34]
	CABP2, NUDT8		↓		

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

	Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
		AIP, NDUFV1*, FAM86C2P, ACY3¶*	↑			Bronchopulmonary dysplasia [34]
		AIP		↑		
		CORO1B, TMEM134, TCIRG1***			↑	*Lung injury [35]; ** T-cell mediated immune response [36]
	chr1: 1,957,385-1,957,806	*MIR3120, RP5-1114G22.2, SNORA77, RP3-329E20.2, GAS5*, RP11-95P13.2*	↓			* Asthma, COPD [20]
		MIR3120, SNORA77, CAMSAP2, IL19, RP11-95P13.2, TGFB2, NBPF3, TPR		↓		
		RNU6-307P, LOC100506801, PTP4A1P7, LYPLAL1-AS1, IPO9-AS1, RNU6-773P, DDOST, RP11-15I11.2, ANGPTL1, C4BPA, CAPZB, CHI3L1, PPP1R11P1, PRDX6, CDA, CYB5R1, RNA5SP71		↑		

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

	Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
		RNU6-307P, PM20D1, RP11-160H22.3, RP11-108M9.4, TNFSF4**, FMO3, LINC01036, LOC101929541,*	↑			*Fetal lung development, FEV_1/FVC [12]; **asthma [21, 22]
	chr1: 2,383,438-2,383,688	*GJC2, LGALS8-AS1, OR2T27, MIR3115, C1QB, GALE, LOC100288175, LYPLAL1-AS1, C1orf35, MORN1, HMGCL, PGBD2, IBA57, ADCK3, CHRM3*, LINC01341, LEFTY1, OR2M3, PEX10, GNG4, RNA5SP78, EPHA8, CAPN9, LOC100129534, LOC339529*	↓			*FEV_1/FVC [37]
		GJC2, LGALS8-AS1, GALE, MIR3115, C1orf35, RP11-275O4.3, RP11-527D7.1, MIR4684, C1QB, ADCK3, CHRM3, MORN1, RNA5SP78, MARC1, HMGCL, LEFTY1, OR2T27, OR2M3		↓		

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

	Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
		MIR320B2, LINC01347, NVL, KDM1A, RP11-488L18.8, OR6F1, OR2L8, ZNF593		↑		
		RER1			↓	
		NVL, KDM1A, MIR320B2, LINC01347, CLIC4, LOC100287497, OR2L8, MIR4742, LINC01061*	↑			*FEV_1 [12]
	chr1: 17,634,543-17,634,717	*PTP4A1P7*	↑			
		ANGPTL1, RABGAP1L*	↓			*Lung development [38]
	chr6: 31,690,998-31,691,540	*TSTD3*	↑			
	chr21: 40,759,534-40,759,695	*BRWD1-IT2,HMGN1, SH3BGR*	↓			
		BRWD1-IT2		↓		
		BRWD1, BRWD1-AS1,BRWD1-IT1*	↑			*FEV_1 [12]
		BRWD1-AS1		↑		
	chr11: 2,721,207-2,721,633	*KCNQ1DN*	↑	↑	↑	
		KIF18A		↑		

S-Table 4.4.7. Associations of Identified Differentially Methylated Regions with Gene Expression in Childhood and Adulthood. (continued)

	Molecular location of the differentially methylated region (Chromosome: start – end)	Expressed gene	DMRs in cord blood and gene expression in children	DMRs in peripheral blood and gene expression in children	DMRs in peripheral blood and gene expression in adults	Previously associated with lung development or respiratory morbidity*
		NAP1L4			↓	
	chr5: 125,931,001 – 125,931,363	*RNUXA*			↓	
	chr17: 44,897,431 – 44,897,513	*ABI3*			↓	

Results present identified differentially methylated regions (DMRs) at birth associated with gene expression. * Association of expressed genes with lung development and respiratory morbidity were explored in previous published studies the OMIM database and UniProt. Gene expressions levels were assessed using the Illumina HumanHT12v4 Expression Beadchip limited to 250kb up- and downstream of the outer border of the DMR. The direction of associations of identified DMRs are marked ↓ if a higher mean methylation of the DMRs was associated with a lower expression of the gene, and ↑ if a higher mean methylation of the DMRs was associated with a higher expression of the gene.

S-Table 4.4.8. Expression of Genes Related to Identified Differentially Methylated Regions in Adult Lung Tissue.

	Molecular location of the differentially methylated region (Chromosome: start – end)	**Gene**
FEV_1	chr1: 1,957,385 - 1,957,806	*PADI4, CHI3L1, CDA, PM20D1, RP11-108M9.4, FMO3*
	chr1:17,222,405-17,222,716	*CROCC**
	chr1:38,461,540-38,461,897	*FHL3**, *INPP5B, YRDC*
	chr3:195,489,708-195,490,310	*MUC4**, *MUC20, MIR570, SDHAP1*
	chr7:76,828,885-76,829,169	*CCDC146**
	chr10: 135,202,522 - 135,203,201	*PAOX**
	chr11:843,897-844,285	*TSPAN4**
	chr12: 120,763,487 - 120,763,870	*SIRT4, COQ5*
	chr15:64,995,133-64,995,502	*OAZ2**
	chr16: 88,803,803 - 88,804,052	*APRT, TRAPPC2L*
	chr19:1,063,624-1,064,219	*ABCA7**
FEV_1/FVC	chr1:86,968,087-86,968,544	*CLCA1**
	chr5:102,898,223-102,898,734	*NUDT12**, *CMBL*
	chr6: 32,305,068 - 32,305,146	*HCG23, HLA-DRB5*
	chr6: 168,533,375 - 168,533,690	*KIF25-AS1, KIF25*
	chr10: 135,051,149 - 135,051,582	*VENTX**, *RP11-122K13.12, ECHS1*
	chr11: 64,064,947 – 64,065,372	*PRDX5*
	chr11: 67,417,958 - 67,418,406	*NUDT8, NDUFV1*
	chr17: 79,485,529 - 79,485,710	*OXLD1*
	chr21:40,759,534-40,759,695	*WRB**, *SH3BGR*
FEF_{75}	chr1: 1,957,385 - 1,957,806	*NBPF3, C4BPA, CAPZB, CHI3L1, CDA, PM20D1, RP11-108M9.4, FMO3*
	chr1: 2,383,438 - 2,383,688	*PLCH2**, *MORN1, IBA57, ADCK3, LEFTY1, CAPN9, NVL, ZNF593*
	chr1: 17,634,543 - 17,634,717	*PADI4**, *RABGAP1L*
	chr2: 54,086,854 - 54,087,553	*GPR75**, *PSME4, ACYP2*
	chr5:125,931,001-125,931,363	*ALDH7A1**
	chr6: 31,650,735 - 31,651,159	*LY6G5C**, *LY6G5C, PRRC2A, BAG6*
	chr17:44,897,431-44,897,513	*WNT3**
	chr21: 40,759,534 - 40,759,695	*WRB**, *SH3BGR*

Results present the expression of genes related to identified differentially methylated regions (DMRs) in adult lung tissue in the Genotype-Tissue Expression (GTEx) database. All annotated and differentially expressed genes were assessed. Molecular locations are based on human GRCh37/hg19 assembly. *Nearest gene to DMR.

S-Table 4.4.9a. Biological Processes Enriched by Genes Related with Identified Differentially Methylated Regions Associated with Childhood FEV_1.

Category	Term	Count	%	Genes	Fold enrichment	Fisher Exact
GOTERM_BP_FAT	GO:0003002~regionalization	5	12.8	*HOXA1; HOXA4; EVX1; HOXA5; HOXA7*	12.7	3.90E-05
GOTERM_BP_FAT	GO:0006355~regulation of transcription; DNA-dependent	12	30.8	*MEF2C; HOXA1; TNFSF4; HOXA4; EVX1; HOXA5; HOXA7; SIRT4; PLA2G1B; BRIP1; PER3; FOXD2*	3.4	6.40E-05
GOTERM_BP_FAT	GO:0051252~regulation of RNA metabolic process	12	30.8	*MEF2C; HOXA1; TNFSF4; HOXA4; EVX1; HOXA5; HOXA7; SIRT4; PLA2G1B; BRIP1; PER3; FOXD2*	3.3	8.00E-05
GOTERM_BP_FAT	GO:0048562~embryonic organ morphogenesis	4	10.3	*HOXA1; HOXA4; HOXA5; HOXA7*	15.1	1.30E-04
GOTERM_BP_FAT	GO:0032583~regulation of gene-specific transcription	4	10.3	*MEF2C; TNFSF4; EVX1; PLA2G1B*	15.0	1.40E-04
GOTERM_MF_FAT	GO:0043565~sequence-specific DNA binding	7	17.9	*MEF2C; HOXA1; HOXA4; EVX1; HOXA5; HOXA7; FOXD2*	5.8	1.40E-04
GOTERM_BP_FAT	GO:0007389~pattern specification process	5	12.8	*HOXA1; HOXA4; EVX1; HOXA5; HOXA7*	9.4	1.60E-04
GOTERM_BP_FAT	GO:0009952~anterior/posterior pattern formation	4	10.3	*HOXA1; HOXA4; HOXA5; HOXA7*	14.3	1.60E-04
GOTERM_BP_FAT	GO:0048704~embryonic skeletal system morphogenesis	3	7.7	*HOXA4; HOXA5; HOXA7*	26.4	1.90E-04
GOTERM_BP_FAT	GO:0048568~embryonic organ development	4	10.3	*HOXA1; HOXA4; HOXA5; HOXA7*	11.7	3.50E-04
GOTERM_BP_FAT	GO:0048706~embryonic skeletal system development	3	7.7	*HOXA4; HOXA5; HOXA7*	19.5	4.70E-04
GOTERM_BP_FAT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	6.8	7.40E-04

S-Table 4.4.9a. Biological Processes Enriched by Genes Related with Identified Differentially Methylated Regions Associated with Childhood FEV_1. (continued)

Category	Term	Count	%	Genes	Fold enrichment	Fisher Exact
GOTERM_BP_FAT	GO:0010551~regulation of specific transcription from RNA polymerase II promoter	3	7.7	*MEF2C; EVX1; PLA2G1B*	16.0	8.40E-04
GOTERM_BP_FAT	GO:0048705~skeletal system morphogenesis	3	7.7	*HOXA4; HOXA5; HOXA7*	13.4	1.40E-03
GOTERM_BP_FAT	GO:0045893~positive regulation of transcription; DNA-dependent	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	5.3	2.30E-03
GOTERM_BP_FAT	GO:0051254~positive regulation of RNA metabolic process	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	5.2	2.30E-03
GOTERM_BP_FAT	GO:0045449~regulation of transcription	12	30.8	*MEF2C; HOXA1; TNFSF4; HOXA4; EVX1; HOXA5; HOXA7; SIRT4; PLA2G1B; BRIP1; PER3; FOXD2*	2.3	2.40E-03
GOTERM_MF_FAT	GO:0003700~transcription factor activity	7	17.9	*MEF2C; HOXA1; HOXA4; EVX1; HOXA5; HOXA7; FOXD2*	3.6	2.40E-03
GOTERM_BP_FAT	GO:0006357~regulation of transcription from RNA polymerase II promoter	6	15.4	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B; BRIP1*	4.1	2.60E-03
GOTERM_BP_FAT	GO:0048598~embryonic morphogenesis	4	10.3	*HOXA1; HOXA4; HOXA5; HOXA7*	6.5	3.00E-03
GOTERM_BP_FAT	GO:0050714~positive regulation of protein secretion	2	5.1	*TNFSF4; PLA2G1B*	24.4	3.00E-03
GOTERM_BP_FAT	GO:0001816~cytokine production	2	5.1	*TNFSF4; PLA2G1B*	21.8	3.80E-03
GOTERM_MF_FAT	GO:0016763~transferase activity; transferring pentosyl groups	2	5.1	*SIRT4; APRT*	21.7	3.80E-03
GOTERM_BP_FAT	GO:0010558~negative regulation of macromolecule biosynthetic process	5	12.8	*MEF2C; TNFSF4; HOXA7; SIRT4; EIF2B3*	4.6	4.10E-03

S-Table 4.4.9a. Biological Processes Enriched by Genes Related with Identified Differentially Methylated Regions Associated with Childhood FEV_1. (continued)

Category	Term	Count	%	Genes	Fold enrichment	Fisher Exact
GOTERM_BP_FAT	GO:0009792~embryonic development ending in birth or egg hatching	4	10.3	*HOXA4; EVX1; HOXA5; HOXA7*	6.0	4.10E-03
GOTERM_BP_FAT	GO:0032582~negative regulation of gene-specific transcription	2	5.1	*MEF2C; TNFSF4*	20.9	4.10E-03
GOTERM_BP_FAT	GO:0031327~negative regulation of cellular biosynthetic process	5	12.8	*MEF2C; TNFSF4; HOXA7; SIRT4; EIF2B3*	4.5	4.60E-03
GOTERM_BP_FAT	GO:0045941~positive regulation of transcription	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	4.4	4.70E-03
GOTERM_BP_FAT	GO:0009890~negative regulation of biosynthetic process	5	12.8	*MEF2C; TNFSF4; HOXA7; SIRT4; EIF2B3*	4.4	5.00E-03
GOTERM_BP_FAT	GO:0045892~negative regulation of transcription; DNA-dependent	4	10.3	*MEF2C; TNFSF4; HOXA7; SIRT4*	5.6	5.10E-03
GOTERM_BP_FAT	GO:0010628~positive regulation of gene expression	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	4.3	5.30E-03
GOTERM_BP_FAT	GO:0051253~negative regulation of RNA metabolic process	4	10.3	*MEF2C; TNFSF4; HOXA7; SIRT4*	5.5	5.40E-03
GOTERM_BP_FAT	GO:0045935~positive regulation of nucleobase; nucleoside; nucleotide and nucleic acid metabolic process	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	4.0	7.10E-03
GOTERM_BP_FAT	GO:0051046~regulation of secretion	3	7.7	*TNFSF4; SIRT4; PLA2G1B*	7.4	7.40E-03
GOTERM_BP_FAT	GO:0051173~positive regulation of nitrogen compound metabolic process	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	3.9	8.10E-03
GOTERM_BP_FAT	GO:0010557~positive regulation of macromolecule biosynthetic process	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	3.8	8.70E-03

S-Table 4.4.9a. Biological Processes Enriched by Genes Related with Identified Differentially Methylated Regions Associated with Childhood FEV_1. (continued)

Category	Term	Count	%	Genes	Fold enrichment	Fisher Exact
GOTERM_BP_FAT	GO:0031328~positive regulation of cellular biosynthetic process	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	3.7	1.00E-02
GOTERM_BP_FAT	GO:0009891~positive regulation of biosynthetic process	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	3.6	1.10E-02
GOTERM_BP_FAT	GO:0016481~negative regulation of transcription	4	10.3	*MEF2C; TNFSF4; HOXA7; SIRT4*	4.4	1.20E-02
GOTERM_BP_FAT	GO:0060341~regulation of cellular localization	3	7.7	*TNFSF4; SIRT4; PLA2G1B*	6.1	1.30E-02
GOTERM_BP_FAT	GO:0010605~negative regulation of macromolecule metabolic process	5	12.8	*MEF2C; TNFSF4; HOXA7; SIRT4; EIF2B3*	3.4	1.40E-02
GOTERM_BP_FAT	GO:0010629~negative regulation of gene expression	4	10.3	*MEF2C; TNFSF4; HOXA7; SIRT4*	4.0	1.70E-02
GOTERM_BP_FAT	GO:0045934~negative regulation of nucleobase; nucleoside; nucleotide and nucleic acid metabolic process	4	10.3	*MEF2C; TNFSF4; HOXA7; SIRT4*	3.9	1.80E-02
GOTERM_BP_FAT	GO:0051172~negative regulation of nitrogen compound metabolic process	4	10.3	*MEF2C; TNFSF4; HOXA7; SIRT4*	3.9	1.90E-02
GOTERM_BP_FAT	GO:0010604~positive regulation of macromolecule metabolic process	5	12.8	*MEF2C; HOXA1; EVX1; HOXA7; PLA2G1B*	2.9	2.50E-02
GOTERM_MF_FAT	GO:0030528~transcription regulator activity	7	17.9	*MEF2C; HOXA1; HOXA4; EVX1; HOXA5; HOXA7; FOXD2*	2.3	2.60E-02

Results present biological pathways enriched for the identified genes. Pathways are based on the Gene Ontology database implemented in DAVID and GeneMania. MF-FAT: molecular function, CC-FAT: Cellular Component, BP-FAT: Biological Process. Count: number of genes enriching the respective Gene Ontology term. %: the % of the identified genes for all genes known for the respective Gene Ontology term. Fold Enrichment: the x-fold enrichment of the respective Gene Ontology term by identified genes. Fisher Exact: p-value for the enrichment of Gene Ontology terms by the identified genes.

S-Table 4.4.9b. Biological Processes Enriched by Genes Related with Identified Differentially Methylated Regions Associated with Childhood FEV_1/FVC.

Category	Term	Count	%	Genes	Fold enrichment	Fisher Exact
GOTERM_BP_FAT	GO:0002504~antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	2	5 .3	*HLA-DRB4; HLA-DRB5*	41 .0	1 .10E-03
GOTERM_CC_FAT	GO:0042613~MHC class II protein complex	2	5 .3	*HLA-DRB4; HLA-DRB5*	33 .9	1 .60E-03
GOTERM_MF_FAT	GO:0046872~metal ion binding	12	31 .6	*RNF112; CLCA1; NUDT12; CABP2; ACY3; MIB2; NDUFV1; NUDT8; HGS; CABP4; CBFA2T3; KCNK4*	1 .7	2 .30E-02
GOTERM_MF_FAT	GO:0043169~cation binding	12	31 .6	*RNF112; CLCA1; NUDT12; CABP2; ACY3; MIB2; NDUFV1; NUDT8; HGS; CABP4; CBFA2T3; KCNK4*	1 .7	2 .50E-02
GOTERM_MF_FAT	GO:0043167~ion binding	12	31 .6	*RNF112; CLCA1; NUDT12; CABP2; ACY3; MIB2; NDUFV1; NUDT8; HGS; CABP4; CBFA2T3; KCNK4*	1 .7	2 .80E-02

Results present biological pathways enriched for the identified genes. Pathways are based on the Gene Ontology database implemented in DAVID and GeneMania. MF-FAT: molecular function, CC-FAT: Cellular Component, BP-FAT: Biological Process. Count: number of genes enriching the respective Gene Ontology term. %: the % of the identified genes for all genes known for the respective Gene Ontology term. Fold Enrichment: the x-fold enrichment of the respective Gene Ontology term by identified genes. Fisher Exact: p-value for the enrichment of Gene Ontology terms by the identified genes.

S-Table 4.4.9c. Associations of Genes Related with Identified Differentially Methylated Regions Associated with Childhood FEV_1/FVC with Human Diseases.

Category	Term	Count	%	Genes	Fold enrichment	Fisher Exact
KEGG_PATHWAY	hsa05416:Viral myocarditis	3	7 .9	*ACTG1; HLA-DRB4; HLA-DRB5*	21 .5	2 .90E-04
KEGG_PATHWAY	hsa05310:Asthma	2	5 .3	*HLA-DRB4; HLA-DRB5*	35 .1	1 .40E-03
KEGG_PATHWAY	hsa05330:Allograft rejection	2	5 .3	*HLA-DRB4; HLA-DRB5*	28 .3	2 .10E-03
KEGG_PATHWAY	hsa05332:Graft-versus-host disease	2	5 .3	*HLA-DRB4; HLA-DRB5*	26 .1	2 .50E-03
KEGG_PATHWAY	hsa04940:Type I diabetes mellitus	2	5 .3	*HLA-DRB4; HLA-DRB5*	24 .2	2 .90E-03
KEGG_PATHWAY	hsa04672:Intestinal immune network for IgA production	2	5 .3	*HLA-DRB4; HLA-DRB5*	20 .8	3 .90E-03
KEGG_PATHWAY	hsa05320:Autoimmune thyroid disease	2	5 .3	*HLA-DRB4; HLA-DRB5*	19 .9	4 .20E-03

Results present the enrichment of KEGG-defined human diseases by the identified genes. Disease terms are based on the KEGG database implemented in DAVID and GeneMania. Count: number of genes enriching the respective Gene Ontology term. %: the % of the identified genes for all genes known for the respective Gene Ontology term. Fold Enrichment: the x-fold enrichment of the respective Gene Ontology term by identified genes. Fisher Exact: p-value for the enrichment of Gene Ontology terms by the identified genes.

S-Table 4.4.9d. Biological Processes Enriched by Genes Related with Identified Differentially Methylated Regions Associated with Childhood FEF_{75}.

Category	Term	Count	%	Genes	Fold enrichment	Fisher Exact
GOTERM_MF_FAT	GO:0050662~coenzyme binding	5	7 .4	*KDM1A; NDUFV1; FMO3; GALE; HMGCL*	8 .2	3 .50E-04
GOTERM_MF_FAT	GO:0048037~cofactor binding	5	7 .4	*KDM1A; NDUFV1; FMO3; GALE; HMGCL*	5 .9	1 .50E-03
GOTERM_MF_FAT	GO:0005254~chloride channel activity	3	4 .4	*GABRD; CLCA1; CLIC4*	12 .3	1 .80E-03
GOTERM_MF_FAT	GO:0031404~chloride ion binding	3	4 .4	*GABRD; CLCA1; CLIC4*	11 .5	2 .20E-03
GOTERM_MF_FAT	GO:0005253~anion channel activity	3	4 .4	*GABRD; CLCA1; CLIC4*	11 .3	2 .30E-03
GOTERM_MF_FAT	GO:0004435~phosphoinositide phospholipase C activity	2	2 .9	*CHRM3; PLCH2*	23 .6	3 .20E-03
GOTERM_MF_FAT	GO:0043168~anion binding	3	4 .4	*GABRD; CLCA1; CLIC4*	9 .6	3 .70E-03
GOTERM_MF_FAT	GO:0004629~phospholipase C activity	2	2 .9	*CHRM3; PLCH2*	19 .0	4 .90E-03
GOTERM_BP_FAT	GO:0032583~regulation of gene-specific transcription	3	4 .4	*KDM1A; TNFSF4; TBX5*	6 .9	9 .40E-03
GOTERM_BP_FAT	GO:0007166~cell surface receptor linked signal transduction	12	17 .6	*OR2L8; WNT3; CHRM3; OR2T27; EPHA8; CTGF; GPR75; ANGPTL1; GPR20; GNG4; OR2M3; LEFTY1*	2 .0	1 .30E-02
GOTERM_MF_FAT	GO:0008509~anion transmembrane transporter activity	3	4 .4	*GABRD; CLCA1; CLIC4*	6 .0	1 .30E-02
GOTERM_BP_FAT	GO:0007167~enzyme linked receptor protein signaling pathway	4	5 .9	*EPHA8; CTGF; ANGPTL1; LEFTY1*	3 .6	2 .50E-02

S-Table 4.4.9d. Biological Processes Enriched by Genes Related with Identified Differentially Methylated Regions Associated with Childhood FEF_{75}. (continued)

Category	Term	Count	%	Genes	Fold enrichment	Fisher Exact
GOTERM_CC_FAT	GO:0005886~plasma membrane	21	30 .9	*GABRD; OR2L8; TNFSF4; CLCA1; CABP2; GPR75; ACY3; PTPRN2; C6ORF25; GJC2; OR2M3; CADPS; CHRM3; OR2T27; EPHA8; CTGF; CLIC4; PLCH2; NEU1; GNG4; GPR20*	1 .5	2 .50E-02
GOTERM_MF_FAT	GO:0005509~calcium ion binding	7	10 .3	*CADPS; CLCA1; CABP2; PLCH2; CAPN9; CABP4; PADI4*	2 .2	3 .40E-02
GOTERM_CC_FAT	GO:0044459~plasma membrane part	13	19 .1	*GABRD; TNFSF4; CLCA1; CABP2; ACY3; GPR75; PTPRN2; GJC2; CADPS; CHRM3; EPHA8; GPR20; GNG4*	1 .6	5 .90E-02

Results present biological pathways enriched for the identified genes. Pathways are based on the Gene Ontology database implemented in DAVID and GeneMania. MF-FAT: molecular function, CC-FAT: Cellular Component, BP-FAT: Biological Process. Count: number of genes enriching the respective Gene Ontology term. %: the % of the identified genes for all genes known for the respective Gene Ontology term. Fold Enrichment: the x-fold enrichment of the respective Gene Ontology term by identified genes. Fisher Exact: p-value for the enrichment of Gene Ontology terms by the identified genes.

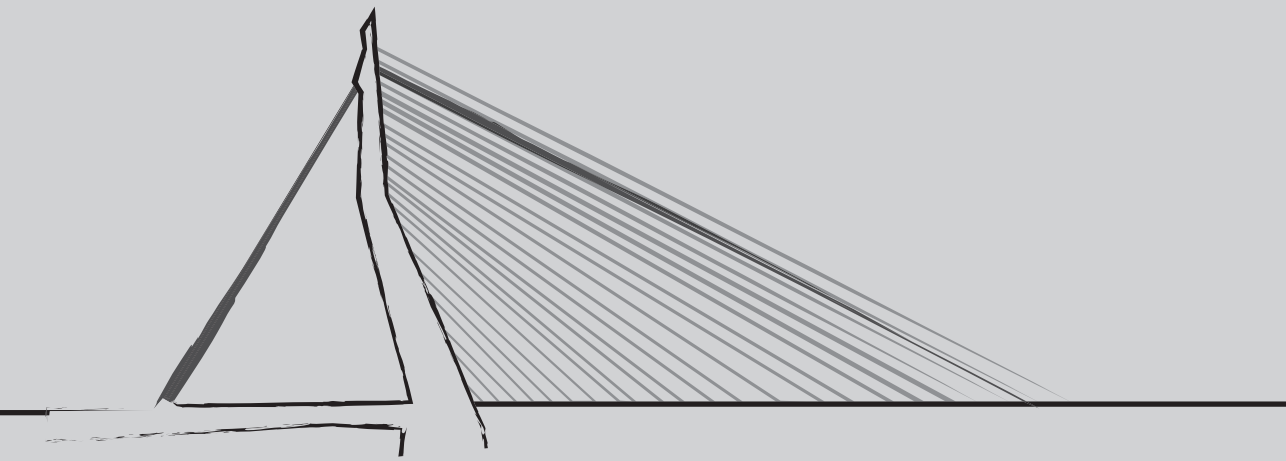
REFERENCES

1. Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. Eur Respir J 2005;26:319-38.
2. Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC bioinformatics 2012;13:86.
3. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics 2010;26:2190-1.
4. Pedersen BS, Schwartz DA, Yang IV, Kechris KJ. Comb-p: software for combining, analyzing, grouping and correcting spatially correlated P-values. Bioinformatics 2012;28:2986-8.
5. Eckhardt F, Lewin J, Cortese R, et al. DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet 2006;38:1378-85.
6. Huang W, Loganantharaj R, Schroeder B, Fargo D, Li L. PAVIS: a tool for Peak Annotation and Visualization. Bioinformatics 2013;29:3097-9.
7. Naeem H, Wong NC, Chatterton Z, et al. Reducing the risk of false discovery enabling identification of biologically significant genome-wide methylation status using the HumanMethylation450 array. BMC Genomics 2014;15:51.
8. Sears MR, Greene JM, Willan AR, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med 2003;349:1414-22.
9. Vonk JM, Postma DS, Boezen HM, et al. Childhood factors associated with asthma remission after 30 year follow up. Thorax 2004;59:925-9.
10. Dai H, Leeder JS, Cui Y. A modified generalized Fisher method for combining probabilities from dependent tests. Front Genet 2014;5:32.
11. Carithers LJ, Ardlie K, Barcus M, et al. A Novel Approach to High-Quality Postmortem Tissue Procurement: The GTEx Project. Biopreserv Biobank 2015;13:311-9.
12. Obeidat M, Hao K, Bosse Y, et al. Molecular mechanisms underlying variations in lung function: a systems genetics analysis. Lancet Respir Med 2015;3:782-95.
13. Rabinovich RA, Drost E, Manning JR, et al. Genome-wide mRNA expression profiling in vastus lateralis of COPD patients with low and normal fat free mass index and healthy controls. Respir Res 2015;16:1.
14. Golpon HA, Geraci MW, Moore MD, et al. HOX genes in human lung: altered expression in primary pulmonary hypertension and emphysema. Am J Pathol 2001;158:955-66.
15. Kim YJ, Park SW, Kim TH, et al. Genome-wide methylation profiling of the bronchial mucosa of asthmatics: relationship to atopy. BMC Med Genet 2013;14:39.
16. Kesimer M, Ehre C, Burns KA, Davis CW, Sheehan JK, Pickles RJ. Molecular organization of the mucins and glycocalyx underlying mucus transport over mucosal surfaces of the airways. Mucosal Immunol 2013;6:379-92.
17. Otulakowski G, Duan W, O'Brodovich H. Global and gene-specific translational regulation in rat lung development. Am J Respir Cell Mol Biol 2009;40:555-67.
18. Duffy SM, Ashmole I, Smallwood DT, Leyland ML, Bradding P. Orai/CRACM1 and KCa3.1 ion channels interact in the human lung mast cell plasma membrane. Cell Commun Signal 2015;13:32.
19. Peel SE, Liu B, Hall IP. ORAI and store-operated calcium influx in human airway smooth muscle cells. Am J Respir Cell Mol Biol 2008;38:744-9.
20. Keenan CR, Schuliga MJ, Stewart AG. Pro-inflammatory mediators increase levels of the noncoding RNA GAS5 in airway smooth muscle and epithelial cells. Can J Physiol Pharmacol 2015;93:203-6.
21. Di C, Lin X, Zhang Y, et al. Basophil-associated OX40 ligand participates in the initiation of Th2 responses during airway inflammation. J Biol Chem 2015;290:12523-36.

22. Siddiqui S, Mistry V, Doe C, Stinson S, Foster M, Brightling C. Airway wall expression of OX40/OX40L and interleukin-4 in asthma. Chest 2010;137:797-804.
23. Lee JH, Cho MH, Hersh CP, et al. Genetic susceptibility for chronic bronchitis in chronic obstructive pulmonary disease. Respir Res 2014;15:113.
24. Ortiz RA, Barnes KC. Genetics of allergic diseases. Immunol Allergy Clin North Am 2015;35:19-44.
25. Hirota T, Takahashi A, Kubo M, et al. Genome-wide association study identifies three new susceptibility loci for adult asthma in the Japanese population. Nat Genet 2011;43:893-6.
26. Tomati V, Sondo E, Armirotti A, et al. Genetic Inhibition Of The Ubiquitin Ligase Rnf5 Attenuates Phenotypes Associated To F508del Cystic Fibrosis Mutation. Sci Rep 2015;5:12138.
27. Kamada F, Mashimo Y, Inoue H, et al. The GSTP1 gene is a susceptibility gene for childhood asthma and the GSTM1 gene is a modifier of the GSTP1 gene. Int Arch Allergy Immunol 2007;144:275-86.
28. Chen L, Wilson R, Bennett E, Zosky GR. Identification of vitamin D sensitive pathways during lung development. Respir Res 2016;17:47.
29. Pastor MD, Nogal A, Molina-Pinelo S, et al. Identification of proteomic signatures associated with lung cancer and COPD. J Proteomics 2013;89:227-37.
30. Bentley AR, Kritchevsky SB, Harris TB, et al. Genetic variation in antioxidant enzymes and lung function. Free Radic Biol Med 2012;52:1577-83.
31. Wan ES, Qiu W, Baccarelli A, et al. Systemic steroid exposure is associated with differential methylation in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2012;186:1248-55.
32. Migita O, Noguchi E, Koga M, et al. Haplotype analysis of a 100 kb region spanning TNF-LTA identifies a polymorphism in the LTA promoter region that is associated with atopic asthma susceptibility in Japan. Clin Exp Allergy 2005;35:790-6.
33. Israel E, Lasky-Su J, Markezich A, et al. Genome-wide association study of short-acting beta2-agonists. A novel genome-wide significant locus on chromosome 2 near ASB3. Am J Respir Crit Care Med 2015;191:530-7.
34. Ahmad A, Bhattacharya S, Sridhar A, Iqbal AM, Mariani TJ. Recurrent copy number variants associated with bronchopulmonary dysplasia. Pediatr Res 2016.
35. Hu P, Wang X, Haitsma JJ, et al. Microarray meta-analysis identifies acute lung injury biomarkers in donor lungs that predict development of primary graft failure in recipients. PLoS One 2012;7:e45506.
36. Smirnova AS, Morgun A, Shulzhenko N, Silva ID, Gerbase-DeLima M. Identification of new alternative splice events in the TCIRG1 gene in different human tissues. Biochem Biophys Res Commun 2005;330:943-9.
37. Wilk JB, Walter RE, Laramie JM, Gottlieb DJ, O'Connor GT. Framingham Heart Study genome-wide association: results for pulmonary function measures. BMC Med Genet 2007;8 Suppl 1:S8.
38. Lai DM, Tu YK, Hsieh YH, et al. Angiopoietin-like protein 1 expression is related to intermuscular connective tissue and cartilage development. Dev Dyn 2007;236:2643-52.

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.

REFERENCES

1. Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 2008; 359:61-73.
2. Barker DJ, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO. Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. BMJ 1991; 303:671-5.
3. Horikoshi M, Beaumont RN, Day FR, Warrington NM, Kooijman MN, Fernandez-Tajes J, et al. Genome-wide associations for birth weight and correlations with adult disease. Nature 2016; 538:248-52.
4. Cai Y, Shaheen SO, Hardy R, Kuh D, Hansell AL. Birth weight, early childhood growth and lung function in middle to early old age: 1946 British birth cohort. Thorax 2016; 71:916-22.
5. Jobe AH. Mechanisms of Lung Injury and Bronchopulmonary Dysplasia. Am J Perinatol 2016; 33:1076-8.
6. Sonnenschein-van der Voort AM, Arends LR, de Jongste JC, Annesi-Maesano I, Arshad SH, Barros H, et al. Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. J Allergy Clin Immunol 2014; 133:1317-29.
7. Pike KC, Crozier SR, Lucas JSA, Inskip HM, Robinson S, Roberts G, et al. Patterns of fetal and infant growth are related to atopy and wheezing disorders at age 3 years. Thorax 2010; 65:1099-105.
8. Turner S, Prabhu N, Danielian P, McNeill G, Craig L, Allan K, et al. First- and Second-Trimester Fetal Size and Asthma Outcomes at Age 10 Years. American Journal of Respiratory and Critical Care Medicine 2011; 184:407-13.
9. Sonnenschein-van der Voort AM, Gaillard R, de Jongste JC, Hofman A, Jaddoe VW, Duijts L. Foetal and infant growth patterns, airway resistance and school-age asthma. Respirology 2016; 21:674-82.
10. Sonnenschein-van der Voort AMM, Howe LD, Granell R, Duijts L, Sterne JAC, Tilling K, et al. Influence of childhood growth on asthma and lung function in adolescence. Journal of Allergy and Clinical Immunology 2015; 135:1435-+.
11. Duijts L, Reiss IK, Brusselle G, de Jongste JC. Early origins of chronic obstructive lung diseases across the life course. Eur J Epidemiol 2014; 29:871-85.
12. Narayanan M, Owers-Bradley J, Beardsmore CS, Mada M, Ball I, Garipov R, et al. Alveolarization Continues during Childhood and Adolescence New Evidence from Helium-3 Magnetic Resonance. American Journal of Respiratory and Critical Care Medicine 2012; 185:186-91.
13. Hsia CCW, Berberich MA, Driscoll B, Laubach VE, Lillehei CW, Massaro C, et al. Mechanisms and limits of induced postnatal lung growth. American Journal of Respiratory and Critical Care Medicine 2004; 170:319-43.
14. Forno E, Acosta-Perez E, Brehm JM, Han Y, Alvarez M, Colon-Semidey A, et al. Obesity and adiposity indicators, asthma, and atopy in Puerto Rican children. J Allergy Clin Immunol 2014; 133:1308-14.
15. Chen Y, Tu Y, Huang K, Chen P, Chu D, Lee YL. Pathway from Central Obesity to Childhood Asthma. Physical Fitness and Sedentary Time Are Leading Factors. Am J Respir Crit Care Med 2014; 189:1194-203.
16. Forno E, Han YY, Muzumdar RH, Celedon JC. Insulin resistance, metabolic syndrome, and lung function in US adolescents with and without asthma. J Allergy Clin Immunol 2015; 136:304-11.
17. Cottrell L, Neal WA, Ice C, Perez MK, Piedimonte G. Metabolic Abnormalities in Children with Asthma. Am J Respir Crit Care Med 2011; 183:441-8.
18. Martinez FD, Vercelli D. Asthma. Lancet 2013; 382:1360-72.
19. Haberg SE, London SJ, Stigum H, Nafstad P, Nystad W. Folic acid supplements in pregnancy and early childhood respiratory health. Archives of Disease in Childhood 2009; 94:180-4.

20. Veeranki SP, Gebretsadik T, Mitchel EF, Tylavsky FA, Hartert TV, Cooper WO, et al. Maternal Folic Acid Supplementation During Pregnancy and Early Childhood Asthma. Epidemiology 2015; 26:934-41.
21. Devakumar D, Stocks J, Ayres JG, Kirkby J, Yadav SK, Saville NM, et al. Effects of antenatal multiple micronutrient supplementation on lung function in mid-childhood: follow-up of a double-blind randomised controlled trial in Nepal. Eur Respir J 2015; 45:1566-75.
22. Sharma S, Litonjua A. Asthma, allergy, and responses to methyl donor supplements and nutrients. J Allergy Clin Immunol 2014; 133:1246-54.
23. Vardavas CI, Hohmann C, Patelarou E, Martinez D, Henderson AJ, Granell R, et al. The independent role of prenatal and postnatal exposure to active and passive smoking on the development of early wheeze in children. Eur Respir J 2016; 48:115-24.
24. den Dekker HT, Sonnenschein-van der Voort AM, de Jongste JC, Reiss IK, Hofman A, Jaddoe VW, et al. Tobacco Smoke Exposure, Airway Resistance, and Asthma in School-age Children: The Generation R Study. Chest 2015; 148:607-17.
25. Wongtrakool C, Wang NS, Hyde DM, Roman J, Spindel ER. Prenatal Nicotine Exposure Alters Lung Function and Airway Geometry through alpha 7 Nicotinic Receptors. American Journal of Respiratory Cell and Molecular Biology 2012; 46:695-702.
26. Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, et al. DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. American Journal of Human Genetics 2016; 98:680-96.
27. Mackay D, Haw S, Ayres JG, Fischbacher C, Pell JP. Smoke-free Legislation and Hospitalizations for Childhood Asthma. New England Journal of Medicine 2010; 363:1139-45.
28. Wolff T, Witkop CT, Miller T, Syed SB, Force USPST. Folic acid supplementation for the prevention of neural tube defects: an update of the evidence for the U.S. Preventive Services Task Force. Ann Intern Med 2009; 150:632-9.
29. Tanaka T, Scheet P, Giusti B, Bandinelli S, Piras MG, Usala G, et al. Genome-wide association study of vitamin B6, vitamin B12, folate, and homocysteine blood concentrations. Am J Hum Genet 2009; 84:477-82.
30. Hazra A, Kraft P, Lazarus R, Chen C, Chanock SJ, Jacques P, et al. Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway. Hum Mol Genet 2009; 18:4677-87.
31. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995; 10:111-3.
32. Fox JT, Stover PJ. Folate-Mediated One-Carbon Metabolism. Folic Acid and Folates 2008; 79:1-44.
33. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A 2007; 104:19351-6.
34. Hollingsworth JW, Maruoka S, Boon K, Garantziotis S, Li Z, Tomfohr J, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. J Clin Invest 2008; 118:3462-9.
35. Brown SB, Reeves KW, Bertone-Johnson ER. Maternal folate exposure in pregnancy and childhood asthma and allergy: a systematic review. Nutrition Reviews 2014; 72:55-64.
36. Dogaru CM, Nyffenegger D, Pescatore AM, Spycher BD, Kuehni CE. Breastfeeding and Childhood Asthma: Systematic Review and Meta-Analysis. American Journal of Epidemiology 2014; 179:1153-67.
37. Friedman NJ, Zeiger RS. The role of breast-feeding in the development of allergies and asthma. Journal of Allergy and Clinical Immunology 2005; 115:1238-48.

38. van Elten TM, van Rossem L, Wijga AH, Brunekreef B, de Jongste JC, Koppelman GH, et al. Breast milk fatty acid composition has a long-term effect on the risk of asthma, eczema, and sensitization. Allergy 2015; 70:1468-76.
39. Walker WA, Iyengar RS. Breast milk, microbiota, and intestinal immune homeostasis. Pediatr Res 2015; 77:220-8.
40. Obermann-Borst SA, Eilers PHC, Tobi EW, de Jong FH, Slagboom PE, Heijmans BT, et al. Duration of breastfeeding and gender are associated with methylation of the LEPTIN gene in very young children. Pediatric Research 2013; 74:344-9.
41. Durmus B, Ay L, Duijts L, Moll HA, Hokken-Koelega ACS, Raat H, et al. Infant diet and subcutaneous fat mass in early childhood: The Generation R Study. European Journal of Clinical Nutrition 2012; 66:253-60.
42. Ferreira MAR, Matheson MC, Duffy DL, Marks GB, Hui JN, Le Souef P, et al. Identification of IL6R and chromosome 11q13.5 as risk loci for asthma. Lancet 2011; 378:1006-14.
43. Gudbjartsson DF, Bjornsdottir US, Halapi E, Helgadottir A, Sulem P, Jonsdottir GM, et al. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. Nat Genet 2009; 41:342-7.
44. Himes BE, Hunninghake GM, Baurley JW, Rafaels NM, Sleiman P, Strachan DP, et al. Genome-wide association analysis identifies PDE4D as an asthma-susceptibility gene. Am J Hum Genet 2009; 84:581-93.
45. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. N Engl J Med 2010; 363:1211-21.
46. Sleiman PM, Flory J, Imielinski M, Bradfield JP, Annaiah K, Willis-Owen SA, et al. Variants of DENND1B associated with asthma in children. N Engl J Med 2010; 362:36-44.
47. Torgerson DG, Ampleford EJ, Chiu GY, Gauderman WJ, Gignoux CR, Graves PE, et al. Meta-analysis of genome-wide association studies of asthma in ethnically diverse North American populations. Nat Genet 2011; 43:887-92.
48. Bonnelykke K, Sleiman P, Nielsen K, Kreiner-Moller E, Mercader JM, Belgrave D, et al. A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. Nat Genet 2014; 46:51-5.
49. Hancock DB, Eijgelsheim M, Wilk JB, Gharib SA, Loehr LR, Marciante KD, et al. Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. Nat Genet 2010; 42:45-52.
50. Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat M, et al. Genome-wide association study identifies five loci associated with lung function. Nat Genet 2010; 42:36-44.
51. Artigas M, Loth DW, Wain LV, Gharib SA, Obeidat M, Tang W, et al. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. Nat Genet 2011; 43:1082-90.
52. Wilk JB, Chen TH, Gottlieb DJ, Walter RE, Nagle MW, Brandler BJ, et al. A genome-wide association study of pulmonary function measures in the Framingham Heart Study. PLoS Genet 2009; 5:e1000429.
53. McGeachie MJ, Yates KP, Zhou X, Guo F, Sternberg AL, Van Natta ML, et al. Patterns of Growth and Decline in Lung Function in Persistent Childhood Asthma. New England Journal of Medicine 2016; 374:1842-52.
54. Lange P, Celli B, Agusti A, Jensen GB, Divo M, Faner R, et al. Lung-Function Trajectories Leading to Chronic Obstructive Pulmonary Disease. New England Journal of Medicine 2015; 373:111-22.
55. Begin P, Nadeau K. Epigenetic regulation of asthma and allergic disease. Allergy Asthma Clin Immunol 2014; 10:27.

56. Godfrey KM, Costello PM, Lillycrop KA. The developmental environment, epigenetic biomarkers and long-term health. J Dev Orig Health Dis 2015:1-8.
57. Krauss-Etschmann S, Meyer KF, Dehmel S, Hylkema MN. Inter- and transgenerational epigenetic inheritance: evidence in asthma and COPD? Clin Epigenetics 2015; 7:53.
58. Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease. Cell Metab 2016.
59. Jaddoe VWV, van Duijn CM, Franco OH, van der Heijden AJ, van Ilzendoorn MH, de Jongste JC, et al. The Generation R Study: design and cohort update 2012. European Journal of Epidemiology 2012; 27:739-56.
60. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol 2006; 21:475-84.
61. Van der Heijden GJ, Donders AR, Stijnen T, Moons KG. Imputation of missing values is superior to complete case analysis and the missing-indicator method in multivariable diagnostic research: a clinical example. J Clin Epidemiol 2006; 59:1102-9.
62. Sterne JA, White IR, Carlin JB, Spratt M, Royston P, Kenward MG, et al. Multiple imputation for missing data in epidemiological and clinical research: potential and pitfalls. BMJ 2009; 29:338.
63. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J 2005; 26:319-38.
64. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J 1995; 8:483-91.
65. Caliskan M, Bochkov YA, Kreiner-Moller E, Bonnelykke K, Stein MM, Du GX, et al. Rhinovirus Wheezing Illness and Genetic Risk of Childhood-Onset Asthma. New England Journal of Medicine 2013; 368:1398-407.
66. Vissing NH, Chawes BLK, Bisgaard H. Increased Risk of Pneumonia and Bronchiolitis after Bacterial Colonization of the Airways as Neonates. American Journal of Respiratory and Critical Care Medicine 2013; 188:1246-52.
67. Pitter G, Ludvigsson JF, Romor P, Zanier L, Zanotti R, Simonato L, et al. Antibiotic exposure in the first year of life and later treated asthma, a population based birth cohort study of 143,000 children. European Journal of Epidemiology 2016; 31:85-94.
68. Stein MM, Hrusch CL, Gozdz J, Igartua C, Pivniouk V, Murray SE, et al. Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children. New England Journal of Medicine 2016; 375:411-21.
69. Haga SB. Impact of limited population diversity of genome-wide association studies. Genet Med 2010; 12:81-4.
70. Robinson MR, Wray NR, Visscher PM. Explaining additional genetic variation in complex traits. Trends in Genetics 2014; 30:124-32.
71. Wang WYS, Barratt BJ, Clayton DG, Todd JA. Genome-wide association studies: Theoretical and practical concerns. Nature Reviews Genetics 2005; 6:109-18.
72. Vilhjalmsson BJ, Nordborg M. The nature of confounding in genome-wide association studies. Nat Rev Genet 2013; 14:1-2.
73. Dedeurwaerder S, Defrance M, Calonne E, Denis H, Sotiriou C, Fuks F. Evaluation of the Infinium Methylation 450K technology. Epigenomics 2011; 3:771-84.
74. Naeem H, Wong NC, Chatterton Z, Hong MK, Pedersen JS, Corcoran NM, et al. Reducing the risk of false discovery enabling identification of biologically significant genome-wide methylation status using the HumanMethylation450 array. BMC Genomics 2014; 15:51.
75. Jaffe AE, Murakami P, Lee H, Leek JT, Fallin MD, Feinberg AP, et al. Bump hunting to identify differentially methylated regions in epigenetic epidemiology studies. Int J Epidemiol 2012; 41:200-9.

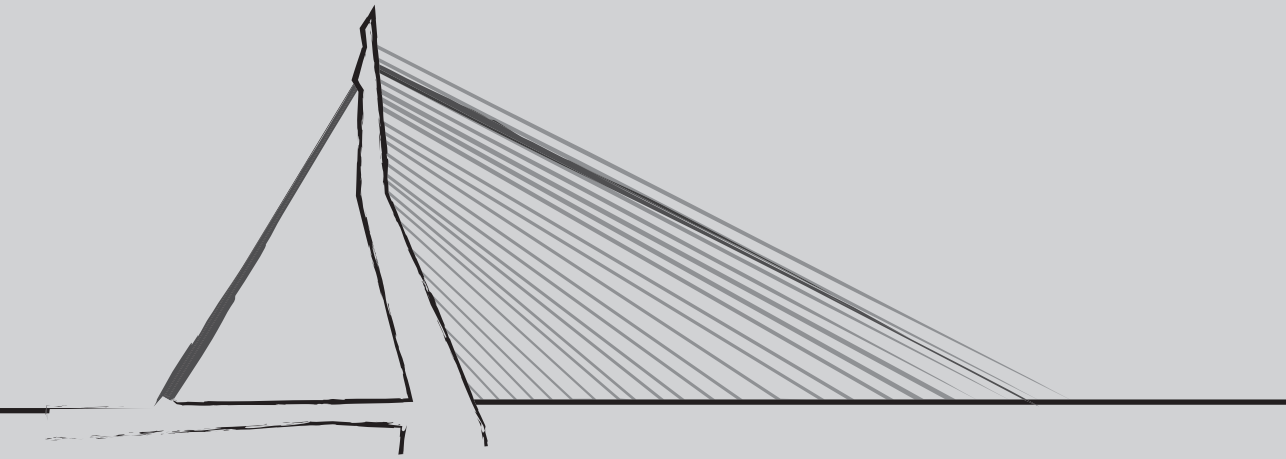
76. Dolzhenko E, Smith AD. Using beta-binomial regression for high-precision differential methylation analysis in multifactor whole-genome bisulfite sequencing experiments. BMC Bioinformatics 2014; 15:215.
77. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 2009; 462:315-22.
78. Leek JT, Scharpf RB, Bravo HC, Simcha D, Langmead B, Johnson WE, et al. Tackling the widespread and critical impact of batch effects in high-throughput data. Nature Reviews Genetics 2010; 11:733-9.
79. Liu Y, Aryee MJ, Padyukov L, Fallin MD, Hesselberg E, Runarsson A, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. Nat Biotechnol 2013; 31:142-7.
80. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. Bmc Bioinformatics 2012; 13.
81. Bakulski KM, Feinberg JI, Andrews SV, Yang J, Brown S, S LM, et al. DNA methylation of cord blood cell types: Applications for mixed cell birth studies. Epigenetics 2016; 11:354-62.
82. Gervin K, Page CM, Aass HC, Jansen MA, Fjeldstad HE, Andreassen BK, et al. Cell type specific DNA methylation in cord blood: a 450K-reference data set and cell count-based validation of estimated cell type composition. Epigenetics 2016:0.
83. Nadif R, Siroux V, Boudier A, le Moual N, Just J, Gormand F, et al. Blood granulocyte patterns as predictors of asthma phenotypes in adults from the EGEA study. Eur Respir J 2016.
84. Barnes PJ. Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. J Allergy Clin Immunol 2016; 138:16-27.
85. Hill AB. The Environment and Disease: Association or Causation? Proc R Soc Med 1965; 58:295-300.
86. Lochte L, Nielsen KG, Petersen PE, Platts-Mills TAE. Childhood asthma and physical activity: a systematic review with meta-analysis and Graphic Appraisal Tool for Epidemiology assessment. Bmc Pediatrics 2016; 16.
87. Forno E, Acosta-Perez E, Brehm JM, Han YY, Alvarez M, Colon-Semidey A, et al. Obesity and adiposity indicators, asthma, and atopy in Puerto Rican children. Journal of Allergy and Clinical Immunology 2014; 133:1308-U516.
88. Kramer MS, Chalmers B, Sevkovskaya Z, Dzikovich I, Shapiro S, Collet JP, et al. Promotion of Breastfeeding Intervention Trial (PROBIT): a randomized trial in the Republic of Belarus. JAMA 2001; 285:413-20.
89. Smith GD, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? International Journal of Epidemiology 2003; 32:1-22.
90. Granell R, Henderson AJ, Evans DM, Smith GD, Ness AR, Lewis S, et al. Effects of BMI, Fat Mass, and Lean Mass on Asthma in Childhood: A Mendelian Randomization Study. Plos Medicine 2014; 11.
91. Gaunt TR, Shibab HA, Hemani G, Min JL, Woodward G, Lyttleton O, et al. Systematic identification of genetic influences on methylation across the human life course. Genome Biol 2016; 17:61.
92. Chadwick LH, Sawa A, Yang IV, Baccarelli AA, Breakefield XO, Deng HW, et al. New insights and updated guidelines for epigenome-wide association studies. Neuroepigenetics 2015; 1:14-9.
93. Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. Nat Rev Genet 2011; 12:529-41.
94. Ng MCY, Barrett LM, Wong A, Kuh D, Smith GD, Relton CL. The role of longitudinal cohort studies in epigenetic epidemiology: challenges and opportunities. Genome Biol 2012; 13:246.

95. Relton CL, Smith GD. Mendelian randomization: applications and limitations in epigenetic studies. Epigenomics 2015; 7:1239-43.
96. Hafkamp-de Groen E, Lingsma HF, Caudri D, Levie D, Wijga A, Koppelman GH, et al. Predicting asthma in preschool children with asthma-like symptoms: Validating and updating the PIAMA risk score. Journal of Allergy and Clinical Immunology 2013; 132:1303.
97. Beasley R, Semprini A, Mitchell EA. Risk factors for asthma: is prevention possible? Lancet 2015; 386:1075-85.
98. WHO. Essential Nutrition Actions. Improving maternal, newborn, infant and young child health and nutrition. 2013.
99. Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. Immunol Rev 2011; 242:205-19.
100. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A Large-Scale, Consortium-Based Genomewide Association Study of Asthma. New England Journal of Medicine 2010; 363:1211-21.
101. Wain LV, Shrine N, Miller S, JAckson VE, Ntalla I, Soler Artigas M, et al. Novel insights into the genetics of smoking behaviour, lung function, and chronic obstructive pulmonary disease (UK BiLEVE): a genetic association study in UK Biobank. Lancet Respir Med 2015; 3:769-81.
102. Wagner MJ. Rare-variant genome-wide association studies: a new frontier in genetic analysis of complex traits. Pharmacogenomics 2013; 14:413-24.
103. Ek WE, Rask-Andersen M, Johansson A. The role of DNA methylation in the pathogenesis of disease: what can epigenome-wide association studies tell? Epigenomics 2016; 8:5-7.
104. Koch L. An epigenetic twist on the missing heritability of complex traits. Nat Rev Genet 2014; 15:218.
105. Ingelman-Sundberg M, Gomez A. The past, present and future of pharmacoepigenomics. Pharmacogenomics 2010; 11:625-7.

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.

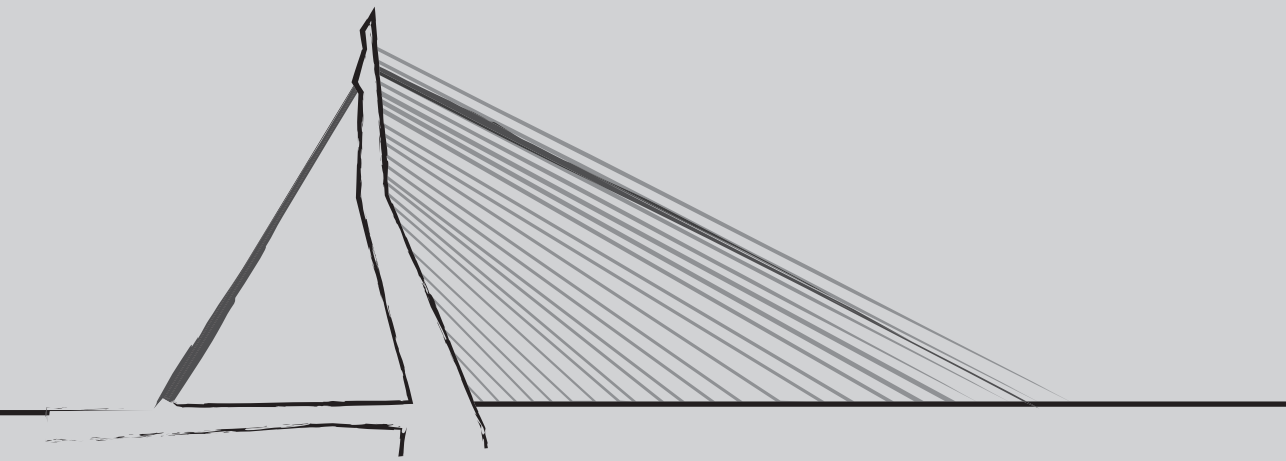
Chapter 7

List of publications

PhD portfolio

About the author

Dankwoord



LIST OF PUBLICATIONS

M Abraham, S Alramadhan, C Iniguez, L Duijts, VWV Jaddoe, **HT Den Dekker**, S Crozier, K Godfrey, P Hindmarsh, T Vik, GW Jacobsen, W Hanke, W Sobala, G Devereux, S Turner: A systematic review of maternal smoking during pregnancy and fetal measurements with meta-analysis. *PLoS ONE* 2017 Jan

N Stratakis, T Roumeliotaki, E Oken, F Ballester, H Barros, M Basterrechea, S Cordier, R de Groot, **HT den Dekker**, L Duijts, M Eggesbø, M Pia Fantini, F Forastiere, U Gehring, M Gielen, D Gori, E Govarts, HM Inskip, N Iszatt, M Jansen, C Kelleher, J Mehegan, C Moltó-Puigmartí, M Mommers, A Oliveira, SF Olsen, F Pelé, C Pizzi, D Porta, L Richiardi, SL Rifas-Shiman, SM Robinson, G Schoeters, M Strøm, J Sunyer, C Thijs, M Vrijheid, TGM Vrijkotte, AH Wijga, M Kogevinas, MP Zeegers, L Chatzi: Fish and seafood consumption during pregnancy and the risk of asthma and allergic rhinitis in childhood: a pooled analysis of 18 European and US birth cohorts. *Int J Epidemiol* 2017 Jan

NJ Elbert, L Duijts, **HT den Dekker**, NW de Jong, TEC Nijsten, VWV Jaddoe, JC de Jongste, R Gert van Wijk, HW Tiemeier, SGMA Pasmans: Maternal psychiatric symptoms during pregnancy and the risk of childhood atopic diseases. *Clin Exp Allergy* 2016 Dec 22

V Gallo, FN Dijk, JW Holloway, SM Ring, GH Koppelman, DS Postma, DP Strachan, R Granell, JC de Jongste, VWV Jaddoe, **HT den Dekker**, L Duijts, AJ Henderson, SO Shaheen: TRPA1 gene polymorphisms and childhood asthma. *Pediatr Allergy Immunol*. 2016 Oct 25

M Casas, **HT den Dekker**, CJ Kruithof, IK Reiss, M Vrijheid, JC de Jongste, VWV Jaddoe, L Duijts: Early childhood growth patterns and school-age respiratory resistance, fractional exhaled nitric oxide and asthma. *Pediatr Allergy Immunol*. 2016 Oct 5

HT den Dekker, KPI Ros, JC de Jongste, IK Reiss, VWV Jaddoe, L Duijts: Body fat mass distribution and interrupter resistance, fractional exhaled nitric oxide and asthma at school-age. *J Allergy Clin Immunol* 2016 Jul 16

O Gruzieva, C Xu, CV Breton, I Annesi-Maesano, JM Antó, C Auffray, S Ballereau, T Bellander, J Bousquet, M Bustamante, M Charles, Y de Kluizenaar, **HT den Dekker**, L Duijts, JF Felix, U Gehring, M Guxens, VWV Jaddoe, SA Jankipersadsing, S Kebede Merid, J Kere, A Kumar, N Lemonnier, J Lepeule, W Nystad, CM Page, S Panasevich, D Postma, R Slama, J Sunyer, C Söderhäll, J Yao, SJ London, G Pershagen, GH Koppelman, Erik Melén; Epigenome-wide meta-analysis of methylation in children related to prenatal air pollution exposure. *Environ Health Perspect* 2016 Jul 22

HT den Dekker, AM Sonnenschein-van der Voort, VWV Jaddoe, IK Reiss, JC de Jongste, L Duijts; Breastfeeding and asthma outcomes at the age of 6 years. The Generation R Study. *Pediatr Allergy Immunol* 2016 Aug;27(5):486-92.

NJ Elbert, L Duijts, **HT den Dekker**, VWV Jaddoe, AM Sonnenschein-van der Voort, JC de Jongste, SG Pasmans; Role of environmental exposures and filaggrin mutations on associations of ethnic origin with risk of childhood eczema. The Generation R Study *Pediatr Allergy Immunol* 2016 Sep;27(6):627-35.

BR Joubert*, **HT den Dekker***, JF Felix, J Bohlin, S Ligthart, E Beckett, H Tiemeier, JB van Meurs, AG Uitterlinden, A Hofman, SE Håberg, SE Reese, MJ Peters, B Kulle Andreassen, EA Steegers, RM Nilsen , SE Vollset, Ø Midttun, PM Ueland, OH Franco, A Dehghan, JC de Jongste, MC Wu, T Wang, SD Peddada, VWV Jaddoe, W Nystad, L Duijts, SJ London; Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. *Nat Commun* 2016 Feb 10;7:10577
*Authors contributed equally

T Gazibara, NJ Elbert, **HT den Dekker**, JC de Jongste, IK Reiss, JJ McGrath, DW Eyles, TH Burne, H Tiemeier, VWV Jaddoe, SG Pasmans, L Duijts; Associations of maternal and fetal 25-hydroxyvitamin D levels with childhood eczema. The Generation R Study. *Pediatr Allergy Immunol* 2016 May;27:283-9

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

L Paternoster, M Standl, J Waage, H Baurecht, M Hotze, DP Strachan, JA Curtin, K Bønnelykke, C Tian, A Takahashi, J Esparza-Gordillo, AC Alves, JP Thyssen, **HT den Dekker**, MA Ferreira, E Altmaier, PM Sleiman, FL Xiao, JR Gonzalez, I Marenholz, B Kalb, M Pino-Yanes, CJ Xu, L Carstensen, MM Groen-Blokhuis, C Venturini, CE Pennell, SJ Barton, AM Levin, I Curjuric, M Bustamante, E Kreiner-Møller, GA Lockett, J Bacelis, S Bunyavanich, RA Myers, A Matanovic, A Kumar, JY Tung, T Hirota, M Kubo M, WL McArdle, AJ Henderson, JP Kemp, J Zheng, GD Smith, F Rüschendorf, A Bauerfeind, MA Lee-Kirsch,

A Arnold, G Homuth, CO Schmidt, E Mangold, S Cichon, T Keil, E Rodríguez, A Peters, A Franke, W Lieb, N Novak, R Fölster-Holst, M Horikoshi, J Pekkanen, S Sebert, LL Husemoen, N Grarup, JC de Jongste, F Rivadeneira, A Hofman, VWV Jaddoe, SG Pasmans, NJ Elbert, AG Uitterlinden, GB Marks, PJ Thompson, MC Matheson, CF Robertson; Australian Asthma Genetics Consortium (AAGC), JS Ried, J Li, XB Zuo, XD Zheng, XY Yin, LD Sun, MA McAleer, GM O'Regan, CM Fahy, L Campbell, M Macek, M Kurek, D Hu, C Eng, DS Postma, B Feenstra, F Geller, JJ Hottenga, CM Middeldorp, P Hysi, V Bataille, T Spector, CM Tiesler, E Thiering, B Pahukasahasram, JJ Yang, M Imboden, S Huntsman, N Vilor-Tejedor, CL Relton, R Myhre, W Nystad, A Custovic, ST Weiss, DA Meyers, C Söderhäll, E Melén, C Ober, BA Raby, A Simpson, B Jacobsson, JW Holloway, H Bisgaard, J Sunyer, NM Probst-Hensch, LK Williams, KM Godfrey, CA Wang, DI Boomsma, M Melbye, GH Koppelman, D Jarvis, WH McLean, AD Irvine, XJ Zhang, H Hakonarson, C Gieger, EG Burchard, NG Martin, L Duijts, A Linneberg, MR Jarvelin, MM Nöthen, S Lau, N Hübner, YA Lee, M Tamari, DA Hinds, D Glass, SJ Brown, J Heinrich, DM Evans, S Weidinger; EArly Genetics and Lifecourse Epidemiology (EAGLE) Eczema Consortium; Multi-ancestry genome-wide association study of 21,000 cases and 95,000 controls identifies new risk loci for atopic dermatitis. *Nat Genet* 2015 Dec;47(12):1449-56

T Gazibara, **HT den Dekker**, JC de Jongste, JJ McGrath, DW Eyles, TH Burne, IK Reiss, OH Franco, H Tiemeier, VWV Jaddoe, L Duijts; Associations of maternal and fetal 25-hydroxyvitamin D levels with childhood lung function and asthma. The Generation R Study. *Clin Exp Allergy* 2016 Feb;46(2):337-46

E Rucci, **HT den Dekker**, JC de Jongste, j Steenweg-de Graaff, R Gaillard, SG Pasmans, A Hofman, H Tiemeier, VWV Jaddoe, L Duijts; Maternal fatty acid levels during pregnancy, childhood lung function and atopic diseases. The Generation R Study. *Clin Exp Allergy* 2016 Mar;46(3);461-71

JE Freund, **MH den Dekker**, AC Blank, F Haas, MW Freund; Midterm Follow-Up After Biventricular Repair of the Hypoplastic Left Heart Complex. *Ann Thorac Surg* 2015 Jun;99(6):2150-6

AC van Berkel, **HT den Dekker**, VWV Jaddoe, IK Reiss, R Gaillard, A Hofman, JC de Jongste, L Duijts; Mode of delivery and childhood fractional exhaled nitric oxide, interrupter resistance and asthma: the Generation R study. *Pediatr Allergy Immunol* 2015 Jun;26(4):330-6

HT den Dekker, AM Sonnenschein-van der Voort, JC de Jongste, IK Reiss, A Hofman, VWV Jaddoe, l Duijts; Tobacco Smoke Exposure, Airway Resistance, and Asthma in School-age Children: The Generation R Study. *Chest* 2015 Sep;148(3):607-17

HT den Dekker*, AM de Grauw*, AC de Mol, S Rombout-de Weerd; The diagnostic value of routine antenatal ultrasound in screening for congenital uropathies. *J Matern Fetal Neonatal Med* 2016 Jan;29(2):237-41
*Authors contributed equally

HT den Dekker, L Duijts; A fortified follow-up formula for 3-4-year-olds reduces episodes of acute respiratory infection and antibiotic use compared with cow's milk. *Evid Based Nurs* 2015 Jul;18(3):80

MW Freund, **MH den Dekker**, AC Blank, F Haas, MG Slieker; Authors reply. *J Am Soc Echocardiogr* 2014 Mar;27(3):340

K Bønnelykke, P Sleiman, K Nielsen, E Kreiner-Møller, JM Mercader, D Belgrave, **HT den Dekker**, A Husby, A Sevelsted, G Faura-Tellez, LJ Mortensen, L Paternoster, R Flaaten, A Mølgaard, DE Smart, PF Thomsen, MA Rasmussen, S Bonàs-Guarch, C Holst, EA Nohr, R Yadav, ME March, T Blicher, PM Lackie, VWV Jaddoe, A Simpson, JW Holloway, L Duijts, A Custovic, DE Davies, D Torrents, R Gupta, MV Hollegaard, DM Hougaard, H Hakonarson, H Bisgaard; A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. *Nat Genet* 2014 Jan;46(1):51-5

M den Dekker, M Slieker, C A Blank, F Haas, M W Freund; Comparability of Z-scores Equations of Cardiac Structures in Hypoplastic Left Heart Complex. *J Am Soc Echocardiogr* 2013 Nov;26(11): 1314-21

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)."

Na vier jaar uitdagingen, barbecues en rib-nights, foute grappen, frustraties, vriendschappen, reisjes variërend van Amsterdam tot Australië, en heel soms hard werken, ligt hier mijn eindresultaat. Al het werk dat in dit proefschrift wordt beschreven is het resultaat van de inspanning van velen. En dit is niet alleen input op het wetenschappelijke vlak; over de volle breedte spelen familie, vrienden en collega's een essentiële rol. Wetenschappelijk onderzoek kan niet worden gedaan zonder de kennis, expertise, input en interesse van de omgeving. Ik wil graag een aantal van de betrokkenen in het bijzonder bedanken.

Allereerst natuurlijk mijn directe begeleiders, die elk woord dat ik in de afgelopen vier jaar op papier heb gezet tot in den treure hebben gelezen. Geachte **prof. dr. V.W.V. Jaddoe**, beste Vincent. Vijf jaar geleden vertelde ik je bij onze eerste kennismaking dat ik kinderarts wilde worden "op de klinische manier", dus zonder vooraf wetenschappelijk onderzoek te verrichten. Nog geen twee maanden later zat ik opnieuw tegenover je, maar nu om te solliciteren voor een promotieplek. Ondanks ons eerste gesprek heb je me de kans gegeven. Heel veel dank daarvoor. Mede dankzij jouw input, betrokken begeleiding, snelle reacties en kritische blik is dit proefschrift geworden wat het is. Geachte **prof. dr. J.C. de Jongste**, beste Johan. Heel veel dank voor al het meedenken, het sparren over manuscripten en uw kritische blik. Bij elk manuscript heeft u een "finishing touch" toegevoegd die met minimale wijzigingen leidde tot een sterke verbetering van de beoogde boodschap. Dank! Geachte **mw. dr. L. Duijts**, beste Liesbeth. Betrokken en bevlogen, 24 uur per dag. Vaak stuurde je mijn manuscripten tot in het kleinste detail nagekeken binnen een dag terug. We hebben veel gediscussieerd over alle manuscripten, studenten en analyses, waarmee je me heel veel hebt geleerd. Nogmaals dank voor hoe je me voor schut zette door mij als vrijwilliger voor de zeeleeuwen-show aan te wijzen in de dierentuin in Singapore. Bedankt voor al je support!

Geachte **prof. dr. I.K. Reiss**, beste Irwin. Vijf jaar geleden had ik de eer aanwezig te zijn bij je oratie. Op dat moment had ik zelf nog niet eens overwogen om een promotietraject te starten. Veel dank dat je nu plaats wilde nemen in de kleine commissie en ook de taak van secretaris op je wilde nemen. Ik zal nooit vergeten hoe je mijn proefschrift in ontvangst nam terwijl je ondertussen een ernstig zieke patiënt intubeerde! Beste **prof dr. G. Brusselle** en **prof. Dr. A. Uitterlinden**. Ik ben vereerd dat u beiden de tijd en moeite heeft willen nemen plaats te nemen in de kleine commissie. Ook dank aan **prof. dr. De Hoog, prof. dr. Postma, prof. dr. Steegers en dr. Turner,** de leden van de **Grote**

commissie, voor de bereidheid met mij van gedachten te willen wisselen tijdens mijn verdediging.

Zoe en **Willem**, mijn paranimfen. Dank dat jullie bij mijn verdediging naast me willen staan. Zoe, heel veel dank voor alle gezelligheid en je zieke gevoel voor humor. Je bent in staat om elke werkdag voor de eerste slok koffie al meerdere vieze verhalen te vertellen. En ook om mijn vertrouwen zo te beschamen dat ik na een vakantie 4000 met water gevulde bekertjes in mijn huis vind. Maar naast de gezelligheid en plagerijen hebben we ook serieuze gesprekken kunnen voeren, en gaf je me na veel zeuren de eer om dag-gast te zijn op je huwelijk. Ik kijk al uit naar het moment dat ik als kinderarts al de problemen die jij als gynaecoloog hebt veroorzaakt weer kan oplossen. Willem, we zijn samen gestart bij Generation R, en hebben samen de statistiek en genetica ontdekt. Vanaf dag 1 was het een topsfeer. We begonnen in de schimmelkamer van het AE-gebouw, waar we liever een biertje dronken op de tuinbank. We hebben samen duizenden kilometers langs de Rotte en de Maas gefietst. En ook duizenden doelpunten gemaakt op de voetbaltafel (waarvan ik het merendeel in eigen doel). Wat hebben we gelachen in de afgelopen jaren. Ik hoop dat we dat in de toekomst blijven doen!

Heel veel dank aan alle **Generation R deelnemers**. Zonder jullie motivatie en inzet zou het hele Generation R project, en daarmee ook mijn proefschrift, nooit mogelijk zijn geweest. Veel dank aan alle **medewerkers van het Focus-centrum**; jullie passie en inzet vormt de basis voor alle mooie studies die binnen Generation R worden gedaan. En in het bijzonder dank aan datamanagers **Claudia** en **Marjolein**, en secretaresses **Patricia** en **Rose**. Ik heb uren bij jullie doorgebracht om data op te vragen of datasets om te zetten, en net zoveel uren op het belletje van Patricia geslagen voor allerlei logistieke dingen. Dank overigens voor het belletje, Patricia.

Ik heb enorm veel plezier beleefd in mijn werk, en dat is voor een groot deel te danken aan de **collega's van Generation R** en de **kinderpulmonologie** met wie ik heb samengewerkt. Ik wil alle collega's met wie ik door de jaren heen heb gewerkt daarom ook heel erg bedanken! Inmiddels zijn dit er echt teveel geworden om allemaal bij naam te noemen. Ik heb genoten van de interdisciplinaire samenwerkingen, de presentaties, discussies en de gezelligheid! Ook een woord van dank aan alle co-auteurs van de manuscripten in dit proefschrift. Een enkeling wil ik daarbij benadrukken. **Agnes Sonnenschein – van der Voort**, jij hebt me vanuit Bristol wegwijs gemaakt in Generation R, en de basis gelegd voor mijn eerste manuscripten. **Janine Felix**, dank voor alle hulp en discussies op het gebied van genetica en epigenetica, maar vooral voor je gezelligheid. Ik kijk uit naar de resultaten van de epigenetische studies!

Amy en **Bas**, ik leerde jullie kennen als goede vrienden van Lotte. Dankzij de wekelijkse etentjes ben ik jullie echter ook steeds meer als goeie vrienden van mezelf gaan beschouwen. Dank voor jullie vriendschap, interesse en kookkunsten. **Kristin**, vakantiemaatje. We hebben samen een aantal hele mooie reizen mogen maken. Je was zelfs gek genoeg om me te komen opzoeken in Australië. Ik hoop ooit nog een keer die walvishaai te kunnen spotten met je! **Kitty** en **Robin**, wat zijn jullie toch twee geweldige vrienden! We hebben samen de Gavia en de Stelvio beklommen, en Lotte en ik hebben genoten van jullie bruiloft. Nieuw Zeeland ligt helaas wel wat verder weg dan Leidschendam, maar de afstand Zuid-Amerika – Australië is ook nooit een probleem geweest voor ons.

De voetbaltafel-mannen; **Ronald**, **Ryan**, **Philip**, **Gerard**, **Tim**, **Gijs**, **Strahinja**. Wat heb ik een geweldige tijd met jullie gehad, met het weekend in Servië als absoluut hoogtepunt. We zijn een hechte groep geworden, en ik hoop dat er nog heel veel rib-nights volgen! **Ronald**, of het nu met de eerste verbouwing van mijn huis was, de fiets-ergometer, of de meest duurzame voetbaltafel, je staat altijd klaar om te helpen. Dank! **Ryan**, na al die jaren vertik ik het je nu nog in het Engels aan te spreken. Ik heb heel veel respect voor hoe je het met ons "knuckleheads" hebt uitgehouden, en zelfs hebt besloten om nog langer in Nederland te blijven. Laten we nog heel veel Fantasy Football competities spelen; misschien leer je het ooit nog wel een keer. **Philip**, je hebt volgens mij nog nooit een vrijmibo overgeslagen, en nog nooit een potje aan de voetbaltafel gewonnen. Je hebt heel indrukwekkende analyses verricht, en ik ben benieuwd naar je resultaten. **Gerard**, onze superman en mister gadget. Ik begrijp nog steeds niet hoe je in dat kleine tasje alle kleding voor Servië kon meenemen. Ik hoop dat je de eervolle titel "pussy for life", die je eerlijk hebt gewonnen aan de voetbaltafel, met trots zal blijven dragen. Inverse U-shaped **Tim**. Ik ga het sparren, je eeuwig kritische blik en cynisme missen op kantoor. En vooral je reacties op weer een schuin balletje dat ook volgens je eigen regels telde. **Gijs**, wat was het mooi dat jij in -20°C in Philadelphia zat, en ik in 40°C in Perth. En dat je de Vredesloop hebt gedaan op een krat bier en een kapsalon. Dank voor je gezelligheid en altijd sportieve reacties op alle grappen die we met je hebben uitgehaald. De baby-shower vond ik een hoogtepunt! **Strahinja**, wat heb ik een respect voor hoe snel jij je hebt aangepast aan Nederland. Nadat je in je eerste jaar nog mijn wasmachine sloopte en mijn handdoeken meenam, was je twee jaar later eigenaar van je eigen Servische sportschool inclusief zwembad en sauna. En zelfs de flauwe Servië-grappen ben je gaan waarderen. Het is heel mooi om te zien hoe je je plek in Nederland hebt gevonden. Ik hoop ooit nog een keer bij je in de tandartsstoel te durven liggen.

Lieve **John**, **Lienet**, **Anne** en **Pieter**, dank voor jullie oprechte belangstelling, humor, zorg en gezelligheid, en de geweldige bootreisjes en skivakanties. Ik ben enorm blij met jullie als schoonfamilie!

Lieve **Jolinde**, **Gert Jan**, **Chantal**, **Erik**, **Diandra**, **Niels**, **Daphne** en **Renske**. Mijn broers en (schoon)zussen, allemaal met een onbegrensd relativeringsvermogen en een (boeren) nuchterheid. Mijn promotie-periode werd gezien als stage, wonen in Rotterdam was bij voorbaat een grove fout en mijn eerste publicatie in *Nature Genetics* was makkelijker te onthouden als *dat stukje in de Naturisten Gids*. Ik heb altijd op jullie kunnen bouwen, en jullie staan klaar op elk moment dat nodig is. We vormen een bijzonder stel met z'n allen, en ik ben blij dat ik daar deel van uit maak.

Lieve **pa** en **ma**. Heel veel dank voor jullie oneindige vertrouwen, onvoorwaardelijke steun en grenzeloze hulp. Jullie laten me mijn eigen richting bepalen, maar staan altijd en overal als vangnet achter mij. De basis die jullie me hebben meegegeven, met de nodige duwtjes in de rug op latere leeftijd, hebben er toe geleid dat ik hier vandaag kan staan.

Lieve **Lotte**, jij bent halverwege dit proefschrift in mijn leven gekomen, en bent sindsdien mijn hulp, steun, luisterend oor en mede-avonturier geweest. Je zal dat ook zijn bij alles wat nog komen gaat. Ik kijk uit naar wat de toekomst ons brengen zal! Ik hou van je!