Exhaled nitric oxide and asthma in childhood

Ralf van der Valk

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*Both authors contributed equally

**These authors jointly directed this work

List of abbreviations

ARG, arginase
ATS, American Thoracic Society
BAL, bronchoalveolar lavage
BMI, body mass index
CHARISM, CHildren with Asthma subjected to Respiratory Inflammatory Status Monitoring
CI, confidence interval
cNOS, constitutive nitric oxide synthase
CV, coefficient of variation
DFA, detrended fluctuation analysis
DOHaD, Developmental Origins of Health and Disease
EAGLE, EArly Genetics and Lifecourse Epidemiology
eQTL, expression quantitative trait locus
ERS, European Respiratory Society
ETS, environmental tobacco smoke
FACS, folic acid-containing supplement
FeNO, fraction of exhaled nitric oxide
FEV1, forced expiratory volume in 1 second
GCTA, genome-wide complex trait analysis
GEE, generalized estimating equation
GLCCI1, glucocorticoid-induced transcript 1
GLMM, generalized linear mixed model
GSDMB, gasdermin B
GWA, genome-wide association
ICS, inhaled corticoid steroids
IL-13, interleukin-13
IL-33, interleukin-33
IL1RL1, interleukin 1 receptor-like 1
iNOS, inducible nitric oxide synthase
ISAAC, International Study of Asthma and Allergies in Childhood
LABA, long-acting beta-agonist
LCLs, lymphoblastoid cell lines
LD, linkage disequilibrium
LGALS9, lectin, galactoside-binding, soluble, 9
LLCA, longitudinal latent class analysis
LYRM9, LYR motif containing 9
MAF, minor allele frequency
MAR, missing at random

MCAR, missing completely at random
MI, multiple imputations
MI-GEE, multiple imputation-based generalized estimating equation
MTHFR, methylenetetrahydrofolate reductase
nNOS, neuronal nitric oxide synthase
NO, nitric oxide
NOS, nitric oxide synthases
NOS2, nitric oxide synthase 2, inducible
NPV, negative predictive value
OR, odds ratio
ORMDL3, ORM1-like 3
PEF, peak expiratory flow
PIAMA, Prevention and Incidence of Asthma and Mite Allergy
ppb, parts per billion
PPV, positive predictive value
Rint, interrupter resistance
ROC, receiver operator curve
SABA, short acting bronchodilator
SD, standard deviation
SE, standard error
SNP, single nucleotide polymorphism
TSLP, thymic stromal lymphopoietin
WGEE, weighted generalized estimating equation
ZPBP2, zona pellucida binding protein 2

Chapter 1

General introduction

General introduction

Asthma was first described in the medical literature of Greek antiquity. It is difficult to determine whether by referring to "asthma", Hippocrates and his school (460-360 B.C.) meant an autonomous clinical entity or a symptom[1]. The clinical presentation of asthma nowadays has probably changed little compared to 200 years ago. However, there are now many more people with asthma[2]. According to the Global Initiative for Asthma: 'Asthma is a disorder defined by its clinical, physiological, and pathological characteristics. The predominant feature of the clinical history is episodic shortness of breath, particularly at night, often accompanied by cough. Wheezing appreciated on auscultation of the chest is the most common physical finding. The main physiological feature of asthma is episodic airway obstruction characterized by expiratory airflow limitation. The dominant pathological feature is airway inflammation, sometimes associated with airway structural changes (www.ginasthma.org).' In Western countries the prevalence of childhood asthma and atopic diseases has increased dramatically during the end of the last century[3,4]. There are large differences in asthma prevalence between Western countries[5], and between the different continents. In Western countries, asthma is one of the most frequent chronic disorders in childhood, with a high burden of morbidity, absenteeism from school, health care costs[6] and reduced quality of life[7].

Assessment of asthma control is important to guide treatment. It is however difficult to predict the temporal pattern and risk of exacerbations in a given patient. Theories derived from sciences dealing with complexity of physiological parameters can explain the seemingly unpredictable nature of bronchial asthma. Fluctuation analysis, a method used in statistical physics, can be used to gain insight into asthma as a dynamic disease of the respiratory system, viewed as a set of interacting subsystems (e.g., inflammatory, immunological, and mechanical). Fluctuation analysis methods can be applied to the quantification of the long-term temporal history of lung function parameters[8]. This information is potentially useful and might help to assess the risk of future asthma episodes, with implications for asthma severity and control[9].

Childhood asthma is a highly dynamic heterogeneous and complex disease, existing in many different phenotypes[10], influenced by many genetic and environmental factors[11]. The mechanisms underlying different asthma phenotypes are still poorly understood[12,13]. Twin and family studies have shown that predisposition to asthma is highly heritable. In the past 4 years a new hypothesis-free methodology has been introduced to study the genetics of complex, non-Mendelian diseases, the genome-wide association (GWA) study[14,15]. Recent GWA studies provided evidence that asthma is a heterogeneous disease by showing that different common genetic variants are associated with different asthma-related outcomes: childhood onset asthma[16,17],

adult asthma[17-19], impaired lung function[20-22], and atopy[23-25]. Identifying genes associated with asthma-related outcomes advances our understanding of biological pathways for asthma and may highlight potential future drug targets for the treatment of asthma[14,15]. For this purpose, GWA studies need to be followed by functional studies of the identified genes to highlight potential drug targets, but also to epidemiological gene-environment studies. Studies examining the interaction between genetic and environmental risk factors that are associated with different phenotypes may help to elucidate the origins of asthma[10,11]. It has been proposed to abolish the term asthma altogether[12] and focus on specific asthma phenotypes or endotypes rather than on asthma as a single disease entity[13].

In this thesis we specifically focus on the fraction of exhaled nitric oxide (FeNO). FeNO is a non-invasive biomarker of eosinophilic airway inflammation[26-28], and is associated with childhood asthma symptoms[29], exacerbations[30], physician-diagnosed asthma[26-28] and atopy[31]. Nitric oxide (NO) is a reactive free-radical gas that is generated in the airway epithelium when L-arginine is oxidized to L-citrulline[32]. This reaction is catalyzed by nitric oxide synthases (NOS)[28]. Three isoforms of NOS have been described: neuronal NOS (nNOS also known as NOS1), inducible NOS (iNOS also known as NOS2) and constitutive NOS (cNOS also known as NOS3)[33]. iNOS is mostly localized in the airways, alveolar epithelium, alveolar macrophages and the vascular endothelium[32,34]. Expression of NOS can be upregulated in the presence of pro-inflammatory cytokines, inflammatory mediators and small rises in intracellular calcium, which could lead to increases in NO levels. NO regulates airway and blood vessel tone and high NO concentrations have antimicrobial effects[28]. Measurements of FeNO are non-invasive, well standardized[27] and suitable for use in large epidemiological studies of children[35].

This thesis focuses on major challenges in the field of childhood asthma that have still been insufficiently addressed: the interpretation of daily fluctuations in FeNO and genetic and environmental risk factors of asthma and FeNO in childhood. To investigate this, we used data of several studies:

The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) Study

The PIAMA study is a prospective birth cohort study in the Netherlands. Recruitment took place in 1996-97 through prenatal clinics. Children were labelled as high-risk and low-risk, based on the atopic status of the mother. Respiratory health and asthma symptoms of the children were assessed yearly by questionnaires, partly based on the International Study of Asthma and Allergies in Childhood (ISAAC) core questionnaires[37], along with data on demographics and a wide range of asthma risk

factors. All high-risk children and a subgroup of low-risk children were invited for FeNO measurement at the age of 4 years and 8 years[38,39]. In this thesis findings of the PIAMA study were used to describe differences in FeNO levels measured at 4 and 8 years for distinct phenotypes of wheeze and atopy. In addition, we used PIAMA data to study the modifying effect of smoke exposure during fetal and early postnatal life on the association between a specific asthma gene and asthma-related outcomes.

The CHildren with Asthma subjected to Respiratory Inflammatory Status Monitoring (CHARISM) Study

The CHARISM study represents the cooperation of 15 clinical research centers. In a prospective, open label, randomized, multicenter, parallel group study, children with atopic asthma were monitored for 30 weeks, and inhaled corticoid steroid (ICS) doses were adjusted every 3 weeks on the basis of either FeNO and symptom scores, or symptom scores alone[36]. In this thesis we used data from this study and performed post hoc analyses of fluctuations in daily FeNO measurements and data on asthma control and exacerbations over 30 weeks in children with atopic asthma.

The EArly Genetics and Lifecourse Epidemiology (EAGLE) Consortium

The EAGLE consortium is a consortium of pregnancy and birth cohorts that collaborate to investigate the genetic basis of a wide range of phenotypes in antenatal and early life and childhood[40]. EAGLE integrates closely with the 'Developmental Origins of Health and Disease' (DOHaD) society. DOHaD is a society of researchers who are interested in developmental epidemiology of chronic diseases throughout the life course. In order to identify genetic variants associated with certain traits, such as FeNO, several thousands of samples are required to achieve the high threshold of significant statistical evidence[14,15]. This is only possible if large cohorts with researchers who share a common goal collaborate in a consortium.

The Generation R Study

The Generation R Study is a population-based prospective cohort study from fetal life until young adulthood. The study is designed to identify early environmental and genetic causes of normal and abnormal growth, development and health during fetal life, childhood and adulthood. The study focuses on four primary areas of research: (1) growth and physical development; (2) behavioural and cognitive development; (3) diseases in childhood; and (4) health and healthcare for pregnant women and children. Recruitment took place from April 2002 until January 2006 in Rotterdam. Data collection in mothers, fathers and preschool children included questionnaires, detailed physical and ultrasound examinations, behavioural observations, and

biological samples. Genome-wide SNP genotyping (Illumina 610K array) of the participating children is available. Regular detailed assessments are performed at various ages. Generation R Study aims to contribute to the development of strategies for optimizing health and healthcare for pregnant women and children[41]. The Generation R Study is one of the leading centers in EAGLE. In this thesis we used Generation R data to examine asthma, FeNO, and relevant gene-environment interactions in childhood.

Aims of this thesis

In the current thesis we aimed to investigate the following aspects of FeNO and asthma in childhood:

- To compare if distinct temporal phenotypes of wheeze and atopy differ with respect to FeNO, a marker of eosinophilic airway inflammation
- To examine the possible clinical relevance of daily fluctuations in FeNO, especially regarding asthma severity, control, and exacerbation risk
- To identify which genetic loci are related to FeNO in childhood, and their relation with asthma
- To examine if gene-environment interaction is important for the effect of a known asthma gene (*GSDMB*) by examining the effect of cigarette smoke exposure during fetal and early postnatal life
- To establish if cord blood folate and homocysteine levels have an impact on the risk of asthma and eczema in childhood, and if this is genetically determined
- To compare different strategies for analysis and imputation of missing data in binary repeated asthma-related outcomes

Outline of this thesis

This thesis examines FeNO in relation to asthma in childhood, focusing on different asthma phenotypes, FeNO fluctuation patterns and the contribution of genetic factors on FeNO and asthma. First, **chapter 2** describes differences in FeNO levels measured at 4 and 8 years for distinct phenotypes of wheeze and atopy. **Chapter 3** presents an analysis of daily fluctuation patterns of FeNO before and after asthma exacerbations compared to a stable control period in children with atopic asthma. **Chapter 4** presents the associations between fluctuations in FeNO and the correlation between daily FeNO and symptoms with asthma control and exacerbation risk in

atopic asthmatic children. In **chapter 5**, we present a genome-wide association study of FeNO in childhood. **Chapter 6** describes the modifying effect of smoke exposure during fetal and early postnatal life on the association between asthma *GSDMB* gene and asthma-related outcomes. In **chapter 7** we explored the associations of cord blood folate, homocysteine and vitamin B12 levels of children at birth with asthma and eczema in childhood and the influence of *MTHFR* variants on these associations. Repeated asthma-related outcomes were used in these studies. Missing data in binary repeated outcomes is complex to analyze and could lead to biased results. Therefore, we examined in **chapter 8** what the impact is of different strategies for analysis and imputation of missing data in binary repeated asthma-related outcomes. **Chapter 9** discusses the main findings of this thesis in context of the literature, methodological considerations and evaluates the clinical implications and future research directions for genetic studies of asthma-related outcomes.

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Chapter 2

Childhood wheezing phenotypes and FeNO in atopic children at age 8

The PIAMA Study

Ralf J.P. van der Valk*, Daan Caudri*, Olga Savenije, Gerard H. Koppelman, Henriette A. Smit, Alet H. Wijga, Dirkje S. Postma, Marjan Kerkhof, Bert Brunekreef and Johan C. de Jongste

**Both authors contributed equally*

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Abstract

Background

Fractional exhaled nitric oxide (FeNO) is a surrogate biomarker of the degree of eosinophilic airway inflammation. Using longitudinal latent class analysis, 5 wheezing phenotypes have been identified, characterized by different age of onset and prognosis.

Objectives

To assess FeNO measured at 4 and 8 years in children with different phenotypes of wheeze and atopy.

Methods

Children participated in the PIAMA study, a prospective birth cohort in The Netherlands. Respiratory health was assessed yearly by questionnaires until the age of 8 years, these data were used to identify 5 wheezing phenotypes. Associations between FeNO and wheezing phenotypes were investigated using weighted linear regression.

Results

Data on wheezing phenotypes and FeNO at 4 and 8 years was available in 588 and 973 children, respectively. Compared to the phenotype of never and transient wheeze, FeNO at 4 years was higher in intermediate onset and persistent wheeze. FeNO at 8 years of age differed significantly between all phenotypes, with highest FeNO values for persistent, intermediate onset, and late onset wheeze. Rise in FeNO from 4 to 8 years in intermediate and late onset wheezers was significantly higher compared to FeNO rise in never and transient wheezers. Stratified analyses showed that the increase in FeNO in persistent, intermediate and late onset wheeze was only present in children with allergic sensitization at 8 years.

Conclusions

FeNO measured at 8 years was associated with specific wheezing phenotypes, only among atopic children.

Introduction

The fraction of nitric oxide in exhaled air (FeNO) is a non-invasive surrogate biomarker of the degree of eosinophilic airway inflammation with excellent reproducibility in childhood[1,2]. Recent studies have shown that FeNO can be used both in large population-based studies and in clinical asthma management studies[3,4]. Elevated FeNO was found in children and adults with asthma and atopy[2,3,5,6], overlapping with the distribution in normals[7,8]. We previously reported on FeNO in 4-year-old children from the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) cohort, and found no association with classic wheezing phenotypes as described by Martinez in preschool children[8,9]. However, FeNO may be influenced by atopy, which can develop later in life[2,10-12]. The PIAMA birth cohort study provided the opportunity to study FeNO in relation to phenotypes of wheeze in a large group of children recruited from the general population. One of the special features of PIAMA is the yearly respiratory health assessment, which can be used to define phenotypes of wheeze. Recently, phenotypes of wheeze were identified by longitudinal latent class analysis (LLCA) in the ALSPAC study, and these phenotypes were differently associated with atopy and lung function[13]. This analysis was repeated in the PIAMA study and resulted in comparable phenotypes with similar associations with doctor's diagnosed asthma, inhaled corticosteroid use, sensitization to common allergens, FEV1 and bronchial responsiveness[14]. FeNO has not been studied in relation to phenotypes identified using this novel approach. We hypothesized that the different wheezing phenotypes are characterized by differences in eosinophilic inflammation, which would be reflected by differences and change in FeNO measured at the age of 4 and 8 years. Because atopy is an important determinant of FeNO, we stratified our analysis for atopy[2].

Methods

Study design

The PIAMA study is a prospective birth cohort study in The Netherlands. Recruitment took place in 1996-97 through prenatal clinics; 7,862 pregnant women were invited to participate, 4,146 (53%) agreed and gave informed consent. Children were labelled as high-risk (n=1,327) and low-risk (n=2,819), based on the atopic status of the mother. Respiratory health and asthma symptoms of the children were assessed yearly by questionnaires, partly based on the ISAAC core questionnaires, along with data on demographics and a wide range of asthma risk factors[15]. All high-risk children and a subgroup of low-risk children were invited for FeNO measurement at the age of 4 years (n=1,808) and 8 years (n=1,554). A detailed description of the PIAMA study

design was previously published[8,16]. The study protocol was approved by the medical ethics committees of the participating medical centers (Groningen: M 4.019912, Rotterdam: MEC 2004-152 and Utrecht: CCMO P04.0071C 04-101/K).

Study population

At the age of 4 years all high-risk (n=1,173) and a random sample of low-risk children (n=635) were invited for a medical examination, including offline FeNO measurement. Of those 1,808 children 1,269 attended the examination, and an exhaled air sample was obtained in 939 children. Off-line FeNO measurements of sufficient quality were obtained in 595 children (63%) at age 4[8]. At 8 years also all high-risk children still in follow-up (n=988) and a similar, random sample of low-risk children (n=566) were invited for a hospital-based medical examination including online FeNO measurement. Of these 1,554 children 1,129 (73%) gave informed consent and attended the examination. In 39 children a FeNO measurement could not be performed due to device failure. Of the remaining 1,090 children at least 1 successful FeNO measurement was obtained in 976 children (90%). The other 114 children were unable to exhale at a constant flow during FeNO measurement. A detailed flowchart of the study population with complete data on confounders, wheezing phenotypes and FeNO at 4 years (n=588) and 8 years (n=973) is presented in Figure 2.1.

Measurements

FeNO in 4-year-old children was measured offline by the balloon method[17], according to European Respiratory Society (ERS) / American Thoracic Society (ATS) guidelines[8,18]. FeNO in 8-year-old children was measured online using the NIOX chemiluminescence analyzer (Aerocrine AB, Solna, Sweden) according to ERS and ATS guidelines[1]. We previously found good agreement between these on- and offline FeNO measurements[19]. At 8 years blood was drawn to assess sensitization to airborne allergens, defined as specific IgE of ≥ 0.70 IU/mL for at least one of the following allergens: house dust mite (Dermatophagoides pteronyssinus), cats, dogs, grass pollen (Dactylis glomerata), birch, Alternaria alternata.

Phenotypes of wheeze

Longitudinal latent class analysis was used by Savenije et al. to define wheezing phenotypes in PIAMA in early childhood, as originally published by Henderson et al[13]. Wheezing phenotypes were previously defined in children with at least data on wheezing at 2 or more occasions, and in a subgroup of children with complete data on wheezing at every age from 1 to 8 years[14]. There were no major differences between these two analyses. In the current analysis phenotypes derived in children with at least 2 wheezing observations were used, in order to minimize the risk of selection

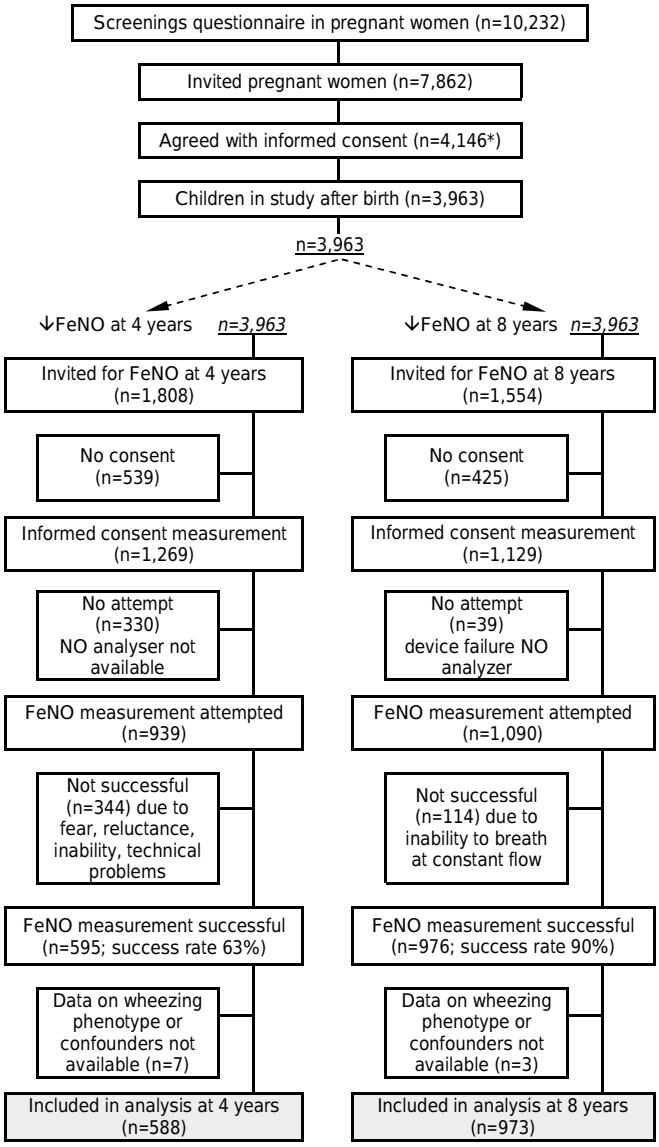

Figure 2.1. Flow chart of study population at 4 and 8 years

Flow chart of the number of children participating in the study. *: These 4,146 consisted of 1,327 atopic (32%) and 2,819 non-atopic mothers (68%), which is a good reflection of the general Dutch population.

bias. Five wheezing phenotypes were identified in the first 8 years of life: never/ infrequent wheeze (73.2%), transient early wheeze (17.3%), intermediate onset wheeze (3.4%), persistent wheeze (4.3%) and late onset wheeze (1.8%). These phenotypes were comparable with those identified in the ALSPAC cohort[14]. The five phenotypes are graphically depicted in Figure 2.2.

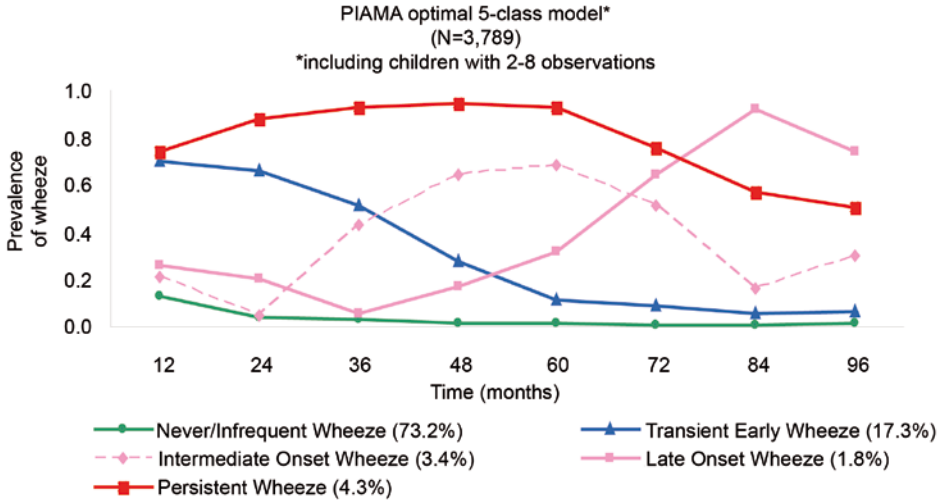

Figure 2.2. Probability of wheeze at each time point from birth to age 8 years for each wheezing phenotype in PIAMA (N=3,789)

The prevalences of the phenotypes are shown next to the phenotypes in the legend. Figure adjusted from Savenije&Granell et al, with copyright permission[14].

Statistical analysis

All analyses were carried out in SAS 9.1 (SAS Institute, Inc., Cary, NC). The associations between FeNO at 4 and 8 years and phenotypes of wheeze were investigated with weighted linear regression models (SAS PROC GENMOD). FeNO data were log-transformed, to achieve a normal distribution for linear regression analyses and back-transformations were used to calculate geometric mean FeNO for the phenotypes of wheeze. Due to the stratified study design, all analyses were performed for the total study population as well as for the high-risk and low-risk children separately. The analyses were also stratified for allergic sensitization at 8 years, because specific IgE is an important determinant of FeNO[2]. Individual membership probabilities (each child gets a probability to belong to each phenotype) derived from LLCA were used as weight factors in the linear regression models to minimize the risk of misclassification of the wheezing phenotypes. Gender, recent symptoms of cold, steroid use, study region, education of the mother and exposure to environmental tobacco smoke were considered as potential confounders. Confounders were included in the models based on their association with wheezing phenotypes, or if they changed the effect estimate by more than 10%.

Results

General characteristics of the study population

Baseline characteristics at 4 and 8 years are given in Table 2.1. Due to the study design, high-risk children were overrepresented in comparison with the total PIAMA population. Compared to those invited for medical examination at 8 years (n=1,554), children with complete FeNO data at 8 years had a higher level of maternal education and lower prevalence of prenatal smoking. However, differences were small, and with respect to other general characteristics the groups were similar (Table 2.1). Among children with complete FeNO data at the age of 4 years, there was an overrepresentation of the Western study region and an underrepresentation of the Northern and Middle study regions due to technical problems with FeNO measurements. This may explain the somewhat lower proportion of never/infrequent wheeze and late onset wheeze compared to the population invited for medical examination.

Associations of FeNO values at 4 years and phenotypes of wheeze

Phenotypes of wheeze were derived from yearly respiratory health assessments from birth up to 8 years. The adjusted geometric mean FeNO was highest in intermediate onset wheeze and persistent wheeze compared to never/infrequent and transient wheeze, but with considerable overlap (Table 2.2).

Associations of FeNO values at 8 years among different phenotypes of wheeze

FeNO at 8 years of age differed significantly between all phenotypes. It should be noted that also at 8 years of age, there was considerable overlap in FeNO between all phenotypes. FeNO was highest when wheeze started later in life and persisted longer, in intermediate onset wheeze, persistent wheeze and late onset wheeze. The adjusted geometric mean FeNO for each wheezing phenotype is given in Table 2.2 and the distributions are shown in Figure 2.3.

Change in FeNO over time was analyzed in the subgroup of children with FeNO measurements both at 4 and 8 years. FeNO in intermediate and late onset wheezers was significantly higher compared to never/infrequent and transient wheezers.

Environmental tobacco smoke and steroid use did not change the association between FeNO and phenotype of wheeze. These variables add up to 6.5% missing data and were not included in the final model in order to increase power. None of the other potential confounders changed the association by 10%. Steroids were mainly used in intermediate-, late onset- and persistent wheeze, and this might lead to underestimation of the differences between these phenotypes and the reference

Table 2.1. General characteristics of study population

	Invited for FeNO at 8 yrs (n=1,554)	Complete wheezing and FeNO data at 4 yrs (n=588)	Complete wheezing and FeNO data at 8 yrs (n=973)
Characteristics			
Gender (% females)	49	48	50
Study region			
West	31	50	32
Middle	37	34	38
North	32	16	29
Maternal education level			
Low	22	21	20
Middle	42	41	42
High	36	38	38
Caesarean section	9	8	10
Atopic mother*	64	66	66
Atopic father*	32	32	32
Exposure to pets in 1st yr	48	47	47
Older siblings (% present)	48	49	47
Daycare attendance in 1st yr	24	28	25
Smoking during pregnancy	16	14	15
Exposure to environmental tobacco smoke†	16	15	16
Inhaled steroid use†	9	9	9
Doctors' diagnosis asthma†	12	14	12
Phenotypes of wheeze‡			
Never/infrequent wheeze	69.9	66.5	68.8
Transient early wheeze	18.6	21.8	19.7
Intermediate onset wheeze	3.9	4.4	4.0
Persistent wheeze	5.1	5.7	5.2
Late onset wheeze	2.5	1.6	2.2
Specific IgE inhalant allergen age 8			
Positive for at least 1 of the 6 tested allergens¶	30	30	29

Values are percentages (%). *: Defined as a positive report of hayfever, allergy and/or asthma. †: Reported at the age of 8 years. ‡: Defined using longitudinal latent class analysis as previously described[14], known in 1,165/1,554 children invited at 8 years. ¶: The following 6 inhalant allergens were tested for: house dust mite (Dermatophagoides pteronyssinus), cats, dogs, grass pollen (Dactylis glomerata), birch, Alternaria alternata. Complete data on FeNO and specific IgE at 8 years in n=792 children.

Table 2.2. Adjusted geometric mean FeNO (ppb) and change in FeNO per phenotype of wheeze at 4 and 8 years

	FeNO at 4 years (N=588)		**FeNO at 8 years (N=973)**		**Difference in FeNO between 4 and 8 years (N=420)**	
Phenotype of wheeze	**n***	**mean (95% CI)†**	**n***	**mean (95% CI)†**	**n***	**mean (95% CI)†**
Never/infrequent	391	8.7 (8.3;9.2)	670	9.5 (9.0;10.1)#	279	+0.6 (-1.4;2.6)
Transient early	128	8.8 (7.9;9.8)	192	8.8 (7.8;9.8)#	95	-0.3 (-4,2;3.9)
Intermediate onset	26	11.2 (9.5;13.2)‡¶	39	15.8 (13.3;18.7)#	18	+5.5 (-1.0;12.7)‡¶
Persistent	33	9.9 (8.5;11.6)‡¶	51	13.0 (11.1;15.3)#	21	+2.7 (-3.2;9.2)
Late onset	10	9.6 (7.6;12.1)	21	20.6 (16.7;25.4)#	7	+9.8 (0.4;20.7)‡¶

Analyses were weighted for the probability that a child belongs to a certain phenotype (individual posterior membership probabilities). *: Frequency (n) of each wheezing phenotype is calculated as the sum of the membership probability of all children for that phenotype. †: Geometric mean FeNO value (95% confidence interval) in ppb per phenotype of wheeze, adjusted for gender, recent symptoms of cold, study region and education of the mother. ‡: p<0.05 for difference in comparison with never/infrequent wheeze. ¶: p<0.05 for difference in comparison with transient wheeze. #: At 8 years FeNO in every phenotype was significantly (p<0.01) different in comparison to all other phenotypes.

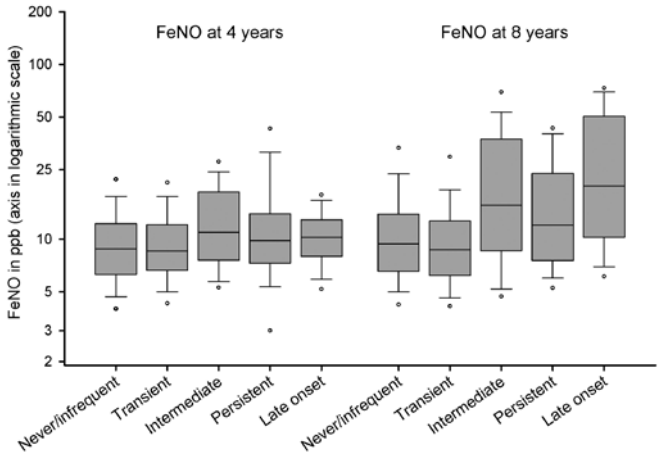

Figure 2.3. Box plots of FeNO at 4 and 8 years per phenotype of wheeze

Horizontal lines indicate the median FeNO. Upper/lower limits of the box, outer lines and dots represent the 25th/75th, the 10th/90th, and the 5th/95th percentiles, respectively. Data were weighted for the probability that a child belongs to a certain phenotype (individual posterior membership probabilities).

group. We think that this is not the case because exclusion of children using steroids at 8 years led to similar results of the phenotypes. In order to investigate whether the association between FeNO and the wheezing phenotypes may be caused solely by the association between FeNO and wheeze at the age that FeNO was measured, we performed a sensitivity analyses adjusting for current wheeze at the ages of 4 and 8 years (ISAAC question: reported wheezing symptoms in the past year). This adjustment did not alter the associations between FeNO and phenotypes of wheeze. All analyses were repeated using wheezing phenotypes defined in the subgroup of children with complete data on wheezing at every age from 1 to 8 years, and this produced similar results (data not shown).

FeNO values and phenotypes of wheeze in atopic and non-atopic children

We performed stratified analyses based on atopy of the mother. The associations between FeNO and phenotypes were similar. However, we found a strong and significant interaction with allergic sensitization of the children themselves at the age of 8 years (overall p-value for interaction < .001). Among children with elevated specific IgE, FeNO levels at 8 years were low in never/infrequent wheeze and transient early wheeze, and significantly elevated in the remaining persistent phenotypes. In children without elevated specific IgE for inhalant allergens, FeNO levels at 8 years were not significantly

Table 2.3. Adjusted geometric mean FeNO (ppb) at 8 years per phenotype of wheeze stratified for atopy

		Atopy – (N=562)		**Atopy + (N=228)**	
Phenotype of wheeze	**% atopy**	**n***	**mean (95% CI)†**	**n***	**mean (95% CI)†**
Never/infrequent	23.9	410	7.9 (7.5;8.4)	129	13.5 (12.0;15.3)
Transient early	27.3	117	7.3 (6.5;8.3)	44	11.8 (9.1;15.2)‡
Intermediate onset	67.7	10	9.0 (7.1;11.4)	21	22.6 (16.7;30.6)‡
Persistent	51.2	20	8.2 (6.8;9.8)	21	20.9 (15.4;28.3)‡
Late onset	72.2	5	8.2 (6.0;11.3)	13	29.4 (20.7;41.8)‡
Combined persistent phenotypes¶	61.1	35	8.4 (7.2;9.8)	55	22.8 (17.8;29.2)‡

We found significant interaction between phenotypes of wheeze and allergic sensitization on FeNO levels (p-interaction < .001). Atopy of the child defined as specific IgE of ≥ 0.70 IU/mL for at least one inhalant allergen at the age of 8 years. Analyses were weighted for the probability that a child belongs to a certain phenotype (individual posterior membership probabilities). *: Frequency (n) of each wheezing phenotype is calculated as the sum of the membership probability of all children for that phenotype. †: Geometric mean FeNO value (95% confidence interval) (ppb) per phenotype of wheeze stratified for atopy at 8 years, adjusted for gender, recent symptoms of cold, study region and education of the mother. ‡: p<0.05 for difference in comparison with never/infrequent wheeze. ¶: Due to the smaller sample size, 3 phenotypes with persistent symptoms (intermediate onset wheeze, persistent wheeze and late onset wheeze) were also combined in this analysis.

associated with phenotypes of wheeze. Because the numbers of children with low specific IgE were small for the wheezing phenotypes with persistent symptoms (intermediate onset wheeze (n=10), persistent wheeze (n=20) and late onset wheeze (n=5)), these phenotypes were also combined for this analysis. Table 2.3 shows that, among atopic children, all three phenotypes with persistent symptoms had a significantly higher FeNO levels than never/infrequent and transient wheeze, while no such association was present in non-atopic children. This interaction is illustrated in Figure 2.4.

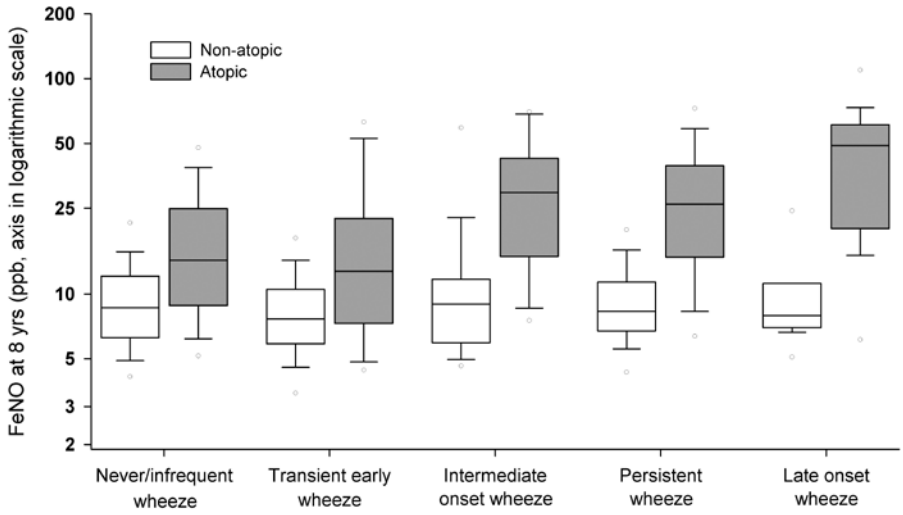

Figure 2.4. Boxplots of FeNO at 8 years per phenotype of wheeze, stratified for atopy
Atopy of the child defined as a specific IgE of ≥ 0.70 IU/mL for at least one of the tested inhalant allergens at the age of 8 years. Horizontal lines indicate the median FeNO. Upper/lower limits of the box, outer lines and dots represent the 25th/75th, the 10th/90th, and the 5th/95th percentiles, respectively. Data were weighted for the probability that a child belongs to a certain phenotype (individual posterior membership probabilities).

Discussion

We examined FeNO at 4 and 8 years in relation to phenotypes of wheeze and atopy. We found that FeNO at 4 years was higher in intermediate onset and persistent wheeze compared to never and transient wheeze. The association between phenotypes of wheeze and FeNO measured at 8 years was much stronger. FeNO at 8 years was significantly higher in persistent phenotypes of wheeze (including intermediate onset, persistent and late onset wheeze) compared to never and transient wheeze, but only among children with allergic sensitization at 8 years. Smaller but significant differences were observed for FeNO at 8 years between the three persistent phenotypes.

FeNO and wheezing phenotypes

Previous studies have reported increased FeNO in asthmatic children[3,5,20-22], while others did not confirm this[23,24]. A possible explanation for these discrepancies is that 'asthma' comprises several phenotypes that may or may not share the same inflammatory mechanisms[9,13,14,25]. Lumping all these phenotypes together as a single disease entity might hamper our efforts to understand the aetiology and pathophysiology of specific phenotypes[25,26]. The presence of such differences has been suggested in studies that assessed airway inflammation using bronchoalveolar lavage (BAL)[27]. BAL is an invasive procedure and hence not feasible for research purposes in children with mild disease. Only few studies investigated the association between non-invasive surrogate markers of airway inflammation, such as FeNO, and wheezing phenotypes in early childhood. Moeller et al. measured higher FeNO levels in wheezing preschool children compared to non-wheezers, in line with our findings. Among wheezing children the authors found higher FeNO levels in children with a positive asthma prediction index, which is suggestive for persistent symptoms[28]. In contrast, we found no differences in FeNO at the age of 4 between transient and late onset wheezers. This may be explained by differences in classification using the asthma predictive index or longitudinal latent class analysis. Brussee et al. found in the PIAMA study only a weak association of FeNO at 4 years with phenotypes of wheeze up to that age, with slightly higher FeNO levels in children who wheezed at the age of 4 years, compared to those who never wheezed[8]. Also in the present study only weak associations between phenotype and FeNO at 4 years were found. This could be explained by an increase in chronic airway inflammation with age[29], with differences in FeNO becoming detectable only after the age of 4 years. However, the early development of eosinophilic inflammation as underlying mechanism of persistent wheeze is not well understood. Alternatively, differences in the methods of FeNO measurement at 4 and 8 years might be involved, but we earlier found that these on- and offline methods give similar results, so this seems unlikely[19]. At 8 years, FeNO levels were increased in the phenotypes with persistent symptoms compared to never and transient wheezers. Differences in FeNO at 8 years between the three persistent phenotypes (intermediate onset, persistent and late onset wheeze) were smaller, but also significant. These results need to be interpreted with some caution, as significant differences may have resulted from multiple testing. We analyzed change in FeNO over time. Despite the small numbers, the rise in FeNO in intermediate onset and late onset wheezers was significantly higher compared to that in never/infrequent and transient wheezers. Possibly the underlying disease process in late onset wheezers leads to a faster increase of eosinophilic inflammation between 4 and 8 years. Elevated FeNO levels were especially pronounced in the phenotypes with onset of wheezing after the age of 2 years.

FeNO and atopy

We found a strong association between atopy and FeNO. This is a consistent finding in earlier studies[3,5,23,24]. Some authors have suggested that the association between asthma and FeNO may be entirely explained by atopy, implying that measuring FeNO is of limited use to assess whether a child has asthma[30]. The present study showed that FeNO is not simply a marker of atopy, but that the presence of atopy modifies the association between wheezing phenotypes and FeNO, which is in line with previous studies[6,23]. Indeed, FeNO levels differed substantially between the wheezing phenotypes in atopic children at 8 years. Furthermore this shows that all wheezing phenotypes occur in atopic and non-atopic children, but that the pathophysiology of wheeze in these two groups is probably different. As FeNO has been shown to correlate with eosinophilic airway inflammation, we speculate that a predominant eosinophilic inflammation might be present selectively in atopic children with persistent phenotypes of wheeze. Other mechanisms may play a role in the pathophysiology of transient wheeze and of persistent wheeze in non-atopic children. Possible mechanisms include smaller airway caliber and/or neutrophilic airway inflammation[27].

Strengths and limitations

A strong point of our study is that we assessed wheezing prospectively, and that the wheezing phenotypes were discovered without pre-specified constraints in two large birth cohorts, using longitudinal latent class analysis[13,14]. Well-standardized FeNO measurements[1], objective assessment of atopy at 8 years, and the large size of the PIAMA cohort with good follow-up allowed us to detect significant differences in FeNO in less common phenotypes, even after stratification for atopy.

A point of consideration in the interpretation of the data is that some children were using inhaled steroids while FeNO was measured, and it has been shown that steroids can decrease FeNO[2]. However, any such effect seems limited because a sensitivity analysis after exclusion of steroid users did not change the results. In addition, one should take into account the possibility that the reported association between FeNO and phenotypes of wheeze solely depends on the relation between FeNO and current wheeze at the age of 8 years. This seems unlikely, because adjustment for current wheeze at 8 years did not alter any of the associations between FeNO and phenotypes of wheeze. The present study used parent-reported wheezing symptoms. This method of assessing symptoms is widely accepted in epidemiological asthma studies[31], but may lead to misclassification. Because parents were unaware of their child's FeNO level, any misclassification of wheezing would be independent of FeNO, resulting in a diluting effect with underestimation of the true differences in FeNO between the wheezing phenotypes. Furthermore, the small sample sizes at the age of

4 years should be noted, which may have decreased the power to detect significant associations between FeNO and the less frequent wheezing phenotypes. This holds true also for the stratified analyses of FeNO at 8 years, where sample sizes were small, especially among the non-atopic children. FeNO was not measured in early life and our data can therefore not confirm earlier findings that FeNO might be increased in transient early wheeze at a time when wheeze was still present[32].

Conclusion

FeNO measured at 8 years differed between wheezing phenotypes, only in atopic children. Hence, we speculate that the pathophysiology of wheezing phenotypes differs between atopic and non-atopic children. Whether or not eosinophilic inflammation is indeed causally involved in the pathogenesis of specific wheezing phenotypes remains to be shown.

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Chapter 3

Daily exhaled nitric oxide measurements and asthma exacerbations in children

The CHARISM Study

Ralf J.P. van der Valk, Eugenio Baraldi, Georgette Stern, Urs Frey and Johan C. de Jongste

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Abstract

Background

Fractional exhaled nitric oxide (FeNO) is a biomarker for eosinophilic airway inflammation and can be measured at home on a daily basis. A short-term increase in FeNO may indicate a higher risk of future asthma exacerbations.

Objectives

To assess changes in FeNO before and after asthma exacerbations compared to a stable control period.

Methods

A post-hoc analysis was performed on daily FeNO measurements over 30 weeks in asthmatic children (n=77). Moderate exacerbations were defined by an increase in symptom scores, severe exacerbations by prescription of prednisone. Individual mean and maximum FeNO, the variability of FeNO assessed by the coefficient of variation (CV) and slopes of FeNO in time were all quantified in 3 weeks blocks. Cross-correlation of FeNO with symptoms and autocorrelation of FeNO were assessed in relation to exacerbations, and examined as predictors for exacerbations compared to reference periods using logistic regression.

Results

FeNO could be assessed in relation to 25 moderate and 12 severe exacerbations. The CV, slope, cross-correlation and autocorrelation of daily FeNO increased before moderate exacerbations. Increases in slope were also randomly seen in 19% of 2-week blocks of children without exacerbations. At least 3 to 5 FeNO measurements in the 3 weeks before an exacerbation were needed to calculate a slope that could predict moderate exacerbations. No specific pattern of FeNO was seen before severe exacerbations.

Conclusions

FeNO monitoring revealed changes in FeNO prior to moderate exacerbations. Whether or not this can be used to prevent loss of asthma control should be further explored.

Introduction

Asthma monitoring is challenging in children with frequent exacerbations. Asthma is commonly monitored on the basis of symptoms, exacerbation frequency, need for rescue treatment and lung function[1]. This approach does not take airway inflammation into account. The fraction of nitric oxide in exhaled air (FeNO) is a non-invasive, feasible biomarker which reflects eosinophilic airway inflammation[2]. FeNO has excellent reproducibility, immediate results and has been validated and studied in relation to asthma control[3,4]. Clinical studies on monitoring of airway inflammation by means of FeNO were until now inconsistent, but some evidence for limited benefits, including less exacerbations, lower steroid doses, reduced airway hyperresponsiveness and improved lung function was found[5-12]. It has been suggested that the combination of spirometry and FeNO allows objective assessment of asthma control status[13]. However, FeNO concentrations during asthma exacerbations did not correlate with other measures of acute severity, suggesting that they might provide additive information[14,15]. Using a hand-held NO-analyzer, it is possible to monitor FeNO at home on a daily basis[16,17]. Recently, the CHildhood Asthma Respiratory Inflammatory Status Monitoring (CHARISM) study examined 77 atopic asthmatic children with daily telemonitoring of symptoms and FeNO for 30 weeks[18]. This follow-up provided the opportunity to study FeNO fluctuation. Similar to fluctuation in lung function over time, we have shown that fluctuations in FeNO are not a random process but show internal long-range correlation[19]. We have previously shown that these correlation properties of daily FeNO are different in a subgroup of children with a high risk of exacerbation[20]. In the present study, we describe FeNO variability in relation to exacerbations of asthma. We hypothesized that exacerbations were preceded by an increase in FeNO.

Methods

We performed a post-hoc analysis of FeNO data from the CHARISM study[18]. Daily FeNO was measured daily in 77 atopic asthmatic children using a hand-held airway inflammation monitor (NIOX MINO, Aerocrine, Solna, Sweden), along with daily symptom scores for 30 weeks at home. The study protocol was approved by the medical ethics committees of the participating centers.

Exacerbations were defined by a prespecified increase in symptom scores during 1 or 2 days (moderate exacerbation), or by prescription of an oral prednisone course (severe exacerbation) (Table 3.1)[21]. Daily FeNO and symptom scores were taken from 3-week blocks before and after the onset of exacerbations, and were included in the analyses if at least 18 FeNO values out of 21 were available. To avoid carry-over

Table 3.1. Criteria for determining moderate and severe exacerbations

Exacerbation	Description	Days
Moderate	3 points above the average daily symptom score* of the 2 preceding weeks	2 or more
	5 points above the average daily symptom score* of the 2 preceding weeks	1 or more
Severe	Prednisone prescription, hospitalisation or emergency visits because of asthma	1 or more
End	Less than 3 points above the average daily symptom score* of the 2 preceding weeks	3 or more

*: Daily symptom score = total sum of wheezing, shortness of breath, coughing and sleep disturbances symptoms. Symptoms could have values between 0 and 3, where 0 = no symptoms, 1 = occasional symptoms, 2 = symptoms most of the day and 3 = asthma very bad, unable to do normal activities.

effects, second moderate exacerbations were excluded when they occurred within 21 days of the previous exacerbation. If a severe exacerbation was preceded by a moderate exacerbation, both were included in our analysis to account for this clinically relevant situation. We related the changes in FeNO to a stable reference period in the same individual. Reference periods were selected from the same patient when stable, and matched for use of same dose of inhaled corticosteroid (ICS) and use of long-acting beta-agonist (LABA). Reference periods were taken at least 6 weeks before or 3 weeks after the exacerbation. We estimated the risk of false negative observations by examining slopes of children without exacerbations. Fluctuation and correlation properties in daily FeNO were quantified by the coefficient of variation (CV), the slope of daily FeNO (best-fit line through FeNO data), cross-correlation (strength of correlation between FeNO and symptoms) and autocorrelation (strength of correlation of FeNO with itself shifted by one or more days)[20,22,23].

Statistical evaluation

Analyses of FeNO were performed after natural log transformation. FeNO percentage was calculated by dividing individual daily values of FeNO by the median of the reference period, and averaged over all children who had an exacerbation. Individual mean and maximum FeNO, as well as CV and slope of FeNO were calculated for periods of 21, 14, 10, 7 and 4 days prior to exacerbations and in matched reference periods. These parameters were then examined as predictors for the outcome of an exacerbation compared to reference periods, using logistic regression to estimate the odds ratios (OR) and the 95% confidence intervals (CI). Cross-correlation and autocorrelation were determined in periods of 21, 14, 10 and 7 days. We performed sensitivity analysis to estimate the number of measurements needed to detect changes in FeNO before an exacerbation by looking at the predictive power of single FeNO values at 21, 14, 10, 7, 4 days and 1 day before an exacerbation. In order to test how many data points were needed to detect an exacerbation, randomly picked days were dropped from the time series and we calculated best-fit slopes for the remaining data points. Analyses were

performed using custom-written software in Matlab (The Mathworks Inc., Natick, MA, USA) and SAS PROC GENMOD was used for logistic regression analyses in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) for Windows.

Results

Five patients were excluded due to missing data over the whole period (n=4) or unknown date of prednisone use (n=1). Twenty-four patients had 1 or more moderate exacerbations, for a total of 38. Thirteen of these were excluded due to missing data (n=9) or overlap with moderate (n=3) or severe exacerbation (n=1). After quality control, 25 moderate exacerbations could be included for 3-week period analysis. Eleven patients had 1 or more severe exacerbations, for a total of 15. Three of these were excluded from analysis due to missing data. In total, 12 severe exacerbations were selected for 3-week period analysis and were analyzed qualitatively, as statistical interference of low numbers could be unreliable. Two patients had moderate

Table 3.2. General characteristics of study population

		Moderate exacerbations	Severe exacerbations
Demographics			
Patients	N	18	9
Exacerbations	N	25	12
Gender (male)	N (%)	8 (44.4)	2 (22.2)
Age (year)	Mean (SD)	11.7 (2.5)	10.1 (2.1)
Weight (kg)	Mean (SD)	43.8 (11.8)	37.6 (11.1)
Height (cm)	Mean (SD)	150.8 (14.4)	142.2 (13.4)
Medication			
ICS dose ($\mu g.day^{-1}$)	Median (IQR)	400 (200-1000)	400 (200-1200)
LABA use per day	%	44.0	63.4
Rescue medication use per day	%	21.0	20.1
Lung function			
FeNO (ppb)*	Median (IQR)	21 (14-32)	19 (10-40)
FEV1[ǂ] (% pred)†	Mean (SD)	86.7 (16.6)	78.7 (24.3)
Post-bronchodilator FEV1** (% pred)‡	Mean (SD)	90.1 (16.3)	85.6 (19.5)

ICS, inhaled corticosteroid; LABA, long acting bronchodilator; Rescue medication, short acting bronchodilator; FEV1, forced expiratory volume in 1 second. *: Median FeNO of total study period. †: Baseline FEV1. ‡: Baseline reversibility of FEV1.

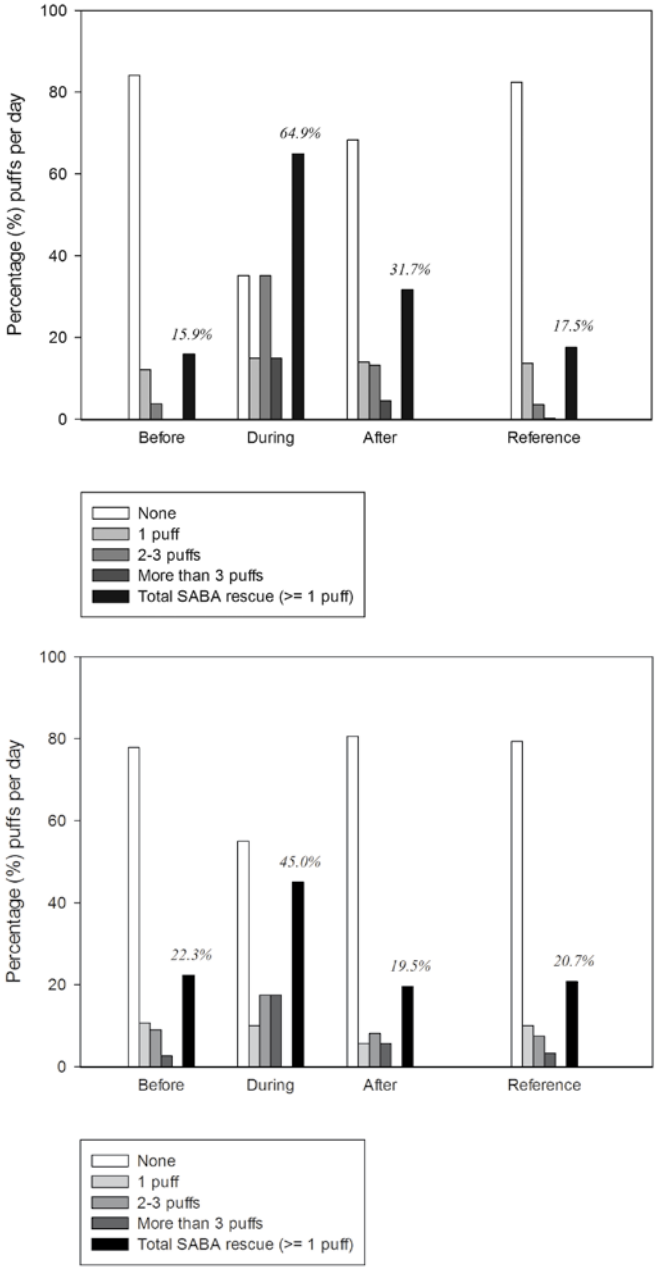

Figure 3.1. SABA rescue medication use around exacerbations and in the reference periods

Percentage short acting bronchodilator (SABA) rescue puffs per day in the periods before, during and after exacerbations and in the reference period. Rescue medication use is depicted for moderate exacerbations (upper panel) and severe exacerbations (lower panel).

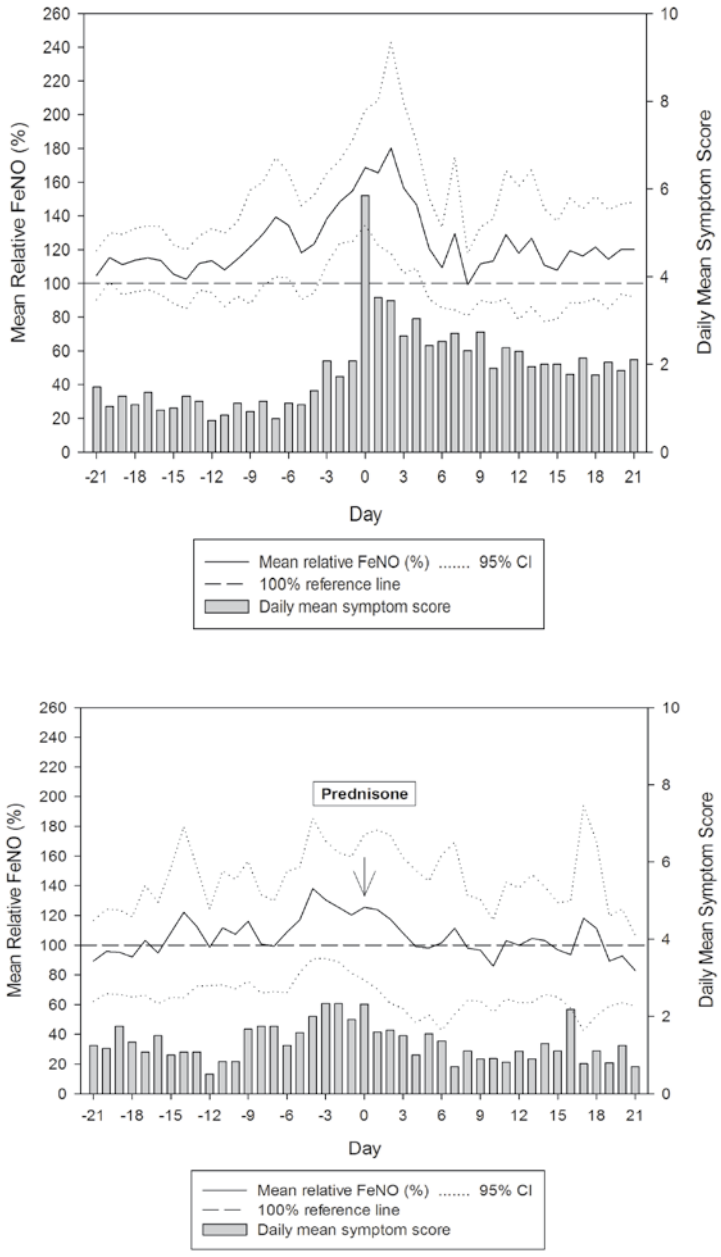

Figure 3.2. Percentage change in FeNO before and after exacerbations

Mean relative FeNO time series for 3 week periods centered around moderate exacerbations (upper panel) and severe exacerbations (lower panel) (onset exacerbation at day 0). Relative FeNO = FeNO divided by the median of the reference period. Bars show average daily symptom scores (sum of wheezing, shortness of breath, coughing and sleep disturbances).

exacerbations preceding a severe exacerbation by 3 and 9 days. Demographics and baseline characteristics of children with moderate and severe exacerbations are given in Table 3.2. Maintenance and reliever medication, used in the periods before, during and after exacerbations and in the reference periods are depicted in Figure 3.1.

Moderate exacerbations

On the average, FeNO started to increase approximately 10 days before the onset of a moderate exacerbation. The mean change of FeNO before and after a moderate exacerbation is displayed in Figure 3.2, upper panel. There was a significant increase of FeNO 3 days prior until 4 days after the exacerbations. Figure 3.1 (upper panel) shows that short acting bronchodilator use was higher during exacerbations compared to the period before the moderate exacerbations (64.9% VS 15.9%, P<.001).

Severe exacerbations

FeNO showed marked variability around severe exacerbations, but no clear rise preceding the onset of prednisone treatment (Figure 3.2, lower panel). Individual plots did not show a clear trend of symptoms in relation to severe exacerbations. There was however a rise in the use of rescue medication (Figure 3.1, lower panel).

Daily FeNO fluctuations and moderate exacerbations

The risk of moderate exacerbation was not associated with geometric mean FeNO or maximum FeNO. A higher CV of FeNO during 14 and 10 days before an exacerbation compared to the reference period was significantly associated with moderate exacerbations (standardized CV 14 and 10 days: OR 2.27 (1.16; 4.44) and 1.93 (1.02; 3.66)) (Table 3.3). The slope of FeNO between 14 and 4 days before moderate exacerbations was associated with the exacerbation (slope in 14, 10, 7 and 4 days before onset of exacerbation: OR 2.98 (1.22; 7.28), 1.58 (0.98; 2.54), 1.25 (0.97; 1.63) and 1.16 (1.00; 1.34), respectively). A higher cross-correlation of FeNO and symptoms during 14 and 10 days before the onset of moderate exacerbations was associated with exacerbation (standardized cross-correlation 14 and 10 days: OR 2.35 (1.12; 4.90) and 1.84 (0.94; 3.59)), respectively). Higher autocorrelation of FeNO was observed during 21, 14 and 10 days before exacerbations (OR 1.97 (1.04; 3.74), 2.09 (1.08; 4.03) and 1.71 (0.93; 3.16), respectively). Increases in slope, with a cut-off defined as mean slope before moderate exacerbations, were randomly seen in 19% of 2-week blocks of children without exacerbations. In a receiver operating curve analysis, we found an area under the curve of 0.71 for slopes of 2-week blocks to predict exacerbations (test cut-off slope = .385; sensitivity, 64.0%; specificity, 74.3%).

Table 3.3. Associations between parameters derived from daily FeNO and moderate exacerbations

	Before moderate exacerbation vs. Reference period OR (95% CI)[a]				
Days before onset of exacerbation	**21**	**14**	**10**	**7**	**4**
Coefficient of Variation*	1.52 (0.85;2.72)	2.27 (1.16;4.44)	1.93 (1.02;3.66)	1.54 (0.85;2.78)	1.07 (0.61;1.87)
Slope	1.74 (0.72;4.17)	2.98 (1.22;7.28)	1.58 (0.98;2.54)	1.25 (0.97;1.63)	1.16 (1.00;1.34)
Cross-Correlation*†	1.50 (0.79;2.83)	2.35 (1.12;4.90)	1.84 (0.94;3.59)	1.54 (0.79;3.00)	-
Autocorrelation*‡	1.97 (1.04;3.74)	2.09 (1.08;4.03)	1.71 (0.93;3.16)	1.32 (0.74;2.34)	-

[a]: Odds ratios (95% confidence interval), parameters derived from daily FeNO before moderate exacerbations were examined as predictors for the outcome of an exacerbation compared to parameters of daily FeNO derived from reference periods. *: Coefficients were divided by the standard deviation to make OR's more directly interpretable[33]. †: Cross-correlation coefficient of daily FeNO with daily sum of symptoms on the same day. ‡: Autocorrelation coefficient of FeNO with FeNO lagged by 1 day.

Single FeNO values were not predictive of exacerbations. At least 3-5 data points in 3 weeks were required to detect a moderate exacerbation (for 3, 4 and 5 data points: OR 1.56 (0.97; 2.50), 1.92 (1.08; 3.41) and 1.79 (1.00; 3.20), respectively).

Discussion

In this proof-of-concept study we examined daily FeNO measurements in relation to exacerbations of childhood asthma, compared to reference periods of the same child when stable. We found an increase in FeNO starting approximately 10 days before moderate, but not before severe exacerbations. Moderate exacerbations were quantitatively analyzed with novel mathematical methods[23]. The CV, slope, cross-correlation and autocorrelation of the FeNO time-series all showed significant changes prior to moderate exacerbations. Increases in slope were also randomly seen in 19% of 2-week blocks of children without exacerbations. Single FeNO values were not predictive of exacerbations and at least 3-5 data points in 3 weeks were required to detect an upcoming exacerbation.

Previous studies on FeNO in relation to asthma management suggested that using FeNO to guide asthma treatment might reduce the risk of exacerbations[18]. Unfortunately, most earlier studies on FeNO-guided asthma management were underpowered to demonstrate a significant effect on exacerbations[7-10]. In the only

study with sufficient power, FeNO monitoring significantly reduced the number of prednisone courses[11]. However, design issues may have clouded the results[24]. FeNO during acute severe exacerbations was studied previously, and did not correlate with other measures of severity[14,15]. These studies concluded that FeNO is not informative for severe exacerbations. Longitudinal daily FeNO measurements in relation to exacerbations have not been studied before.

In our study we found no evidence that daily FeNO measurements could predict severe exacerbations. However, our study sample of children with severe exacerbations was relatively small. Surprisingly, daily symptoms were not strongly associated with severe exacerbations. We speculate that the symptom diaries might be insensitive for symptoms of severe exacerbations, e.g. because severe obstruction would not be recognized as wheeze, or severe symptoms might have been misinterpreted and hence not properly labelled and scored. That a clinically relevant worsening of symptoms had occurred was evident from the increased use of rescue medication (Figure 3.1). Unfortunately, diary questionnaires are not commonly validated for severe episodes. Spirometric measurements, which could have helped defining both moderate and severe exacerbations, were not obtained concurrently with FeNO. We did not measure PEF or FEV1 at home after ample consideration, because this would require the use of two different measurement devices with essentially different blowing techniques, and this was considered not feasible.

Asthma exacerbations are characterized by increases in airway inflammation, which can differ in type, depending on the pathogenesis that may involve infective or allergic stimuli[25]. FeNO provides indirect information on eosinophilic airway inflammation[2]. Respiratory viruses are associated with neutrophilic airway inflammation, and are responsible for the majority of severe asthma exacerbations with emergency visits, hospitalization and prednisone prescriptions[26,27]. This could explain why FeNO did not show an increase before such exacerbations in the present study. Indeed, experimental rhinovirus infection caused only a small increase in FeNO 1-2 days before an increase in symptoms[28,29]. In contrast, allergen exposure may cause elevated FeNO many days or even weeks before symptoms worsen[30]. In our study, FeNO could detect moderate exacerbations 1-2 weeks before symptoms occurred. We therefore speculate that moderate exacerbations were preceded by increased eosinophilic airway inflammation.

Interestingly, the majority of children with exacerbations had a strong, positive cross-correlation between FeNO and symptoms and autocorrelation before exacerbations compared to the reference periods. We recently found that the level of cross-correlation between FeNO and symptoms in the whole study period was stronger in children with- than in those without exacerbations, and speculated that the level of cross-correlation may be useful to identify children at risk for exacerbations[20,31].

Treatment with inhaled corticosteroids may affect FeNO levels. The magnitude of the change in FeNO and its time course are dose-dependent[32]. A point of consideration is therefore that our monitoring strategy was coupled to therapeutic intervention in the CHARISM study, and might have modified the association of FeNO and exacerbations. Our study was not designed to evaluate individual FeNO changes as a result of steroid dose changes. As an increase in ICS would have reduced FeNO, we would have underestimated the effect[32].

We examined the possibility of false negative episodes by looking at the number of periods in which we found a slope that was higher than the average 2-week blocks slope preceding moderate exacerbations and found higher slopes in 19% of all 2-week blocks in children without exacerbations. However, daily FeNO slopes preceding moderate exacerbations were significantly higher compared to slopes throughout the whole study period in children without exacerbations ($p<.001$). This observation shows that FeNO slope changes are not highly specific for children with imminent exacerbations, though indicative of an increased risk.

Clinical implications

This is a proof-of-concept study, where different types of mathematical techniques were used in a dataset designed for another hypothesis. Long-term daily measurements of FeNO enabled us to look at periods of exacerbations relative to reference periods in the same subject. This analysis showed that there are indeed changes in FeNO before exacerbations compared to reference periods, and quantifies some of these changes. The study sample size was small, 25 moderate exacerbations in 18 patients. There were only 12 severe exacerbations in 9 patients, which may have decreased the power to detect significant associations between daily FeNO and severe exacerbations. We believe however that our sample size was sufficient for a proof of concept study for moderate exacerbations and think that our findings warrant further studies looking at changes in FENO over time in selected populations, which may be better than looking at single and averaged values for monitoring and risk prediction in asthma[23]. Our findings also suggest that regular FeNO measurements in the home setting could help to detect and perhaps even help prevent loss of asthma control. Such monitoring could be especially useful in a selected population with frequent moderate exacerbations.

In conclusion, daily FeNO monitoring revealed changes in FeNO prior to moderate exacerbations of asthma. Whether or not this can be used to prevent loss of asthma control should be further explored.

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Chapter 4

Fluctuation phenotyping based on daily fraction of exhaled nitric oxide values in asthmatic children

The CHARISM Study

Georgette Stern, Johan C. de Jongste, Ralf J.P. van der Valk, Eugenio Baraldi, Silvia Carraro, Cindy Thamrin and Urs Frey

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Abstract

Background

Fractional concentration of exhaled nitric oxide (FeNO), a marker of airway inflammation, has been proposed to be useful for asthma management, but conclusions are inconsistent. This may be due to the failure of mean statistics to characterize individual variability in FeNO, possibly a better indicator of asthma control than single measurements.

Objectives

We characterized fractal fluctuations in daily FeNO over time and the relationship between FeNO and symptoms. We investigated whether these are associated with asthma severity, control, and exacerbation risk.

Methods

Daily FeNO and symptom scores over 192 days in 41 atopic asthmatic children from the CHARISM study were analyzed. Two methods of time series analysis were used: detrended fluctuation analysis to quantify fractal patterns in fluctuations in daily FeNO (α) and cross-correlation to quantify the strength of the relationship between daily FeNO and symptoms. The associations of α and cross-correlation with markers of asthma severity and control were assessed via regression analysis.

Results

Daily fluctuations in FeNO exhibited fractal-type long-range correlations. Those subjects on higher doses of inhaled corticosteroids (ICS) at study entry had a significantly lower α, corresponding to more random fluctuations in FeNO in those with greater ICS need. The cross-correlation between FeNO and symptoms was significantly higher in those subjects that had exacerbations.

Conclusions

Fluctuation in FeNO and its cross-correlation to symptoms contains information on asthma severity and control. Methods that quantify the complexity of asthma over time may assist in identifying asthmatics with concordance between eosinophilic inflammation and symptoms and thus elevated exacerbation risk.

Introduction

The heterogeneous expressions of asthma make it a complex disease to characterize[1]. Clinically, the goal is to achieve asthma control, which may be facilitated by identifying functional asthma phenotypes and reliable markers of disease, and to design a targeted treatment strategy with minimal inhaled corticosteroid (ICS) dose. The fractional concentration of exhaled nitric oxide (FeNO), a biomarker of airway inflammation, has been shown to reflect asthma control[2-4] and therapy response[5-7] and has been proposed to be useful for asthma management[8-11]. Longitudinal randomized controlled trials in which ICS were titrated based on FeNO showed only limited benefits[12,13], but several studies found that longitudinal measurements of FeNO in asthmatics are helpful to predict deterioration[2,14,15]. Furthermore, studies with positive results utilizing FeNO as a predictor of deterioration concluded that the change in FeNO was more predictive than isolated FeNO measurements[2], especially in those with medium to high-dose ICS treatment[14]. This provides support for studying the day-to-day changes in FeNO[16,17] for long-term asthma management.

The study of variability in physiological systems over time allows patients to be considered as nonlinear dynamic systems that are constantly adapting to changes in their environment. Utilizing methods from time series analysis, different aspects of dynamic systems can be quantified. Detrended fluctuation analysis (DFA)[18] is a technique which quantifies the extent to which signal fluctuations are correlated over different time scales, a property consistent with fractal systems. Using DFA, it has been shown that fluctuations in heart-rate dynamics[18-20], tidal breathing[21,22], as well as lung function in adult asthmatics [23,24] exhibit fractal behaviour. Specifically, fractal correlations in fluctuations of peak expiratory flows in adult asthmatics were shown to be related to asthma severity and control[24] and could be used to predict the risk of future obstructive events[24,25] as well as β2-agonist treatment response[23]. Therefore, the quantification of fractal properties in fluctuations in FeNO over long periods of time may provide information on an individual's asthma severity in relation to asthma control and exacerbation risk.

Recent investigations have advocated the need to focus on the multidimensional nature of asthma phenotypes and their temporal pattern in the assessment of asthma control[1,26]. It has been shown that the concordance between FeNO and symptoms may identify a phenotype of adult asthma that has a greater exacerbation risk, with possible therapeutic implications[26]. One method to quantify this relationship between FeNO and symptoms is cross-correlation, which calculates the correlation between two fluctuating signals as a function of a time-lag applied to one of them. This type of analysis can reveal the extent to which different expressions of asthma change together.

In this study, we investigated the fluctuations of FeNO and symptoms over 30 weeks using DFA and cross-correlation, and examined their association with clinical status and asthma severity and control in atopic asthmatic children who participated in a previous study[12]. We hypothesized that information contained within fluctuations in FeNO over time and its relationship to symptoms may be an additional marker to describe individual asthma severity and control.

Methods

Study population and design

We retrospectively analyzed data from the CHildhood Asthma Respiratory Inflammatory Status Monitoring (CHARISM) study[12]; a prospective, open label, randomized, multicenter, parallel-group study in Italy and the Netherlands. Asthma was monitored daily over 30 weeks in 151 mild to moderate atopic asthmatic children aged 6-18 years. The aim of the original study was to investigate whether ICS doses are better titrated based on FeNO and symptoms rather than symptoms only. We analyzed data from the FeNO arm of the trial, where ICS doses were adjusted every 3 weeks based on FeNO and symptoms assessed daily in 77 randomly-assigned children. Prior to analysis we defined a technical inclusion criterion of less than 10% of FeNO data missing out of the first 192 days of the study (<19 days missing), to minimize errors in the DFA[22]. Missing days were filled in with values of the previous day, as detailed previously[24].

FeNO measurements

Daily FeNO telemonitoring was performed using a portable airway inflammation monitor (NIOX MINO®, Aerocrine, Solna, Sweden) with a constant flow of 50 ml/s according to ERS/ATS criteria[27]. Measurements were transferred daily to the study centre via a palm-top computer.

Clinical status

Subjects recorded daily symptom scores, ICS, rescue bronchodilator use, and adverse events in a palm-top electronic diary (PalmOne Tungsten W TrialMax, CRF Inc., Helsinki, Finland). Similar to the Santanello score[28], sleep disturbance and symptom scores of cough, wheeze, and shortness of breath were scored from 0 to 3 based on increasing severity (0 = no symptoms, 1 = occasional symptoms, 2 = symptoms most of the day, and 3 = very bad asthma, unable to perform normal activities). Asthma control was estimated weekly and then averaged over the observation period according to the GINA guidelines[29,30], where symptoms, bronchodilator use,

exacerbations, and lung function contributed to an asthma control rating of controlled (code = 0), partly controlled (= 1), and uncontrolled (= 2). Forced expiratory volume in 1 s (FEV1) and reversibility was measured at clinical visits 5 times during the study. The lung function results were applied to all weeks following the measurement until the next measurement. Weeks were excluded if they did not have at least 6 of 7 days of symptom data in their electronic diary. Symptoms, medications, and asthma control were summarized over the entire study period, at baseline (asthma activity in the first 3 weeks of the study) and at the end of the study (asthma activity in the last 12 weeks).

Exacerbations

In accordance with the 2009 ATS/ERS statement[31], severe exacerbations were defined as one or more days of oral prednisone prescription. Moderate exacerbations were defined as 2 or more days with a total symptom score of greater than or equal to 3 above the average total symptom score of the preceding 2 weeks or 1 or more days with a symptom score greater than 5 above the preceding 2 week average and increased rescue bronchodilator use.

Comorbidities

All subjects had a positive radioallergosorbent test class 2 or higher or a positive skin prick test for at least 1 airborne allergen. Comorbidities were recorded at each clinic visit and entered into the electronic diary by the study nurses. Reports of cold, pharyngitis, fever, flu, otitis, bronchitis, and pneumonia during the study period were classified as 'infectious comorbidities'. Allergic rhinitis, conjunctivitis and eczema, were classified as 'allergic comorbidities'.

Detrended fluctuation analysis

The DFA was used to quantify the extent to which fractal-type correlations were present in the FeNO signal with a single correlation exponent, α[18]. The details of the method have been previously published [24]. Briefly, to calculate α, a FeNO time-series of day-by-day FeNO measurements was integrated and divided into non-overlapping time windows of size n. For each window, the fluctuation function $F(n)$ was calculated by taking the root-mean-square values of the detrended signal[32]. This process was repeated for increasing n and plotted on a log-log plot, where a linear relationship implies $F(n)$ follows a power law functional form $F(n) = A\, n^{\alpha}$, where A is the amplitude of the power law fluctuation function and α is the correlation exponent, which indicates the extent of long-range correlation in the original signal.

One can think of the presence of correlations in a signal as a form of memory, since fluctuations at any certain time point are related to those at previous points

in time. An uncorrelated, random signal such as white noise has an $\alpha = 0.5$. As α increases above 0.5, fluctuations have stronger long-range correlations, i.e. there is a stronger relationship between values at different short- or long-range time scales (e.g. days or weeks).

We chose to analyze the first 192 days of data, which was a compromise between the data length requirements of the DFA calculation[22] and inclusion of subjects with sufficient data points. In order to account for long-term linear trends present in the FeNO time series, a modified detrended fluctuation analysis algorithm[32] was used.

Cross-correlation of FeNO and symptoms

Cross-correlation measures the degree to which two signals are linearly correlated when one of these signals is lagged in time. Similar to a regression coefficient, the strength and direction of this relationship is quantified by a correlation coefficient, where a positive coefficient indicates that as one variable increases, so does the other, and a negative coefficient indicates an inverse relationship between the variables. In this study, FeNO was the reference signal, and symptoms were shifted in time. Thus, when a maximum positive cross-correlation is seen at a lag of 2, the strongest relationship between FeNO at present is with symptoms measured 2 days later. We calculated the cross-correlation between daily FeNO and daily sum of symptom scores, where symptoms were lagged by FeNO within ± 7 days to allow for the possibility that increases in symptoms could precede or follow increases in FeNO.

Statistical analysis

Associations between parameters of clinical asthma control status (exposures) and a or cross-correlation of symptoms/FeNO (outcomes) were examined separately using multiple linear regression models adjusted for age, height and sex. Associations between occurrence of exacerbation (exposure) and α or cross-correlations (outcome) were examined using multiple linear regressions adjusted for mean ICS dose. Severe and moderate exacerbations were also examined separately. The potential confounding effect of infectious and allergic comorbidities as well as medications on the above associations was investigated. First, univariate associations of each confounder with mean FeNO, α and cross-correlation coefficient were investigated with linear regression. Second, significantly associated parameters from these univariate investigations were included as confounders in the above multiple linear regression analysis. In addition, we examined the contribution of treatment decisions on fluctuations in FeNO by calculating the cross-correlation between FeNO and ICS in three-week windows. Statistical analyses were performed using Intercooled Stata 10 for Windows (Stata Corporation, College Station, Texas, USA).

Results

Forty-one children met the inclusion criterion of less than 10% data missing of daily FeNO and symptoms out of the first 192 days of the study, resulting in a total of 7,820 measurements of FeNO for analysis. Table 4.1 summarizes the subject characteristics

Table 4.1. Characteristics of study population

	All Included Subjects (N = 41)	**Asthma Exacerbations Severe (N = 5) Moderate (N = 10)**	**No Asthma Exacerbation (N = 26)**	**P Value***
Characteristics				
Sex, male, N (%)	20 (49)	4 (27)	16 (62)	0.03
Age, years	10.9 ± 2.4	10.9 ± 2.4	10.8 ± 2.4	0.92
Weight, kg	39.6 ± 12.1	40.6 ± 12.5	39.1 ± 12.1	0.53
Height, cm	146.3 ± 15.5	146.2 ± 14.7	146.4 ± 16.2	0.90
Medication, N (%)				
Antihistamine	19 (46)	7 (47)	12 (47)	0.98
Montelukast	11 (27)	5 (33)	6 (23)	0.48
Nasal-steroid	15 (37)	5 (33)	10 (38)	0.75
Long-acting β-agonist	30 (73)	11 (73)	19 (73)	0.46
Mean ICS dose: $\mu g \cdot day^{-1}$, median[l]	309 (90-1080)	469 (90-1080)	308 (89-1160)	0.77[†]
Mean bronchodilator use: puffs/day, median[l]	0.88 (0.16-3.29)	1.32 (0.34-5.05)	0.59 (0.11-3.23)	0.02[†]
Lung function and symptoms				
Geometric mean FeNO, ppb	19.8 ± 8.6	18.8 ± 6.4	20.4 ± 9.6	0.79
Mean FEV1, % predicted[‡]	97.6 ± 11.3	96.0 ± 9.8	98.6 ± 12.1	0.49
Mean reversibility of FEV1, % pred[§]	6.2 ± 6.7	6.9 ± 5.5	5.9 ± 7.3	0.65
Mean asthma control[ll]	0.83 ± 0.46	1.12 ± 0.38	0.66 ± 0.42	0.0005
Symptom free days, median[l]	88 (2-176)	75 (2-166)	106 (1-182)	0.19[†]
Mean daily sum symptom score, median[l]	0.87 (0.15-3.34)	1.32 (0.29-5.33)	0.62 (0.10-3.27)	0.02[†]
Comorbidities, N (%)				
Infectious comorbidities	25 (61)	11 (73)	14 (54)	0.22
Allergic comorbidities	13 (32)	5 (33)	8 (31)	0.87
FeNO fluctuation				
α	1.03 ± 0.13	1.05± 0.13	1.02 ± 0.14	0.51
Cross-correlation	0.15 ± 0.21	0.27 ± 0.18	0.09 ± 0.21	0.01

Mean (standard deviation) values are provided unless otherwise indicated. *: Assessed via student's t test. †: Assessed via Wilcoxon rank-sum test. l: Expressed as 95% confidence intervals. ‡: Average of 5 FEV1 tests in weeks 1, 3, 12, 21 and 30. §: Average of 5 reversibility FEV1 tests. ll: Mean asthma control classified weekly as controlled = 0, partly controlled = 1, or uncontrolled = 2.

stratified by severe, moderate, or no asthma exacerbation during the study period. In the whole study group, FeNO measured over 192 days displayed a considerable amount of fluctuation. Two examples of a FeNO time series over 192 days from subjects with no changes in steroid dose can be seen in Figure 4.1.

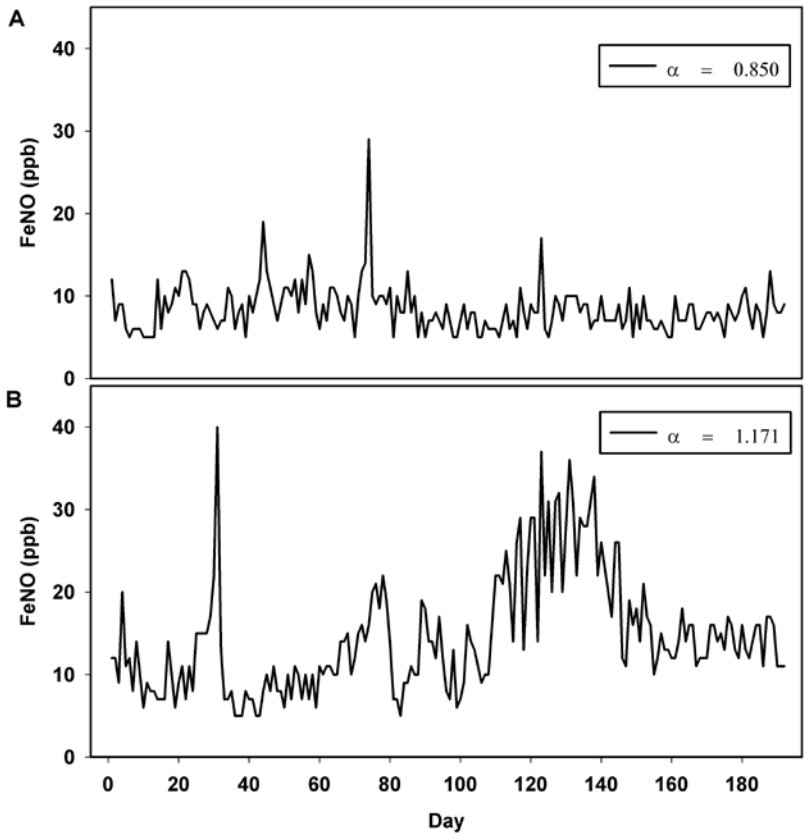

Figure 4.1. Exhaled nitric oxide over 192 days from two subjects with no changes in ICS dose during study period

Upper panel: Subject with relatively small range of FeNO [5 - 30 parts per billion (ppb)], constant ICS dose of 1000 µg/day throughout the study, and weak long-range correlation in FeNO reflected by an α of 0.850. Lower panel: Subject with relatively large range of FeNO [6 - 40 ppb], constant ICS dose of 400 µg/day throughout the study, and stronger long-range correlations reflected by an α of 1.171.

Detrended fluctuation analysis

Fluctuations in FeNO values recorded daily over 192 days were correlated on time scales of more than a month (long-range correlation) with a mean long-range correlation exponent of (α = 1.03 (SD 0.13)).

Cross-correlation of FeNO and symptoms

More subjects (n = 10) had their strongest correlation between FeNO and symptoms on the same day (lag 0) than for any other day, whereas the rest of the subjects had maximum correlation when symptoms were shifted with respect to FeNO within ± 5 days. In order to maintain comparability, we used the cross-correlation at lag 0. The cross-correlation coefficient of FeNO and symptoms ranged from -0.31 to 0.59, where 23 subjects had a significantly positive relationship between FeNO and symptoms ($p < 0.05$), 4 subjects had a significantly negative relationship, and 14 had a weak relationship (not significant). An example of a subject with strong positive correlation can be seen in Figure 4.2. In this subject, the strongest cross-correlation occurred when FeNO preceded symptoms by 2 days (lag=2).

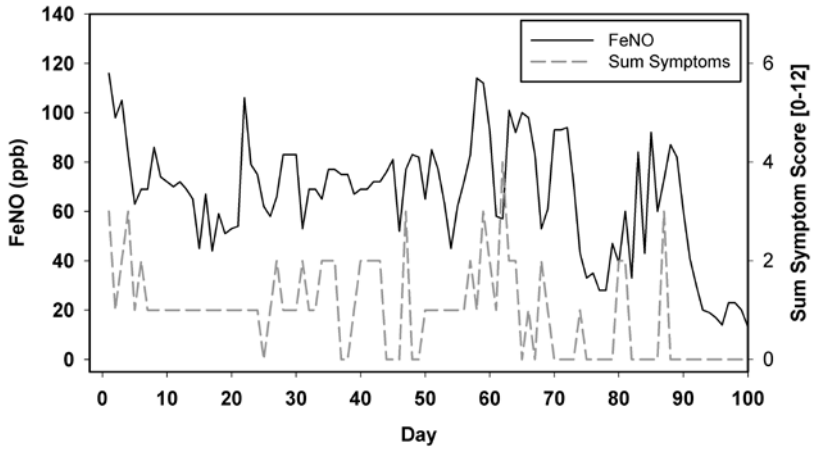

Figure 4.2. Cross-correlation of daily FeNO and symptoms

Day-by-day FeNO and symptoms in a subject with a strong concordance between the FeNO and symptoms. Here, the maximum correlation occurred when increases in FeNO preceded increases in symptoms by 2 days (lag 2): cross-correlation; (lag 0) 0.558, (lag 1) 0.607, (lag 2) 0.620.

Relationship with clinical status

The long-range correlation exponent α was negatively associated with ICS dose at baseline (Table 4.2), but was not found to be associated with ICS, symptoms, and asthma control averaged over the whole period, or in the last 12 weeks of the study. Furthermore, when stratified by mean asthma control over the study period, there was a significant decrease in α with ICS dose only in the uncontrolled children (mean asthma control score >1 over the study period, n = 14, 71% of whom had asthma exacerbations) of 0.03 per 100mg/day increase in ICS (95%CI -0.05 to -0.01, p

Table 4.2. Association of α and cross-correlation with clinical status

	Multivariable Models*					
Effect	**Long-range correlation α†**			**Cross-Correlation‡**		
	Coeff.	**95% CI**	**P value**	**Coeff.**	**95% CI**	**P value**
Baseline§ Mean FeNOǁ	0.0007	-0.0019 to 0.0034	0.58	0.0038	-0.0006 to 0.0082	0.09
Baseline Symptom Score	-0.017	-0.057 to 0.021	0.36	0.019	-0.048 to 0.086	0.58
Baseline ICS Dose**	-0.013	-0.025 to -0.001	0.04	0.002	-0.020 to 0.024	0.86
Baseline Asthma Control	-0.013	-0.095 to 0.069	0.76	0.12	-0.02 to 0.25	0.09
Mean FeNO	0.001	-0.004 to 0.006	0.74	0.004	-0.004 to 0.012	0.35
Mean Sum Symptoms	-0.016	-0.053 to 0.021	0.38	0.0004	-0.0638 to 0.0647	0.99
Mean ICS Dose**	-0.009	-0.021 to 0.004	0.15	0.02	0.00 to 0.04	0.11
Mean Asthma Control	0.010	-0.082 to 0.102	0.82	0.08	-0.08 to 0.23	0.32
Mean FeNO at study end††	0.001	-0.004 to 0.006	0.76	-0.001	-0.009 to 0.008	0.91
Mean symptom score at study end	-0.017	-0.052 to 0.018	0.33	0.018	-0.043 to 0.079	0.55
Mean ICS dose at study end**	-0.006	-0.018 to 0.006	0.32	0.008	-0.013 to 0.029	0.44
Mean asthma control at study end	0.016	-0.067 to 0.098	0.70	0.08	-0.06 to 0.22	0.23

*: Model was adjusted for age, height, and sex. †: Long-range correlations calculated using detrended fluctuation analysis adjusted for linear trend. ‡: Cross-correlation between FeNO and symptoms on the same day (lag 0). §: Clinical status parameters assessed in the first 3 weeks of the study. ǁ: Geometric mean of FeNO. **: Calculated as per 100 mg increase in ICS. ††: Clinical status parameters assessed in the last 12 weeks of the study.

= 0.007). The strength of cross-correlation between FeNO and symptoms was not related to clinical status or ICS dose throughout the study.

Relationship with exacerbations

Of the included subjects, 5 had a severe asthma exacerbation and 10 had moderate exacerbations. Two of those with severe exacerbations had more than 1 prednisone course, and 4 of those with moderate exacerbations had more than 1 moderate exacerbation. There was no difference in mean FeNO in the exacerbation groups (Table 4.1). Both α and cross-correlation were able to distinguish between the exacerbation group (severe and moderate) and the group with no exacerbations. The strength of long-range correlations, α tended to be higher in those children that had severe exacerbations, but the differences were not significant. Even when adjusted for mean ICS dose during the study, the strength of cross-correlation between FeNO and symptoms (Figure 4.3) was greater in those who had either a severe or moderate exacerbation by 0.17 (95% CI 0.018 to 0.30, p = 0.030) compared to those who did not have an exacerbation.

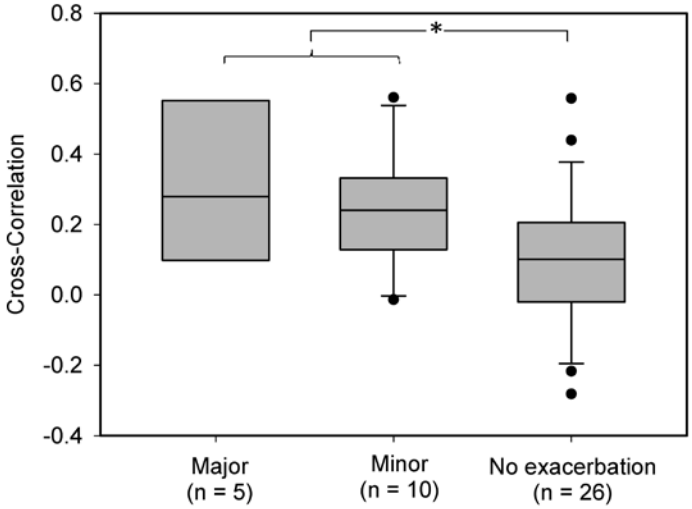

Figure 4.3. Cross-correlation of daily FeNO and symptoms in exacerbation groups
Box plots of cross-correlation between symptoms and FeNO stratified by severe, moderate or no exacerbation during the study period. Boxes represent 25th and 75th percentiles with median line and error bars representing 10th and 90th percentiles.

Effect of comorbidities and medications

Children who took antihistamines had a borderline increased geometric mean FeNO of 5.13 ppb (95% CI -0.10 to10.37, p = 0.054) and a stronger of cross-correlation of FeNO and symptoms by 0.13 (95% CI 0.00 to 0.26, p = 0.043) compared to those that did not take antihistamines in a univariate model. These effects disappeared in a multivariable model, while the relationship between exacerbations and cross-correlation remained significant. In addition, there was a significant feedback of changes in ICS on mean FeNO in 71% of the subjects, but this was not related to the long-range correlation exponent, α. In a receiver operator curve analysis (ROC), the cross-correlation coefficient of FeNO and symptoms was the best predictor for exacerbations (Figure 4.4) compared to mean FeNO (p = 0.033), mean symptoms (p = 0.80), and α (p = 0.16). A cut-off in cross-correlation above which significant cross-correlation between FeNO and symptoms was found across all subjects (cross-correlation = 0.134) had a positive predictive value (PPV) of 54% and a negative predictive value (NPV) of 84% (sensitivity 80%, specificity 61%).

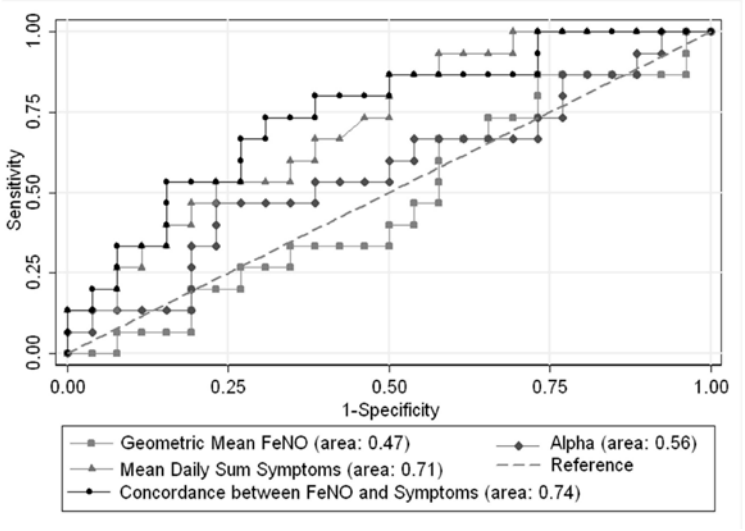

Figure 4.4. Receiver operator curves

Receiver operator curves for mean FeNO, mean sum symptoms, the cross-correlation between FeNO and symptoms, and the long-range correlation coefficient α to predict exacerbations. The cross-correlation coefficient of FeNO and symptoms was the best predictor for exacerbations compared to mean FeNO (p=0.033), mean symptoms (p=0.80), and α (p=0.16).

Discussion

In our study of FeNO recorded daily over 192 days in atopic asthmatic children[12], we found that fluctuations in FeNO were not random, but were correlated over time scales of more than a month (long-range correlation). The long-range correlation exponent, α, was found to be lower in those subjects requiring higher ICS doses at study entry. Thus, fluctuations in FeNO were more random in children requiring higher ICS doses. However, α was not related to mean asthma control as defined by the GINA guidelines. Although mean FeNO levels were not predictive, the day-by-day cross-correlation of FeNO and symptoms was significantly stronger in those subjects that had severe or moderate exacerbations, even when adjusting for mean ICS dose.

Long-range correlations

There is accumulating evidence that there is a healthy amount of variability in physiologic systems with a complex, but organized structure. The outputs of such systems have been shown to contain long-range, fractal correlations, a property which may render them more adaptive to external perturbations. These fractal correlations have been shown to break down with disease, possibly reflecting a reduction in the adaptive ability of the system and prospective critical events. In asthmatics, the study of long-range, fractal correlations in lung function has been shown to provide valuable

information on the complex nature of asthma[23,24]. We have previously shown that patients with a more random pattern in twice-daily peak expiratory flows had more severe asthma[24], poor treatment response[23], and higher probability for an obstructive event[24]. Our present findings indicate that long-range, fractal correlations are also manifested in fluctuations of an inflammatory marker of asthma, FeNO.

We found that long-range correlations in fluctuations in daily FeNO were associated with ICS doses, but were not related to clinical status, and were not predictive of treatment response throughout the study or at the end of the study. This could be due to the constant adaptation of ICS to FeNO levels every 3 weeks, as a consequence of the original study design. This association between α and mean ICS dose, particularly when stratified by asthma control, is likely to be complex. While GINA guidelines proposed that asthma severity could best be determined in the untreated patient[30], recent guidelines[33,34] suggest that asthma severity may be a function of asthma control, as it is related to ICS treatment that can mask the underlying disease process. Our data provide evidence that the relationship between ICS dose, severity of asthma and long range correlations in FeNO were most obvious in poorly controlled asthmatics. This might be due to the amount of the disease process that is not masked by treatment.

The characterizations of long-range correlations in twice-daily peak-expiratory flows in asthmatics in combination with variability potentially offer the possibility to predict future obstructive events [24,25]. In this study we provide the first evidence that fluctuations in daily inflammatory markers also contain long-range correlations. Based on the present findings it is not possible to draw conclusions on the predictive ability of long-range correlations in FeNO, as our data only show a trend for higher α in those that had exacerbations. Future trials of fluctuation analysis of daily FeNO in steroid naïve asthmatics before and after ICS treatment could help to elucidate the relationship between severity of the underlying disease process and asthma control in relation to ICS dose.

Concordance of FeNO and symptoms

Although the relationship between FeNO and symptoms has been investigated[35-37], this is the first study that compares day-by-day FeNO and symptoms over such a long period of time using time-series analysis techniques in asthmatic children. In our study population, we found that the majority of subjects had the strongest positive relationship between FeNO and symptoms on the same day. Subjects who had severe or moderate exacerbations had a stronger positive cross-correlation between FeNO and symptoms, suggesting that concordance of FeNO and symptoms is an indicator of elevated risk of exacerbation.

This concept of concordance between FeNO and symptoms has recently been advocated by Haldar *et al*[26] via phenotype cluster analysis of an asthma cohort. They found that within a group of adult asthma patients, there are subjects in whom FeNO is strongly associated with asthma symptoms, whereas in others, this relationship is weak. They suggested a new concept of 'concordant' and 'discordant' phenotypes of asthma. In our study population of atopic asthmatic children, such a distinction may explain why in some patients FeNO and symptoms are dissociated in time. Cross-correlation analysis of biomarkers and symptoms fluctuating over time might be a new way of quantifying such concordance and may help to identify new 'fluctuation phenotypes' of asthma. This is particularly important since there is increasing evidence that asthma therapy might benefit from a more individualized and phenotype-specific approach[26,38-40]. Therefore, the temporal concordance between fluctuations in FeNO and symptoms, quantified by cross-correlation methods, could help to identify an elevated risk of asthma instability and exacerbation risk. Moreover, asthmatics in which FeNO and symptoms are discordant might specifically benefit from FeNO monitoring.

In a ROC analysis, we see that the cross-correlation between FeNO and symptoms is the best predictor of exacerbations compared to the other predictor variables, but only slightly better than symptoms. Since the definition of exacerbations depends on symptoms, the usefulness of symptoms as a predictor is limited. Therefore the cross-correlation of FeNO and symptoms is attractive as an independent predictor of exacerbations. If we use a cut-off cross-correlation coefficient of 0.134, we find a PPV of 54% and a NPV of 84% (sensitivity 80%, specificity 61%). This is similar to the predictive value of FeNO to predict loss of control found in other studies [2,41]. As the cut-off value for concordance is derived from this relatively specific dataset, with no information on physiological meaningfulness, the predictive ability should be taken with caution.

Limitations of the study

In human studies over long periods of time, it is impossible to control the external environment. In this study where asthma was monitored over 30 weeks, not only were children exposed to various undocumented external factors such as the school environment, local pollution, allergens, but their asthma medication was potentially changed every 3 weeks, depending on FeNO and symptoms. Since ICS have an effect on FeNO, one could presume that this would have an effect on FeNO variability and asthma control, as well as contribute to the intrinsic correlations in FeNO. While we could show that there was a strong cross-correlation between FeNO and ICS dose in most subjects, we did not find an impact of this feedback on long-range correlations in FeNO. We also did not find an impact of the average ICS dose or the number of ICS step changes, implying that the amount of ICS dose that a subject received did

not seem to impact the level of internal correlation. Better insight in FeNO behaviour might be possible in a study in which ICS doses do not depend on FeNO.

We excluded 34 children due to the inclusion criterion of less than 10% missing data. These children tended to be older and have higher FeNO values. They did not differ in terms of ICS doses at study entry, but due to titration of ICS based on FeNO, tended to have higher ICS doses throughout the study. Whereas the exclusion of this population did not affect our main findings, one should be cautious when extrapolating these results to a more general asthma population.

Clinical implications and future hypotheses

As proposed in a recent review[1], monitoring multiple disease parameters over time and characterizing their fluctuations may provide new tools to characterize the dynamic, complex nature of asthma better than mean values. In our proof of concept study we show that by monitoring fluctuations in symptoms and FeNO, we can better identify which children are at risk for exacerbations compared to cut-off or mean FeNO values. Furthermore, by studying long-range patterns in FeNO, we may be able to better characterize of asthma severity and control, which could aid in treatment decisions. We speculate that the application of time-series analysis to clinical parameters could help to define new fluctuation phenotypes of asthma. Although this dataset was not ideal to answer all our questions on the clinical utility of such methods, future studies with a prospective design may be able to disentangle the effect of ICS on correlations in FeNO, and help to determine which treatment will be most effective for a given patient. Presently, FeNO analyzers are still relatively expensive and complex, but with the foreseen development of cheaper, simpler devices, daily monitoring of FeNO may become a reality for clinical practice. Such methods are attractive as we move towards patient-initiated personalized medicine.

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Chapter 5

Common variants at 17q11.2-q12 and 17q12-q21 are associated with fractional exhaled nitric oxide in childhood

EAGLE Consortium Study

Ralf J.P. van der Valk*, Liesbeth Duijts*, Nicholas J. Timpson, Muhammad T. Salam, Marie Standl, John A. Curtin, Jon Genuneit, Marjan Kerhof, Eskil Kreiner-Møller, Alejandro Cáceres, Anna Gref, Liming L. Liang, Rob H. Taal, Emmanuelle Bouzigon, Florence Demenais, Rachel Nadif, Carole Ober, Emma E. Thompson, Karol Estrada, Albert Hofman, André G. Uitterlinden, Cornélia van Duijn, Fernando Rivadeneira, Xia Li, Sandrah P. Eckel, Kiros Berhane, W. James Gauderman, Raquel Granell, David M. Evans, Beate St Pourcain, Wendy McArdle, John P. Kemp, George Davey Smith, Carla M.T. Tiesler, Claudia Flexeder, Angela Simpson, Clare S. Murray, Oliver Fuchs, Dirkje S. Postma, Klaus Bønnelykke, Maties Torrent, Martin Andersson, Patrick Sleiman, Hakon Hakonarson, William O. Cookson, Miriam F. Moffatt, Lavinia Paternoster, Erik Melén, Jordi Sunyer, Hans Bisgaard, Gerard H. Koppelman, Markus Ege, Adnan Custovic, Joachim Heinrichs, Frank D. Gilliland, Alexander J. Henderson**, Vincent W.V. Jaddoe**, Johan C de Jongste**
for the EArly Genetics & Lifecourse Epidemiology (EAGLE) Consortium

**Both authors contributed equally*
***These authors jointly directed this work*

Submitted

Abstract

Background

The fractional concentration of nitric oxide in exhaled air (FeNO) is a biomarker of eosinophilic airway inflammation and associated with childhood asthma. Identification of common genetic variants associated with childhood FeNO may suggest biological mechanisms related to specific asthma phenotypes.

Objectives

To identify common genetic variants associated with childhood FeNO, and their relation with asthma.

Methods

In 14 independent pediatric discovery genome-wide association (GWA) studies of FeNO (N = 8,858), we examined association statistics of ~2.5 million single nucleotide polymorphisms (SNPs). Subsequently, we assessed whether significant SNPs were expression quantitative trait loci (eQTLs) in genome-wide expression datasets of lymphoblastoid cell lines (N = 1,830). Significant SNPs and their relation with asthma were tested in a previously published GWA dataset of physician-diagnosed asthma (cases: n=10,365; controls: n=16,110).

Results

FeNO was measured online in children aged 5 to 15 years. We identified 3 SNPs associated with FeNO: rs3751972 in *LYR motif containing 9* (*LYRM9*) ($P = 1.97x10^{-10}$) and rs944722 in *nitric oxide synthase 2, inducible* (*NOS2*) ($P = 1.28x10^{-9}$) both located at 17q11.2-q12, and rs8069176 near *gasdermin B* (*GSDMB*) ($P = 1.88x10^{-8}$) at 17q12-q21. We found a *cis* eQTL for the transcript *lectin, galactoside-binding, soluble, 9* (*LGALS9*) that is in linkage disequilibrium with rs944722. rs8069176 was associated with *GSDMB* and *ORM1-like 3* (*ORMDL3*) gene expression. rs8069176 at 17q12-q21, not rs3751972 and rs944722 at 17q11.2-q12, were associated with physician-diagnosed asthma.

Conclusions

This study highlights that both shared and distinct genetic factors affect FeNO and childhood asthma.

Introduction

Asthma is a complex disease with different phenotypes, influenced by many genetic and environmental factors[1]. The mechanisms leading to specific asthma phenotypes are poorly understood[2,3]. Recent genome-wide association (GWA) studies provided evidence that different common genetic variants are associated with specific asthma-related outcomes such as childhood onset asthma[4-6], adult asthma[5-7], impaired lung function[8-11], and atopy[12-14].

The fractional concentration of nitric oxide in exhaled air (FeNO) is a non-invasive biomarker of eosinophilic airway inflammation[15-17]. Higher FeNO is associated with childhood asthma symptoms[18], exacerbations[19], physician-diagnosed asthma[15-17] and atopy[20]. Nitric oxide (NO) is a reactive free-radical gas generated in the airway epithelium when L-arginine is oxidized to L-citrulline[17]. This reaction is catalyzed by nitric oxide synthases (NOS), that are upregulated in the presence of pro-inflammatory cytokines and inflammatory mediators[17]. NO regulates airway and blood vessel tone and high NO concentrations have antimicrobial effects[17]. Although 60% of the variation of FeNO in adults can be explained by heritability[21], the genetic loci that influence FeNO are largely unknown. Identification of common genetic variants associated with childhood FeNO may help to define biological mechanisms related to specific asthma phenotypes[2,3,22,23].

To identify common genetic variants associated with childhood FeNO, we examined the association of ~2.5 million directly genotyped and imputed single nucleotide polymorphisms (SNPs) and FeNO in 14 independent pediatric discovery GWA studies (N = 8,858).

Methods

FeNO was measured online in children aged 5 to 15 years according to European Respiratory Society (ERS) and American Thoracic Society (ATS) guidelines[16]. FeNO was natural-log transformed to obtain a normal distribution. We applied linear regression between allele dosages obtained from imputations and natural-log FeNO adjusted for sex and age at time of measurement. Details on the discovery analysis and additional analyses are presented in the Supplementary Methods section, and an overview of our study design is outlined in Figure 5.1. Details on individual study characteristics, SNP genotyping platforms and study association analyses are provided in Supplementary Table 5.1.

We assessed whether significant SNPs or SNPs in linkage disequilibrium (LD) with our lead SNPs were expression quantitative trait loci (eQTLs) in genome-wide

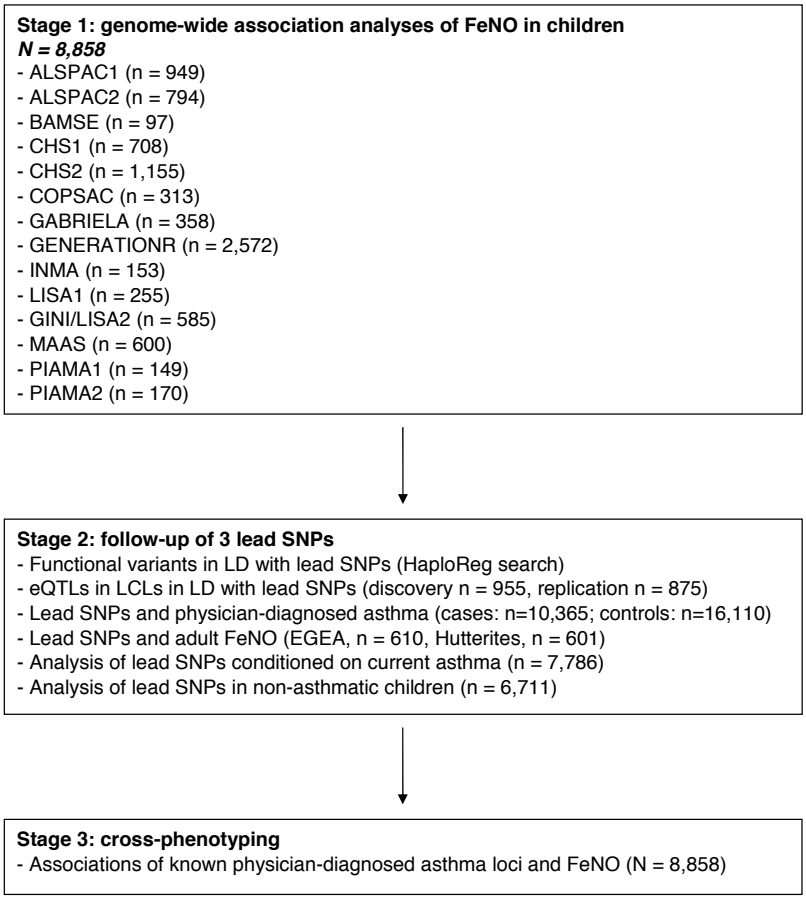

Figure 5.1. Study design

SNPs, single nucleotide polymorphisms; LD, linkage disequilibrium; eQTLs, expression quantitative trait loci; LCLs, lymphoblastoid cell lines.

expression datasets of lymphoblastoid cell lines (LCLs) (N = 1,830)[24,25]. eQTLs are genomic loci that regulate expression levels of messenger RNAs. We also tested the relation of significant SNPs with asthma in a previously published GWA dataset of physician-diagnosed asthma (cases: n=10,365; controls: n=16,110)[5]. We explored whether the identified SNPs were related with FeNO in adults in the Epidemiological study on the Genetics and Environment of Asthma (EGEA) and in Hutterites (N=1,211). We also explored whether common genetic variants known to be associated with physician-diagnosed asthma[5] were related with childhood FeNO. The institutional review boards for human studies approved the protocols and written consent was obtained from the participating subjects or their caregivers if required by the institutional review board.

Results

We identified genome-wide significant ($P < 5x10^{-8}$) association of childhood FeNO and SNPs at 3 loci. Two of these were located at chromosome 17q11.2-q12 (Table 5.1): rs3751972 in *LYR motif containing 9* (*LYRM9*) and rs944722 in *nitric oxide synthase 2, inducible* (*NOS2*). Each C allele of rs3751972 was associated with higher ln(FeNO) (β = 0.086 ppb; S.E. = 0.014; $P = 1.97x10^{-10}$; explained variance = 0.23%), and each C allele of rs944722 was associated with lower ln(FeNO) (β = -0.073 ppb; S.E. = 0.012; $P = 1.28x10^{-9}$; explained variance = 0.30%). rs3751972 and rs944722 are in neighbouring loci with low LD (HapMap pairwise LD (phase II release 22 CEU); D' = 0.237, $r^2 = 0.014$). A third SNP (rs8069176) near *gasdermin B* (*GSDMB*) at 17q12-q21 was also associated with childhood FeNO. Each A allele of rs8069176 was associated with lower ln(FeNO) (β = -0.066 ppb; S.E. = 0.012; $P = 1.88x10^{-8}$; explained variance = 0.41%). Figure 5.2-5.4 show the QQ-, Manhattan-, regional association- and forest plots of the 3 signals.

Table 5.1. Summary statistics of the 3 SNPs at $P < 5x10^{-8}$

Marker	**MAF**	***β***	**S.E.**	***P***	I^2	***HetP***
rs3751972[C] at 17q11.2 (*LYRM9*)	0.25	0.086	0.014	$1.97x10^{-10}$	27.4	0.161
rs944722[C] at 17q11.2-q12 (*NOS2*)	0.38	-0.073	0.012	$1.28x10^{-09}$	37.8	0.075
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.43	-0.066	0.012	$1.88x10^{-08}$	0.0	0.668

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Independent SNPs with a genome-wide significant effect on FeNO levels in children are shown ($P < 5x10^{-8}$). The total sample includes data of 14 independent GWA datasets (N = 8,858). MAF, minor allele frequency; S.E., standard error. β reflects differences in natural log-transformed FeNO per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for sex and age at time of measurement (fixed-effect additive genetic model). Derived inconsistency statistic I^2 and *HetP* values reflect heterogeneity across studies with the use of Cochran's Q tests.

We performed a conditional analysis using the genome-wide complex trait analysis (GCTA) tool conditioning on all SNPs of the meta-analysis[26] and showed that rs3751972 and rs944722 were indeed independent signals (Supplementary Table 5.2). After conditioning on all SNPs of the meta-analysis, rs3751972 and rs2274894 showed the strongest association in *LYRM9* ($P = 2.06x10^{-9}$) and in *NOS2* ($P = 1.50x10^{-8}$), respectively. Using the same approach, rs8069176 showed the strongest association at 17q12-q21 ($P = 2.14x10^{-8}$). The conditioned SNP effect estimates were largely unaffected (all SNPs < 6% difference in effect estimate as compared to unconditioned SNP effect estimates).

The 3 genome-wide significant SNPs showed low heterogeneity between studies (all $P \geq 0.075$, $I^2 = 0 - 37.8\%$). The 3 SNPs together explained 0.95% of the variance

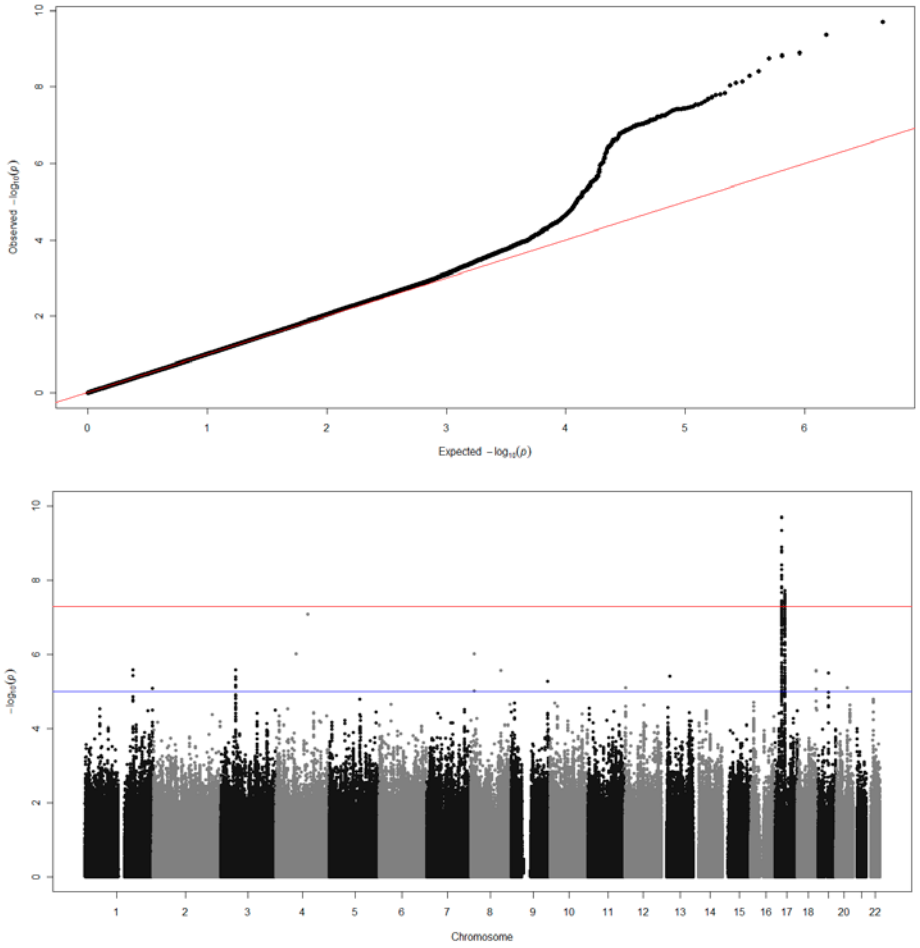

Figure 5.2. QQ and Manhattan plot of 2,253,077 SNPs of 14 GWA studies (N = 8,858)

Quantile-Quantile (QQ) plot (upper panel) of 2,253,077 single nucleotide polymorphisms (SNPs) of 14 genome-wide association (GWA) studies (N = 8,858). The black dots represent observed *P* values and the red line represents the expected *P* values under the null distribution. Manhattan plot (lower panel) showing the association *P* values of FeNO from the 14 studies. The $-\log_{10}$ of the *P* value for each of 2,253,077 SNPs (y-axis) is plotted against the genomic position (NCBI build 36; x-axis). Model: ln(FeNO) = SNP + sex + age. For two samples with non-Caucasian children adjustment of principal components was applied. SNP filters: minor allele frequency 5%, imputation accuracy, SNP had to be available in at least 8 cohorts.

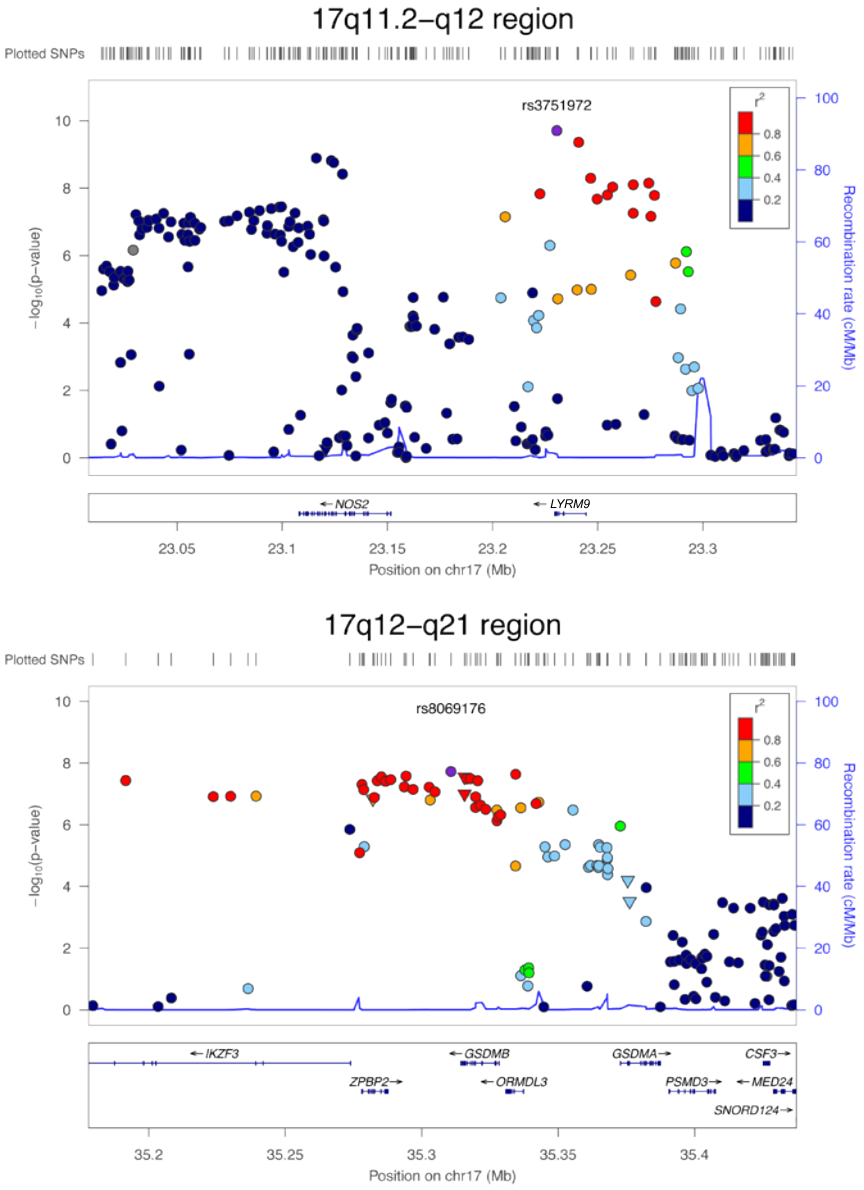

Figure 5.3. Association plots of the 17q11.2-q12 and 17q12-q21 regions

For each of the 17q11.2-q12 (upper panel) and 17q12-q21 (lower panel) regions, single nucleotide polymorphisms (SNPs) are plotted with their *P* values (as –log10 values; left y-axis) as a function of genomic position (NCBI Build 36; x-axis). Estimated recombination rates (right y-axis) taken from HapMap are plotted to reflect the local linkage disequilibrium (LD) structure around the top associated SNP (represented by a purple circle) and their correlated proxies (according to a blue to red scale from $r^2 = 0$ to 1, based on pairwise r^2 values from HapMap CEU). Triangles represent nonsynonymous SNPs in the region.

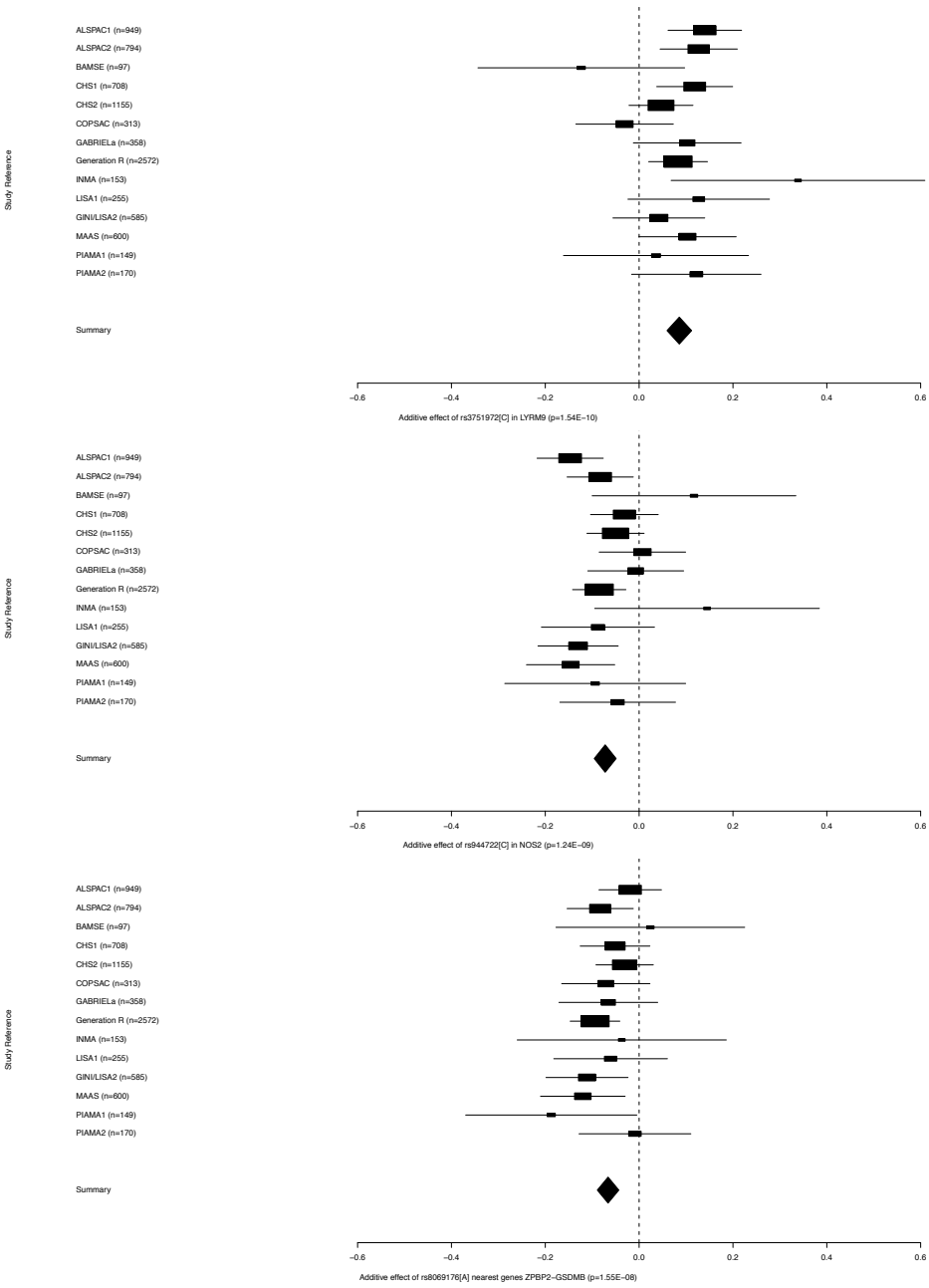

Figure 5.4. Forest plots of the associations between FeNO and the 3 SNPs associated with FeNO at $P < 5x10^{-8}$

Forest plots of the associations between FeNO and single nucleotide polymorphisms (SNPs) in *LYRM9* (a), *NOS2* (b) and near *ZPBP2-GSDMB* (c) at $P < 5x10^{-8}$. In each plot, the triangle indicates the effect size and the confidence interval of the 14 studies. The *P* values in the plots are without genomic control correction.

in FeNO. Other suggestive loci, that did not reach genome-wide significance ($P < 1x10^{-5}$), associated with FeNO in childhood, are given in Supplementary Table 5.3 and 5.4.

We assessed whether there were common non-synonymous functional variants in LD ($r^2 > 0.80$) with our 3 genome-wide significant SNPs using HaploReg[27]. We found 3 variants, rs11557467, rs2305480 and rs2305479 in high LD with rs8069176 at 17q12-q21 (HapMap; $r^2 = 0.84$, 1.00 and 0.90, respectively). Rs11557467 is located in *zona pellucida binding protein 2* (*ZPBP2*), and rs2305480 and rs2305479 in *GSDMB*.

Subsequently, we assessed whether the identified 3 loci were eQTLs in genome-wide expression datasets of LCLs (N = 1,830)[24,25]. We found a *cis* eQTL for the transcript *lectin, galactoside-binding, soluble, 9* (*LGALS9*) in LD with rs944722 in two independent datasets (Supplementary Tables 5.5 and 5.6). rs8069176 was associated with both *GSDMB* and *ORM1-like 3* (*ORMDL3*) gene expression. We did not find eQTLs for rs3751972.

Table 5.2. Association of the 3 SNPs related to childhood FeNO with physician-diagnosed asthma and adult FeNO

Physician-diagnosed asthma (cases = 10,365 : controls = 16,110)[5]			
Marker	**OR (95% CI)**		***P***
Proxy for rs3751972: rs4796222[A] (r^2=1.000; D'=1.000) at 17q11.2 (*LYRM9*)	0.98 (0.93-1.02)		0.303
Proxy for rs944722: rs2274894[T] (r^2=0.967; D'=1.000) at 17q11.2-q12 (*NOS2*)	1.00 (0.96-1.04)		0.983
Proxy for rs8069176: rs2305480[A] (r^2=1.000; D'=1.000) at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.85 (0.81-0.88)		$7.93x10^{-17}$
Adult FeNO			
Marker (EGEA, n = 610)	***β***	**S.E.**	***P***
rs3751972[C] at 17q11.2 (*LYRM9*)	0.125	0.065	0.057
rs944722[C] at 17q11.2-q12 (*NOS2*)	-0.015	0.061	0.802
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	-0.113	0.062	0.067
Marker (Hutterites, n = 601)	**Z score**		***P***
Proxy for rs3751972: rs4796228[G] (r^2=0.659; D'=1.000) at 17q11.2 (*LYRM9*)	-1.536		0.125
Proxy for rs944722: rs2314809[T] (r^2=0.967; D'=1.000) at 17q11.2-q12 (*NOS2*)	-2.322		0.020
Proxy for rs8069176: rs11078927[T] (r^2=1.000; D'=1.000) at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.505		0.613

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Independent SNPs with a genome-wide significant effect on FeNO levels in children are shown ($P < 5x10^{-8}$) in relation to physician-diagnosed asthma[5] and adult FeNO. S.E., standard error. Odds ratios (OR) with 95% confidence intervals (CI) for physician-diagnosed asthma[5]. *β* reflects differences in natural log-transformed FeNO per minor allele for adult FeNO in EGEA. Z-score reflects the strength of the association between SNP and natural log-transformed FeNO and the direction of the effect of the minor allele in Hutterites.

We tested the associations of the 3 FeNO-associated SNPs with physician-diagnosed asthma in a previously published GWA dataset (cases: n=10,365; controls: n=16,110)[5]. The rs8069176[A] minor allele at the 17q12-q21 locus was associated with a decreased risk of asthma. We used rs2305480[A] as a proxy for rs8069176[A] (odds ratio (OR) 0.85; 95% CI = 0.81 to 0.88; $P = 7.93 \times 10^{-17}$; Table 5.2). This is in line with the association with lower FeNO that we found for rs8069176[A]. The SNPs rs3751972 and rs944722 were not associated with an asthma diagnosis ($P \geq 0.3$). The 3 SNPs were not associated with adult FeNO (N = 1,211, Table 5.2).

Finally, we explored whether common genetic variants known to be associated with physician-diagnosed asthma[5] were related with childhood FeNO. We observed that asthma SNPs rs2305480 at 17q12 (*GSDMB*), rs3894194 at 17q21.1 (*GSDMA*), rs744910 at 15q22.33 (*SMAD3*) and rs1295686 at 5q31 (*IL13*) were indeed associated with childhood FeNO, after Bonferroni correction (all $P \leq 0.005$; Table 5.3). The directions of the SNP effects were as expected.

Table 5.3. Association of known physician-diagnosed asthma loci, from a previous GWA study[5] with childhood FeNO

Physician-diagnosed asthma[5]						
Marker	**MAF**	***β***	**S.E.**	***P***	I^2	***HetP***
rs2305480[A] decreasing risk-allele at 17q12 (*GSDMB*)	0.42	-0.065	0.012	2.83×10^{-08}	0.0	0.731
rs3894194[A] increasing risk-allele at 17q21.1 (*GSDMA*)	0.47	0.048	0.012	6.35×10^{-05}	9.5	0.349
rs744910[A] decreasing risk-allele at 15q22.33 (*SMAD3*)	0.49	-0.039	0.012	8.41×10^{-04}	0.0	0.491
rs1295686[T] increasing risk-allele at 5q31 (*IL13*)	0.27	0.044	0.014	1.25×10^{-03}	4.6	0.401
rs1342326[C] increasing risk-allele at 9p24.1 (*IL33*)	0.17	0.025	0.016	0.119	0.0	0.515
rs9273349[T] decreasing risk-allele at 6p21.3 (*HLA-DQ*)	0.37	-0.022	0.022	0.310	0.0	0.802
rs11071559[T] decreasing risk-allele at 15q22.2 (*RORA*)	0.14	-0.014	0.017	0.415	0.0	0.651
rs3771166[A] decreasing risk-allele at 2q12 (*IL18R1*)	0.35	-0.009	0.012	0.463	7.4	0.371
rs2284033[A] decreasing risk-allele at 22q13.1 (*IL2RB*)	0.42	0.005	0.012	0.705	0.0	0.633
rs2073643[T] increasing risk-allele at 5q23.3 (*SLC22A5*)	0.47	0.000	0.012	0.993	0.0	0.590

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). We explored whether common genetic variants known to be related with physician-diagnosed asthma[5] were associated with childhood FeNO. The total sample includes data of 14 independent GWA datasets (N = 8,858). MAF, minor allele frequency; S.E., standard error. *β* reflects differences in natural log-transformed FeNO per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for sex and age at time of measurement (fixed-effect additive genetic model). Derived inconsistency statistic I^2 and *HetP* values reflect heterogeneity across studies with the use of Cochran's Q tests.

Discussion

We identified association with FeNO and genetic variants at 3 loci. The common variants in and near *LYRM9* and *NOS2* were located at 17q11.2-q12. The function of *LYRM9* is unknown. In earlier studies, variants in *NOS* encoded by *NOS1*, *NOS2* and *NOS3* and *arginase* genes jointly contributed to differences in FeNO[28-31], and variation in *arginase* genes to asthma severity[32]. Inducible NOS2 is expressed in lung epithelium and is synthesized in response to pro-inflammatory cytokines and mediators. Expression of inducible NOS2 may be beneficial in host defense or in modulating the immune response[17,33]. In our study genetic variants in *NOS2,* but not *NOS1* and *NOS3,* were robustly associated with childhood FeNO. The associations of genetic variants in *NOS* or *arginase* genes might be different among asthmatic versus non-asthmatic children[28]. Therefore, we conditioned for current asthma, leading to comparable results for the SNPs in *LYRM9* and *NOS2* and a slightly lower effect for the SNP in the 17q12-q21 locus (Supplementary Tables 5.7). In addition we showed that the 3 lead SNPs were also associated with FeNO in non-asthmatic children (Supplementary Tables 5.8).

We found a *cis* eQTL for the transcript *LGALS9* in LD with rs944722, downstream of *NOS2,* and this suggests that the protein Gal-9 may be involved in the regulation of FeNO. Gal-9 plays a crucial role in immune responses, including allergic inflammation. Gal-9 was shown to inhibit allergic airway inflammation, and airway hyperresponsiveness by modulating CD44-dependent leukocyte recognition of the extracellular matrix in mice[34]. Results in guinea pigs showed that Gal-9 might be involved in prolonged eosinophil accumulation in the lung[35]. A recent study suggested a novel function of Gal-9 in mast cells and suggested that Gal-9 might be an interesting new target for the treatment of allergic disorders including asthma[36].

The 17q12-q21 asthma locus, harbouring the *ZPBP2*, *GSDMB*, and *ORMDL3* genes, is a complex region with high LD[4,5,37,38]. *GSDMB* may be involved in the regulation of the growth and differentiation of epithelial cells[39,40]. The function of upstream *ORMDL3* gene in humans is not completely clear. The *ORMDL* family genes encode for transmembrane proteins located in the endoplasmic reticulum membrane. In mice, double knockout of the *ORMDL* genes leads to slower growth and higher sensitivity to toxic compounds in mice[41]. The function of the downstream *ZPBP2* gene is not known. Hence, the mechanism by which 17q12-q21 variants regulate FeNO remains to be elucidated.

Some authors have suggested that the association between asthma and FeNO may be entirely explained by atopy[42]. We found an association between the 17q12-q21 childhood asthma locus and FeNO. This suggests that FeNO might be causally related with asthma: as far as we know the variants at the 17q12-q21 locus are not associated with specific atopic outcomes.

In summary, we identified 3 independent signals that were associated with childhood FeNO levels in *LYRM9* (rs3751972), in *NOS2* (rs944722), which are both at 17q11.2-q12, and one signal near *GSDMB* (rs8069176) at 17q12-q21. The 17q11.2-q12 and 17q12-q21 loci are both complex regions with high LD, and may harbour multiple independent signals that influence FeNO and asthma. This study provides novel insights in the regulation of FeNO and highlights that both shared and distinct genetic factors affect FeNO and childhood asthma.

Supplementary Methods

Stage 1: genome-wide association (GWA) analyses of FeNO in children. We combined 10 population-based cohorts with GWA data and FeNO in children available (total N = 8,858 individuals). Four of our discovery cohorts had two independent sub-samples within their study leading to a total of 14 independent GWA sub-samples for our analysis: two sub-samples from the Avon Longitudinal Study of Parents and Children (ALSPAC1, *n* = 949; ALSPAC2, *n* = 794); BAMSE (BAMSE, *n* = 97); two sub-samples from the Children's Health Study (CHS1, *n* = 708; CHS2, *n* = 1,155); Copenhagen Study on Asthma in Childhood (COPSAC, *n* = 313); GABRIELA Advanced Surveys (GABRIELA, *n* = 358); Generation R Study (GENERATIONR, *n* = 2,572); Infancia y Medio Ambiente (INMA, *n* = 153); two sub-samples from Lifestyle Immune System Allergy Study (LISA1, *n* = 255; GINI/LISA2, *n* = 585); Manchester Asthma and Allergy Study (MAAS, *n* = 600); and two sub-samples from the Prevention and Incidence of Asthma and Mite Allergy birth cohort study (PIAMA1, , *n* = 149; PIAMA2, *n* = 170). While no systematic phenotypic differences were observed between the sub-samples of the Avon Longitudinal Study of Parents and Children, Children's Health Study, Lifestyle Immune System Allergy Study and Prevention and Incidence of Asthma and Mite Allergy birth cohort study, they were analyzed separately due to genotyping on different platforms and/or at different time periods. Genotypes within each study were obtained using high-density SNP arrays and then imputed for ~2.5M HapMap SNPs (Phase II, release 22; http://hapmap.ncbi.nlm.nih.gov/). The basic characteristics, exclusions applied (for example, individuals of non-European ancestry for the European samples, family related individuals), genotyping, quality control and imputation methods for each discovery study are presented in Supplementary Table 5.1.

Statistical analysis within discovery studies. Fractional exhaled Nitric Oxide (FeNO) was natural log-transformed to obtain a normal distribution. Multiple births and twins were excluded from all analyses. The association between each SNP and FeNO was

assessed in each study sample using linear regression of natural log-transformed FeNO against genotype allelic-dosage using an additive genetic model, with sex and age at the time of measurement as covariates. Twelve out of the fourteen sub-samples contained children from European descent and the remaining two cohorts, Children's Health Study sub-sample 2 and the Generation R study, had an admix population. Therefore, we applied correction for principal components (PCs) in these two datasets. No population stratification was observed in the two datasets (study λ values: CHS2 = 1.031 and GENERATIONR = 0.977). We performed an additional sensitivity analysis excluding the non-European samples, which led to comparable results Supplementary Table 5.4. Excluding the two admix datasets led to comparable results, according to effect size and direction of effect, but higher standard errors were observed. Standard errors were most likely higher due to the lower number of study subjects (N = 3,727). Details of any additional corrections for study specific population structure are given in the Supplementary Table 5.1. The GWA analysis per cohort was performed using MaCH2qtl[43], SNPTEST[44], PLINK[45] or PropABEL[46]. The secured data exchange and storage were facilitated by the Erasmus Medical Center, Department of Internal Medicine[47].

Meta-analysis of discovery studies. Prior to meta-analysis, SNPs with a minor allele frequency (MAF) ≤ 0.01 and poorly imputed SNPs (r2hat ≤ 0.3 (MaCH); proper_info ≤ 0.4 (IMPUTE2)) were filtered. Genomic control (GC)[48] was applied to adjust the statistics generated within each cohort (see Supplementary Table 5.1 for individual study λ values). Inverse variance fixed-effects meta-analyses were analyzed using METAL (released 2010-08-01)[49] by two meta-analysts in parallel and blinded to obtain identical results. After the METAL meta-analysis, we filtered SNPs with a MAF ≤ 0.05 and SNPs that were not available in at least eight sub-samples to avoid false-positive findings. We used Cochran's *Q* test and the derived inconsistency statistic *I2* to assess evidence of between-study heterogeneity of the effect sizes. The meta-analysis results were obtained for a total of 2,253,077 SNPs. SNPs that crossed the widely accepted genome-wide significance threshold $P \leq 5x10^{-8}$ were considered to represent robust evidence of association with FeNO. SNPs which surpassed a *P* value threshold of $P \leq 1x10^{-5}$ were considered to represent suggestive evidence for association with FeNO (see Supplementary Table 5.3 and 5.4). The explained variance of the SNPs was calculated in the Generation R Study (n = 2,572). The genome-wide complex trait analysis (GCTA) tool[26] was used to condition on all SNPs of the meta-analysis to determine independent genome-wide SNPs. Summary statistics of the meta-analysis and individual genotype data of the Generation R study from European ancestry only (N = 2,661, also children without FeNO measurements) have been used as reference dataset in the GCTA tool.

Stage 2: follow-up of the 3 lead SNPs and additional analyses. *Analysis of functional SNPs and eQTLs in LD with the 3 lead signals.* We assessed whether there were common non-synonymous functional variants in linkage disequilibrium (r^2 > 0.80) with our 3 genome-wide significant SNPs using HaploReg[27]. Subsequently, we assessed whether our 3 genome-wide significant SNPs were expression quantitative trait loci (eQTLs). Genome-wide expression data of lymphoblastoid cell lines (LCLs) was used to search for the eQTLs[24]. Gene expression in LCLs was characterized in two independent datasets, one sample of 405 siblings using Affymetrix HG U133 Plus 2.0 chips and the other sample of 550 siblings using Illumina Human6V1 array. Among these individuals, 928 were also genotyped at > 300,000 SNPs using the Illumina HumanHap300 arrays, with additional genotypes for 2.4 millions SNPs in HapMap (release II) and 8 million SNPs in the 1000 Genomes Project filled in using imputation. We defined genome-wide significant *cis* eQTL as false discovery rate (FDR) < 1% account for all SNP-probe pairs that were within 1Mb of each other (Supplementary Table 5.5). Discovery eQTLs were followed-up in an ALSPAC expression dataset of LCLs of 875 individuals (Supplementary Table 5.6)[25].

Analysis of lead SNPs conditioned on current asthma and effect of lead SNPs for non-asthmatic children. Associations of genetic variants might be different among asthmatic versus non-asthmatic children[28]. Therefore, we assessed whether current asthma was a confounder by conditioning on current asthma for our 3 lead SNPs. In addition, we explored the effects of the 3 lead SNPs for non-asthmatic children (see Supplementary Tables 5.7 and 5.8).

Association of the 3 lead signals related to childhood FeNO with physician-diagnosed asthma and adult FeNO. We searched in the previously published GWA meta-analysis dataset if the lead index SNPs of FeNO were available and associated with FeNO related outcome physician-diagnosed asthma. This dataset include independent samples of cohorts participating in the GABRIEL consortium[5]. The lead SNPs were substituted in with a closely correlated proxy from the HapMap, because the index SNPs were not available. The 3 independent genome-wide significant SNPs were taken forward for *in silico* replication in a GWA of FeNO in adults (EGEA, n = 610 and Hutterites, n = 601). The lead SNPs were substituted in Hutterites with a closely correlated proxy, because the index SNPs were not available. Details of the replication studies are presented in Supplementary Table 5.1. Within the replication studies, we analyzed the association between each SNP and natural log-transformed FeNO adjusted for age, sex, height, smoking status, study center and principal components.

Stage 3: cross-phenotyping. *Association of known physician-diagnosed asthma loci with childhood FeNO.* We looked up and assessed whether previously identified SNPs associated with FeNO related outcome physician-diagnosed asthma[5] were also associated with childhood FeNO. Bonferroni correction was applied to correct for multiple testing (asthma: $P = 0.05 / 10$ selected loci = 0.005).

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Supplementary materials

Supplementary Table 5.1. Basic characteristics, exclusions, genotyping, quality control and imputation in GWA studies per cohort

This table is available on request (rjpvandervalk@gmail.com).

Supplementary Table 5.2. Summary statistics of the 3 lead SNPs at $P < 5x10^{-8}$ and their independent effects using GCTA tool

Discovery analysis				**GCTA**			
Marker	***β***	**S.E.**	***P***	**Marker**	***β***	**S.E.**	***P***
rs3751972[C]	0.086	0.014	$1.97x10^{-10}$	rs3751972[C]	0.081	0.014	$2.06x10^{-09}$
rs944722[C]	-0.073	0.012	$1.28x10^{-09}$	rs2274894[T]*	-0.068	0.012	$1.50x10^{-08}$
rs8069176[A]	-0.066	0.012	$1.88x10^{-08}$	rs8069176[A]	-0.066	0.012	$2.14x10^{-08}$

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Independent SNPs with a genome-wide significant effect on FeNO levels in children are shown ($P < 5x10^{-8}$). In the left column independent signals were determined with HapMap pairwise LD (phase II release 22 CEU) and in the right column with the GCTA tool. The total sample includes data of 14 independent GWA datasets (N = 8,858). GCTA, genome-wide complex trait analysis; S.E., standard error. *β* reflects differences in natural log-transformed FeNO per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for sex and age at time of measurement (fixed-effect additive genetic model). *: Using GCTA, the strongest and independent signal in *NOS2* is rs2274894.

Supplementary Table 5.3. Summary statistics of the 16 independent SNPs at $P < 1x10^{-5}$

Marker	MAF	β	S.E.	P	DIRECTION	I^2	*HetP*
rs3751972[C] at 17q11.2 (*LYRM9*)	0.25	0.086	0.014	$1.97x10^{-10}$	++-++-+++++++++	27.4	0.161
rs944722[C] at 17q11.2-q12 (*NOS2*)	0.38	-0.073	0.012	$1.28x10^{-09}$	--+--+--+-----	37.8	0.075
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.43	-0.066	0.012	$1.88x10^{-08}$	--+----------	0.0	0.668
rs12500579[G] at 4q25-q27 (*ANK2*)	0.06	-0.194	0.036	$8.15x10^{-08}$	-+?+++---??+-?	89.8	$4.13x10^{-15}$
rs2016444[G] at 8p22 (near *DLC1*)	0.09	-0.130	0.026	$9.30x10^{-07}$	++?--+-++---?+	87.5	$3.71x10^{-14}$
rs7685921[G] at 4q21 (near *SLC4A4*)	0.13	0.116	0.024	$9.46x10^{-07}$	++-??-+?++++++	49.3	0.032
rs1820616[T] at 3p14.3 (*LRTM1*)	0.06	0.113	0.024	$2.62x10^{-06}$	+++++++++++++	0.0	0.997
rs4471226[C] at 1q23-q25 (near *PAPPA2*)	0.10	-0.097	0.021	$2.64x10^{-06}$	----------	0.0	0.797
rs2156107[C] at 18q22.2 (near *NETO1*)	0.05	0.144	0.031	$2.66x10^{-06}$	++?++++++++++	0.0	0.710
rs2163870[G] at 8q23.1 (*ANGPT1*)	0.35	0.057	0.012	$2.74x10^{-06}$	-++++++++++++	33.4	0.108
rs7245959[C] at 19q12 (near *TSHZ3*)	0.43	0.059	0.013	$3.16x10^{-06}$	++++++-+++++++	0.8	0.440
rs12429252[G] at 13q12.3 (nearest gene *KATNAL1*)	0.19	0.117	0.025	$3.82x10^{-06}$	-+?+++++++-+++	17.6	0.267
rs3802344[A] at 9q33-q34 (near *PRDM12*)	0.07	0.111	0.024	$5.41x10^{-06}$	++++++++++++--	25.3	0.182
rs13042473[G] at 20q12-q13 (*PTPRT*)	0.19	0.069	0.016	$7.74x10^{-06}$	+++++++++++++	0.0	0.941
rs216026[T] at 12p13.3 (*CACNA1C*)	0.21	-0.067	0.015	$7.80x10^{-06}$	-+----------	0.0	0.480
rs10802346[C] at 1q44 (*SMYD3*)	0.15	0.076	0.017	$7.92x10^{-06}$	+++++++++++++	0.0	0.816

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Sixteen independent SNPs with a suggestive effect on FeNO levels in children are shown ($P < 1x10^{-5}$). The total sample includes data of 14 independent GWA datasets (N = 8,858). MAF, minor allele frequency; S.E., standard error. β reflects differences in natural log-transformed FeNO per minor allele. P values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for sex and age at time of measurement (fixed-effect additive genetic model). Direction of effect per GWA study (order of datasets: ALSPAC1, ALSPAC2, BAMSE, CHS1, CHS2, COPSAC, GABRIELA, GENERATIONR, INMA, LISA1, GINI/LISA2, MAAS, PIAMA1 and PIAMA2). Derived inconsistency statistic I^2 and *HetP* values reflect heterogeneity across studies with the use of Cochran's Q tests.

Supplementary Table 5.4. Summary statistics of the 16 independent SNPs at $P < 1x10^{-5}$ in European children only

Marker	MAF	β	S.E.	P	DIRECTION	I^2	HetP
rs3751972[C] at 17q11.2 (*LYRM9*)	0.26	0.095	0.016	$6.83x10^{-09}$	++-+-+++++++	32.8	0.128
rs944722[C] at 17q11.2-q12 (*NOS2*)	0.40	-0.075	0.015	$2.77x10^{-07}$	--+-+-+-----	45.5	0.043
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.45	-0.066	0.014	$4.97x10^{-06}$	--+---------	0.0	0.712
rs12500579[G] at 4q25-q27 (*ANK2*)	0.01	-0.420	0.058	$5.33x10^{-13}$	-+?++--??+-?	87.9	$4.03x10^{-10}$
rs2016444[G] at 8p22 (near *DLC1*)	0.01	-0.292	0.041	$1.28x10^{-12}$	++?-+-+---?+	85.1	$1.13x10^{-09}$
rs7685921[G] at 4q21 (near *SLC4A4*)	0.13	0.116	0.024	$9.46x10^{-07}$	++-?-+++++++	49.3	0.032
rs1820616[T] at 3p14.3 (*LRTM1*)	0.06	0.128	0.029	$1.27x10^{-05}$	++++++++++++	0.0	0.997
rs4471226[C] at 1q23-q25 (near *PAPPA2*)	0.09	-0.091	0.025	$2.96x10^{-04}$	----------	0.0	0.682
rs2156107[C] at 18q22.2 (near *NETO1*)	0.03	0.203	0.042	$1.25x10^{-06}$	++?+++++++++	0.0	0.916
rs2163870[G] at 8q23.1 (*ANGPT1*)	0.36	0.060	0.015	$5.24x10^{-05}$	-++++++++++	40.8	0.069
rs7245959[C] at 19q12 (near *TSHZ3*)	0.44	0.069	0.015	$6.14x10^{-06}$	+++++-++++++	0.0	0.490
rs12429252[G] at 13q12.3 (nearest gene *KATNAL1*)	0.03	0.147	0.046	$1.22x10^{-03}$	-+?+++++-+++	15.5	0.296
rs3802344[A] at 9q33-q34 (near *PRDM12*)	0.07	0.128	0.030	$2.14x10^{-05}$	++++++++++--	32.8	0.128
rs13042473[G] at 20q12-q13 (*PTPRT*)	0.18	0.090	0.019	$1.90x10^{-06}$	++++++++++++	0.0	0.997
rs216026[T] at 12p13.3 (*CACNA1C*)	0.21	-0.068	0.018	$1.43x10^{-04}$	-+----------	0.0	0.457
rs10802346[C] at 1q44 (*SMYD3*)	0.14	0.082	0.021	$7.17x10^{-05}$	++++++++++++	0.0	0.804

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Sixteen independent SNPs with a suggestive effect on FeNO levels in children are shown ($P < 1x10^{-5}$). The total sample includes data of 12 independent GWA datasets from children of European descent (N = 5,131). MAF, minor allele frequency; S.E., standard error. *β* reflects differences in natural log-transformed FeNO per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for sex and age at time of measurement (fixed-effect additive genetic model). Direction of effect per GWA study (order of datasets: ALSPAC1, ALSPAC2, BAMSE, CHS1, COPSAC, GABRIELA, INMA, LISA1, GINI/LISA2, MAAS, PIAMA1 and PIAMA2). Derived inconsistency statistic I^2 and *HetP* values reflect heterogeneity across studies with the use of Cochran's Q tests.

Supplementary Table 5.5. Discovery eQTLs in LCLs of the 3 lead FeNO SNPs and SNPs in LD

Transcripts associated with FeNO lead signals					**Peak association of gene expression and LD with FeNO lead signals**					
					LD					
Marker	**MAF**	**H^2**	***P***	**TRANSCRIPT**	**D'**	**r^2**	**Peak marker**	**MAF**	**H^2**	***P***
rs3751972[C]	-	-	-	-	-	-	-	-	-	-
rs944722[C]	0.39	11.2%	$9.2x10^{-09}$	*LGALS9* (203236_s_at)	NA	<0.3	rs4239242[C]	0.39	18.1%	$1.7x10^{-12}$
rs8069176[G]	0.43	30.7%	$1.8x10^{-33}$	*ORMDL3* (GI_27544926-S)	1.00	0.82	rs8067378[A]	0.48	36.2%	$1.4x10^{-39}$
rs8069176[G]	0.43	34.8%	$1.2x10^{-37}$	*GSDML* (GI_8924175-S)	1.00	0.82	rs8067378[A]	0.48	43.9%	$1.9x10^{-47}$
rs8069176[A]	0.38	20.9%	$1.1x10^{-15}$	*GSDML* (219233_s_at)	1.00	0.82	rs12936231[G]	0.41	24.0%	$7.2x10^{-18}$
rs8069176[A]	0.38	15.4%	$7.4x10^{-12}$	*GSDML* (215659_at)	1.00	0.79	rs3816470[G]	0.42	18.5%	$7.0x10^{-14}$
rs8069176[A]	0.38	8.8%	$1.3x10^{-07}$	*ORMDL3* (240701_at)	1.00	0.82	rs7216389[C]	0.41	12.2%	$6.0x10^{-10}$
rs8069176[A]	0.38	35.8%	$1.8x10^{-25}$	*ORMDL3* (223259_at)	0.96	0.79	rs7359623[T]	0.40	41.5%	$4.3x10^{-29}$
rs8069176[A]	0.38	18.8%	$2.8x10^{-14}$	*ORMDL3* (235136_at)	0.96	0.79	rs7359623[T]	0.40	21.1%	$1.0x10^{-15}$

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Genes within 1Mb of the lead signals that were significantly associated with FeNO are shown. eQTLs, expression quantitative trait loci; LCLs, lymphoblastoid cell lines; LD, linkage disequilibrium; MAF, minor allele frequency. H^2 is the % variance in expression after adjusting for batch effects explained by SNP. *P* values are obtained from a score test taking into account the family relatedness of the sample.

Supplementary Table 5.6. Replication of eQTLs in an independent dataset of LCLs

Marker	**MAF**	**H^2**	***P***	**TRANSCRIPT**
rs944722[C]	0.40	0.7%	0.014	*LGALS9*
rs8069176[A]	0.48	16.4%	$7.8x10^{-36}$	*ORMDL3*
rs8069176[A]	0.48	16.6%	$3.1x10^{-36}$	*GSDML*
Peak expression markers*				
rs4239242[C]	0.37	3.4%	$2.4x10^{-08}$	*LGALS9*
rs8067378[A]	0.48	19.9%	$6.4x10^{-44}$	*ORMDL3*
rs8067378[A]	0.48	20.0%	$3.9x10^{-44}$	*GSDML*
rs12936231[C]	0.48	19.7%	$1.4x10^{-43}$	*ORMDL3*
rs12936231[C]	0.48	19.8%	$8.3x10^{-44}$	*GSDML*
rs3816470[A]	0.46	18.5%	$1.2x10^{-40}$	*ORMDL3*
rs3816470[A]	0.46	19.0%	$6.3x10^{-42}$	*GSDML*
rs7216389[T]	0.48	19.1%	$4.3x10^{-42}$	*ORMDL3*
rs7216389[T]	0.48	18.4%	$1.9x10^{-40}$	*GSDML*
rs7359623[C]	0.49	19.7%	$1.5x10^{-43}$	*ORMDL3*
rs7359623[C]	0.49	19.6%	$2.2x10^{-43}$	*GSDML*

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Discovery eQTLs were followed-up in the ALSPAC expression dataset. *: Peak expression markers from the eQTL discovery analysis. eQTLs, expression quantitative trait loci; LCLs, lymphoblastoid cell lines; MAF, minor allele frequency. H^2 is the % variance in expression after adjusting for batch effects explained by SNP. *P* values are obtained from a score test.

Supplementary Table 5.7. Summary statistics of the 3 lead SNPs conditioned on current asthma

SNP effects conditioned on current asthma (n = 7,786)			
Marker	***β***	**S.E.**	***P***
rs3751972[C] at 17q11.2 (*LYRM9*)	0.092	0.014	$7.44x10^{-11}$
rs944722[C] at 17q11.2-q12 (*NOS2*)	-0.071	0.012	$1.37x10^{-08}$
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	-0.051	0.012	$3.23x10^{-05}$

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). The total sample includes data of 14 independent datasets where current asthma information was available (n = 7,786). S.E., standard error. *β* reflects differences in natural log-transformed FeNO per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for current asthma, sex and age at time of measurement (additive genetic model).

Supplementary Table 5.8. Summary statistics of the 3 lead SNPs for non-asthmatic children

SNP effects for non-asthmatic children (n = 6,711)			
Marker	***β***	**S.E.**	***P***
rs3751972[C] at 17q11.2 (*LYRM9*)	0.100	0.015	$1.09x10^{-11}$
rs944722[C] at 17q11.2-q12 (*NOS2*)	-0.075	0.013	$6.10x10^{-09}$
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	-0.046	0.013	$2.87x10^{-04}$

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Data of 14 independent datasets. S.E., standard error. *β* reflects differences in natural log-transformed FeNO per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for sex and age at time of measurement (additive genetic model).

Chapter 6

Interaction of a 17q12 variant with both fetal and infant smoke exposure in the development of childhood asthma-like symptoms

The Generation R and PIAMA Study

Ralf J.P. van der Valk, Liesbeth Duijts, Marjan Kerkhof, Sten P. Willemsen, Albert Hofman, Henriette A. Moll, Henriette A. Smit, Bert Brunekreef, Dirkje S. Postma, Vincent V.W. Jaddoe, Gerard H. Koppelman and Johan C. de Jongste,

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Abstract

Background

Gene variants on chromosome 17q12-21 are associated with an increased risk of childhood-onset asthma, a risk known to be modified by environmental tobacco smoke.

Objectives

To assess whether the association of rs2305480 on chromosome 17q12 in the *GSDMB* gene with asthma-like symptoms in the first 4 years of life is modified by smoke exposure during fetal and early postnatal life.

Methods

We used data from two independent prospective cohort studies from fetal life onwards in The Netherlands. We genotyped rs2305480 and assessed maternal smoking during pregnancy and environmental tobacco smoke (ETS) exposure at the age of 2 years. Asthma-like symptoms, defined as any reported wheezing, shortness of breath or dry nocturnal cough, were reported by parents when the children were 1, 2, 3 and 4 years. Analyses were based on a total group of 4,461 Caucasian children participating in the Generation R and PIAMA studies.

Results

The G risk-allele of rs2305480 was associated with asthma-like symptoms (overall odds ratio 1.17 (1.11, 1.24), $p=2.66x10^{-9}$). The effect of rs2305480 on asthma-like symptoms was stronger among children who were exposed to smoke during fetal life (p-interaction= 0.04). Smoke exposure in early postnatal life was also associated with an increased effect of the 17q12 SNP on asthma-like symptoms (p-interaction=$5.06x10^{-4}$). These associations were consistent in both cohorts.

Conclusions

A 17q12 variant, rs2305480, was associated with asthma-like symptoms in preschool children, and this association was modified by smoke exposure already during fetal life and in infancy. Further investigation regarding SNPs in linkage disequilibrium with rs2305480 in relation to pathophysiological pathways is needed.

Introduction

Genome-wide association (GWA) studies identified single nucleotide polymorphisms (SNPs) on chromosome 17q12-21 that are associated with childhood asthma[1-3]. The associations have been replicated in independent populations[1-9]. Rs2305480 in *gasdermin B* (*GSDMB*) gene on 17q12 showed the strongest association with childhood asthma[3] and was associated with transcript levels of *ORM1-like 3* (*ORMDL3*)[1] and *GSDMB*[10]. Gene-environment interactions contribute to the development of asthma[2]. An interaction between 17q12-21 variants, including rs2305480, and environmental tobacco smoke (ETS) exposure in the first years of life with asthma phenotypes has been published previously[8,9]. Whether the effect of rs2305480 variants on asthma-like symptoms is modified by fetal smoke exposure is unknown[2]. Exploration of a possible interaction between rs2305480, fetal smoke exposure and ETS exposure in infancy may identify specific early critical time periods of increased susceptibility[2,9]. In the present study, we examined in two independent prospective birth cohort studies whether the association between rs2305480 and asthma-like symptoms was modified by smoke exposure already during fetal life, and by ETS exposure in infancy.

Methods

We used data from two independent prospective birth cohort studies from fetal life onwards in The Netherlands. In the Generation R Study[11], recruitment by midwifes and obstetricians from the Rotterdam area took place between July 2001 and January 2006, and for the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study[12] recruitment took place in 1996-97 through prenatal clinics in Groningen, Rotterdam and Utrecht. Both studies have been approved by the local medical ethics committees. Analyses were restricted to singleton live-births with Caucasian ethnicity and subjects from whom DNA was available for genotyping[13]. Caucasian ethnicity was defined as having principal components within 4 SD values of the CEU cluster of HapMap[14]. We genotyped rs2305480 in the *GSDMB* gene. Causal SNP(s) may lie elsewhere in the block of linkage disequilibrium (LD)[2]. In the Generation R Study, genotyping was performed using Illumina 610k Quad arrays (San Diego, CA, USA) and in the PIAMA study by KBioscience KASP SNP genotyping technology (Hoddesdon, Herts, England). The genotype frequencies of rs2305480 were 22.2%(AA), 48.3%(GA) and 29.5%(GG) (Hardy-Weinberg p=0.16) in the Generation R Study and in PIAMA 19.2%(AA), 49.4%(GA) and 31.4% (GG) (Hardy-Weinberg p-value=0.56).

Respiratory symptoms were assessed by questionnaires at the ages of 1, 2, 3 and 4 years. Questions were taken from the International Study of Asthma and Allergies

in Childhood (ISAAC)[15]. For this analysis we defined asthma-like symptoms as any reported wheezing, shortness of breath or dry nocturnal cough without a cold in a given year[16]. Information about fetal smoke exposure and ETS exposure in infancy was assessed by postal questionnaires during pregnancy and at the age of 2 years, asking the mothers whether they had smoked during pregnancy, and if people smoked occasionally in the house, respectively[12,17]. In this analysis first trimester smokers and mothers who continued smoking during pregnancy were combined.

Analyses were based on a total group of 4,461 subjects. We evaluated the strength of association (SNP effect) assuming an additive genetic model. The associations of rs2305480 with asthma-like symptoms in children at the ages of 1, 2, 3 and 4 years and the overall effects at all ages were analyzed using multiple imputation-based generalized estimating equation (MI-GEE) models[18,19]. To test for effect modification, we calculated terms for interactions between rs2305480 and smoke exposure status in the MI-GEE model. All models were adjusted for maternal age, education and parity, children's sex, gestational age, birth weight, familial history of asthma and allergy, breastfeeding, daycare attendance and pet keeping, based on the significance of their associations with asthma-like symptoms ($p<0.05$), or a change in effect estimate of >10%. Missing values in covariates were low (highest 9.6%). To handle missing values in binary repeated outcomes[18,19] and covariates, but not for the determinants 'fetal smoke exposure' and 'ETS exposure in infancy', multiple imputations were used for all analyses. Ten independent datasets were generated and calculations of pooled estimates were performed. Imputations were based on the relationships between all potential confounders. No differences in results were observed between analyses with imputed data or complete cases (data not shown). Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC). Combined effect estimates and heterogeneity between cohorts was calculated using fixed effects meta-analyses in R Version 2.8.1 (The R foundation for Statistical Computing, library rmeta).

Results

Descriptive baseline characteristics of mothers and children were comparable in both cohorts, with the exception of education levels of the mother and daycare attendance, which were higher in the Generation R Study (Table 6.1). Early life smoke exposure was more frequent in the PIAMA cohort. The prevalence of asthma-like symptoms according to fetal and infant smoke exposure categories varied between 25 and over 50%, and was higher in children who were exposed compared to those who were not exposed to smoke (Table 6.2).

Table 6.1. Baseline characteristics of mothers and children (n=4,461)

	Generation R (n=2,438)	**PIAMA (n=2,023)**
Maternal characteristics		
Age, years	31.4 (4.2)	30.7 (3.8)
Highest completed education (%)		
Primary, or secondary	37.6 (916)	62.2 (1,259)
Higher	62.4 (1,522)	37.8 (764)
Child characteristics		
Female (%)	49.0 (1,195)	48.7 (986)
Gestational age at birth (weeks)	40.3 (37.4-42.1)	40.1 (37.1-41.9)
Birth weight (grams)	3,534 (520)	3,531 (530)
Familial history of asthma or atopy (%)		
No	47.3 (1,153)	43.3 (875)
Yes	52.7 (1,285)	56.7 (1,148)
Breastfed (%)		
No	11.2 (274)	15.6 (315)
Yes	88.8 (2,164)	84.4 (1,708)
Day care attendance 1st year (%)		
No	40.2 (979)	75.2 (1,521)
Yes	59.8 (1,459)	24.8 (502)
Pets in house (%)		
No	56.2 (1,370)	48.8 (987)
Yes	43.8 (1,068)	51.2 (1,036)
Smoke exposure (%)		
No smoke	50.2 (1,225)	55.6 (1,125)
Fetal smoke only	9.1 (222)	4.6 (93)
Infant smoke only	4.8 (117)	22.5 (455)
Fetal and infant smoke	5.8 (142)	15.2 (308)
Missing	*30.0 (732)*	*2.1 (42)*
Asthma-like symptoms (%)		
Year 1*	50.1 (1,222)	32.4 (656)
Year 2*	41.3 (1,006)	27.1 (548)
Year 3	33.8 (825)	34.5 (698)
Year 4	34.3 (836)	33.1 (671)

Values are means (standard deviation), medians (5-95th percentiles) or percentages (absolute numbers). Pooled estimates were calculated of the 10 multiple imputed datasets. *: Shortness of breath question not available in PIAMA at age of 1 and 2 years.

Table 6.2. Prevalence of asthma-like symptoms according to fetal and infant smoke exposure

	Generation R					PIAMA				
	No Smoke (n=1,225)	Fetal Only (n=222)	Infant Only (n=117)	Fetal and Infant (n=142)	Missing (n=732)	No Smoke (n=1125)	Fetal Only (n=93)	Infant Only (n=445)	Fetal and Infant (n=308)	Missing (n=42)
Year 1*	49.1 (602)	53.8 (120)	41.8 (49)	51.1 (73)	51.7 (379)	33.6 (378)	45.2 (42)	27.0 (123)	32.5 (100)	33.3 (14)
Year 2*	38.5 (472)	44.9 (100)	52.0 (61)	42.3 (60)	42.9 (314)	26.3 (296)	35.5 (33)	25.9 (118)	27.6 (85)	35.7 (15)
Year 3	31.8 (390)	36.6 (81)	34.4 (40)	34.4 (49)	36.2 (265)	33.4 (376)	49.5 (46)	32.7 (149)	35.1 (108)	47.6 (20)
Year 4	32.5 (398)	37.1 (82)	42.1 (49)	33.1 (47)	35.4 (259)	32.2 (363)	44.1 (41)	29.7 (135)	38.0 (117)	35.7 (15)
Overall	38.0 (465)	43.1 (96)	42.6 (50)	40.2 (57)	41.6 (304)	31.4 (353)	43.6 (41)	28.8 (131)	33.3 (103)	38.1 (16)

Values are percentages N/total of stratum (N = number of asthma-like symptom cases). Pooled estimates were calculated of the 10 multiple imputed datasets. *: Shortness of breath question not available in PIAMA at age of 1 and 2 years.

Table 6.3. Association between rs2305480 and asthma-like symptoms

	Generation R (n=2,438)	PIAMA (n=2,023)	Combined (n=4,461)	
Asthma-like symptoms at age	**OR (95% CI) for asthma-like symptoms[a]**	**OR (95% CI) for asthma-like symptoms[a]**	**OR (95% CI) for asthma-like symptoms[a]**	**Hetero-geneity**
Year 1*	1.05 (0.92-1.19)	1.10 (0.96-1.27)	1.07 (0.98-1.18)	p=.595
Year 2*	1.17 (1.02-1.34)	1.13 (0.98-1.31)	1.15 (1.05-1.27)	p=.760
Year 3	1.25 (1.07-1.46)	1.31 (1.15-1.50)	1.28 (1.16-1.42)	p=.658
Year 4	1.17 (1.00-1.37)	1.20 (1.05-1.38)	1.19 (1.07-1.32)	p=.779
Overall	1.15 (1.06-1.26)	1.19 (1.11-1.27)	1.17 (1.11-1.24)	p=.554

[a]: Odds ratios (OR) and 95% confidence intervals (CI) for asthma-like symptoms are given for the risk-allele G. *: Shortness of breath question not available in PIAMA. OR were given (allowing for a time trend) for each year of age separately, and for the overall effect. Models are adjusted for gender, maternal age, familial atopy history, breastfeeding and daycare attendance.

The G risk-allele of rs2305480 was associated with asthma-like symptoms from birth until 4 years of age in both the Generation R Study and PIAMA (overall combined odds ratio (OR) 1.17 (95% confidence interval: 1.11, 1.24), $p=2.66x10^{-9}$)) (Table 6.3). The associations were significant for each year separately for age 2 to 4 years, but not for the first year. The strongest associations were seen in children aged 3 and 4 years with both the risk-allele and exposure to ETS in fetal and early life (Table 6.4 and 6.5).

Table 6.4. Association between rs2305480, fetal smoke exposure and asthma-like symptoms

	Generation R (E- n=1,696 : E+ n=540)		**PIAMA (E- n=1,600 : E+ n=411)**		**Combined (E- n=3,296 : E+ n=951)**			
Asthma-like symptoms at age	**OR (95% CI) for asthma-like symptoms[a]**		**OR (95% CI) for asthma-like symptoms[a]**		**OR (95% CI) for asthma-like symptoms[a]**			
	E-	**E+**	**E-**	**E+**	**E-**	**Hetero-geneity**	**E+**	**Hetero-geneity**
Year 1*	1.01 (0.87-1.18)	1.05 (0.77-1.42)	1.14 (0.98-1.33)	0.94 (0.69-1.27)	1.07 (0.97-1.19)	p=.278	0.99 (0.80-1.22)	p=.614
Year 2*	1.15 (0.99-1.35)	1.06 (0.76-1.48)	1.09 (0.93-1.28)	1.21 (0.89-1.67)	1.12 (1.01-1.26)	p=.626	1.14 (0.91-1.43)	p=.557
Year 3	1.19 (1.00-1.41)	1.33 (0.97-1.82)	1.28 (1.10-1.49)	1.38 (1.03-1.86)	1.24 (1.11-1.39)	p=.531	1.36 (1.09-1.68)	p=.846
Year 4	1.12 (0.95-1.32)	1.26 (0.86-1.84)	1.16 (0.99-1.35)	1.32 (0.98-1.79)	1.14 (1.02-1.28)	p=.800	1.29 (1.02-1.64)	p=.832
Overall	1.11 (1.02-1.22)	1.16 (0.93-1.44)	1.17 (1.08-1.27)	1.21 (1.03-1.41)	1.15 (1.08-1.21)	p=.390	1.19 (1.05-1.35)	p=.757

[a]: Odds ratios (OR) and 95% confidence intervals (CI) for asthma-like symptoms are given for the risk-allele G in all children where fetal smoke exposure information was available (E- not exposed : E+ exposed children). *: Shortness of breath question not available in PIAMA. OR were given (allowing for a time trend) for each year of age separately, and for the overall effect. Models are adjusted for gender, maternal age, familial atopy history, breastfeeding and daycare attendance.

Table 6.5. Association between rs2305480, infant smoke exposure and asthma-like symptoms

	Generation R (E- n=1,582 : E+ n=284)		PIAMA (E- n=1,225 : E+ n=768)		Combined (E- n=2,807 : E+ n=1,052)			
Asthma-like symptoms at age	**OR (95% CI) for asthma-like symptoms[a]**		**OR (95% CI) for asthma-like symptoms[a]**		**OR (95% CI) for asthma-like symptoms[a]**			
	E-	**E+**	**E-**	**E+**	**E-**	**Hetero-geneity**	**E+**	**Hetero-geneity**
Year 1*	1.06 (0.92-1.22)	1.06 (0.72-1.57)	1.10 (0.93-1.31)	1.10 (0.87-1.38)	1.08 (0.97-1.20)	p=.719	1.09 (0.89-1.33)	p=.882
Year 2*	1.22 (1.06-1.40)	0.98 (0.69-1.40)	1.01 (0.84-1.21)	1.36 (1.07-1.72)	1.13 (1.01-1.27)	p=.111	1.23 (1.01-1.50)	p=.134
Year 3	1.23 (1.05-1.44)	1.91 (1.25-2.92)	1.26 (1.06-1.49)	1.39 (1.11-1.74)	1.24 (1.10-1.39)	p=.847	1.49 (1.23-1.81)	p=.190
Year 4	1.18 (1.01-1.37)	1.27 (0.86-1.87)	1.08 (0.91-1.28)	1.46 (1.16-1.83)	1.13 (1.01-1.27)	p=.454	1.41 (1.15-1.71)	p=.552
Overall	1.16 (1.08-1.25)	1.23 (1.01-1.49)	1.11 (1.02-1.22)	1.32 (1.18-1.48)	1.14 (1.08-1.21)	p=.466	1.30 (1.18-1.43)	p=.513

[a]: Odds ratios (OR) and 95% confidence intervals (CI) for asthma-like symptoms are given for the risk-allele G in all children where infant smoke exposure information was available (E- not exposed : E+ exposed children). *: Shortness of breath question not available in PIAMA. OR were given (allowing for a time trend) for each year of age separately, and for the overall effect. Models are adjusted for gender, maternal age, familial atopy history, breastfeeding and daycare attendance.

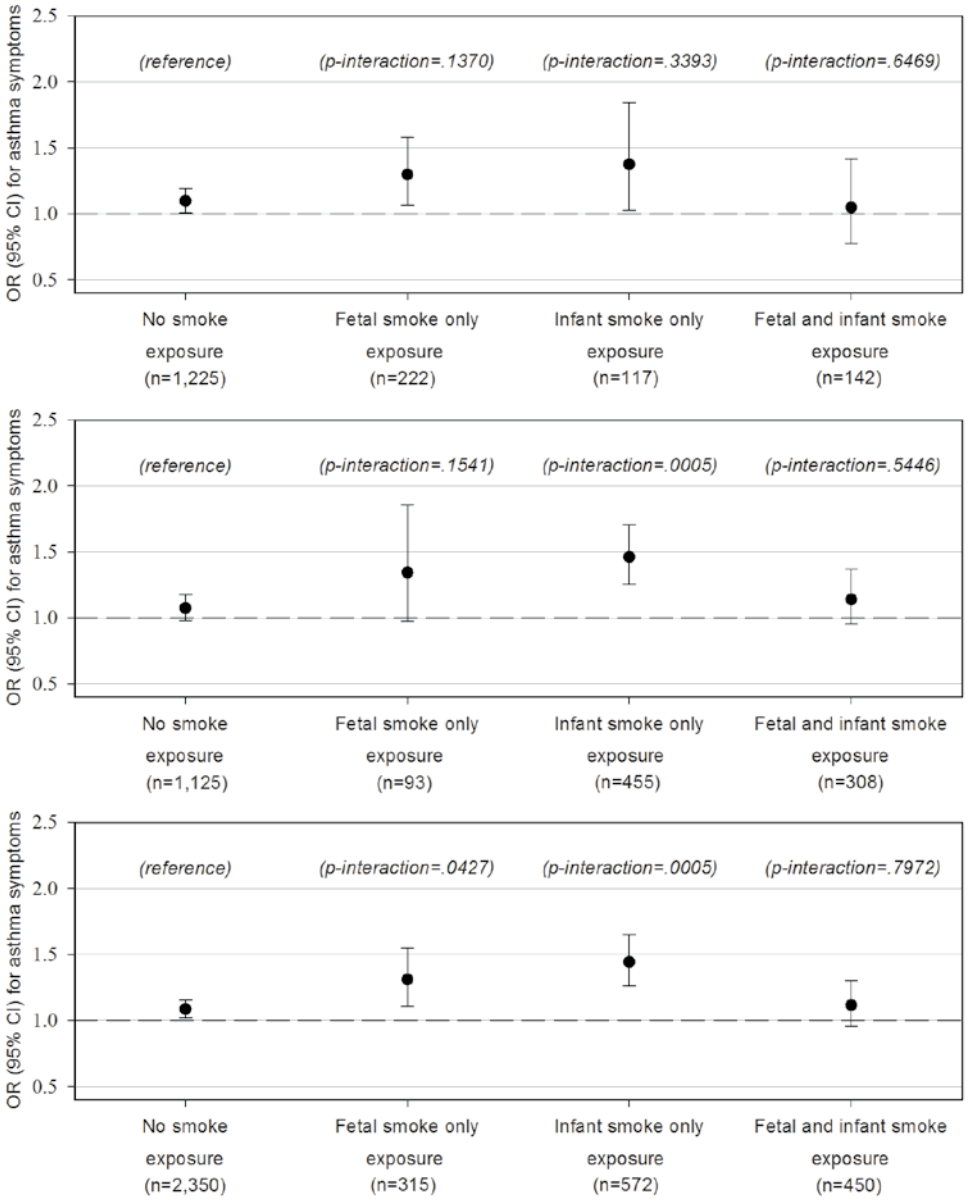

Figure 6.1. Overall association between rs2305480 and asthma-like symptoms, according to fetal and infant smoke exposure

Overall odds ratios (OR) and 95% confidence intervals (CI) for asthma-like symptoms are given for the risk-allele G in a) the Generation R study, b) PIAMA and c) combined. Models are adjusted for gender, maternal age, familial atopy history, breastfeeding and daycare attendance. For this analysis it was required to have information available for both fetal and infant smoke exposure. The following strata were depicted: No smoke (the Generation R Study n=1,225, PIAMA n=1,125); Fetal smoke only (the Generation R Study n=222, PIAMA n=93); Infant smoke only (the Generation R Study n=117, PIAMA n=455); Fetal and infant smoke exposure (the Generation R Study n=142, PIAMA n=308).

The associations between rs2305480 and asthma-like symptoms were modified by fetal smoke exposure (combined p-interaction=0.04) and ETS exposure in infancy (combined p-interaction=$5.06x10^{-4}$) (Figure 6.1). We did not find an overall SNP effect in the group with both fetal and early life smoke exposure in the Generation R and PIAMA study. In the Generation R Study complete data on fetal and early life smoke exposure was missing in 30%, and in PIAMA 2.1%. A sensitivity analysis for missing data in Generation R showed that the effect of rs2305480 on asthma-like symptoms was comparable in children of whom complete smoking data were not available and in children that were not exposed (OR 1.19 (.95-1.47) and OR 1.10 (1.01-1.19) respectively, p-interaction=.52). The directions of the SNP effects were consistent between the Generation R Study and PIAMA for all analyses (heterogeneity p-values > 0.05).

Discussion

We found that a common 17q12-21 variant, rs2305480, was associated with asthma-like symptoms in preschool children and for the first time demonstrated that smoke exposure modified this effect of rs2305480 already during fetal life.

Previous studies have shown associations between 17q12-21 variants and early-onset asthma[1-9]. We replicated these findings for asthma-like symptoms in preschool children in two independent birth cohorts. The increased risk of asthma conferred by rs2305480 was enhanced by early childhood ETS exposure[8,9], which was also observed in the present study. To our knowledge, there are no reports on the interaction between 17q12-21 variants and fetal smoke exposure on any asthma outcome. We found a modifying effect of fetal smoke exposure on the association between rs2305480 and asthma-like symptoms. We did not find an association for the first year of life, which may partly be explained by the specific, transient wheezing phenotype that has its highest prevalence at this age, and relates to viral infection rather than asthma[20]. In children with 17q21 risk genotypes and early-life ETS exposure, associations between infection and asthma were further enhanced[21].

Fetal smoke exposure affects future respiratory health by mechanisms that may include interference with fetal overall- and lung growth, and reduction of fetal breathing movements[22]. Maternal smoking during pregnancy decreases expression of genes that are involved in lung development in neonatal mice, and increases airway remodelling and hyperresponsiveness in the offspring[23,24]. Evidence of epigenetic interactions with in utero smoke exposure in humans is emerging[25,26], but has not yet been described for *GSDMB.* A common disease allele in 17q12-21 was linked to changes in insulator protein CTCF binding and nucleosome occupancy leading

to altered domain-wide *cis*-regulation and *cis*-regulatory haplotypes were strongly associated with asthma[27]. Previous studies showed that *GSDMB* is involved in epithelial barrier function in mice[28,29]. The mechanisms by which ETS affects respiratory health are complex, and may involve gene-ETS interactions[26,30]. Indeed, airway inflammation may result from a genetically compromised barrier function of the airway mucosa, allowing airborne allergens to penetrate the mucosa[31].

There are some methodological issues that could have influenced our findings. Firstly, parent-reported asthma-like symptoms were the outcome in our study, and asking for symptoms that occurred in the previous year may not produce accurate results. However, in preschool children a diagnosis of asthma is based on symptoms[32]. This method of assessing symptoms and exposures is widely accepted in epidemiological asthma studies, and we used well-validated questionnaires[15,16]. We were not able to assess the association of rs2305480 and its interaction with fetal and infant smoke exposure with specific childhood asthma phenotypes, including transient, intermediate, late onset, persistent or other wheezing phenotypes[33,34], due to the availability of data in the Generation R Study until the age of 4 years only. Follow-up studies at older ages which include more detailed assessments of childhood asthma phenotypes are needed. Although assessing smoke exposure by questionnaires is valid, misclassification may occur due to underreporting leading to bias towards the null [35,36]. We addressed this in a previous study, which showed good agreement between the PIAMA questions after ETS exposure and actual measurements of nicotine in indoor air [37]. Misclassification may still differ between symptomatic and non-symptomatic children. The impact of this cannot be measured, but differential misclassification is unlikely since we used data of two prospective birth cohorts. Assessment of smoke exposure in early life could have introduced misclassification, since this was measured at a single point in time, at the age of 2 years, while variation in levels and duration of exposure could have changed over time. This could not be examined as smoking was not assessed annually. Baseline demographic characteristics including social status, day care attendance and smoke exposure prevalences differed between the two cohorts, but were controlled for in the analysis. Heterogeneity tests showed that there existed no different effects between the two cohorts. In general, the previously shown effect estimates for the associations of common genetic variants with doctor's diagnosis of childhood asthma were small with odds ratios ranging from 1.09 to 1.32[3]. In our study we found a SNP effect of OR 1.09 overall in unexposed children, 1.31 in the fetal only-exposed group, and 1.44 in the infant only-exposed group. It was puzzling to find a smaller SNP effect in the group with both pre- and postnatal smoke exposure than with either pre- or postnatal separate exposures. We think that this could result from different mechanisms. Heavy smoking parents may underreport respiratory symptoms of their children[38], and 'continuation of

parents' smoking after pregnancy might be more likely if children have no respiratory symptoms. Hence, underestimation of symptoms and/or reverse causation might have specifically affected this subgroup. We genotyped rs2305480 in the *GSDMB* gene, but causal SNP(s) may lie elsewhere in the block of linkage disequilibrium[2]. Further investigation regarding SNPs in LD with rs2305480 in relation to pathophysiological pathways is therefore needed.

We conclude that 17q12 variant, rs2305480, is associated with asthma-like symptoms from the age of 2 to 4 years in preschool children and that smoke exposure already during fetal life, and exposure to ETS in infancy enhanced the effect of rs2305480. These effects were consistent in two independent, prospective, prenatally recruited birth cohorts.

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Chapter 7

Neonatal folate, homocysteine, vitamin B12 levels and *methylenetetrahydrofolate reductase* variants in childhood asthma and eczema

The Generation R Study

Ralf J.P. van der Valk, Jessica C. Kiefte-de Jong, Agnes M.M. Sonnenschein-van der Voort, Liesbeth Duijts, Esther Hafkamp-de Groen, Henriette A. Moll, Henning Tiemeier, Eric A.P. Steegers, Albert Hofman, Vincent W.V. Jaddoe and Johan C. de Jongste

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Abstract

Background

Higher maternal folate levels during pregnancy may lead to higher risks of asthma and eczema in childhood.

Objectives

Our aim was to assess the associations of folate, homocysteine and vitamin B12 levels of children at birth and their *methylenetetrahydrofolate reductase* (*MTHFR*) variants with asthma and eczema in childhood.

Methods

This study was embedded in a population-based prospective cohort study (N=2,001). Neonatal cord blood folate, homocysteine, and vitamin B12 levels were measured, and *MTHFR* C677T and A1298C genotyped. Wheezing and physician-diagnosed eczema were annually obtained by questionnaire until 4 years. At 6 years, we collected information on physician-diagnosed asthma ever and self-reported eczema ever, measured fractional exhaled nitric oxide (FeNO), and interrupter resistance (Rint). Data were analysed with generalized estimating equations or logistic regression: continuous outcomes with linear regression models.

Results

Folate, homocysteine and vitamin B12 levels of children at birth were not associated with wheezing or eczema until 4 years, asthma and eczema ever, or FeNO or Rint at 6 years. In children carrying C677T mutations in *MTHFR*, higher folate levels were associated with an increased risk of eczema (repeated eczema until 4 years: OR 1.40 (95% CI 1.09-1.80) (SD change) *P*-interaction=0.003, eczema ever at 6 years: OR 1.41 (0.97-2.03) *P*-interaction=0.011). No interactions between *MTHFR* and child folate and homocysteine levels were observed for wheezing and asthma.

Conclusions

Folate, homocysteine and vitamin B12 levels of children at birth did not affect asthma- and eczema-related outcomes up to the age of 6 years. Further studies are warranted to establish the role of *MTHFR* variants in these associations.

Introduction

Children of mothers who used folic acid-containing supplements (FACSs) during pregnancy may have a higher risk of asthma[1-4]. However, previous studies showed conflicting results[1-7]. A study using objective measures of maternal folate showed that higher folate during pregnancy was associated with higher risks of asthma in the offspring[8]. In our previous study, we found that higher maternal folate was associated with eczema, not wheeze[9]. One explanation was that higher folate, along with higher vitamin B12, and lower homocysteine levels, was associated with increased DNA methylation, and hypermethylation may lead to atopic disease[10]. Previous observations in mice showed that a methyl-rich diet in pregnancy leads to an allergic asthma phenotype via epigenetic mechanisms[11]. Hence, methyldonors could influence programming of the fetal immune system in favour of development of allergic disease[9,10]. *Methylenetetrahydrofolate reductase* (*MTHFR*) variants are genetic determinants of folate and homocysteine status. Specific *MTHFR* variants may modify the effects of folate and homocysteine on atopic disease[10]. Nucleotide mutations in *MTHFR* at position 677 and 1298 lead to amino acid changes[12]. *MTHFR* C677T influences folate and homocysteine levels more than *MTHFR* A1298C[12-16]. *MTHFR* C677T, the well-studied functional polymorphism in *MTHFR*, has also been identified by previous genome-wide association studies on folate, homocysteine and vitamin B12[15,16]. No other functional variants in *MTHFR* have been identified. To our knowledge, there are no reports on the associations of neonatal folate, homocysteine and vitamin B12 levels with development of asthma and eczema. The role of *MTHFR* variants in these associations is not clear. Therefore, in a population-based prospective cohort study among Caucasian children, we assessed the associations between cord blood folate, homocysteine and vitamin B12 levels, and *MTHFR* variants, with asthma- and eczema-related outcomes in childhood.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective birth cohort study in The Netherlands[17]. For the present study, we included a selection of Caucasian children, with cord blood available (N=2,001).

Neonatal folate, homocysteine, and total vitamin B12 levels

In cord blood were analyzed using an immunoelectrochemoluminesence assay (Abbott Diagnostics B.V., Hoofddorp, the Netherlands)[17].

MTHFR variants

C677T (rs1801133) and A1298C (rs1801131) were genotyped. Caucasian ethnicity was defined as having principal components within 4 SD values of the CEU cluster of HapMap[18]. Genotype data was 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).

Asthma- and eczema-related outcomes

Wheezing and physician-diagnosed eczema in the past 12 months were assessed yearly by questionnaires, until 4 years using questions from the International Study of Asthma and Allergies in Childhood (ISAAC)[19]. Information on physician-diagnosed asthma ever and self-reported eczema ever was obtained at 6 years. Fractional exhaled nitric oxide (FeNO) was measured at 6 years using the NIOX chemiluminescence analyzer (Aerocrine AB, Solna, Sweden). Lung function (interrupter resistance, MicroRint, MicroMedical, Rochester, Kent, UK) was measured during tidal breathing, with occlusion of the airway at tidal peak expiratory flow. Median values for at least 5 acceptable Rint measurements were calculated and these were used to calculate Z-scores[20]. Due to technical issues we had to replace the MicroRint during the study period. This resulted in stepwise variation in the median, which was corrected for.

Statistical analysis

The associations of folate, homocysteine, and vitamin B12 at birth with repeated wheezing and eczema in children aged 1 to 4 years, were modelled per year (allowing for a time trend) and the overall effects were analyzed using multiple imputation-based generalized estimating equation (MI-GEE) models allowing for correction for the within-subject dependence as a result of the repeated measurements, while physician-diagnosed asthma and self-reported eczema ever, and FeNO and Rint at 6 years were analysed using logistic and linear regression models. FeNO was ^{e}log transformed to obtain a normal distribution. We explored effect modification by *MTHFR*

variants for the associations with repeated wheezing and eczema, and asthma and eczema ever, by calculating interaction terms between *MTHFR* variants with folate and homocysteine levels. We assumed additive SNP effects. All models were adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, parity, smoking, folic acid supplement use and pet keeping during pregnancy, and children's sex, gestational age, birth weight and daycare attendance, based on the significance of their associations with repeated wheezing ($P<0.05$), or a change in effect estimate of >10%. We performed sensitivity analyses where we additionally adjusted for complementary measures, folate, homocysteine and total vitamin B12, as these could influence the metabolic pathway[10]. Multiple imputations were used for all analyses of binary outcomes and covariates. Details are available in Supplementary Table 7.1. Data management was performed in SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Statistical modelling using MI-GEE (SAS PROC GENMOD) was performed in SAS 9.2 (SAS Institute, Cary, NC, USA).

Results

Subject characteristics

The descriptives of mothers and children were similar in the observed and multiple imputed datasets (Table 7.1 and Supplementary Table 7.2).

Folate, homocysteine and vitamin B12 levels at birth and childhood outcomes

We found an association between homocysteine and physician-diagnosed eczema at age 1 year (increase homocysteine per standard deviation (SD): odds ratio (OR) 1.13 (95% confidence interval (CI), 1.01-1.27)). Folate, homocysteine and vitamin B12 were not associated with repeatedly measured wheezing or eczema for each year separately, and from birth until 4 years (all $P>0.05$) (Table 7.2). The levels were also not associated with physician-diagnosed asthma ever and self-reported eczema ever, FeNO or Rint at 6 years (Table 7.3).

Folate and homocysteine levels, *MTHFR* variants and childhood outcomes

We found a significant interaction between *MTHFR* C677T and folate on eczema until 4 years (P-interaction=0.003), and of *MTHFR* A1298C and homocysteine on eczema until 4 years (P-interaction=0.033). We stratified for *MTHFR* C677T and A1298C genotype to explore the association between folate or homocysteine and eczema in children with genetic mutations (Table 7.4). Among children carrying the polymorphic

Table 7.1. Descriptive characteristics of mothers and children (N=2,001)

Maternal characteristics	
Age at enrolment (years)	31.8 (23.8-37.9)
BMI at enrolment (kg/m^2)	23.3 (19.4-32.0)
Smoking during pregnancy	
None (%)	76.4
First trimester only (%)	9.3
Continued (%)	14.3
Educational level	
Primary/secondary education (%)	37.2
Higher education (%)	62.8
Parity	
0 (%)	59.0
1 (%)	31.8
≥2 (%)	9.2
History of asthma or atopy (%)	37.8
Self reported folic acid use during pregnancy	
None (%)	10.5
Suboptimal (first 10 weeks) (%)	33.5
Optimal (periconceptional) (%)	56.0
Pets keeping during pregnancy (%)	42.2
Child characteristics	
Sex (female %)	48.6
Gestational age at birth (weeks)	40.3 (37.6-42.1)
Birth weight (grams)	3550 (2735-4380)
Day care attendance at 1 year (%)	63.3
Child cord blood measures	
Folate (nmol/L)	21.2 (11.9-35.9)
Homocysteine (umol/L)	8.9 (5.6-14.5)
Vitamin B12 (pmol/L)	293 (138-690)
Child outcomes	
Wheezing 1st year (%)	29.8
Wheezing 2nd year (%)	18.9
Wheezing 3rd year (%)	12.2
Wheezing 4th year (%)	11.5
Physician-diagnosed eczema 1st year (%)	22.0

Table 7.1. Descriptive characteristics of mothers and children (N=2,001) (continued)

Physician-diagnosed eczema 2nd year (%)	13.4
Physician-diagnosed eczema 3rd year (%)	9.0
Physician-diagnosed eczema 4th year (%)	7.4
Physician-diagnosed asthma ever at 6 years (%)	7.3
Self-reported eczema ever at 6 years (%)	32.4
FeNO (ppb)*	7.1 (3.5-18.3)
Rint (kPa/l)*	0.85 (0.41-1.36)

Values are percentages for categorical variables and for continuous variables median (95% range). *: Continuous outcomes were not imputed (FeNO, n=1,009; Rint, n=1,059).

allele of C677T in *MTHFR*, higher folate levels were associated with an increased risk of eczema until 4 years (increase folate per SD for the heterozygous mutant TC: OR 1.18 (95%, 1.02-1.37); homozygous mutant TT: OR 1.40 (95%, 1.09-1.80)). These effects were independent of homocysteine and vitamin B12. Children with wild type *MTHFR* A1298C and higher homocysteine levels had an increased risk of eczema until 4 years (increase homocysteine SD for the homozygous wild type AA: OR 1.19 (95%, 1.05-1.36). This effect was independent of folate and vitamin B12 levels. We repeated the analysis for eczema ever at 6 years. We found similar results for *MTHFR* C677T, folate and eczema, but did not find a significant interaction between *MTHFR* A1298C and homocysteine on eczema ever at 6 years (Table 7.5). No interactions between *MTHFR* C677T and A1298C and folate or homocysteine were observed for wheezing and asthma.

Table 7.2. Associations of cord blood folate, homocysteine and vitamin B12 with repeatedly measured wheezing and eczema in childhood (N=2,001)

	OR's of wheezing (95% CI)[a]*					OR's of eczema (95% CI)[a]*				
Levels	**1st year**	**2nd year**	**3rd year**	**4th year**	**Overall**	**1st year**	**2nd year**	**3rd year**	**4th year**	**Overall**
Folate nmol/L										
Change in SD (7.5)	1.02 (0.92-1.14)	1.00 (0.89-1.13)	1.03 (0.89-1.20)	0.92 (0.78-1.09)	1.00 (0.94-1.08)	1.06 (0.93-1.20)	1.08 (0.93-1.27)	1.00 (0.84-1.19)	0.97 (0.77-1.22)	1.05 (0.95-1.15)
Homocysteine umol/L										
Change in SD (2.9)	0.97 (0.87-1.08)	0.96 (0.84-1.09)	0.96 (0.80-1.14)	1.05 (0.89-1.25)	0.98 (0.91-1.05)	1.13 (1.01-1.27)	1.04 (0.89-1.21)	1.01 (0.83-1.22)	1.03 (0.82-1.28)	1.08 (0.99-1.17)
Vitamin B12 pmol/L										
Change in SD (188)	1.03 (0.92-1.15)	1.09 (0.98-1.22)	0.93 (0.80-1.09)	1.00 (0.86-1.16)	1.03 (0.96-1.10)	0.95 (0.83-1.09)	1.02 (0.88-1.18)	0.98 (0.83-1.17)	0.92 (0.75-1.14)	0.97 (0.89-1.07)

Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation. [a]: Odds ratios (95% confidence intervals) were given (allowing for a time trend) for each year of age separately, and for the overall effects using multiple imputation-based generalized estimating equation models. *: Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity, and children's sex, gestational age and birth weight. Pet keeping during pregnancy and daycare attendance were not significantly associated with repeatedly measured wheezing and did not alter the effect estimates of the determinants with more than 10%. Sensitivity analyses adjusting for complementary measures showed similar results.

Table 7.3. Associations of cord blood folate, homocysteine and vitamin B12 with asthma- and eczema- related outcomes at 6 years

	Asthma N=2,001	**Eczema N=2,001**	**FeNO n=1,009**	**Rint n=1,059**
Levels	**OR (95% CI)[a]***	**OR (95% CI)[a]***	**Ratio change (95% CI)[b]***	**Z-score change (95% CI)[b]***
Folate nmol/L				
Change in SD (7.5)	1.02 (0.83-1.25)	1.05 (0.95-1.18)	1.02 (0.98-1.06)	-0.11 (-0.29-0.08)
Homocysteine umol/L				
Change in SD (2.9)	0.96 (0.77-1.19)	0.94 (0.84-1.05)	1.01 (0.97-1.05)	0.01 (-0.16-0.19)
Vitamin B12 pmol/L				
Change in SD (188)	0.84 (0.62-1.14)	0.97 (0.87-1.08)	1.00 (0.96-1.03)	-0.01 (-0.19-0.18)

Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation. [a]: Odds ratios (95% confidence intervals) from logistic regression models. [b]: Ratio changes and change in standardized z-scores (95% confidence intervals) from linear regression models. *: Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity, and children's sex, gestational age and birth weight. Sensitivity analyses adjusting for complementary measures showed similar results.

Table 7.4. Associations of cord blood folate and homocysteine stratified for *MTHFR* C677T and A1298C with repeatedly measured eczema in childhood (N=2,001)

	OR's of eczema until 4 years (95% CI)[a]	
***MTHFR* genotype C677T (rs1801133)**	**Crude**	**Adjusted***
	Folate change in SD (7.5 nmol/L)	
CC genotype (n=921)	0.93 (0.82-1.06)	0.90 (0.79-1.02)
TC genotype (n=873)	1.18 (1.02-1.36)	1.18 (1.02-1.37)
TT genotype (n=207)	1.50 (1.17-1.91)	1.40 (1.09-1.80)
	P-interaction = 0.002	*P*-interaction = 0.003
	OR's of eczema until 4 years (95% CI)[a]	
***MTHFR* genotype A1298C (rs1801131)**	**Crude**	**Adjusted***
	Homocysteine change in SD (2.9 umol/L)	
AA genotype (n=912)	1.21 (1.07-1.37)	1.19 (1.05-1.36)
CA genotype (n=874)	1.04 (0.92-1.18)	1.05 (0.93-1.20)
CC genotype (n=215)	0.88 (0.67-1.16)	0.88 (0.67-1.17)
	P-interaction = 0.026	*P*-interaction = 0.033

Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation. [a]: Overall odds ratios (95% confidence intervals) from multiple imputation-based generalized estimating equation models. *: Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity, and children's sex, gestational age and birth weight. Folate, homocysteine and vitamin B12 levels were in one model. The non significant crude *P*-interaction values for repeated eczema: A1298C*folate, *P*-interaction = 0.489; C677T*homocysteine, *P*-interaction = 0.064. There were no interactions with repeated wheezing: C677T*folate, *P*-interaction = 0.099; A1298C*folate, *P*-interaction = 0.498; C677T*homocysteine, *P*-interaction = 0.131; A1298C*homocysteine, *P*-interaction = 0.298.

Table 7.5. Associations of cord blood folate stratified for *MTHFR* C677T with eczema ever in childhood (N=2,001)

	OR's of eczema ever at 6 years (95% CI)[a]	
***MTHFR* genotype C677T (rs1801133)**	**Crude**	**Adjusted***
	Folate change in SD (7.5 nmol/L)	
CC genotype (n=921)	0.90 (0.77-1.05)	0.88 (0.75-1.04)
TC genotype (n=873)	1.16 (0.98-1.38)	1.14 (0.95-1.37)
TT genotype (n=207)	1.47 (1.05-2.06)	1.41 (0.97-2.03)
	P-interaction = 0.009	*P*-interaction = 0.011

Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation. [a]: Overall odds ratios (95% confidence intervals) from logistic regression models. *: Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity, and children's sex, gestational age and birth weight. Folate, homocysteine and vitamin B12 levels were in one model. The non significant crude *P*-interaction values for eczema ever: A1298C*folate, *P*-interaction = 0.208; C677T*homocysteine, *P*-interaction = 0.446; A1298C*homocysteine, *P*-interaction = 0.439. There were no interactions with asthma ever: C677T*folate, *P*-interaction = 0.057; A1298C*folate, *P*-interaction = 0.983; C677T*homocysteine, *P*-interaction = 0.584; A1298C*homocysteine, *P*-interaction = 0.586.

Discussion

Neonatal folate, homocysteine and vitamin B12 were not associated with repeatedly measured wheezing or eczema until 4 years, or with physician-diagnosed asthma ever and self-reported eczema ever, FeNO or Rint at 6 years. In children carrying the polymorphic allele of C677T in *MTHFR*, higher folate was associated with an increased risk of eczema.

Previous studies have shown conflicting results for the associations of FACSs use during pregnancy and asthma and allergy-related outcomes in children[1-7]. Differences in measurement methods and the timing and amount of folate exposure between studies make it difficult to compare studies. Biological markers are objective and less prone to reporter bias. Higher maternal folate measured in blood during pregnancy have been suggested to affect the risk of pre-school asthma at age 3 years[8], and eczema in the first 4 years[9]. We previously reported no association between maternal folate during pregnancy and wheezing in the child up to the age of 4 years[9]. To our knowledge, the associations of neonatal folate, homocysteine and vitamin B12 levels and *MTHFR* variants with asthma and eczema in childhood have not been studied before. One study of folate levels at 2 years of age found that higher levels were inversely associated with IgE, atopy and wheezing[21]. A recent study showed that higher folate measured at ages 2, 4, 6, and 8 years was significantly associated with both food and aeroallergen sensitization, but not with total IgE, wheezing or asthma. This study suggested that folate status in early life may influence the risk of specific

allergic sensitization rather than IgE production in general[22]. No association between *MTHFR* C677T and asthma or atopy was found in 7-8 years olds[3]. These studies did not explore the effects of *MTHFR* variants and biochemical measures of folate and homocysteine.

Folate metabolism is influenced by multiple factors, including homocysteine and vitamin B12[10,23]. *MTHFR* variants are genetic determinants of folate and homocysteine[10], of which *MTHFR* C677T influences folate and homocysteine more than *MTHFR* A1298C[12-16]. We previously showed that maternal *MTHFR* C677T did not alter the associations of maternal folate or homocysteine and wheezing or eczema in the first 4 years[9]. Interestingly, in the current study we did observe a significant interaction between child *MTHFR* C677T and child folate levels on eczema. We can think of two hypotheses to explain this. Maternal folate levels may be associated with an increased risk of allergic outcomes in the offspring as a result of epigenetic mechanisms in utero[8,9]. Regulation of neonatal Th1/Th2 balance is under epigenetic control[24]. Folate provides methyl donors for methylation of DNA[10,25], which may determine when and where a gene is expressed, and this could play a role during neonatal immune development[24,26]. In mice, prenatal exposure to methyldonors skewed the fetal immune system toward a Th2 profile, in favour of allergic disease[11]. In dendritic cells that process antigens, altered DNA methylation patterns have been shown in neonates at high risk for allergy[27]. Increased DNA methylation may play a role during early immune development when naive T-cells differentiate into Th2 cells[28,29], and decreased methylation may support the switch of T-cells into a Th1 phenotype[30]. Thus, our results may be explained by increased methylation of fetal DNA as a result of high maternal folate levels. However, maternal and child folate levels were not strongly correlated (Supplementary Table 7.3), and this favours another hypothesis: adverse effect of higher folate on the development of eczema in childhood may depend on the child's genetic mutations in *MTHFR* C677T. Mutations in *MTHFR* C677T, but not *MTHFR* A1298C, seem to be associated with mild hyperhomocysteinemia and decreased folate in adults[12,13]. In our study, the child's TT genotype was not associated with folate or homocysteine levels (Supplementary Table 7.4). In healthy adults, folate has a major role in the homocysteine homeostasis by stimulating the transmethylation and remethylation of homocysteine into methionine which may subsequently provide methyldonors for DNA methylation[31]. Mutations in *MTHFR* C677T may render individuals more susceptible to DNA hypomethylation, for example in response to low folate status[32]. Also, studies in adults showed that folate supplementation leading to decreased homocysteine levels reduces homocysteine levels in those with the TT genotype[33,34]. However, in newborns, homocysteine levels were not affected by an increased folate, suggesting that the homocysteine metabolism and response to folate is different in newborns[35]. In addition, we found

that the effect of *MTHFR* C677T on folate metabolism is also different in newborns. With this in mind, we speculate that newborns with *MTHFR* C677T variants and high folate may be particularly vulnerable to increased DNA methylation as a result of a continuously altered transmethylation and remethylation flux rate, irrespective of their homocysteine levels. Studies on methylation of genes involved in asthma and allergic disease and the role of *MTHFR* mutations in these associations are needed.

In the current study we found an interaction between child *MTHFR* A1298C and homocysteine on eczema until 4 years. Homocysteine converts into methionine which may provide methyldonors leading to increased DNA methylation[10] and this may depend on genetic mutations in *MTHFR* A1298C. We did not find an interaction between *MTHFR* A1298C and homocysteine on eczema ever at 6 years. We considered that this interaction might be a chance finding, because we did not find an effect for both repeated physician-diagnosed eczema until 4 years and self-reported eczema ever at 6 years.

There are some methodological issues that could have influenced our findings. Wheezing, asthma and eczema outcomes were obtained by questionnaires, and asking for symptoms and diagnosis that occurred in previous years may not produce accurate results. However, this method of assessing symptoms and diagnosis is widely accepted in epidemiological studies, and we used well-validated questionnaires[19]. Assessment of biochemical measures occurred at a single point in time, at birth, while variation in levels could have been different during pregnancy. *MTHFR* C677T and A1298C may not cover all genetic variation across *MTHFR*. As far as we know, there are no additional functional variants in *MTHFR* known in the literature that may influence folate or homocysteine status. However, there are variants in other genes that may affect one carbon metabolism, the pathway that is centered around folate, but these variants are not related to both homocysteine and folate status, the pathway of interest[14]. The children with genetic data were not selected randomly, as cord blood had to be available. The numbers of missing cord blood samples were higher for lower social economic status. Therefore, we have to be cautious with the generalizabilty of our findings. Baseline characteristics of mothers and children were comparable in the observed and multiple imputed datasets. To reduce attrition bias, we performed the analyses after multiple imputations. We performed additional sensitivity analyses without multiple imputations of the outcomes and the effect estimates were slightly stronger (Supplementary Table 7.5 and 7.6). Neonatal folate, homocysteine and vitamin B12 levels did not affect asthma- and eczema-related outcomes. In general, the effects of common genetic variants on complex traits are small. In our study we found that children with genetic mutations in *MTHFR* C677T and higher folate levels, and children with wild type *MTHFR* A1298C and higher homocysteine levels had an increased risk of eczema. Gene-environment interactions might well be more

important than single environmental exposure or genetic risk factors, in particular for asthma.

In conclusion, folate, homocysteine and vitamin B12 levels of children at birth did not affect asthma- and eczema-related outcomes in childhood. We found a significant interaction between *MTHFR* C677T and folate levels, and *MTHFR* A1298C and homocysteine levels on eczema. Any adverse effects of folate and homocysteine on the development of eczema may depend on genetic mutations in *MTHFR*. These findings warrant replication in larger groups.

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Supplementary materials

Supplementary Table 7.1. Details of multiple imputations modelling

Software used and key setting: SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) – Imputation Method determined by SPSS (automatic option) with 10 iterations.
Number of imputed datasets created: 10
Variables included in the imputation procedure:
Imputed asthma-related variables:
wheezing year 1 (binary: no, yes)
wheezing year 2 (binary: no, yes)
wheezing year 3 (binary: no, yes)
wheezing year 4 (binary: no, yes)
eczema year 1 (combination of 2 binary questions: eczema 1-6 months, eczema 7-12 months)*
eczema year 2 (binary: no, yes)
eczema year 3 (binary: no, yes)
eczema year 4 (binary: no, yes)
asthma ever birth till year 6 (binary: no, yes)
eczema ever birth till year 6 (binary: no, yes)
age mother at intake (continuous)
bmi mother at intake (continuous)
maternal prenatal smoking (nominal: no, first trimester, continued smoking)
maternal education (nominal: primary, secundary, higher)
number of deliveries (nominal: 0, 1 >=2)
maternal history (combination of 4 binary questions: house dust, hay faver, eczema, asthma)*
pets keeping during pregnancy (combination of 3 binary questions: cat, dog and bird)*
gender child (binary: male, female)
gestational age (continuous)
birth weight (continuous)
daycare attendance (binary: no, yes)
folic acid supplement use during pregnancy (nominal: none, suboptimal, optimal)
Predictor variables:
wheezing year 6 (binary: no, yes)
respiratory tract infections severity year 1 (binary: no, yes)
respiratory tract infections severity year 2 (binary: no, yes)
respiratory tract infections severity year 3 (binary: no, yes)
respiratory tract infections severity year 4 (binary: no, yes)
respiratory tract infections severity year 6 (binary: no, yes)
shortness of breath year 1 (binary: no, yes)
shortness of breath year 2 (binary: no, yes)

shortness of breath year 3 (binary: no, yes)
shortness of breath year 4 (binary: no, yes)
cough year 1 (binary: no, yes)
cough year 2 (binary: no, yes)
cough year 3 (binary: no, yes)
cough year 4 (binary: no, yes)
cough year 6 (binary: no, yes)
phlegm year 1 (binary: no, yes)
phlegm year 2 (binary: no, yes)
phlegm year 3 (binary: no, yes)
phlegm year 4 ((binary: no, yes)
weight year 1 (continuous)
height year 1 (continuous)
weight year 2 (continuous)
height year 2 (continuous)
weight year 3 (continuous)
height year 3 (continuous)
weight year 4 (continuous)
height year 4 (continuous)
weight - year 6 (continuous)
height - year 6 (continuous)
breastfeeding ever (binary: no, yes)
paternal house dust mite allergy (binary: no, yes)
paternal hay fever (binary: no, yes)
paternal eczema (binary: no, yes)
paternal asthma (binary: no, yes)
maternal prenatal alcohol (binary: no, yes)
paternal education (nominal: primary, secundary, higher)
paternal prenatal smoking (binary: no, yes)
bmi father at intake (continuous)
age father at intake (continuous)
maternal prenatal stress (continuous)
parenting stress score (continuous)
cows allergy (binary: no, yes)
income household (binary: < €2000,-, > €2000,-)
postnatal environmental smoke year 2 (binary: no, yes)
Treatment of binary/categorical variables: Logistic/multinomial models
Statistical interactions included in imputation models: None

*: Each binary variable was imputed first and combination of variables was done after MI procedure.

Supplementary Table 7.2. Descriptive characteristics of mothers and children in observed and multiple imputed dataset

	Observed dataset	**Multiple imputed dataset**
	N=2,001	**N=2,001**
Maternal characteristics		
Age at enrolment (years)	31.8 (23.8-37.9)	31.8 (23.8-37.9)
*Missing**	*0.0*	-
BMI at enrolment (kg/m^2)	23.3 (19.4-32.0)	23.3 (19.4-32.0)
*Missing**	*0.3*	-
Smoking during pregnancy		
None (%)	76.3	76.4
First trimester only (%)	9.2	9.3
Continued (%)	14.5	14.3
*Missing**	*8.0*	-
Educational level		
Primary/secondary education (%)	37.0	37.2
Higher education (%)	63.0	62.8
*Missing**	*1.2*	-
Parity		
0 (%)	59.0	59.0
1 (%)	31.8	31.8
≥2 (%)	9.2	9.2
*Missing**	*0.0*	-
History of asthma or atopy (%)	38.4	37.8
*Missing**	*12.0*	-
Self reported folic acid use during pregnancy		
None (%)	9.6	10.5
Suboptimal (first 10 weeks) (%)	33.2	33.5
Optimal (periconceptional) (%)	57.2	56.0
*Missing**	*17.7*	-
Pets keeping during pregnancy (%)	44.7	42.2
*Missing**	*9.3*	-
Child characteristics		
Sex (female %)	48.6	48.6
*Missing**	*0.0*	-
Gestational age at birth (weeks)	40.3 (37.6-42.1)	40.3 (37.6-42.1)
*Missing**	*0.0*	-
Birth weight (grams)	3550 (2735-4380)	3550 (2735-4380)
*Missing**	*0.1*	-
Day care attendance at 1 year (%)	68.1	63.3
*Missing**	*29.4*	-

Supplementary Table 7.2. Descriptive characteristics of mothers and children in observed and multiple imputed dataset (continued)

	Observed dataset	**Multiple imputed dataset**
	N=2,001	**N=2,001**
Child cord blood measures		
Folate (nmol/L)	21.2 (11.9-35.9)	21.2 (11.9-35.9)
*Missing**	-	-
Homocysteine (umol/L)	8.9 (5.6-14.5)	8.9 (5.6-14.5)
*Missing**	-	-
Vitamin B12 (pmol/L)	293 (138-690)	293 (138-690)
*Missing**	-	-
Child outcomes		
Wheezing 1st year (%)	28.8	29.8
*Missing**	*23.1*	-
Wheezing 2nd year (%)	17.1	18.9
*Missing**	*23.3*	-
Wheezing 3rd year (%)	10.5	12.2
*Missing**	*29.3*	-
Wheezing 4th year (%)	9.7	11.5
*Missing**	*27.6*	-
Physician-diagnosed eczema 1st year (%)	25.9	22.0
*Missing**	*43.0*	-
Physician-diagnosed eczema 2nd year (%)	12.9	13.4
*Missing**	*24.0*	-
Physician-diagnosed eczema 3rd year (%)	8.0	9.0
*Missing**	*30.3*	-
Physician-diagnosed eczema 4th year (%)	6.1	7.4
*Missing**	*30.0*	-
Physician-diagnosed asthma ever at 6 years (%)	6.2	7.3
*Missing**	*27.6*	-
Self-reported eczema ever at 6 years (%)	31.7	32.4
*Missing**	*27.8*	-
FeNO (ppb)	7.1 (3.5-18.3)	7.1 (3.5-18.3)
*Missing**	*49.6*	*49.6*
Rint (kPa/l)	0.85 (0.41-1.36)	0.85 (0.41-1.36)
*Missing**	*47.1*	*47.1*

Values are percentages for categorical variables and for continuous variables median (95% range). *: Percentage of valid missing in the total group (N=2,001).

Supplementary Table 7.3. Relation of maternal folate levels during pregnancy and child cord blood levels (N=1,554)

		Child folate		
Mother folate		**T1**	**T2**	**T3**
	T1	51.2%	34.9%	13.9%
	T2	31.1%	35.9%	33.0%
	T3	17.3%	32.0%	50.7%

Abbreviations: T1, first tertile; T2, second tertile; T3, third tertile (highest folate levels). Values are percentages of children that remain or move to another tertile compared to the tertile of maternal folate during pregnancy. Maternal and child folate levels were not strongly correlated (r = 0.365).

Supplementary Table 7.4. Associations of *MTHFR* C677T and A1298C and cord blood folate and homocysteine (N=2,001)

	β (95% CI)	
***MTHFR* genotype C677T (rs1801133)**	**Folate**	**Homocysteine**
CC genotype (n=921)	Reference	Reference
TC genotype (n=873)	0.36 (-0.34 - 1.06), *P* = 0.311	0.04 (-0.23 - 0.31), *P* = 0.768
TT genotype (n=207)	0.78 (-0.35 - 1.92), *P* = 0.175	0.23 (-0.21 - 0.67), *P* = 0.302
	β (95% CI)	
***MTHFR* genotype A1298C (rs1801131)**	**Folate**	**Homocysteine**
AA genotype (n=912)	Reference	Reference
CA genotype (n=874)	-0.88 (-1.58 - -0.19), *P* = 0.013	0.09 (-0.18 - 0.37), *P* = 0.499
CC genotype (n=215)	-0.65 (-1.76 - 0.47), *P* = 0.256	0.41 (-0.03 - 0.84), *P* = 0.065

Abbreviations: CI, confidence interval. b = Changes in folate/homocysteine (95% confidence intervals) from linear regression models.

Supplementary Table 7.5. Sensitivity analysis without multiple imputations of the outcomes for the associations of cord blood folate and homocysteine stratified for *MTHFR* C677T and A1298C with repeatedly measured eczema in childhood (N=1,643)

	OR's of eczema until 4 years (95% CI)[a]	
***MTHFR* genotype C677T (rs1801133)**	**Crude**	**Adjusted***
	Folate change in SD (7.5 nmol/L)	
CC genotype (n=757)	0.86 (0.72-1.02)	0.80 (0.70-0.93)
TC genotype (n=709)	1.23 (1.06-1.44)	1.23 (1.07-1.41)
TT genotype (n=177)	1.51 (1.08-2.12)	1.45 (1.09-1.92)
	P-interaction = 0.003	*P*-interaction = 0.0002
	OR's of eczema until 4 years (95% CI)[a]	
***MTHFR* genotype A1298C (rs1801131)**	**Crude**	**Adjusted***
	Homocysteine change in SD (2.9 umol/L)	
AA genotype (n=752)	1.30 (1.09-1.54)	1.31 (1.14-1.49)
CA genotype (n=716)	1.08 (0.92-1.28)	1.09 (0.96-1.23)
CC genotype (n=175)	0.82 (0.61-1.09)	0.63 (0.46-0.85)
	P-interaction = 0.031	*P*-interaction = 0.009

Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation. [a]: Overall odds ratios (95% confidence intervals) from multiple imputation-based generalized estimating equation models. *: Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity, and children's sex, gestational age and birth weight. Folate, homocysteine and vitamin B12 levels were in one model.

Supplementary Table 7.6. Sensitivity analysis without multiple imputations of the outcome for the association of cord blood folate stratified for *MTHFR C677T* with eczema ever in childhood (N=1,445)

	OR's of eczema ever at 6 years (95% CI)a	
MTHFR genotype C677T (rs1801133)	**Crude**	**Adjusted***
	Folate change in SD (7.5 nmol/L)	
CC genotype (n=671)	0.90 (0.76-1.07)	0.88 (0.73-1.06)
TC genotype (n=619)	1.19 (1.00-1.42)	1.15 (0.96-1.39)
TT genotype (n=155)	1.49 (1.05-2.12)	1.37 (0.92-2.06)
	P-interaction = 0.015	*P*-interaction = 0.027

Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation. [a]: Overall odds ratios (95% confidence intervals) from logistic regression models. *: Adjusted for maternal age, BMI, educational level at intake, history of maternal atopy or asthma, smoking and folic acid supplement use during pregnancy, parity, and children's sex, gestational age and birth weight. Folate, homocysteine and vitamin B12 levels were in one model.

Chapter 8

Imputation and analysis strategies of missing binary repeated outcome measurements in a prospective birth cohort study

The Generation R Study

Ralf J.P. van der Valk, Rachel Bakker, Jessica C. Kiefte-de Jong, Caspar W. Looman, Albert Hofman, Henning Tiemeier, Johan C. de Jongste, Ewout W. Steyerberg, Vincent W.V. Jaddoe

Submitted

Abstract

Background

Binary repeated outcome measurements are complex to analyze. Missing values in determinants, confounders and outcomes further complicate analyses and may lead to inefficiency.

Objectives

We aimed to explore differences in the associations of maternal atopy with childhood wheeze from birth to 4 years using different strategies for analysis and imputation of missing values.

Methods

This study was performed on data from 7,696 parents and children participating in a population-based prospective cohort. Information on determinants and outcomes and potential confounders was available from questionnaires. We compared generalized estimating equations (GEE) and generalized linear mixed models (GLMM) in complete cases (N=2,866) to multiple imputations based GEE (MI-GEE) and GLMM in three different population selections, in which missing confounders, determinants and outcomes were multiple imputed. These analyses were based on 4,780 to 7,696 children.

Results

We observed largely similar estimates using MI-GEE and GLMM and applying different imputation methods of missing values. Slightly weaker effect estimates and larger standard errors were observed for complete case analysis as compared to analyses of datasets where multiple imputations were used. Imputation of determinants and outcomes had no benefit over models without imputation.

Conclusions

Our results suggest that MI-GEE and GLMM lead to similar effect estimates in a study of maternal atopy and childhood wheeze. Imputation of determinants and outcomes did not lead to different effect estimates, as compared to only imputing missing covariates.

Introduction

Missing data may occur for determinants, confounders and outcomes[1]. Imputation of missing values allows for analyses of more subjects than a simple complete case analysis. Multiple imputations (MI) is increasingly used in cohort studies to avoid loss of information that may occur due to restriction to study participants with complete data and to adjust for bias caused by missing data[2-4]. Imputation of confounders is frequently used and generally accepted as a strategy in epidemiological studies[5]. Although several analysis strategies have been described for dealing with missing longitudinal outcome data, missing data in binary repeated outcome measurements are more complex to analyse[6-9]. Data are missing completely at random (MCAR) when there are no systematic differences between a missing variable and other observed and missing study variables. This may occur due to e.g. technical failure during measurements. When determinants and confounders are not measured at the same time point as the outcomes, generalized estimating equations (GEE) models work under the MCAR assumption[6]. With the MI-GEE approach, MI can be used to pre-process incomplete data, including outcome data, after which GEE can be applied[6]. Alternatively, generalized linear mixed models (GLMM) can be used to deal with missing binary repeated outcome measurements, due to their validity under the assumption of missing at random (MAR) and flexibility of dealing with missing data in repeated outcome measures[6,8]. Data are MAR when any systematic differences between the missing and observed data can be explained by differences in the observed data. The target of inference of MI-GEE is a population-average effect; for GLMM it is a subject-specific effect[10]. Use of different population selections for multiple imputations may lead to different results. MI of a subgroup for analysis, which requires observed information on the determinant and at least one outcome measurement in time, might be more prone to bias as compared to MI of the total original study cohort. The MI assumption of MAR may be violated due to population selection in the first option. Imputation of confounders with the determinant and outcome in the imputation model may minimize bias in the relationship between determinant and outcome[11]. When the determinant and outcome carry information about the missing values of other covariates, this information can be used to impute missing values to increase the plausibility of the MAR assumption[2,3].

We aimed to explore the differences in effect estimates for the associations of maternal atopy with childhood wheezing after applying (MI-)GEE and GLMM analyses. We used data from a population-based prospective cohort study among pregnant women and their children. Analyses were performed in complete cases and three different population selections, in which missing confounders, determinants and outcomes were imputed.

Methods

Design

We used data from the Generation R Study, a population-based prospective cohort study from fetal life onwards in the Netherlands[12]. Recruitment by midwifes and obstetricians from the Rotterdam area took place between July 2001 and January 2006. The study was approved by the medical ethics committee (MEC 217.595/2002/202). For the present study, we included only singleton live births, which led to a total population of 7,696 children. A participant flow chart is given in Figure 8.1.

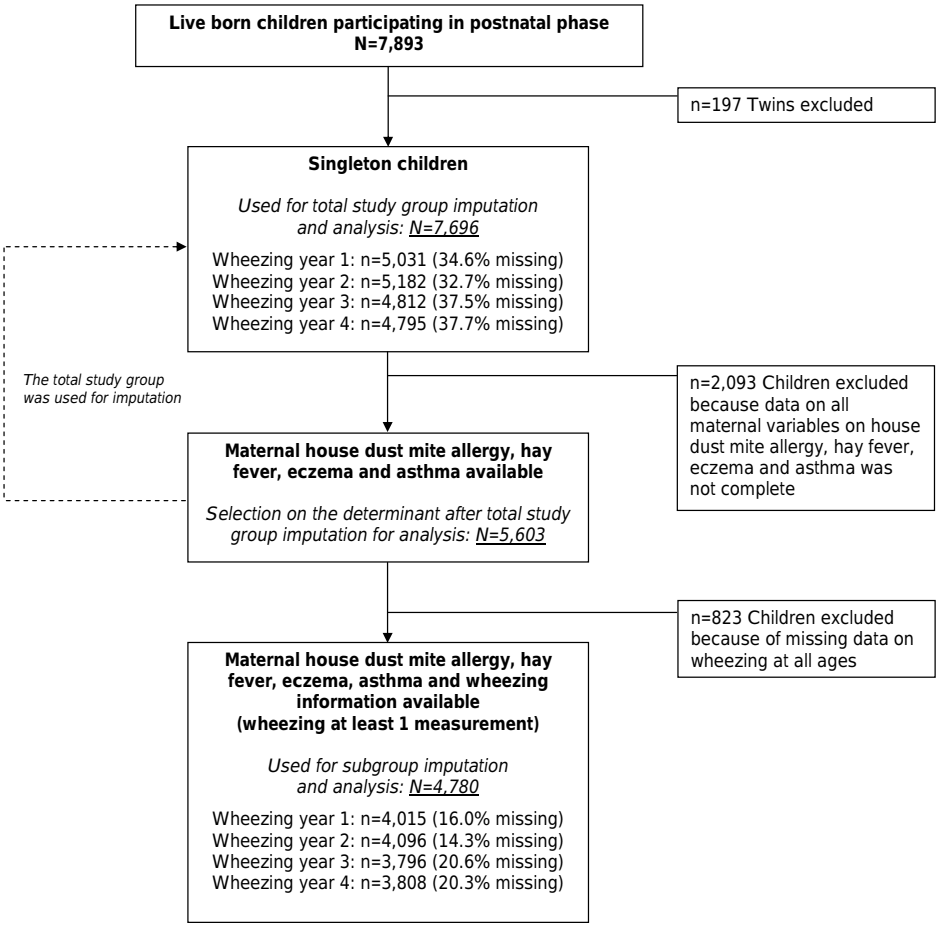

Figure 8.1. Flowchart of participants and population selections for multiple imputations

Determinants and outcomes

We used the self-reported maternal doctor's diagnosis of atopy or asthma as determinant. We defined maternal atopy or asthma using the following questions, obtained by questionnaires at enrolment: (1) 'Are you allergic to house dust mite?' (2) 'Do you have any kind of nose allergy such as hay fever?' (3) 'Do you have chronic eczema?' (4) 'Have you ever had asthma?'[12]. The mother was considered a case when at least one out of the four questions was answered with yes. When one of the answers was missing, it was set to missing, to avoid selection towards positive answers. In datasets where determinants were multiple imputed, we first imputed the answers on the questions separately and then defined maternal atopy or asthma. Childhood wheezing was used as repeated outcome and was assessed by parental reports in questionnaires at the ages of 1, 2, 3, and 4 years ('Has your child had problems with a wheezing chest during the last year?'). Questions about wheeze were taken from the International Study of Asthma and Allergies in Childhood (ISAAC)[13].

Covariates

Information on maternal anthropometrics, age and body mass index (BMI), parental educational level (primary, secondary, and higher education), children's ethnicity (European and non-European) and pet keeping was obtained by questionnaires completed by the mother at enrolment[12]. Information on active maternal smoking during pregnancy was obtained by postal questionnaires in the first, second and third trimester and was combined into three smoking categories: none, first trimester only, and continued smoking[14]. We used parity as a proxy for siblings. Information on birth weight, gestational age and sex of the children was obtained from midwife and hospital registries at birth. Postal questionnaires at the ages of 6 and 12 months provided information about breastfeeding and day care attendance, respectively[12,15].

Statistical analysis

We performed comparative analyses by analysing the associations of maternal atopy and childhood wheeze using different population selections, and statistical techniques for imputation and analysis. The population selections and imputation strategies are depicted in Table 8.1. First, we explored differences in wheezing-related characteristics using (1) observed data (complete cases); (2) multiple imputed data of a subgroup with observed information on at least the determinant and one outcome measurement; (3) imputed data of the total study group invited at baseline with an analysis afterwards on subjects with observed information on the determinant; and (4) multiple imputed data of the total study group without any selection. To observe differences resulting from MI, we compared characteristics of the imputed dataset of the subgroup with the imputed dataset of the total study group and

Table 8.1. Population selections and multiple imputations strategies

	Non imputed *required observed data*				**Multiple imputed** *data available after MI procedure*				
	D	**C**	**O**	**N used for MI**	**D**	**C**	**O**	**Selection after MI**	**Analysis N**
1. Complete case analysis									
Analysis 1a: Crude model	Y*	N	Y†	NA	NA	NA	NA	NA	n=2,866
Analysis 1b: Adjusted model	Y*	Y	Y†	NA	NA	NA	NA	NA	n=1,934
2. Subgroup selection for imputation and analysis									
Analysis 2: MI of confounders	Y*	N	Y‡	N=4,780	NA	Y	N	NA	n=4,780
Analysis 3: MI of confounders + outcomes	Y*	N	Y‡	N=4,780	NA	Y	Y	NA	n=4,780
3. Total study group for imputation, subgroup for analysis									
Analysis 4: MI of confounders; for analysis selection on determinant	N	N	N	N=7,696	NA	Y	N	Det*	n=4,780
Analysis 5: MI of confounders + outcomes; for analysis selection on determinant	N	N	N	N=7,696	NA	Y	Y	Det*	n=5,603
4. Total study group for imputation and analysis									
Analysis 6: MI of determinants + confounders	N	N	N	N=7,696	Y	Y	N	NA	n=6,165
Analysis 7: MI of determinants + confounders + outcomes	N	N	N	N=7,696	Y	Y	Y	NA	n=7,696

Abbreviations: MI, multiple imputations; D, determinants; C, confounders; O, outcomes; Y, yes, data required for specific analysis; N, no, data not required for specific analysis; NA, not applicable for specific analysis. *: Data on all four maternal history variables (house dust mite allergy, hay fever, eczema and asthma) was required for this specific analysis. When one of the answers was missing, the determinant was set to missing, to avoid selection towards positive answers. †: Data on wheezing for all ages was required for this specific analysis. ‡: Information on wheezing for at least 1 year was required for this analysis.

considered a change of 10% as relevant. Next, we performed GEE analyses using complete cases. Estimates were given for the overall effect on wheezing, allowing for a time trend, from birth until 4 years of age. MI-GEE models[6,9], were used for the different population selections and imputation strategies. For each imputation, n=10 independent datasets were generated with multiple imputations. Calculations of pooled estimates were performed according to Rubin's rules[16]. Imputations were based on the relationships between all potential confounders and additional predictor variables associated with the outcomes (Supplementary Table 8.1). As excluding the outcome and determinant in the imputation model may falsely weaken the association between determinant and outcome in some cases[2]. We used the compound symmetry matrix as covariance structure in the MI-GEE models, as we assumed that the correlation between two separate measures would be constant regardless of how far apart the measurements are. Finally, we repeated the analyses with the same datasets using GLMM. If an effect estimate from one model was outside the 95% confidence interval (CI) of another model, we considered the difference in models as relevant. All models were adjusted for maternal age at intake, BMI at enrolment and parity, pet keeping and maternal smoking during pregnancy, maternal and paternal educational level, and children's sex, ethnicity, gestational age, birth weight, breastfeeding and day care attendance, based on the significance of their associations with wheezing ($p<0.05$), or a change in effect estimate of more than 10% within the total study population. In order to examine the relation between missing study variables we examined descriptive confounder characteristics in relation to observed and missing data of determinants and outcomes of our study. Data management and the multiple imputations procedures were performed in SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Statistical modelling of GEE and MI-GEE (SAS PROC GENMOD) was performed in SAS 9.2 (SAS Institute, Cary, NC, USA). The GLMM (Library lme4) analyses were performed in R Version 2.13.0 (The R foundation for Statistical Computing).

Results

Subject characteristics

Descriptive characteristics of mothers and children related to childhood wheezing in the observed and MI datasets are given in Table 8.2. MI led to higher percentages of prenatal smoke exposure, lower paternal educational level, higher parity, more often non-European ethnicity, less breastfeeding and daycare attendance of the children (Table 8.2). Table 8.3 shows observed and MI frequencies of wheezing symptoms at the ages of 1, 2, 3 and 4 years. MI led to higher frequencies of wheezing symptoms as compared to the complete case group. Supplementary Table 8.2 shows confounders

Table 8.2. Childhood wheezing-related variables in the observed and the multiple imputed datasets

	Observed dataset	**Imputed subgroup**[a]	**Imputed total study group and analysis in subgroup**[b]	**Imputed total study group**[c]
	N=7,696	**N=4,780**	**N=5,603**	**N=7,696**
Maternal characteristics				
Age at enrolment (years)	31.0 (21.0-38.5)	31.5 (22.4-38.5)	31.1 (21.4-38.3)	31.0 (21.0-38.5)
*Missing**	*0*	-	-	-
BMI at enrolment (kg/m2)	23.7 (19.4-33.3)	23.6 (19.3-32.6)	23.6 (19.2-33.1)	23.8 (19.2-33.2)
*Missing**	*11.9*	-	-	-
House dust mite allergy, hay fever, eczema or asthma				
No	61.7	65.5	65.8	63.3
Yes	38.3	34.5	34.2	36.7
*Missing**	*22.4*	-	-	-
Smoking during pregnancy				
None	75.9	78.3	76.6	75.7
First trimester only	8.6	8.6	8.4	8.5
Continued	15.5	13.1	15.0	15.8
*Missing**	*23.2*	-	-	-
Education level				
Primary education	9.8	6.5	9.0	10.9
Secondary education	42.8	39.3	42.1	43.8
Higher education	47.4	54.2	48.9	45.3
*Missing**	*11.2*	-	-	-
Parity				
0	55.1	57.6	56.0	54.8
1	30.4	30.4	30.7	30.6
≥2	14.4	12.0	13.3	14.6
*Missing**	*4.0*	-	-	-
Paternal characteristics				
Education level				
Primary education	6.8	7.8	9.9	11.4
Secondary education	38.9	38.7	40.8	42.3
Higher education	54.3	53.5	49.2	46.3
*Missing**	*42.8*	-	-	-

Table 8.2. Childhood wheezing-related variables in the observed and the multiple imputed datasets (continued)

	Observed dataset	**Imputed subgroup**[a]	**Imputed total study group and analysis in subgroup**[b]	**Imputed total study group**[c]
	N=7,696	**N=4,780**	**N=5,603**	**N=7,696**
Familial characteristics				
Pets keeping during pregnancy				
No	67.6	65.7	67.0	67.8
Yes	32.4	34.3	33.0	32.2
*Missing**	*25.1*	-	-	-
Child characteristics				
Sex				
Female	49.5	50.3	49.9	49.5
Male	50.5	49.7	50.1	50.5
*Missing**	*0*	-	-	-
Ethnicity				
European	65.3	73.3	68.2	64.0
Non-European	34.7	26.7	31.8	36.0
*Missing**	*8.7*	-	-	-
Gestational age at birth (weeks)	40.0 (37.0-42.0)	40.1 (37.1-42.1)	40.1 (37.1-42.0)	40.0 (37.0-42.0)
*Missing**	*0*	-	-	-
Birth weight (grams)	3440 (2524-4300)	3480 (2585-4325)	3460 (2560-4320)	3440 (2524-4300)
*Missing**	*0.2*	-	-	-
Breastfeeding ever				
No	8.1	10.4	12.7	13.9
Yes	91.9	89.6	87.3	86.1
*Missing**	*14.0*	-	-	-
Day care attendance at 1 year				
No	42.1	42.8	47.5	50.6
Yes	57.9	57.2	52.5	49.4
*Missing**	*40.9*	-	-	-

Values are percentages for categorical variables and for continuous variables median (95% range). [a]: Subgroup (N=4,780) was used for multiple imputations, possible confounders and outcomes were imputed. [b]: Total study group (N=7,696) was used for multiple imputations, possible confounders and outcomes were imputed, selection was made on observed determinants after the multiple imputations procedure (N=5,603). [c]: Total study group (N=7,696) was used for multiple imputations, determinants, possible confounders and outcomes were imputed. *: Percentage of valid missing in the total study group (N=7,696).

Table 8.3. Wheezing symptoms in the observed and the multiple imputed datasets

	Observed dataset	**Imputed subgroup**[a]	**Imputed total study group and analysis in subgroup**[b]	**Imputed total study group**[c]
	N=7,696	**N=4,780**	**N=5,603**	**N=7,696**
Wheezing symptoms				
1st year				
No	70.6	71.1	70.4	69.4
Yes	29.4	28.9	29.6	30.6
*Missing**	*34.6*	-	-	-
2nd year				
No	80.1	80.2	79.4	78.4
Yes	19.9	19.8	20.6	21.6
*Missing**	*32.7*	-	-	-
3rd year				
No	87.3	87.4	86.8	86.0
Yes	12.7	12.6	13.2	14.0
*Missing**	*37.5*	-	-	-
4th year				
No	87.1	87.0	86.1	85.1
Yes	12.9	13.0	13.9	14.9
*Missing**	*37.7*	-	-	-

Values are percentages. [a]: Subgroup (N=4,780) was used for multiple imputations, possible confounders and outcomes were imputed. [b]: Total study group (N=7,696) was used for multiple imputations, possible confounders and outcomes were imputed, selection was made on observed determinants after the multiple imputations procedure (N=5,603). [c]: Total study group (N=7,696) was used for multiple imputations, determinants, possible confounders and outcomes were imputed. *: Percentage of valid missing in the total study group (N=7,696).

in relation to observed and missing data of determinants and outcomes. In general, missing determinants were associated with repeated outcomes and most confounders. Missing outcomes were also associated with most confounders, but not consistently with determinants.

Comparative analyses

Effect estimates were not different between (MI-)GEE and GLMM (Table 8.4 and 8.5). Higher effect estimates, but also larger standard errors were observed in the unadjusted GLMM compared to (MI-)GEE. After adjustment for confounders

Table 8.4. Associations of maternal history of atopy or asthma with childhood wheezing using different population selections and imputation strategies with (MI-)GEE models

Analysis 1a + b		**Analysis 2**		**Analysis 3**		**Analysis 4**		**Analysis 5**		**Analysis 6**		**Analysis 7**	
Complete cases		MI of C and analysis in subgroup		MI of C + O and analysis in subgroup		MI of C in total study group and analysis in subgroup		MI of C + O in total study group and analysis in subgroup		MI of D + C in total study group		MI of D + C + O in total study group	
OR wheezing (95% CI)[a]		**OR wheezing (95% CI)**[b]		**OR wheezing (95% CI)**[b]		**OR wheezing (95% CI)**[b]		**OR wheezing (95% CI)**[b]		**OR wheezing (95% CI)**[b]		**OR wheezing (95% CI)**[b]	
Crude (n=2,866)		**Crude (n=4,780)**†		**Crude (n=4,780)**		**Crude (n=4,780)**†		**Crude (n=5,603)**		**Crude (n=6,165)**		**Crude (n=7,696)**	
No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*
Yes	1.25 (1.09-1.43)	Yes	1.31 (1.17-1.46)	Yes	1.31 (1.20-1.42)	Yes	1.31 (1.17-1.46)	Yes	1.29 (1.17-1.42)	Yes	1.36 (1.26-1.47)	Yes	1.34 (1.22-1.47)
Adjusted (n=1,934)*		**Adjusted (n=4,780)***		**Adjusted (n=4,780)***		**Adjusted (n=4,780)***		**Adjusted (n=5,603)***		**Adjusted (n=6,165)***		**Adjusted (n=7,696)***	
No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*
Yes	1.26 (1.07-1.49)	Yes	1.35 (1.24-1.47)	Yes	1.35 (1.24-1.47)	Yes	1.35 (1.24-1.47)	Yes	1.33 (1.22-1.46)	Yes	1.40 (1.29-1.51)	Yes	1.38 (1.26-1.51)

Abbreviations: MI, multiple imputations; D, determinants; C, confounders; O, outcomes; OR, odds ratio; CI, confidence interval. [a]: Overall wheezing (1st-4th year) odds ratio (95% confidence interval) from generalized estimating equation (GEE) models and [b]: multiple imputation-based generalized estimating equation (MI-GEE) models. Modelling the probability that children are wheezing. *: Adjusted for maternal age at intake, BMI at enrolment, parity, and smoking during pregnancy, paternal educational level, and children's sex, ethnicity, gestational age, birth weight, and day-care attendance. Maternal educational level, pet keeping during pregnancy and breastfeeding were not significantly associated with wheezing and did not alter the effect estimates of the determinants with more than 10%, and were therefore not included in the model. †: The crude models of analysis 2 and analysis 4 are the same, as no variables are imputed for these analyses.

Table 8.5. Associations of maternal history of atopy or asthma with childhood wheezing using different population selections and imputation strategies with GLMM models

Analysis 1a + b		**Analysis 2**		**Analysis 3**		**Analysis 4**		**Analysis 5**		**Analysis 6**		**Analysis 7**	
Complete cases		MI of C and analysis in subgroup		MI of C + O and analysis in subgroup		MI of C in total study group and analysis in subgroup		MI of C + O in total study group and analysis in subgroup		MI of D + C in total study group		MI of D + C + O in total study group	
OR wheezing (95% CI)[a]		**OR wheezing (95% CI)[a]**		**OR wheezing (95% CI)[a]**		**OR wheezing (95% CI)[a]**		**OR wheezing (95% CI)[a]**		**OR wheezing (95% CI)[a]**		**OR wheezing (95% CI)[a]**	
Crude (n=2,866)		**Crude (n=4,780)†**		**Crude (n=4,780)**		**Crude (n=4,780)†**		**Crude (n=5,603)**		**Crude (n=6,165)**		**Crude (n=7,696)**	
No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*
Yes	1.40 (1.15-1.70)	Yes	1.50 (1.28-1.76)	Yes	1.49 (1.27-1.74)	Yes	1.50 (1.28-1.76)	Yes	1.47 (1.25-1.72)	Yes	1.59 (1.38-1.84)	Yes	1.55 (1.34-1.79)
Adjusted (n=1,934)*		**Adjusted (n=4,780)***		**Adjusted (n=4,780)***		**Adjusted (n=4,780)***		**Adjusted (n=5,603)***		**Adjusted (n=6,165)***		**Adjusted (n=7,696)***	
No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*	No	*Reference*
Yes	1.25 (1.07-1.46)	Yes	1.34 (1.20-1.50)	Yes	1.35 (1.22-1.49)	Yes	1.34 (1.20-1.49)	Yes	1.33 (1.20-1.48)	Yes	1.39 (1.26-1.54)	Yes	1.38 (1.26-1.52)

Abbreviations: MI, multiple imputations; D, determinants; C, confounders; O, outcomes; OR, odds ratio; CI, confidence interval. [a]: Overall wheezing (1st–4th year) odds ratio (95% confidence interval) from generalized linear mixed models (GLMM). *: Adjusted for maternal age at intake, BMI at enrolment, parity, and smoking during pregnancy, paternal educational level, and children's sex, ethnicity, gestational age, birth weight, and day-care attendance. Maternal educational level, pet keeping during pregnancy and breastfeeding were not significantly associated with wheezing and did not alter the effect estimates of the determinants with more than 10%, and were therefore not included in the model. †: The crude models of analysis 2 and analysis 4 are the same, as no variables are imputed for these analyses.

the differences in results from the models remained similar. The estimates of the MI subgroup, total group with selection on observed determinant after MI and the MI total study group were comparable for both MI-GEE and GLMM (Tables 8.4 and 8.5). Slightly lower effect estimates and larger standard errors were observed for complete case GEE and GLMM analyses compared to the MI subgroup, the total group with selection on determinant and the total study group. However, the results were not different. MI of determinants and outcomes did not lead to differences in effect estimates, as compared to models without imputation, with MI-GEE or GLMM (Tables 8.4 and 8.5). Slightly stronger estimates were observed when determinants were imputed. All estimates were within the 95% CI of the other models.

Discussion

We performed a study focused on associations of a determinant (maternal history of atopy or asthma) with an outcome (repeatedly measured childhood wheezing from birth to 4 years). We explored differences in effect estimates after applying GEE and GLMM analyses in complete cases and MI-GEE and GLMM. We did this in three different population selections for MI. Missing confounders, determinants and outcomes were imputed. The results showed no overall differences in effect estimates for the associations of the determinant and outcomes for all different strategies of analysis and MI. Slightly weaker effect estimates and larger standard errors were observed for complete case analysis as compared to analyses of datasets where MI were used. Imputation of determinants and outcomes did not lead to differences in effect estimates as compared to models without imputation of determinants and outcomes.

Statistical approaches

We observed no differences in effect estimates with GLMM, irrespective of whether outcomes were imputed. GLMM are flexible in handling repeated missing outcome data[6]. Both (MI-)GEE and GLMM were equivalent to study the association between maternal history of atopy or asthma and wheezing within this cohort. Alternative statistical methods to deal with missing binary repeated outcome data are available, including the weighted GEE (WGEE) procedure. This is not widely available, however, and requires advanced statistical knowledge[5]. WGEE models produce accurate results, but might be biased and less accurate in small to moderate sample sizes compared to MI-GEE[6]. Covariates and determinants were not repeatedly assessed in our study, which may have led to larger differences in estimates when missings are related to different time periods.

Population selection

We observed that differences in estimates based on MI of a subgroup, of the total study group with selection on the observed determinant afterwards or of the total study group were negligible. Complete case analysis showed slightly lower effect estimates as compared to the MI analysis. This is in line with previous studies[17,18]. Inclusion of variables associated with the outcome in the MI might have increased odds ratios and reduced standard errors[3]. We note that differences in complete case analyses and MI should be interpreted with caution and should not only be based on comparison of standard errors, as standard errors after MI reflects the uncertainty of the data rather than study precision[17]. Adjustment for confounders reduced the number of complete cases dramatically leading to reduced power. Hence, complete case analysis is not to be preferred for our particular analysis. Indeed, imputation of missing confounder values has been advised for analyses of the determinant – outcome relation[11]. Using the total study population invited at baseline for MI takes the uncertainty of all missing data in the dataset into account, instead of only those missing data that remain after selecting a particular subgroup[19]. However, if the number of missing data is high, too much uncertainty may be created for the studied association, leading to a reduced model fit[20].

Multiple imputations strategies

Missing data on determinants were weakly related to repeated outcomes and confounders in this study. As a result, we observed a very small increase in the effect estimates when determinants were imputed. It has been shown previously that MI of confounders should include outcome and determinant in the imputation model to minimize bias in the relationship between determinant and outcome[11]. Also, when outcome and determinant carry information about the missing values of other covariates, this information must be used for MI to increase the plausibility of the MAR assumption[2,3]. Excluding the outcome and determinant in the MI model may falsely weaken the association between determinant and outcome[2]. Higher effect estimates, but also larger standard errors were observed for univariate GLMM compared to (MI-)GEE. Multivariate models performed similarly. After adjustment for confounders no differences were found suggesting that the covariates may sufficiently capture the pattern of missing data on the outcome variables. Inclusion of more variables associated with the outcome in the MI procedure could increase odds ratios and reduce standard errors[3], and would increase the plausibility of the MAR assumption[2,3]. Different strategies for dealing with missing binary repeated outcome had no effect on the associations between determinant and outcome. However, it should be noted that we did not observe clear patterns for the missing variables. When missing data on the outcomes are related to several confounders and the determinant, imputation of

both the confounders and outcome can be necessary. Also, when missing determinants are not random, imputation on the determinant variable may be needed[17]. Relations between missing study variables is recommended to always be carefully explored and appropriate methods should be based on the missing patterns[17].

Conclusion

Our results show that (MI-)GEE and GLMM lead to comparable associations of determinant and outcome. No differences were observed when we imputed not only covariates, but also determinants and outcomes. The major advantage of MI is a larger study population.

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Supplementary materials

Supplementary Table 8.1. Details of multiple imputations modelling

Software used and key setting: SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) – Imputation Method determined by SPSS (automatic option) with 10 iterations.
Number of imputed datasets created: 10
Variables included in the imputation procedure:
Imputed wheezing-related variables:
wheezing year 1 (binary: no, yes)
wheezing year 2 (binary: no, yes)
wheezing year 3 (binary: no, yes)
wheezing year 4 (binary: no, yes)
maternal history (combination of 4 binary questions: house dust, hay faver, eczema, asthma)*
age mother at intake (continuous)
bmi mother at intake (continuous)
maternal prenatal smoking (nominal: no, first trimester, continued smoking)
maternal education (nominal: primary, secundary, higher)
number of deliveries (nominal: 0, 1 >=2)
paternal education (nominal: primary, secundary, higher)
pets keeping during pregnancy (combination of 3 binary questions: cat, dog and bird)*
gender child (binary: male, female)
ethnicity child (nominal: Dutch, European, Turkish, Surinamese, Moroccan, Asian, Other)†
gestational age (continuous)
birth weight (continuous)
breastfeeding ever (binary: no, yes)
day care attendance (binary: no, yes)
Predictor variables:
respiratory tract infections severity year 1 (binary: no, yes)
respiratory tract infections severity year 2 (binary: no, yes)
respiratory tract infections severity year 3 (binary: no, yes)
respiratory tract infections severity year 4 (binary: no, yes)
eczema year 1 (combination of 2 binary questions: eczema 1-6 months, eczema 7-12 months)*
eczema year 2 (binary: no, yes)
eczema year 3 (binary: no, yes)
eczema year 4 (binary: no, yes)
shortness of breath year 1 (binary: no, yes)
shortness of breath year 2 (binary: no, yes)

shortness of breath year 3 (binary: no, yes)
shortness of breath year 4 (binary: no, yes)
cough year 1 (binary: no, yes)
cough year 2 (binary: no, yes)
cough year 3 (binary: no, yes)
cough year 4 (binary: no, yes)
phlegm year 1 (binary: no, yes)
phlegm year 2 (binary: no, yes)
phlegm year 3 (binary: no, yes)
phlegm year 4 ((binary: no, yes)
weight 10-13 months – year 1 (continuous)
height 10-13 months – year 1 (continuous)
weight 23-29 months – year 2 (continuous)
height 23-29 months – year 2 (continuous)
weight 35-44 months – year 3 (continuous)
height 35-44 months – year 3 (continuous)
weight 44-56 months – year 4 (continuous)
height 44-56 months – year 4 (continuous)
paternal house dust mite allergy (binary: no, yes)
paternal hay fever (binary: no, yes)
paternal eczema (binary: no, yes)
paternal asthma (binary: no, yes)
postnatal environmental smoke year 2 (binary: no, yes)
breastfeeding duration (nominal: never, <3 months, 3-6 months, > 6 months)
breastfeeding exclusivity (nominal: never, partial 4 months, exclusive until 4 months)
maternal prenatal alcohol (binary: no, yes)
paternal prenatal smoking (binary: no, yes)
bmi father at intake (continuous)
age father at intake (continuous)
maternal prenatal stress (continuous)
parenting stress score (continuous)
cows allergy (binary: no, yes)
income household (binary: < €2000,-, > €2000,-)
folic acid supplementation during pregnancy (nominal: no, suboptimal, optimal)
Treatment of binary/categorical variables: Logistic/multinomial models
Statistical interactions included in imputation models: None

*: Each binary variable was imputed first and combination of variables was done after multiple imputations procedure; †: The seven ethnicities were imputed, after the multiple imputations procedure we defined European and non-European children.

Supplementary Table 8.2. Confounder characteristics in relation to observed and missing data of the determinants and outcomes

	Maternal age (years)	Maternal BMI (kg/m^2)	Parity	Prenatal smoke exposure	Paternal education	Child gender	Child ethnicity	Gestational age (weeks)	Birth weight (grams)	Daycare attendance	Maternal house dust	Maternal hay fever	Maternal eczema	Maternal asthma	Wheezing at 1 year	Wheezing at 2 years	Wheezing at 3 years	Wheezing at 4 years
	Median	Median	% ≥2	% Continued	% Highest	% Female	% European	Median	Median	% Yes	% Yes	% Yes	% Yes	% Yes	% Yes	% Yes	% Yes	% Yes
DETERMINANTS																		
House dust																		
Observed	31.0	23.6	13.4	15.2	55.4	49.7	67.2	40.1	3450	59.5	-	-	-	-	28.8	19.3	12.0	12.4
Missing	30.5	24.2	18.2	18.3	48.8	48.7	55.0	40.0	3390	49.6	-	-	-	-	32.7	23.0	15.9	15.4
Hay fever																		
Observed	31.0	23.7	13.3	15.2	54.9	49.9	66.2	40.1	3450	58.9	-	-	-	-	28.7	19.5	12.3	12.5
Missing	30.5	24.2	19.6	19.2	50.5	47.7	59.0	40.0	3400	51.1	-	-	-	-	33.9	22.3	15.0	15.0
Eczema																		
Observed	31.0	23.7	13.1	15.3	54.9	49.6	66.7	40.1	3450	59.4	-	-	-	-	28.8	19.5	12.4	12.6
Missing	30.4	24.4	20.8	20.1	50.2	48.9	54.8	40.0	3380	47.2	-	-	-	-	32.7	22.7	14.5	15.1
Asthma																		
Observed	31.0	23.7	13.2	15.3	54.6	49.6	66.0	40.1	3450	58.9	-	-	-	-	28.9	19.6	12.3	12.6
Missing	30.6	24.4	21.3	24.4	51.8	48.7	59.1	40.0	3370	49.5	-	-	-	-	33.8	22.5	15.7	14.9

Supplementary Table 8.2. Confounder characteristics in relation to observed and missing data of the determinants and outcomes (continued)

	Maternal age (years)	Maternal BMI (kg/m^2)	Parity	Prenatal smoke exposure	Paternal education	Child gender	Child ethnicity	Gestational age (weeks)	Birth weight (grams)	Daycare attendance	Maternal house dust	Maternal hay fever	Maternal eczema	Maternal asthma	Wheezing at 1 year	Wheezing at 2 years	Wheezing at 3 years	Wheezing at 4 years
	Median	Median	% ≥2	% Continued	% Highest	% Female	% European	Median	Median	% Yes	% Yes	% Yes	% Yes	% Yes	% Yes	% Yes	% Yes	% Yes
OUTCOMES																		
Wheezing																		
Year 1																		
Observed	31.6	23.6	11.6	12.4	58.7	50.2	74.1	40.1	3475	57.8	16.9	28.8	5.1	7.5	-	-	-	-
Missing	29.1	24.2	20.1	23.0	37.4	48.1	44.9	40.0	3375	60.3	14.7	25.2	4.7	8.4	-	-	-	-
Year 2																		
Observed	31.7	23.6	11.7	13.0	59.2	49.9	74.3	40.1	3480	60.8	16.5	28.4	5.3	7.5	-	-	-	-
Missing	28.3	24.2	20.3	21.1	35.3	48.5	42.6	40.0	3360	37.4	15.7	26.1	4.3	8.2	-	-	-	-
Year 3																		
Observed	31.8	23.5	11.6	12.0	59.6	49.8	74.3	40.1	3480	61.3	16.2	27.9	5.3	7.2	-	-	-	-
Missing	28.6	24.2	19.3	21.9	39.7	48.9	47.4	40.0	3380	42.5	16.3	27.3	4.4	8.7	-	-	-	-
Year 4																		
Observed	31.8	23.6	11.6	12.0	59.3	49.9	74.9	40.1	3480	62.2	15.9	27.9	5.1	7.4	-	-	-	-
Missing	28.6	24.1	19.3	21.8	39.7	48.8	46.4	40.0	3390	40.0	16.9	27.4	5.0	8.3	-	-	-	-

Values are percentages for categorical variables and medians are depicted for continuous variables. We examined descriptive characteristics of confounders in relation to observed and missing data of the determinants and outcomes in the total study group (N=7,69).

Chapter 9

General discussion

General discussion

In this thesis, we focussed on the fractional concentration of exhaled nitric oxide (FeNO), a biomarker of eosinophilic airway inflammation, in children with asthma. FeNO exhibits daily fluctuations, and we examined these fluctuations in relation to asthma control and exacerbations in children participating in the CHARISM study. We also measured FeNO at 4 and 8 years in the PIAMA birth cohort and examined FeNO in distinct phenotypes of wheeze and atopy. Identification of common genetic variants associated with complex traits, such as FeNO, may help to further elucidate biological mechanisms related to a specific asthma phenotype in childhood[1-4]. Hence, an important objective of this thesis was to identify novel genetic variants associated with childhood FeNO using the hypothesis-free genome-wide association (GWA) study approach in the EAGLE Consortium. Previously identified genetic risk factors of asthma and the influence of environmental risk factors on the development of childhood asthma were examined in the Generation R Study.

This chapter summarizes the main findings of this thesis and provides a discussion on methodological considerations and clinical applications. Finally, recommendations for future research are presented.

Main findings

Fluctuations in exhaled nitric oxide measurements

Assessment of asthma control is important to guide treatment. It is however difficult to predict the temporal pattern and risk of exacerbations in a given patient. Theories derived from sciences dealing with complexity of physiological parameters can be applied to (partly) explain the 'unpredictable' nature of asthma. Fluctuation analysis, a method used in statistical physics, can be used to gain insight into asthma as a dynamic disease of the respiratory system, viewed as a set of interacting subsystems (e.g., inflammatory, immunological, and mechanical). Fluctuation analysis methods can be applied to the quantification of the long-term temporal history of lung function parameters (e.g., daily peak expiratory flows (PEFs), measurements of airway patency)[5]. This information is potentially useful and might help to assess the risk of future asthma episodes, with implications for asthma severity and control[6,7]. We found similarly informative fluctuation patterns of FeNO and this implicates that childhood asthma is indeed a highly dynamic and heterogeneous disease.

Previous studies on FeNO in relation to asthma management suggested that using FeNO to guide asthma treatment might reduce the risk of exacerbations[8].

Unfortunately, most earlier studies on FeNO-guided asthma management were underpowered to demonstrate a significant effect on exacerbations[9-12]. In the only study with sufficient power, FeNO monitoring significantly reduced the number of children that needed multiple prednisone courses[13]. However, design issues may have flawed the results[14]. FeNO during acute severe exacerbations was studied previously, and no correlation was found between FeNO and other measures of severity[15,16]. These studies concluded that FeNO is not informative for severe exacerbations.

Single FeNO measurements differed for distinct phenotypes of wheeze and atopy, but there is considerable overlap between the distributions of FeNO for the different phenotypes[17]. Patterns of daily FeNO measurements in relation to asthma control and exacerbations have not been studied before. Fluctuations in FeNO might provide better information than looking at single and averaged FeNO for monitoring and risk prediction of asthma[6]. FeNO is not constant over time, but is a highly fluctuating dynamic parameter (Figure 9.1) that can be influenced by many factors, e.g. inhaled corticosteroids (ICS) use, sputum induction, exercise, smoke and allergen exposure[18].

Interestingly, the majority of children with exacerbations had an increase in FeNO, a strong positive cross-correlation between FeNO and symptoms and autocorrelation before exacerbations compared to reference periods[19]. The level of cross-correlation between FeNO and symptoms in the whole study period of 192 days was stronger in children with- than in those without exacerbations, and we speculated that the level

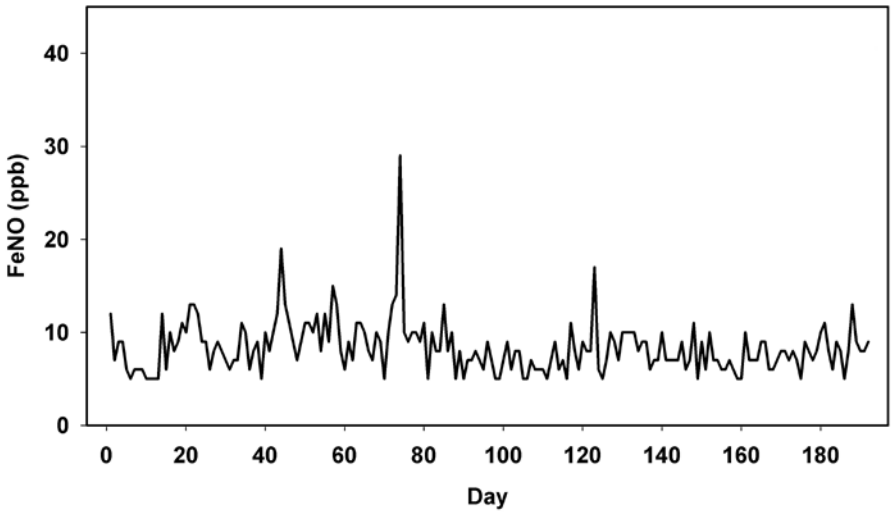

Figure 9.1. Daily exhaled nitric oxide measurements over 192 days from a child participating in the CHARISM study

This child had a constant ICS dose during the study period.

of cross-correlation may be useful to identify children at risk for exacerbations[20]. In addition, we showed that daily FeNO measurements exhibited fractal-type long-range correlations. Lower fractal-type correlation, indicating more random fluctuations in FeNO, was associated with greater ICS need[21].

Cross-correlation between daily FeNO and symptoms and fractal-type fluctuations in daily FeNO might contain information on asthma severity and control. Identifying asthmatic children with discordance between high FeNO, suggestive of eosinophilic inflammation, and symptoms is clearly important, as such children may have an increased exacerbation risk[6,21]. Our findings also suggest that regular FeNO measurements in the home setting could help to detect and perhaps even help prevent loss of asthma control. Such monitoring with advanced algorithms could be especially useful in a selected population with frequent moderate exacerbations[19]. However, daily FeNO measurements in a home setting are costly and not practical and it remains to be shown if daily FeNO measurements are cost-effective in reducing asthma exacerbations and treatment prescriptions in any patient population. In addition, we calculated cut-off values to predict exacerbations for our specific dataset, with no information available on the physiological origins of the fluctuations in daily FeNO. Therefore, the predictive ability of our studies should be taken with caution.

Although, FeNO is fluctuating, FeNO is on average higher in children with allergic asthma who have eosinophilic airway inflammation[17]. The mechanisms underlying different asthma phenotypes are still poorly understood[1,2]. FeNO is a trait that is influenced by an interplay of genetic and environmental factors. A Norwegian twin study showed that genetic effects accounted for 60% for the variation in FeNO in adults[22]. We aimed to identify common genetic variants associated with FeNO, because this may help to further elucidate biological mechanisms related to specific asthma phenotypes in childhood[1-4].

Genetics of exhaled nitric oxide and asthma in childhood

We performed a GWA study on FeNO in childhood, and found a novel independent signal of rs3751972 in *LYRM9*, next to the signal of rs944722 in the downstream neighbouring gene *NOS2* at 17q11.2-q12 to be associated with FeNO. The function of *LYRM9* is unknown. Variants in the nitric oxide synthases (NOS) pathway genes and arginase (ARG) genes seem to contribute to differences in FeNO concentrations[23-26] and, perhaps, asthma severity[27]. Inducible NOS2 is expressed in lung epithelium and is synthesized in response to proinflammatory mediators. Expression of inducible NOS2 may be beneficial in host defense or in modulating the immune response[18,28]. In our study only *NOS2*, but not *NOS1* and *NOS3*, was robustly associated with childhood FeNO, suggesting that inducible NOS2 is indeed the most important NOS enzyme in

the production of NO[23]. Furthermore, we found a *cis* expression quantitative trait locus (eQTL) for the transcript *LGALS9* in LD with rs944722 in *NOS2*. *LGALS9* is downstream of *NOS2* and might be involved in prolonged eosinophil accumulation in the lung[29]. We identified a third genome-wide signal for FeNO in the 17q12-q21 asthma locus harbouring *ZPBP2*, *GSDMB*, and *ORMDL3* genes and this locus is a complex region with high LD. The 17q12-q21 locus is the most consistent and strongest associated locus for childhood asthma[30-34]. The exact functions of *ZPBP2*, *GSDMB*, and *ORMDL3* genes are not completely clear[35-37]. Surprisingly, the functions of the 17q12-q21 genes seem not to influence allergic type of pathways. The study of Verlaan *et al* implicates one of the potential causative SNPs of the 17q12-q21 locus[34], but further follow-up studies are needed.

Signals in *LYRM9* and *NOS2* were not associated with childhood asthma. This study highlights that different genetic factors act on childhood asthma through specific phenotypic pathways, such as FeNO. GWA studies learned us that different common genetic variants are associated with different asthma-related outcomes: childhood onset asthma[30,31], adult asthma[31,38,39], impaired lung function[40-42], and atopy[43-45]. The 17q11.2-q12 and 17q12-q21 loci are both complex regions with high LD and seem to harbour multiple independent signals. Our GWA study explained only a small portion of the heritability of childhood FeNO. It is expected that loci can harbour multiple independent signals (both common and rare) that are independently associated with the outcome of interest and adding those variants will increase the percentage of variation explained[46-48]. We performed a conditional analysis of all single nucleotide polymorphisms (SNPs) using genome-wide complex trait analysis (GCTA) for the GWA study of FeNO[48], showing that the signals in *LYRM9* and *NOS2* are independent. This tool could be used for future GWA studies of asthma to discover additional new variants independently associated with asthma and to explore independent effects in the 17q12-q21 asthma locus. Further explanations of missing heritability for FeNO and asthma are described in the methodological considerations and clinical application paragraphs.

Methodological considerations

Improvements in identifying genetic variants of exhaled nitric oxide and asthma

Clearly the key factor in identifying more novel genetic variants is increasing the sample size. To our knowledge, there are no further child cohort studies with GWA and FeNO data available. Future research to identify more loci for asthma is currently underway in the TAGC consortium, a large consortium of all available consortia with

GWA and both child and adult asthma data available. That increasing the sample size is important has been shown for other traits, such as height, a meta-analysis with an increasingly larger sample size, yielding an ever increasing number of genetic loci to be found. Data of these large collaborations can also be used to determine specific biological pathways incorporating multiple genes[49].

Genetic variants could have an effect on two or more phenotypic traits (pleiotropy). Gene pleiotropic effects is a common feature of human traits[50]. In this thesis we reported that the 17q12-q21 locus was associated with childhood FeNO. Moffatt *et al* previously showed that the same 17q12-q21 locus is associated with childhood asthma[30,31]. Pleiotropic properties can be exploited to identify additional risk variants by combining two GWA studies of disease-related traits. Combining GWA study data of two related traits may help to identify more pleitropic loci beyond those already identified at genome-wide significance level of standard GWA studies[51].

To increase genomic coverage of the GWA study presented in this thesis we performed imputation using HapMap II release 22 CEU as reference panel. This reference panel contains ~2.5 Million, mostly common SNPs, and the SNPs are derived from only 60 sequenced individuals. There is a debate going on of human geneticists considering that rare variants would have a greater impact on phenotype variation[47], whereas others still believe in the common variants approach yielding to more important new biological insights[52]. However, in the future reference panels from an international collaboration the 1000 Genomes project (1000G, http://www.1000genomes.org/) and also from regional projects, such as The Genome of the Netherlands (GoNL, http://www.dutchgenomeproject.com/) can be used to improve genomic coverage by increasing the density and sample size of imputation reference panels.

A point of consideration in the interpretation of the data of the GWA study of FeNO is that some children were using inhaled corticosteroids (ICS) while FeNO was measured, and it has been shown that ICS can decrease FeNO[18]. Unfortunately, not all cohorts had information on ICS use on time of FeNO measurement available and therefore it was not possible to adjust for ICS use in our analysis. However, any such effect of ICS seems limited because a sensitivity analysis after exclusion of steroid users on the association between wheezing phenotypes and FeNO in chapter 2 showed no change in results. If ICS would have reduced FeNO, we would have underestimated the effects.

Associations of genetic variants in *NOS* and *ARG* genes, and perhaps also in other genes with childhood FeNO might be different among asthmatic versus non-asthmatic children[23,26]. Unfortunately, due to the prevalence of asthma in our population-based studies, stratifying for asthma or atopy on genome-wide level was not possible due to lack of power. A previous study suggested that DNA methylation in promotor regions

of *ARG1* and *ARG2* is associated with FeNO in children with asthma[24]. Thus, DNA methylation could play an important role of epigenetic regulation of other genes for nitric oxide production.

Atopy is an important determinant for FeNO[17,53-56]. Some authors have suggested that the association between asthma and FeNO may be entirely explained by atopy, implying that measuring FeNO is of limited use to assess whether a child has asthma[57]. We found an association between the 17q12-q21 childhood asthma locus and FeNO, implying that FeNO might be causally related with asthma. However, this association can still be driven by atopy. Unfortunately, it was not possible to examine a causal relationship between asthma and FENO in a Mendelian randomization approach since most cohorts had no information on atopic status available. In addition, associations of genetic variants with childhood FeNO might also be different for atopic and non-atopic children.

Gene-environment interactions and childhood asthma development

The term 'gene-environment interaction' refers to a situation where an environmental risk factor acts as an effect modifier of a genetic risk factor. Different exposures of genetic and environmental risk factors and the interaction between genetic and environmental risk factors may result in the existence of many different asthma phenotypes (Figure 9.2)[58].

It has been proposed to abolish the term asthma altogether[1] and focus on specific asthma phenotypes or endotypes rather than on asthma as a single disease entity[2].

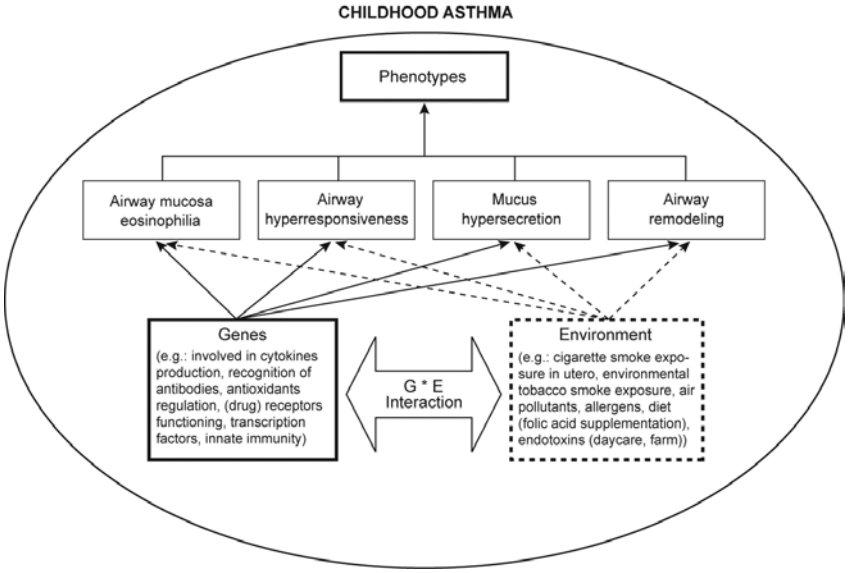

Figure 9.2. Gene-environment interaction and childhood asthma

Studies examining the interaction between genetic and environmental risk factors that are associated with different phenotypes may help to elucidate the origins of asthma[58,59]. That gene-environment interactions in humans are important for asthma was clearly shown by Bouzigon *et al*. Their study showed that the increased risk of asthma conferred by 17q12-q21 genetic variants is restricted to early-onset asthma and that the risk is further increased by early-life exposure to environmental tobacco smoke (ETS)[32]. Exploration of interactions between 17q12-q21 variants, fetal smoke exposure and ETS exposure in infancy may identify specific early critical time periods of increased susceptibility[33,60]. In this thesis we showed that a 17q12 variant, rs2305480, was associated with asthma-related outcomes in preschool children, and this association was modified by smoke exposure already during fetal life, and in infancy.

Misclassification of exposures is a major limitation of environmental epidemiology. Differences in measurement methods and the timing make it difficult to compare study results. Many environmental exposures are assessed by questionnaires, e.g. smoking and folic acid-containing supplementations used during pregnancy, postnatal environmental tobacco smoke exposure and breastfeeding and are subjected to reporter bias. Objective biomarkers, e.g. biochemical measures of cotinine representing smoke exposure or biochemical measures of folate status are less prone to reporter bias. We tried to show independent effects of fetal and postnatal ETS exposure by stratifying on smoke exposure. However, an ERS task-force concluded that stratifying on smoke exposure is not sufficient to claim independent effects of fetal and postnatal ETS exposure. Even with stratifying, it is still possible that we were detecting an effect of postnatal smoke.

Many genetic factors and their interaction with environmental factors still need to be discovered. These interactions may explain a large portion of the missing disease heritability of asthma[46]. The EAGLE consortium is a good basis for future gene-environment studies on asthma and allergic diseases. EAGLE can be used for discovery and replication efforts. Uniform exposure assessment will be challenging and expected differences in results should be discussed in advance. Currently, many gene-environment studies are using tagSNPs to determine genetic variation of certain genes. Further investigation regarding causal SNPs in linkage disequilibrium with tagSNPs in relation to pathophysiological pathways is needed[61].

Repeated asthma-related outcomes were used in our gene-environment studies. Missing data in binary repeated outcome measurements are complex to analyse[62-64]. Multiple imputations (MI) can be used to pre-process incomplete data, including outcome data, after which generalized estimating equations (GEE) is applied (MI-GEE)[62]. Alternatively, generalized linear mixed models (GLMM) can be used to deal with missing binary repeated outcome measurements[62,64]. We explored differences

in effect estimates after applying different strategies for analysis and imputation of missing values, using data from a study on the associations of maternal atopy with childhood wheeze from birth to 4 years. Our results suggest that MI-GEE and GLMM lead to similar effect estimates for the associations of maternal atopy with childhood wheeze. No differences were observed when we imputed not only covariates, but also determinants and outcomes. Relations between missing study variables is recommended to always be carefully explored and appropriate methods should be based on the missing patterns[65]. The major advantage is that imputation leads to larger populations for analysis. Genetic studies have the advantage of random assignment of an individual's genotype from his or her parental genotypes that occurs before conception[66], meaning that associations between genetic variants and asthma-related outcomes are less likely confounded by missing data of genetic risk factors. However, we still need appropriate methods to impute confounders, environmental effect modifiers and methods to deal with missing binary repeated asthma-related outcomes.

Clinical applications

Asthma risk prediction

The advent of GWA studies has led to the discovery of common risk loci for the majority of common diseases including childhood asthma[31]. These discoveries raise the possibility of using these variants for risk prediction in a clinical setting[67]. Predictive value of a single gene test in a complex disease such as asthma is of limited significance for diagnostic or preventive purposes, as only a small proportion of the risk can be explained[68]. It has been shown that multiple common variants explain a large proportion of the heritability, such as human height[69]. However, the predictive accuracy from genetic models incorporating multiple genes varies greatly across diseases. Thus far the results of most models incorporating multiple genes have still been disappointing, but the range is similar to that of non-genetic risk-prediction models[67]. This can be explained by several factors, the most important of which is that the identified genetic variants explain a small proportion of the phenotypic variance. Currently we are mostly studying tagSNPs and the causal variants may explain more. In addition, other types of genetic variation may be missed with the GWA approach of SNPs[46]. It is however also impossible to accurately predict with clinical prediction rules which child will develop asthma and which not[70]. This may be explained by the fact that asthma is a heterogeneous disease, existing in many different phenotypes[59], influenced by many genetic and environmental factors[58]. That genetic origins of asthma are diverse, and some pathways are specific to wheezing

syndromes, whereas others are shared with atopy and bronchial hyperresponsiveness was demonstrated with a genome-wide prediction study of childhood asthma and related phenotypes in a longitudinal birth cohort[71]. GWA studies also provided evidence that asthma is a heterogeneous disease by showing that different common genetic variants are associated with different asthma-related outcomes: childhood onset asthma[30,31], adult asthma[31,38,39], impaired lung function[40-42], and atopy[43-45].

The increase in the prevalence of asthma in the last two decades can not be explained by the small changes in the genetic constitution of the Western population that occur over time[72,73]. It rather occurs by changing of environmental factors, such as indoor and outdoor pollution, allergen exposure, tobacco smoke in utero and in postnatal life and other factors related to the 'Western lifestyle' like eating habits, including vitamin supplementation and contact with microbial products. This thesis showed that genetic and environmental factors interact in the development of asthma. Many genetic factors and their interaction with environmental factors still need to be discovered. Gene-gene interaction and gene-personal risk factors, such as sex-specific genetic effects may also play an important role. Based on data of simulation studies and other complex diseases, the use of genetic profiling that incorporates multiple genetic risk factors holds promise for clinical application[67,74]. The results of GWA studies will be crucial in establishing genetic risk profiles for asthma. In the future, asthma prediction may be possible, based on a prediction model that incorporates genes, personal factors and environmental risk factors[68].

Identifying potential therapeutic targets of asthma

There is heterogeneity in patient responses to current asthma medications. Significant progress has been made in identifying genetic polymorphisms that influence the efficacy and potential for adverse effects to asthma drugs[75]. Recent GWA studies begun to shed light on both common and distinct pathways that contribute to asthma and allergic diseases[76]. Moffatt *et al* identified *interleukin-13* (*IL-13*) with a GWA study approach as a genetic risk factor for physician-diagnosed asthma[31]. *IL-13* is a relevant target for asthma treatment[77], but it is only one of the pathways that can lead to the expression of an asthma phenotype[78]. Anti-*IL-13* therapy targeted to susceptible adults with asthma who had a pre-treatment profile consistent with IL-13 activity showed improved lung function[79]. Another GWA study identified a functional *glucocorticoid-induced transcript 1* (*GLCCI1*) gene variant to be associated with a reduced response to inhaled glucocorticoids in children with asthma[80]. Associations with variation in genes encoding the epithelial cell-derived cytokines, *interleukin-33* (*IL-33*) and *thymic stromal lymphopoietin* (*TSLP*), and the *interleukin 1 receptor-like 1* (*IL1RL1*) gene encoding the IL-33 receptor, ST2, highlight the central roles for innate immune response pathways that promote the activation and differentiation

of T-helper 2 cells in the pathogenesis of both asthma and allergic diseases[76]. The 17q12-q21 asthma locus, encoding the *ZPBP2*, *GSDMB*, and *ORMDL3* genes, is the most consistent and strongest associated locus for childhood asthma[30,31]. These genes, identified by GWA studies, represent potential future therapeutic targets. Identification of common genetic variants associated with a specific phenotype of asthma, such as FeNO, may help to further elucidate biological mechanisms related to specific asthma phenotypes in childhood[1-4]. FeNO and asthma are conditions with multiple etiologies involving both genetic and environmental contributions. Multiple independent signals in and near *LYRM9* and *NOS2* at 17q11.2-q12 were found to be associated with childhood FeNO. Our findings show that the 17q12-q21 locus is associated with both FeNO and asthma. Identification of potential functional SNPs and haplotypes in both the 17q11.2-q12 and 17q12-q21 regions through deep sequencing and functional studies (e.g., knock-out mice, cell-expression) are needed to elucidate the biological mechanisms responsible for childhood asthma and its phenotypes, and this information may well lead to the development of better therapies.

Future research directions

Most of the findings in this thesis have no immediate clinical relevance. Earlier studies on FeNO-guided asthma management did not consistently demonstrate significant benefits. However, design issues may have flawed the results[14]. Fluctuations in FeNO might provide better information than looking at single and averaged FeNO for monitoring and risk prediction of asthma[6]. Advanced algorithms including fluctuation analyses could be useful to indentify children with unstable asthma that are in need of changes in ICS doses. Clinical trials and cost-effectiveness studies are needed to show effectiveness of measuring daily FeNO and improving asthma control and reducing exacerbations. Furthermore, studies on physiological origins of fluctuations in FeNO and other lung function parameters are needed to improve the understanding of asthma as a systemic and dynamic disease.

Common genetic risk variants identified by GWA studies over the past decade have explained a small portion of disease heritability of childhood asthma[31,46]. In this thesis we explained only a small part of the variance in FeNO with common genetic variants. Rare variants and maybe even multiple independent rare variants in the same locus or across the whole genome may have a greater impact on phenotype variation[47]. Next generation sequencing technologies (whole genome, exome and targeted region sequencing) at low costs are necessary to identify all rare variants across a population. Sequence data can also be used to detect structural variation, such as indels and deletions, which have not been studied before in relation to childhood

asthma-related outcomes. Data analysis approaches for sequence data and methods to impute sequence data into a GWA dataset are still under development. Sequencing of individuals with an extreme phenotype of asthma could be in particular useful to indentify novel variants and to impute the detected variants back into a full GWA cohort[81,82]. To avoid false-positive findings of rare variants de-novo re-sequencing data will be required. Challenges lie ahead in applying these technologies, but for many diseases including childhood asthma, rare variants are likely to be a critical piece of the puzzle that needs to be solved to understand genetic basis of complex traits and to use this information to develop better therapies[83].

Rare variants are not the only answer to the missing heritability of childhood asthma[46]. Epigenetics refers to the study of heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence. Epigenetic mechanisms are grouped in three main classes: DNA methylation, modifications of histone tails, and noncoding RNAs (such as MicroRNAs). These classes may be influenced by environmental factors in certain periods in life, but also in utero and even transgenerational, and are also influenced by diseases and aging. Epigenetic marks have been shown to influence immune cell maturation. Evidence is emerging that these epigenetic marks affect gene expression in the lung and are associated with asthma[84-86]. It has already been shown that transcriptional changes in airway epithelia and inflammatory cells of patients with allergic asthma are influenced by asthma phenotype as well as environmental exposures[87]. Whole genome methylation arrays can be used to study epigenetic marks. Epigenetic marks do not have to change gene expression by definition and therefore also gene expression arrays are needed. These novel technologies make it feasible to study epigenetic marks and gene expression, and it is anticipated that this knowledge will enhance our understanding of the dynamic biology in the lung and lead to the development of novel diagnostic and therapeutic approaches for our patients with asthma[84-86].

The results of GWA studies imputed with sequence data will be crucial in establishing genetic risk profiles for childhood asthma. Many genetic factors and their interaction with environmental factors still need to be discovered. Gene-gene interactions and gene-personal risk factor interactions, such as sex-specific genetic effects may also play an important role in the development of childhood asthma. The above described studies should be performed and epidemiological results should be replicated in large consortia, such as EAGLE. In the future, asthma prediction may be possible, based on a prediction model that incorporates genes, personal factors and environmental risk factors[68].

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Summary & Samenvatting

Summary

In Western countries, asthma is one of the most frequent chronic disorders in childhood, with a high burden of morbidity. Childhood asthma exists in various forms, influenced by many genetic and environmental factors. In this thesis, we focus on a biomarker that is relevant for a subtype of asthma: the fraction of exhaled nitric oxide (FeNO). FeNO is a non-invasive biomarker of a distinct type of airway inflammation, and is associated with childhood asthma-like symptoms, asthma attacks, physician-diagnosed asthma and allergy. This thesis focuses on major challenges in the field of childhood asthma which have been insufficiently addressed: The interpretation of daily fluctuations in FeNO, and genetic and environmental risk factors of asthma and FeNO in childhood.

Chapter 1 begins with introducing FeNO and asthma in childhood and provides a background of previous studies, and describes the aims of this thesis. **Chapter 2** describes that FeNO measured at 8 years is differentially associated with five distinct types of wheezing. The differences in FeNO were only observed in allergic children. Assessment of asthma control is important to guide treatment. FeNO-guided asthma management was suggested to improve asthma control and reduce asthma attacks. In a previous project, we have measured FeNO in children at home on a daily basis. Data of this study were used to analyze the importance of daily fluctuations in FeNO. **Chapter 3** revealed changes in daily FeNO prior to asthma attacks and **Chapter 4** describes correlations between daily FeNO and symptoms. Fluctuations in FeNO measured over a long period of time contain information on asthma severity and control. Whether changes in daily FeNO can be used to prevent loss of asthma control should be further explored.

FeNO is on average higher in children with allergic asthma who have a distinct type of airway inflammation. In **Chapter 5** we identified three genetic regions that influence FeNO levels. This study also highlights that both shared and distinct genetic factors affect FeNO and childhood asthma, with still unknown biological mechanisms. **Chapter 6** shows that an important genetic factor of childhood asthma is associated with asthma-like symptoms in preschool children, and this association is modified by smoke exposure already during fetal life and in infancy. In **Chapter 7** we show a possible effect of folate serum levels on the development of eczema in early childhood, that may depend on genetic factors in a folate metabolism gene. Repeatedly measured asthma-related outcomes were used in our gene-environment studies.

Missing data in repeated 'yes/no' outcome measurements are complex to analyse and could lead to false findings. **Chapter 8** compares different strategies of analysis and methods to deal with missing data, and showed equivalence of several recommended strategies.

Finally, we discuss the results of this thesis in a general manner and place our findings in a broader context. The methodological considerations of our studies and clinical applications for asthma risk prediction and identifying potential therapeutic targets of asthma are discussed and we present our future research perspectives at the end of **Chapter 9**.

Samenvatting

Astma is de meest voorkomende aandoening op de kinderleeftijd in Westerse landen. Astma bestaat in verschillende soorten en wordt beïnvloed door vele genetische- en omgevingsfactoren. In dit proefschrift is specifiek gericht op een marker, te weten de fractie van stikstofmonoxide (FeNO) in uitgeademde lucht, die relevant is voor een bepaald type astma, waarbij allergie een rol speelt. FeNO meet de mate van luchtwegontsteking en kan door kinderen zelf worden gemeten. Hogere waarden van FeNO zijn geassocieerd met door een arts vastgestelde astma diagnose, maar ook met astmaklachten, astma aanvallen en allergieën. Dit proefschrift richt zich op een onbelicht gebied van astma bij kinderen: Enerzijds de interpretatie van dagelijkse fluctuatie van FeNO en anderzijds op de genetische- en omgevingsrisicofactoren van astma en FeNO op de kinderleeftijd.

Het proefschrift begint met een introductie van FeNO en astma op de kinderleeftijd. **Hoofdstuk 1** geeft achtergrondinformatie van eerdere studies en beschrijft de doelstellingen van dit proefschrift. **Hoofdstuk 2** beschrijft dat FeNO, gemeten bij 8 jarigen, verschilt tussen verschillende soorten van astma bij kinderen. De verschillen in FeNO werden alleen waargenomen bij allergische kinderen.

Het volgen van de mate van controle bij astma is belangrijk voor de behandeling. Dagelijkse FeNO metingen zijn voorgesteld om astmaklachten beter te kunnen begrijpen, en daarmee astma-aanvallen te voorkomen. **Hoofdstuk 3** beschrijft hoe een stijging in dagelijkse FeNO metingen voorafgaat aan een astma aanval. Wij speculeerden dat hiermee astma-aanvallen mogelijk te voorspellen, en wellicht geheel of deels te voorkomen zijn. **Hoofdstuk 4** analyseert fluctuatie van FeNO over een lange periode en toont de verbanden tussen dagelijkse FeNO metingen met astmaklachten. De schommelingen van FeNO geven informatie over de ernst en mate van controle van astma. Toekomstig onderzoek moet uitwijzen in hoeverre astma beter gecontroleerd kan worden en aanvallen voorkomen kunnen worden door medicatie aan te passen op basis van schommelingen van dagelijkse FeNO metingen.

FeNO bij astmatische kinderen met een specifiek type van luchtwegontsteking is gemiddeld hoger dan bij niet-astmatische kinderen. In **hoofdstuk 5** worden drie genetische regio's beschreven die FeNO kunnen beïnvloeden. Ons onderzoek toont eveneens aan dat zowel gemeenschappelijke als verschillende genetische factoren FeNO en astma bij kinderen kunnen beïnvloeden. De mechanismen hierbij zijn grotendeels nog onopgehelderd. In toekomstig onderzoek moeten deze biologische mechanismen verder worden uitgezocht. De resultaten zouden kunnen leiden tot het ontwikkelen van nieuwe, mogelijk betere therapieën voor astma.

Hoofdstuk 6 toont aan dat een belangrijke genetische risicofactor voor astma bij kinderen geassocieerd is met beginnende astmaklachten bij kleuters, waarbij

blootstelling aan sigarettenrook tijdens de zwangerschap of in het vroege leven het risico op klachten verder verhoogt. In **hoofdstuk 7** laten we zien dat foliumzuur in het bloed het risico kan verhogen op de ontwikkeling van eczeem, en dat dit mogelijk afhangt van een gen dat het niveau van foliumzuur in het lichaam bepaalt. Vele andere genetische factoren en hun interactie met omgevingsfactoren, die kunnen leiden tot een verhoogd risico voor astma, moeten nog onderzocht worden. In hoofdstuk 6 en 7 is gekeken naar astmaklachten die jaarlijks zijn gemeten. Ontbrekende gegevens bij herhaalde meetmomenten leveren problemen op bij dit soort onderzoek en kunnen leiden tot foute resultaten. **Hoofdstuk 8** onderzoekt het effect van verschillende analysestrategieën en verschillende methodes die gewoonlijk gebruikt worden om ontbrekende gegevens in te vullen. De resultaten laten zien dat deze methoden ongeveer gelijkwaardig zijn.

Tot slot wordt in **hoofdstuk 9** besproken wat de resultaten in het algemeen waren van dit proefschrift en worden de bevindingen in een bredere context uitgelegd. De methodologische overwegingen van onze studies en klinische toepassingen voor het voorspellen van astmarisico en het identificeren van potentiële aangrijpingspunten voor de behandeling van astma worden toegelicht. Als laatste worden onze ideeën voor toekomstig onderzoek gepresenteerd.

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Prof.dr. A.G. Uitterlinden, Prof.dr. E.W. Steyerberg en Prof.dr. A.J. van der Heijden wil ik graag bedanken voor hun bereidheid zitting te nemen in de kleine promotie commissie en het beoordelen van mijn proefschrift. Beste Andre, bedankt voor de leerzame genetische cursussen, de discussies over 'astma genetica' en de leuke tijd in San Francisco (ASHG 2012). Beste Ewout, bedankt voor de begeleiding bij het 'multiple imputeren' van missende waarden in het Generation R cohort. Ik vind het jammer dat er geen tijd meer over is om een simulatie studie toe te voegen aan hoofdstuk 8 van dit proefschrift. Beste Prof. van der Heijden, bedankt dat u als secretaris plaats wilde nemen in mijn leescommissie. De overige leden van de grote promotie commissie wil ik graag bedanken voor hun bereidheid om van gedachte te wisselen over de inhoud van dit proefschrift.

I would like to thank Prof.dr. U. Frey and Dr. G. Stern for inspiring training in fluctuation analysis. Dear Urs, thank you for hosting me at your department. Our work marks the onset of my scientific career and represents a great learning experience (and a lot of fun) during my MSc Clinical Research period. Dear Georgette, you were a wonderful host as well. Thank you very much for the great learning experiences and the nice dinners we had. I would also like to thank the 'Berne Gang' (Oliver, Cindy, Philip etc.) for all the fun we had (not for the obligated 'after-work swim' in the cold River Aare).

Prof.dr. G.H. Koppelman en Prof.dr. D.S. Postma wil ik graag bedanken voor de diepgaande inzichten in genetica en dan met name in de functionaliteit van het genoom. Francis Collins quote (ASHG 2012): 'Gene seekers' moeten samenwerken met andere (bv: biologische) labs om hun bevindingen verder functioneel uit te werken. Dit is jullie allang bekend - GRIAC.

Graag wil ik alle kinderen en hun ouders bedanken die deel hebben genomen aan het CHARISM, PIAMA en het Generation R onderzoek. I gratefully acknowledge and thank Prof.dr. E. Baraldi and Dr. S. Carraro for conducting the CHARISM Study. Ik wil de PIAMA (Prof.dr. H.A. Smit, Dr. A.H. Wijga, Dr. M. Kerkhof, Prof.dr. B. Brunekreef) en Generation R (Prof.dr. H.A. Moll, Prof.dr. H. Tiemeijer, Prof.dr. E.A.P. Steegers) coauteurs bedanken voor het kritisch doornemen van mijn manuscripten. Ik wil de overige PIAMA en Generation R PI's bedanken voor het generen van de data voor deze twee prachtige cohorten. Daarnaast wil ik de coauteurs Drs. O. Savenije, Drs. S.P. Willemsen en Dr. C.W. Looman hartelijk danken voor het mee schrijven en meedenken aan mijn manuscripten. I gratefully acknowledge my international 'genetic colleagues' of the EAGLE consortium. Using your valuable data we were able to submit the first genome-wide association meta-analysis study of childhood FeNO. In particular I would like to thank Prof.dr. A.J. Henderson of the ALSPAC cohort for his participation in the writing team and Prof.dr. H. Bisgaard for his efforts as chairman of the 'EAGLE asthma & atopy working group' and for hosting me several times at his department.

Dankzij Irma en Patricia lukte het altijd om een afspraak met Johan en Vincent te plannen. Daarnaast wil ik jullie hartelijk danken voor al jullie ondersteunend werk. De 'focus dames' dank ik voor de gezelligheid op het onderzoekscentrum en het verzamelen van de data. Michael, Pascal, Jeannette en lab co voor hun hulp bij het genotyperen van SNPs. Claudia bedankt voor alle data die zo snel naar mij toe kwam. Alwin bedankt voor computer ondersteuning. In het bijzonder wil ik Daan Caudri, Jessica Kiefte – De Jong, Rachel Bakker, Rob Taal en Karol Estrada bedanken. Beste Daan, ik vond het geweldig om onder jouw begeleiding aan mijn MSc onderzoek te werken. Het was erg fijn om tijdens mijn onderzoek bij Generation R te kunnen rekenen op jouw epidemiologische- en statistische hulp. Ik vind het jammer dat we nooit meer in de kroeg tot vijf of zes uur 's morgens over statistiek kunnen praten. Komt daar nog verandering in? Beste Jessica, wij delen veel passies op het vakgebied. Wat hebben wij toch veel plezier gehad om over 'multiple imputations' te praten en wat leuk dat we uiteindelijk een manuscript hebben kunnen schrijven. Daarnaast mag jouw bijdrage aan het folaat manuscript niet onbenoemd blijven. Beste Rachel, schaduwnimf, ik vind dat paranimfen toch wel mannen zijn (in tegenstelling tot wat jij in je proefschrift schrijft), bedankt voor je hulp bij het imputatie hoofdstuk, maar ook bedankt voor je hulp bij het afronden van dit proefschrift (planning, sponsoring, het lezen van Nederlandse stukjes en lay-out). Hi Rob, bedankt voor het kunnen stellen van 'makkelijke genetische vragen'. Dear Karol, I am extremely grateful for all the times you have helped me with my genetic analyses. There was not a single question you could not answer. Even in the last stage of your career in Rotterdam I received valuable comments for the GWAS FeNO manuscript. The above described qualities resemble your department. I would also like to gratefully acknowledge the help of your colleagues Dr. F. Rivadeneira and Caro, and Prof.dr. Van Duijn and Najaf. It was a long time ago, but I would like to acknowledge Dr. D. Rizopoulos who helped with the statistics of chapter 2 and 3. My dear friend Signe, we met in Boston and knew that we would be friends forever. Thank you for our special friendship and hosting me in CPH for several times. Please do not worry about not coming to my defense if you need to spend time with your lovely baby. My dear friend Eskil, we met in CPH. You might not have liked me in the first place as I proposed to your boss Prof. Bisgaard that you should come to Rotterdam and start a new study for a MSc at NIHES the day after finishing the last exam at medical school. We became very close friends, as with Daan we have discussed epidemiological designs, statistics and genetics in many bars with too many beers till early in the morning. I would like to thank you for inviting me to your lovely wedding. Søde Julie, jeg værdsætter højt din kærlige støtte i den sidste del af min PhD.

Al mijn Generation R en kinderpulmo collega's, bedankt voor de gezellige borrels en de feestjes. Denise en Romy bedankt voor het snoep en het roddelen op jullie

kamer. Liesbeth, bedankt voor het aandringen om nogmaals naar het FeNO schonen te kijken. Mede dankzij jouw hulp hebben we nu drie in plaats van twee 'FeNO hits'. Mijn kamergenoten, Agnes, Akghar, Esther en Ilse, bedankt voor jullie gezelligheid, de theekransjes en jullie nuttige feedback. Speciale dank naar Agnes die in 5 minuten vanuit Bristol een thesis lay-out probleem kon oplossen (ik was het al vijf uur aan het proberen!). Graag wil ik het hele 'kinderpulmo-team' bedanken voor al jullie advies tijdens de vrijdagmiddag research meetings. Mariëlle in het bijzonder voor al de 'FeNO tips' en Harm voor de altijd boeiende gesprekken over uiteenlopende onderwerpen die liepen van subsidies schrijven tot ziekenhuis politiek en complexheid van monogenetische ziekten. Roderick wil ik hartelijk danken voor het ontwerpen van de voorkant. Indrukwekkend hoe snel je dat voor elkaar hebt gekregen en leuk dat de lezers van dit proefschrift nu kunnen zien dat mijn onderzoek ook over genetica gaat (wat mist in de titel). Charlotte bedankt voor de inspiratie voor mijn leukste stelling. Ik wil mijn lieve vrienden en in het bijzonder Vasco bedanken voor al hun interesses in mijn onderzoek. Nick het spijt me dat je mijn paranimf niet mocht zijn. Rutger bedankt voor je interesses in mooie vrouwen (GiGi Ravelli). Nen Xavier voor kleding style advies voor een onderzoeker (Nen Xavier, van Oldebarneveltstraat 121C, Rotterdam). Herman en Maria (Panta Rheyn) voor training in onderhandelen. The 'Wine Ponces' voor te veel geblaat en te dure wijnen.

Ik wil mijn paranimfen John Twigt en Peter-Paul le Conge Kleyn bedanken. Beste John, wat fijn dat ik je paranimf mag zijn en daarvoor niets hoef te doen. Ik hoop dat ik jou ook in taken kan ontzien met al de drukte rond jouw promotie en coschappen. Carrièretechnisch bewandelen we hetzelfde pad en daarom kunnen we elkaar goed versterken. Bedankt voor je tekst 'About the author'. Je bent een lieve en betrouwbare vriend. Naast mijn paranimf ben je ook mijn professionele tuinder geworden. Mijn excuses dat ik je soms uitbuit, maar volgens mij vind je dat wel prettig. Ik kijk uit naar onze verse groentes op de BGE. Wanneer eten we courgettes? Lieve Peter-Paul, je bent al jaren mijn vriend. Je staat altijd voor me klaar op elk gebied. We hebben veel vrijdag avondjes doorgebracht met rode port. Daarbij hebben wij interessante discussies over kunst en wetenschap gehad. Excuses voor de keren dat ik te stellig overkwam zonder gegronde argumentatie. Ik had je gevraagd een gedicht te schrijven/uit te zoeken om dit proefschrift mee af te sluiten. Je hebt een mooi gedicht uitgekozen wat (door jouw aanpassing) goed aansluit bij de inhoud van dit proefschrift, maar ook bij onze vriendschap en onze 'vrijdag avond discussies'. Ik vind het fijn dat we de voor mij bijzondere dag samen zullen delen.

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bijgebracht zijn terug te vinden in mijn werk als wetenschapper. Pa, wat fijn dat je mijn oneindige ‘gezeur’ over mijn onderzoek wilde aanhoren. Het betekent veel voor me dat je altijd de tijd wilde nemen om het te begrijpen en dat je net zo lang bleef doorvragen tot je het begreep. Lieve mamma, ook jij stond bij alle hoogte- en diepte punten voor mij klaar. Ik kan me nog goed herinneren dat jij mijn vuile was in Rotterdam kwam ophalen, omdat ik de lay-out voor mijn thesis voor de kleine cie niet in orde kreeg. Hierdoor zou ik zonder schoon ondergoed naar San Francisco gevlogen zijn voor een congres. Pap, mam, zonder jullie onvoorwaardelijke steun was dit proefschrift nooit afgerond.

About the author

Ralf van der Valk was born on October 2nd 1984 in Voorburg, the Netherlands. After completing high school in 2003, he could not yet pursue his ambitions in medicine, due to the numerus clausus. Post-pubertal reluctance to study anything else than medicine made sure he had enough time to engage in rowing. Consequently, perseverance, iron discipline and a healthy appetite helped him achieve a silver medal at the FISU World Rowing Championships in the men's eight in Brive-La-Gaillarde in 2004. It was not until 2005 that Ralf got the opportunity to start his studies in medicine. The rowing lifestyle soon disappeared when he was offered the chance to do a Master of Science in Clinical Research, parallel to his medicine studies. His all-or-nothing attitude enforced by a newly discovered passion, allowed him to become a skilled biometrician to whom many people turn to for statistical advice. After finishing his Master thesis in Berne, Switzerland, he continued his research in a PhD project under the supervision of Professors J.C. de Jongste, A. Hofman and Doctor V.W.V. Jaddoe in the Generation R study. During this time he was successful in obtaining several grants. The PhD project allowed Ralf to discover and deepen his passion for (genetic) epidemiology. Like all PhD-students, Ralf attended many laborious tasks during his PhD-period. These included becoming a skilled pizza baker, acquiring a taste for fine wine and food and cultivating his vegetable patch and orchard. He also finished his second Master of Science, in Genetic Epidemiology, was a scientific advisor for the non-profit organization 'SamenLerenLezen', and finished the research of the thesis lying before you. Per February 25th 2013 Ralf has started his clinical internships at the Erasmus MC in Rotterdam.

John Twigt (Paranymph)
Rotterdam, January 20 2013

List of publications

van der Valk RJP, Kiefte-de Jong JC, Sonnenschein-van der Voort AMM, Duijts L, Hafkamp-de Groen E, Moll HA, Tiemeier H, Steegers EAP, Hofman A, Jaddoe VWV, de Jongste JC. **Neonatal folate, homocysteine, vitamin B12 levels and methylenetetrahydrofolate reductase variants in childhood asthma and eczema**. *Allergy in Press*.

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GD, Henderson J, Kemp JP, Timpson NJ, Deloukas P, Ring SM, Wichmann HE, Müller-Nurasyid M, Novak N, Klopp N, Rodríguez E, McArdle W, Linneberg A, Menné T, Nohr EA, Hofman A, Uitterlinden AG, van Duijn CM, Rivadeneira F, de Jongste JC, **van der Valk RJ**, Wjst M, Jogi R, Geller F, Boyd HA, Murray JC, Kim C, Mentch F, March M, Mangino M, Spector TD, Bataille V, Pennell CE, Holt PG, Sly P, Tiesler CM, Thiering E, Illig T, Imboden M, Nystad W, Simpson A, Hottenga JJ, Postma D, Koppelman GH, Smit HA, Söderhäll C, Chawes B, Kreiner-Møller E, Bisgaard H, Melén E, Boomsma DI, Custovic A, Jacobsson B, Probst-Hensch NM, Palmer LJ, Glass D, Hakonarson H, Melbye M, Jarvis DL, Jaddoe VW, Gieger C; Genetics of Overweight Young Adults (GOYA) Consortium, Strachan DP, Martin NG, Jarvelin MR, Heinrich J, Evans DM, Weidinger S; EArly Genetics & Lifecourse Epidemiology (EAGLE) Consortium. **Meta-analysis of genome-wide association studies identifies three new risk loci for atopic dermatitis**. *Nat Genet. 2011 Dec 25;44(2):187-92*.

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Duijts L, Jaddoe VW, **van der Valk RJ**, Henderson JA, Hofman A, Raat H, Steegers EA, Moll HA, de Jongste JC. **Fetal exposure to maternal and paternal smoking and the risks of wheezing in preschool children: the Generation R Study**. *Chest. 2012 Apr;141(4):876-85*.

Sonnenschein-van der Voort AM, Jaddoe VW, **van der Valk RJ**, Willemsen SP, Hofman A, Moll HA,de Jongste JC, Duijts L. **Duration and exclusiveness of breastfeeding and childhood asthma-related symptoms**. *Eur Respir J. 2012 Jan;39(1):81-9*.

Stern G, de Jongste J, **van der Valk R**, Baraldi E, Carraro S, Thamrin C, Frey U. **Fluctuation phenotyping based on daily fraction of exhaled nitric oxide values in asthmatic children**. *J Allergy Clin Immunol. 2011 Aug;128(2):293-300*.

Research portfolio summary

Summary of PhD training and teaching activities

Name PhD student:	R.J.P. van der Valk
Erasmus MC Departments:	The Department of Paediatrics, Division of Respiratory Medicine, the Generation R Study Group and the Department of Epidemiology
Medical School:	Erasmus MC: University Medical Center Rotterdam
Medical School period:	September 2005 – Expect to graduate December 2014
Research School:	NIHES: Erasmus University
Msc Clinical Research:	August 2007 – September 2010
Msc Health Sciences – Genetic Epidemiology:	August 2010 – September 2011
PhD period:	September 2009 – February 2013
Promotors:	Prof.dr. J.C. de Jongste, Prof.dr. A. Hofman
Supervisor:	Dr. V.W.V. Jaddoe

PhD training

	Year	Workload (ECTS)
RESEARCH SKILLS		
✓ **Harvard School of Public Health, Boston, USA:**		
Current Issues in Health Policy	2009	2.8
Environmental and Occupational Epidemiology	2009	2.8
✓ **MSc Clinical Research, NIHES:**	2007-2010	120
Principles of Research in Medicine and Epidemiology	2007	0.7
Clinical Trials	2007	0.7
Methods of Clinical Research	2007	0.7
Pharmaco-epidemiology	2007	0.7
Case-control Studies	2007	0.7
Introduction to Decision Making in Medicine	2007	0.7
Study Design	2007	4.3
Introduction to Clinical Research	2008	0.9
Intervention Research and Clinical Trial	2008	0.9
Prognosis Research	2008	0.9
Advance Topics in Decision Making in Medicine	2008	1.4
Diagnostic Research	2008	0.9

	Year	Workload (ECTS)
Topics in Meta-analysis	2008	0.7
Introduction to Data-Analysis	2008	1.0
Regression Analysis	2008	1.9
Survival Analysis	2008	1.9
Modern Statistical Methods	2008	4.3
Pharmaco-epidemiology and Drug Safety	2010	1.9
Advanced Topics in Clinical Trials	2010	1.9
Principles of Epidemiologic Data-analysis	2010	0.9
Advanced Analysis of Prognosis Studies	2010	0.9
✓ **MSc Health Sciences – Genetic Epidemiology, NIHES:**	2010-2011	70
Principles of Genetic Epidemiology	2010	0.7
Genomics in Molecular Medicine	2010	1.4
Advances in Genomic Research	2010	0.4
Genetic-epidemiologic Research Methods	2010	5.7
Introduction to Clinical and Public Health Genomics	2011	1.9
Family-based Genetic Analysis	2011	1.4
Advances in Genome-Wide Association Studies	2011	1.4
Mendelian Randomization	2011	0.9
Conceptual Foundation of Epidemiologic Study Design	2011	0.7
Introduction to Global Public Health	2011	0.7
Causal Inference	2011	0.7
History of Epidemiologic Ideas	2011	0.7
Social Epidemiology	2011	0.7
The Practice of Epidemiologic Analysis	2011	0.7
IN-DEPTH COURSES		
✓ **Genome Wide Association Analysis, NIHES**	2009	1.4
✓ **SNP's and Human Diseases, Mol Med**	2009	1.9
NATIONAL AND INTERNATIONAL CONFERENCES, SEMINARS, AND WORKSHOPS		
✓ **Department of Paediatrics, Division of Respiratory Medicine Research Meetings**	2008-2012	2.0
✓ **General Paediatrics Research Meeting**	2008	1.0
Invited speaker: Complexity of chronic asthma and daily exhaled Nitric Oxide measurements		
✓ **The Generation R Study Group Research Meetings**	2009-2012	2.0

	Year	Workload (ECTS)
✓ **Instellingsgebonden regelgeving en stralingshygiëne niveau 5R, Erasmus MC, Rotterdam, The Netherlands**	2009	0.7
✓ **Generation R Symposium, Epidemiology of Childhood Asthma, Rotterdam, The Netherlands**	2009	1.0
Invited speaker: Genes of wheezing in infancy		
✓ **40 years Epidemiology at Erasmus MC, Rotterdam, The Netherlands**	2009	0.4
✓ **EGG/EAGLE Symposium, Imperial College, London, UK**	2010	1.0
✓ **ATS International Conference, New Orleans, USA**	2010	2.0
Oral presentation: Daily exhaled nitric oxide measurements and asthma exacerbations in children		
Poster discussion: Phenotypes of wheeze and FeNO measurements at the age of 8 years		
✓ **EAGLE and joint birth cohort conference, Oslo, Norway**	2010	1.5
Invited speaker: Genome-wide association scan on Fractional exhaled Nitric Oxide in Childhood		
✓ **EAGLE asthma working group meeting, Copenhagen, Denmark**	2010	1.5
Invited speaker: Genome-wide association scan on Fractional exhaled Nitric Oxide in Childhood		
✓ **Generation R Symposium, Genetics in Child Cohort Studies, Rotterdam, The Netherlands**	2010	1.0
Invited speaker: Genome-wide association scan on Fractional exhaled Nitric Oxide in Childhood		
✓ **GRIAC seminars at UMCG, Groningen, The Netherlands**	2011	1.0
✓ **Grand Round Paediatrics Department**	2011	1.0
Invited speaker: Pulmo goes Genetics		
✓ **Advanced GWAS workshop, Erasmus MC, Department of Epidemiology, Rotterdam, The Netherlands**	2011	0.5
✓ **ERS ANNUAL Congress, Amsterdam, The Netherlands**	2011	2.0
Oral presentation: Interaction of 17q12-21 variants with fetal and early life smoke exposure in the development of asthma symptoms		
✓ **NRS Annual Congress, Amsterdam, The Netherlands**	2011	1.0
Poster discussion: Genome-Wide Association Scan on serum IL-1 receptor-like 1-a levels		
✓ **BBMRI, Connecting Biobanks, Rotterdam, The Netherlands**	2011	1.0
Poster: Genome-Wide Association Scan on serum IL-1 receptor-like 1-a levels		
✓ **EGG/EAGLE Symposium, Imperial College, London, UK**	2012	1.0

	Year	Workload (ECTS)
✓ **ATS International Conference, San Francisco, USA**	2012	1.0
Poster discussion: Genome-Wide Association Scan on serum IL-1 receptor-like 1-a levels		
✓ **Dutch Society Paediatric Pulmonology Site Visit, Imperial College, London, UK**	2012	1.5
Invited speaker: Genetics of FeNO		
✓ **ASHG Annual Congress, San Francisco, USA**	2012	2.0
Poster: A genome-wide meta-analysis identifies common variants in *LOC201229* and *NOS2A* associated with fractional exhaled nitric oxide in childhood		
✓ **DoHad Satellite Meeting, Rotterdam, The Netherlands**	2012	1.5
Poster: The associations between child folate, homocysteine, *MTHFR* C667T, and respiratory health and eczema in childhood		
✓ **MeDALL Meeting, Berlin, Germany**	2013	1.5
Invited speaker: Genetics of Childhood Asthma and FeNO		
INTERNATIONAL WORK EXPERIENCE		**Year**
✓ **Clinical Research internship, Paediatric Respiratory Medicine, Inselspital Universitätsspital, Berne, Switzerland**		2008-2009 (3 months)
Training: Fluctuation analysis of biological signals		
✓ **PhD internship, Copenhagen Prospective Studies on Asthma in Childhood, Faculty of Health Sciences, Copenhagen University Hospital, Gentofte, Denmark**		2010 (3 weeks)
Training: Short term research visit to strengthen collaborations of genetic research projects within the EArly Genetics and Lifecourse Epidemiology EAGLE Consortium		
✓ **PhD internship, Pediatric Pulmonology and Pediatric Allergology, Beatrix Children's Hospital - University Medical Center Groningen, GRIAC Research Institute, Groningen, The Netherlands**		2011 (5 months)
Training: In depth characterization of *Interleukin 1 receptor-like 1* asthma gene		
✓ **EArly Genetics & Lifecourse Epidemiology (EAGLE) consortium**		2009-2012
Working group asthma, allergy and atopy		
GRANTS		
✓ **Sophia Kinderziekenhuis Fund, Junior Research Fellowship**		2008
Project: Daily exhaled nitric oxide measurements and asthma exacerbations in children		
✓ **Sophia Kinderziekenhuis Fund, Short Term Minor Fellowship**		2011

		Year
Project: In depth characterization of *Interleukin 1 receptor-Like 1* asthma gene		
✓ **Stichting Astma Bestrijding Fund, Grand Project**		2011
Project: Modeling and predicting asthma with demographic, environmental and genetic factors		
✓ **ATS International Conference, Travel Grant**		2012
Poster discussion: Genome-Wide Association Scan on serum IL-1 receptor-like 1-a levels		
✓ **GlaxoSmithKline, Supporting Research Grant**		2012
Project: Replication of Results from a Genome-wide Association Scan on Fractional exhaled Nitric Oxide in Childhood to highlight potential drug targets for treatment of asthma		

Teaching activities

	Year	Workload (ECTS)
Supervising practicals and excursions		
✓ **Principles of Research in Medicine, NIHES course**	2010	1.4
✓ **Statistical advice/consultations**	2009-2012	3.0
Other skills		**Year**
✓ **Clinical work Generation R, Research center**		2009-2011
✓ **Peer review of scientific articles**		2009-2012

Gene und Kunst

Gene und Kunst, sie scheinen sich zu fliehen,
Und haben sich, eh‘ man es denkt, gefunden;
Der Widerwille ist auch mir verschwunden,
Und beide scheinen gleich mich anzuziehen.

Es gilt wohl nur ein redliches Bemühen!
Und wenn wir erst in abgemeßnen Stunden;
Mit Geist und Fleiß uns an die Gene gebunden,
Mag frei Gene im Herzen wieder glühen.

So ist‘s mit aller Bildung auch beschaffen:
Vergebens werden ungebundne Geister
Nach der Vollendung reiner Höhe streben.

Wer Großes will, muß sich zusammenraffen;
In der Beschränkung zeigt sich erst der Meister,
Und das Gesetz nur kann uns Freiheit geben.

Johann Wolfgang von Goethe, *Lyrisches*
Adapted by Peter-Paul Nicolaas le Conge Kleyn (Paranymph)