Respiratory Morbidity and Exhaled Nitric Oxide in the First 2 Years of Life
The Generation R Study

Carmelo Gabriele

Acknowledgements

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Respiratory Morbidity and Exhaled Nitric Oxide in the First 2 Years of Life
The Generation R Study

Luchtwegklachten en uitgeademd stikstofmonoxide in de eerste twee levensjaren:
het 'Generation R' onderzoek

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1

General Introduction

Asthma is a lung disease characterized by chronic inflammation of the airways associated with bronchial hyperresponsiveness. The airflow obstruction within the lungs is responsible of recurrent episodes of wheezing, breathlessness, chest tightness and coughing. The physiopathological changes of the airways in asthma are mediated by many cells and cellular elements [1]. It has been argued that asthma is a syndrome comprising a number of separate conditions, rather than a single disease with a broad range of severity. [2]. It has long been thought that the inappropriate response of the specific immune system to harmless antigens leading to the polarization of T-cells toward a T-helper 2 (Th2) phenotype was the central mechanism of asthma. However, in the last years the hypothesis of asthma as one unifying disease concept has disappeared [3, 4]. Novel disease and bronchial inflammation pathways, many of which are independent of adaptive immunity, have been reported. The concept of disease endotypes has been recently introduced and subtypes of asthma with different and specific pathophysiology, immunology, clinical features and response to treatment have been described [4]. A simple categorization of the different inflammatory patterns in asthmatics based on sputum eosinophil and neutrophil proportions has been provided and four inflammatory subtypes have been identified: neutrophilic, eosinophilic, mixed granulocytic and paucigranulocytic asthma [5]. A recent review by Haldar and Pavord suggested that non-eosinophilic asthma represents a stable phenotype associated with distinct etiologic factors and less airway pathology [6]. Also, it has been suggested that severe asthma should no longer be considered as the result of a progressive process, but rather as a separate pathological entity with distinct physiologic and clinical characteristics [7]. These heterogeneous inflammatory patterns have been also reported by Brasier et al, who evaluated bronchoalveolar lavage (BAL) samples in mild to severe asthmatic patients. They showed that cytokine expression patterns in BAL could be used to identify distinct types of asthma and identify distinct subsets of methacholine hyperresponders [8]. Despite improved understanding of pathophysiology, immunology and genetics of asthma in childhood, we still do not know the basic mechanisms underlying the development of the disease.

Most asthma begins early in life [9], but it is still unclear when the pathologic features of asthma first appear [10, 11]. The Tucson children's Respiratory Study showed that only a minority of wheezing infants will develop asthma at school age, as

most wheezing infants will have transient symptoms associated with reduced lung function in the first years of life [12]. The same group has reported follow-up studies of the cohort, demonstrating that the individual level of lung function is established by age 6 years and tracks to age 22 years in children who start having asthma-like symptoms during the preschool years [9, 13]. Therefore, in children with persistent wheezing, lung function changes already occur during the first 6 years of life. A longitudinal study from Germany showed that the allergic component in asthma begins in the first 3 years of life, persists beyond the age of 3 years and is associated with reduced lung function at school age [14]. Also, a recent prospective cohort study conducted in Australia has narrowed the time window during which the changes in the airways develop, by showing that airways hyperresponsiveness in asthmatics appears at the end of the first years of life [15]. Endobronchial biopsy studies in selected infants and preschoolers showed that atopic wheezers with documented bronchodilator reversibility have no evidence of increased airway wall inflammation or reticular basement membrane thickness at a median age of 12 months [16]. However, a subsequent report of the same authors showed that the characteristic pathologic and inflammatory features of asthma in adults and school-aged children develop in preschool children with confirmed wheeze between the ages of 1 and 3 years, a time when intervention may modify the natural history of asthma [11]. However, we cannot yet discriminate with certainty the future individual with asthma from the individual with transient wheeze [17].

Eosinophilic bronchial inflammation and structural changes of the airway wall may play an important role in the pathophysiology of asthma [18, 19]. However, the relationships between inflammation, bronchial hyperresponsiveness, reduced lung function and asthma symptoms are still unclear. The presence of genetic predisposition, the timing and the nature of the environmental risk factors, and their interactions determine whether and how atopic disease will develop.

1.1 Epidemiology

Asthma and other wheezing disorders are the most common chronic health problems in childhood and place a large burden on children, their families and society [20]. The world health organization (WHO) has recently estimated that about 300 million people currently suffer from asthma [21]. The diagnosis of asthma is based on medical history, physical examination and measurements of lung function, often over

a period of time. However, in epidemiological studies a symptom-based rather than a diagnosis-based approach has been used and asthma has commonly been defined as self-reported asthma, doctor-diagnosed asthma or asthma-like symptoms, such as wheezing, whistling, cough, breathlessness and chest tightness [22-25]. The International Study of Asthma and Allergies in Childhood (ISAAC) showed that the prevalence of asthma symptoms in schoolchildren varies worldwide, with a general trend of higher prevalence in children of Western lifestyle countries compared to children of developing countries, suggesting that environmental factors may have a strong influence on the development of asthma in childhood [26]. In particular, the 12-month prevalence of self-reported asthma symptoms from written questionnaires in 6-7 year old children ranged from 2% in Indonesia to 36% in the UK [26]. Recent data from the ISAAC phase 3 showed that in the mid-1990s the prevalence of wheezing increased in most centers where the prevalence had been low and decreased or remained unchanged in most centers where the prevalence had been high [27]. However, the ISAAC reported on the worldwide prevalence of self-reported asthma in school children, but did not study preschool children. The limited data on children younger than 6 years has shown that the prevalence of wheezing episodes in the first year of life is 20-60%, depending on the definition used and the population studied [28-30] (table 1). The Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC) assessed the occurrence of wheezing by means of questionnaires in 1,954 infants in three District Health Authorities of Bristol and found that the prevalence of wheezing in the first 6 months was 21.5% [29]. In the first 3 years of life, about one third of children has at least one episode of wheezing and the cumulative prevalence of wheeze is almost 50% at the age of 6 years [12, 31]. Although wheezing is the most important symptom for the early identification of asthma, in infants the clinical manifestations of atopic diseases may include also other symptoms [32-34]. Dodge et al described the prevalence of cough, wheezing, shortness of breath and chest colds in children below the age of 4 and evaluated the association between symptoms reported in infancy and asthma up to 11 years [30]. They assessed the occurrence of the symptoms every 1-2 years and reported that the prevalence of at least one respiratory symptom at 6-11 months, 1-2 years and 3-4 years was 26%, 38% and 48%, respectively. These authors also showed that children under 1 year of age with either cough or wheeze were more likely to be later labeled asthmatics, when compared with infants without any of the symptoms. Cough, wheeze and frequent chest colds all

significantly related to a future diagnosis of asthma among children aged 3 to 4 years. Recently, Caudri et al reported the prevalence of respiratory symptoms in the first 7 years of life in 3,628 children participating in the Dutch Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study [32]. In this prospective birth cohort, the prevalence at 1 year of any wheezing, self reported doctor diagnosis of current asthma and cough at night was 21%, 7% and 17%, respectively. At 2 years, these prevalences were 17%, 6% and 15%, respectively. The authors showed that after the age of 2 years, cough was the most frequently reported symptom. Between the ages of 4 and 7 about 70% of the cases with any respiratory symptom reported cough. The overall prevalence of any wheezing, self reported doctor diagnosis of current asthma and cough at night in the first 7 years of life was 39%, 14% and 52%, respectively [32]. The Prevention of Asthma in Children (PREVASC) study is another birth cohort in the Netherlands in which a total of 443 high-risk children recruited during the prenatal period by general practitioners were followed for 2 years [33]. Infants were randomized to receive either usual care or received instruction from nurses on how to reduce exposure of newborns to allergens and passive smoking. The prevalence of wheezing at least once was reported in 64% and 57% of infants in the intervention and control group, and night cough without a cold was reported in 48% and 53%, respectively [33]. Only one study previously compared the prevalence of asthma symptoms between countries in a large cohort of children (n=9,490) aged 1-5 years [31]. This survey was conducted in US and in 6 European countries (Denmark, France, Germany, Italy, Spain and UK) by means of telephone interview and showed that 3077 children (32%) had recurrent cough, wheeze or breathlessness in the preceding six winter months. The prevalence of symptoms was 27% in the US and varied from 29% in Northern Europe to 48% in Southern Europe. In the households with at least one child with any symptom, recurrent days with cough were reported by 87%, wheeze by 42%, breathlessness by 21%, and all three symptoms by 15% of children [31]. A previous study in the UK showed that in children aged 5 years and under the overall cumulative prevalence of doctor diagnosed asthma, symptomatic wheeze, recurrent cough with and without colds was 11% , 16%, 69% and 22% respectively, and the prevalence of wheezing attacks during the previous 12 months was 12% [35]. This survey was repeated 8 year later and showed a significant increase in the prevalence of wheeze ever, wheeze in the past year and cough with colds [36]. The increase occurred not only in children wheezing with multiple triggers but also in those

wheezing only during colds, suggesting that not only atopic status, but also changes in environmental exposures account for the rising prevalence of wheeze and asthma [36].

Table 1 Prevalence of respiratory symptoms in children

Study	Respiratory symptom	Age	Prevalence (%)
ALSPAC [29]	Wheezing	<6 mo	21.5
Tucson Children's Respiratory Study [12]	Wheezing	< 3 yrs	34
		< 6 yrs	49.5
PIAMA [32]	Wheezing	<1 yr	21
	Asthma		7
	Cough at night		17
	Wheezing	2 yrs	17
	Asthma		6
	Cough at night		15
	Wheezing	<7 yrs	39
	Asthma		14
	Cough at night		25
Tucson Epidemiologic Study of Airways Obstructive Disease [30]	Cough, wheezing, shortness of breath or chest colds	6-11 mo	26
		1-2 yrs	38
		3-4 yrs	48
Leicester respiratory cohort [36]	Wheezing	< 5 yrs	34
	Cough with colds		76
	Cough without colds		22

There is a lack of large population-based prospective studies evaluating respiratory symptoms in the first 2 years of life and their association with the future development of asthma. Therefore, it is difficult to determine the true prevalence of asthma-like symptoms in this age group. Indeed, infants represent a problematic group to study as there is no suitable and well validated questionnaire for use in children aged 0-2 years [34], there is a lack of standardized criteria to diagnose asthma and the use of objective measures of bronchial responsiveness and bronchial inflammation are often invasive, require specialized staff, and cannot be easily applied to large-scale epidemiological studies [37-39].

1.2 Risk factors for asthma

There is an important hereditary contribution to asthma and the mode of inheritance does not follow the classical Mendelian patterns, suggesting that asthma is a complex genetic disorder. Moreover, it is clear that the development of asthma can be attributed to both genetic and environmental factors [40]. Hence, a multifactorial approach has been suggested for the genetics of asthma [41].

In the next paragraph we will report the most important risk factors associated with the development of asthma with special emphasis on the role of ethnic background. Risk factors for the development of asthma will be divided in host and environmental factors [1] (table 2), bearing in mind that the timing and the nature of the exposures and their interactions with genetic components account for the onset of atopy and asthma.

Table 2 Risk factors for asthma (adapted from [42]).

Host Factors
Genetic predisposition
Ethnic background
Atopy
Environmental Factors
Socioeconomic status
Fetal and postnatal tobacco smoke exposure
Infections
Siblings
Day care attendance
Microbial exposure
Air pollution

1.2.1 Host factors

Genetics

Asthma has a strong genetic component. The inheritance of asthma is polygenic and genetic influences are important in the pathogenesis of asthma and allergy, although non-genetic factors, such as environmental exposures, are also important in the expression of the disease. Indeed, the interactions between environmental and genetic factors play an important role in the development of asthma as the presence

or absence of environmental exposures can result in opposite effects of the same gene [40]. The main approaches previously used to identify genes predisposing to diseases are represented by linkage analysis and genome search. Linkage analysis tests candidate genes by linkage or association with the disease selected, in order to find causative mutations or polymorphisms. In the genome search or 'positional cloning' the entire genome is screened in order to identify regions linked to a specific phenotype. Several studies have tried to find asthma-susceptibility genes using a genome-wide scan approach and refined genetic mapping methods and the number of asthma candidate genes has increased quickly in the last decade [43, 44], as extensively summarized by previous reviews [45, 46]. However, it is remarkable that the attributable risk of the candidate genes was generally lower than 5% and the results have not always been replicated [47]. Since asthma cannot be considered as a single disease, but rather as a syndrome with different pathways and phenotypes, it is likely that a specific gene variant plays a role in some, but not in other wheezing (or asthma) phenotypes.

Ethnicity

A worldwide variation of the prevalence of asthma has been shown, with a general trend of higher prevalence in children of Western countries compared to children of developing countries [26]. Several cross-sectional surveys have shown a variation in the prevalence of asthma and asthma-like symptoms among children with different ethnic background living in the same urban area [48-51]. Migrants from developing to industrialized countries seem to be at increased risk of asthma, and changes in environmental factors and lifestyle rather than genetic factors are likely to be responsible for such a trend [52-55].

In Australia it has been shown that the prevalence of 'wheeze' or 'asthma ever' was higher in Australian-born non-Asians and Australian-born Asians than in Asian immigrants and that the prevalence of asthma in Asian immigrants was strongly associated with length of stay in Australia, in subjects younger than 20 years [56]. In a similar way, schoolchildren born outside Australia had less asthma than children born inside Australia and migrant children tended to develop asthma several years after arriving in Australia [57]. These results were confirmed also in adolescents coming from the Sydney area that completed a video symptom questionnaire and underwent hypertonic saline challenge, sputum induction and allergy skin testing. The prevalence

of asthma symptoms was related to residence time in Australia with an 11% increase in prevalence of current wheeze for every year of residence in Australia. Altogether, these studies found substantial differences in the prevalence of asthma between immigrants and the local population in Australia, with a higher prevalence in Australians, either from Asian or non-Asian origin, as compared to recent immigrants. Besides, a prolonged stay in Australia was found to be associated with increased prevalence of asthma, suggesting that prolonged environmental exposures are required for the development of asthma in immigrants [58, 59].

Studies in the US have shown that the prevalence of childhood respiratory diseases, especially asthma, is increasing in African-American children and this trend was only partly explained by differences in socio-economic factors [60-62]. Rose et al evaluated the data from the 1998 through 2000 US National Health Interview Surveys on lifetime history of asthma and asthma in the past year in 95,615 adults. Asthma diagnosis rates were highest among Puerto Ricans, intermediate among non-Hispanic Blacks and non-Hispanic Whites and lowest among Mexican-Americans [63]. This suggests that genetic/ethnic factors could play an important role in determining the risk of asthma in those populations. A study conducted among 1,770 Mexican-American children aged 12-19 years showed that Mexican-American adolescents born in the United States and those with high acculturation levels reported significantly higher prevalence rates of asthma, wheezing, and hay fever than their peers with low acculturation levels and born in Mexico [64]. Another study based on US mortality records from 1991 through 1996 showed that black race/ethnicity was associated, independently from low income and low education, with an elevated risk of asthma mortality [60]. Joseph et al reported that the prevalence of physician-diagnosed asthma among 6-8 years old children was 10% for both African-American and European-American. However, African-American children were more reactive to methacholine than European-American children and had significantly higher total IgE than European-Americans, suggesting that African-American children might be predisposed for asthma [61]. Asthma prevalence has been evaluated also among US children in ethnic minority subgroups underrepresented in the pediatric asthma literature, including American Indian/Alaska Native, Chinese, Filipino and Asian Indian children. Data on all 51,944 children aged 2 to 17 years from the 2001-2005 National Health Interview Survey were aggregated and analyzed to estimate the prevalence of current asthma and lifetime asthma according to race and place of birth. Current

asthma prevalence ranged from 4.4% for Asian Indian children to 13.3% for black children, with estimates of 13.0% for American Indian/Alaska Native children, 10.7% for Filipino children, 8.4% for white children, and 5.1% for Chinese children. A similar pattern among the race categories was observed for lifetime asthma prevalence. Children born in the United States had a higher prevalence of both current and lifetime asthma than children born outside the United States [65]. These studies show that there is a variation in the prevalence of asthma symptoms among ethnic minorities in the US, which is only partly explained by difference in socio-economic factors. Both acculturation and country of birth are linked with the risk of asthma and wheezing, with acculturation having a stronger effect.

A similar trend of progressive symptoms of allergy and asthma developing after immigration has been reported among other ethnic groups of immigrants to European industrialized countries. In particular, a cross-sectional survey conducted in 7,445 families with children 9-11 yrs of age attending primary schools in Munich (Germany) between September 1989 and July 1990 showed that Turkish children had a significantly lower prevalence of asthma, atopy and bronchial hyperresponsiveness than their German peers [50]. However, another study was conducted in preschool children born in Germany with double German or double Turkish parental citizenship. Cultural adaptation of Turkish children was assessed by the language parents used to communicate with their child: only Turkish, Turkish and German and only German. It was found that higher cultural adaptation was correlated with higher rates of allergic sensitization and disease among children of Turkish origin living in Germany [55].

A recent systematic review on ethnic variations in asthma frequency, morbidity and health-service use in the United Kingdom concluded with the paradox that the prevalence of wheeze and asthma was lower in south Asians than in white children aged 5 years and older, but medical consultations and hospital admissions were more common among south Asian children [53]. However, this review summarized studies that did not distinguish different asthma phenotypes or controlled for varying environmental exposures [53]. Kuehni et al compared the prevalence of wheeze and related health-service use in south Asian and white preschool children in the United Kingdom, taking into account wheeze phenotype (viral and multiple trigger wheeze) and environmental exposures [66]. Postal questionnaires were completed by parents of a population-based sample of 4,366 white and 1,714 south Asian children aged 1-4 years in Leicestershire, UK. Authors found that the prevalence of current wheeze was

35.6% in white and 25.5% in south Asian 1-year-olds. Also, reduced risk of any wheezing was found in 1-year-old south Asian infants, but this trend was inverted when considering only 2-4 years old children with multiple wheeze [66]. Authors speculated that the associations seen in 1-year-olds could be due to a lower prevalence of innate bronchial hyper-responsiveness in south Asian than in white children. Another study of the same authors, evaluated the prevalence of asthma in 2,380 south Asian and 5,796 white young mothers randomly sampled in Leicestershire, by means of postal questionnaires [51]. South Asian women who migrated to the United Kingdom aged 5 years or older reported less asthma than those born in the United Kingdom or who migrated before age 5. For those who migrated after the age of 5 years, the prevalence of asthma was not associated with the duration of residence in the United Kingdom. These data from a large population-based study support the hypothesis that early life environmental factors and duration of residence in the host country might influence the risk of developing asthma [51].

Also in Sweden a difference in the prevalence of atopic disorders has been shown between ethnic minorities. Hjern et al evaluated the prevalence of atopic disorders in 1,734 adults 27-60 years of age and their 2,964 children aged 3-15 and found that the Chilean-born parents and their children had the highest risk of allergic asthma and rhino-conjunctivitis, as compared with the Swedish-born parents and their children. Children of Turkish-born parents had the lowest risk of allergic rhino-conjunctivitis and eczema. The risk for atopic disorders was lower in the Turkish group compared with the Chileans. This study also showed a lower rate of allergic diseases among children of Turkish immigrants and, although the duration of permanence in Sweden by ethnic minorities was not reported, authors suggested that ethnicity is an important determinant of atopic disorder independent of the external childhood environment [48].

Only one study prospectively assessed respiratory and skin symptoms in the first 2 years of life in a large cohort of children with different ethnic background. Children were born in the Netherlands between May 1996 and December 1997 and participated in the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study. Based on maternal country of birth and maternal self-reported ethnicity, infants were grouped in Dutch and non-Dutch, with the latter group including the most representative ethnic minorities in the Netherlands (Indonesian, Antillean Surinamese, Turkish, North African, Eastern, European and Others). Parents

completed questionnaires on respiratory and skin symptoms, ethnic background, and other potential confounders during pregnancy, and at 3 months, 1 year, and 2 years of age. Authors showed that, compared to Dutch, non-Dutch children had a higher prevalence of runny nose with itchy/watery eyes, wheeze at least once, night cough without a cold and runny nose without a cold in the first 2 years. However, adjustment for socioeconomic factors reduced most associations between ethnicity and respiratory symptoms, as only runny nose with itchy/watery eyes in the second year of life was independently associated with non-Dutch ethnicity. Furthermore, the short term follow-up and the relatively small number of children in the subgroups of ethnic minorities did not allow to determine whether this higher prevalence of respiratory symptoms in children with non-Dutch ethnicity represented an increased risk of developing allergic disease rather than non-specific or infection related respiratory symptoms [67].

In conclusion, ethnic differences in atopy and respiratory morbidity between and within countries have been partly attributed to intrinsic susceptibility in certain ethnic groups. However, cultural adaptation, duration of permanence in the host country and early exposure to a new environment are positively associated with the risk of asthma in migrants, suggesting that environmental exposures play a crucial role in explaining large part of the associations found. Prospective birth cohort studies are needed to evaluate the onset of asthma and allergies in large groups of children with different ethnic background. As the timing and intensity of environmental exposures in the first years of life are crucial for the development of atopic diseases, further studies should evaluate to what extent the interactions between ethnic background and pre- and early postnatal environmental exposures can lead to the development of asthma later in life.

Atopy

Atopy represents one of the most important risk factors for the development of asthma. Atopy is defined as the genetic propensity to develop immunoglobulin E antibodies (IgE) in response to exposure to allergens and can be assessed by skin prick test responses to common allergens [68]. Allergens are small antigens that normally enter the body at very low doses by diffusion across mucosal surfaces, and can trigger a T-helper 2 (Th2) lymphocyte response. The differentiation of naive allergen-specific T cells into Th2 cells is stimulated by an early burst of interleukin (IL)-4. Allergen-

specific Th2 cells produce IL-4 and IL-13, which drive allergen-specific B cells to produce IgE [69]. The specific IgE produced in response to the allergen binds to the high-affinity receptor for IgE (FcεRI) on mast cells, which causes the release of several mediators leading to the development of the acute phase of the immune response [70]. The recruitment of activated eosinophils, monocytes and T cells follows the acute phase and determines the late inflammatory response through the release of several cytokines and mediators [71]. A number of studies found that allergic sensitization to common environmental allergens is a major risk factor for the subsequent development of childhood asthma [14, 72-74]. Results of the Third National Health and Nutrition Examination Survey (NHANES III) showed that 56% of asthma cases in the USA were attributable to atopy, in people aged between 6 years and 59 years [72]. In 2001, Arshad et al reported the results of a birth cohort study in children followed up to the age of 4 years. Authors found that an independent effect of allergen sensitization on asthma was observed only with house dust mite with an odds ratio of 8.07. The prevalence of atopy in asthmatic children was 44% and the population-attributable risk was calculated to be 35% [68]. This is in agreement with a review that estimated the proportion of asthma cases attributable to atopy in cross-sectional studies exclusively or predominantly in children to vary from 25% to 63% with a weighted mean of about 38% [75].

1.2.2 Environmental factors

Socio economic status

Socio economic status (SES) is a main determinant for health outcomes [76]. SES can be ascertained at the individual level by the assessment of education, occupation and measures that estimate wealth or financial assets. SES can also be measured at an area level, which incorporates income measures, education patterns and employment rates, but may also include measures of wealth and deprivation, including average home values and rates of social-assistance provision [77]. With regard to atopic diseases, the phase one of the International Study of Asthma and Allergies in Childhood (ISAAC) has showed that the prevalence of asthma, allergic rhinoconjunctivitis and eczema is higher in western lifestyle countries as compared with low income countries [22]. A recent report of the ISAAC phase three conducted ≥5 years after the phase one, has shown that for the younger age-group (6-7 years), the centers that showed increases in all three disorders more frequently than centers

with decreases in all three disorders were Asia-Pacific, Indian subcontinent (India), North America, eastern Mediterranean and western Europe, whereas in the older age-group (13-14 years) they were Africa, Asia-Pacific, India, Latin America, northern and eastern Europe [27]. These findings suggest that the SES of countries and individual families may influence the development of childhood asthma, with high SES being associated with a higher prevalence of atopic diseases. Low prevalence rates of asthma in rural and poor areas of Africa seem to support this hypothesis, as urban living and higher material standards of living have been associated with higher prevalence of reversible airways obstruction in children in Zimbabwe [78]. Similar results were reported by Lewis et al in the 16-years follow up of the British birth cohort on over 6,000 children, showing an increased risk for atopic diseases (hay fever, eczema and wheeze) in children from high social class families compared to those from lower classes [79]. However, other studies that investigated the association between SES and asthma reported inconsistent results and there is growing evidence that in many affluent countries the prevalence of asthma is higher among those in low SES [80-84]. Hedlund et al [85] reported an inverse association between SES and asthma in a 10-yr follow-up of a population-based postal survey in Northern Sweden. The study comprised 4,754 adults and the SES classification used was based on occupation. Authors showed that people in the lowest SES groups (manual workers in industry) had a significantly increased risk of developing asthma, recurrent wheeze, attacks of shortness of breath or a combination of the two, and chronic productive cough, with a corresponding population attributable risks between 8.9 and 11% [85]. A study in Great Britain was conducted in 17,677 children aged 5-11 years and linked survey data for asthma symptoms to census data. Lower SES was associated with higher childhood asthma prevalence and persistent wheeze was more prevalent in poor areas than in less deprived areas, suggesting that poverty is associated with severe asthma [86]. One ecological analysis was performed by retrieving population-based surveys of asthma conducted among children aged 6–7 years or 13–14 years in Brazil between 1994 and 2003 and showed evidence that health and socioeconomic indicators were associated with asthma prevalence at ecological level, prevalence increasing with worsening of socio-economic conditions [87]. While it is still contradictory whether and to what extent SES influences the incidence of asthma, there is a large body of studies showing an influence of poor social conditions on the severity of asthma. An Italian study of childhood asthma

prevalence analyzed individual SES data (using educational level) combined with a census-based approach for SES, including educational level, occupational category, unemployment, one-person families, large families, persons per room and rental versus ownership. This study indicated an association between low socio-economic level and asthma in 3,917 schoolchildren in Rome for both individual and area-based indicators, with the strongest association observed for low SES and hospitalization for asthma and weaker for prevalence of severe asthma and weaker still for prevalence of asthma [80]. A study performed in Colombia (US) reported that, for 1–17 yr olds, the rates of both asthma-related emergency department visits and admissions increased logarithmically with the percentage of children living below the poverty threshold, slowing when this percentage exceeded 30% [88]. Another study in the US by McConnochie et al analyzed all asthma hospitalizations between 1991 and 1995 for children (aged >1 month and <19 years) dwelling in Rochester, New York. Authors found a marked socioeconomic and ethnicl disparity in Rochester's asthma hospitalization rates, which was largely attributable to higher incidence of severe acute asthma exacerbations among inner-city children [89]. Strachan et al reported the results of a nationwide household survey in Great Britain, in which parents of children aged 5-17 years were interviewed. Authors showed that the prevalence of wheeze varied little by socioeconomic group, but there were marked trends in all three indices of severity (woken more than once a week by wheezing, doctor diagnosed asthma, prescription of antiasthmatic drugs in the past year) towards increased morbidity in poorer families [90].

In conclusion, while there is a clear influence of low SES on the severity of asthma, it is still unclear whether socio-economic variables play a role in the development of asthma. In fact, some studies demonstrated a positive relationship between higher socio-economic levels and mild asthma, and some papers showed the reverse, while others also reported no effect of SES on asthma. Socioeconomic status may be a surrogate for living conditions and lifestyle rather than a risk factor for asthma by itself.

Tobacco smoke exposure

The detrimental effects of tobacco smoke exposure in utero and in early childhood on different health outcomes have been demonstrated by a large number of studies [91-94]. Maternal smoking during pregnancy and environmental tobacco smoke (ETS)

exposure after birth have been associated with impaired lung growth and diminished lung function [95, 96]. Gilliland et al evaluated the association between pre- and postnatal smoke exposure and pulmonary function in 3,357 school children in California. Authors found that in utero exposure to maternal smoking was associated with reduced lung function parameters, such as peak expiratory flow rate (PEFR), mean mid expiratory flow (MMEF) and forced expiratory flow (FEF75), also after adjustment for postnatal smoke exposure, suggesting that exposure to maternal smoking during pregnancy is independently associated with decreased lung function, especially for small airway caliber, in children of school age [97]. Li et al reported similar findings by showing that in utero exposure to maternal smoking was independently associated with reduced lung function, especially in children with asthma [98]. Also, reduced flows were associated with postnatal smoke exposure in children with and without asthma, significant only among children without asthma [98]. The results of a longitudinal study on 5,933 children aged 6-18 years showed that exposure to prenatal maternal smoking and early onset asthma are both associated with persistent deficits in lung function and that children with early onset asthma may be at increased risk of subsequent respiratory diseases [99]. Moshammer et al reported the results of a large study involving more than 20,000 children aged 6-12 years from nine countries in Europe and North America. These authors showed that smoking during pregnancy was associated with decreases in forced expiratory volume in 1 second (FEV1) and endexpiratory flows. Associations with current passive smoking were weaker though still measurable, with effects ranging from -0.5% (FEV1) to -2% (MEF50) [100]. These studies provide evidence that maternal smoking during pregnancy may play a greater role than later ETS exposure on the observed effects on lung function in children. The effects of maternal prenatal smoking on airway caliber may be an important factor predisposing infants to the occurrence of wheezing illness later in childhood [101]. Martinez et al showed that in the longitudinal Tucson Cohort Study maternal smoking was related to transient early wheezing and persistent wheezing [12]. A recent study from Norway comprising 22,390 children born between 2000 and 2004 could disentangle the effects of prenatal and postnatal smoking on early childhood respiratory health, as prenatal smoke exposure was assessed prospectively at various stages of pregnancy. Authors found that prenatal maternal smoking was an independent risk factor for wheeze and respiratory infection in the first 18 months and that postnatal paternal smoking was also associated with these

outcomes, independently of maternal smoking in pregnancy [102]. A meta-analysis of 51 publications investigating the effect of ETS exposure on the development of asthma showed that maternal smoking was associated with an increased incidence of wheezing illness up to age 6 (pooled odds ratio 1.31), but less strongly thereafter (odds ratio 1.13) [103]. Authors concluded that parental smoking is likely to be causally related to acute lower respiratory tract illnesses in infancy and to childhood asthma and wheezing.

Several immunological mechanisms have been proposed to explain the relationship between smoke exposure and respiratory symptoms. Maternal smoking has been associated with impaired neonatal Toll-like receptor (TLR) -mediated immune responses [104], which is implicated in susceptibility to respiratory infection as well as in the potential regulation of allergic responses [105]. TLR activation is also important for the activation of T-regulatory cells, which are involved in the suppression of allergic Th2 responses. Together, these effects could contribute to increased allergic risk [105]. Other studies have shown that nicotine can exert its immunosuppressive effects on immune surveillance through functional impairment of the dendritic cells system [106]. Recent studies have also identified a smoking-induced disruption in antioxidant systems that could lead to further disruptions of local immune function in the placenta and in the fetus [107, 108]. Oxidative stress is associated with reduced IL-2 production [109], which promotes pro-Th2 signaling by dendritic cells. Hence, modification of the oxidative function could represent a possible pathway for smoking in modifying the developing immune responses [105].

The association between smoke exposure and atopy is not conclusive [110], although a recent report of the German Multicenter Allergy Study (MAS) reported an increased risk of food allergen sensitization in children who were pre- and postnatally exposed to tobacco smoke by the age of 3 years [111].

In conclusion, there is an association between parental smoking and respiratory symptoms, and this relationship seems to be causal. Considering that in the Netherlands the estimated prevalence of children exposed to tobacco smoke during pregnancy ranges between 15% and 35% [112, 113] and that 40 to 50% children are exposed to passive smoking after birth [114], this risk factor for reduced lung function and respiratory symptoms remains a serious pediatric and public health issue.

Exposure to infections, siblings and day care attendance

In 1989 Strachan studied the epidemiology of hay fever in a national sample of 17,414 British children followed up to the age of 23 years and showed that hay fever was inversely related to family size and to the number of older children at age 11 and 23 years. The author speculated that repeated infections in early life transmitted by contact with older siblings could exert a protective effect against the development of allergic diseases [115]. The underlying immunological mechanism hypothesized was that natural immunity to bacterial and viral infections induces a Th1 pattern of cytokine release that can suppress the Th2 immune responses involved in IgE mediated allergy [116-118]. Although the recent discovery and characterization of different subtypes of T-cells has improved our understanding of the mechanisms involved in the immunological homeostasis of the airways, the 'hygiene hypothesis' has been supported for several years by studies showing the same inverse association of birth order with markers of atopy [119-125]. Von Mutius et al reported a low prevalence of atopy and asthma in the former East Germany shortly after the German reunification, which markedly increased in a survey conducted 5 years later. This suggested that changes in lifestyle, improved living conditions, the declining family size and the subsequent reduced exposure to infections early in life through siblings could in part contribute to the increased prevalence of atopic diseases [126-128]. However, inconsistent results have been reported with regard to the association between siblings and asthma symptoms [129-132]. Another important exposure to infections in early life is represented by child day care. However, no consistent association has been found between day care attendance and asthma [129, 133-137]. Day care attendance was characteristic of the former East German lifestyle, where the prevalence of asthma and bronchial hyperresponsiveness was significantly lower as compared with West Germany [136, 138]. Yet, it has been shown that the timing of exposure to day care is important in the development of asthma, as children from East German families entering day nursery in the first year of life were less likely to develop asthma and atopy than children attending day care after their second birthday [139].

The role of viral infections early in life on the development of asthma has been intensely debated [140]. It has been reported that bronchiolitis due to respiratory syncytial virus (RSV) can further increase the risk of subsequent wheezing episodes up to school age [141]. Stein et al reported the results of the 13-years follow-up of the

Tucson children's Respiratory Study and showed than RSV lower respiratory tract illnesses in early childhood (before 3 years of age) were an independent risk factor for the subsequent development of wheezing up to age 11 years but not at age 13, and this association was not caused by an increased risk of allergic sensitization [141]. A possible explanation for these findings is that infants with reduced lung function at birth are more likely to have severe symptoms during viral lower respiratory tract infections, suggesting that the interactions between infections and the innate predisposition contribute to the development of asthma. In the multicentre allergy study (MAS), a longitudinal birth cohort in Germany, Illi et al showed that lower respiratory tract infections early in life (before the age of 3 years) were positively associated with subsequent development of asthma, wheeze, and bronchial hyperreactivity by the age of 7. In contrast, early episodes of other infections, such as herpes type, were inversely related to development of asthma and respiratory symptoms [142]. Also, orofecal microbes have been associated with a reduced risk of atopy and, to a lesser extent, asthma, whereas airborne viruses, such as measles, mumps, rubella, chickenpox, the transmission of which is less affected by hygiene, were not associated with atopy or asthma [143, 144]. Shaheen et al reported the results of a follow up study in Guinea-Bissau children firstly surveyed in 1978-80 at age 0-6 years and reassessed 14 years later [145]. The subjects with childhood measles had about half the rate of atopic sensitization compared to those who had been vaccinated [145]. However, such findings should be interpreted with caution as one third of the children were lost to follow-up. Parasitic infestations are common in developing countries and have been considered as an indirect measure of poor hygiene. A recent meta-analysis of 33 epidemiologic studies found that infestation with any parasite was associated with a non significant increase in asthma risk, but hookworm infestation was associated with a reduced odds of asthma related to infection intensity [146]. Studies in rural Ecuador have shown that active infestation with geohelminth parasites and the presence of serologic markers of chronic infections are independent protective factors against allergen skin test reactivity among school-age children living in an endemic region [147]. Similar results were reported by a study in southern Brazil among 1,011 children aged between 8.2 and 13.3 years. Pereira et al showed that helminthes infestation were inversely associated with positive skin prick test results and represented a risk factor for non-atopic asthma [148]. Studies in Gabon [149] and Ethiopia [150, 151] have consistently

shown that protection against asthma may be greater with parasite species that have a host systemic phase in their life cycle, and may also be related to the intensity of infection. In fact, chronic infestation with helminthes may confer protection or an attenuation of asthma-related symptoms [152], but short-lived episodes of infestation may exacerbate atopic disorders [153].

In conclusion, several studies have shown an inverse association between birth order and atopic sensitization, but the role of infections and day care attendance on the development of atopic diseases is still controversial. Indeed, the immunological mechanisms that might explain the hygiene hypothesis are complex and cannot be entirely attributed to the unbalanced Th1/Th2 phenotype. Other cells and mediators play a central role in modulating the immune response within the airways. The timing of exposure to infections, the total burden of microbial stimuli and their interaction with the immune system seem relevant in determining the onset and the severity of atopic diseases.

Exposure to microbial substances in the environment

The primary source of microbial stimulation is represented by the gut, which is rapidly colonized by commensal organisms during infancy [154-156]. The gastrointestinal flora modulates mucosal physiology, barrier function and systemic immunologic and inflammatory responses [157-159]. Sepp et al showed that in Estonian infants, lactobacilli were more frequently found and the counts were higher than in Swedish infants, whereas clostridia counts were higher in Swedish than in Estonian infants. These differences were associated with the prevalence of atopy in each country [160]. The same authors extended the findings by showing that the allergic children in Estonia and Sweden were less often colonized with lactobacilli, as compared with the non-allergic children in the two countries. Besides, the proportion of aerobes was higher in allergic as compared with non-allergic children, while the proportion of anaerobes was lower [161]. Authors further investigated this association with a prospective study and showed that, in comparison with healthy infants, babies who developed allergy were less often colonized with enterococci during the first month of life and with bifidobacteria during the first year of life, suggesting that the intestinal microflora might have a role in the development of and protection from allergy [162]. Similar results were recently reported by Mah et al [163], showing that, in the tropical region of South East Asia, toddlers with eczema

harbored significantly lower counts of Bifidobacterium and Clostridium, but significantly higher counts of enterococci.

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [164]. Probiotics have anti-inflammatory properties associated with changes in cytokine expression. It was speculated that this could play a role in Th1/Th2 polarization, potentially facilitating T1-helper cell immune response [165]. Also, an effect of probiotics on regulatory T cells has been recently proposed [166, 167]. Therefore, in the last years several studies have addressed whether probiotics could be used for the treatment of allergic diseases. Vliagoftis et al recently published a review of randomized controlled trials evaluating the clinical evidence for the use of probiotics as a therapeutic modality for allergic rhinitis and asthma [168]. The authors concluded that probiotics may have a beneficial effect in allergic rhinitis by reducing symptom severity and medication use, but no positive effect was found on asthma. In a Cochrane database review, Osborn and Sinn evaluated the role of probiotics in infants for prevention of allergic disease and food hypersensitivity [169]. Authors showed that several randomized studies have demonstrated efficacy from the use of probiotics in infants with active eczema [170-172]. Not all studies have shown conclusive benefits [173]. Furthermore, the most consistent finding in these studies is a reduced proportion of bifidobacteria species in the faeces of infants with eczema [174] and atopic sensitization [175], but not in children with symptoms of asthma [174]. Authors concluded that there is insufficient evidence to recommend the addition of probiotics to infant feeds for prevention of allergic disease or food hypersensitivity [169].

High levels of microbial products can be found in stables and barns of traditional dairy farms, where animals such as cattle, pigs and poultry are kept [176]. Farming animals are an important source of endotoxin, which comprises the outer lipopolysaccharide (LPS) component of gram-negative bacteria cell walls [177]. Many bacterial surface molecules interact with pattern-recognition receptors, such as Toll-like receptors (TLRs) and CD14, which are part of the innate immune system [178, 179]. TLR are an evolutionarily conserved group of molecules expressed in antigen-presenting cells and epithelial cells, whereas CD14 is constitutively expressed primarily on the surface of monocytes, macrophages, and neutrophils as membrane CD14. After the interaction with antigen-presenting cells, endotoxin and other bacterial wall components strongly stimulate IL-12 responses [180]. IL-12, in turn, is

an obligatory signal for the maturation of naive T-cells into Th1-type cells, which leads to an upregulation of IFN-γ [181, 182]. Several studies have shown that exposure to farm environment is associated with lower prevalence of asthma and atopy [176, 183]. This association was stronger for long-term and early-life exposure to stables and farm milk [184-186]. However, endotoxin is also present at lower levels in normal indoor environments as a component of house dust. A number of studies have consistently reported inverse associations between exposure to endotoxin in house dust and atopy in children and adults, also in nonfarming environments [187-190], whereas conflicting results have been reported in studies that assessed the relationship between exposure to endotoxin and asthma [191-193].

The ISAAC Phase II showed between-countries variation in the associations between house dust endotoxin in living room floor dust and health outcomes and, combined across countries, endotoxin levels were inversely associated with asthma ever and current wheeze, in agreement with the findings of other cohort studies [187, 191]. In early infancy, most wheezing is unrelated to atopy and several studies have shown a positive association between endotoxin exposure and non-atopic asthma and wheezing [192, 193]. A longitudinal study by Litonjua et al in 226 preschool children of parents with asthma or allergy showed that high endotoxin was a risk factor for wheezing in the first year of life. However, over the follow-up period the risk rapidly decreased and by the age of 4 years the proportion of children with wheezing episodes was no longer higher in children heavily exposed to endotoxin compared with those not heavily exposed [194]. Further follow up of this cohort will show whether this exposure will protect children against other episodes of wheezing or asthma as the children get older. Other studies have found inverse associations between objective measures of bacterial and fungal exposure and asthma and wheezing [195, 196]. However, in a recent prospective birth cohort study, Bisgaard et al have evaluated the association between bacterial airway colonization in asymptomatic neonates born to mothers with asthma and the development of recurrent wheeze and asthma from birth through the first 5 years of life [197]. At one month, 21% of the infants were colonized with Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, or a combination of these organisms. Infants colonized with one or more of these organisms, had an increased cumulative risk of persistent wheeze (hazard ratio 2.40), acute severe exacerbation of wheeze (hazard ratio 2.99), and hospitalization for wheeze (hazard ratio 3.85). Furthermore,

children colonized with these organisms at one month had increased prevalence of asthma and reversibility of airway resistance after beta2-agonist administration at 5 years of age, compared with the children without such colonization. Such associations were not seen for Staphylococcus aureus colonization. These authors estimated that the population attributable risk in this high-risk population was 4.6%, suggesting that elimination of the risk associated with bacterial airway colonization should lead to a drop in overall asthma prevalence by the age of 5 years from 14.2 to 9.4% in similar high-risk populations. However, the generalizability of these findings is restricted to high-risk children and validation in unselected populations is needed.

Previous studies also evaluated whether alterations of the immunological mechanisms underlying the recognition of microbial products were associated with different risks of atopic diseases. Vercelli et al showed that a polymorphism associated with higher expression of CD14 was related to reduced risk of developing allergic diseases, suggesting that children with that polymorphism would be more sensitive to environmental microbial exposures that would protect against allergy [198]. Results of further studies were not always consistent with the original findings of Vercelli [199, 200], suggesting more complex mechanisms underlying the described associations. In particular, Eder et al demonstrated a strong gene-environment interaction showing that the polymorphism found could be either protective or a risk factor, depending on the degree of exposure to environmental microbial products [201]. Therefore, it has been suggested that the risk of asthma and allergies depends on the responses to a complex array of environmental exposures, which influence the development of the disease, in the genetically determined context [178]. A recent study confirmed the hypothesis of gene–environment interactions, showing that variation in CD14, TLR2, TLR4, and TLR9 genes modified associations between country living during childhood and asthma, particularly among atopics [202]. Furthermore, it has been recently shown that the risk of early-onset asthma in individuals with certain genetic variants may be enhanced by the exposure to tobacco smoke exposure in early life [203].

Another mechanism that may alter the balance of the normal colonization of the gut is represented by antibiotic use. An association between the use of antibiotics and the risk of asthma has been previously found in retrospective studies [204-206]. However, asthma symptoms, especially in young children, are often treated with antibiotics, and a potential bias by reverse causation cannot be excluded, as infants

with more asthma symptoms will receive more courses of antibiotic treatment for their symptoms[206]. Therefore, only studies taking into account the indication for prescribing antibiotics and the use of antibiotics before the onset of asthma can adequately address the question of the association between antibiotic use and asthma. Illi et al reported the results of the longitudinal German MAS, in which the number of antibiotic courses received by the children was recorded, but only antibiotic courses not related to the treatment of lower respiratory tract infections were included in statistical analysis. Authors found no association between antibiotic use in the first 3 yrs of life for indications other than lower respiratory tract illnesses and asthma or atopy at the age of 7 yrs [142]. Similarly, there is no conclusive evidence that routine vaccinations affect the onset of asthma or atopy [207-210].

The use of acetaminophen has also been linked to the onset of asthma [211] and a recent international study found that use of paracetamol in the first year of life and in later childhood, is associated with risk of asthma, rhinoconjunctivitis, and eczema at age 6 to 7 years, suggesting that exposure to paracetamol might be a risk factor for the development of asthma in childhood [212]. The authors also showed that the use of paracetamol was associated with the risk of severe asthma symptoms, with population-attributable risks between 22% and 38%.

In conclusion, it has been suggested that the intestinal microflora plays a role in allergic sensitization and in the subsequent development of atopy and asthma. However, large randomized controlled trials are still needed in order to recommend the addition of probiotics to infant feeds for prevention of allergic disease. The exposure to environmental microbial products is associated with lower prevalence of hay fever, atopic sensitization, atopic asthma and atopic wheeze in childhood, depending on timing and duration of the exposure. However, in high-risk infants the exposure to endotoxin and early bacterial colonization of the hypopharynx are associated with an increased risk of early wheezing and asthma. There is no conclusive evidence that antibiotics or paracetamol use and routine vaccinations modify the risk to develop atopy or asthma.

Exposure to outdoor air pollution

Although the nature and concentration of outdoor air pollutants vary from one area to another, the most abundant pollutants in the atmosphere of urban areas are ozone (O3), nitrogen dioxide (NO2) and particulate matter (PM). O3 is generated at

ground level by photochemical reactions involving ultraviolet radiation on atmospheric mixtures of NO2 and hydrocarbons derived from vehicle emissions. Approximately 40–60% of inhaled O3 is absorbed in the nasal airways, the remainder reaching the lower airways. NO2 is found in outdoor air in urban and industrial regions and, in conjunction with sunlight and hydrocarbons, results in the production of O3. Automobile exhaust is the most important source of outdoor NO2. NO2 is often used as an indicator for traffic related air pollution components, and the spatial variation in NO2 concentration is strongly related to motorized vehicle traffic. Indoors the most significant exposure to NO2 occurs in conjunction with the use of gas cooking stoves, geysers without exhaust pipes and kerosene space heaters. Like O3, NO2 is an oxidant pollutant although it is less chemically reactive. Carbon monoxide (CO) is a colorless and odorless, yet highly toxic gas produced from the partial oxidation of carbon-containing compounds, notably in internal-combustion engines. Carbon monoxide forms in preference to the more usual carbon dioxide when there is a reduced availability of oxygen during the combustion process. Airborne PM, which is a major component of urban air pollution, is a mixture of solid and liquid particles of different origin, size and composition, among which pollen grains and other organic particles carrying allergens and mould spores are included. Inhalable PM is measured as PM10 and PM2.5 (particles with an aerodynamic diameter < 10 μm and < 2.5 μm, respectively) [213]. PM2.5 can deposit in the human lung peripheral airways and alveoli, while particles >5 μm and <10 μm only reach the proximal airways, where they are eliminated by mucociliary clearance. High concentrations of air pollution can have serious adverse effects on health [214], particularly in children [215]. Several studies conducted in Europe and US have shown that the exposure to outdoor air pollution may decrease lung function, trigger exacerbations of asthma and increase rates of hospitalization for asthma [216-237] (table 3).

Traffic-related air pollution and, in particular, living close to streets with high traffic intensity have been associated with asthma, wheezing and cough [238-244], also in the first years of life [245], but not with bronchial hyperresponsiveness [239, 240, 246]. Recently, studies evaluated the contribution of air pollution to the initial development of asthma and atopy [215, 247, 248]. A recent birth cohort study conducted among 205 children living in Copenhagen for the first 3 years of life evaluated the association between incident wheezing symptoms and air pollution on the concurrent and previous 4 days. Parents recorded their child’s wheezing

symptoms daily with diary records for the first 3 years of life and daily air pollution levels were available from a central background monitoring station in Copenhagen. Authors showed significant positive associations between concentrations of pollutants and wheezing symptoms in infants (aged 0-1 year) with a delay of 3-4 days and showed significant effects throughout the 3 first years of life [249]. Another birth cohort study conducted in the Netherlands evaluated the relationship between traffic-related air pollution and the development of asthma, atopy and respiratory infections in 4,000 children. Outdoor concentrations of traffic-related air pollutants were assessed at the home of each subject and questionnaire-derived data on respiratory symptoms and eczema in the first 2 years were analyzed in relation to air pollutants.

Table 3 Types, sources and respiratory health effects of common outdoor pollutants (adapted from [223]).

Type	Source	Main health effect
Ozone (O_3)	Photochemical reaction from autovehicle traffic	Airway obstruction Bronchial hyperresponsiveness Increased prevalence of respiratory symptoms Increased hospitalization rate for respiratory disease Reduced exercise tolerance
Nitrogen dioxide (NO_2)	Automobile exhaust	Airway obstruction Bronchial hyperresponsiveness Increased prevalence of respiratory symptoms Reduced exercise tolerance
Carbon Monoxide (CO)	Fuel combustion	Reduced exercise tolerance
Particulate Matter (PM_{10}, $PM_{2.5}$)	Autovehicle traffic Industrial activity	Airway obstruction Increased prevalence of respiratory symptoms/diseases Increased mortality from cardiorespiratory diseases Asthma exacerbations

The authors found that wheezing, physician-diagnosed asthma, ear/nose/throat infections, and flu/serious colds were positively associated with air pollutants [250]. Miller et al. evaluated whether important components of diesel exhaust and other

combustion sources, such as aromatic hydrocarbons (PAH), were associated with respiratory symptoms in young children. For this purpose, 303 pregnant women from northern Manhattan were recruited, the 48-hours personal PAH exposure measurements were collected and their children were prospectively monitored. These authors found that early exposure to airborne PAH increased respiratory symptoms and probable asthma by age 12 to 24 months [251]. Similar results were obtained by a longitudinal study which evaluated the association between traffic-related air pollution and incident asthma. A total of 217 children were followed from 10 to 18 years and new asthma incidence was reported annually through questionnaires during 8 years of follow-up. As a marker of traffic-related air pollution, nitrogen dioxide monitors were placed outside the children's home for 2 weeks in the summer and 2 weeks in the fall-winter season. Authors found that incident asthma was positively associated with traffic pollution, with a hazard ratio of 1.29 across the average within-community interquartile range of 6.2 ppb in annual residential NO2 [252]. Furthermore, Mortimer et al. showed that exposure to several air pollutants in the pre- and early postnatal period was associated with lower pulmonary function in 232 children with asthma aged 6-11 years. However, these authors found that most of the effects were limited to subgroups of the population with characteristics typically associated with a greater prevalence or severity of asthma, such as children of African-American ethnicity, early asthma diagnosis, prenatal smoke exposure, and history of atopy or steroid use [253]. In a different study, Mortimer et al also evaluated the association between prenatal and early-life exposures to outdoor air pollutants and allergic sensitization in a cohort of 170 children ages 6-11 years with asthma, living in the Central Valley of California [254]. Allergic sensitization was assessed by skin-prick tests and prenatal and early-life exposure to O3, NO2, CO and PM10 was reconstructed for each child. Authors found that higher exposure to CO during pregnancy was associated with an increased risk of sensitization to at least one outdoor allergen [254]. In a recent prospective birth cohort study, Latzin et al [255] evaluated the association between pre and postnatal exposure to pollutants (PM10, NO2 and O3) and lung function in 241 healthy newborns. They found that minute ventilation was higher in infants with higher prenatal PM10 exposure and postnatal exposure to air pollution did not modify this association, suggesting that prenatal exposure to air pollution might be associated with respiratory symptoms in newborns [255]. Other cross-sectional studies also found that traffic-related air pollution leads

to increased prevalence of sensitization and atopic symptoms [256] and that sensitization to pollen increased in relation to truck but not car traffic counts[239]. Mechanisms by which exposure to air pollution might induce new cases of allergic asthma include damage of the airway epithelium, stimulation of inflammatory cell activity, increased release of inflammatory mediators and, thereby, enhanced penetration of allergens through the airway epithelium. Furthermore, oxidant exposure may increase airway inflammation, thereby, inducing bronchial hyperresponsiveness in susceptible, e.g. atopic subjects and adding to the prevalence of asthma in polluted areas. Evidence of the role of air pollution on bronchial inflammation has been supported by studies that found increased fractional exhaled nitric oxide (FeNO), a marker of eosinophilic bronchial inflammation, in adults and children exposed to outdoor air pollutants [244, 257-259].

In conclusion, there is evidence that outdoor air pollution is associated with increased prevalence and incidence of asthma and atopy and with increased levels of bronchial inflammation. Children with bronchial hyperresponsiveness and/or sensitization to common allergens are the most sensitive subgroup among all children for these effects. Further large prospective studies with longer follow up are needed in order to disentangle the association between pre- and postnatal pollutant exposures and the inception of asthma and allergy. This would help identify the time window in which a possible intervention could modify the natural course of atopic diseases.

1.3 Exhaled nitric oxide in infants

(*Based on:* Exhaled nitric oxide in infants – what is a nice test like FeNO doing in a place like this? van Mastrigt E, Gabriele C and de Jongste JC. *Seminars in Respiratory and Critical Care Medicine 2007 Jun;28(3):264-71. Reprinted with permission of the Thieme Publishing Group*)

Markers of bronchial inflammation can be measured with invasive and non-invasive procedures, such as bronchoscopy, bronchial biopsy, analysis of the bronchoalveolar lavage fluid, examination of sputum, blood, urine, exhaled gases and breath condensate. In adults and older children the measurement of fractional exhaled nitric oxide (FeNO) has shown to be useful as a tool to diagnose and monitor eosinophilic airway inflammation. However, the recommended method to measure FeNO in school age children is not suited for use in preschool children and infants. In

the following section, methodological and practical aspects of the FeNO measurements in the first 2 years of life will be illustrated. Also, available data on factors influencing FeNO and the relationships between FeNO, respiratory diseases and atopy will be reported.

1.4 Background

Nitric oxide (NO) is a mediator with a multitude of important regulatory functions and is present in exhaled air in humans [260]. It is formed in biological systems from L-arginine and oxygen by the nitric oxide synthases (NOS) [261-263]. Three different types of NOS have been described: type I and III which are constitutive (cNOS) have been found respectively, type I in human airway nerves and type III in lung epithelial cells and the vascular endothelium; type II which is an inducible isoform (iNOS) has been mainly localized to the airway and alveolar epithelium, alveolar macrophages and the vascular endothelium. Cytokines (i.e. IFN, interleukines, TNF) and endotoxins may up-regulate the iNOS gene expression, causing an increase in NO synthesis [264].

Increased levels of the fractional concentration of nitric oxide in exhaled air (FeNO) have been found in asthmatic adults and children more than a decade ago [265-267]. In the past years a well-standardized methodology for FeNO measurement has been developed [268] and normative values for school children and adolescents have been published [269]. Several studies have evaluated the role of FeNO as a tool to diagnose and monitor eosinophilic airway inflammation in atopic asthmatic children and adults [270-272]. However, scanty information is available on FeNO in infants, as difficulties in obtaining suitable exhaled air samples in uncooperative children have until now impaired the development of standardized, feasible techniques. This is unfortunate, as wheezing is an extremely common symptom during infancy and often of a transient nature. Also, the clinical diagnosis of asthma is unreliable in wheezing infants and is based on symptoms, physical examination and a family history of asthma or atopy, all of which are non-specific [1]. An early asthma diagnosis is important for timely institution of adequate therapy, although the effectiveness of the current available treatment in controlling the disease progression has still to be shown [273]. Indeed, it would be of critical importance a bedside test that could predict steroid-responsive airways disease. Chronic airway inflammation is an important factor in the pathogenesis of asthma and recent data suggest that eosinophilic inflammation seen in asthmatic adults is present already in wheezy preschool children [11] and in atopic

infants [274]. Therefore, the assessment of FeNO is also potentially useful for the diagnosis of asthma in young children.

Guidelines for the measurement of FeNO in adults and children have been published and recently updated [268]. Practical recommendations for the measurement of FeNO young children are also available [275]. Nevertheless, the interpretation of FeNO in young children is still problematic, because of the lack of standardized techniques for infants and the different anatomy and pathology compared to older children and adults.

1.4.1 FeNO measurements in infants: a methodological nightmare

FeNO is critically dependent on exhalation flow [276] and in adults and older children it is recommended that FeNO is measured during a single, slow exhalation from total lung capacity at a constant flow of 50 mLxsec-1. Exhalation against a resistance leads to elevated mouth pressure, which ensures soft palate closure, thereby preventing contamination with nasal NO [268, 275]. However, this method is not suitable in uncooperative patients and the proportion of reliable measurements drops dramatically in children younger than 4 years. As a result of these limitations, FeNO measurements in infants have been performed during tidal breathing or during a single forced passive expiration.

Tidal breathing measurements

The tidal breathing method uses a facemask that covers mouth (oral FeNO) or both nose and mouth (mixed FeNO). The facemask can be connected to a 2-way, non-rebreathing valve that allows inspiration of NO-free air from a reservoir. The expiratory port is coupled to either an NO-inert bag, in which the expired air can be stored for later analyses (off-line, figure 1) or directly sampled by the NO analyser (on-line). The main disadvantage of this technique is that the measurement is performed with variable expiratory flow [275, 277]. However, simultaneous recording of FeNO and tidal flow has been employed to allow control for the potential effect of expiratory flow on exhaled NO [277, 278]. This method requires a pneumotachograph attached to the facemask to measure respiratory rate and expiratory flow. Tidal breathing FeNO measurements showed fair reproducibility [279]. With a fast response NO analyser small samples of exhaled air are sufficient for analysis. The commercially

available NO chemiluminescence analyzers fulfill this requirement, whereas analyzers using photochemical sensors are still too slow.

Single breath technique

Wildhaber et al [39] and Martinez et al [280] described similar methods to measure FeNO on-line with the single breath technique. Sedated infants undergo passive lung inflation followed by a controlled slow thoracoabdominal compression by means of an inflatable jacket, leading to a single forced expiration while FeNO is sampled via a facemask. A two-compartment facemask was used in order to sample exhaled air from the mouth only. This single-breath technique requires sedation, specialized equipment and well-trained personnel. The measurements can be combined with other measures of lung-function, but are cumbersome and time-consuming and therefore less suited for routine testing or large epidemiological studies.

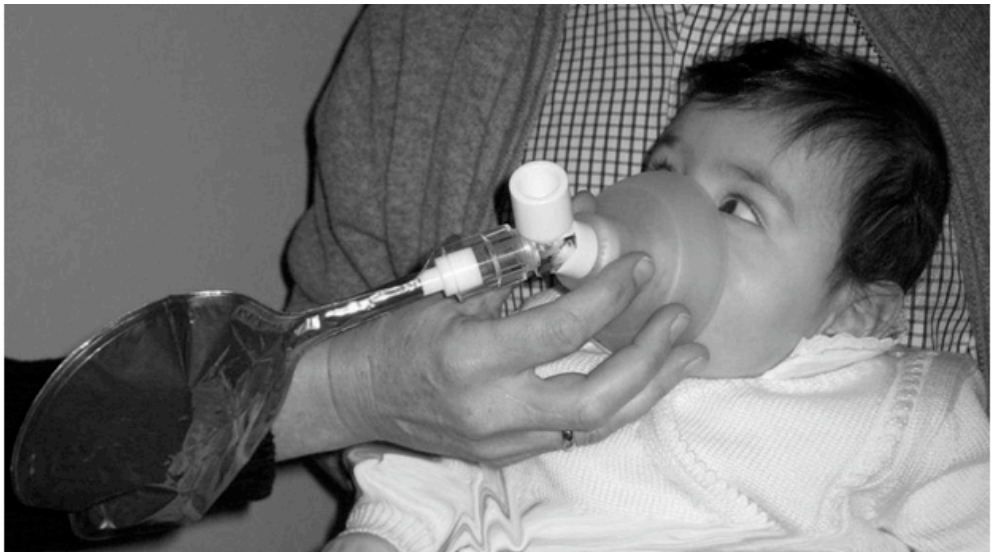

Figure 1 Measurement of fractional exhaled nitric oxide (FeNO) in an infant using the tidal breathing technique. Mixed oral and nasal expired air is collected via a face mask, connected to an NO-inert balloon via a nonrebreathing valve. The inspiratory port can be fitted to a source of NO-free air (e.g., a large Mylar balloon), to avoid contamination with ambient NO (not shown). For a successful measurement, the child should tolerate the facemask and breathe quietly. Air sampled during four to five tidal breaths can be stored for 6 to 9 hours at room temperature and analyzed offline.

Single-breath versus tidal breathing technique

Franklin et al found poor agreement between tidal breathing and single-breath FeNO in infants with recurrent wheeze, recurrent cough or without respiratory symptoms. FeNO levels obtained by the single-breath technique were higher than levels collected by tidal breathing and the difference increased with higher levels of FeNO. Indeed, Franklin et al reported significantly different FeNO levels between healthy and wheezing infants with the single-breath technique, but not with the tidal breathing technique [281]. However, other studies have demonstrated that tidal breathing FeNO may well discriminate between children [282] and adults [283] with and without asthma symptoms. Hence, it can be questioned whether the single breath method is indeed worth pursuing. Obviously, there is no simple way out of this problem and there is need for clinical studies showing the merits and limitations of the different methodologies, aiming for standardization of FeNO measurements in infants and normal reference values for this age group.

1.4.2 Measurement conditions – do they matter in infants too?

According to the American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines, there are many factors that may influence FeNO measurements both in children and adults [268]. There is scanty information on the effect of such factors on FeNO values in infants.

Ambient NO

Atmospheric ambient NO levels can be highly variable from 0 up to exceeding 200 ppb, dependent on several environmental factors, such as traffic emissions and climate [284]. Hence, standardized techniques must prevent the contamination of samples with ambient NO. Contamination of FeNO levels with ambient NO is mainly a problem with the tidal breathing methods and can be minimized by inspiration of NO free air from a NO-inert reservoir. Ambient contamination can also be prevented by only measuring at ambient levels < 10 ppb [275]. However, because contamination may even occur with ambient levels as low as 5-10 ppb [277], it may be better to use NO free air for all FeNO measurements in infants.

Nasal NO

The nasal mucosa and paranasal sinuses physiologically produce large amounts of NO that will contaminate NO exhaled from the lung if no measures are taken to isolate the oral cavity from the nose. In adults, levels of NO in upper airways are an order of 10-100 times higher than NO concentrations from lower airways [285]. In infants paranasal sinuses are not yet developed and nasal contamination could therefore be less important. However, Schedin et al showed that within a few minutes after birth significant amounts of NO are excreted from the upper airways in both preterm and term infants [286, 287]. Theoretically, contamination with nasal NO can be prevented by nasal suctioning and by exhalation against a resistance [268]. Nasal suction is unpleasant, cumbersome and therefore not often applied, especially in infants who are generally nose breathers. Exhalation against a positive mouth pressure ≥ 5 cm H2O will ensure closure of the velum. Although the velum will not stay closed during inspiration, there is evidence that inhaled NO during inspiration contributes little to FeNO in infants [288]. In an attempt to avoid nasal contamination Baraldi et al measured offline tidal FeNO with the facemask simply placed over the mouth while closing the nostrils. Infants are preferential nose breathers, therefore this method induces variable breathing patterns that may influence FeNO values [289]. During a single forced exhalation, nasal contamination is unlikely [39].

Expiration flow

FeNO is highly flow-dependent, with higher flow resulting in lower FeNO [290, 291]. In infants, exhaled NO concentrations are significantly affected by breath-to-breath variation in expiratory flow during online tidal breathing [277]. Flow dependence was also evident when using the single-breath technique [39, 280, 281]. Hall and Frey reported similar findings and found that the intrasubject variability of both FeNO and NO output (FeNO x Tidal flow) was dependent on the phase of expiration. In order to adjust for the expiratory flow dependence of tidal FeNO measurements, they determined exhaled NO in the time based 3rd quartile of the expiration. At this phase, FeNO showed the lowest intraindividual variability [39, 281]. Simultaneous recording of flow and FeNO makes it possible to assess NO output. However, NO output did not distinguish better between children with and without parental smoke exposure than FeNO [292], suggesting that it might not be worthwhile to measure and correct for expiratory flow.

Sedation

Lack of cooperation is a major determinant of FeNO variability in awake infants. Sedation may overcome this problem but markedly affects breathing patterns and hence FeNO. Indeed, higher FeNO and smaller intrasubject variability were found in sedated infants [277]. FeNO in infants during natural sleep was comparable to that obtained under sedation [292]. Clearly, a need for sedation would drastically limit the feasibility of FeNO measurements for relatively minor disease and for large scale epidemiological studies.

Lung Function Tests

Spirometric manoeuvres transiently reduce FeNO levels in asthmatic adults [293] and children [294] and it is recommended that FeNO measurements are performed prior to forced expiratory manoeuvres.

Food and beverages

The ATS/ERS guidelines recommend refraining from eating and drinking for 1 h before FeNO measurement [268]. Several foods and beverages, in particular nitrate-reach meals, have been shown to transiently influence FeNO [295].

1.4.3 Other factors influencing FeNO in infants

Gender

In adults, slightly higher FeNO has been reported in men compared to women [290]. Inconsistent and varying gender effects on FeNO have been reported in infants, both with single-breath and tidal breathing methods, with significantly higher FeNO levels in girls compared to boys, or the reverse [281]. Higher FeNO has been found in boys than in girls in the first month after birth, selectively in infants of atopic mothers [279]. Most studies in older children found no significant difference in FeNO between boys and girls, with the exception of a single study in 4-7 year olds [269, 296]. The available data suggest that gender differences may be present in infants and may be modified by genetic factors.

Gestational and postnatal age

FeNO levels in term and preterm infants shortly after birth vary greatly between studies and show conflicting results. According to Biban et al term infants have

significantly lower levels of exhaled NO at birth then preterm infants. This difference was no longer present at 24 and 48 hours after birth [297]. In contrast Colnagni et al found a peak of FeNO in the first hours of life in term infants, while in preterm infants FeNO was low at birth and then gradually increased [298]. Schedin et al measured FeNO in the upper airways of healthy term newborns [287] and premature infants [286]. They found a 30% increase between 1 hour and 24 hours in healthy term newborns and a significant increase with postconceptional age in prematures. FeNO, measured at a median postnatal age of 5 days was not significantly different between preterm and term infants, using an online method with sampling via a nasal cannula [288]. Furthermore, one group reported that tidal breathing FeNO levels increase with age in infants[281], although most other studies found no such relationship [39, 288, 289]. Although all these studies used different measurement techniques for FeNO and the variation in results may well depend on methodology, the data seem to indicate that time after birth may be an important determinant of FeNO and should be taken into account.

Age-dependency of FeNO in children may be anticipated as a result of developmental and material changes, including increased lung volume and airway surface area or changes in airway NO diffusion coefficients. Furthermore, the use of a fixed standardized exhalation flow in children with different airway sizes may also be a factor explaining the age-dependency of FeNO: the same flow is relatively higher for younger children, probably resulting in lower FeNO levels.

Maternal atopy

Maternal atopy is a strong risk factor for the development of atopic disease and asthma in children. Frey et al found that flow-corrected tidal breathing FeNO tended to be higher in 36 days-old infants from atopic mothers [279]. Similar findings were reported by Baraldi et al [289]. Using the single breath technique, Wildhaber et al found significantly higher levels of exhaled NO in infants with a family history of atopy, independent of whether infants wheezed. The mean levels of FeNO were highest in infants with both maternal and paternal atopy, lower in infants with one atopic parent and lowest in infants with no family history of atopy [39]. It remains to be shown whether this means that eosinophilic airway inflammation is already present in these infants before the onset of airway symptoms.

Maternal smoking

Two recent studies in infants evaluated the association between prenatal tobacco exposure and FeNO. Frey et al found that the effect of prenatal smoking on FeNO in infants was modified by atopic constitution in the mother, with elevated FeNO if the mother was atopic and reduced FeNO with non-atopic mothers [279]. Furthermore, a recent study showed a positive association between nitric oxide and subsequent respiratory symptoms in infants of smoking mothers [299]. These data suggest that, apart from being a marker of eosinophilic inflammation at least in older children and adults, FeNO seems to reflect a toxic effect of cigarette smoke exposure in infants.

1.4.4 FeNO - a marker of airway disease in infants?

In older subjects, FeNO has been studied in relation to a large number of pulmonary and other diseases. In infants, there are few studies exploring FeNO as a possible disease marker and the majority is cross-sectional. As asthma is uncommon in infants and wheezing is often related to viral infection, considerable differences in clinical utility of FeNO between infants and older children could be expected and this is indeed what the limited data seem to show.

Wheezing

Baraldi et al first described that infants with recurrent wheeze had significantly elevated tidal breathing FeNO during an exacerbation compared to healthy controls and to infants with a first time viral-induced wheezy episode [289]. Several other studies found FeNO significantly higher in wheezy infants compared to healthy infants [39, 281]. Hence, FeNO seems consistently higher in wheezing infants, in particularly those with an atopic predisposition. Interestingly, a recent study by Latzin et al showed that a high FeNO after birth was associated with more respiratory symptoms in the first year of life if the mother had an atopic disease or had been smoking during pregnancy, with the strongest association when both factors were present [299]. Until now, there is no evidence that FeNO in early infancy predicts later asthma, but follow-up studies should be able to answer this question. Moreover, the reported differences seem relatively small compared to those of asthmatic and healthy older children. Often, the reported levels of FeNO in wheezing and non-wheezing infants do not exceed the within-subject reproducibility and high FeNO, as seen in older asthmatics, is apparently rare in infants and preschool children. Whether or not this means that

the pathogenesis of wheezing in atopic infants is of another nature than in older children is presently unclear; a previous biopsy study suggested that eosinophilic inflammation may not develop before the age of 1 year in wheezing infants [16]. However, the same authors later showed evidence of eosinophilic bronchial inflammation in infants with confirmed wheeze between the age of 1 and 3 years [11].

Eczema

Children with eczema have an increased risk of developing other atopic disease, such as asthma. It has been found that children with eczema but without a history of wheezing had significantly higher FeNO values than healthy controls [300] Also, children with doctor-diagnosed eczema had significantly elevated levels of FeNO [277]. Whether or not such elevated FeNO reflects subclinical airway eosinophilia and may even be predictive of asthma, is at present unclear.

Respiratory tract infections

Baraldi et al found similar levels of exhaled NO in infants with first time wheeze due to an upper respiratory tract infection and in healthy controls [289]. However, others reported that FeNO was temporarily reduced in infants with symptoms of an upper respiratory tract infection compared to healthy controls [278, 301]. It has been speculated that a decrease in FeNO may reflect a downregulation of NO production during acute viral infection [278]. An alternative hypothesis is that the epithelial damage and the increased airway secretions in infants with upper respiratory infections could result in impaired NO diffusion into the airway, leading to reduced FeNO [301].

Other applications of FeNO

In older subjects, FeNO has been explored as a marker of a wide variety of obstructive diseases of upper and lower airways, infections, gastrointestinal and liver diseases and metabolic disease. Much less is known about infancy, partly because many of these disorders are uncommon at a young age. There are however a number of areas where promising results have been reported.

Primary ciliary dyskinesia

Primary ciliary dyskinesia (PCD) is a genetic disease characterized by lack of effective ciliary motility, which results in chronic infection in the upper and lower airways. Exhaled and especially nasal NO are much reduced in PCD, due to increased NO metabolism in mucus and perhaps to reduced NO formation [302]. Nasal NO measurement have been proposed as a screening test for the diagnosis of PCD with nearly perfect sensitivity and specificity [303]. FeNO, although lower in groups of PCD patients, is less useful as a diagnostic test because of overlap between patients and controls. The diagnostic value of low FeNO and nasal NO for PCD in infants has not been shown convincingly, partly because of problematic methodology at this age, partly because the contrast with healthy children may be less due to undeveloped paranasal sinuses in infants. Still, it is to be expected that also in infancy NO measurements may be helpful in diagnosing PCD and larger series of patients and controls should be studied to confirm this.

Bronchopulmonary dysplasia (BPD), chronic lung disease (CLD)

Low FeNO has been found in school-age BPD survivors [304] and it has been speculated that a defective NO synthesis and/or diffusion into the airway lumen could be either a consequence of the epithelial damage occurring in the early phases of BPD or could be attributed to a reduction in the pulmonary vascular bed [305]. Bronchial inflammation dominated by neutrophils is present in BPD early in life. Chronic lung disease (CLD) is defined as an oxygen dependency after a post-conceptional age of 36 weeks and is common after very premature birth. Data on FeNO in CLD are inconsistent. In one study, premature infants with CLD had significantly higher FeNO than premature infants without CLD and healthy term infants [306]. Another study found no differences in FeNO between CLD and non-CLD patients [307]. And a single study reported lower NO output in infants with CLD compared to healthy infants [307]. From these data it is unclear if FeNO measurements could be useful in neonatal lung disease and are indicative of airway pathology. More studies into the time course of FeNO changes and on treatment effects, in BPD and CLD are needed.

Cystic fibrosis

Cystic Fibrosis (CF) airway disease is characterized by chronic neutrophilic airway inflammation, mucus plugging and bronchial infection with specific bacterial

pathogens. Older CF patients have reduced FeNO despite massive airway inflammation [308, 309]. The mechanism underlying reduced FeNO in CF is unknown, but may be due either the decreased expression of iNOS or to NO metabolism in mucus. Indeed, Moeller et al recently found that inducible NO synthase expression is low in airway epithelium from young children with cystic fibrosis [310]. Compared with healthy infants, reduced FeNO values have been shown in CF infants [311], but this finding was not replicated by a subsequent study [312]. However, a recent cross-sectional study has shown that children with CF had lower FeNO and increased nitrite levels in the exhaled breath condensate as compared with children without CF, suggesting a role of these inflammatory markers in the management of CF [313]. Although the reported differences are relatively small and often within the range of reproducibility, the contrast between FeNO levels of infants with CF and other obstructive airways disease seem sufficient to be helpful in the diagnostic process, but further work is needed to test this.

1.4.5 Conclusions

In summary, both tidal breathing and single breath methods have been used successfully to measure FeNO in infants, and both approaches have attractive and problematic features. Measurement conditions seem as important as in older subjects and should be standardized. Issues to be resolved include the effect of nasal contamination and the importance of correction for expiratory flow. Whether or not technically demanding methods pay off in terms of feasibility, discriminatory power and reproducibility remains to be shown.

1.5 Aims of the study

The studies presented in this thesis focus on respiratory morbidity in the first 2 years of life. Within the framework of the Generation R Study, we sought to determine ethnicity-specific risk factors for respiratory morbidity and evaluated feasibility and usefulness of exhaled nitric oxide (FeNO) measurements in infants. The Generation R Study is a large prenatally recruited population-based multicultural birth cohort study in Rotterdam (The Netherlands), designed to identify early environmental and genetic determinants of growth, development and health [314-316] (2).

Fetal and postnatal exposures to risk factors for respiratory morbidity have been prospectively assessed with questionnaires and the occurrence of respiratory symptoms has been investigated at the age of 2, 6, 12 and 24 months. More detailed assessments were performed in a subgroup of Dutch children and their parents, referred to as the Generation R Focus cohort (figure 3).

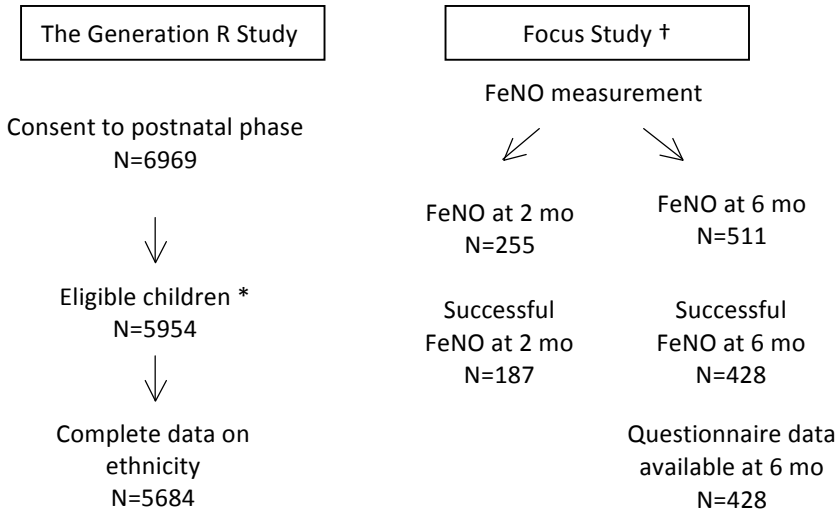

Figure 2 Study design.

* Full consent to the use of the data; no twin pregnancy; if the mother participated with more than one pregnancy, only the first pregnancy was included. † Eligibility criteria: enrolment before 25 wks of gestation; Dutch ethnicity; delivery date between February 2003 and August 2005.

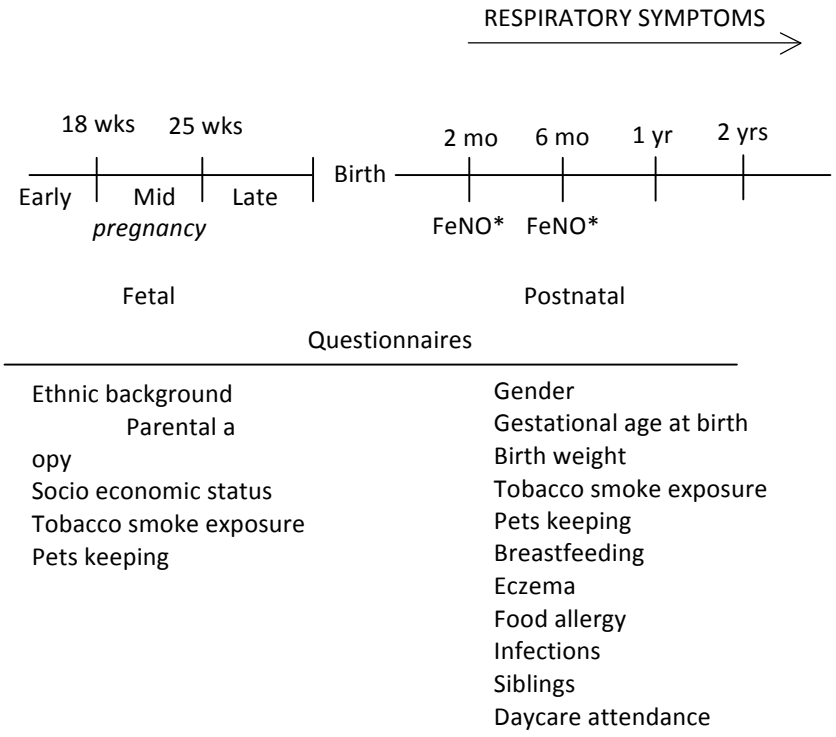

Figure 3 Assessment of fetal and postnatal variables in the Generation R Study

* Only performed in the Focus cohort

We addressed the following issues:

1. Evaluate the prevalence of respiratory morbidity in the first 2 years of life in association with ethnic background

2. Examine to what extent differences in respiratory morbidity between ethnic groups are mediated by fetal and early postnatal environmental factors.

3. Determine feasibility, reproducibility and methodological aspects of FeNO measurements in infants below the age of 2 years.

4. Evaluate if FeNO in infants is associated with pre- and early postnatal environmental exposures and with allergy/respiratory symptoms in the first 2 years of life.

Data from the Generation R Study were used to describe the effect of ethnic background on asthma-related symptoms in the first 2 years of life (chapter 2). Exhaled nitric oxide (FeNO) measurements were conducted in a subgroup of Dutch children and their parents, referred to as the Generation R Focus cohort. We assessed success rate, reproducibility and methodological issues related to FeNO measurements in infants (chapter 3).

In separate populations of infants, we evaluated whether FeNO measurements could differentiate various airways diseases in the first 2 years of life (chapter 4). We evaluated FeNO values during cow's milk food challenge in infants (chapter 5). We assessed the association between environmental exposures, respiratory symptoms and FeNO measurements in infants (chapter 6). Finally, we evaluated whether increased FeNO values precede respiratory symptoms (chapter 7).

References

1. Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, et al. Global strategy for asthma management and prevention: GINA executive summary. Eur Respir J. 2008 Jan;31(1):143-78.
2. Kuehni CE, Brooke AM, Strippoli MP, Spycher BD, Davis A, Silverman M. Cohort profile: the Leicester respiratory cohorts. International journal of epidemiology. 2007 Oct;36(5):977-85.
3. Asthma: still more questions than answers. Lancet. 2008 Sep 20;372(9643):1009.
4. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. Lancet. 2008 Sep 20;372(9643):1107-19.
5. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. Respirology (Carlton, Vic. 2006 Jan;11(1):54-61.
6. Haldar P, Pavord ID. Noneosinophilic asthma: a distinct clinical and pathologic phenotype. J Allergy Clin Immunol. 2007 May;119(5):1043-52; quiz 53-4.
7. Lemiere C, Ernst P, Olivenstein R, Yamauchi Y, Govindaraju K, Ludwig MS, et al. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. J Allergy Clin Immunol. 2006 Nov;118(5):1033-9.
8. Brasier AR, Victor S, Boetticher G, Ju H, Lee C, Bleecker ER, et al. Molecular phenotyping of severe asthma using pattern recognition of bronchoalveolar lavage-derived cytokines. J Allergy Clin Immunol. 2008 Jan;121(1):30-7 e6.
9. Morgan WJ, Stern DA, Sherrill DL, Guerra S, Holberg CJ, Guilbert TW, et al. Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence. Am J Respir Crit Care Med. 2005 Nov 15;172(10):1253-8.
10. Warner JO, Naspitz CK. Third International Pediatric Consensus statement on the management of childhood asthma. International Pediatric Asthma Consensus Group. Pediatr Pulmonol. 1998 Jan;25(1):1-17.
11. Saglani S, Payne DN, Zhu J, Wang Z, Nicholson AG, Bush A, et al. Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. Am J Respir Crit Care Med. 2007 Nov 1;176(9):858-64.
12. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med. 1995 Jan 19;332(3):133-8.
13. Stern DA, Morgan WJ, Halonen M, Wright AL, Martinez FD. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly diagnosed asthma in early adulthood: a longitudinal birth-cohort study. Lancet. 2008 Sep 20;372(9643):1058-64.
14. Illi S, von Mutius E, Lau S, Niggemann B, Gruber C, Wahn U. Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. Lancet. 2006 Aug 26;368(9537):763-70.
15. Turner SW, Young S, Goldblatt J, Landau LI, Le Souef PN. Childhood Asthma and Increased Airway Responsiveness-- A Relationship that Begins in Infancy. Am J Respir Crit Care Med. 2009 Jan 15;179(2):98-104.
16. Saglani S, Malmstrom K, Pelkonen AS, Malmberg LP, Lindahl H, Kajosaari M, et al. Airway remodeling and inflammation in symptomatic infants with reversible airflow obstruction. Am J Respir Crit Care Med. 2005 Apr 1;171(7):722-7.
17. Castro-Rodriguez JA, Holberg CJ, Wright AL, Martinez FD. A clinical index to define risk of asthma in young children with recurrent wheezing. Am J Respir Crit Care Med. 2000 Oct;162(4 Pt 1):1403-6.
18. Redington AE. Fibrosis and airway remodelling. Clin Exp Allergy. 2000 Jun;30 Suppl 1:42-5.

19. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 2):S28-38.
20. Stevens CA, Turner D, Kuehni CE, Couriel JM, Silverman M. The economic impact of preschool asthma and wheeze. Eur Respir J. 2003 Jun;21(6):1000-6.
21. World Health Organization (WHO). Bronchial asthma. 2008.WHO Fact Sheet N° 307. http://www.who.int/mediacentre/factsheets/fs307/en/index.html.
22. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. Eur Respir J. 1995 Mar;8(3):483-91.
23. Brunekreef B, Smit J, de Jongste J, Neijens H, Gerritsen J, Postma D, et al. The prevention and incidence of asthma and mite allergy (PIAMA) birth cohort study: design and first results. Pediatr Allergy Immunol. 2002;13 Suppl 15:55-60.
24. Koopman LP, Brunekreef B, de Jongste JC, Neijens HJ. Definition of respiratory symptoms and disease in early childhood in large prospective birth cohort studies that predict the development of asthma. Pediatr Allergy Immunol. 2001 Jun;12(3):118-24.
25. Sistek D, Tschopp JM, Schindler C, Brutsche M, Ackermann-Liebrich U, Perruchoud AP, et al. Clinical diagnosis of current asthma: predictive value of respiratory symptoms in the SAPALDIA study. Swiss Study on Air Pollution and Lung Diseases in Adults. Eur Respir J. 2001 Feb;17(2):214-9.
26. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Lancet. 1998 Apr 25;351(9111):1225-32.
27. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet. 2006 Aug 26;368(9537):733-43.
28. Silverman M. Clinical Diagnosis and Assessment in Infants. . Philadelphia: Lipincott-Raven. 1997.
29. Baker D, Henderson J. Differences between infants and adults in the social aetiology of wheeze. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. Journal of epidemiology and community health. 1999 Oct;53(10):636-42.
30. Dodge R, Martinez FD, Cline MG, Lebowitz MD, Burrows B. Early childhood respiratory symptoms and the subsequent diagnosis of asthma. J Allergy Clin Immunol. 1996 Jul;98(1):48-54.
31. Bisgaard H, Szefler S. Prevalence of asthma-like symptoms in young children. Pediatr Pulmonol. 2007 Aug;42(8):723-8.
32. Caudri D, Wijga A, Gehring U, Smit HA, Brunekreef B, Kerkhof M, et al. Respiratory symptoms in the first 7 years of life and birth weight at term: the PIAMA Birth Cohort. Am J Respir Crit Care Med. 2007 May 15;175(10):1078-85.
33. Schonberger HJ, Dompeling E, Knottnerus JA, Maas T, Muris JW, van Weel C, et al. The PREVASC study: the clinical effect of a multifaceted educational intervention to prevent childhood asthma. Eur Respir J. 2005 Apr;25(4):660-70.
34. Strippoli MP, Silverman M, Michel G, Kuehni CE. A parent-completed respiratory questionnaire for 1-year-old children: repeatability. Arch Dis Child. 2007 Oct;92(10):861-5.
35. Luyt DK, Burton PR, Simpson H. Epidemiological study of wheeze, doctor diagnosed asthma, and cough in preschool children in Leicestershire. Bmj. 1993 May 22;306(6889):1386-90.
36. Kuehni CE, Davis A, Brooke AM, Silverman M. Are all wheezing disorders in very young (preschool) children increasing in prevalence? Lancet. 2001 Jun 9;357(9271):1821-5.
37. Sly PD, Tepper R, Henschen M, Gappa M, Stocks J. Tidal forced expirations. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/American Thoracic Society. Eur Respir J. 2000 Oct;16(4):741-8.
38. Stocks J, Godfrey S, Beardsmore C, Bar-Yishay E, Castile R. Plethysmographic measurements of lung volume and airway resistance. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/ American Thoracic Society. Eur Respir J. 2001 Feb;17(2):302-12.
39. Wildhaber JH, Hall GL, Stick SM. Measurements of exhaled nitric oxide with the single-breath technique and positive expiratory pressure in infants. Am J Respir Crit Care Med. 1999 Jan;159(1):74-8.
40. von Mutius E. Gene-environment interactions in asthma. J Allergy Clin Immunol. 2009 Jan;123(1):3-11; quiz 2-3.

41. Guerra S, Martinez FD. Asthma genetics: from linear to multifactorial approaches. Annual review of medicine. 2008;59:327-41.
42. Global Initiative for Asthma. www.ginasthma.com. 2008.
43. Vercelli D. Discovering susceptibility genes for asthma and allergy. Nature reviews. 2008 Mar;8(3):169-82.
44. Zhang J, Pare PD, Sandford AJ. Recent advances in asthma genetics. Respir Res. 2008;9:4.
45. Ober C, Hoffjan S. Asthma genetics 2006: the long and winding road to gene discovery. Genes and immunity. 2006 Mar;7(2):95-100.
46. Vercelli D. Advances in asthma and allergy genetics in 2007. J Allergy Clin Immunol. 2008 Aug;122(2):267-71.
47. Hersh CP, Raby BA, Soto-Quiros ME, Murphy AJ, Avila L, Lasky-Su J, et al. Comprehensive testing of positionally cloned asthma genes in two populations. Am J Respir Crit Care Med. 2007 Nov 1;176(9):849-57.
48. Hjern A, Haglund B, Hedlin G. Ethnicity, childhood environment and atopic disorder. Clin Exp Allergy. 2000 Apr;30(4):521-8.
49. Hunninghake GM, Weiss ST, Celedon JC. Asthma in Hispanics. Am J Respir Crit Care Med. 2006 Jan 15;173(2):143-63.
50. Kabesch M, Schaal W, Nicolai T, von Mutius E. Lower prevalence of asthma and atopy in Turkish children living in Germany. Eur Respir J. 1999 Mar;13(3):577-82.
51. Kuehni CE, Strippoli MP, Low N, Silverman M. Asthma in young south Asian women living in the United Kingdom: the importance of early life. Clin Exp Allergy. 2007 Jan;37(1):47-53.
52. Drake KA, Galanter JM, Burchard EG. Race, ethnicity and social class and the complex etiologies of asthma. Pharmacogenomics. 2008 Apr;9(4):453-62.
53. Netuveli G, Hurwitz B, Levy M, Fletcher M, Barnes G, Durham SR, et al. Ethnic variations in UK asthma frequency, morbidity, and health-service use: a systematic review and meta-analysis. Lancet. 2005 Jan 22-28;365(9456):312-7.
54. Rottem M, Szyper-Kravitz M, Shoenfeld Y. Atopy and asthma in migrants. International archives of allergy and immunology. 2005 Feb;136(2):198-204.
55. Gruber C, Illi S, Plieth A, Sommerfeld C, Wahn U. Cultural adaptation is associated with atopy and wheezing among children of Turkish origin living in Germany. Clin Exp Allergy. 2002 Apr;32(4):526-31.
56. Leung RC, Carlin JB, Burdon JG, Czarny D. Asthma, allergy and atopy in Asian immigrants in Melbourne. Med J Aust. 1994 Oct 3;161(7):418-25.
57. Peat JK, Woolcock AJ, Leeder SR, Blackburn CR. Asthma and bronchitis in Sydney schoolchildren. II. The effect of social factors and smoking on prevalence. American journal of epidemiology. 1980 Jun;111(6):728-35.
58. Gibson PG, Henry RL, Shah S, Powell H, Wang H. Migration to a western country increases asthma symptoms but not eosinophilic airway inflammation. Pediatr Pulmonol. 2003 Sep;36(3):209-15.
59. Powell CV, Nolan TM, Carlin JB, Bennett CM, Johnson PD. Respiratory symptoms and duration of residence in immigrant teenagers living in Melbourne, Australia. Arch Dis Child. 1999 Aug;81(2):159-62.
60. Grant EN, Lyttle CS, Weiss KB. The relation of socioeconomic factors and racial/ethnic differences in US asthma mortality. American journal of public health. 2000 Dec;90(12):1923-5.
61. Joseph CL, Ownby DR, Peterson EL, Johnson CC. Racial differences in physiologic parameters related to asthma among middle-class children. Chest. 2000 May;117(5):1336-44.
62. Shapiro GG, Stout JW. Childhood asthma in the United States: urban issues. Pediatr Pulmonol. 2002 Jan;33(1):47-55.
63. Rose D, Mannino DM, Leaderer BP. Asthma prevalence among US adults, 1998-2000: role of Puerto Rican ethnicity and behavioral and geographic factors. American journal of public health. 2006 May;96(5):880-8.
64. Eldeirawi KM, Persky VW. Associations of acculturation and country of birth with asthma and wheezing in Mexican American youths. J Asthma. 2006 May;43(4):279-86.
65. Brim SN, Rudd RA, Funk RH, Callahan DB. Asthma prevalence among US children in underrepresented minority populations: American Indian/Alaska Native, Chinese, Filipino, and Asian Indian. Pediatrics. 2008 Jul;122(1):e217-22.
66. Kuehni CE, Strippoli MP, Low N, Brooke AM, Silverman M. Wheeze and asthma prevalence and related health-service use in white and south Asian pre-schoolchildren in the United Kingdom. Clin Exp Allergy. 2007 Dec;37(12):1738-46.
67. Koopman LP, Wijga A, Smit HA, De Jongste JC, Kerkhof M, Gerritsen J, et al. Early respiratory and skin symptoms in relation to ethnic background: the importance of socioeconomic status; the PIAMA study. Arch Dis Child. 2002 Dec;87(6):482-8.
68. Arshad SH, Tariq SM, Matthews S, Hakim E. Sensitization to common allergens and its association with allergic disorders at age 4 years:

a whole population birth cohort study. Pediatrics. 2001 Aug;108(2):E33.
69. Kabesch M, Schedel M, Carr D, Woitsch B, Fritzsch C, Weiland SK, et al. IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma. J Allergy Clin Immunol. 2006 Feb;117(2):269-74.
70. Nielsen GD, Hansen JS, Lund RM, Bergqvist M, Larsen ST, Clausen SK, et al. IgE-mediated asthma and rhinitis I: a role of allergen exposure? Pharmacology & toxicology. 2002 May;90(5):231-42.
71. Oettgen HC, Geha RS. IgE regulation and roles in asthma pathogenesis. J Allergy Clin Immunol. 2001 Mar;107(3):429-40.
72. Arbes SJ, Jr., Gergen PJ, Vaughn B, Zeldin DC. Asthma cases attributable to atopy: results from the Third National Health and Nutrition Examination Survey. J Allergy Clin Immunol. 2007 Nov;120(5):1139-45.
73. Lau S, Illi S, Sommerfeld C, Niggemann B, Bergmann R, von Mutius E, et al. Early exposure to house-dust mite and cat allergens and development of childhood asthma: a cohort study. Multicentre Allergy Study Group. Lancet. 2000 Oct 21;356(9239):1392-7.
74. Platts-Mills T, Vaughan J, Squillace S, Woodfolk J, Sporik R. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. Lancet. 2001 Mar 10;357(9258):752-6.
75. Pearce N, Pekkanen J, Beasley R. How much asthma is really attributable to atopy? Thorax. 1999 Mar;54(3):268-72.
76. Isaacs SL, Schroeder SA. Class - the ignored determinant of the nation's health. N Engl J Med. 2004 Sep 9;351(11):1137-42.
77. Blanc PD, Yen IH, Chen H, Katz PP, Earnest G, Balmes JR, et al. Area-level socio-economic status and health status among adults with asthma and rhinitis. Eur Respir J. 2006 Jan;27(1):85-94.
78. Keeley DJ, Neill P, Gallivan S. Comparison of the prevalence of reversible airways obstruction in rural and urban Zimbabwean children. Thorax. 1991 Aug;46(8):549-53.
79. Lewis SA, Britton JR. Consistent effects of high socioeconomic status and low birth order, and the modifying effect of maternal smoking on the risk of allergic disease during childhood. Respiratory medicine. 1998 Oct;92(10):1237-44.
80. Cesaroni G, Farchi S, Davoli M, Forastiere F, Perucci CA. Individual and area-based indicators of socioeconomic status and childhood asthma. Eur Respir J. 2003 Oct;22(4):619-24.
81. Eagan TM, Gulsvik A, Eide GE, Bakke PS. The effect of educational level on the incidence of asthma and respiratory symptoms. Respiratory medicine. 2004 Aug;98(8):730-6.
82. Gold DR, Wright R. Population disparities in asthma. Annual review of public health. 2005;26:89-113.
83. Lindbaek M, Wefring KW, Grangard EH, Ovsthus K. Socioeconomical conditions as risk factors for bronchial asthma in children aged 4-5 yrs. Eur Respir J. 2003 Jan;21(1):105-8.
84. Rona RJ. Asthma and poverty. Thorax. 2000 Mar;55(3):239-44.
85. Hedlund U, Eriksson K, Ronmark E. Socio-economic status is related to incidence of asthma and respiratory symptoms in adults. Eur Respir J. 2006 Aug;28(2):303-10.
86. Duran-Tauleria E, Rona RJ. Geographical and socioeconomic variation in the prevalence of asthma symptoms in English and Scottish children. Thorax. 1999 Jun;54(6):476-81.
87. da Cunha SS, Pujades-Rodriguez M, Barreto ML, Genser B, Rodrigues LC. Ecological study of socio-economic indicators and prevalence of asthma in schoolchildren in urban Brazil. BMC public health. 2007;7:205.
88. Babin SM, Burkom HS, Holtry RS, Tabernero NR, Stokes LD, Davies-Cole JO, et al. Pediatric patient asthma-related emergency department visits and admissions in Washington, DC, from 2001-2004, and associations with air quality, socio-economic status and age group. Environ Health. 2007;6:9.
89. McConnochie KM, Russo MJ, McBride JT, Szilagyi PG, Brooks AM, Roghmann KJ. Socioeconomic variation in asthma hospitalization: excess utilization or greater need? Pediatrics. 1999 Jun;103(6):e75.
90. Strachan DP, Anderson HR, Limb ES, O'Neill A, Wells N. A national survey of asthma prevalence, severity, and treatment in Great Britain. Arch Dis Child. 1994 Mar;70(3):174-8.
91. Bush PG, Mayhew TM, Abramovich DR, Aggett PJ, Burke MD, Page KR. Maternal cigarette smoking and oxygen diffusion across the placenta. Placenta. 2000 Nov;21(8):824-33.
92. Stathis SL, O'Callaghan DM, Williams GM, Najman JM, Andersen MJ, Bor W. Maternal cigarette smoking during pregnancy is an independent predictor for symptoms of middle ear disease at five years' postdelivery. Pediatrics. 1999 Aug;104(2):e16.
93. Weitzman M, Byrd RS, Aligne CA, Moss M. The effects of tobacco exposure on children's behavioral and cognitive functioning: implications for clinical and public health policy and future research. Neurotoxicology and teratology. 2002 May-Jun;24(3):397-406.

94. Wisborg K, Kesmodel U, Henriksen TB, Olsen SF, Secher NJ. A prospective study of smoking during pregnancy and SIDS. Arch Dis Child. 2000 Sep;83(3):203-6.
95. Sheikh S, Goldsmith LJ, Howell L, Parry L, Eid N. Comparison of lung function in infants exposed to maternal smoking and in infants with a family history of asthma. Chest. 1999 Jul;116(1):52-8.
96. Wang L, Pinkerton KE. Detrimental effects of tobacco smoke exposure during development on postnatal lung function and asthma. Birth Defects Res C Embryo Today. 2008 Mar;84(1):54-60.
97. Gilliland FD, Berhane K, McConnell R, Gauderman WJ, Vora H, Rappaport EB, et al. Maternal smoking during pregnancy, environmental tobacco smoke exposure and childhood lung function. Thorax. 2000 Apr;55(4):271-6.
98. Li YF, Gilliland FD, Berhane K, McConnell R, Gauderman WJ, Rappaport EB, et al. Effects of in utero and environmental tobacco smoke exposure on lung function in boys and girls with and without asthma. Am J Respir Crit Care Med. 2000 Dec;162(6):2097-104.
99. Gilliland FD, Berhane K, Li YF, Rappaport EB, Peters JM. Effects of early onset asthma and in utero exposure to maternal smoking on childhood lung function. Am J Respir Crit Care Med. 2003 Mar 15;167(6):917-24.
100. Moshammer H, Hoek G, Luttmann-Gibson H, Neuberger MA, Antova T, Gehring U, et al. Parental smoking and lung function in children: an international study. Am J Respir Crit Care Med. 2006 Jun 1;173(11):1255-63.
101. Martinez FD, Morgan WJ, Wright AL, Holberg CJ, Taussig LM. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. N Engl J Med. 1988 Oct 27;319(17):1112-7.
102. Haberg SE, Stigum H, Nystad W, Nafstad P. Effects of pre- and postnatal exposure to parental smoking on early childhood respiratory health. American journal of epidemiology. 2007 Sep 15;166(6):679-86.
103. Strachan DP, Cook DG. Health effects of passive smoking. 6. Parental smoking and childhood asthma: longitudinal and case-control studies. Thorax. 1998 Mar;53(3):204-12.
104. Noakes PS, Hale J, Thomas R, Lane C, Devadason SG, Prescott SL. Maternal smoking is associated with impaired neonatal toll-like-receptor-mediated immune responses. Eur Respir J. 2006 Oct;28(4):721-9.
105. Prescott SL. Effects of early cigarette smoke exposure on early immune development and respiratory disease. Paediatric respiratory reviews. 2008 Mar;9(1):3-9; quiz 10.
106. Nouri-Shirazi M, Guinet E. Evidence for the immunosuppressive role of nicotine on human dendritic cell functions. Immunology. 2003 Jul;109(3):365-73.
107. de la Chica RA, Ribas I, Giraldo J, Egozcue J, Fuster C. Chromosomal instability in amniocytes from fetuses of mothers who smoke. Jama. 2005 Mar 9;293(10):1212-22.
108. Macaubas C, de Klerk NH, Holt BJ, Wee C, Kendall G, Firth M, et al. Association between antenatal cytokine production and the development of atopy and asthma at age 6 years. Lancet. 2003 Oct 11;362(9391):1192-7.
109. Utsugi M, Dobashi K, Ishizuka T, Endou K, Hamuro J, Murata Y, et al. c-Jun N-terminal kinase negatively regulates lipopolysaccharide-induced IL-12 production in human macrophages: role of mitogen-activated protein kinase in glutathione redox regulation of IL-12 production. J Immunol. 2003 Jul 15;171(2):628-35.
110. Strachan DP, Cook DG. Health effects of passive smoking .5. Parental smoking and allergic sensitisation in children. Thorax. 1998 Feb;53(2):117-23.
111. Kulig M, Luck W, Lau S, Niggemann B, Bergmann R, Klettke U, et al. Effect of pre- and postnatal tobacco smoke exposure on specific sensitization to food and inhalant allergens during the first 3 years of life. Multicenter Allergy Study Group, Germany. Allergy. 1999 Mar;54(3):220-8.
112. Adriaanse HP, Knottnerus JA, Delgado LR, Cox HH, Essed GG. Smoking in Dutch pregnant women and birth weight. Patient education and counseling. 1996 Jun;28(1):25-30.
113. Hannover W, Thyrian JR, Ebner A, Roske K, Grempler J, Kuhl R, et al. Smoking during pregnancy and postpartum: smoking rates and intention to quit smoking or resume after pregnancy. Journal of women's health (2002). 2008 May;17(4):631-40.
114. Hofhuis W, de Jongste JC, Merkus PJ. Adverse health effects of prenatal and postnatal tobacco smoke exposure on children. Arch Dis Child. 2003 Dec;88(12):1086-90.
115. Strachan DP. Hay fever, hygiene, and household size. Bmj. 1989 Nov 18;299(6710):1259-60.
116. Krug N, Jung T, Napp U, Wagner K, Schultze-Werninghaus G, Heusser C, et al. Frequencies of T cells expressing interleukin-4 and interleukin-5 in atopic asthmatic children. Comparison with atopic asthmatic adults. Am J Respir Crit Care Med. 1998 Sep;158(3):754-9.

117. Martinez FD, Stern DA, Wright AL, Holberg CJ, Taussig LM, Halonen M. Association of interleukin-2 and interferon-gamma production by blood mononuclear cells in infancy with parental allergy skin tests and with subsequent development of atopy. J Allergy Clin Immunol. 1995 Nov;96(5 Pt 1):652-60.
118. Romagnani S. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. International archives of allergy and immunology. 1992;98(4):279-85.
119. Forastiere F, Agabiti N, Corbo GM, Dell'Orco V, Porta D, Pistelli R, et al. Socioeconomic status, number of siblings, and respiratory infections in early life as determinants of atopy in children. Epidemiology (Cambridge, Mass. 1997 Sep;8(5):566-70.
120. Strachan DP, Harkins LS, Johnston ID, Anderson HR. Childhood antecedents of allergic sensitization in young British adults. J Allergy Clin Immunol. 1997 Jan;99(1 Pt 1):6-12.
121. Strachan DP, Taylor EM, Carpenter RG. Family structure, neonatal infection, and hay fever in adolescence. Arch Dis Child. 1996 May;74(5):422-6.
122. von Mutius E, Martinez FD, Fritzsch C, Nicolai T, Reitmeir P, Thiemann HH. Skin test reactivity and number of siblings. Bmj. 1994 Mar 12;308(6930):692-5.
123. Jarvis D, Chinn S, Luczynska C, Burney P. The association of family size with atopy and atopic disease. Clin Exp Allergy. 1997 Mar;27(3):240-5.
124. Matricardi PM, Franzinelli F, Franco A, Caprio G, Murru F, Cioffi D, et al. Sibship size, birth order, and atopy in 11,371 Italian young men. J Allergy Clin Immunol. 1998 Apr;101(4 Pt 1):439-44.
125. Svanes C, Jarvis D, Chinn S, Burney P. Childhood environment and adult atopy: results from the European Community Respiratory Health Survey. J Allergy Clin Immunol. 1999 Mar;103(3 Pt 1):415-20.
126. von Mutius E, Fritzsch C, Weiland SK, Roll G, Magnussen H. Prevalence of asthma and allergic disorders among children in united Germany: a descriptive comparison. Bmj. 1992 Dec 5;305(6866):1395-9.
127. von Mutius E, Martinez FD, Fritzsch C, Nicolai T, Roell G, Thiemann HH. Prevalence of asthma and atopy in two areas of West and East Germany. Am J Respir Crit Care Med. 1994 Feb;149(2 Pt 1):358-64.
128. von Mutius E, Weiland SK, Fritzsch C, Duhme H, Keil U. Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. Lancet. 1998 Mar 21;351(9106):862-6.
129. Infante-Rivard C, Amre D, Gautrin D, Malo JL. Family size, day-care attendance, and breastfeeding in relation to the incidence of childhood asthma. American journal of epidemiology. 2001 Apr 1;153(7):653-8.
130. Wickens KL, Crane J, Kemp TJ, Lewis SJ, D'Souza WJ, Sawyer GM, et al. Family size, infections, and asthma prevalence in New Zealand children. Epidemiology (Cambridge, Mass. 1999 Nov;10(6):699-705.
131. Wickens K, Crane J, Pearce N, Beasley R. The magnitude of the effect of smaller family sizes on the increase in the prevalence of asthma and hay fever in the United Kingdom and New Zealand. J Allergy Clin Immunol. 1999 Sep;104(3 Pt 1):554-8.
132. Goldberg S, Israeli E, Schwartz S, Shochat T, Izbicki G, Toker-Maimon O, et al. Asthma prevalence, family size, and birth order. Chest. 2007 Jun;131(6):1747-52.
133. Celedon JC, Wright RJ, Litonjua AA, Sredl D, Ryan L, Weiss ST, et al. Day care attendance in early life, maternal history of asthma, and asthma at the age of 6 years. Am J Respir Crit Care Med. 2003 May 1;167(9):1239-43.
134. Koopman LP, Smit HA, Heijnen ML, Wijga A, van Strien RT, Kerkhof M, et al. Respiratory infections in infants: interaction of parental allergy, child care, and siblings-- The PIAMA study. Pediatrics. 2001 Oct;108(4):943-8.
135. Svanes C, Jarvis D, Chinn S, Omenaas E, Gulsvik A, Burney P. Early exposure to children in family and day care as related to adult asthma and hay fever: results from the European Community Respiratory Health Survey. Thorax. 2002 Nov;57(11):945-50.
136. Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. N Engl J Med. 2000 Aug 24;343(8):538-43.
137. Celedon JC, Litonjua AA, Ryan L, Weiss ST, Gold DR. Day care attendance, respiratory tract illnesses, wheezing, asthma, and total serum IgE level in early childhood. Arch Pediatr Adolesc Med. 2002 Mar;156(3):241-5.
138. von Mutius E. Pro: the increase in asthma can be ascribed to cleanliness. Am J Respir Crit Care Med. 2001 Oct 1;164(7):1106-7; discussion 8-9.
139. Kramer U, Heinrich J, Wjst M, Wichmann HE. Age of entry to day nursery and allergy in later childhood. Lancet. 1999 Feb 6;353(9151):450-4.
140. von Mutius E. Infection: friend or foe in the development of atopy and asthma? The epidemiological evidence. Eur Respir J. 2001 Nov;18(5):872-81.

141. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet. 1999 Aug 14;354(9178):541-5.
142. Illi S, von Mutius E, Lau S, Bergmann R, Niggemann B, Sommerfeld C, et al. Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. Bmj. 2001 Feb 17;322(7283):390-5.
143. Matricardi PM, Rosmini F, Ferrigno L, Nisini R, Rapicetta M, Chionne P, et al. Cross sectional retrospective study of prevalence of atopy among Italian military students with antibodies against hepatitis A virus. Bmj. 1997 Apr 5;314(7086):999-1003.
144. Matricardi PM, Rosmini F, Riondino S, Fortini M, Ferrigno L, Rapicetta M, et al. Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. Bmj. 2000 Feb 12;320(7232):412-7.
145. Shaheen SO, Aaby P, Hall AJ, Barker DJ, Heyes CB, Shiell AW, et al. Measles and atopy in Guinea-Bissau. Lancet. 1996 Jun 29;347(9018):1792-6.
146. Leonardi-Bee J, Pritchard D, Britton J. Asthma and current intestinal parasite infection: systematic review and meta-analysis. Am J Respir Crit Care Med. 2006 Sep 1;174(5):514-23.
147. Cooper PJ, Chico ME, Rodrigues LC, Ordonez M, Strachan D, Griffin GE, et al. Reduced risk of atopy among school-age children infected with geohelminth parasites in a rural area of the tropics. J Allergy Clin Immunol. 2003 May;111(5):995-1000.
148. Pereira MU, Sly PD, Pitrez PM, Jones MH, Escouto D, Dias AC, et al. Nonatopic asthma is associated with helminth infections and bronchiolitis in poor children. Eur Respir J. 2007 Jun;29(6):1154-60.
149. van den Biggelaar AH, van Ree R, Rodrigues LC, Lell B, Deelder AM, Kremsner PG, et al. Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10. Lancet. 2000 Nov 18;356(9243):1723-7.
150. Scrivener S, Yemaneberhan H, Zebenigus M, Tilahun D, Girma S, Ali S, et al. Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study. Lancet. 2001 Nov 3;358(9292):1493-9.
151. Dagoye D, Bekele Z, Woldemichael K, Nida H, Yimam M, Hall A, et al. Wheezing, allergy, and parasite infection in children in urban and rural Ethiopia. Am J Respir Crit Care Med. 2003 May 15;167(10):1369-73.
152. Medeiros M, Jr., Figueiredo JP, Almeida MC, Matos MA, Araujo MI, Cruz AA, et al. Schistosoma mansoni infection is associated with a reduced course of asthma. J Allergy Clin Immunol. 2003 May;111(5):947-51.
153. Yazdanbakhsh M, Wahyuni S. The role of helminth infections in protection from atopic disorders. Curr Opin Allergy Clin Immunol. 2005 Oct;5(5):386-91.
154. Holt PG. Environmental factors and primary T-cell sensitisation to inhalant allergens in infancy: reappraisal of the role of infections and air pollution. Pediatr Allergy Immunol. 1995 Feb;6(1):1-10.
155. Holt PG, Sly PD, Bjorksten B. Atopic versus infectious diseases in childhood: a question of balance? Pediatr Allergy Immunol. 1997 May;8(2):53-8.
156. Holt PG, Macaubas C. Development of long-term tolerance versus sensitisation to environmental allergens during the perinatal period. Current opinion in immunology. 1997 Dec;9(6):782-7.
157. Adlerberth I, Wold AE. Establishment of the gut microbiota in Western infants. Acta Paediatr. 2009 Feb;98(2):229-38.
158. Macdonald TT, Monteleone G. Immunity, inflammation, and allergy in the gut. Science (New York, NY. 2005 Mar 25;307(5717):1920-5.
159. Vighi G, Marcucci F, Sensi L, Di Cara G, Frati F. Allergy and the gastrointestinal system. Clinical and experimental immunology. 2008 Sep;153 Suppl 1:3-6.
160. Sepp E, Julge K, Vasar M, Naaber P, Bjorksten B, Mikelsaar M. Intestinal microflora of Estonian and Swedish infants. Acta Paediatr. 1997 Sep;86(9):956-61.
161. Bjorksten B. Environmental influence on the development of childhood immunity. Nutrition reviews. 1998 Jan;56(1 Pt 2):S106-12.
162. Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol. 2001 Oct;108(4):516-20.
163. Mah KW, Bjorksten B, Lee BW, van Bever HP, Shek LP, Tan TN, et al. Distinct pattern of commensal gut microbiota in toddlers with eczema. International archives of allergy and immunology. 2006;140(2):157-63.
164. Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. Journal of gastroenterology. 2009;44(1):26-46.
165. Heller F, Duchmann R. Intestinal flora and mucosal immune responses. Int J Med Microbiol. 2003 Apr;293(1):77-86.

166. Askenasy N, Kaminitz A, Yarkoni S. Mechanisms of T regulatory cell function. Autoimmunity reviews. 2008 May;7(5):370-5.
167. Mantovani A, Garlanda C, Locati M, Rodriguez TV, Feo SG, Savino B, et al. Regulatory pathways in inflammation. Autoimmunity reviews. 2007 Nov;7(1):8-11.
168. Vliagoftis H, Kouranos VD, Betsi GI, Falagas ME. Probiotics for the treatment of allergic rhinitis and asthma: systematic review of randomized controlled trials. Ann Allergy Asthma Immunol. 2008 Dec;101(6):570-9.
169. Osborn DA, Sinn JK. Probiotics in infants for prevention of allergic disease and food hypersensitivity. Cochrane Database Syst Rev. 2007(4):CD006475.
170. Majamaa H, Isolauri E. Probiotics: a novel approach in the management of food allergy. J Allergy Clin Immunol. 1997 Feb;99(2):179-85.
171. Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. Clin Exp Allergy. 2000 Nov;30(11):1604-10.
172. Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH, et al. Effect of probiotic Lactobacillus strains in children with atopic dermatitis. J Allergy Clin Immunol. 2003 Feb;111(2):389-95.
173. Viljanen M, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, et al. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. Allergy. 2005 Apr;60(4):494-500.
174. Murray CS, Tannock GW, Simon MA, Harmsen HJ, Welling GW, Custovic A, et al. Fecal microbiota in sensitized wheezy and non-sensitized non-wheezy children: a nested case-control study. Clin Exp Allergy. 2005 Jun;35(6):741-5.
175. Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. Lancet. 2001 Apr 7;357(9262):1076-9.
176. von Mutius E, Braun-Fahrlander C, Schierl R, Riedler J, Ehlermann S, Maisch S, et al. Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin Exp Allergy. 2000 Sep;30(9):1230-4.
177. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. Nature reviews. 2003 Feb;3(2):169-76.
178. Martinez FD. CD14, endotoxin, and asthma risk: actions and interactions. Proceedings of the American Thoracic Society. 2007 Jul;4(3):221-5.
179. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. J Allergy Clin Immunol. 2004 Mar;113(3):482-8.
180. Martinez FD. Maturation of immune responses at the beginning of asthma. J Allergy Clin Immunol. 1999 Mar;103(3 Pt 1):355-61.
181. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol. 1995 May 15;154(10):5071-9.
182. Herz U, Lacy P, Renz H, Erb K. The influence of infections on the development and severity of allergic disorders. Current opinion in immunology. 2000 Dec;12(6):632-40.
183. Braun-Fahrlander C, Lauener R. Farming and protective agents against allergy and asthma. Clin Exp Allergy. 2003 Apr;33(4):409-11.
184. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet. 2001 Oct 6;358(9288):1129-33.
185. Douwes J, Travier N, Huang K, Cheng S, McKenzie J, Le Gros G, et al. Lifelong farm exposure may strongly reduce the risk of asthma in adults. Allergy. 2007 Oct;62(10):1158-65.
186. Douwes J, Cheng S, Travier N, Cohet C, Niesink A, McKenzie J, et al. Farm exposure in utero may protect against asthma, hay fever and eczema. Eur Respir J. 2008 Sep;32(3):603-11.
187. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med. 2002 Sep 19;347(12):869-77.
188. Celedon JC, Milton DK, Ramsey CD, Litonjua AA, Ryan L, Platts-Mills TA, et al. Exposure to dust mite allergen and endotoxin in early life and asthma and atopy in childhood. J Allergy Clin Immunol. 2007 Jul;120(1):144-9.
189. Gereda JE, Leung DY, Thatayatikom A, Streib JE, Price MR, Klinnert MD, et al. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet. 2000 May 13;355(9216):1680-3.
190. Gehring U, Bischof W, Schlenvoigt G, Richter K, Fahlbusch B, Wichmann HE, et al. Exposure to house dust endotoxin and allergic sensitization in adults. Allergy. 2004 Sep;59(9):946-52.
191. Douwes J, van Strien R, Doekes G, Smit J, Kerkhof M, Gerritsen J, et al. Does early indoor microbial exposure reduce the risk of asthma? The Prevention and Incidence of Asthma and

Mite Allergy birth cohort study. J Allergy Clin Immunol. 2006 May;117(5):1067-73.
192. Gehring U, Bolte G, Borte M, Bischof W, Fahlbusch B, Wichmann HE, et al. Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. J Allergy Clin Immunol. 2001 Nov;108(5):847-54.
193. Park JH, Gold DR, Spiegelman DL, Burge HA, Milton DK. House dust endotoxin and wheeze in the first year of life. Am J Respir Crit Care Med. 2001 Feb;163(2):322-8.
194. Litonjua AA, Milton DK, Celedon JC, Ryan L, Weiss ST, Gold DR. A longitudinal analysis of wheezing in young children: the independent effects of early life exposure to house dust endotoxin, allergens, and pets. J Allergy Clin Immunol. 2002 Nov;110(5):736-42.
195. van Strien RT, Engel R, Holst O, Bufe A, Eder W, Waser M, et al. Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health. J Allergy Clin Immunol. 2004 May;113(5):860-7.
196. Eder W, von Mutius E. Hygiene hypothesis and endotoxin: what is the evidence? Current opinion in allergy and clinical immunology. 2004 Apr;4(2):113-7.
197. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, et al. Childhood asthma after bacterial colonization of the airway in neonates. N Engl J Med. 2007 Oct 11;357(15):1487-95.
198. Vercelli D, Baldini M, Martinez F. The monocyte/IgE connection: may polymorphisms in the CD14 gene teach us about IgE regulation? International archives of allergy and immunology. 2001 Jan-Mar;124(1-3):20-4.
199. Ober C, Tsalenko A, Parry R, Cox NJ. A second-generation genomewide screen for asthma-susceptibility alleles in a founder population. American journal of human genetics. 2000 Nov;67(5):1154-62.
200. Kedda MA, Lose F, Duffy D, Bell E, Thompson PJ, Upham J. The CD14 C-159T polymorphism is not associated with asthma or asthma severity in an Australian adult population. Thorax. 2005 Mar;60(3):211-4.
201. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, et al. Opposite effects of CD 14/-260 on serum IgE levels in children raised in different environments. J Allergy Clin Immunol. 2005 Sep;116(3):601-7.
202. Smit LA, Siroux V, Bouzigon E, Oryszczyn MP, Lathrop M, Demenais F, et al. CD14 and toll-like receptor gene polymorphisms, country living, and asthma in adults. Am J Respir Crit Care Med. 2009 Mar 1;179(5):363-8.
203. Bouzigon E, Corda E, Aschard H, Dizier MH, Boland A, Bousquet J, et al. Effect of 17q21 variants and smoking exposure in early-onset asthma. N Engl J Med. 2008 Nov 6;359(19):1985-94.
204. Farooqi IS, Hopkin JM. Early childhood infection and atopic disorder. Thorax. 1998 Nov;53(11):927-32.
205. Wickens K, Pearce N, Crane J, Beasley R. Antibiotic use in early childhood and the development of asthma. Clin Exp Allergy. 1999 Jun;29(6):766-71.
206. Mattes J, Karmaus W. The use of antibiotics in the first year of life and development of asthma: which comes first? Clin Exp Allergy. 1999 Jun;29(6):729-32.
207. Alm JS, Lilja G, Pershagen G, Scheynius A. Early BCG vaccination and development of atopy. Lancet. 1997 Aug 9;350(9075):400-3.
208. Henderson J, North K, Griffiths M, Harvey I, Golding J. Pertussis vaccination and wheezing illnesses in young children: prospective cohort study. The Longitudinal Study of Pregnancy and Childhood Team. Bmj. 1999 May 1;318(7192):1173-6.
209. Nilsson L, Kjellman NI, Bjorksten B. A randomized controlled trial of the effect of pertussis vaccines on atopic disease. Arch Pediatr Adolesc Med. 1998 Aug;152(8):734-8.
210. Strannegard IL, Larsson LO, Wennergren G, Strannegard O. Prevalence of allergy in children in relation to prior BCG vaccination and infection with atypical mycobacteria. Allergy. 1998 Mar;53(3):249-54.
211. Allmers H. Frequent acetaminophen use and allergic diseases: is the association clear? J Allergy Clin Immunol. 2005 Oct;116(4):859-62.
212. Beasley R, Clayton T, Crane J, von Mutius E, Lai CK, Montefort S, et al. Association between paracetamol use in infancy and childhood, and risk of asthma, rhinoconjunctivitis, and eczema in children aged 6-7 years: analysis from Phase Three of the ISAAC programme. Lancet. 2008 Sep 20;372(9643):1039-48.
213. Churg A, Brauer M. Human lung parenchyma retains PM2.5. Am J Respir Crit Care Med. 1997 Jun;155(6):2109-11.
214. Pope CA, 3rd. Air pollution and health - good news and bad. N Engl J Med. 2004 Sep 9;351(11):1132-4.
215. Schwartz J. Air pollution and children's health. Pediatrics. 2004 Apr;113(4 Suppl):1037-43.
216. Tatum AJ, Shapiro GG. The effects of outdoor air pollution and tobacco smoke on asthma. Immunology and allergy clinics of North America. 2005 Feb;25(1):15-30.

217. Barnett AG, Williams GM, Schwartz J, Neller AH, Best TL, Petroeschevsky AL, et al. Air pollution and child respiratory health: a case-crossover study in Australia and New Zealand. Am J Respir Crit Care Med. 2005 Jun 1;171(11):1272-8.
218. Lee SL, Wong WH, Lau YL. Association between air pollution and asthma admission among children in Hong Kong. Clin Exp Allergy. 2006 Sep;36(9):1138-46.
219. O'Connor GT, Neas L, Vaughn B, Kattan M, Mitchell H, Crain EF, et al. Acute respiratory health effects of air pollution on children with asthma in US inner cities. J Allergy Clin Immunol. 2008 May;121(5):1133-9 e1.
220. Schildcrout JS, Sheppard L, Lumley T, Slaughter JC, Koenig JQ, Shapiro GG. Ambient air pollution and asthma exacerbations in children: an eight-city analysis. American journal of epidemiology. 2006 Sep 15;164(6):505-17.
221. Slaughter JC, Lumley T, Sheppard L, Koenig JQ, Shapiro GG. Effects of ambient air pollution on symptom severity and medication use in children with asthma. Ann Allergy Asthma Immunol. 2003 Oct;91(4):346-53.
222. Wilhelm M, Qian L, Ritz B. Outdoor air pollution, family and neighborhood environment, and asthma in LA FANS children. Health & place. 2009 Mar;15(1):25-36.
223. Health effects of outdoor air pollution. Committee of the Environmental and Occupational Health Assembly of the American Thoracic Society. Am J Respir Crit Care Med. 1996 Jan;153(1):3-50.
224. Delfino RJ, Staimer N, Tjoa T, Gillen D, Kleinman MT, Sioutas C, et al. Personal and ambient air pollution exposures and lung function decrements in children with asthma. Environ Health Perspect. 2008 Apr;116(4):550-8.
225. Mortimer KM, Neas LM, Dockery DW, Redline S, Tager IB. The effect of air pollution on inner-city children with asthma. Eur Respir J. 2002 Apr;19(4):699-705.
226. Gauderman WJ, Avol E, Gilliland F, Vora H, Thomas D, Berhane K, et al. The effect of air pollution on lung development from 10 to 18 years of age. N Engl J Med. 2004 Sep 9;351(11):1057-67.
227. Jedrychowski W, Flak E, Mroz E. The adverse effect of low levels of ambient air pollutants on lung function growth in preadolescent children. Environ Health Perspect. 1999 Aug;107(8):669-74.
228. Horak F, Jr., Studnicka M, Gartner C, Spengler JD, Tauber E, Urbanek R, et al. Particulate matter and lung function growth in children: a 3-yr follow-up study in Austrian schoolchildren. Eur Respir J. 2002 May;19(5):838-45.
229. Frischer T, Studnicka M, Gartner C, Tauber E, Horak F, Veiter A, et al. Lung function growth and ambient ozone: a three-year population study in school children. Am J Respir Crit Care Med. 1999 Aug;160(2):390-6.
230. Peters JM, Avol E, Gauderman WJ, Linn WS, Navidi W, London SJ, et al. A study of twelve Southern California communities with differing levels and types of air pollution. II. Effects on pulmonary function. Am J Respir Crit Care Med. 1999 Mar;159(3):768-75.
231. Raizenne M, Neas LM, Damokosh AI, Dockery DW, Spengler JD, Koutrakis P, et al. Health effects of acid aerosols on North American children: pulmonary function. Environ Health Perspect. 1996 May;104(5):506-14.
232. Schwartz J. Lung function and chronic exposure to air pollution: a cross-sectional analysis of NHANES II. Environmental research. 1989 Dec;50(2):309-21.
233. Dockery DW, Speizer FE, Stram DO, Ware JH, Spengler JD, Ferris BG, Jr. Effects of inhalable particles on respiratory health of children. Am Rev Respir Dis. 1989 Mar;139(3):587-94.
234. Ware JH, Ferris BG, Jr., Dockery DW, Spengler JD, Stram DO, Speizer FE. Effects of ambient sulfur oxides and suspended particles on respiratory health of preadolescent children. Am Rev Respir Dis. 1986 May;133(5):834-42.
235. Gauderman WJ, Gilliland GF, Vora H, Avol E, Stram D, McConnell R, et al. Association between air pollution and lung function growth in southern California children: results from a second cohort. Am J Respir Crit Care Med. 2002 Jul 1;166(1):76-84.
236. Avol EL, Gauderman WJ, Tan SM, London SJ, Peters JM. Respiratory effects of relocating to areas of differing air pollution levels. Am J Respir Crit Care Med. 2001 Dec 1;164(11):2067-72.
237. Gauderman WJ, McConnell R, Gilliland F, London S, Thomas D, Avol E, et al. Association between air pollution and lung function growth in southern California children. Am J Respir Crit Care Med. 2000 Oct;162(4 Pt 1):1383-90.
238. Hwang BF, Lee YL, Lin YC, Jaakkola JJ, Guo YL. Traffic related air pollution as a determinant of asthma among Taiwanese school children. Thorax. 2005 Jun;60(6):467-73.
239. Janssen NA, Brunekreef B, van Vliet P, Aarts F, Meliefste K, Harssema H, et al. The relationship between air pollution from heavy traffic and allergic sensitization, bronchial hyperresponsiveness, and respiratory symptoms in Dutch schoolchildren. Environ Health Perspect. 2003 Sep;111(12):1512-8.

240. Nicolai T, Carr D, Weiland SK, Duhme H, von Ehrenstein O, Wagner C, et al. Urban traffic and pollutant exposure related to respiratory outcomes and atopy in a large sample of children. Eur Respir J. 2003 Jun;21(6):956-63.
241. Oosterlee A, Drijver M, Lebret E, Brunekreef B. Chronic respiratory symptoms in children and adults living along streets with high traffic density. Occup Environ Med. 1996 Apr;53(4):241-7.
242. Preutthipan A, Udomsubpayakul U, Chaisupamongkollarp T, Pentamwa P. Effect of PM10 pollution in Bangkok on children with and without asthma. Pediatr Pulmonol. 2004 Mar;37(3):187-92.
243. van Vliet P, Knape M, de Hartog J, Janssen N, Harssema H, Brunekreef B. Motor vehicle exhaust and chronic respiratory symptoms in children living near freeways. Environmental research. 1997;74(2):122-32.
244. Barraza-Villarreal A, Sunyer J, Hernandez-Cadena L, Escamilla-Nunez MC, Sienra-Monge JJ, Ramirez-Aguilar M, et al. Air pollution, airway inflammation, and lung function in a cohort study of Mexico City schoolchildren. Environ Health Perspect. 2008 Jun;116(6):832-8.
245. Gehring U, Cyrys J, Sedlmeir G, Brunekreef B, Bellander T, Fischer P, et al. Traffic-related air pollution and respiratory health during the first 2 yrs of life. Eur Respir J. 2002 Apr;19(4):690-8.
246. Hirsch T, Weiland SK, von Mutius E, Safeca AF, Grafe H, Csaplovics E, et al. Inner city air pollution and respiratory health and atopy in children. Eur Respir J. 1999 Sep;14(3):669-77.
247. Thurston GD, Bates DV. Air pollution as an underappreciated cause of asthma symptoms. Jama. 2003 Oct 8;290(14):1915-7.
248. Zmirou D, Gauvin S, Pin I, Momas I, Sahraoui F, Just J, et al. Traffic related air pollution and incidence of childhood asthma: results of the Vesta case-control study. Journal of epidemiology and community health. 2004 Jan;58(1):18-23.
249. Andersen ZJ, Loft S, Ketzel M, Stage M, Scheike T, Hermansen MN, et al. Ambient air pollution triggers wheezing symptoms in infants. Thorax. 2008 Aug;63(8):710-6.
250. Brauer M, Hoek G, Van Vliet P, Meliefste K, Fischer PH, Wijga A, et al. Air pollution from traffic and the development of respiratory infections and asthmatic and allergic symptoms in children. Am J Respir Crit Care Med. 2002 Oct 15;166(8):1092-8.
251. Miller RL, Garfinkel R, Horton M, Camann D, Perera FP, Whyatt RM, et al. Polycyclic aromatic hydrocarbons, environmental tobacco smoke, and respiratory symptoms in an inner-city birth cohort. Chest. 2004 Oct;126(4):1071-8.
252. Jerrett M, Shankardass K, Berhane K, Gauderman WJ, Kunzli N, Avol E, et al. Traffic-related air pollution and asthma onset in children: a prospective cohort study with individual exposure measurement. Environ Health Perspect. 2008 Oct;116(10):1433-8.
253. Mortimer K, Neugebauer R, Lurmann F, Alcorn S, Balmes J, Tager I. Air pollution and pulmonary function in asthmatic children: effects of prenatal and lifetime exposures. Epidemiology (Cambridge, Mass. 2008 Jul;19(4):550-7; discussion 61-2.
254. Mortimer K, Neugebauer R, Lurmann F, Alcorn S, Balmes J, Tager I. Early-lifetime exposure to air pollution and allergic sensitization in children with asthma. J Asthma. 2008 Dec;45(10):874-81.
255. Latzin P, Roosli M, Huss A, Kuehni CE, Frey U. Air pollution during pregnancy and lung function in newborns: a birth cohort study. Eur Respir J. 2009 Mar;33(3):594-603.
256. Kramer U, Koch T, Ranft U, Ring J, Behrendt H. Traffic-related air pollution is associated with atopy in children living in urban areas. Epidemiology (Cambridge, Mass. 2000 Jan;11(1):64-70.
257. Delfino RJ, Staimer N, Gillen D, Tjoa T, Sioutas C, Fung K, et al. Personal and ambient air pollution is associated with increased exhaled nitric oxide in children with asthma. Environ Health Perspect. 2006 Nov;114(11):1736-43.
258. Adamkiewicz G, Ebelt S, Syring M, Slater J, Speizer FE, Schwartz J, et al. Association between air pollution exposure and exhaled nitric oxide in an elderly population. Thorax. 2004 Mar;59(3):204-9.
259. Steerenberg PA, Snelder JB, Fischer PH, Vos JG, van Loveren H, van Amsterdam JG. Increased exhaled nitric oxide on days with high outdoor air pollution is of endogenous origin. Eur Respir J. 1999 Feb;13(2):334-7.
260. Gustafsson LE, Leone AM, Persson MG, Wiklund NP, Moncada S. Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans. Biochemical and biophysical research communications. 1991 Dec 16;181(2):852-7.
261. Ricciardolo FL, Sterk PJ, Gaston B, Folkerts G. Nitric oxide in health and disease of the respiratory system. Physiological reviews. 2004 Jul;84(3):731-65.
262. Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. Am J Respir Crit Care Med. 2001 Jun;163(7):1693-722.

263. Gaston B, Drazen JM, Loscalzo J, Stamler JS. The biology of nitrogen oxides in the airways. Am J Respir Crit Care Med. 1994 Feb;149(2 Pt 1):538-51.
264. Morris SM, Jr., Billiar TR. New insights into the regulation of inducible nitric oxide synthesis. Am J Physiol. 1994 Jun;266(6 Pt 1):E829-39.
265. Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. Eur Respir J. 1993 Oct;6(9):1368-70.
266. Artlich A, Hagenah JU, Jonas S, Ahrens P, Gortner L. Exhaled nitric oxide in childhood asthma. Eur J Pediatr. 1996 Aug;155(8):698-701.
267. Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet. 1994 Jan 15;343(8890):133-5.
268. ATS/ERS Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005. Am J Respir Crit Care Med. 2005 Apr 15;171(8):912-30.
269. Buchvald F, Baraldi E, Carraro S, Gaston B, De Jongste J, Pijnenburg MW, et al. Measurements of exhaled nitric oxide in healthy subjects age 4 to 17 years. J Allergy Clin Immunol. 2005 Jun;115(6):1130-6.
270. Pijnenburg MW, Bakker EM, Hop WC, De Jongste JC. Titrating steroids on exhaled nitric oxide in children with asthma: a randomized controlled trial. Am J Respir Crit Care Med. 2005 Oct 1;172(7):831-6.
271. Smith AD, Cowan JO, Brassett KP, Herbison GP, Taylor DR. Use of exhaled nitric oxide measurements to guide treatment in chronic asthma. N Engl J Med. 2005 May 26;352(21):2163-73.
272. de Jongste JC, Carraro S, Hop WC, Baraldi E. Daily telemonitoring of exhaled nitric oxide and symptoms in the treatment of childhood asthma. Am J Respir Crit Care Med. 2009 Jan 15;179(2):93-7.
273. Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F. Intermittent inhaled corticosteroids in infants with episodic wheezing. N Engl J Med. 2006 May 11;354(19):1998-2005.
274. Tepper RS, Llapur CJ, Jones MH, Tiller C, Coates C, Kimmel R, et al. Expired nitric oxide and airway reactivity in infants at risk for asthma. J Allergy Clin Immunol. 2008 Oct;122(4):760-5.
275. Baraldi E, de Jongste JC. Measurement of exhaled nitric oxide in children, 2001. Eur Respir J. 2002 Jul;20(1):223-37.
276. Deykin A, Massaro AF, Drazen JM, Israel E. Exhaled nitric oxide as a diagnostic test for asthma: online versus offline techniques and effect of flow rate. Am J Respir Crit Care Med. 2002 Jun 15;165(12):1597-601.
277. Franklin PJ, Turner SW, Mutch RC, Stick SM. Measuring exhaled nitric oxide in infants during tidal breathing: methodological issues. Pediatr Pulmonol. 2004 Jan;37(1):24-30.
278. Ratjen F, Kavuk I, Gartig S, Wiesemann HG, Grasemann H. Airway nitric oxide in infants with acute wheezy bronchitis. Pediatr Allergy Immunol. 2000 Nov;11(4):230-5.
279. Frey U, Kuehni C, Roiha H, Cernelc M, Reinmann B, Wildhaber JH, et al. Maternal atopic disease modifies effects of prenatal risk factors on exhaled nitric oxide in infants. Am J Respir Crit Care Med. 2004 Aug 1;170(3):260-5.
280. Martinez T, Weist A, Williams T, Clem C, Silkoff P, Tepper RS. Assessment of exhaled nitric oxide kinetics in healthy infants. J Appl Physiol. 2003 Jun;94(6):2384-90.
281. Franklin PJ, Turner SW, Mutch RC, Stick SM. Comparison of single-breath and tidal breathing exhaled nitric oxide levels in infants. Eur Respir J. 2004 Mar;23(3):369-72.
282. Artlich A, Busch T, Lewandowski K, Jonas S, Gortner L, Falke KJ. Childhood asthma: exhaled nitric oxide in relation to clinical symptoms. Eur Respir J. 1999 Jun;13(6):1396-401.
283. Rutgers SR, Meijer RJ, Kerstjens HA, van der Mark TW, Koeter GH, Postma DS. Nitric oxide measured with single-breath and tidal-breathing methods in asthma and COPD. Eur Respir J. 1998 Oct;12(4):816-9.
284. Baraldi E, Azzolin NM, Dario C, Carra S, Ongaro R, Biban P, et al. Effect of atmospheric nitric oxide (NO) on measurements of exhaled NO in asthmatic children. Pediatr Pulmonol. 1998 Jul;26(1):30-4.
285. Stark H, Purokivi M, Kiviranta J, Randell J, Tukiainen H. Short-term and seasonal variations of exhaled and nasal NO in healthy subjects. Respir Med. 2006 Jun 21.
286. Schedin U, Norman M, Gustafsson LE, Jonsson B, Frostell C. Endogenous nitric oxide in the upper airways of premature and term infants. Acta Paediatr. 1997 Nov;86(11):1229-35.
287. Schedin U, Norman M, Gustafsson LE, Herin P, Frostell C. Endogenous nitric oxide in the upper airways of healthy newborn infants. Pediatr Res. 1996 Jul;40(1):148-51.
288. Artlich A, Jonsson B, Bhiladvala M, Lonnqvist PA, Gustafsson LE. Single breath analysis of endogenous nitric oxide in the newborn. Biol Neonate. 2001 Jan;79(1):21-6.
289. Baraldi E, Dario C, Ongaro R, Scollo M, Azzolin NM, Panza N, et al. Exhaled nitric oxide concentrations during treatment of wheezing exacerbation in infants and young children. Am J

Respir Crit Care Med. 1999 Apr;159(4 Pt 1):1284-8.
290. Olivieri M, Talamini G, Corradi M, Perbellini L, Mutti A, Tantucci C, et al. Reference values for exhaled nitric oxide (reveno) study. Respir Res. 2006;7:94.
291. Deykin A. Exhaled nitric oxide as a diagnostic test for asthma; online versus offline techniques and effect of flow rate. Am J Respir Crit Care Med. 2002;165:1597-601.
292. Hall GL, Reinmann B, Wildhaber JH, Frey U. Tidal exhaled nitric oxide in healthy, unsedated newborn infants with prenatal tobacco exposure. J Appl Physiol. 2002 Jan;92(1):59-66.
293. Deykin A, Massaro AF, Coulston E, Drazen JM, Israel E. Exhaled nitric oxide following repeated spirometry or repeated plethysmography in healthy individuals. Am J Respir Crit Care Med. 2000 Apr;161(4 Pt 1):1237-40.
294. Gabriele C, Pijnenburg MW, Monti F, Hop W, Bakker ME, de Jongste JC. The effect of spirometry and exercise on exhaled nitric oxide in asthmatic children. Pediatr Allergy Immunol. 2005 May;16(3):243-7.
295. Vints AM, Oostveen E, Eeckhaut G, Smolders M, De Backer WA. Time-dependent effect of nitrate-rich meals on exhaled nitric oxide in healthy subjects. Chest. 2005 Oct;128(4):2465-70.
296. Pijnenburg MW, Lissenberg ET, Hofhuis W, Ghiro L, Ho WC, Holland WP, et al. Exhaled nitric oxide measurements with dynamic flow restriction in children aged 4-8 yrs. Eur Respir J. 2002 Oct;20(4):919-24.
297. Biban P, Zangardi T, Baraldi E, Dussini N, Chiandetti L, Zacchello F. Mixed exhaled nitric oxide and plasma nitrites and nitrates in newborn infants. Life Sci. 2001 May 11;68(25):2789-97.
298. Colnaghi M, Condo V, Pugni L, Fumagalli M, Mosca F. Endogenous nitric oxide production in the airways of preterm and term infants. Biol Neonate. 2003;83(2):113-6.
299. Latzin P, Kuehni CE, Baldwin DN, Roiha HL, Casaulta C, Frey U. Elevated exhaled nitric oxide in newborns of atopic mothers precedes respiratory symptoms. Am J Respir Crit Care Med. 2006 Dec 15;174(12):1292-8.
300. Dinakar C, Craff M, Laskowski D. Infants and toddlers without asthma with eczema have elevated exhaled nitric oxide levels. J Allergy Clin Immunol. 2006 Jan;117(1):212-3.
301. Franklin PJ, Turner SW, Hall GL, Moeller A, Stick SM. Exhaled nitric oxide is reduced in infants with rhinorrhea. Pediatr Pulmonol. 2005 Feb;39(2):117-9.
302. Mahut B. Impairment of nitric oxide output of conducting airways in primary ciliary dyskinesia. Pediatr Pulmonol. 2006;41:158-63.
303. Corbelli R. Nasal nitric oxide measurement to screen children for primary ciliary dyskinesia. Chest. 2004;126:1054-9.
304. Baraldi E, Bonetto G, Zacchello F, Filippone M. Low exhaled nitric oxide in school-age children with bronchopulmonary dysplasia and airflow limitation. Am J Respir Crit Care Med. 2005 Jan 1;171(1):68-72.
305. Abman SH. Bronchopulmonary dysplasia: "a vascular hypothesis". Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 1):1755-6.
306. Leipala JA, Williams O, Sreekumar S, Cheeseman P, Rafferty GF, Hannam S, et al. Exhaled nitric oxide levels in infants with chronic lung disease. Eur J Pediatr. 2004 Sep;163(9):555-8.
307. Roiha HL, Kuehni CE, Zanolari M, Zwahlen M, Baldwin DN, Casaulta C, et al. Alterations of exhaled nitric oxide in preterm infants with chronic lung disease. Eur Respir J. 2006 Oct 18.
308. Dotsch J, Demirakca S, Terbrack HG, Huls G, Rascher W, Kuhl PG. Airway nitric oxide in asthmatic children and patients with cystic fibrosis. Eur Respir J. 1996 Dec;9(12):2537-40.
309. Grasemann H, Michler E, Wallot M, Ratjen F. Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. Pediatr Pulmonol. 1997 Sep;24(3):173-7.
310. Moeller A, Horak F, Jr., Lane C, Knight D, Kicic A, Brennan S, et al. Inducible NO synthase expression is low in airway epithelium from young children with cystic fibrosis. Thorax. 2006 Jun;61(6):514-20.
311. Elphick HE, Demoncheaux EA, Ritson S, Higenbottam TW, Everard ML. Exhaled nitric oxide is reduced in infants with cystic fibrosis. Thorax. 2001 Feb;56(2):151-2.
312. Franklin PJ, Hall GL, Moeller A, Horak F, Jr., Brennan S, Stick SM. Exhaled nitric oxide is not reduced in infants with cystic fibrosis. Eur Respir J. 2006 Feb;27(2):350-3.
313. Robroeks CM, Rosias PP, van Vliet D, Jobsis Q, Yntema JB, Brackel HJ, et al. Biomarkers in exhaled breath condensate indicate presence and severity of cystic fibrosis in children. Pediatr Allergy Immunol. 2008 Nov;19(7):652-9.
314. Jaddoe VW, Bakker R, van Duijn CM, van der Heijden AJ, Lindemans J, Mackenbach JP, et al. The Generation R Study Biobank: a resource for epidemiological studies in children and their parents. Eur J Epidemiol. 2007;22(12):917-23.
315. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The

Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006;21(6):475-84.
316. Jaddoe VW, van Duijn CM, van der Heijden AJ, Mackenbach JP, Moll HA, Steegers EA, et al. The Generation R Study: design and cohort update until the age of 4 years. Eur J Epidemiol. 2008;23(12):801-11.

Part I

Ethnic differences in early respiratory morbidity

2

Early respiratory morbidity in a multicultural birth cohort

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Submitted

Abstract

Background: Ethnic disparities in the prevalence of asthma symptoms in children have been described.

Objectives: We evaluated the association between ethnic background and respiratory symptoms during the first 2 years of life. We also evaluated to what extent ethnic differences could be explained by the mediating effect of risk factors for respiratory morbidity.

Methods: The Generation R Study is a multi-ethnic, population-based birth cohort study. Pre- and postnatal risk factors for respiratory morbidity were prospectively assessed by questionnaires. Information about ethnicity was available for 5684 infants. The associations between ethnic background and lower respiratory symptoms at 12 and 24 months were evaluated with logistic regression. Odds ratios and 95% confidence intervals (OR [95% CI]) were computed for Cape Verdean, Moroccan, Antillean, Surinamese and Turkish ethnicity with Dutch ethnicity as the reference category.

Results: We found an increased risk of lower respiratory symptoms at 24 months in Antillean infants (1.84 [1.19-2.84]) that was mediated by socioeconomic variables. Infants of Turkish ethnicity had an increased risk of lower respiratory symptoms at 12 and 24 months (1.37 [1.02-1.83] and 1.48 [1.12-1.97], respectively), partly explained by previous morbidity (eczema, infections and upper respiratory symptoms). Moroccan ethnicity was associated with reduced risk of lower respiratory symptoms at 24 months (0.67 [0.46-0.97]), after adjustment for potential mediators and confounders.

Conclusion: Ethnic background is associated with respiratory symptoms during the first 2 years of life and this association is only partly explained by the mediating effect of pre- and postnatal risk factors for respiratory morbidity.

Introduction

The prevalence of asthma varies worldwide, with a higher prevalence in children of Western lifestyle-countries compared to children of developing countries (1). A variation in the prevalence of asthma and asthma-like symptoms has also been shown among children with different ethnic background living in the same urban area (2-4). Previous cross-sectional studies have shown that in the US some ethnic groups, such as African-Americans and Porto Ricans, are more prone to develop asthma compared to Caucasian and Mexican Americans and that these differences are mostly independent of socioeconomic variables (5-8). Studies on migrants in Europe showed that children of Turkish ethnicity had a lower prevalence of atopic diseases compared to their German and Swedish peers [3, 12]. A striking association between higher levels of cultural adaptation in Turkish migrants and the prevalence of atopic diseases was reported: the prevalence of atopic sensitization and disease increased with the parental use of German language, up to the level of their German peers (9). Furthermore, studies from US, Europe and Australia have demonstrated that the risk of asthma symptoms in migrants increases in case of immigration early in life and is positively associated with the duration of residence, suggesting that environmental rather than genetic factors determine the risk of atopy and asthma (10-12). This hypothesis had been previously suggested by a comparison of the prevalence of asthma symptoms in ethnically similar populations after the German reunification, indicating that environmental factors associated with Western lifestyle and living conditions were mostly responsible of atopic sensitization and the subsequent development of asthma (13, 14). In a previous study conducted in the Netherlands, Koopman et al (15) prospectively assessed the prevalence of respiratory symptoms in the first 2 years of life in a cohort of children with different ethnic background. These authors showed that there was a higher prevalence of lower respiratory symptoms in non-Dutch children compared to Dutch, which could largely be explained by differences in socioeconomic status (15). Until now, this was the only longitudinal study on the association between ethnicity and respiratory symptoms from early infancy onwards. Therefore, within the framework of a large prospective multiethnic birth cohort, we examined the associations between ethnic background and symptoms of the lower respiratory tract in the first 2 years of life. We also assessed whether these associations could be explained by the mediating effects of known risk factors for respiratory symptoms.

Some of the results of the present study have been previously reported in the form of an abstract (16).

Methods

Study design

The Generation R Study is a prenatally recruited population-based multicultural birth cohort study in which 9778 pregnant women and their children were enrolled in the city of Rotterdam (the Netherlands) (17, 18). Pre- and postnatal risk factors for respiratory morbidity were prospectively assessed by questionnaires. If needed, ethnic minorities were approached in their own language. The Medical Ethical Committee of the Erasmus Medical Center, Rotterdam, approved the study and parents gave written informed consent.

Definition of ethnicity A child was considered to be of Dutch ethnicity if both parents were born in the Netherlands and from migrant origin if one parent was born abroad. If both parents were born abroad, mother's country of origin was leading (19).

Outcomes Wheezing, breathlessness, dry cough without a cold, persistent phlegm and doctor-diagnosed asthma in the past year were assessed at 12 and 24 months by means of postal questionnaires. The combined variables 'lower respiratory symptoms (LRS) at 0-12 months' and 'LRS at 12-24 months' were considered positive if at least one of the above symptoms was reported at 12 or at 24 months, respectively.

Mediators and confounders The following risk factors for LRS, considered as intermediate in possible causal pathways between ethnicity and LRS, are referred to as potential mediators (20):

- Socioeconomic status (SES) defined by net monthly income of the household, maternal education and marital status.
- Prenatal environment and perinatal characteristics. Prenatal smoke exposure and parental atopy were assessed by questionnaire; birth weight (kg) and gestational age (wks) were obtained from hospital registries.
- Postnatal environment and nutrition. Based on the answer to pre-and postnatal questionnaires, the variable 'pets keeping' was grouped in 4 mutually exclusive categories (never exposed, prenatal exposure only, postnatal exposure only or pre- and postnatal exposure). Breastfeeding and the number of siblings in the household were recorded at 6 months. Daycare attendance in the past 12 months was investigated at 12 and at 24 months. Postnatal environmental tobacco smoke exposure was assessed at 24 months.

- Previous morbidity. At 12 and 24 months doctor-diagnosed child allergy, upper respiratory symptoms (URS), eczema, respiratory and non-respiratory tract infections were assessed.

Gender and age of the child at the time of completion of the 12 and 24 months' questionnaires were included in all logistic regression models as confounders.

Statistical analysis

Categorical variables were compared with Chi-square tests. Continuous variables were not normally distributed and were compared with Mann-Whitney U-test or Kruskal-Wallis test, as appropriate. Multiple imputation was used to impute missing values of the outcomes, mediators and confounders (21). Logistic regression was used to evaluate the association between ethnic background and each respiratory symptom reported at 12 and 24 months, adjusting for age and gender. The associations between ethnic background and LRS during the first (0-12 months) or the second year (12-24 months) were evaluated in two separate logistic regression models adjusting for age and gender (basic model). Each potential mediator was separately added to the basic model. Variables that individually caused a change ≥ 10% in the odds ratio (OR) of any ethnic group compared to the Dutch in the basic model were included in subsequent logistic regression models, taking into account the hierarchical relationship between the investigated mediators (22). Results of the logistic regression models are reported as odds ratio and 95% confidence intervals (OR [95% CI]). Figure 1 shows a hierarchical framework, in which variables near the top of the figure influence those below. Statistical analyses were performed using the Statistical Package of Social Sciences version 15 for Windows (SPSS Inc, Chicago, IL, USA) and the Statistical Analysis System (SAS) for Windows, version 9.1.3.

Results

Study population Of the 9778 women enrolled, 8880 were included prenatally, 6969 agreed to participate to the postnatal phase and 6492 gave full consent to the use of postnatal data. Twin pregnancies (n=50) were excluded and if the mother participated with more than one pregnancy, only the first pregnancy was included in the analyses (n=5954) to avoid clustering. As ethnic background was the main determinant of the study, infants with missing data on ethnicity (n=270) were excluded, leaving 5684 infants for the analyses. The ethnic background of the study

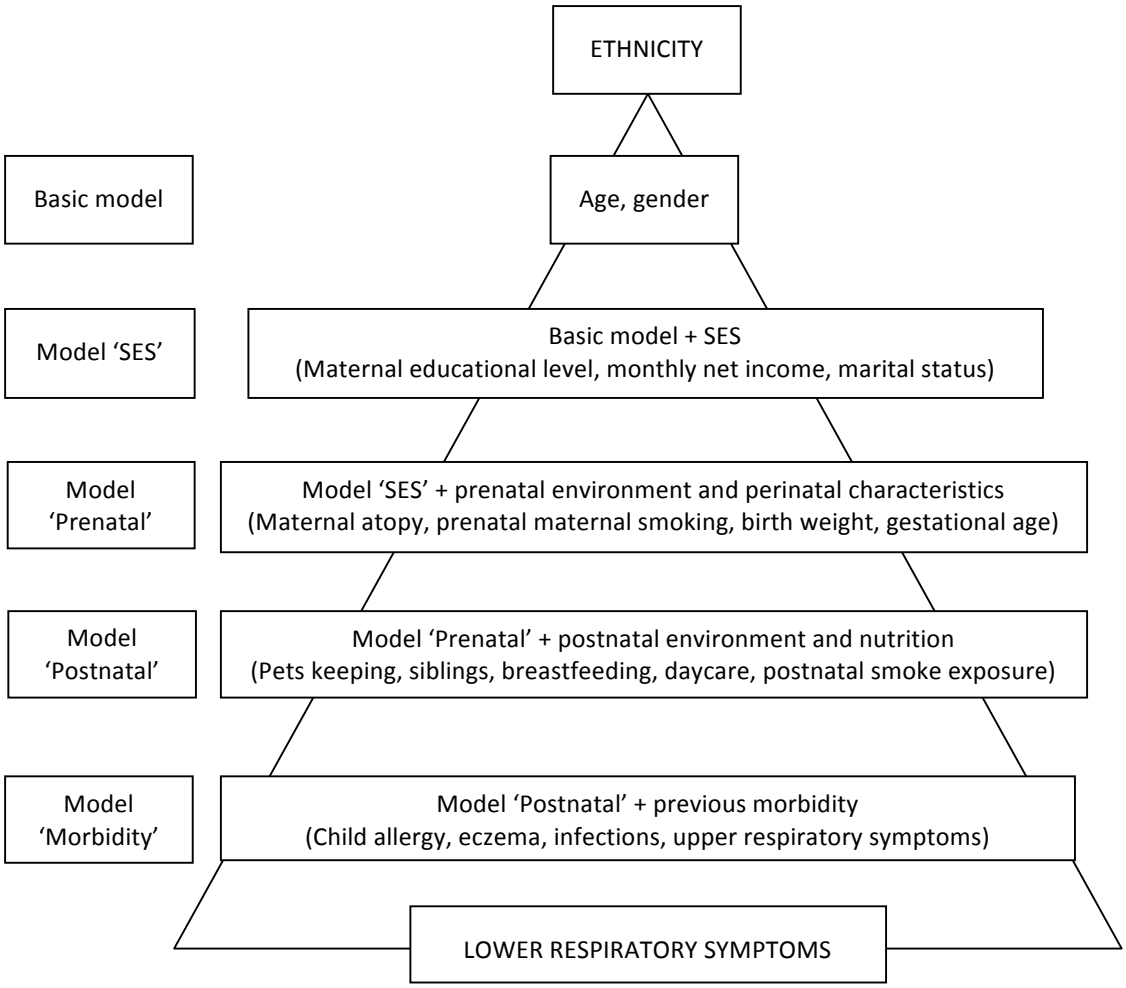

Figure 1 Hierarchical framework

population was Dutch in 56.6%, Cape Verdean in 2.9%, Moroccan in 6.2%, Antillean in 3.1%, Surinamese in 7%, Turkish in 7.6% and 'others' in 16.6%. As the group 'others' represented a heterogeneous population of infants, the results were reported, but could not be meaningfully interpreted. The general characteristics of the different ethnic groups are presented in table 1.

Risk factors for lower respiratory symptoms The 12 months' survey showed that, compared with infants without LRS, infants with LRS were more likely boys, allergic, used more daycare, had more often an atopic mother, a mother with a high educational level and a higher net income. Infants with LRS also had more respiratory tract infections, URS and eczema, and were less often breastfed in the first 6 months. The 24 months' survey showed that, compared with infants without LRS, infants with LRS in the past 12 months were more likely boys, allergic, used more daycare, were more exposed to prenatal and postnatal smoke had more often an atopic mother, and a mother who lived alone.

Table 1 General characteristics of the study population

	Dutch N=3217	Cape Verdean N=163	Moroccan N=350	Antillean N=178	Surinamese N=400	Turkish N=434
Male gender	50%	49%	49%	60%†	46%	47%
Educational level mother						
high	59%	13%	14%	19%	19%	14%
intermediate	38%	63%	59%	67%	68%	51%
low	3%	24%*	27%*	14%*	13%*	35%*
Monthly family net income						
>2200 Euro	75%	11%	11%	20%	33%	14%
900-2200 Euro	21%	51%	68%	40%	45%	62%
<900 Euro	4%	38%*	21%*	40%*	22%*	23%*
Mother married/living together	92%	58%‡	91%	46%‡	66%‡	95%
Prenatal maternal smoking	24%	22%	7%‡	31%†	29%†	35%‡
Maternal atopy	39%	35%	37%	46%	36%	33% †
Birth weight: mean (SD) gr	3.485 (0.5)§	3.303 (0.5)	3.523 (0.5)	3.174 (0.6)	3.184 (0.6)	3.387 (0.5)
Gestational age: mean (SD) wks	40.0 (1.7)§	39.9 (1.4)	40.3 (1.5)	39.5 (1.8)	39.5 (1.8)	39.9 (1.8)
Pets' keeping						
Never	55%	80%*	92%*	77%*	75%*	85%*
Only prenatal	7%	8%	4%	7%	9%	7%
Only postnatal	6%	7%	1%	4%	5%	3%
Pre- and postnatal	32%	5%	3%	12%	11%	5%
Breastfeeding	28%	19%†	27%	15%‡	19%‡	40%‡
Siblings	34%	50%‡	57%‡	34%	40%†	51%‡
Daycare at 0-12 months	92%	87%	66%‡	79%‡	85%‡	59%‡
Daycare at 12-24 months	89%	88%	51%‡	82%†	81%‡	48%‡
Postnatal smoke exposure	30%	25%	24%	40%†	42%‡	56%‡
Child allergy	6%	6%	7%	8%	6%	3%
URS at 0-12 months	38%	50%	46%†	40%	50%‡	44%
URS at 12-24 months	30%	39%	31%	36%	35%	37%†
Eczema at 0-12 months	31%	34%	48%‡	37%	48%‡	36%
Eczema at 12-24 months	22%	27%	16%	30%	31%‡	14%‡
Infections at 0-12 months						
no	18%	23%	20%	19%	20%	14%
other infections	14%	14%	4%	5%	10%	2%
URTI or LRTI	68%	63%	76%	76%	70%	84%
Infections at 12-24 months						
no	10%	5%	11%	6%	11%	5%
other infections	19%	9%	11%	9%	11%	6%
URTI or LRTI	71%	86%*	78%*	85%*	78%*	89%*

SD: Standard deviation; URS: upper respiratory symptoms; URTI: upper respiratory tract infections; LRTI: lower respiratory tract infections. †p<0.05 compared to Dutch (Chi-square test); ‡p<0.01 compared to Dutch (Chi-square test); *p<0.001, Chi-square trend test. § p<0.01, Kruskal-Wallis test.

They were also more likely to have eczema, respiratory tract infections and URS and were less likely to have siblings, to keep pets pre-and postnatally and were less often breastfed in the first 6 months.

Ethnicity and lower respiratory symptoms The prevalence of respiratory symptoms in different ethnic groups at 12 and 24 months is reported in table 2.

Table 2 Prevalence of respiratory symptoms at 12 and 24 months stratified by ethnicity of the child

	Dutch	Cape Verde	Morocco	Dutch Antilles	Suriname	Turkey	Total
Respiratory symptoms during the first year							
Wheezing	31%	36%	26%	32%	28%	37%	30%
Breathlessness	25%	19%	22%	22%	18%	18%	23%
Dry cough without a cold	24%	18%	25%	22%	20%	25%	23%
Persistent phlegm	12%	23%	20%	26%	18%	25%	15%
Doctor-diagnosed asthma	2%	1%	2%	5%	2%	3%	2%
LRS at 0-12 months	54%	58%	51%	61%	52%	62%	53%
Respiratory symptoms during the second year							
Wheezing	20%	23%	19%	31%	25%	25%	21%
Breathlessness	20%	18%	16%	26%	14%	14%	18%
Dry cough without a cold	25%	28%	21%	30%	23%	33%	25%
Persistent phlegm	8%	25%	8%	19%	15%	18%	11%
Doctor-diagnosed asthma	2%	3%	0.3%	3%	3%	2%	2%
LRS at 12-24 months	46%	55%	42%	61%	48%	56%	47%

The logistic regression models adjusted for gender and age showed that Turkish children had a reduced risk of breathlessness at 12 and 24 months and an increased risk of dry cough without a cold at 24 months, whereas Antilleans were more likely to have doctor-diagnosed asthma at 12 months and wheezing at 24 months, as compared to Dutch. Also, all non-Dutch ethnic groups had increased risk of persistent phlegm at 12 months and all except Moroccans also at 24 months (figures 2 and 3).

Next, the basic model (including ethnicity, age and gender) was fitted on LRS at 0-12 months (table 3) and showed that infants with Turkish background had increased risk of LRS compared to their Dutch peers (OR [95% CI] 1.37 [1.02-1.83]). Variables associated with a >10% change in OR of LRS at 0-12 months for any ethnic group were maternal education, net income, marital status, prenatal smoke exposure, birth weight, gestational age, pets' keeping, breastfeeding, daycare attendance, eczema,

respiratory and non-respiratory infections and URS. Following the hierarchical levels, socioeconomic factors were added to the basic model (model 'SES') and this caused a 38% increase of the OR of Turkish vs. Dutch (1.51 [1.11-2.06]), suggesting that the relatively low SES in the Turkish subgroup suppressed part of their increased risk of LRS between 0 and 12 months. Adding prenatal exposures and birth data had no consistent effect on the OR. The model that included also nutrition and postnatal exposures (model 'Postnatal') caused a further increase of the OR of LRS of Turkish vs. Dutch (1.66 [1.17-2.36]), due to less daycare in Turkish infants, as daycare is a risk factor for LRS at 0-12 months (1.92 [1.58-2.33]).

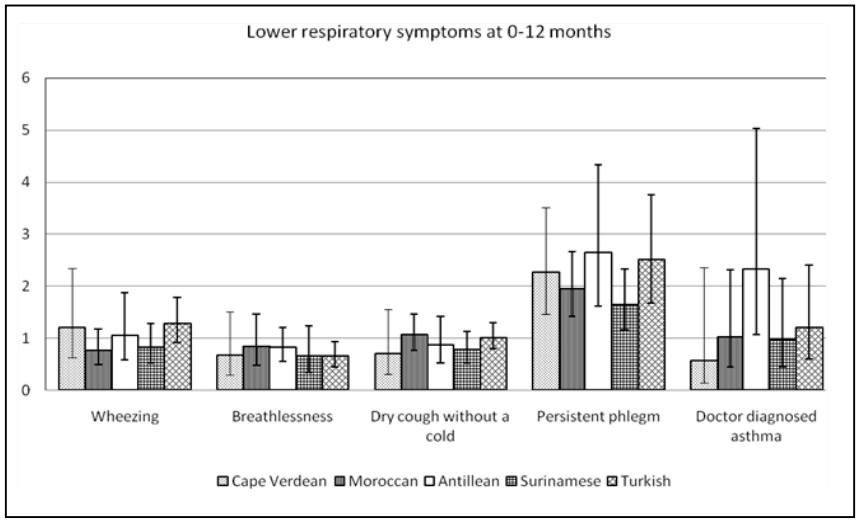

Figure 2 Odds ratio (OR) of respiratory symptoms at 0-12 months for each ethnic group compared to Dutch. Bars represent 95% CI.

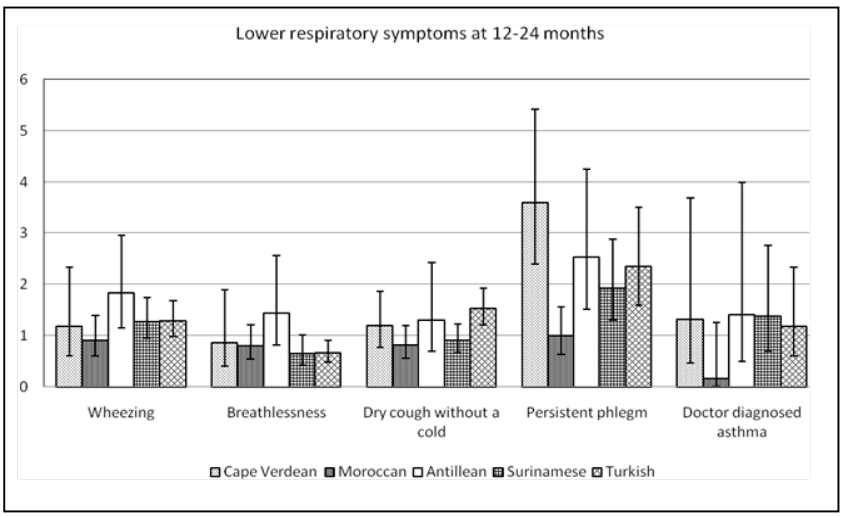

Figure 3 Odds ratio (OR) of respiratory symptoms at 12-24 months for each ethnic group compared to Dutch. Bars represent 95% CI.

Table 3 Hierarchical logistic regression models fitted on LRS at 0-12 months (n=5684).

	Basic model	Model 'SES'	Model 'Prenatal'	Model 'Postnatal'	Model 'Morbidity'
Ethnicity					
Dutch	Reference	Reference	Reference	Reference	Reference
Cape Verdean	1.19 (0.74-1.91)	1.19 (0.74-1.91)	1.22 (0.75-1.98)	1.08 (0.66-1.76)	1.03 (0.63-1.68)
Moroccan	0.90 (0.64-1.28)	1.00 (0.70-1.43)	1.04 (0.75-1.46)	1.06 (0.73-1.53)	0.81 (0.58-1.13)
Antillean	1.34 (0.76-2.3)	1.27 (0.73-2.19)	1.27 (0.73-2.21)	1.26 (0.71-2.24)	1.30 (0.80-2.10)
Surinamese	0.91 (0.67-1.23)	0.92 (0.69-1.23)	0.91 (0.68-1.24)	0.88 (0.64-1.21)	0.78 (0.57-1.09)
Turkish	1.37 (1.02-1.83)	1.51 (1.11-2.06)	1.51 (1.10-2.06)	1.66 (1.17-2.36)	1.58 (1.17-2.12)
SES					
Maternal educational level					
High		Reference	Reference	Reference	Reference
Intermediate		0.88 (0.76-1.01)	0.86 (0.74-0.99)	1.02 (0.77-1.05)	0.83 (0.71-0.97)
Low		0.87 (0.60-1.25)	0.85 (0.58-1.23)	0.82 (0.67-1.33)	0.73 (0.52-1.03)
Monthly net income					
>2200 Euro		Reference	Reference	Reference	Reference
900-2200 Euro		0.89 (0.77-1.04)	0.89 (0.77-1.04)	1.00 (0.84-1.16)	0.96 (0.80-1.14)
<900 Euro		1.08 (0.81-1.45)	1.07 (0.80-1.44)	1.26 (0.92-1.73)	1.08 (0.77-1.52)
Marital status – unmarried/living alone		1.24 (0.96-1.60)	1.22 (0.95-1.57)	1.1 (0.85-1.43)	1.17 (0.88-1.55)
Prenatal environment and perinatal characteristics					
Prenatal maternal smoking			1.1 (0.92-1.31)	1.08 (0.90-1.29)	1.04 (0.87-1.25)
Birth weight			1.03 (0.91-1.18)	1.05 (0.92-1.19)	1.06 (0.92-1.23)
Gestational age			0.95 (0.91-0.99)	0.95 (0.90-0.99)	0.94 (0.90-0.99)
Postnatal environment and nutrition					
Pets' keeping					
Never				Reference	Reference
Only prenatal				0.98 (0.77-1.24)	1.00 (0.75-1.33)
Only postnatal				0.83 (0.62-1.12)	0.9 (0.65-1.23)
Pre- and postnatal				0.84 (0.73-0.96)	0.91 (0.78-1.06)
Breastfeeding				0.8 (0.70-0.91)	0.85 (0.74-0.98)
Daycare at 0-12 months				1.92 (1.58-2.33)	1.80 (1.47-2.21)
Previous morbidity					
Eczema at 0-12 months					1.17 (0.99-1.37)
Infections at 0-12 months					
No					Reference
Respiratory infections					2.94 (2.48-3.49)
Non-respiratory infections					1.64 (1.30-2.06)
Upper respiratory symptoms at 0-12 months					2.00 (1.75-2.28)

Results are reported as OR (95% CI)

Basic model: Ethnicity, age, gender. Model 'SES': Basic model + educational level of the mother, income, marital status. Model 'Prenatal': Model 'SES' + maternal smoking during pregnancy, birth weight, gestational age. Model 'Postnatal': Model 'Prenatal' + pets keeping, breastfeeding, daycare attendance at 1 year. Model 'Morbidity': Model 'Postnatal' + eczema at 1 year, infections at 1 year, upper respiratory symptoms at 1 year.

Adding previous morbidity variables reduced the OR of LRS at 0-12 months of Turkish vs. Dutch to 1.58 [1.17-2.12] as infections at 12 months, which were a risk factor for LRS, were more frequently reported by Turkish than by Dutch parents. The

other ethnic groups did not show any different risk of LRS at 0-12 months compared to Dutch (table 3).

Variables associated with a change >10% in OR of LRS at 12-24 months for any ethnic group included maternal education, net income, marital status, prenatal smoke exposure, birth weight, gestational age, pets' keeping, siblings, breastfeeding, daycare attendance, postnatal smoke exposure, eczema, respiratory and non-respiratory infections and URS. The basic model fitted on LRS at 12-24 months showed that Antillean and Turkish children had an increased risk of LRS in the second year compared to Dutch (table 4).

The association between Moroccan, Cape Verdean or Surinamese ethnicity and LRS at 12-24 months was not significant in the basic model. Adding socioeconomic factors (model 'SES') caused a 36.8% reduction of the OR for Antillean to 1.53 [0.97-2.41], as Antillean mothers were more likely unmarried or living alone, this being a risk factor for LRS at 12-24 months. Prenatal exposures and birth data did not change the OR across ethnic groups. The model 'Postnatal' included nutrition and postnatal exposures, which increased the OR of Turkish vs. Dutch to 1.57 [1.11 2.22], as Turkish infants received less daycare, which was a risk factor for LRS at 12-24 months. Previous morbidity mediated part of the increased risk of LRS in Turkish and Antillean children as adding this set of variables reduced the OR across all ethnic groups (model 'Morbidity'). The association between Turkish ethnicity and increased risk of LRS did not reach statistical significance in the second year (1.33 [0.98-1.80]).The mediating effect of previous morbidity was related to the higher prevalence of risk factors for LRS at 12-24 months in Turkish compared to Dutch infants. Moroccan ethnicity was associated with a reduced risk of LRS to 0.67 [0.46-0.97], only in the model including previous morbidity. No difference was observed in the risk of LRS at 12-24 months between Dutch and Cape Verdean or Surinamese infants.

Discussion

In the present prospective birth cohort study we showed marked effects of ethnicity on the risk of lower respiratory symptoms. Compared to Dutch, Antillean infants had an increased risk of lower respiratory symptoms at 24 months, which was mediated by socioeconomic variables. Infants of Turkish ethnicity had higher risk of respiratory symptoms both at 12 and 24 months as compared to Dutch, which was

Table 4 Hierarchical logistic regression models fitted on LRS at 12-24 months (n=5684)

	Basic model	Model 'SES'	Model 'prenatal'	Model 'postnatal'	Model 'morbidity'
Ethnicity					
Dutch	Reference	Reference	Reference	Reference	Reference
Cape Verdean	1.40 (0.81-2.44)	1.23 (0.69-2.22)	1.28 (0.70-2.34)	1.15 (0.63-2.09)	0.90 (0.55-1.47)
Moroccan	0.86 (0.61-1.19)	0.85 (0.58-1.24)	0.89 (0.61-1.31)	0.91 (0.63-1.33)	0.67 (0.46-0.97)
Antillean	1.84 (1.19-2.84)	1.53 (0.97-2.41)	1.54 (0.97-2.45)	1.47 (0.91-2.37)	1.31 (0.82-2.10)
Surinamese	1.06 (0.83-1.36)	0.97 (0.75-1.24)	0.97 (0.76-1.25)	0.92 (0.71-1.20)	0.81 (0.60-1.10)
Turkish	1.48 (1.12-1.97)	1.49 (1.08-2.06)	1.49 (1.08-2.05)	1.57 (1.11-2.22)	1.33 (0.98-1.80)
SES					
Maternal educational level					
High		Reference	Reference	Reference	Reference
Intermediate		1.09 (0.95-1.23)	1.06 (0.93-1.21)	1.10 (0.96-1.26)	1.06 (0.90-1.24)
Low		0.88 (0.67-1.14)	0.85 (0.65-1.11)	0.98 (0.76-1.26)	0.93 (0.67-1.28)
Monthly net income					
>2200 Euro		Reference	Reference	Reference	Reference
900-2200 Euro		0.95 (0.81-1.11)	0.94 (0.80-1.11)	1.02 (0.87-1.20)	0.91 (0.76-1.09)
<900 Euro		1.32 (0.99-1.76)	1.31 (0.99-1.73)	1.46 (1.05-2.02)	1.21 (0.85-1.73)
Marital status – unmarried/living alone		1.19 (0.97-1.47)	1.17 (0.95-1.43)	1.05 (0.85-1.30)	1.12 (0.86-1.46)
Prenatal environment and perinatal characteristics					
Prenatal maternal smoking			1.17 (1.01-1.34)	1.08 (0.93-1.25)	0.99 (0.83-1.18)
Birth weight			1.039 (0.87-1.24)	1.07 (0.89-1.29)	1.08 (0.93-1.26)
Gestational age			0.96 (0.90-1.02)	0.96 (0.90-1.02)	0.96 (0.91-1.01)
Postnatal environment and nutrition					
Pets' keeping					
Never				Reference	Reference
Only prenatal				1.05 (0.79-1.41)	0.96 (0.71-1.29)
Only postnatal				0.84 (0.65-1.10)	0.81 (0.60-1.09)
Pre- and postnatal				0.81 (0.70-0.93)	0.81 (0.69-0.94)
Siblings				0.93 (0.81-1.07)	0.93 (0.81-1.07)
Breastfeeding				0.86 (0.76-0.98)	0.92 (0.79-1.07)
Daycare at 12-24 months				1.67 (1.38-2.02)	1.66 (1.37-2.02)
Postnatal smoke exposure				1.16 (0.96-1.41)	1.18 (1.01-1.38)
Previous morbidity					
Eczema at 12-24 months					1.30 (1.11-1.52)
Infections at 12-24 months					
No					Reference
Respiratory infections					2.96 (2.31-3.79)
Non-respiratory infections					1.45 (1.08-1.93)
Upper respiratory symptoms at 12-24 months					2.23 (1.94-2.56)

Results are reported as OR (95% CI)

Basic model: Ethnicity, age, gender. Model 'SES': Basic model + educational level of the mother, income, marital status. Model 'Prenatal': Model 'SES' + maternal smoking during pregnancy, birth weight, gestational age. Model 'Postnatal': Model 'Prenatal' + pets keeping, breastfeeding, daycare attendance at 2 years. Model 'Morbidity': Model 'Postnatal' + eczema at 2 years, infections at 2 years, upper respiratory symptoms at 2 years.

partly mediated by previous morbidity. Moroccan ethnicity was associated with reduced risk of lower respiratory symptoms at 24 months, only after adjustment for all the potential mediators and confounders. The risk of lower respiratory symptoms in the first 2 years of life was not different in Cape Verdean or Surinamese infants, as compared to Dutch infants.

This is the first birth cohort study specifically focusing on differences in respiratory morbidity between ethnic groups coming from a multicultural urban society, who were approached in their own language. The results of our study are consistent with the finding of an increased risk of respiratory symptoms before the age of 2 years in Turkish infants living in the Netherlands, as reported previously from the PIAMA birth cohort (15). However, the relatively small size of the non-Dutch groups in the previous study did not allow the comparison between ethnic minorities and the differences observed were abolished by adjustment for socioeconomic indices (15). In our study, SES variables suppressed only part of the association between Turkish ethnicity and LRS, and only at 0-12 months. Turkish children had mothers with lower educational level and lower net income of the household. Taking this into account reduced the risk of LRS at 0-12 months. These results suggest that the lower SES in the Turkish subgroup is likely to attenuate their increased risk of LRS at 0-12 months, whereas reported respiratory tract infections, URS and eczema largely explained the association between Turkish ethnicity and LRS both at 12 and 24 months. Our results are apparently in contrast with previous European studies that found Turkish migrants to have a lower risk of asthma (3, 23). However, we could evaluate the effect of ethnicity on respiratory symptoms up to the age of 2 years and it is likely that the majority of symptomatic 2-year olds will not develop asthma later in life (24). Therefore, as the cohort matures, we will be able to evaluate whether Turkish ethnicity is indeed associated with an increased risk of developing asthma. In Antillean children, SES mediated the increased risk of LRS at 12-24 months. Antillean mothers were more likely to be unmarried/living alone, which we found to be a risk factor for LRS at 12-24 months. The few data available in literature have shown that the prevalence of asthma in Moroccan children is lower than that in other African countries, and lower than in most Western and Northern European countries (25). Also, a recent study in the Netherlands has shown that Moroccan adults are less likely to have asthma than their Dutch peers (26). We found consistent results in our study, showing that Moroccan ethnicity was associated with a reduced risk of respiratory symptoms in the period up to 24 months, compared to Dutch. However, this

difference was statistically significant only in the model which included all the mediators and confounders at 24 months. Possible explanations are that either Moroccans have genetic factors that confer protection against the development of respiratory symptoms in infancy, or that variables not measured and/or not included in the analyses could explain this association. The Tucson Children's Respiratory Study showed that daycare attendance was associated with frequent wheezing at the age of 2 years, but not at 6 to 13 years (27). We also found a positive association between daycare attendance and LRS at 0-12 and at 12-24 months. In our study, children of non-Dutch ethnicity reported less daycare attendance than Dutch. Daycare attendance suppressed part of the association between Turkish ethnicity and LRS at 12 and at 24 months, suggesting that the lower use of daycare in the non-Dutch children is likely to reduce their risk of LRS. However, the effect of daycare attendance on LRS is mediated by respiratory infections, which may trigger respiratory symptoms (28). When we added previous morbidity variables to the hierarchical logistic regression model, we found that a history of infections, eczema and URS reduced the OR of LRS at 12 and 24 months across all the ethnic groups. This finding suggests that infections, eczema and URS were intermediate variables in the pathway between ethnicity and LRS, and also mediated part of the effect of variables previously added to the model. We found that both respiratory and non-respiratory infections were associated with increased risk of LRS in the first 2 years of life and, as the cohort matures and more clear patterns of respiratory diseases will become evident, we will be able to assess whether the overall burden of infections may predispose towards a certain phenotype.

Although the prospective design and the large scale of our study allowed us to evaluate the temporal relationship between exposure to several risk factors for respiratory morbidity and the occurrence of LRS, some possible limitations to our study have to be considered. We defined the ethnic background of infants according to the Dutch standard classification (19). This classification is objective, reproducible and can be easily applied in epidemiological studies, allowing comparison with future studies. However, some misclassification of ethnicity might have occurred as third generation migrants were labeled Dutch and were not distinguished. Hence, it is possible that we underestimated the effect size of Turkish ethnicity on LRS.

In the current study we used standard respiratory questionnaires for schoolchildren, which have shown satisfactory repeatability but may not be entirely appropriate for infants and preschool children (29). A recent study by Strippoli et al

(30), showed poorer repeatability in infants for questions regarding cough and upper respiratory symptoms compared to wheeze and shortness of breath, probably as the latter symptoms are recalled more consistently because parents are more concerned (30). However, the outcomes of our study, including the combined variables LRS at 0-12 and LRS at 12-24 months, were considered positive if at least one of the investigated symptoms occurred. Therefore, we consider it unlikely that misclassification of the combined variables occurred.

Our findings contribute to the understanding of respiratory diseases early in childhood in ethnic diverse populations, by identifying ethnicity-specific risk factors for respiratory morbidity in the first 2 years of life. We showed that postnatal exposures mediated part of the associations found between ethnicity and respiratory symptoms, whereas socioeconomic variables mainly suppressed such associations. However, the effect of pre- and postnatal environmental exposures could not entirely explain the association between ethnic background and risk of respiratory symptoms in infancy, suggesting that genetic factors may play an important role in determining the risk of respiratory symptoms in ethnically diverse populations. The follow up of our cohort will determine whether the increased prevalence of respiratory symptoms in certain ethnic groups represents a temporary association with respiratory infections in early childhood or predicts progression to chronic persistent symptoms, and perhaps asthma.

References

1. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: Isaac. The international study of asthma and allergies in childhood (isaac) steering committee. Lancet 1998;351:1225-1232.
2. Hjern A, Haglund B, Hedlin G. Ethnicity, childhood environment and atopic disorder. Clin Exp Allergy 2000;30:521-528.
3. Kabesch M, Schaal W, Nicolai T, von Mutius E. Lower prevalence of asthma and atopy in turkish children living in germany. Eur Respir J 1999;13:577-582.
4. Kuehni CE, Strippoli MP, Low N, Brooke AM, Silverman M. Wheeze and asthma prevalence and related health-service use in white and south asian pre-schoolchildren in the united kingdom. Clin Exp Allergy 2007;37:1738-1746.
5. Grant EN, Lyttle CS, Weiss KB. The relation of socioeconomic factors and racial/ethnic differences in us asthma mortality. American journal of public health 2000;90:1923-1925.
6. Joseph CL, Ownby DR, Peterson EL, Johnson CC. Racial differences in physiologic parameters related to asthma among middle-class children. Chest 2000;117:1336-1344.
7. Rose D, Mannino DM, Leaderer BP. Asthma prevalence among us adults, 1998-2000: Role of puerto rican ethnicity and behavioral and geographic factors. American journal of public health 2006;96:880-888.
8. Shapiro GG, Stout JW. Childhood asthma in the united states: Urban issues. Pediatr Pulmonol 2002;33:47-55.
9. Gruber C, Illi S, Plieth A, Sommerfeld C, Wahn U. Cultural adaptation is associated with atopy and wheezing among children of turkish origin living in germany. Clin Exp Allergy 2002;32:526-531.

10. Eldeirawi KM, Persky VW. Associations of acculturation and country of birth with asthma and wheezing in mexican american youths. J Asthma 2006;43:279-286.
11. Powell CV, Nolan TM, Carlin JB, Bennett CM, Johnson PD. Respiratory symptoms and duration of residence in immigrant teenagers living in melbourne, australia. Arch Dis Child 1999;81:159-162.
12. Kuehni CE, Strippoli MP, Low N, Silverman M. Asthma in young south asian women living in the united kingdom: The importance of early life. Clin Exp Allergy 2007;37:47-53.
13. von Mutius E, Martinez FD, Fritzsch C, Nicolai T, Roell G, Thiemann HH. Prevalence of asthma and atopy in two areas of west and east germany. Am J Respir Crit Care Med 1994;149:358-364.
14. von Mutius E, Fritzsch C, Weiland SK, Roll G, Magnussen H. Prevalence of asthma and allergic disorders among children in united germany: A descriptive comparison. Bmj 1992;305:1395-1399.
15. Koopman LP, Wijga A, Smit HA, De Jongste JC, Kerkhof M, Gerritsen J, Vos AP, Van Strien RT, Brunekreef B, Neijens HJ. Early respiratory and skin symptoms in relation to ethnic background: The importance of socioeconomic status; the piama study. Arch Dis Child 2002;87:482-488.
16. Gabriele C, Jaddoe VW, Hofman A, Merkus PJ, de Jongste JC. Early respiratory morbidity in a multicultural birth cohort: The Generation R study. Eur Respir J 2007;30:398s.
17. Jaddoe VW, Bakker R, van Duijn CM, van der Heijden AJ, Lindemans J, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Uitterlinden AG, et al. The Generation R study biobank: A resource for epidemiological studies in children and their parents. Eur J Epidemiol 2007;22:917-923.
18. Jaddoe VW, van Duijn CM, van der Heijden AJ, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Uitterlinden AG, Verhulst FC, Hofman A. The Generation R study: Design and cohort update until the age of 4 years. Eur J Epidemiol 2008;23:801-811.
19. Voorburg/Heerlen. Standaard onderwijsindeling 2003. Statistics Netherlands 2004.
20. McNamee R. Confounding and confounders. Occup Environ Med 2003;60:227-234; quiz 164, 234.
21. Rubin DB. Multiple imputation for nonresponse in surveys. Wiley & Sons, New York 1987.
22. Victora CG, Huttly SR, Fuchs SC, Olinto MT. The role of conceptual frameworks in epidemiological analysis: A hierarchical approach. International journal of epidemiology 1997;26:224-227.
23. Hjern A, Rasmussen F, Johansson M, Aberg N. Migration and atopic disorder in swedish conscripts. Pediatr Allergy Immunol 1999;10:209-215.
24. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The group health medical associates. N Engl J Med 1995;332:133-138.
25. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, Williams H. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: Isaac phases one and three repeat multicountry cross-sectional surveys. Lancet 2006;368:733-743.
26. Uijen AA, Schermer TR, van den Hoogen HJ, Mulder J, Zantinge EM, Bottema BJ. Prevalence of and health care consumption for asthma and copd in relation to ethnicity. Ned Tijdschr Geneeskd 2008;152:1157-1163.
27. Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. N Engl J Med 2000;343:538-543.
28. Koopman LP, Smit HA, Heijnen ML, Wijga A, van Strien RT, Kerkhof M, Gerritsen J, Brunekreef B, de Jongste JC, Neijens HJ. Respiratory infections in infants: Interaction of parental allergy, child care, and siblings-- the piama study. Pediatrics 2001;108:943-948.
29. Brunekreef B, Groot B, Rijcken B, Hoek G, Steenbekkers A, de Boer A. Reproducibility of childhood respiratory symptom questions. Eur Respir J 1992;5:930-935.
30. Strippoli MP, Silverman M, Michel G, Kuehni CE. A parent-completed respiratory questionnaire for 1-year-old children: Repeatability. Arch Dis Child 2007;92:861-865.

Part II

Exhaled nitric oxide in infants

3

Methodological aspect of exhaled nitric oxide measurements in infants

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Abstract

Background: Guidelines for the measurement of fractional exhaled nitric oxide (FeNO) recommend refraining from lung function tests and certain foods and beverages before performing FeNO measurements, since they may lead to transiently altered FeNO levels. Little is known of such factors in infants.

Objectives: The aim of the present study was to evaluate whether forced expiratory maneuvers, sedation, nasal contamination and breastfeeding affect FeNO values in infants.

Methods: FeNO was measured off-line during tidal breathing by means of a facemask covering nose and mouth. FeNO measurements were performed in 45 sedated infants (mean age 12.1 months) who underwent lung function tests (LFT) because of airway diseases and in 83 unsedated healthy infants (mean age 4.3 months).

Results: In infants with airway diseases, no difference was found in FeNO values before and 5 min after LFT (N=19 infants, p=0.7) and FeNO values before sedation did not differ from FeNO values during sedation (N=10 infants, p=0.2).Oral FeNO values were significantly lower than mixed (nasal + oral) FeNO (N=42 infants, p<0.001). FeNO values before and 5 min after breastfeeding were not different (N=11 healthy infants, p=0.57). The short-term reproducibility in healthy infants (N=54) was satisfactory (intraclass correlation coefficient=0.94).

Conclusion: In infants with airway diseases, lung function tests prior to FeNO measurement did not influence FeNO values and FeNO values did not change after sedation. Oral FeNO values were significantly lower than mixed (oral + nasal) FeNO, and breastfeeding did not influence FeNO. Short-term reproducibility in awake healthy infants was good.

Introduction

Guidelines for the measurement of fractional exhaled nitric oxide (FeNO) in older subjects recommend performing measurements before lung function tests (1, 2), because repeated spirometric maneuvers have been shown to transiently reduce FeNO levels in asthmatic adults (3-5) and children (6). However, it is not known whether such an effect is also present in infants.

In the last few years there has been an increasing interest in measuring FeNO in infants, as it might provide a useful tool to study and monitor bronchial inflammation in airway diseases early in life (7-11). Several methodological issues related to the measurements of FeNO have been identified in children below the age of 2 years (12). FeNO measurements in infants should be performed during quiet, regular tidal breathing, a condition that can be easily achieved with the infants sedated. However, only one study previously showed differences in FeNO values in sedated and conscious infants, with the latter having higher FeNO than the former (12). Current guidelines also suggest to perform FeNO measurements in infants excluding the nose, as nasal NO concentrations are higher than orally exhaled lower airway NO (13, 14). However, paranasal sinuses are not yet completely developed at a young age and the effect of nasal contamination may be less than in older children. Several foods and beverages have been shown to transiently influence FeNO (15-21). However, it has never been investigated whether breastfeeding immediately preceding FeNO measurements might influence FeNO values.

The aim of this study was to explore a number of methodological issues in infants' FeNO measurements. These included forced expiratory maneuvers immediately preceding FeNO measurements, sedation, the differences between oral and mixed FeNO values and whether breastfeeding influences FeNO values.

Methods

We evaluated 45 infants (20 boys) with airway diseases (mean [SD] age = 12.1 [4.1] months), who were referred to the department of pediatric respiratory medicine of the Sophia Children's Hospital in Rotterdam to perform lung function tests between August 2003 and August 2005. None of the infants used corticosteroids in the week prior to the measurements or beta-2 agonists within 24 hours of the measurements. At the time of testing all children with airway diseases were clinically stable and there were no signs of acute airway infections. FeNO was also measured in 83 healthy

infants (41 boys; mean [SD] age = 4.3 [4.3] months) participating in an ongoing birth cohort study (22). These infants were not sedated for ethical reasons.

Infant Lung Function Tests

Lung function measurements were performed when the infants were free from acute respiratory symptoms. To prevent the infants from waking up during the measurements, they were sedated with choral hydrate (50–100 mg/kg). Forced expiratory flow (VmaxFRC) was assessed using the end-tidal rapid thoracoabdominal compression technique (custom-made equipment; Department for Experimental Medical Instrumentation, Erasmus University Medical Center, Rotterdam, The Netherlands). The mean VmaxFRC of 3 to 5 technically acceptable measurements was recorded and expressed as Z score. Equipments and procedure were in accordance with published guidelines (23, 24).

FeNO measurements

Exhaled air samples were collected with a facemask placed over the infants' nose and mouth during tidal breathing. The facemask was connected to a two-way non-rebreathing valve (Hans Rudolph inc., Kansas City, Mo, USA) that allows inspiration of NO-free air from an NO-inert 750 mL Mylar balloon to avoid contamination by ambient NO. This balloon was connected to the inspiratory port if ambient levels were >10 parts per billion (ppb), allowing washout of the dead space of the lungs. Exhaled breath samples were collected into an NO-inert 150 mL Mylar balloon fitted with the expiratory port and 5 breaths were collected. Then FeNO was analyzed by a fast response NO analyzer (Sievers 280 B, Boulder, Co. USA) within 1 hour. The analyzer was calibrated according to the manufacturer instructions with 0 ppb and 200 ppb NO certified calibration gas (Hoek Loos, Barendrecht, the Netherlands). A FeNO measurement was considered successful if exhaled air was sampled during quiet tidal breathing, if the facemask was tightly fitted to nose and mouth during the whole procedure and if at least 5 breaths were obtained. Before each FeNO measurement, the ambient NO concentration was recorded.

Study design

FeNO was measured in 45 infants with airway diseases: the effect of forced expirations was evaluated in 19, the effect of sedation in 10 and the comparison between oral and mixed FeNO was performed in 24 of these infants. In 8 infants, 3

different measurements were performed in the same session: oral FeNO was followed by the measurement of mixed FeNO before and after LFT. Eighty-three healthy awake infants underwent FeNO measurements: the comparison between oral and mixed FeNO was performed in 18, the effect of breastfeeding on FeNO was evaluated in 11 and the short-term reproducibility of the method was assessed in 54 infants.

To examine the effects of forced expirations, FeNO measurements were performed before and within 5 minutes after lung function testing in 19 infants with airway diseases. The effect of sedation was studied in 10 infants in whom FeNO measurements were first performed prior to sedation and compared to the measurements performed during sedation. The effect of nasal contamination was examined in 42 infants (N=24 diseased and N=18 healthy infants). FeNO was measured with the mask placed over the mouth only (oral FeNO) or over nose and mouth (mixed oral + nasal FeNO).

In 11 healthy infants, FeNO was measured before and 5 min after breastfeeding.

In 54 healthy infants (20 boys, mean [SD] age=4.6 [4.1] months) FeNO was measured twice within 10 min in order to assess the short-term reproducibility of the method.

Throughout the paper, the term FeNO indicates mixed oral + nasal expired NO.

Statistical analysis

FeNO values were log-transformed prior to the analysis in order to obtain a near-normal distribution and analyzed by means of parametric tests. Then FeNO values were back transformed and presented as geometric mean with their 95% confidence interval [95% CI].

Comparisons between the different methods used were performed with the repeated measurements ANOVA. Regression analysis was used to evaluate the relation between FeNO and ambient NO concentrations and VmaxFRC (z-score).

The intraclass correlation coefficient was calculated and a Bland and Altman plot (25) was made to assess the short-term reproducibility of the FeNO measurements in healthy infants. Two-tailed p values < 0.05 were considered significant.

Results

Demographic and lung function characteristics of infants are presented in table 1.

Table 1. Study population.

	Healthy controls (N=100)	Recurrent wheezing (N=74)	Bronchopulmonary dysplasia (N=24)	Cystic fibrosis (N=20)
Age, months	3.9 [1.1-7.7]	13.4 [5.6-25.2]	10.8 [4.6-24.0]	12.5 [6.1-19.6]
Males/Females	62/38	48/26	12/12	9/11
Weight (kg)	6.0 [0.2]	10.4 [0.2]	7.3 [0.3]	8.6 [0.4]
Length (cm)	61.3 [0.7]	77.4 [0.7]	68.8 [1.3]	72.9 [1.2]

Age, expressed in months, is reported as mean [range]. Weight and length are presented as mean [SEM]. Age, weight and length were significantly different between the 4 groups of infants ($p<0.001$ for all characteristics on univariate analysis of variance).

Geometric mean [95% CI] FeNO values before and 5 min after LFT were 13.3 [8.9 – 19.8] ppb and 13.8 [10.0 – 19.1] ppb, respectively (p=0.7) (figure 1). No significant difference was observed in FeNO levels collected before and during sedation. Geometric mean [95% CI] FeNO values were 11.6 [6.1-22.2] ppb before and 14.8 [9.9-21.9] ppb during sedation (p=0.2) (figure 2).

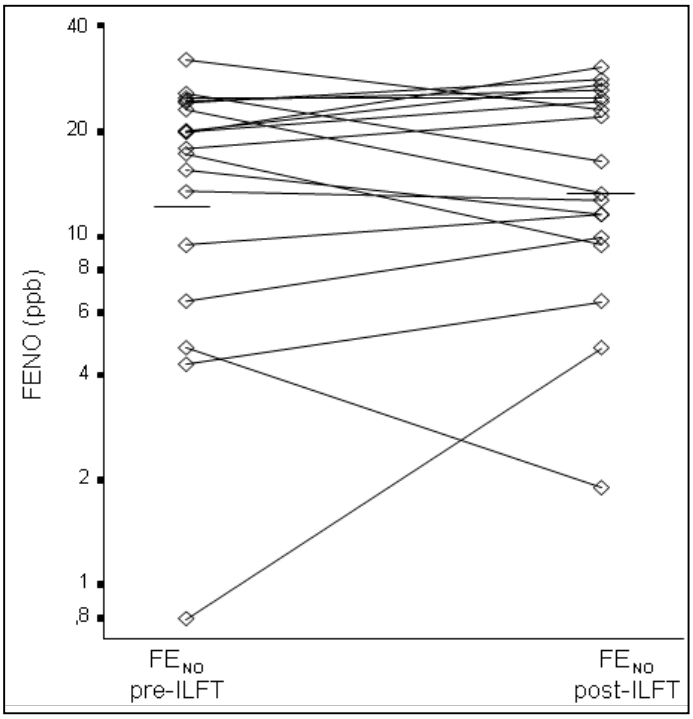

Figure 1 Individual FeNO values (represented on a log-scale) pre- and post- lung function tests (LFT) in 19 infants with airway diseases. The difference was not significant. Bars represent geometric mean FeNO.

The geometric mean [95% CI] of oral FeNO was significantly lower than mixed (oral + nasal) FeNO (4.5 [3.2-61] ppb and 10.5 [8.1-13.7] ppb, respectively; p<0.001). Such difference between oral and mixed FeNO remained significant in the group of 24 diseased infants (5.5 [3.4-8.8] ppb and 15.9 [12.1-21.1] ppb, respectively; p<0.001) and in the group of 18 healthy infants (3.4 [2.3-4.9] ppb and 6.1 [4.2-8.7] ppb, respectively; p=0.009) (figure 3). Geometric mean [95% CI] FeNO values before and 5 min after breastfeeding were not different (8.9 [4.5-17.5] ppb and 9.2 [4.7-17.9] ppb, respectively; p=0.57).

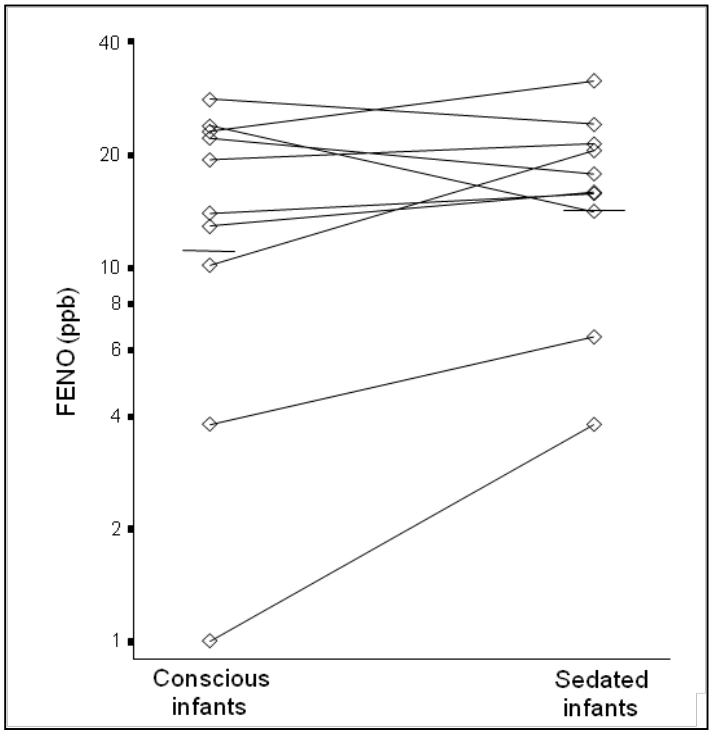

Figure 2 Individual FeNO values (represented on a log-scale) measured with the infants conscious and sedated (N=10 infants with airway diseases). The difference was not significant. Bars represent geometric mean FeNO.

No correlation was found between VmaxFRC and FeNO values in the group of diseased infants. No correlation between ambient NO concentration and FeNO values was observed.

FeNO measurements in healthy infants were highly reproducible (intraclass correlation coefficient=0.94) (figure 4). Although beyond the scope of this paper, we observed that mixed (oral + nasal) FeNO showed suggestive differences between diagnostic groups as have been found earlier with more sophisticated methodologies

(7, 10, 26) (table 1). Because of the small numbers, no statistical evaluations of these findings were done.

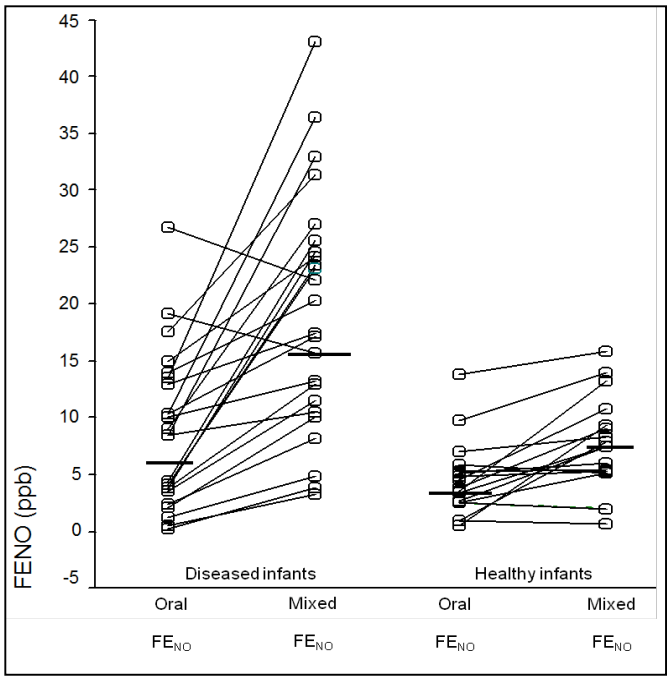

Figure 3 Individual oral and mixed (oral + nasal) FeNO values in diseased (left graph) and healthy infants (right graph). Oral FeNO was significantly lower than mixed FeNO in 24 sedated infants with airway diseases ($p<0.001$) and in 18 awake healthy infants ($p=0.009$). Bars represent geometric mean FeNO.

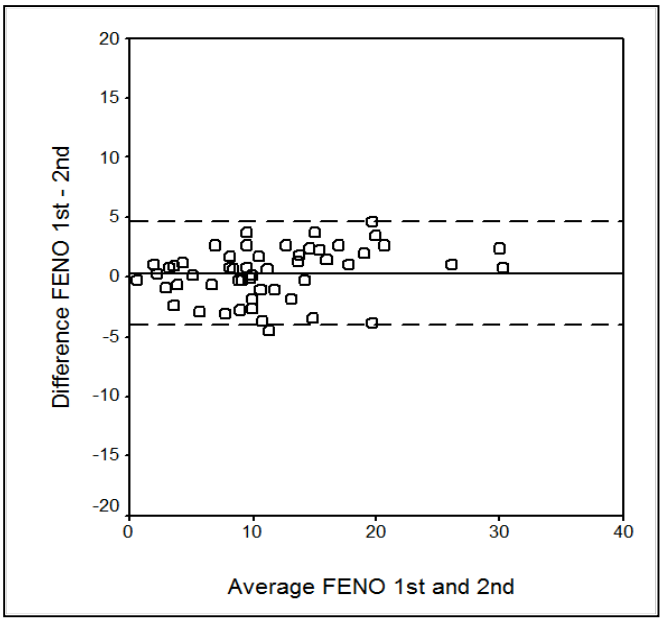

Figure 4 Bland and Altman plot showing good short-term reproducibility of the tidal breathing off-line FeNO measurements in a group of 54 healthy infants (mean difference [SD] of the 2 measurements=0.29 [2.16] ppb).

Table 1 Demographic characteristics of the study population.

Diagnosis	Boys / Girls	Age (months)	Weight (kg)	Length (cm)	FE_{NO} (ppb) *	V_{maxFRC} (ml/sec)	V_{maxFRC} Z-score
Post-ECMO (n=10)	5 / 5	10.9 [3.2]	8.5 [1.2]	73.2 [4.8]	17 [11.4-25.5]	200 [82.9]	-0.75 [0.09]
Esophageal atresia (n=7)	4 / 3	12.2 [3.8]	9.0 [1.2]	75.2 [3.8]	15.8 [9.5-26.2]	180 [16.0]	-1.23 [0.25]
CF (n=4)	0 / 4	19.2 [0.4]	9.5 [0.8]	77.6 [4.2]	3.7 [1.9-7.3]	243 [15.1]	-1.12 [0.18]
CHD (n=9)	5 / 4	11.5 [4.1]	8.9 [2.8]	74.8 [10.2]	13.9 [8.9-21.8]	149 [10.2]	-1.66 [0.14]
Wheezing (n=5)	3 / 2	14.5 [4.9]	10.0 [2.3]	77.0 [5.4]	20.8 [11.5-37.9]	143 [23.9]	-1.95 [0.34]
CLD (n=3)	1 / 2	12.4 [0.7]	8.1 [0.7]	72.9 [2.6]	17.7 [8.2-38.5]	106 [7.4]	-2.22 [0.13]
MAS (n=7)	2 / 5	9.6 [3.0]	8.5 [2.1]	72.2 [5.4]	21.6 [13.1-35.9]	129 [6.5]	-1.52 [0.09]
Healthy (n=83)	41 / 42	4.3 [4.3]	7.1 [2.6]	64.4 [9.2]	8.4 [7.0-10.1]	ND	ND

Data are reported as mean [SD]. FeNO values are presented as geometric mean [95% CI]. Definition of abbreviations: ECMO: extracorporeal membrane oxygenation received for different reasons; CF: cystic fibrosis; CHD: congenital heart disease; CLD: chronic lung disease; MAS: meconium aspiration. ND: not done.* FeNO was obtained during sedation except for the healthy infants.

Discussion

We evaluated several methodological aspects of FeNO measurements in infants. We could not demonstrate any effects of forced expiratory maneuvers immediately preceding FeNO measurements on FeNO in sedated infants with airway diseases. In addition, we did not observe an effect of sedation with chloral hydrate but we did demonstrate that oral FeNO values are significantly lower than mixed (oral + nasal) FeNO values in both diseased and healthy infants. Furthermore, we showed that breastfeeding did not systematically influence FeNO values in healthy infants. Also, we showed that tidal breathing off-line FeNO measurements are reproducible in healthy non-sedated infants.

It has been previously shown that the reproducibility of the off-line tidal breathing FeNO measurements in sedated infants was satisfactory, due to the reduced variability of the expiratory flow in infants when sedated (12). Previous studies investigated the effect of lung function tests on FeNO values in adults and children, but reached conflicting results (3-6, 27, 28). Current guidelines recommend refraining from forced expiratory maneuvers before FeNO measurements, as lower FeNO values have been reported after spirometric maneuvers in adults and children (3-6), but this

was not confirmed by others (27, 28). It has been hypothesized that the drop in FeNO values after lung function testing may be related to the reduction in NO production caused by an effect on neural mechanisms within the airways (3) rather than to a reduced airway caliber after such maneuvers (5). The end-tidal rapid thoracoabdominal compression technique does not require active cooperation by the infants and the driving force for the forced expiration is the externally applied jacket pressure, and this might explain why such passive maneuvers have a different effect on the source of NO production within the airways than active forced expirations.

It was previously reported that FeNO measured while infants were awake was significantly higher than when sedated (12). We could not demonstrate such a difference. Franklin et al (12) reported that of the 39 infants from whom they tried to collect breaths while awake, only 11 were considered fully cooperative, therefore a selection bias might have occurred. Moreover, it is unclear whether these findings pertain to diseased or healthy infants, or both. In our study we evaluated only infants with airway diseases and in those, sedation did not systematically influence FeNO values.

Although the paranasal sinuses are not yet fully developed in newborns and infants, the nasal cavity and sinuses have been shown to produce relatively large amounts of NO (29, 30) compared to the lower respiratory tract and a substantial NO production in the nose has been demonstrated early in the development and even in preterm infants (13, 14). Our results support the role of nasal mucosa and/or paranasal sinuses as an important source of NO production already in the first 2 years of life. This phenomenon raises an important methodological issue in the measurement of FeNO in infants. In fact, placing the facemask only on the infants' mouth was associated in our study with a considerable reduction of the success rate of the FeNO measurements, since infants tended either to wake up or to breathe irregularly when the nose was occluded. It is difficult for infants to breathe quietly when the nose is occluded. A possible alternative is the use of a facemask with a septum which can separate the nose from the mouth in order to measure oral FeNO selectively. The merits of avoiding nasal contamination for obtaining meaningful FeNO data remain to be elucidated (31). However, although this study was not powered to this purpose, it seems clear that mixed (oral + nasal) FeNO differentiates between infants with different airway diseases and healthy infants (table 1) in a similar way as has been described with more complicated methods (7, 10, 26).

The effect of foods and beverages on FeNO values has been previously investigated and guidelines recommend to refrain from eating and drinking before NO analysis (1, 2). An increase in FeNO has been found after the ingestion of nitrate or nitrate-containing foods, such as lettuce (15, 16, 21). Drinking of water and ingestion of caffeine may lead to transiently decreased FeNO levels (17, 18). We showed that breastfeeding immediately preceding FeNO measurements did not systematically influence FeNO values in healthy infants. This is an issue of practical relevance, as an infant who just received breastfeeding is more likely to cooperate and to maintain a tidal quiet breathing during the FeNO measurement, especially if awake.

The main limitation of our study was the relatively small number of infants in whom the effects of sedation and breastfeeding on FeNO values were evaluated. Further studies with larger groups of infants might be necessary in order to investigate any effect of such variables on FeNO values early in life.

Our study adds important methodological information related to the measurement of FeNO in infants. We propose that forced expiratory or tidal lung function tests with sedation are not to be avoided prior to FeNO measurements in infants with airway diseases. However, oral FeNO values are significantly lower than mixed (oral + nasal) FeNO already in the first 2 years of life, and techniques to avoid nasal contamination in this age group should be developed.

We conclude that offline FeNO measurement in infants is feasible, reproducible and seems not affected by forced expiratory maneuvers in infants with airway diseases. Also, FeNO values did not change after sedation in infants with airway disease. Oral FeNO values are significantly lower than mixed (oral + nasal) FeNO, indicating that the nose is a source of contamination of oral FeNO in both diseased and healthy infants. Furthermore, we observed no important effects of breastfeeding on FeNO in healthy infants.

References

1. ATS/ERS Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005. Am J Respir Crit Care Med 2005;171(8):912-930.

2. Baraldi E, de Jongste JC. Measurement of exhaled nitric oxide in children, 2001. Eur Respir J 2002;20(1):223-37.

3. Deykin A, Halpern O, Massaro AF, Drazen JM, Israel E. Expired nitric oxide after bronchoprovocation and repeated spirometry in patients with asthma. Am J Respir Crit Care Med 1998;157(3 Pt 1):769-75.

4. Deykin A, Massaro AF, Coulston E, Drazen JM, Israel E. Exhaled nitric oxide following repeated spirometry or repeated plethysmography in

healthy individuals. Am J Respir Crit Care Med 2000;161(4 Pt 1):1237-40.
5. Silkoff PE, Wakita S, Chatkin J, Ansarin K, Gutierrez C, Caramori M, et al. Exhaled nitric oxide after beta2-agonist inhalation and spirometry in asthma. Am J Respir Crit Care Med 1999;159(3):940-4.
6. Gabriele C, Pijnenburg MW, Monti F, Hop W, Bakker ME, de Jongste JC. The effect of spirometry and exercise on exhaled nitric oxide in asthmatic children. Pediatr Allergy Immunol 2005;16(3):243-7.
7. Franklin PJ, Turner SW, Hall GL, Moeller A, Stick SM. Exhaled nitric oxide is reduced in infants with rhinorrhea. Pediatr Pulmonol 2005;39(2):117-9.
8. Moeller A, Franklin P, Hall GL, Turner S, Straub D, Wildhaber JH, et al. Inhaled fluticasone dipropionate decreases levels of nitric oxide in recurrenty wheezy infants. Pediatr Pulmonol 2004;38(3):250-5.
9. Mieskonen ST, Malmberg LP, Kari MA, Pelkonen AS, Turpeinen MT, Hallman NM, et al. Exhaled nitric oxide at school age in prematurely born infants with neonatal chronic lung disease. Pediatr Pulmonol 2002;33(5):347-55.
10. Ratjen F, Kavuk I, Gartig S, Wiesemann HG, Grasemann H. Airway nitric oxide in infants with acute wheezy bronchitis. Pediatr Allergy Immunol 2000;11(4):230-5.
11. Baraldi E, Dario C, Ongaro R, Scollo M, Azzolin NM, Panza N, et al. Exhaled nitric oxide concentrations during treatment of wheezing exacerbation in infants and young children. Am J Respir Crit Care Med 1999;159(4 Pt 1):1284-8.
12. Franklin PJ, Turner SW, Mutch RC, Stick SM. Measuring exhaled nitric oxide in infants during tidal breathing: methodological issues. Pediatr Pulmonol 2004;37(1):24-30.
13. Artlich A, Busch T, Lewandowski K, Schaible T, Falke KJ, Gortner L. Exhaled nitric oxide in preterm infants. Respir Physiol 1998;114(2):195-200.
14. Schedin U, Norman M, Gustafsson LE, Jonsson B, Frostell C. Endogenous nitric oxide in the upper airways of premature and term infants. Acta Paediatr 1997;86(11):1229-35.
15. Zetterquist W, Pedroletti C, Lundberg JO, Alving K. Salivary contribution to exhaled nitric oxide. Eur Respir J 1999;13(2):327-33.
16. Olin AC, Aldenbratt A, Ekman A, Ljungkvist G, Jungersten L, Alving K, et al. Increased nitric oxide in exhaled air after intake of a nitrate-rich meal. Respir Med 2001;95(2):153-8.
17. Byrnes CA, Dinarevic S, Busst CA, Shinebourne EA, Bush A. Effect of measurement conditions on measured levels of peak exhaled nitric oxide. Thorax 1997;52(8):697-701.
18. Bruce C, Yates DH, Thomas PS. Caffeine decreases exhaled nitric oxide. Thorax 2002;57(4):361-3.
19. Yates DH, Kharitonov SA, Robbins RA, Thomas PS, Barnes PJ. The effect of alcohol ingestion on exhaled nitric oxide. Eur Respir J 1996;9(6):1130-3.
20. Persson MG, Cederqvist B, Wiklund CU, Gustafsson LE. Ethanol causes decrements in airway excretion of endogenous nitric oxide in humans. Eur J Pharmacol 1994;270(4):273-8.
21. Vints AM, Oostveen E, Eeckhaut G, Smolders M, De Backer WA. Time-dependent effect of nitrate-rich meals on exhaled nitric oxide in healthy subjects. Chest 2005;128(4):2465-70.
22. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol 2004;18(1):61-72.
23. Sly PD, Tepper R, Henschen M, Gappa M, Stocks J. Tidal forced expirations. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/American Thoracic Society. Eur Respir J 2000;16(4):741-8.
24. Tepper RS, Reister T. Forced expiratory flows and lung volumes in normal infants. Pediatr Pulmonol 1993;15(6):357-61.
25. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;1(8476):307-10.
26. Hall GL, Reinmann B, Wildhaber JH, Frey U. Tidal exhaled nitric oxide in healthy, unsedated newborn infants with prenatal tobacco exposure. J Appl Physiol 2002;92(1):59-66.
27. Tee AK, Hui KP. Effect of spirometric maneuver, nasal clip, and submaximal inspiratory effort on measurement of exhaled nitric oxide levels in asthmatic patients. Chest 2005;127(1):131-4.
28. Kissoon N, Duckworth LJ, Blake KV, Murphy SP, Lima JJ. Effect of beta2-agonist treatment and spirometry on exhaled nitric oxide in healthy children and children with asthma. Pediatr Pulmonol 2002;34(3):203-8.
29. Lundberg JO, Farkas-Szallasi T, Weitzberg E, Rinder J, Lidholm J, Anggaard A, et al. High nitric oxide production in human paranasal sinuses. Nat Med 1995;1(4):370-3.
30. Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Nitric oxide in exhaled air. Eur Respir J 1996;9(12):2671-80.

31. Martinez T, Weist A, Williams T, Clem C, Silkoff P, Tepper RS. Assessment of exhaled nitric oxide kinetics in healthy infants. J Appl Physiol 2003;94(6):2384-90.

4

Exhaled nitric oxide differentiates airway diseases in the first 2 years of life

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Abstract

Background: Fractional exhaled nitric oxide (FeNO) levels are increased in children and adults with asthma, while low levels have been found in cystic fibrosis and primary ciliary dyskinesia.

Objectives: The aim of this study was to investigate whether FeNO measurements could distinguish between children below the age of 2 with different airway diseases.

Methods: FeNO measurements were performed in 118 infants aged between 4.6 and 25.2 months: 74 infants with recurrent wheezing (RW), 24 with bronchopulmonary dysplasia (BPD) and 20 with cystic fibrosis (CF). FeNO was measured also in 100 healthy controls aged between 1.1 and 7.7 months.

Results: Geometric mean [95% CI] FeNO values were 10.4 [9.1-12.0] parts per billion (ppb) in healthy infants, 18.6 [15.6-22.2] ppb in wheezy infants, 11.7 [8.2-16.8] ppb in BPD infants and 5.9 [3.4-10.1] ppb in CF infants. FeNO in wheezers was higher than in controls, BPD and CF ($p=0.009$; $p=0.038$; $p<0.001$, respectively). Atopic wheezers showed higher FeNO than non-atopic wheezers ($p=0.04$). CF infants had lower FeNO than healthy controls and BPD infants ($p=0.003$ and $p=0.043$, respectively). FeNO values in BPD and control infants were not different.

Conclusion: FeNO is helpful to differentiate various airway diseases already in the first 2 years of life.

Introduction

The fractional concentration of nitric oxide in exhaled air (FeNO) has been suggested as a marker of bronchial eosinophilic inflammation. Increased FeNO levels have been found in asthmatic adults [1] and children with symptoms of asthma and atopy [2]. Guidelines for the measurement of FeNO are available for both adults and children [3, 4] and normal values for healthy children between 4 and 17 years of age have been recently published [5]. Although in the last decade there has been a growing interest in measuring FeNO in non-cooperative young children as well, few studies have investigated FeNO as marker of bronchial inflammation in children below the age of 2 years. It has been shown that infants with recurrent wheeze have elevated levels of FeNO during exacerbations that rapidly decrease after steroid therapy [6], suggesting that eosinophilic airway inflammation is present in early childhood wheeze. Low FeNO levels have been found in infants with cystic fibrosis [7], primary ciliary diskinesia [8] and rhinorrhea [9]. A recent study by Baraldi and coworkers showed that school-age children with bronchopulmonary dysplasia (BPD) and airflow limitation had lower FeNO levels than healthy matched controls and asthmatic children, suggesting that airflow limitation in children with BPD might not be related to ongoing inflammation as is the case in asthma [10]. It is well known that infants with BPD have an early inflammatory response followed by chronic inflammation and airways remodeling [11-13]. Only one study previously reported high FeNO levels in infants with chronic lung disease [14].

The aim of the present study was to measure FeNO in infants below the age of 2 years and to evaluate whether FeNO could be used to differentiate airways diseases in the first 2 years of life.

Methods

Subjects

FeNO measurements were conducted in 118 infants with different respiratory diseases, who either participated in other clinical trials [15] or were referred to the department of pediatric respiratory medicine, Sophia Children's Hospital in Rotterdam to perform lung function tests as part of the routine patient care. As control group we took a random sample of 100 healthy infants who participate in an ongoing birth cohort study [16]. All these infants had been free of significant respiratory symptoms

since birth and FeNO measurements were performed at about 6 weeks or 6 months of age.

Parents gave written informed consent. The Medical Ethical Committee of the Erasmus University Medical Centre approved the study.

Infants with recurrent wheezing (N=74). Recurrent wheezing was defined as 3 or more reported wheezing episodes, or at least 1 period of persistent wheezing longer than 2 months, but not continuously present from birth on. Fifty-three infants had a high risk for atopy, defined as parental history of allergy, asthma, eczema or hay fever, or had eczema, and 21 were non-atopic. Five infants had been treated with inhaled corticosteroids prior to the study.

Infants with bronchopulmonary dysplasia (N=24). BPD was defined as clinical signs of respiratory distress, chest radiograph abnormalities, and oxygen dependence at 28 days [11]. Infants were born at gestational age < 32 weeks and had a birth weight < 2000 g. During the month prior to the study, 5 infants had been using both inhaled corticosteroids and beta-2 agonists and 3 used corticosteroids only. At the time of the study, none of the infants required oxygen treatment.

Infants with cystic fibrosis (N=20). Cystic fibrosis (CF) was diagnosed on the basis of typical symptoms, a positive sweat test and two DNA CF mutations. Eight infants had been receiving antibiotic treatment during the week prior to the measurements. At the time of testing all children were clinically stable and there were no signs of acute airway infection.

Measurements

Infant Lung Function Tests

Lung function measurements were performed between 6 and 25 months corrected age, when the infants were free from acute respiratory symptoms. Infants refrained from using beta-2 agonists in the 24 hours prior to testing. Infants were sedated with choral hydrate (50–100 mg/kg). Functional residual capacity (FRCp) was measured by means of a modified whole body plethysmograph (Jaeger, Würzburg, Germany). The mean FRCp of 3 to 5 technically acceptable measurements was recorded and expressed as Z score [17]. Forced expiratory flow at functional residual capacity (VmaxFRC) was assessed using the end-tidal rapid thoracoabdominal compression technique (custom-made equipment; Department for Experimental Medical Instrumentation, Erasmus University Medical Center, Rotterdam, The

Netherlands). The mean VmaxFRC of 3 to 5 technically acceptable measurements was recorded and expressed as Z score [18, 19]. Equipment and procedures were in accordance with recently published guidelines [17, 18]. Measurement of airway resistance was performed by means of the interrupter technique (MicroRint; MicroMedical Ltd, Rochester, UK).

FeNO measurements

Exhaled air samples were collected with a facemask placed over infants' nose and mouth during tidal breathing while infants with lung diseases were sedated (50-100 mg/kg chloral hydrate). Because of medical ethical reasons, no chloral hydrate was used in healthy control infants. The facemask was connected to a two-way non-rebreathing valve (Hans Rudolph inc., Kansas City, Mo, USA) that allows inspiration of NO-free air from an NO-inert 750 mL Mylar balloon to avoid contamination by ambient NO. This balloon was connected to the inspiratory port if ambient levels were >10 parts per billion (ppb), allowing washout of the dead space of the lungs. Ten NO-free breaths were sufficient for this purpose as suggested by current guidelines [4]. Exhaled breath samples were collected into an NO-inert 150 mL Mylar balloon fitted with the expiratory port, and 5 breaths were collected in the sampling balloon. Then FeNO was analyzed by a fast response NO analyzer (Sievers 280 B, Boulder, Co. USA) within 1 hour of the measurement. The analyzer was calibrated in accordance to the manufacturer instructions with 0 ppb and 200 ppb NO certified calibration gas (Hoek Loos, Barendrecht, the Netherlands). All FeNO measurements were performed prior to lung function tests. In healthy infants FeNO measurements were also performed with the facemask placed over infants' nose and mouth, during quiet tidal breathing with infants in supine position, but without the use of sedation. A FeNO measurement was considered successful if exhaled air was sampled during quiet tidal breathing, if the facemask was tightly fitted to nose and mouth during the whole procedure and if at least 5 breaths were collected.

Before each FeNO measurement, the ambient NO concentration was recorded.

Data analysis

FeNO values were log-transformed in order to obtain a near-normal distribution and analyzed by means of parametric tests. The log-FeNO values were subsequently backtransformed and expressed as geometric means with their 95% confidence intervals [95% CI]. Univariate analysis of variance was used to compare demographic

characteristics and lung function parameters in the groups of infants. Regression analysis was used to evaluate the relation between FeNO and ambient NO concentrations. Comparisons between the groups of diseased infants and the healthy control group were performed by linear regression analysis, controlling for age, length and weight. As infants with lung diseases had different degrees of airflow limitation, a linear regression analysis was performed with log-FeNO as dependent variable, while controlling for age, weight, length and lung function parameters, including tidal volume and breathing frequency, of the 3 groups of diseased infants.

The area under the Receiver Operating Characteristic curve (ROC curve) was calculated in order to evaluate the accuracy of FeNO measurements in differentiating the diagnostic groups.

Proportions were compared by chi-square test. Correlations were evaluated by Pearson's test if the variables were normally distributed, otherwise the Spearman's coefficient was calculated. Student t-test was used to compare FeNO values in infants with high risk of atopy and non-atopic infants and to compare FeNO between infants who used medications and infants who did not. Two-tailed p-values of <0.05 were considered significant.

Results

All anthropometric characteristics, except for gender ratio, were significantly different between the 4 groups of infants as outlined in table 1.

Table 1 Study population

	Healthy controls (N=100)	Recurrent wheezing (N=74)	Bronchopulmonary dysplasia (N=24)	Cystic fibrosis (N=20)
Age, months	3.9 [1.1-7.7]	13.4 [5.6-25.2]	10.8 [4.6-24.0]	12.5 [6.1-19.6]
Males/Females	62/38	48/26	12/12	9/11
Weight (kg)	6.0 [0.2]	10.4 [0.2]	7.3 [0.3]	8.6 [0.4]
Length (cm)	61.3 [0.7]	77.4 [0.7]	68.8 [1.3]	72.9 [1.2]

Age, expressed in months, is reported as mean [range]. Weight and length are presented as mean [SEM]. Age, weight and length were significantly different between the 4 groups of infants ($p<0.001$ for all characteristics on univariate analysis of variance).

Lung function tests and FeNO measurements were successfully performed in all infants. Lung function parameters differed between the groups and are reported in table 2 together with FeNO values.

Table 2 Lung function parameters and FeNO values in different groups of infants.

	Healthy controls (N=100)	Recurrent wheezing (N=74)	Bronchopulmonary dysplasia (N=24)	Cystic fibrosis (N=20)
FE_{NO} (ppb)	10.4 [9.1-12.0]	18.6 [15.6-22.2]	11.8 [8.2-16.8]	5.9 [3.4-10.1]
FRC_p, ml/kg	ND	23.4 [0.5]	29 [1.6]	27.6 [1.3]
FRC_p, Z score		- 0.86 [0.1]*	0.26 [0.3]**	0.12 [0.3]
V_{maxFRC}, ml/s	ND	164.2 [8.6]	81.8 [7.3]	191.6 [20.6]
V_{maxFRC}, Z-score		-1.6 [0.1]*	-2.1 [0.1]§	-0.9 [0.2]
Rint, kPa/L per s	ND	2.9 [0.1]	4.0 [0.3]	2.7 [0.3]

Values are expressed as mean [SEM]. FeNO values are reported as geometric mean [95% CI].

Definition of abbreviations: FRCp = functional residual capacity; VmaxFRC = forced expiratory flow at FRC; Rint = airway resistance measured by means of the interrupter technique. ND = measurement not done.

*p=0.005 compared to CF; **p=0.001 compared to RW; §p=0.02 compared to RW and p<0.001 compared to CF.

Log-FeNO values were significantly related to weight (Pearson's coefficient=0.243; p=0.008) and length (Pearson's coefficient=0.221; p=0.016) only within groups of infants with wheeze, BPD and CF (figure 1a and 1b). Age was significantly related to FeNO when considering the groups altogether (Spearman's coefficient 0.208; p=0.002). No relation was found between ambient NO and measured FeNO in any group of infants.

Adjusting for age, length and weight, and in patient groups also for lung function parameters, the linear regression model showed that FeNO values in wheezy infants were higher than in controls (mean difference [95% CI] FeNO=1.6 [1.1 – 2.3] ppb; p=0.009), higher than in BPD infants (mean difference [95% CI] FeNO=1.9 [1.0 – 3.5] ppb; p=0.038) and higher than in CF infants (mean difference [95% CI] FeNO=3.6 [2.2 – 6.0] ppb; p<0.001). Atopic and high-risk wheezy infants (n=53) had higher geometric mean FeNO than non-atopic wheezy infants (n=21) (20.8 ppb and 14.0 ppb respectively; p=0.04), whereas FeNO in non-atopic wheezers was not different from healthy controls and BPD infants. CF infants showed lower FeNO than healthy controls and BPD infants (mean difference [95% CI] FeNO=2.0 [1.3 – 3.1] ppb; p=0.003 and 1.9 [1.0 – 3.6]; p=0.043, respectively), whereas FeNO was similar between BPD infants and the control group (mean difference [95% CI] FeNO=1.0 [0.7 – 1.5] ppb; p=0.8) (figure 2).

Figure 1a ***Figure 1b***

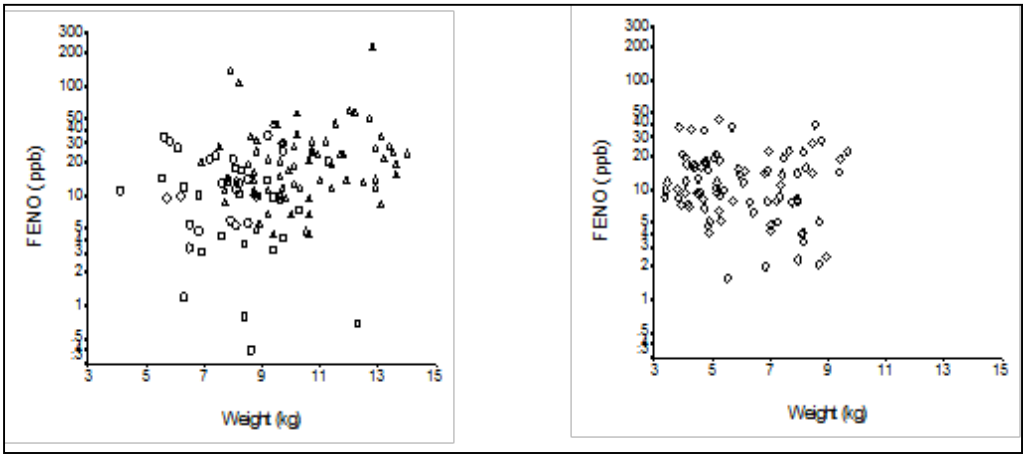

Figure 1a-b Relation between FeNO (represented on a log-scale) and weight within groups of diseased (**figure 1a**) and healthy (**figure 1b**) infants. Δ=Wheezy; ○=BPD; □=CF; ◊=Healthy

The area under the ROC curve of wheezy infants compared to healthy controls was 0.710 ($p<0.001$). A cut-off value of 14.1 ppb would give a sensitivity of 61% and specificity of 63%, whereas 10.1 ppb would give a sensitivity of 82% and specificity of 51% (figure 3). The area under the ROC curves for the other diagnostic groups were: 0.567 for BPD versus controls ($p=0.3$) and 0.374 for CF versus controls ($p=0.075$).

A significant correlation was found between FeNO and FRCp Z-score in the recurrent wheezers (Pearson's coefficient=0.252; $p=0.037$) (figure 4). No correlation was found between FeNO and lung function parameters within all other groups of infants.

No difference was obvious between FeNO of infants who used either inhaled corticosteroids (RW=18.5 ppb and BPD=14.4 ppb) or antibiotics (CF=5.3 ppb), when compared to the same category group of infants who did not use such medications (RW=18.6 ppb, BPD=10.6 ppb and CF=6.3 ppb). However, numbers were small and no further calculation was performed.

Discussion

We evaluated FeNO in infants with various airway diseases. We found that FeNO values in wheezy infants were higher and in CF infants lower than in controls. FeNO values in BPD and control infants were not significantly different. We also found that FeNO was significantly higher in wheezy infants with high-risk of atopy, compared to non-atopic wheezy infants.

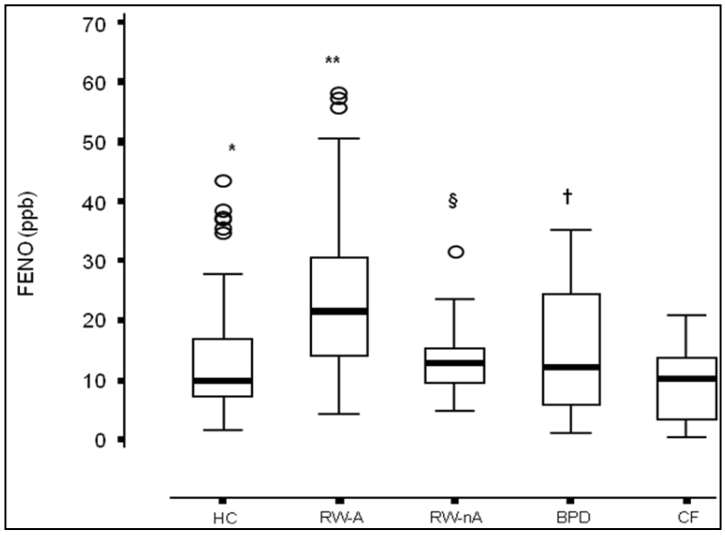

Figure 2 FeNO levels in different groups of infants. Median is line in box, while box limits represent 25th and 75th percentiles. Whiskers extend to 10th and 90th percentiles. Circles represent outliers.

Definition of abbreviations: HC=healthy controls (n=100); RW-A= recurrent wheezing, atopic (n=53); RW-nA= recurrent wheezing, non atopic (n=21); BPD=bronchopulmonary dysplasia (n=24); CF=cystic fibrosis (n=20).

* p=0.003 compared to RW-A and to CF; ** p=0.04 compared to RW-nA; p=0.005 compared to BPD; p<0.001 compared to CF; § p<0.001 compared to CF; † p=0.043 compared to CF.

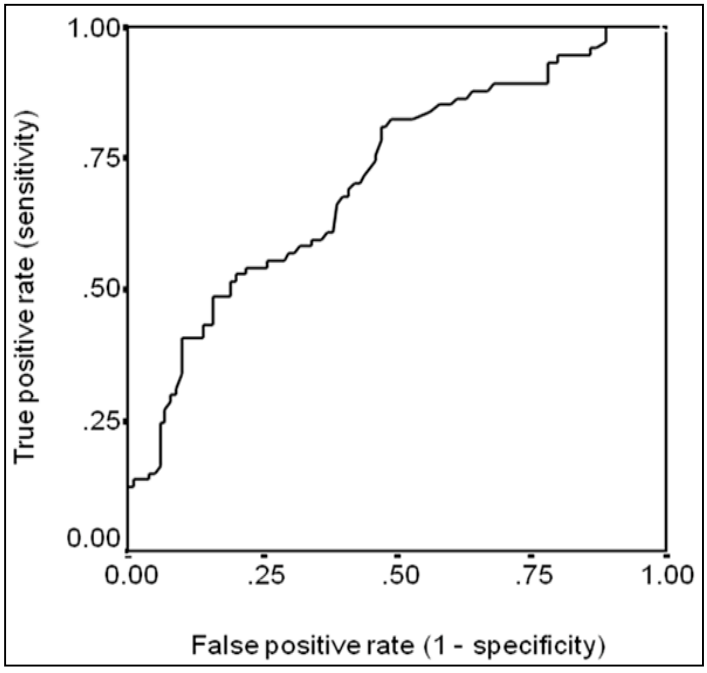

Figure 3 Receiver operating characteristic curve analysis of wheezy infants compared to controls.

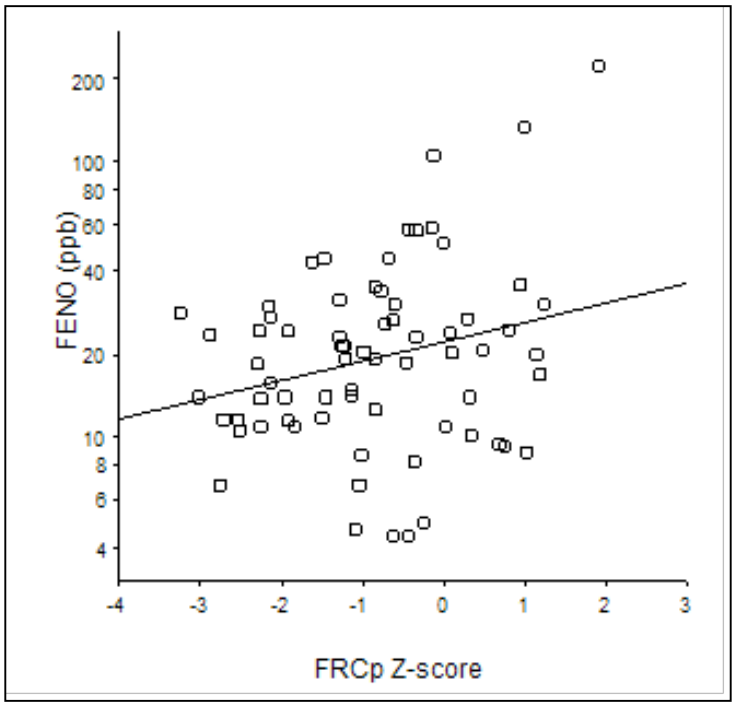

Figure 4 FeNO values (represented on a log scale) were correlated to FRCp Z-score in infants with RW (n=74). FeNO=1.346 + 0.069 FRCp Z-score (p=0.037).

The relation between FeNO and various airway diseases has extensively been studied in adults and older children. However, few studies evaluated FeNO in relatively large groups of infants with different airway diseases, while taking lung function parameters, tidal volume and breathing frequency into account. Moreover, we used a simple technique to collect exhaled air that may easily be applied in practice and does not require a sophisticated infant lung function lab and specialized staff [20].

Despite the fact that we used a relatively simple method, our results confirm the findings of Wildhaber et al [21], and Franklin et al [22] who showed that wheezy infants had higher FeNO than healthy controls. We found that atopic wheezers had higher FeNO than non-atopic wheezers and healthy controls, whereas no difference was obvious between non-atopic wheezers and healthy controls. This suggests that eosinophilic airway inflammation is already present in these infants with atopic wheeze. In agreement with the study of Mappa and coll. [23], we also found a correlation between FeNO levels and FRCp in wheezy infants, suggesting that air trapping in the lungs is related to airway inflammation.

In a recent study, Baraldi and coworkers found low levels of FeNO in school-age BPD survivors [10], and speculated that the defective NO synthesis and/or diffusion in the airway lumen could be either a consequence of the epithelial damage occurring in the early phases of BPD or could be attributed to a disturbed vascular growth, with a reduction in the pulmonary vascular bed [24]. Although it has been shown that BPD children have bronchial inflammation early in life, in our study we found that FeNO did not differ between BPD infants and healthy controls. The inflammation pathway in the airways of BPD seems to be mediated by neutrophilic granulocytes [25, 26], and this may explain our findings, as FeNO has been shown to reflect eosinophilic rather than neutrophilic inflammation of the airways.

Our results confirm the findings of Elphick et al showing that CF infants have lower FeNO levels than healthy controls [7]. The mechanisms underlying the low FeNO levels in CF subjects have been hypothesized to be due either to excess secretions in CF airways that might inhibit the diffusion of NO into the airway lumen, or to a primary defect in NO production. Since we measured FeNO in an early stage of the disease and only 8 CF infants had needed antibiotics for previous lung infections, our results would support the findings of Steagall et al that nitric oxide synthase 2 (NOS2) expression is regulated by the presence of active CFTR [27].

It has been previously shown that FeNO correlates with age of children between 4 and 17 years [5]. A correlation between FeNO and age has been also reported in children below the age of 2 only when FeNO was measured off-line during tidal breathing, with an increase of 0.1 ppb per week of age [22]. We confirm the previous findings, although the regression model showed a higher increase of FeNO per week of age in our study than previously reported (0.2 ppb per week of age). As healthy infants in our study had FeNO measured at the age of 6 weeks or 6 months, age-differences between controls and diseased infants were present. Significant differences were also present in other characteristics between diagnostic groups, such as length and weight, and these could have affected the results of our study. However, the regression model showed that differences in FeNO values between groups remained significant also after controlling for such variables, indicating that the variability of FeNO between groups cannot be attributed only to the differences in anthropometric characteristics of the study subjects.

A possible limitation of our study is the use of sedation for the FeNO measurements in the diseased groups, but not in healthy controls. In a previous, study Franklin and coworkers found a significant difference in FeNO levels between awake

and sedated infants, with the latter group having lower FeNO values than the former [28]. If this is the case, the FeNO values of the control group in our study might have been overestimated. However, in this case the differences in our study between controls and wheezy infants might have been underestimated. Another limitation of our study was that we collected mixed oral and nasal FeNO by collecting expired gas in a single-compartment facemask, therefore we could not distinguish between NO derived from upper and lower airways [29]. This might introduce variability and explain some of the overlap between groups. This would also imply that our study underestimates any differences between groups. However, it has been shown that both nasal and oral NO levels reflect mixed exhaled NO in infants [28] and techniques to avoid nasal contamination of exhaled air in infants require complicated and demanding equipment, unlikely to become useful in practice [30, 31]. Also, standardization for expiratory flow in infants is technically possible, but difficult [20] and may be unnecessary. Moreover, such overlap is a characteristic of any lung function test in a cross-sectional study, but this does not per definition invalidate the findings which may well be clinically useful and should be further evaluated for their merits, e.g. in follow-up studies. We did not measure lung function in healthy infants, therefore we could not compare lung function parameters in the control group to the other groups of diseased infants. The healthy infants participate in an ongoing birth cohort study and it was not ethically permitted to sedate before lung function tests. However, in this study we also found that FeNO values were significantly different between groups of infants with pulmonary diseases, showing the potential utility of FeNO in differentiating airway diseases already in the first 2 years of life.

As in the last decade there has been a growing interest in measuring FeNO also in non-cooperative children, our findings have important implications. The early detection of the inflammatory pattern underlying different airway diseases, could lead to a better targeted management of such children already in the first years of life. This is especially important for BPD infants, who share some clinical and spirometric features with bronchial asthma and are often treated with asthma medication including inhaled corticosteroids.

We conclude that FeNO measurements can be performed early in life using a simple methodology and can be considered potentially useful to differentiate between various airway diseases in children below the age of 2 years. It may be possible to improve the technique in order to evaluate the utility of FeNO measurements at individual level. Further studies should explore feasible techniques

to optimize FeNO measurements in infants, and the role of FeNO in follow-up studies of disease monitoring or guiding treatment in infants and young children with pulmonary diseases.

References

1. Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. Eur Respir J. 1993 Oct;6(9):1368-70.
2. Brussee JE, Smit HA, Kerkhof M, Koopman LP, Wijga AH, Postma DS, et al. Exhaled nitric oxide in 4-year-old children: relationship with asthma and atopy. Eur Respir J. 2005 Mar;25(3):455-61.
3. ATS/ERS Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005. Am J Respir Crit Care Med. 2005 Apr 15;171(8):912-30.
4. Baraldi E, de Jongste JC. Measurement of exhaled nitric oxide in children, 2001. Eur Respir J. 2002 Jul;20(1):223-37.
5. Buchvald F, Baraldi E, Carraro S, Gaston B, De Jongste J, Pijnenburg MW, et al. Measurements of exhaled nitric oxide in healthy subjects age 4 to 17 years. J Allergy Clin Immunol. 2005 Jun;115(6):1130-6.
6. Baraldi E, Dario C, Ongaro R, Scollo M, Azzolin NM, Panza N, et al. Exhaled nitric oxide concentrations during treatment of wheezing exacerbation in infants and young children. Am J Respir Crit Care Med. 1999 Apr;159(4 Pt 1):1284-8.
7. Elphick HE, Demoncheaux EA, Ritson S, Higenbottam TW, Everard ML. Exhaled nitric oxide is reduced in infants with cystic fibrosis. Thorax. 2001 Feb;56(2):151-2.
8. Karadag B, James AJ, Gultekin E, Wilson NM, Bush A. Nasal and lower airway level of nitric oxide in children with primary ciliary dyskinesia. Eur Respir J. 1999 Jun;13(6):1402-5.
9. Franklin PJ, Turner SW, Hall GL, Moeller A, Stick SM. Exhaled nitric oxide is reduced in infants with rhinorrhea. Pediatr Pulmonol. 2005 Feb;39(2):117-9.
10. Baraldi E, Bonetto G, Zacchello F, Filippone M. Low exhaled nitric oxide in school-age children with bronchopulmonary dysplasia and airflow limitation. Am J Respir Crit Care Med. 2005 Jan 1;171(1):68-72.
11. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001 Jun;163(7):1723-9.
12. Allen J, Zwerdling R, Ehrenkranz R, Gaultier C, Geggel R, Greenough A, et al. Statement on the care of the child with chronic lung disease of infancy and childhood. Am J Respir Crit Care Med. 2003 Aug 1;168(3):356-96.
13. Ozdemir A, Brown MA, Morgan WJ. Markers and mediators of inflammation in neonatal lung disease. Pediatr Pulmonol. 1997 Apr;23(4):292-306.
14. Leipala JA, Williams O, Sreekumar S, Cheeseman P, Rafferty GF, Hannam S, et al. Exhaled nitric oxide levels in infants with chronic lung disease. Eur J Pediatr. 2004 Sep;163(9):555-8.
15. Hofhuis W, van der Wiel EC, Nieuwhof EM, Hop WC, Affourtit MJ, Smit FJ, et al. Efficacy of fluticasone propionate on lung function and symptoms in wheezy infants. Am J Respir Crit Care Med. 2005 Feb 15;171(4):328-33.
16. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol. 2004 Jan;18(1):61-72.
17. Stocks J, Godfrey S, Beardsmore C, Bar-Yishay E, Castile R. Plethysmographic measurements of lung volume and airway resistance. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/ American Thoracic Society. Eur Respir J. 2001 Feb;17(2):302-12.
18. Sly PD, Tepper R, Henschen M, Gappa M, Stocks J. Tidal forced expirations. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/American Thoracic Society. Eur Respir J. 2000 Oct;16(4):741-8.
19. Tepper RS, Reister T. Forced expiratory flows and lung volumes in normal infants. Pediatr Pulmonol. 1993 Jun;15(6):357-61.
20. Hall GL, Reinmann B, Wildhaber JH, Frey U. Tidal exhaled nitric oxide in healthy, unsedated newborn infants with prenatal tobacco exposure. J Appl Physiol. 2002 Jan;92(1):59-66.
21. Wildhaber JH, Hall GL, Stick SM. Measurements of exhaled nitric oxide with the single-breath technique and positive expiratory

pressure in infants. Am J Respir Crit Care Med. 1999 Jan;159(1):74-8.
22. Franklin PJ, Turner SW, Mutch RC, Stick SM. Comparison of single-breath and tidal breathing exhaled nitric oxide levels in infants. Eur Respir J. 2004 Mar;23(3):369-72.
23. Mappa L, Cardinale F, Camodeca R, Tortorella ML, Pietrobelli A, Armenio L, et al. Exaled nitric oxide and air trapping correlation in asthmatic children. Allergy. 2005 Nov;60(11):1436-9.
24. Abman SH. Bronchopulmonary dysplasia: "a vascular hypothesis". Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 1):1755-6.
25. Carlton DP, Albertine KH, Cho SC, Lont M, Bland RD. Role of neutrophils in lung vascular injury and edema after premature birth in lambs. J Appl Physiol. 1997 Oct;83(4):1307-17.
26. Ferreira PJ, Bunch TJ, Albertine KH, Carlton DP. Circulating neutrophil concentration and respiratory distress in premature infants. J Pediatr. 2000 Apr;136(4):466-72.
27. Steagall WK, Elmer HL, Brady KG, Kelley TJ. Cystic fibrosis transmembrane conductance regulator-dependent regulation of epithelial inducible nitric oxide synthase expression. Am J Respir Cell Mol Biol. 2000 Jan;22(1):45-50.
28. Franklin PJ, Turner SW, Mutch RC, Stick SM. Measuring exhaled nitric oxide in infants during tidal breathing: methodological issues. Pediatr Pulmonol. 2004 Jan;37(1):24-30.
29. Artlich A, Busch T, Lewandowski K, Jonas S, Gortner L, Falke KJ. Childhood asthma: exhaled nitric oxide in relation to clinical symptoms. Eur Respir J. 1999 Jun;13(6):1396-401.
30. Martinez T, Weist A, Williams T, Clem C, Silkoff P, Tepper RS. Assessment of exhaled nitric oxide kinetics in healthy infants. J Appl Physiol. 2003 Jun;94(6):2384-90.
31. Artlich A, Jonsson B, Bhiladvala M, Lonnqvist PA, Gustafsson LE. Single breath analysis of endogenous nitric oxide in the newborn. Biol Neonate. 2001 Jan;79(1):21-6.

5

Fractional exhaled nitric oxide in infants during cow's milk food challenge

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Abstract

Background: Cow's milk allergy is the most common food allergy in early childhood. The golden standard for the diagnosis of cow's milk allergy is a food challenge after a period of elimination. Increased levels of exhaled nitric oxide (FeNO) have been shown after bronchial allergen provocation.

Objectives: We evaluated whether FeNO may also be a predictor of a positive reaction during cow's milk challenge in infants.

Methods: Forty-four infants (mean age [range]: 4.2 [3.7-4.6] months) suspected of cow's milk allergy underwent an open food challenge with cow's milk formula administered in ascending quantities, starting with 2ml and then 6, 20, 60 and 200ml until a clinical reaction occurred. Off-line FeNO samples were obtained during tidal breathing by means of a facemask covering infants' nose and mouth. FeNO was measured twice before the challenge (baseline), immediately before each new dose of milk and after a positive reaction or after the last dose of milk.

Results: Eleven children showed immediate positive clinical responses to cow's milk, whereas 13 infants presented only a late-type reaction. FeNO values before or after a positive reaction (either immediate or late) were not different from FeNO values at baseline. Baseline FeNO in infants with a positive reaction did not differ from FeNO in infants without a reaction at any time point.

Conclusion: FeNO values are not predictive and not related to the occurrence of a positive reaction during a cow's milk challenge in infants.

Introduction

Cow's milk allergy (CMA) is the most common food allergy in early childhood [1]. From prospective studies, the incidence is estimated to be 2-5% [2]. It has been hypothesized that CMA is due to immaturity of local and systemic immune responses combined with an increased permeability of the gut [3]. This could lead to presentation of large milk peptides to the immune system, triggering an allergic immune reaction in susceptible infants [4]. Although the majority of infants with CMA become tolerant in the first years of life, many continue to have CMA throughout childhood [5].

To date, the golden standard for the diagnosis of CMA is a food challenge after a period of elimination [6]. The food challenge includes subjective assessment, is time consuming and carries a small risk of anaphylactic shock. The allergic response to a food challenge can occur in different organ systems. The skin, upper and lower respiratory system and the gastrointestinal tract might be involved in case of a positive reaction to food challenge. How and in which organ an individual reacts is therefore dependent on systemic and local circumstances. Sub-clinical inflammation could be present in several organs and may well remain undetected during a food challenge. An objective marker of a positive response would therefore be desirable.

Fractional exhaled nitric oxide (FeNO) is a marker of bronchial inflammation in atopic asthma [7] and correlates with IgE levels of atopic wheezing infants [8]. It has been shown that FeNO values increase after bronchial allergen provocation in adults [9-11], whereas in children no change in FeNO has been reported after a nasal allergen challenge [12]. Furthermore, in mouse models it has also been shown that induced gastrointestinal allergy enhances allergic airway responses also to unrelated allergens, indicating the systemic nature of an allergic reaction [13].

Since no data is available on infants, we explored the relation between food allergy, airway inflammation and respiratory symptoms in atopic infants. Specifically, we sought to evaluate if changes in FeNO values during a cow's milk challenge test would reflect underlying immune responses and subsequently allow us to differentiate between tolerant and allergic infants.

Methods

The CAMEL-study (Cow's Milk Allergy with Elimination and Lactobacilli) explores the effects of non-pathogenic bacteria of the gut (probiotics) on the development of

the immune system in infants with CMA. Parents were informed and asked to participate as soon as an infant suspected of CMA was seen by the healthy baby clinics or by the general practitioner. Then infants were referred to the Sophia Children's Hospital in order to perform an open cow milk challenge test at the start of the study according to the guidelines of ESPGHAN and ESPACI and criteria of Bock and Sampson [2, 14]. Verum formula was Allergycare® (Royal Friesland Foods, Leeuwarden) and Protifar® (Nutricia, Zoetermeer) in an 11:3 mixture, resulting in 1.8 gram of cow milk protein per 100 ml. During the food challenge the formula was administered in ascending quantities, starting with 2ml and then 6, 20, 60 and 200 ml. The time between two consecutive doses ranged between 30 and 60 min. Infants were scored for 9 items divided into four main categories (general, skin, gastro-intestinal, respiratory) in a 0-3 system (0=none, 1=light, 2=moderate, 3=severe). A test was considered positive if one item scored 3 or if 2 or more items scored 2 (table 1).

One to 2 hours after the final dose patients were discharged and contacted by telephone the next morning to assess whether a late type reaction had occurred, such as vomiting and diarrhoea. Patients attended the outpatient clinic the next day only if parents reported skin problems that needed to be evaluated by a physician. Only infants with a positive challenge underwent a skin prick test using fresh cow's milk and venous IgE assessment (Pharmacia, Uppsala, Sweden).

FeNO measurements were performed off-line, during tidal breathing and without the use of sedation, with infants in supine position, as previously described in detail [15]. Briefly, we collected mixed exhaled air during quiet breathing via a silicon facemask covering infant's nose and mouth. A measurement was considered successful if at least 5 breaths could be sampled during quiet tidal breathing with the facemask tightly fitted to nose and mouth. Before each measurement, ambient NO was recorded. FeNO was measured twice within 10 min before the challenge and the mean of the two measurements was taken as baseline. Then FeNO was measured immediately before every new dose of cow's milk. The last FeNO measurement was performed 1 hour after the highest dose, or after a positive reaction. Infants were included in the analysis only if they successfully performed at least 75% of the attempted FeNO measurements including baseline and final FeNO measurement.

The medical ethical committee of the Sophia Children's Hospital approved the study. Parents gave written informed consent.

Table 1 Scoring system for response to cow's milk food challenge.

Score	Subjective symptoms	Anaphylaxis	Urticaria	Rash	Itch	SCORAD increase	Gastro-intestinal: vomiting, diarrhoea	Sneezing	Wheezing
0	Absent	no change	none	absent	absent	0-10%	absent	absent	absent
1	Minimal nausea or pain, no change in activity	n/a	< 3	minimal, small areas of faint erythema (<20% surface)	minimal, occasional scratching moderate	10-20%	minimal, 1 episode	minimal, sneezes rarely	minimal, expiratory wheezing to auscultation
2	moderate, frequent nausea or pain, decreased activity	n/a	> 3 and < 10	moderate, areas of erythema and macula (>20% and <50% surface)	scratching continuously for > 2 minutes at a time	20-30%	moderate, 2-3 episodes (or 1 episode of vomiting and 1 episode of diarrhoea)	moderate, sneezes < 10; intermittent rubbing of nose and/or eyes	moderate, dyspnoea; inspiratory and expiratory wheezing
3	severe, notably distressed, continuous crying	Anaphylactic shock	> 10 generalized	severe, generalized erythema (>50%) or >25% of the surface erythemato-squamous lesions / vesicles	continuous scratching with excoriations	>30%	severe, > 3 episodes (or 2 episode of vomiting and 2 episode of diarrhoea)	severe, continuous rubbing of nose / eyes; periorbital swelling	severe, dyspnoea, use of accessory muscles

Infants were scored for 9 items on a 0-3 scale each. A test was considered positive if one item scored 3 or if 2 or more items scored 2.

Statistical Analysis

FeNO values were log-transformed and then analyzed by means of parametric tests. FeNO reproducibility was assessed according to Bland and Altman [16] and was quantified by the intraclass correlation coefficient (ICC). The Cox proportional hazards regression model, with FeNO changes as a time dependent variable, was used in order to investigate whether these changes were related to a positive clinical reaction to cow's milk. Additionally, baseline FeNO values of infants with a positive reaction were compared to those of infants without reaction at any time point by means of the t-test. Regression analysis was used to evaluate the influence of ambient NO on baseline FeNO. FeNO values are reported in parts per billion (ppb).

For all statistical tests, a two-tailed p value < 0.05 was considered significant.

Results

Fifty-eight children underwent the open challenge with cow's milk between September 2004 and March 2005. Forty-four infants (83%, 32 boys, mean age [range]=4.2 [3.7-4.6] months) successfully performed FeNO measurements during the open food challenge (figure 1).

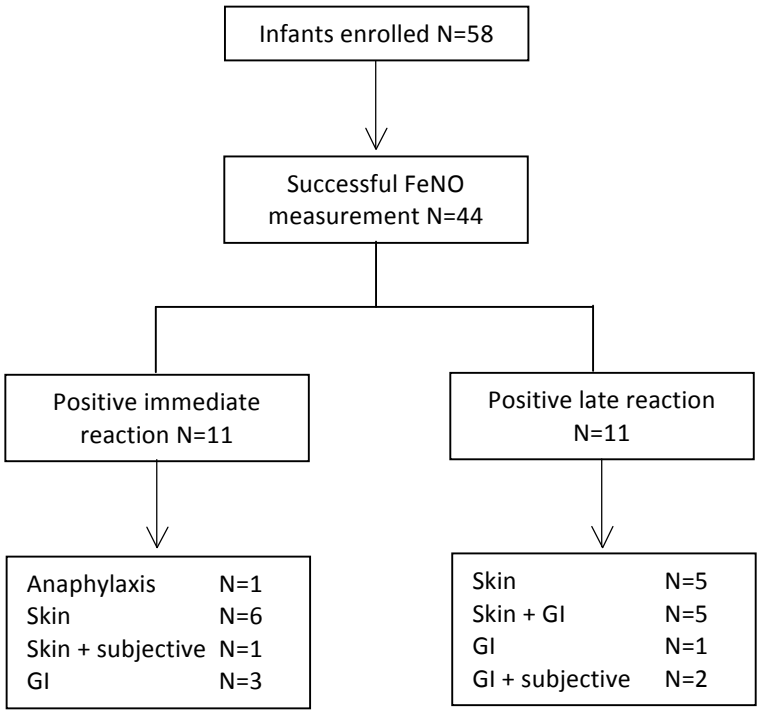

Figure 1 Flow chart of the study population.

The two baseline FeNO measurements were successfully performed in 39 infants and showed good reproducibility (intraclass correlation coefficient=0.88, mean difference [SD]: -0.29 [4.6] ppb) (fig 2). Hence, the geometric mean of the 2 baseline values was calculated and used as the individual baseline for the analysis. Immediate reactions occurred in 11 infants (25%), whereas in 13 infants (29%) a late-type reaction was recorded. Subjective symptoms, whether during the challenge or reported by parents, are difficult to interpret and debatable. Therefore we performed a separate analysis after excluding all patients who reported only subjective symptoms during and after the challenge. Such exclusion did not modify our results.

FeNO values before and after a positive reaction did not differ from FeNO at baseline. FeNO was not different in infants with a positive immediate clinical reaction compared to infants without reaction at any time point (fig 3). The results did not change when infants with a late type reaction were considered in the analysis.

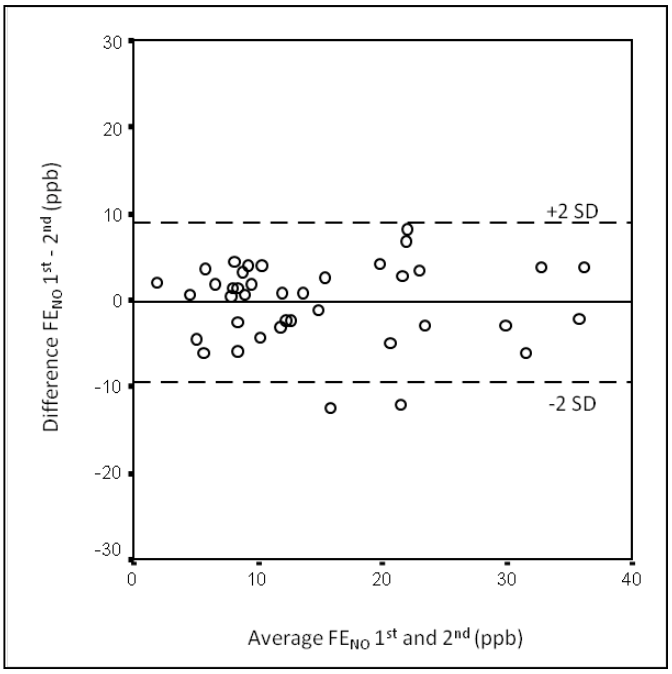

Figure 2 Bland and Altman plot showing good agreement of the two baseline FeNO measurements in 39 infants (mean difference [SD] of the two mesurements = -0.29 [4.6] ppb)

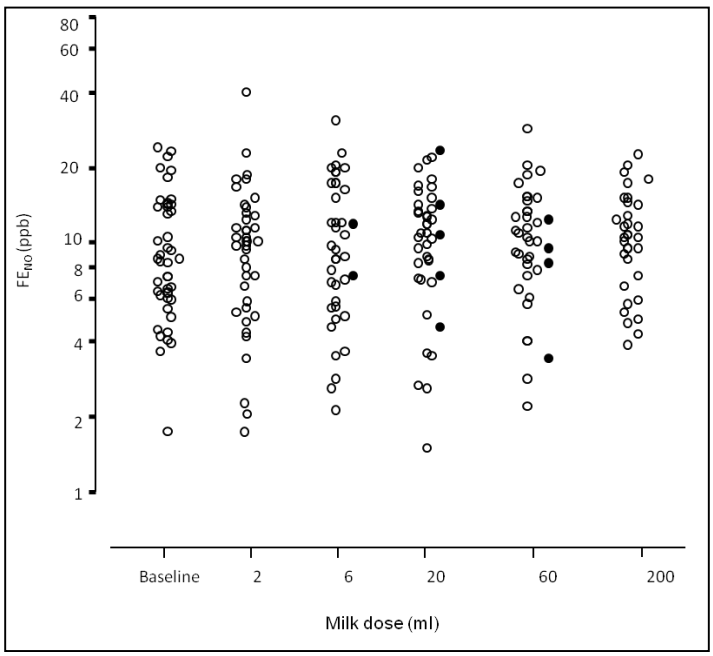

Figure 3 Individual FeNO values of 44 infants suspected of CMA. Open circles: children witout immediate clinical response; dark circles: children with immediate clinical response (n=11) to the subsequent cow's milk dose. There was no difference between FeNO of children with or without positive immediate reaction.

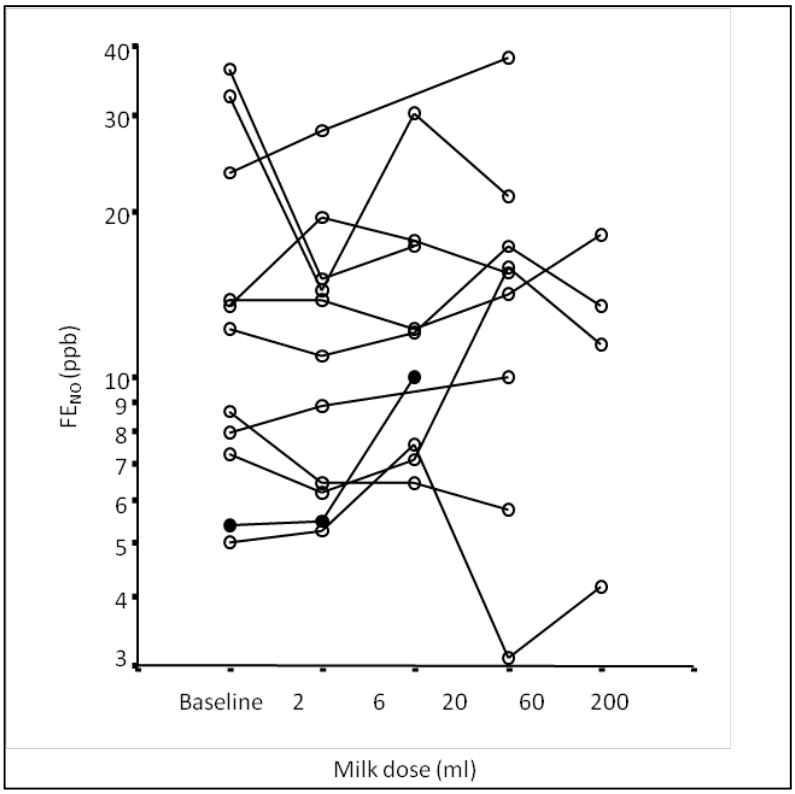

Figure 4 Individual FeNO values of infants with immediate positive reaction (N=11) at different time points, until a reaction occurred. Dark circles represent individual FeNO values of the infant who experienced anaphylactic shock.

Individual FeNO values of infants with a positive immediate reaction at different time points are reported in fig 4. A single infant developed a severe, systemic immediate response (anaphylaxis). In this case we found rise in FeNO from 5.4 ppb at baseline to 9.9 ppb after the last dose of milk was introduced (fig 4). Due to the design of the CAMEL-study cow's milk specific IgE and skin prick test were only performed in a subset of 29 patients. Total IgE levels ranged from 0 to 270 kU/L, mean 29 kU/L. Seven infants (24%) had a positive skin prick test and IgE directed against cow's milk. No correlation was found between IgE (total or cow's milk specific) and FeNO. No relation was found between FeNO and ambient NO.

Discussion

We evaluated whether changes in FeNO levels were predictive of a positive response in infants undergoing cow's milk challenge. This study was set up based on previous data that link FeNO production to (the severity of) airway inflammation, mucosal recruitment and activation of eosinophils in particular. To our knowledge,

this is the first study that investigated the possible role of FeNO during a food challenge in infants. The results do not support the hypothesis that FeNO can be used as a predictor or marker of a positive clinical reaction to cow's milk in an open food challenge.

In the last decade several studies evaluated the effect of allergen challenge on FeNO in asthmatics. Kharitonov et al. demonstrated that the late asthmatic response to bronchial allergen provocation in adults is associated with elevated FeNO, while the early response showed no significant increase in FeNO [9]. Olin et al found that atopic subjects have elevated levels of FeNO only if they had recently been exposed to the relevant allergen [17]. Atopic subjects not exposed to a relevant allergen or without symptoms of asthma or rhinitis showed normal FeNO. The relevant allergens of this study however did not include food allergens. Pedroletti et al demonstrated that a single nasal allergen challenge with cat dander did not induce bronchial inflammation and increase in FeNO in a pediatric population [12]. Although the challenge caused a local reaction in the nose, the authors speculated that a single nasal allergen challenge might have been insufficient to induce bronchial inflammation. Our study is in line with the findings of Kharitonov and Pedroletti, since we did not find any change in FeNO related to the early reaction to cow's milk challenge. We could not evaluate a late FeNO response to the allergen, since infants were discharged within 2 hours after the challenge was completed, and FeNO assessment did not take place at the time of the late responses. Apparently, FeNO was not predictive of a late response on the day of the challenge.

The lack of association between FeNO and the positive reaction to cow's milk in our study might be due to the absence of eosinophilic infiltration in the lower airway mucosa. Indeed, none of the 11 infants with immediate positive reactions showed respiratory symptoms during allergen challenge. Baseline FeNO did not differ between infants with or without immediate or late response to cow's milk, suggesting no pre-existing eosinophilic airway inflammation. It could be argued that FeNO is not a suitable marker in case of absence of airway symptoms. However, we don't think this is valid as eosinophilic airway inflammation and airway symptoms correlate weakly or not at all [18].

Most infants with a positive challenge presented a late-type reaction only with a combination of gastrointestinal and subjective symptoms. It has been hypothesized that delayed gastrointestinal symptoms due to CMA are related to a variant Th-2-type immune response, mainly characterized by the production of IgG4 rather than an IgE

mediated immune response [19]. In 5-year-old atopic children sensitized, to either an aeroallergen or food, Pleiss et al. found significantly higher FeNO values when compared to non-sensitized children [20]. Since FeNO is a marker of eosinophilic inflammation, which is unlikely in non-IgE mediated intolerance, an IgG-mediated response might explain why no change in FeNO values was observed in infants with a late-type positive reaction to cow's milk.

A single infant developed a severe systemic reaction (anaphylaxis), and this child showed a consistent increase in FeNO values preceding the response. This rise might have been useful in characterizing the systemic reaction at individual level, but the magnitude of the change was such that it could escape detection due to within-subject short-term variability.

Could our results be due to selection bias? All doctors collaborating in the CAMEL-study were trained to recognize and refer children with a high probability of CMA based on history and/or physical examination. Hence, in our study group a high percentage of the cow's milk challenges were positive. Any selection bias would therefore have led to an overestimation of the association. As we found no association at all, it seems unlikely that the selection of the population might have biased the results.

The method used for the measurement of FeNO in non-sedated infants might have introduced variability, since no control for the expiratory flow neither for the breathing frequency was performed, and it could be argued that this would reduce the possibility of detecting FeNO changes. This seems unlikely, however, as our method showed good reproducibility within infants and could differentiate between infants with different airway diseases [15, 21].

In summary, our data indicate that no correlation exists between changes in FeNO and the outcome of a food challenge in allergic children, suggesting that a positive reaction may not result from eosinophilic activation. We conclude that FeNO measurements cannot be used to predict or characterize a positive reaction to cow's milk in infants.

References

1. Sampson HA. Update on food allergy. J Allergy Clin Immunol. 2004 May;113(5):805-19; quiz 20.
2. Host A. Cow's milk protein allergy and intolerance in infancy. Some clinical, epidemiological and immunological aspects. Pediatr Allergy Immunol. 1994;5(5 Suppl):1-36.
3. Strobel S. Neonatal oral tolerance. Ann N Y Acad Sci. 1996 Feb 13;778:88-102.

4. Wal JM. Bovine milk allergenicity. Ann Allergy Asthma Immunol. 2004 Nov;93(5 Suppl 3):S2-11.
5. Iacono G, Cavataio F, Montalto G, Soresi M, Notarbartolo A, Carroccio A. Persistent cow's milk protein intolerance in infants: the changing faces of the same disease. Clin Exp Allergy. 1998 Jul;28(7):817-23.
6. Bruijnzeel-Koomen C, Ortolani C, Aas K, Bindslev-Jensen C, Bjorksten B, Moneret-Vautrin D, et al. Adverse reactions to food. European Academy of Allergology and Clinical Immunology Subcommittee. Allergy. 1995 Aug;50(8):623-35.
7. Barnes PJ, Kharitonov SA. Exhaled nitric oxide: a new lung function test. Thorax. 1996 Mar;51(3):233-7.
8. Cardinale F, de Benedictis FM, Muggeo V, Giordano P, Loffredo MS, Iacoviello G, et al. Exhaled nitric oxide, total serum IgE and allergic sensitization in childhood asthma and allergic rhinitis. Pediatr Allergy Immunol. 2005 May;16(3):236-42.
9. Kharitonov SA, O'Connor BJ, Evans DJ, Barnes PJ. Allergen-induced late asthmatic reactions are associated with elevation of exhaled nitric oxide. Am J Respir Crit Care Med. 1995 Jun;151(6):1894-9.
10. Obata H, Dittrick M, Chan H, Chan-Yeung M. Sputum eosinophils and exhaled nitric oxide during late asthmatic reaction in patients with western red cedar asthma. Eur Respir J. 1999 Mar;13(3):489-95.
11. Ricciardolo FL, Timmers MC, Sont JK, Folkerts G, Sterk PJ. Effect of bradykinin on allergen induced increase in exhaled nitric oxide in asthma. Thorax. 2003 Oct;58(10):840-5.
12. Pedroletti C, Lundahl J, Alving K, Hedlin G. Exhaled nitric oxide in asthmatic children and adolescents after nasal allergen challenge. Pediatr Allergy Immunol. 2005 Feb;16(1):59-64.
13. Brandt EB, Scribner TA, Akei HS, Rothenberg ME. Experimental gastrointestinal allergy enhances pulmonary responses to specific and unrelated allergens. J Allergy Clin Immunol. 2006 Aug;118(2):420-7.
14. Bock SA, Sampson HA, Atkins FM, Zeiger RS, Lehrer S, Sachs M, et al. Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. J Allergy Clin Immunol. 1988 Dec;82(6):986-97.
15. Gabriele C, van der Wiel EC, Nieuwhof EM, Moll HA, Merkus PJ, de Jongste JC. Methodological aspects of exhaled nitric oxide measurements in infants. Pediatr Allergy Immunol. 2007 Feb;18(1):36-41.
16. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986 Feb 8;1(8476):307-10.
17. Olin AC, Alving K, Toren K. Exhaled nitric oxide: relation to sensitization and respiratory symptoms. Clin Exp Allergy. 2004 Feb;34(2):221-6.
18. van den Toorn LM, Overbeek SE, de Jongste JC, Leman K, Hoogsteden HC, Prins JB. Airway inflammation is present during clinical remission of atopic asthma. Am J Respir Crit Care Med. 2001 Dec 1;164(11):2107-13.
19. Sletten GB, Halvorsen R, Egaas E, Halstensen TS. Changes in humoral responses to beta-lactoglobulin in tolerant patients suggest a particular role for IgG4 in delayed, non-IgE-mediated cow's milk allergy. Pediatr Allergy Immunol. 2006 Sep;17(6):435-43.
20. Pleiss LE, Evans M, Anderson EL, Da Silva DF, Pappas TE, Roberg KA, et al. Fractiona exhaled nitric oxide (FeNO) is a marker of allergic inflammation in high risk 5 year old children. J Allergy Clin Immunol. 2006(117(2)):S216.
21. Gabriele C, Nieuwhof EM, Van Der Wiel EC, Hofhuis W, Moll HA, Merkus PJ, et al. Exhaled nitric oxide differentiates airway diseases in the first two years of life. Pediatr Res. 2006 Oct;60(4):461-5.

6

Smoke exposure, airway symptoms and exhaled nitric oxide in infants: The Generation R Study

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Abstract

Objectives: We evaluated the effect of pre- and postnatal smoke exposure on exhaled nitric oxide (FeNO) in infants and investigated the association between respiratory symptoms and FeNO in the first 2 months of life.

Methods: The Generation R Study is a population-based prenatally recruited birth cohort. Exposures were assessed by means of questionnaires prospectively administered during pregnancy and after birth. Successful off-line FeNO measurements during tidal breathing were obtained in 187 infants (median age 6.9 weeks). The association between possible determinants and logFeNO was investigated with multiple linear regression analysis.

Results: Infants exposed pre- and postnatally to smoke showed lower FeNO than infants exposed only after birth (difference: 1.5 [1.0-2.1] ppb; p=0.042) and than never exposed infants (difference: 1.4 [1.0-1.8] ppb; p=0.052). FeNO was reduced in infants with severe upper respiratory symptoms compared to infants with non-severe symptoms (difference: 1.6 [1.0-2.4] ppb; p=0.047). Infants with symptoms of the lower respiratory tract had lower FeNO than asymptomatic infants (difference: 1.2 [1.0-1.5] ppb; p=0.046).

Conclusion: The nature of the association between smoke exposure and FeNO is dependent on timing and intensity of exposure. The occurrence and the severity of respiratory symptoms in the first 2 months of life are associated with lower FeNO.

Introduction

Fractional exhaled nitric oxide (FeNO) is increased in asthmatic adults [1], children [2], and infants with eczema [3] and recurrent wheezing[4], and has been proposed as a noninvasive marker of eosinophilic airway inflammation. Compared to healthy infants, lower FeNO levels have been found in infants with virus-associated acute wheezy bronchitis [5] and in infants with upper respiratory symptoms such as rhinorrhea [6]. Several pre- and postnatal factors have been shown to influence the levels of FeNO in infants, such as tobacco smoke exposure [7-9], coffee consumption during pregnancy [8], maternal atopic disease [8, 10], birth weight [11], gestational age [11, 12], gender [8] and infections [13]. However, the influence of risk factors for respiratory morbidity on FeNO in infancy is not clear. Previous studies investigating the association between smoke exposure, one of the best known risk factors for respiratory morbidity in infants, and FeNO have given conflicting results. Hall et al found lower FeNO in infants exposed to smoking during pregnancy than in unexposed infants [9]. In a subsequent report of the same authors, this difference was only significant in infants of mothers without atopic disease [8]. Also, the role of postnatal exposure to tobacco smoke in infants has been investigated, but the results are not consistent. In a recent paper, Franklin et al [7] reported higher FeNO in infants exposed to postnatal tobacco smoking, whereas previous studies did not show such an effect, or a lower FeNO in exposed asthmatics and healthy subjects [14, 15].

Previous studies that sought to investigate the effect of different determinants of FeNO levels in early infancy retrospectively assessed prenatal exposure variables after birth, rather than prospectively during pregnancy. Therefore, the temporality or succession of events is not documented and the exposure assessment is more prone to recall bias. The aim of this prospective birth cohort study was to evaluate whether and to what extent pre- and early postnatal exposures influence FeNO in early infancy. We also investigated the effect of upper and lower respiratory symptoms (URS and LRS) in the first 2 months of life on FeNO.

Subjects and methods

The Generation R Study is a prospective population-based prenatally recruited birth cohort study in Rotterdam. A randomly selected group of 1,232 Dutch pregnant women and their children have been enrolled in the Generation R focus study. In the focus study more detailed assessments of fetal and postnatal growth and

development are performed [16, 17]. Women were enrolled during pregnancy. Sociodemographic factors and exposure to risk factors for respiratory diseases were assessed by means of questionnaires, administered to the mother in early (gestational age < 18 weeks), mid- (gestational age 18 - 25 weeks) and late (gestational age ≥ 25 weeks) pregnancy and to the partner at 20 weeks. Information was gathered on the following exposure variables for both the mother and the partner: sociodemographic factors, smoking habits, atopy and siblings. A questionnaire was administered to the parents when the children were 2 months old and exposure variables were again assessed together with onset, occurrence and severity of URS and LRS.

Between November 2004 and September 2005, FeNO measurements were attempted in 225 infants participating in the focus study at a median [range] age of 6.7 weeks [3.7-16.9]. Mixed oral/nasal FeNO was measured off-line during tidal breathing with a facemask covering nose and mouth without the use of sedation as previously described [18]. A FeNO measurement was considered successful if exhaled air was sampled during quiet tidal breathing, if the facemask was tightly fitted to nose and mouth during the whole procedure and if at least five breaths were obtained. All FeNO measurements were conducted with the infants awake. Ambient NO was measured before each FeNO measurement (Sievers 280 B, Boulder, CO, USA) and in case of ambient NO concentration above 10 parts per billion (ppb), the infant inhaled at least 2 tidal breaths of NO-free air from an NO-inert 750 ml balloon in order to permit the washout of the dead space of the lungs [19]. However, as FeNO showed a positive significant association with ambient NO levels, we always included ambient NO in the multivariable models.

All infants were free of respiratory symptoms and had no clinical evidence of airways infection at the time of the measurement. The Medical Ethical Committee of the Erasmus Medical Center, Rotterdam, approved the study. Mothers and their partners received written and verbal information about the study and gave written informed consent.

Definition of variables considered in the analysis

Educational level of the mother was divided into 3 categories (lower, intermediate, higher vocational training) according to the classification of Statistics Netherlands [20]. Parental atopy was defined as self-reported or doctor diagnosed allergy or atopic disease (allergic asthma, hay fever, eczema). Prenatal maternal smoking was assessed in the first questionnaire by asking the mother whether she smoked during the

pregnancy (no, smoked until the pregnancy was known, or continued smoking after the pregnancy was known). In the second and third questionnaires, the mother was asked whether she had smoked in the past 2 months (no, yes). If the answer was positive at least at 2 time points, the infant was classified as prenatally exposed. Exposure to passive tobacco smoking was also assessed by asking whether people smoked regularly in the house or in the working environment of the mother during pregnancy. Gestational age, birth weight and length were obtained from midwife and hospital registries.

Postnatal exposure to tobacco smoke was assessed by asking whether the infant had been exposed to smoke by the mother or by any of the members of the household at least once a week. Also, parents were asked whether their child had had runny and/or blocked nose (URS), breathlessness, a whistling noise when breathing, wheezing, panting, difficult breathing and/or cough (LRS) in the past 2 months. Symptoms were considered severe if they required a visit to a physician, as reported by the parents.

Statistical Analysis

FeNO values were log-normally distributed. Univariable analyses using Student t test and simple linear regression were used to determine associations between logFeNO and the following explanatory variables: ambient NO, birth weight, gestational age, gender, breastfeeding, maternal educational level, maternal and paternal atopy, prenatal and postnatal tobacco smoke exposure, siblings, weight, length and age at the study date, upper and lower respiratory symptoms. Factors that had a significance level ≤ 0.1 from univariate analyses were included in the multiple linear regression models in order to evaluate the relation between logFeNO (dependent variable) and pre- and postnatal exposures while controlling for other relevant factors. Although not directly associated to FeNO in our study population, atopic status of the mother, birth weight, gender and age at the study date were included in all regression models as covariates, since these have been shown to influence FeNO or the occurrence of respiratory symptoms [4, 8]. Effect modification by maternal atopy and gender was investigated by adding interaction terms in the final models.

FeNO values were backtransformed after the analysis and are reported as geometric mean and 95% confidence interval [95% CI] in ppb. Comparisons of FeNO between groups are presented as geometric mean of the difference [95% CI]. Two-

tailed p value < 0.05 was considered significant. Data analyses were performed using the Statistical Package of Social Sciences version 11 for Windows (SPSS Inc, Chicago, IL, USA).

Due to the paucity of data in the literature, no power calculation could be performed in order to evaluate the size of the study needed to detect a difference in FeNO values between groups of infants.

Results

FeNO measurements were attempted in 225 infants and succeeded in 187 infants (success rate 83%). Thirty-eight measurements were excluded because a quiet tidal breathing pattern was not maintained during the whole procedure (n=31) or because less than 5 breaths could be collected in the sampling balloon (n=7).

Excluded infants had younger mothers (median age [range] 30.3 [18.5 – 40] yrs) and fathers (32.8 [25.3 – 39.6] yrs) than infants with successful FeNO measurements (p=0.003 and p=0.017, respectively; t-test), but the other baseline characteristics and anthropometrics at the study date did not differ between the two groups (table 1).

Table 1 Baseline characteristics and anthropometrics in the study population (n=187)

	Median	Range
Age mother (yrs)	32.5	18.5 - 42.9
Age father (yrs)	33.8	16-58.2
Gestational age at enrolment (wks)	13	8.5-23.2
Gestational age at birth (wks)	40.3	34.6-43.0
Birth weight (gr)	3520	1958-5170
Age at the study date (wks)	6.9	3.7-16.9
Weight at the study date (gr)	4890	3350-8230
Length at the study date (cm)	56.9	50.6-66.3

Pre- and postnatal exposures and FeNO

On univariable analysis, anthropometrics were not related to FeNO (table 2), whereas prenatal maternal smoking affected FeNO, with lower levels in exposed infants (p=0.047) (table 3). Paternal smoking and maternal passive tobacco smoke exposure during pregnancy did not affect FeNO, therefore an infant was considered prenatally exposed if the mother smoked during pregnancy, independent of other sources of smoke. With regard to prenatal smoke, 44 infants were exposed (23 only

prenatally and 21 pre- and postnatally), whereas 51 infants were exposed to environmental smoke after birth by the mother or by other members of the household (30 only postnatally and 21 pre- and postnatally). Gender, parental atopic status and maternal asthma were not related to FeNO (table 3). In order to compare the different smoke exposure categories directly, we created one variable resulting from the combination of prenatal and postnatal smoke exposure, with four mutually exclusive categories: never exposed, exposed only prenatally, exposed only postnatally and continuously exposed both pre- and postnatally. The association between the combined variable 'smoke exposure' and FeNO was significant in the univariable analysis (table 3) and also in the multivariable regression model (table 4). Infants exposed pre- and postnatally to smoke showed lower FeNO than infants exposed only after birth (difference: 1.5 [1.0-2.1] ppb; p=0.042) and than never exposed infants (1.4 [1.0-1.8] ppb; p=0.052) (figure 1). This association was independent of respiratory symptoms and not modified by gender (p for interaction = 0.69) or by maternal atopy (p for interaction = 0.46). However, among the 60 infants of atopic mothers, only 22 were exposed to smoke (6 only prenatally, 10 only postnatally and 6 pre- and postnatally). The association between ambient NO and FeNO remained significant (p<0.001) also in the multivariable model.

Table 2 Univariable analyses: FeNO and ambient NO and anthropometrics in the study population

Variables	Beta coefficient	95% CI
Ambient NO (ppb)	0.0069*	**0.004 – 0.01**
Gestational age (wks)	0.0145	**-0.016 – 0.045**
Birth weight (kg)	-0.0388	**-0.12 – 0.042**
Age at study date (wks)	0.0025	**-0.017 – 0.022**
Weight at study date (kg)	-0.0054	**-0.06 – 0049**
Length at study date (cm)	0.0054	**-0.011 – 0.021**

*p<0.001. Beta coefficients were estimated by linear regression analysis and should be judged as the change of logFeNO per unit change in the variables

Respiratory symptoms and FeNO

LRS were reported for 83 infants (63 non-severe and 20 severe), whereas 130 infants had URS (120 non-severe and 10 severe symptoms). In the multivariable analysis,

Table 3 Univariable analyses: pre and postnatal variables and FeNO

Variable	Geometric mean FE_{NO} [95%CI] (ppb)
Gender	
Boys (n=95)	10.6 [9.2 – 12.2]
Girls (n=92)	11.2 [9.7 – 12.9]
Exclusive breastfeeding	
No (n=123)	10.5 [9.2 – 11.8]
Yes (n=64)	11.7 [9.9 – 14.0]
Maternal education	
Low (n=5)	9.8 [5.2 – 18.3]
Intermediate (n=72)	10.9 [9.3 – 12.8]
High (n=110)	10.9 [9.6 – 12.5]
Maternal atopy	
No (n=127)	10.8 [9.6 – 12.2]
Yes (n=60)	11.0 [9.2 – 13.1]
Maternal asthma	
No (n=175)	10.8 [9.7 – 12.0]
Yes (n=12)	12.1 [8.1 – 18.2]
Paternal atopy	
No (n=143)	10.9 [9.7 – 12.2]
Yes (n=44)	10.8 [8.8 – 13.3]
Paternal smoking	
No (n=130)	10.6 [9.4 – 11.9]
Yes (n=57)	11.6 [9.7 – 13.9]
Maternal passive smoke exposure during pregnancy	
No (n=69)	11.1 [9.4 – 13.1]
Yes (n=118)	10.8 [9.5 – 12.2]
Maternal smoking during pregnancy	
No (n=143)	11.5 [10.3 – 12.9]
Yes (n=44)	9.1 [7.4 – 11.1] †
Postnatal environmental smoke exposure	
No (n=136)	11.1 [9.9 – 12.5]
Yes (n=51)	10.4 [8.6-12.6]
Smoke exposure ¶	
Never (n=113)	11.3 [9.9 – 12.8]
Prenatal only (n=23)	10.1 [7.6 – 13.4]
Postnatal only (n=30)	12.3 [9.6 – 15.8]
Pre- and postnatal (n=21)	8.1 [6.0 – 10.9] §
Siblings	
No (n=121)	11.1 [9.8 – 12.6]
Yes (n=66)	10.4 [8.8 – 12.4]
Upper respiratory Symptoms	
No (n=57)	11.7 [9.8 – 14.0]
Yes (n=130)	10.5 [9.4 – 11.9]
Lower Respiratory Symptoms	
No (104)	11.7 [10.3 – 13.4]
Yes (83)	9.9 [8.5 – 11.5] ‡

† p=0.047 compared to unexposed; ¶ this variable is the combination of the two variables above; § p=0.042 compared to never exposed and p=0.033 compared to exposed only postnatally; ‡ p=0.09 compared to asymptomatic infants; LogFeNO values were compared by using the Student t test

FeNO values were lower in infants with LRS (n=83) than in asymptomatic (n=104) infants (difference: 1.2 [1.0-1.5] ppb; p=0.046) (table 4), but no association was found between the severity of LRS and FeNO.

Gender and atopy did not modify this association (p for interaction=0.68 and p=0.88, respectively). Such difference was not found for URS in univariate analysis (p=0.3), nor when URS was added into the multivariable model as a binary outcome (p=0.9). However, considering the severity of the symptoms (coded as 0=no symptoms; 1=non-severe symptoms; 2=severe symptoms), infants with URS that required a visit by a doctor (n=10) had lower FeNO compared to infants with non-severe symptoms (difference: 1.6 [1.0-2.4] ppb; p=0.047) and tended to have lower FeNO than asymptomatic infants (difference 1.5 [0.9-1.2] ppb; p=0.075) (figure 2). This effect was independent of smoke exposure and LRS, still significantly associated to FeNO (table 4). Tobacco smoke exposure or parental atopy, as well as the other investigated determinants, were not associated with occurrence or severity of respiratory symptoms.

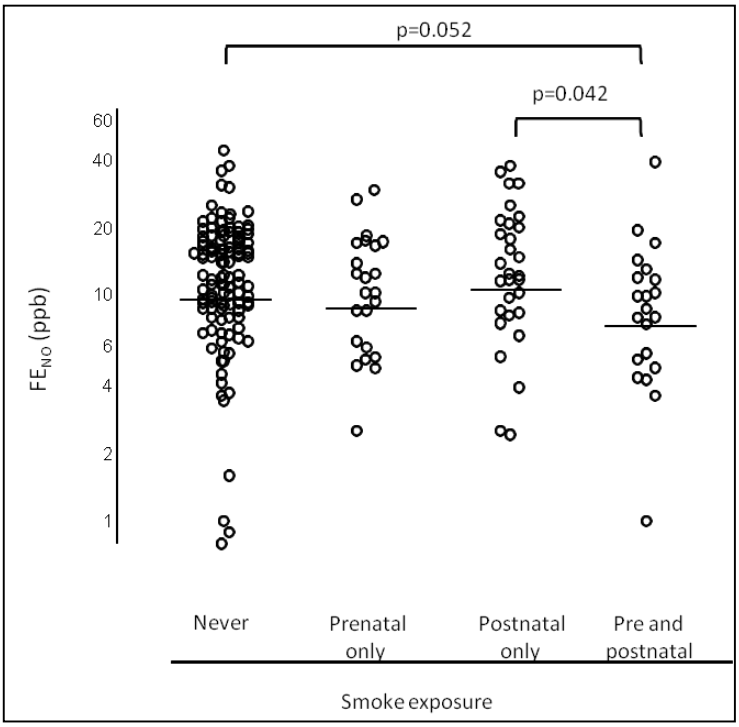

Figure 1 Pre- and postnatal maternal smoking and FeNO values in infants. Never exposed n=113; exposed only prenatally n=23; exposed only postnatally n=30; exposed pre- and postnatally n=21. Bars represent geometric means FeNO estimated with multivariable linear regression and adjusted for gender, birth weight, maternal atopy, age at study date, ambient NO, LRS and URS.

Table 4 Multivariable linear regression model with logFeNO as dependent variable

Variable	Geometric mean FENO	[95% CI] ppb
Smoke exposure		
Never (n=113)	9.8	[8.2-11.8]
Prenatal only (n=23)	9.6	[7.2-12.7]
Postnatal only (n=30)	10.5	[8.0-13.9]
Pre- and postnatal (n=21)	7.2	[5.3-9.8] †
Lower Respiratory Symptoms		
No (n=104)	10.2	[8.5-12.2]
Yes (n=83)	8.3	[6.7-10.2] ‡
Upper Respiratory Symptoms		
No (n=57)	10.4	[8.6-12.7]
Yes, no doctor (n=120)	10.8	[9.3-12.5]
Yes, doctor (n=10)	6.9	[4.6-10.4] §

The FeNO values have been adjusted in the regression model for all the listed factors and for gender, birth weight, maternal atopy, age at study date and ambient NO. †p=0.052 compared to never exposed and p=0.042 compared to exposed only postnatally; ‡p=0.046 compared to asymptomatic infants; §p=0.047 compared to non-severe symptoms

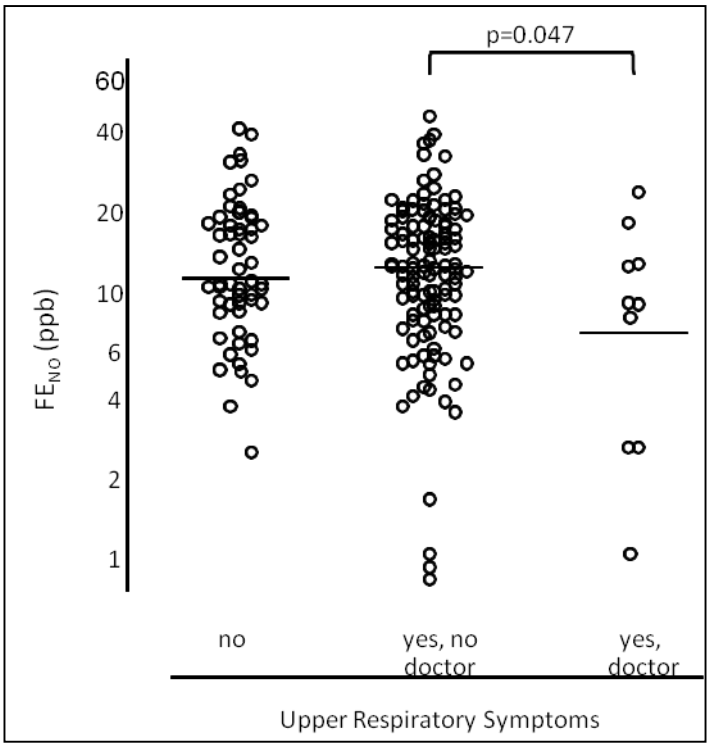

Figure 2 Upper respiratory symptoms and FeNO values in infants. No symptoms n=57; yes, did not visit a doctor n=120; yes, did visit a doctor n=10. Bars represent geometric means FeNO estimated with multivariable linear regression and adjusted for gender, birth weight, maternal atopy, age at study date, ambient NO, smoke exposure and LRS.

Discussion

In the present birth cohort study we found an association between tobacco smoke exposure and FeNO values in infants. Infants continuously exposed to smoke both in utero and after birth had lower FeNO than never exposed infants and than infants exposed only postnatally. None of the other investigated risk factors for respiratory morbidity affected FeNO. The association between respiratory symptoms and lower FeNO was significant in infants with severe upper and lower respiratory symptoms.

Few studies addressed the separate effects of pre-and postnatal tobacco smoke exposure in infants [7-9, 14]. Frey et al [8] measured FeNO in a selected group of healthy infants and assessed pre-and postnatal environmental tobacco smoke exposure after birth. They found that maternal smoking in pregnancy was associated with lower FeNO, but only in infants of mothers without asthma, whereas the same exposure in mothers with atopic disease was associated with higher levels. In univariable analysis, we also found that FeNO was lower in infants of mothers who smoked during pregnancy, whereas in the multivariable analysis infants exposed to smoke prenatally had lower FeNO than never exposed only if smoke exposure was protracted also after birth. In our study neither parental atopy nor gender modified this association, confirming earlier findings of Hall et al [9]. However, due to the small numbers of infants per group, there was insufficient power to adequately investigate such interactions. Our study is embedded in a larger population-based birth cohort, and infants were not selected depending on their health status. Furthermore, the repeated assessment of exposure variables during pregnancy gave us the opportunity to study prenatal smoke exposures in greater detail and reduced the likelihood of recall bias, strengthening the validity of our findings. In a recent study, Franklin et al [7], found increased FeNO in infants exposed to parental smoking with evidence of a dose-response relationship. We could not demonstrate a clear effect of postnatal smoke exposure on FeNO as we found that FeNO was higher in infants exposed to smoke only postnatally than in infants exposed both pre- and postnatally, but not different from never exposed infants. The mechanisms for increased FeNO in infants exposed to postnatal maternal smoking, as observed in this and previous studies are not clear. A possible explanation may be a direct irritant effect of smoke on the airways [7].

The lower FeNO found in infants exposed to prenatal tobacco smoking would support the hypothesis that smoke exposure during pregnancy inhibits inducible NO

synthase [21]. Possible implications of such suppression is hypothetical, but as NO may serve important functions in local defense and in maintenance of normal vaso- and bronchomotor tone, any factor that modifies baseline NO generation in the airways of young infants should be reason for concern and further study.

The occurrence and the severity of respiratory symptoms were associated with lower FeNO in infants. Franklin et al also found low FeNO in infants with ongoing rhinorrhea, but FeNO increased 4-12 weeks after the initial assessment, when symptoms had resolved [6]. Although children in our study were free of respiratory symptoms and had no evidence of respiratory infection at the time of testing, they might have had symptoms in the weeks preceding the FeNO measurement. Therefore, our results should be interpreted with caution, as the age at which symptoms occurred and the timing of symptoms in relation to the FeNO measurements might have influenced the findings. We cannot exclude that a reduced FeNO in infants with respiratory symptoms is related to a delayed effect of acute symptoms on the NO generation or diffusion through the airways. Another reason for caution when interpreting our results is that we showed this association when comparing FeNO between the relatively small group of infants with severe upper respiratory symptoms and infants with non-severe symptoms. A child may be taken to the doctor for many reasons, symptom severity being one of those, but also anxiety of the parents could have influenced the decision, and this might have led to misclassification. However, if such misclassification occurred, this is not likely to bias the direction of our results as this would mean that we underestimated the effect size.

We found lower FeNO in infants who had had lower respiratory symptoms, but no association was found between wheezing and FeNO. Previous studies found an association between wheezing and FeNO in selected populations of infants with high risk of developing atopic disease or with recurrent wheeze [4, 22]. Also, in a prospective study [10], higher FeNO at 1 month was predictive of the development of respiratory symptoms in the first year only in infants of atopic mothers, who are at higher risk of developing asthma. In contrast, in the same study, a trend toward a negative association between FeNO and severe respiratory symptoms in infants of nonatopic mothers was found. Infants in our study came from an unselected population and maternal atopy did not modify any of the associations that we found, and this may explain the discrepancies. Although there is evidence that most asthma starts in early life [23], respiratory morbidity in the preschool child is mostly related to

neutrophilic airway inflammation [23] and there is very little evidence of chronic eosinophilic bronchial inflammation in the first months [24].

One could argue that measuring mixed oral/nasal FeNO without controlling for expiratory flow might introduce variability [25], especially in infants exposed to tobacco smoke that may have abnormal airway mechanics. We previously demonstrated that FeNO measured with variable flow was reproducible [18] and able to differentiate between infants with different respiratory diseases in a similar way as more sophisticated techniques taking into account also lung function parameters and breathing pattern [4]. However, differences between groups may still be due to differences in tidal flows, particularly when comparing groups with potentially different tidal breathing patterns. Indeed, differences in tidal flows might introduce variability and explain some of the overlap and the relatively small differences between groups in our study.

In the present study we found a positive correlation between FeNO and ambient NO, in agreement with a previous study by Pijnenburg et al [26]. Although significant also in the multivariable model, we showed that such correlation did not affect the results of our study as the associations that we found were independent of ambient NO concentrations. Using NO-free air always when measuring FeNO in infants could reduce the influence of ambient NO on FeNO, as suggested by recently published guidelines [27]. However, such recommendations were not yet available when we started the study, and we used NO-free air only if room concentrations were above 10 ppb, in agreement with previously published guidelines for infants [19]. Our findings suggest that infants should inhale more than 2 tidal breaths of NO-free air, as this would reduce the contamination by ambient NO, but on the other hand this might reduce the success rate of the measurements, as awake infants might not tolerate the facemask for a longer time.

A possible limitation to our study is that smoke exposure was assessed by means of questionnaires and was not confirmed with the measurement of specific biomarkers. Although a good agreement between parental report of smoking and air nicotine concentration has been shown [28], some misclassification might have occurred, as parents would underreport smoking. If this is the case, we would underestimate the effect of smoke on FeNO, as smoking parents would be classified in the group of non-smokers. Therefore, we hypothesize that the size of this effect could be greater than reported in our study.

We conclude that pre- and postnatal tobacco smoke exposure are associated with mixed oral/nasal FeNO in early infancy, with lower FeNO in prenatally exposed infants and higher FeNO in case of postnatal exposure. Reported airway symptoms, depending on their frequency and severity, were associated with lower FeNO already in the first 2 months of life. The meaning of changes in FeNO for respiratory health in infancy needs to be further elucidated.

References

1. Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet. 1994 Jan 15;343(8890):133-5.
2. Brussee JE, Smit HA, Kerkhof M, Koopman LP, Wijga AH, Postma DS, et al. Exhaled nitric oxide in 4-year-old children: relationship with asthma and atopy. Eur Respir J. 2005 Mar;25(3):455-61.
3. Dinakar C, Craff M, Laskowski D. Infants and toddlers without asthma with eczema have elevated exhaled nitric oxide levels. J Allergy Clin Immunol. 2006 Jan;117(1):212-3.
4. Gabriele C, Nieuwhof EM, Van Der Wiel EC, Hofhuis W, Moll HA, Merkus PJ, et al. Exhaled nitric oxide differentiates airway diseases in the first two years of life. Pediatr Res. 2006 Oct;60(4):461-5.
5. Ratjen F, Kavuk I, Gartig S, Wiesemann HG, Grasemann H. Airway nitric oxide in infants with acute wheezy bronchitis. Pediatr Allergy Immunol. 2000 Nov;11(4):230-5.
6. Franklin PJ, Turner SW, Hall GL, Moeller A, Stick SM. Exhaled nitric oxide is reduced in infants with rhinorrhea. Pediatr Pulmonol. 2005 Feb;39(2):117-9.
7. Franklin PJ, Turner S, Mutch R, Stick SM. Parental smoking increases exhaled nitric oxide in young children. Eur Respir J. 2006 Oct;28(4):730-3.
8. Frey U, Kuehni C, Roiha H, Cernelc M, Reinmann B, Wildhaber JH, et al. Maternal atopic disease modifies effects of prenatal risk factors on exhaled nitric oxide in infants. Am J Respir Crit Care Med. 2004 Aug 1;170(3):260-5.
9. Hall GL, Reinmann B, Wildhaber JH, Frey U. Tidal exhaled nitric oxide in healthy, unsedated newborn infants with prenatal tobacco exposure. J Appl Physiol. 2002 Jan;92(1):59-66.
10. Latzin P, Kuehni CE, Baldwin DN, Roiha HL, Casaulta C, Frey U. Elevated exhaled nitric oxide in newborns of atopic mothers precedes respiratory symptoms. Am J Respir Crit Care Med. 2006 Dec 15;174(12):1292-8.
11. Biban P, Zangardi T, Baraldi E, Dussini N, Chiandetti L, Zacchello F. Mixed exhaled nitric oxide and plasma nitrites and nitrates in newborn infants. Life Sci. 2001 May 11;68(25):2789-97.
12. Roiha HL, Kuehni CE, Zanolari M, Zwahlen M, Baldwin DN, Casaulta C, et al. Alterations of exhaled nitric oxide in preterm infants with chronic lung disease. Eur Respir J. 2006 Oct 18.
13. Kharitonov SA, Yates D, Barnes PJ. Increased nitric oxide in exhaled air of normal human subjects with upper respiratory tract infections. Eur Respir J. 1995 Feb;8(2):295-7.
14. Dinakar C, Lapuente M, Barnes C, Garg U. Real-life environmental tobacco exposure does not affect exhaled nitric oxide levels in asthmatic children. J Asthma. 2005 Mar;42(2):113-8.
15. Yates DH, Breen H, Thomas PS. Passive smoke inhalation decreases exhaled nitric oxide in normal subjects. Am J Respir Crit Care Med. 2001 Sep 15;164(6):1043-6.
16. Jaddoe VW, Bakker R, van Duijn CM, van der Heijden AJ, Lindemans J, Mackenbach JP, et al. The Generation R Study Biobank: a resource for epidemiological studies in children and their parents. Eur J Epidemiol. 2007;22(12):917-23.
17. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006;21(6):475-84.
18. Gabriele C, van der Wiel EC, Nieuwhof EM, Moll HA, Merkus PJFM, de Jongste JC. Methodological aspects of exhaled nitric oxide measurements in infants. Pediatr Allergy Immunol. 2007(18):36-41.
19. Baraldi E, de Jongste JC. Measurement of exhaled nitric oxide in children, 2001. Eur Respir J. 2002 Jul;20(1):223-37.

20. Voorburg/Heerlen. Standaard onderwijsindeling 2003. Statistics Netherlands. 2004.
21. Hoyt JC, Robbins RA, Habib M, Springall DR, Buttery LD, Polak JM, et al. Cigarette smoke decreases inducible nitric oxide synthase in lung epithelial cells. Exp Lung Res. 2003 Jan-Feb;29(1):17-28.
22. Wildhaber JH, Hall GL, Stick SM. Measurements of exhaled nitric oxide with the single-breath technique and positive expiratory pressure in infants. Am J Respir Crit Care Med. 1999 Jan;159(1):74-8.
23. Oommen A, Patel R, Browning M, Grigg J. Systemic neutrophil activation in acute preschool viral wheeze. Arch Dis Child. 2003 Jun;88(6):529-31.
24. Saglani S, Malmstrom K, Pelkonen AS, Malmberg LP, Lindahl H, Kajosaari M, et al. Airway remodeling and inflammation in symptomatic infants with reversible airflow obstruction. Am J Respir Crit Care Med. 2005 Apr 1;171(7):722-7.
25. Deykin A, Massaro AF, Drazen JM, Israel E. Exhaled nitric oxide as a diagnostic test for asthma: online versus offline techniques and effect of flow rate. Am J Respir Crit Care Med. 2002 Jun 15;165(12):1597-601.
26. Pijnenburg MW, Lissenberg ET, Hofhuis W, Ghiro L, Ho WC, Holland WP, et al. Exhaled nitric oxide measurements with dynamic flow restriction in children aged 4-8 yrs. Eur Respir J. 2002 Oct;20(4):919-24.
27. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med. 2005 Apr 15;171(8):912-30.
28. Gehring U, Leaderer BP, Heinrich J, Oldenwening M, Giovannangelo MECA, Nordling E, et al. Comparison of parental reports of smoking and resudential air nicotine concentrations in children. Occup Environ Med. 2006(63):766-72.

7

Elevated exhaled nitric oxide increases the risk of wheezing in infancy. The Generation R Study

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Submitted

Abstract

Background: There is a lack of prospective studies investigating the association between exhaled nitric oxide (FeNO) and development of wheezing in infancy.

Objectives: To assess whether FeNO was predictive of respiratory symptoms in the first 2 years of life.

Methods: In a subsample of the Generation R birth cohort we prospectively assessed pre- and postnatal risk factors for respiratory morbidity and respiratory symptoms by means of questionnaires at 6 and 24 months. Mixed oral/nasal FeNO was measured off-line during tidal breathing at 6 months.

Results: FeNO was higher in boys and was positively associated with age, weight and length at the study date. Infants in the high tertile of FeNO at 6 months had a 3 fold increased risk of wheezing at 2 years as compared to those in the low tertile (adjusted odds ratio(aOR)[95% CI] 3.04[1.19-7.74]). Infants in the high tertile of FeNO were more likely to have persistent wheezing than infants in the low tertile (aOR[95% CI] 5.85[1.46-23.3]). Infants in the mid and in the high tertile of FeNO obtained at 6 months were at lower risk of cough in the first 6 months compared to infants in the low tertile (aOR[95% CI] 0.26[0.12-0.55] and 0.43[0.19-0.97]).

Conclusion: High FeNO at 6 months increases the risk of wheezing in the second year of life. Follow up studies are needed to assess whether this leads to an increased risk of asthma at older age.

Introduction

In the last decade there has been a growing interest in measuring the fraction of nitric oxide in exhaled air (FeNO) in young children as it might provide a useful tool to monitor eosinophilic bronchial inflammation early in life. Increased FeNO levels have been found in asthmatic adults and children (1, 2). In previous studies in infants, higher FeNO has been found in association with recurrent wheezing (3-5), whereas lower FeNO has been shown in virus-associated wheezing (6) and in infants with severe upper and lower respiratory symptoms (7, 8). Franklin et al. (9) evaluated the association between FeNO and symptoms other than wheezing in the first year of life and they found similar FeNO levels in infants with and without cough. However, previous studies in young children are difficult to compare as different methods to measure FeNO have been used. Furthermore, it is important to take possible confounders into account, such as pre- and postnatal smoke exposure (8, 10), parental atopy (11), gender (11), birth weight (12) and anthropometrics (3), which have been shown to influence FeNO values in infants. In a prospective study, Latzin et al. (13) measured FeNO on-line in 1 month old infants and found that increased FeNO was associated with the development of severe respiratory symptoms in the first year of life, but only if the mother had an atopic disease or had been smoking during pregnancy. These findings suggest that FeNO is increased at a very young age in children who are at high risk of developing asthma and that some degree of airway inflammation might be present already the first months of life, even before the onset of symptoms. We hypothesized that an early, non-invasive marker of bronchial inflammation like FeNO could predict the development of asthma early in life.

In the present prospective birth cohort study, we assessed FeNO in infants at the age of 6 months and we investigated whether FeNO was predictive of respiratory symptoms in the first 2 years of life.

Methods

Study population

The Generation R Study is a prenatally recruited population-based birth cohort in Rotterdam, the Netherlands (14, 15). Detailed assessments of fetal and postnatal growth and development are conducted in the Generation R Focus Cohort, a subgroup of 1,232 Dutch children and their parents. The Medical Ethical Committee of the Erasmus Medical Center, Rotterdam, approved the study. Mothers and their

partners received written and verbal information about the study and gave written informed consent.

Pre- and postnatal exposure variables

Family history of atopy and prenatal exposure to tobacco smoke were assessed prospectively by means of questionnaires administered to the mother and to the partner. Gestational age, birth weight and birth length were obtained from midwife and hospital registries. Postnatal exposures were investigated with a questionnaire administered at 6 months and tobacco smoke exposure, eczema and upper respiratory symptoms in the first 6 months of life were assessed. Respiratory tract infections were assessed at 6 months and parents were asked to report the occurrence of flu, ear or throat infection and bronchiolitis.

FeNO measurements

FeNO was measured off-line in awake infants during tidal breathing (8, 16). A facemask covering nose and mouth was connected to a two-way non-rebreathing valve (Hans Rudolph Inc., Kansas City, MO, USA) with the expiratory port attached to a 150 ml Mylar balloon. The NO concentration in the sampling balloon was measured by chemiluminescence (Sievers 280 B, Boulder, CO, USA). Ambient NO was determined before each FeNO measurement. If ambient NO was above 10 parts per billion (ppb), then a 750 ml NO-free air balloon was connected to the inspiratory port of the valve and infants inhaled two breathes of NO-free air. Measurements were excluded if a quiet tidal breathing pattern was not maintained during the whole procedure, if the facemask was not tightly fitted to infants' nose and mouth and if less than 5 breaths were collected in the sampling balloon. All infants were free of respiratory symptoms and had no clinical evidence of airways infection at the time of testing.

Lower respiratory symptoms

The occurrence of lower respiratory symptoms in the first 6 months of life and in the second year was assessed by questionnaires administered at 6 and 24 months, respectively. Symptoms included wheezing, breathlessness and cough at 6 months and wheezing, breathlessness, dry cough without a cold and persistent phlegm in the previous 12 months at 24 months. Based on longitudinal data on wheezing at 6 and 24 months, infants were classified in 4 wheezing phenotypes as follows: never wheeze,

wheezing at 6, but not at 24 months, wheezing at 24, but not at 6 months and wheezing both at 6 and 24 months (17).

Analysis

Continuous variables were normally distributed, with the exception of FeNO values that were right-skewed and were logtransformed in order to achieve a near-normal distribution. Log-FeNO values were backtransformed after the analysis and reported as geometric mean and 95% confidence interval [95% CI]. Student's t-test and chi-square test were used to evaluate the difference in baseline characteristics between infants with and without complete data at 6 months. Correlations between age, weight and length at study date were evaluated with Pearson's coefficient.

Based on our previous findings (8), we first evaluated the relationship between ambient NO and logFeNO with linear regression and we found a significant association (logFeNO=0.928 + 0.0066 * ambient NO; $p<0.001$). Therefore, multiple linear regression analysis was used to evaluate the associations between logFeNO (dependent variable) and each of the following pre- and postnatal variables, controlling for ambient NO: gestational age, birth weight, age, weight and length at the study date, gender, pre and postnatal smoke exposure, maternal atopy and eczema, respiratory tract infections and upper respiratory symptoms at 6 months. Results of the linear regression models are presented as beta coefficient [95% CI], which should be judged as the change of log FeNO per unit change in the independent variables.

Logistic regression analysis was used to investigate whether FeNO measured at 6 months was associated with lower respiratory symptoms in the first 6 months and whether FeNO was predictive of the development of wheezing in the second year of life. For this purpose, FeNO values were divided in tertiles, with the lowest tertile as the reference category, and each lower respiratory symptom at 6 and at 24 months was used as outcome variable in separate models. Pre and postnatal variables that were associated with FeNO were included in the final logistic regression models as potential confounders. As we and others previously showed an association between FeNO and tobacco smoke exposure (8, 11, 18), maternal atopy (3, 11, 13), birth weight (12), upper respiratory symptoms (8) and respiratory tract infections (6) these variables were added in the final regression models. Based on previous studies (11, 13), we investigated effect modification by maternal atopy and prenatal smoke exposure by adding interaction terms into the final models (maternal atopy x 'tertiles

of FeNO' and prenatal smoke exposure x 'tertiles of FeNO') for each of the respiratory symptoms assessed at 6 and at 24 months. The risk estimates are reported as adjusted odds ratio (aOR) [95% CI]. A multinomial logistic regression analysis was performed in which wheezing phenotype (dependent variable) was predicted by high vs. low and mid vs. low tertiles of FeNO, controlling for pre-and postnatal variables associated with FeNO at 6 months. The dependent outcome consisted of 4 nominal categories: 1=never wheeze, 2=wheezing at 6 months only, 3=wheezing at 24 months only, 4=wheezing at 6 and 24 months. As we were interested in the ability to discriminate the groups of infants with wheezing from the group of infants who never wheezed, risk estimates were computed for the comparison of infants with wheezing at 6 months only, at 24months only and at 6 and 24 months vs. never wheeze. Risk estimates of multinomial regression analysis are reported as aOR and represent the odds of an infant in the high and in the mid tertile of FeNO having wheezing at 6 months only, at 24 months only or at 6 and at 24 months vs. never wheeze compared to the odds of an infant in the low tertile. For all statistical tests, two tailed p values ≤ 0.05 were considered statistically significant. Data analyses were performed using the Statistical Package of Social Sciences version 11 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Study population

Between March 2004 and March 2006, FeNO measurements were attempted in 511 infants (boys=53%) participating in the Focus Study at a mean (range) age of 27.7 (22-48) weeks. The measurements were successful in 428 infants: 56 measurements were excluded as tidal breathing was not maintained during the whole procedure and 27 because less than 5 breaths were collected in the sampling balloon. As the 6 months questionnaire was implemented after the start of the FeNO measurements, the analysis was limited to 294 infants. General characteristics of the study population are presented in table 1. Infants with available data compared to excluded infants were less exposed to prenatal maternal smoke (16.3 % and 24.6 %, respectively; p=0.042). FeNO, gestational age, birth weight, age, weight and length at the study date, gender and maternal atopy did not differ. Also, there was no difference between infants with and without available data at 6 months with regard to the prevalence of lower respiratory symptoms at 24 months.

Table 1 General characteristics of the study population (n=294)

	Mean (SD)
Gestational age (wks)	40.1 (1.63)
Birth weight (kg)	3.543 (0.55)
Age at the study date (wks)	27.3 (2.92)
Weight at the study date (kg)	7.936 (0.86)
Length at the study date (cm)	68.8 (2.75)
FeNO (ppb)	10.3 [9.3-11.3] §
Gender (% boys)	53
Prenatal smoke exposure (% yes)	16
Postnatal smoke exposure (% yes)	28
Smoke exposure (%) †	
Never	65
Prenatal only	7
Postnatal only	19
Pre- and postnatal	9
Maternal atopy (% yes)	34
Eczema at 6 months (% yes)	20
Respiratory tract infections at 6 months (% yes)	37
Upper respiratory symptoms at 6 months (% yes)	94
Lower respiratory symptoms at 6 months (% yes)	
Cough	72
Wheezing	20
Breathlessness	8
Lower respiratory symptoms at 24 months (% yes)	
Wheezing	16
Breathlessness	16
Dry cough without a cold	22
Persistent phlegm	6

§ Geometric mean [95% CI]

† combination of prenatal smoke exposure and postnatal smoke exposure

Pre- and postnatal exposures and FeNO at 6 months

The linear regression analysis, controlling for ambient NO, showed a positive association between FeNO and age (beta coefficient [95% CI] 0.014 [0.001 – 0.028]; p=0.039), weight (0.047 [0.002 – 0.093]; p=0.041) and length (0.014 [-0.001 – 0.028]; p=0.061) at the study date and boys showed higher FeNO values than girls (11.2 [9.9-12.7] ppb and 9.3 [8.1-10.6] ppb, respectively; p=0.04). None of the other investigated variables was associated with FeNO. Anthropometrics at the study date were highly correlated (p<0.001 for all correlations) and in order to avoid collinearity, weight and

length were dropped from the final models and only age was included as covariate, as this showed to be the best predictor of FeNO.

FeNO and respiratory symptoms in the first 6 months

The association between FeNO and each respiratory symptom in the first 6 months was evaluated in separate logistic regression models, which included ambient NO, gender, age, smoke exposure, maternal atopy, birth weight, respiratory tract infections and upper respiratory symptoms as covariates. FeNO measured at 6 months was divided in tertiles as follows: low (range=0.5-8.4 ppb; n=97 infants), mid (8.5-15.7 ppb; n=100) and high (15.7-78.2 ppb; n=97) tertile. FeNO was not associated with wheezing or breathlessness at 6 months (table 2). Compared to infants in the low tertile of FeNO, infants in the mid and in the high tertiles were at lower risk of cough at 6 months (aOR [95% CI] 0.26 [0.12-0.55], p<0.001 and 0.43 [0.19-0.97], p=0.043, respectively) (table 2). This association was not modified by maternal atopy (p for interaction = 0.86) or by prenatal smoke exposure (p for interaction = 0.74).

Table 2 FeNO measured at 6 months and risk of lower respiratory symptoms in the first 6 months of life.

	Cough	Wheezing	Breathlessness
Tertiles of FeNO	aOR [95% CI]	aOR [95% CI]	aOR [95% CI]
Low	Reference	Reference	Reference
Mid	0.26 [0.12-0.55] †	1.34 [0.63-2.84]	1.61 [0.69-3.74]
High	0.43 [0.19-0.97] ‡	1.52 [0.52-4.44]	1.44 [0.39-5.30]

Odds ratios were adjusted for ambient NO, gender, age, smoke exposure, maternal atopy, birth weight, respiratory tract infections and upper respiratory symptoms at 6 months. † p<0.001; ‡ p=0.043.

FeNO at 6 months and lower respiratory symptoms in the first 2 years

At 24 months wheezing and breathlessness were reported in 46 infants (15.6%), and dry cough without a cold and persistent phlegm were reported in 66 (22.4%) and 17 (5.8%) infants, respectively. The occurrence of at least one lower respiratory symptom in the second year was reported in 117 infants (39.8%).

Ambient NO, gender, age, smoke exposure, maternal atopy, birth weight, respiratory tract infections and upper respiratory symptoms were included as covariates in the logistic regression models used to evaluate whether FeNO at 6 months was predictive of lower respiratory symptoms in the second year. Also cough at 6 months was included in the models, as this variable was associated with FeNO.

Infants in the highest tertile of FeNO had a three fold risk of wheezing in the second year of life compared to infants in the lowest tertile (aOR [95% CI] 3.04 [1.19-7.74]) (table 3). This association was not modified by maternal atopy (p for interaction = 0.53) or by prenatal smoke exposure (p for interaction = 0.43). A separate regression model was made with FeNO as continuous variable and this model showed that the risk of wheezing in the second year [95% CI] increased by 5.4 % [1.2 – 9.8%] per ppb increase of FeNO at 6 months (p=0.012). No association was observed between FeNO and any of the other lower respiratory symptoms at 24 months (table 3) and in these models the interaction terms tested were not statistically significant.

Table 3 FeNO measured at 6 months and risk of lower respiratory symptoms in the second year of life

	Wheezing	Breathlessness	Dry cough without a cold	Persistent phlegm
Tertiles of FeNO	aOR [95% CI]	aOR [95% CI]	aOR [95% CI]	aOR [95% CI]
Low	Reference	Reference	Reference	Reference
Mid	1.98 [0.82-4.78]	1.25 [0.55-2.85]	0.75 [0.37-1.49]	0.64 [0.19-2.16]
High	3.04 [1.19-7.74] †	1.83 [0.76-4.42]	0.66 [0.30-1.43]	0.37 [0.08-1.79]

Odds ratios were adjusted for ambient NO, gender, age, smoke exposure, maternal atopy, birth weight, respiratory tract infections, upper respiratory symptoms and cough at 6 months. † p=0.02

The longitudinal data at 6 and 24 months showed that wheezing was never reported in 208 infants (71%). In 40 (13%) wheezing was reported only at 6 months, in 26 (9%) only at 24 months and in 20 (7%) at 6 and 24 months. The comparison of infants with wheezing at 6 and 24 months vs. never wheeze showed that infants in the high tertile of FeNO had increased risk of persistent wheezing compared to infants in the low tertile (aOR [95% CI] 5.85[1.47-23.4]) (table 4). This association was not modified by maternal atopy or by prenatal smoke exposure (p for interaction = 0.87 and 0.84, respectively).

Discussion

In the present prospective birth cohort study we found that infants with high FeNO values at 6 months had a 3 fold increased risk of wheezing in the second year. High FeNO was associated with an increased likelihood of wheezing at 6 and at 24 months.

Table 4 FeNO measured at 6 months and wheezing phenotype in the first 2 years of life

	Wheezing at 6 months only	*Wheezing at 24 months only*	*Wheezing at 6 and 24 months*
	aOR [95% CI]	aOR [95% CI]	aOR [95% CI]
Tertiles of FeNO			
Low	Reference	Reference	Reference
Mid	1.14 [0.49 – 2.66]	1.23 [0.39 – 3.78]	2.20 [0.59 – 8.27]
High	0.9 [0.32 – 2.52]	1.50 [0.44 – 5.16]	5.85 [1.47 – 23.4] †

Multinomial logistic regression analysis. Odds ratios represent the odds of an infant in the high and in the mid tertile of FeNO having wheezing at 6 months only, at 24 months only or at 6 and 24 months vs. never wheeze compared to the odds of an infant in the low tertile. Odds ratios were adjusted for ambient NO, gender, age, smoke exposure, maternal atopy, birth weight, respiratory tract infections and upper respiratory symptoms at 6 months.

† p=0.012

Only few studies investigated the association between FeNO and respiratory symptoms in infants and they were performed with a cross-sectional design in selected groups of infants with respiratory diseases (3, 7, 19). It has been shown that FeNO is increased in infants at high risk of developing asthma, such as offspring of atopic parents or infants with recurrent wheezing (3, 4, 20, 21). Infants in our study participate in a large prenatally recruited population-based prospective birth cohort and their selection was not based on an increased risk of developing asthma. In a recent prospective study, Latzin et al (13) investigated the association between FeNO measured in healthy infants at the age of 1 month and the development of respiratory symptoms in the first year of life. They found that increased FeNO levels after birth were associated with increased risk of subsequent severe respiratory symptoms, but only in infants of atopic mothers or in infants of mothers who smoked during pregnancy, with the strongest association in infants of mothers with both risk factors. The study by Latzin et al focused on more severe symptoms, such as awakening because of airway symptoms or GP consultations, in order to identify those children most likely to develop severe asthma (22, 23). The prospective design of our study enabled us to investigate the temporal relationship between increased FeNO and occurrence of respiratory symptoms as well. We found a positive association between

FeNO measured at 6 months and risk of wheezing in the second year of life irrespective of maternal atopy and smoke exposure, whereas no association was found with other respiratory symptoms. As wheezing is the best predictor of asthma later in life (24), our findings of the association between high FeNO at 6 months and wheezing in the second year are in agreement with Latzin et al and support the hypothesis that eosinophilic inflammation is present in high-risk infants before the onset of symptoms. Furthermore, we showed that the odds of an infant in the high tertile of FeNO having wheezing at 6 and 24 months compared to never wheeze is 5.85 times the odds an infant in the low tertile would, strengthening the observation that FeNO is increased in infants with persistent symptoms. Hence, FeNO might be useful to identify those infants who are at increased risk of developing childhood asthma, at least at group level.

Despite the prospective assessment of exposure variables and the repeated assessment of respiratory symptoms, some methodological aspects should be considered.

The 6 months questionnaire data were not available for 134 infants. This was due to a delayed implementation of this questionnaire and was not related either to the exposures or to the outcomes of interest. Compared to infants with complete data available, excluded infants were more often exposed to prenatal maternal smoking, but the other baseline characteristics and the prevalence of respiratory symptoms in the first 2 years of life were not different between the two groups. As the associations between FeNO and respiratory symptoms were not different for infants with and without prenatal smoke exposure, we consider it unlikely that selection bias influenced the results.

The questionnaires were completed by the parents a few days after the study date (mean difference -10.2 days) and infants could have developed symptoms after the FeNO measurement and prior to the completion of the questionnaire. Therefore, misclassification of the outcome might have occurred as some infants would have been classified as symptomatic, although respiratory symptoms occurred after the FeNO measurement. Although we could not investigate the etiology or the exact timing of respiratory infections in relation to the FeNO measurement, we consider it unlikely that most infants had the first respiratory tract infection in the short time period between FeNO measurement and completion of the questionnaire. Also, such misclassification would have caused the effects of FeNO to be underestimated rather

than overestimated in our study. Hence, it is unlikely that misclassification explains our results.

Infants participating in our study had their FeNO measured off-line during tidal breathing. This method is non-invasive, does not require specialized staff and can be easily applied in large epidemiological studies, but does not allow to control for tidal flow or for breathing frequency, which have been shown to influence FeNO values (18, 25). We found good short-term reproducibility of the method in healthy infants (16) and the results of the current study are consistent with our previous findings of lower FeNO in 2 month old infants with lower respiratory symptoms (8). We cannot exclude that differences in expiratory flow between infants with and without respiratory symptoms might have influenced the association between FeNO and symptoms at 6 months. However, in that case infants with symptoms and low FeNO at 6 months would have higher risk of symptoms at 2 years, whereas we found that higher FeNO at 6 months was predictive of wheezing in the second year of life. Hence, we consider it unlikely that any difference in respiratory flow as a result of airway symptoms would explain the positive association between FeNO at 6 months and the subsequent development of wheezing at 2 years.

Our results may have clinical and epidemiological implications. As most childhood asthma begins in infancy (26), FeNO may be helpful to identify children at risk of asthma in an early stage of the disease. The results of our study need to be confirmed, and the children of the cohort will need to be reassessed as they grow up, in order to have a better definition of wheezing phenotype (17). There is a lack of studies investigating the relationship between FeNO and eosinophilic bronchial inflammation in infants. It is therefore difficult to evaluate whether eosinophilic bronchial inflammation indeed precedes the onset of asthma. Further prospective studies might help to understand how increased FeNO in infancy should be interpreted.

We conclude that high FeNO at 6 months is predictive of wheezing in the second year and increases the risk of persistent wheezing in the first 2 years of life.

References

1. Alving K, Weitzberg E, Lundberg JM. Increased amount of nitric oxide in exhaled air of asthmatics. Eur Respir J 1993;6:1368-1370.
2. Artlich A, Hagenah JU, Jonas S, Ahrens P, Gortner L. Exhaled nitric oxide in childhood asthma. Eur J Pediatr 1996;155:698-701.
3. Gabriele C, Nieuwhof EM, Van Der Wiel EC, Hofhuis W, Moll HA, Merkus PJ, De Jongste JC. Exhaled nitric oxide differentiates airway diseases in the first two years of life. Pediatr Res 2006;60:461-465.

4. Moeller A, Diefenbacher C, Lehmann A, Rochat M, Brooks-Wildhaber J, Hall GL, Wildhaber JH. Exhaled nitric oxide distinguishes between subgroups of preschool children with respiratory symptoms. J Allergy Clin Immunol 2008;121:705-709.
5. Baraldi E, Dario C, Ongaro R, Scollo M, Azzolin NM, Panza N, Paganini N, Zacchello F. Exhaled nitric oxide concentrations during treatment of wheezing exacerbation in infants and young children. Am J Respir Crit Care Med 1999;159:1284-1288.
6. Ratjen F, Kavuk I, Gartig S, Wiesemann HG, Grasemann H. Airway nitric oxide in infants with acute wheezy bronchitis. Pediatr Allergy Immunol 2000;11:230-235.
7. Franklin PJ, Turner SW, Hall GL, Moeller A, Stick SM. Exhaled nitric oxide is reduced in infants with rhinorrhea. Pediatr Pulmonol 2005;39:117-119.
8. Gabriele C, Asgarali R, Jaddoe VW, Hofman A, Moll HA, de Jongste JC. Smoke exposure, airway symptoms and exhaled nitric oxide in infants: The generation r study. Eur Respir J 2008;32:307-313.
9. Franklin PJ, Turner SW, Mutch RC, Stick SM. Comparison of single-breath and tidal breathing exhaled nitric oxide levels in infants. Eur Respir J 2004;23:369-372.
10. Franklin PJ, Turner S, Mutch R, Stick SM. Parental smoking increases exhaled nitric oxide in young children. Eur Respir J 2006;28:730-733.
11. Frey U, Kuehni C, Roiha H, Cernelc M, Reinmann B, Wildhaber JH, Hall GL. Maternal atopic disease modifies effects of prenatal risk factors on exhaled nitric oxide in infants. Am J Respir Crit Care Med 2004;170:260-265.
12. Biban P, Zangardi T, Baraldi E, Dussini N, Chiandetti L, Zacchello F. Mixed exhaled nitric oxide and plasma nitrites and nitrates in newborn infants. Life Sci 2001;68:2789-2797.
13. Latzin P, Kuehni CE, Baldwin DN, Roiha HL, Casaulta C, Frey U. Elevated exhaled nitric oxide in newborns of atopic mothers precedes respiratory symptoms. Am J Respir Crit Care Med 2006;174:1292-1298.
14. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, Witteman JC, Hofman A. The generation r study: Design and cohort profile. Eur J Epidemiol 2006;21:475-484.
15. Jaddoe VW, Bakker R, van Duijn CM, van der Heijden AJ, Lindemans J, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Uitterlinden AG, et al. The generation r study biobank: A resource for epidemiological studies in children and their parents. Eur J Epidemiol 2007;22:917-923.
16. Gabriele C, van der Wiel EC, Nieuwhof EM, Moll HA, Merkus PJFM, de Jongste JC. Methodological aspects of exhaled nitric oxide measurements in infants. Pediatr Allergy Immunol 2007:36-41.
17. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The group health medical associates. N Engl J Med 1995;332:133-138.
18. Hall GL, Reinmann B, Wildhaber JH, Frey U. Tidal exhaled nitric oxide in healthy, unsedated newborn infants with prenatal tobacco exposure. J Appl Physiol 2002;92:59-66.
19. Elphick HE, Demoncheaux EA, Ritson S, Higenbottam TW, Everard ML. Exhaled nitric oxide is reduced in infants with cystic fibrosis. Thorax 2001;56:151-152.
20. Franklin PJ, Turner SW, Mutch RC, Stick SM. Measuring exhaled nitric oxide in infants during tidal breathing: Methodological issues. Pediatr Pulmonol 2004;37:24-30.
21. Wildhaber JH, Hall GL, Stick SM. Measurements of exhaled nitric oxide with the single-breath technique and positive expiratory pressure in infants. Am J Respir Crit Care Med 1999;159:74-78.
22. Holt PG, Macaubas C, Stumbles PA, Sly PD. The role of allergy in the development of asthma. Nature 1999;402:B12-17.
23. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, Cowan JO, Herbison GP, Silva PA, Poulton R. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med 2003;349:1414-1422.
24. Castro-Rodriguez JA, Holberg CJ, Wright AL, Martinez FD. A clinical index to define risk of asthma in young children with recurrent wheezing. Am J Respir Crit Care Med 2000;162:1403-1406.
25. Deykin A, Massaro AF, Drazen JM, Israel E. Exhaled nitric oxide as a diagnostic test for asthma: Online versus offline techniques and effect of flow rate. Am J Respir Crit Care Med 2002;165:1597-1601.
26. Wright AL, Taussig LM. Lessons from long-term cohort studies. Childhood asthma. The European respiratory journal 1998;27:17s-22s.

8

General Discussion

There is a large body of evidence suggesting that the events occurring during the first years of life, the timing and intensity of certain environmental exposures and their interactions with genetic predisposition play a central role in the development of lung function, bronchial inflammation, respiratory morbidity, asthma and atopic diseases. Studies in this thesis focused on the first 2 years of life and aimed to assess ethnicity-specific risk factors for respiratory symptoms, and the association between eosinophilic bronchial inflammation and respiratory symptoms.

Data from the Generation R study were used to evaluate whether the prevalence of lower respiratory symptoms was associated with ethnic background in a multicultural population-based birth cohort. Furthermore, we evaluated whether and to what extent fetal and postnatal exposures could explain the associations between ethnicity and respiratory symptoms in infants, in order to identify those factors that could modify the natural history of asthma. We explored several methodological issues related to the measurement of fractional exhaled nitric oxide (FeNO) in infants and also evaluated the associations between fetal and postnatal exposures and FeNO values in the first 2 years of life. Also, we assessed whether FeNO measurements could be useful to differentiate airways diseases early in life.

This chapter provides a critical synthesis of the main findings of the studies presented in the current thesis. The methodological issues that could have affected the findings will also be addressed. We will report to what extent the findings described in this thesis may be useful for clinical or epidemiological purposes. Finally, directions for future research will be outlined.

8.1 Ethnic background and respiratory morbidity in the first 2 years of life

Main findings

The Generation R Study is a multiethnic population based birth cohort study in Rotterdam. The ethnic background of the study population reflected the ethnic composition of the population in the city of Rotterdam, but showed a higher number of Dutch and a lower number of non-Dutch children, than expected from the population ethnicity distributions among neonates born between January 2002 and December 2005 in Rotterdam (figures 1 and 2). The first aim of this thesis was to evaluate the prevalence of respiratory symptoms in different ethnic groups.

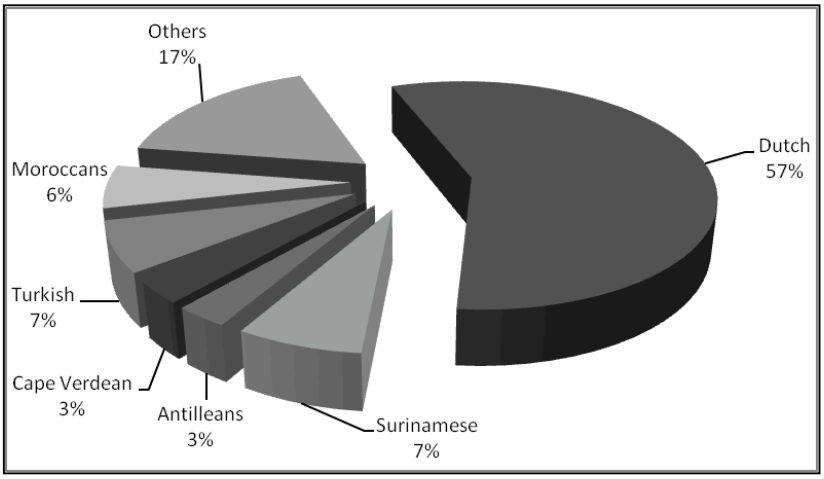

Figure 1 Ethnic distribution of newborns in Rotterdam (average 2002-2005)

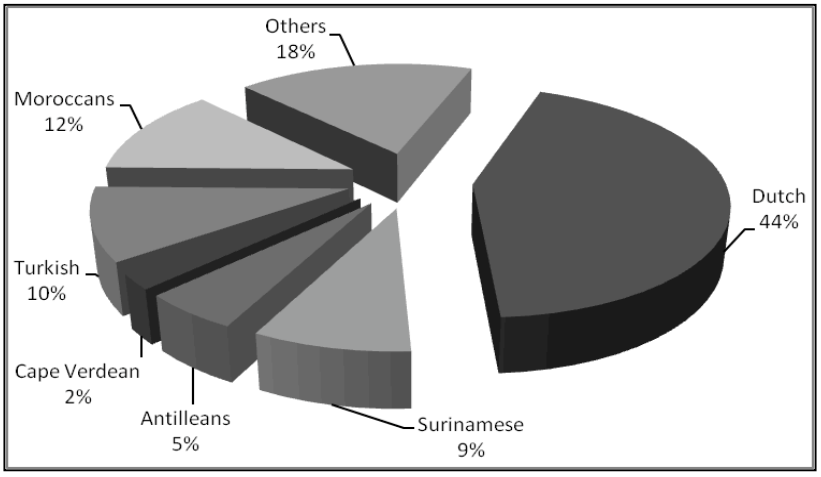

Figure 2 Ethnic distribution of the generation R cohort (n=5684)

We showed that infants with non-Dutch ethnic background had different prevalences of respiratory symptoms during the first 2 years of life as compared with Dutch infants. The occurrence of at least one lower respiratory symptom (wheezing, breathlessness, dry cough, persistent phlegm or doctor-diagnosed asthma) was more often reported in Cape Verdean, Antillean and Turkish infants and less often in Moroccan infants, than in Dutch infants. Then, we investigated if the different prevalence of LRS between ethnic groups could be explained by the mediating effect of fetal and postnatal exposures. We hypothesized that the effect of ethnic background on LRS was partly direct and partly mediated by fetal and postnatal

exposures, as schematically shown in figure 3. Therefore, we used a conceptual hierarchical framework in order to evaluate the interrelationships between ethnicity and mediators, and their combined effects on LRS [1].

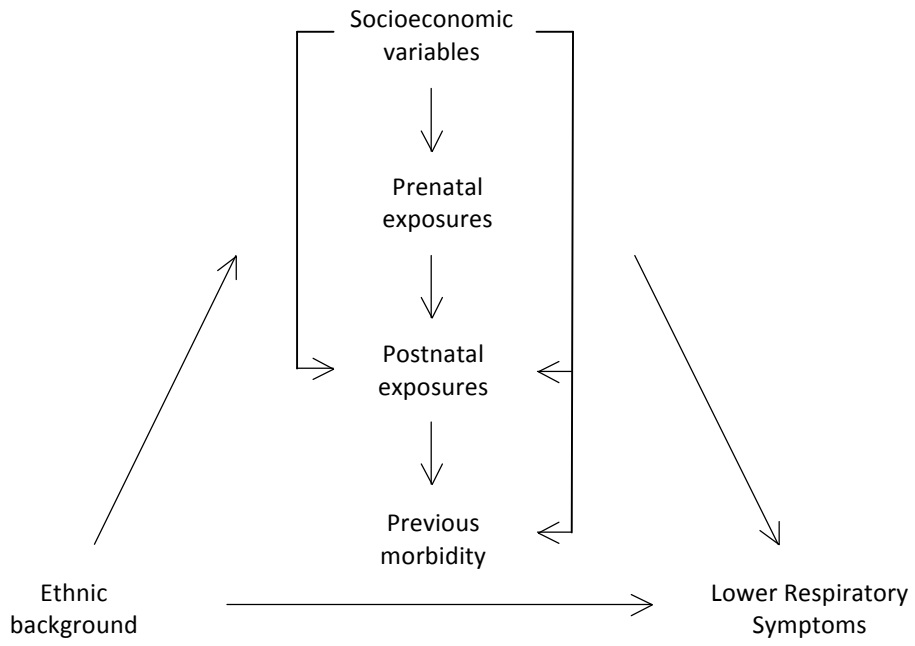

Figure 3 The relation of ethnic background with the outcome and the mediating variables.The arrows indicate the direction of the associations.

The basic model included gender and age of the child at the time of completion of the questionnaire, which were considered as potential confounders of the relationship between ethnicity and LRS. The use of a conceptual framework allowed us to manage the complex hierarchical interrelationships between the investigated determinants of LRS. This is in contrast to most previous studies that used multivariate analyses for prediction of respiratory symptoms based on statistical associations with some degree of biological or social plausibility, with all explanatory variables treated as if belonging to the same hierarchical level. We found that the associations between Turkish ethnicity and increased risk of LRS at 0-12 and 12-24 months as compared to Dutch infants were partly mediated by previous morbidity (eczema, infections and upper respiratory symptoms (URS)). In the mediational context, the reduction of the effect size suggests that the mediator explains part of

the relationship because it is in the causal path between the independent and dependent variables [2]. This was the case for infections at 12 months, which were a risk factor for LRS and were more frequently reported by Turkish than by Dutch parents. In the second year, the mediating effect of previous morbidity was mostly related to the higher prevalence of risk factors for LRS at 12-24 months, such as infections and URS, in Turkish as compared to Dutch infants. On the other hand, the inclusion in the regression equation of socioeconomic status (SES) variables and postnatal factors increased the magnitude of the relationship between Turkish ethnicity and LRS, suggesting that such variables partly masked these associations. Our results are in agreement with the findings of the 'Dutch Prevention and Incidence of Asthma and Mite Allergy' (PIAMA) study, in which Turkish infants were at increased risk of respiratory symptoms in the first 2 years of life as compared to their Dutch peers [3]. In contrast to the PIAMA study, we showed that this risk remained significantly higher in Turkish infants after adjustment for socio-economic variables, which only partly masked this association. This could be explained by the fact that we studied larger groups of infants with different ethnic background, whereas the PIAMA study might not have had enough power to discriminate between subgroups of infants with non-Dutch ethnic background. Other studies in Europe found that children of Turkish migrants have lower risk of asthma as compared to their Europe-born peers [4, 5]. Our study evaluated only the first 2 years of life and we cannot yet establish how many and which infants will develop asthma by the time they reach the school age.

In addition, we found that the increased risk of Antillean infants of LRS at 12-24 months compared to Dutch (1.84 [1.19-2.84]) was entirely mediated by socioeconomic factors, as the inclusion of the SES variables produced a considerable reduction of the odds ratio (OR) of Antilleans vs. Dutch, which was no longer statistically significant. This effect was due to the fact that, compared to Dutch, Antillean mothers were more likely single or not married and this was a risk factor for LRS at 12-24 months. A further reduction of the OR of Antilleans vs. Dutch was observed after the inclusion in the regression model of postnatal exposures (pets' keeping, siblings, breastfeeding, day care attendance and postnatal smoke exposure) and previous morbidity (eczema, infections and URS), respectively. The associations between Moroccan ethnicity and LRS at 0-12 and 12-24 months were not significant in the basic model. However, Moroccan ethnicity was associated with a reduced risk of LRS at 12-24 months in the model including previous morbidity, as Moroccan infants

reported a higher prevalence of infections at 24 months, which were risk factors for LRS at 12-24 months. Although our findings of reduced risk of LRS in Moroccans are in agreement with previous international [6] and Dutch studies [7], these results were statistically significant only in the model including all fetal and postnatal factors. Possible explanations are that either Moroccans have genetic factors that confer protection against the development of respiratory symptoms in infancy, or that variables not measured and/or not included in the analyses could explain this association. It is remarkable that prenatal exposure variables, such as maternal smoking during pregnancy, gestational age at birth and birth weight did not produce a change in the risk estimates across the strata of ethnic groups.

Previous studies have shown ethnic differences in the prevalence of atopic diseases and asthma between and within countries [5, 8-10]. Migration studies have provided information on the role of environmental factors in the development of atopy and asthma [11, 12]. Migration involves exposure to a new environment, which may influence the development of atopic diseases. However, it is still unclear whether such differences are related to an increased intrinsic risk of asthma in certain ethnic groups or to the effect of fetal and/or postnatal environmental exposures [13-16]. Several factors such as socio-economic status, social and cultural integration in the host country, housing condition, changes in lifestyle and accessibility of health care may influence migrants' health. In addition, race, ethnicity and social class are important proxies for unmeasured factors that influence health outcomes. Studies on migrants support the notion that lifestyle and environmental factors in Western industrialized countries facilitate atopy and asthma [5, 17]. Immigration to 'Western lifestyle countries' with high prevalence of atopic diseases causes more allergies and asthma in immigrants as compared to the prevalence of atopy in their countries of origin [18]. This effect depends largely on the age at the time of immigration and on the length of stay in the host country. These findings confirm that genetic and environmental factors, their interactions and the timing of exposure to risk factors play a crucial role in determining the onset of asthma.

Limitations – Methodological considerations

The Generation R cohort was recruited in the city of Rotterdam and of all eligible children at birth, 61% participated. It is difficult to perform a detailed non-response analysis as in national and regional registries there is a lack of subject characteristics for children and their parents. However, there is evidence of a selection toward a

healthier and more affluent study population as the percentages of mothers from ethnic minorities and lower socio-economic status and of mothers or children with medical complications are lower than expected from the population data in Rotterdam [19].

Infants included in the present study were more often Dutch as compared with the average distribution of ethnicity among neonates born in Rotterdam during the enrolment phase of the Generation R Study (figures 1 and 2). This selection would be a concern for the generalizability of the findings if the participation to the study was associated with both the determinants and the outcomes of the studies. As the Generation R Study aims to study growth, development and health from fetal life until young adulthood and is not specifically focused to evaluate respiratory morbidity in infancy, we consider it unlikely that non-participation was related to the occurrence of respiratory symptoms. Furthermore, there was a fair response rate of postnatal questionnaires in the first 2 years of life, which ranged between 71% and 82% of the questionnaires sent to the participants [20].

With regard to the study of ethnic differences in early respiratory morbidity, missing information on ethnicity was present in 4.5% of 5954 eligible infants. Among the 5684 infants with available data on ethnicity, non-Dutch ethnic groups were more likely to have missing values for the investigated lower respiratory symptoms at 12 and 24 months. Because missing data on the outcome variables were not missing at random, complete-case-analysis was likely to introduce biased results [21] and would also lead to loss of a large number of study subjects in the multivariate analyses. It has been shown that imputation of outcome variables using the predictors under study minimizes the bias [22]. Therefore, we imputed missing values of the outcomes, mediators and confounders, using ‘multiple imputation’; this simulation-based approach creates a number of imputed (complete) data sets by “filling in” plausible values for the missing data [23]. The method of multiple imputation allowed us to analyze a complete dataset with a flexible, convenient mean of adjustment for non-response.

As in any large-scale epidemiological study, information on determinants and outcomes under study has been collected by means of questionnaires. Indeed, the prospective design of the study reduced the likelihood of recall bias and the use of translated questionnaires reduced the comprehension problems in ethnic minorities. Moreover, in the postnatal phase of the study further support for verbal translation of questionnaires was available in Arabic, Portuguese and French. Yet, the translation of

the questionnaires could not be used by Moroccans who spoke Berber languages and a selection of Moroccan parents fluent in the Dutch language was therefore unavoidable. If this reflected also a selection of mothers with higher educational level, then we might have underestimated the protective effect of Moroccan ethnicity on respiratory symptoms, as high educational level was associated with increased risk of respiratory symptoms in our study population. Also, it is possible that bias occurred in exposure assessment, as we did not use biological markers to confirm the environmental exposures reported by the parents. A good agreement between parental report of smoking and air nicotine concentration has been shown [24], and several studies have shown that this biomarker is not superior to self-report when studying the effect of maternal smoking on respiratory symptoms of their offspring [25, 26]. Hence, we feel that questionnaire information on smoking may well have provided reliable results. Also, we did not use any biological marker of respiratory infections, such as specific immunoglobulin levels or the detection of the pathogens in saliva or stools. Concern and awareness of respiratory infections by parents may have lead to a differential reporting of infections. Moreover, reversed causation is an important issue, as most children with respiratory infections will also have symptoms of the upper and/or lower respiratory tract, leading to an overestimation of the effects of infections on the risk of respiratory symptoms. In order to reduce the possible bias due to misclassification, we considered reported symptoms accompanied by fever as infections. Also, in our studies, we included respiratory and non-respiratory infections in the multivariable models, in order to control for their possible influence on respiratory symptoms. Measurement of objective biomarkers of environmental exposures and of infections might reduce the misclassification of the determinants, but was not feasible in the context of the Generation R study, due to logistic restraints.

Another possible limitation is that we assessed respiratory symptoms in infants through questionnaires that were partly derived from the ISAAC project, developed and standardized for use in school-age children. A previous study showed poorer repeatability in infants for questions regarding cough and upper respiratory symptoms compared to wheeze and shortness of breath [27]. However, the main outcome variable in most studies reported in this thesis was a combined variable ‘lower respiratory symptoms’, which included different symptoms. Therefore, we consider misclassification of the outcomes less likely.

8.2 FeNO in infants

Methodological issues of FeNO measurements

Guidelines for the standardization of FeNO measurements in adults and children have been published [28, 29]. However, there are still several methodological aspects of FeNO measurements in infants and children younger than 4 years of age that need to be addressed in order to meaningfully interpret FeNO values in this age group. The reproducibility of the off-line mixed nasal/oral FeNO measurements was evaluated in awake infants during tidal breathing without the use of sedation. We assessed the short-term reproducibility of the FeNO measurements in healthy infants participating in the Generation R Focus study and in infants suspected of cow's milk allergy (CMA) participating to the Cow's Milk Allergy with Elimination and Lactobacilli (CAMEL) study. In both studies FeNO was measured twice within 10 minutes. We found good agreement between the two FeNO measurements both in infants participating to the Focus study and in those enrolled in the CAMEL study.

FeNO values are inversely associated with respiratory flow [30, 31] and methods that could help infants to maintain a quiet tidal breathing during the FeNO measurement would be of great value.

As the expiratory flow is less variable in infants when sedated, we investigated the effect of sedation on FeNO values in infants with respiratory diseases and found that geometric mean FeNO collected before and during sedation were not different. However, sedation is not an option in healthy infants and could be used only in selected groups of infants who undergo lung function testing. Another method that could facilitate the cooperation of infants is measuring FeNO immediately after breastfeeding, as an infant who has just been breastfed is more likely to have a constant quiet breathing pattern. In healthy infants participating in the Focus study, FeNO was measured before and 5 minutes after breastfeeding and we found no evidence of an effect of breastfeeding on FeNO values. Therefore, breastfeeding an infant before FeNO measurement could increase the reproducibility of the FeNO measurements within and between infants.

The nasal mucosa and the paranasal sinuses are a source of variable NO excretion. In infants it is recommended to measure FeNO while avoiding contamination with nasal air. However, infants are nasal breathers and the occlusion of the nostrils during the FeNO measurement could lead to a disturbed breathing pattern, influencing FeNO values. Therefore, we compared oral with mixed oral/nasal FeNO values and we found that oral FeNO values were lower than mixed oral/nasal FeNO values in diseased and

healthy infants. It still remains to be evaluated whether the exclusion of nasal NO would improve the ability of FeNO to differentiate between groups of infants with various airways diseases.

FeNO measurements are increasingly used next to lung function tests and forced expiratory maneuvers have been shown to transiently reduce FeNO levels both in asthmatic adults [32-34] and children [35, 36]. We could not confirm such findings in infants, as we found similar geometric mean FeNO before and 5 minutes after lung function tests in infants with various airway diseases. Hence, it seems unnecessary to standardize the sequence of these tests.

We also showed that FeNO measurements were feasible in infants participating in a large epidemiological study, with a comparable success rate at the age of 6 weeks and 6 months in the Focus study (83% and 84%, respectively). The success rate of the FeNO measurements was also evaluated at 4 months in infants participating to the CAMEL study and we found that the large majority of the infants successfully performed the measurements.

Determinants of FeNO

The effects of several fetal and postnatal exposures on FeNO values were evaluated in infants participating in the Focus study. Smoke exposure is one of the most important risk factors for early respiratory morbidity and for the later development of asthma. Previous studies have found that exposure to smoke affects FeNO values in infants [37]. The prospective design of our study allowed us to disentangle the association between fetal and postnatal smoke exposure and FeNO values in infants. We found that, compared with no exposure, post-natal smoke exposure alone led to an increase in FeNO, whereas exposure to smoke both in utero and after birth led to a decrease in FeNO. These findings suggest that direct exposure to smoke after birth may induce inflammation in the airways in a larger extent than indirect exposure through the placenta. However, these differences were observed at 6.9 weeks, but not at 27.3 weeks, suggesting that FeNO may be sensitive to detect an effect of postnatal smoke exposure only during the first 2 months of life. We also found that ambient NO concentrations were related to FeNO in both age groups, whereas age, weight and length at the study date and male gender were positively associated with FeNO only in infants at 27.3 weeks. Therefore, these factors should always be recorded, a possible association with FeNO in the specific population under the study should be investigated and, eventually, controlled for in multivariable

analyses. In contrast with a previous study [37], we found that FeNO values were not modified by maternal atopy and that only within the group of infants with recurrent wheezing, atopy was associated with increased FeNO, suggesting that the association between atopy and FeNO atopy may be significant only in infants at increased risk of asthma. None of the other investigated exposures, such as birth weight, gestational age, breastfeeding, maternal educational level, maternal and paternal atopy and siblings was associated with FeNO values in infants, suggesting that risk factors for wheezing and asthma do not necessarily influence FeNO values in infancy.

FeNO and respiratory symptoms

In a cross-sectional study, we compared the FeNO levels of 118 infants younger than 2 years with various airways diseases (recurrent wheezing (RW), bronchopulmonary dysplasia (BPD) and cystic fibrosis (CF)) with FeNO measured in 100 healthy infants participating to the Generation R Focus study, who served as controls. We found that infants with RW had higher FeNO than healthy controls and further showed that atopic wheezers had higher FeNO than nonatopic wheezers. Several studies have been conducted in selected groups of infants with airways diseases and, although different methodologies have been used to measure FeNO, an association between increased FeNO levels and recurrent wheezing (RW) has been consistently found also in children below the age of 2 years [38-40]. We confirmed that infants with CF have lower FeNO as compared as compared with controls, RW and BPD infants. Although it seems biologically plausible that infants with CF have lower FeNO due to a reduced diffusion of NO through the excess of secretions within the airways, other mechanisms have been hypothesized to be responsible of such findings, such as a primary defect in NO production or a reduced expression of nitric oxide synthase [41, 42]. Yet, in infants with CF either lower or normal FeNO levels have been reported in previous studies [43, 44]. In school-age children with BPD [45] lower FeNO has been reported, but we could not observe any difference in FeNO values between BPD infants and healthy controls in the first 2 years of life.

The association between FeNO and respiratory symptoms was further evaluated in the infants participating in the Generation R Focus study. We evaluated the association between symptoms (breathlessness, a whistling noise when breathing, wheezing, panting, difficulty breathing and/or cough) and FeNO. We showed that infants with at least one symptom had reduced FeNO values as compared with infants without reported symptoms. These results are apparently in contrast with the findings

of higher FeNO in wheezy infants. However, in our study we evaluated the association between FeNO and a combined variable 'lower respiratory symptoms', which included not only wheezing, but also other respiratory symptoms that may be due to other mechanisms, including infection. Moreover, we did not find any evidence of effect modification by maternal atopy or maternal smoking. This could explain the discrepant results with previous prospective studies [46]. Infants with symptoms of the upper respiratory tract (runny and/or blocked nose) severe enough to visit a doctor had lower FeNO than infants with non-severe symptoms, in agreement with a previous study that showed lower FeNO in infants with rhinorrhea [47]. Hence we feel that the finding of reduced FeNO in children with respiratory symptoms could well be explained by an effect of viral infections.

We evaluated also the association between FeNO at a mean age of 27.3 weeks and the development of wheezing in the first 2 years of life. FeNO values were divided in tertiles and infants in the lowest tertile at 6 months showed a three-fold increased risk of wheezing at 24 months, as assessed by questionnaires. The longitudinal data regarding respiratory symptoms allowed us to evaluate whether FeNO at 6 months was associated with persistent symptoms in the first 2 years of life. Interestingly, the comparison of infants with wheezing at 6 and 24 months vs. those who never wheezed showed that infants in the high tertile of FeNO had an almost six-fold increased risk of persistent wheezing compared with infants in the low tertile. Our results would be in agreement with the only other birth cohort study that prospectively evaluated the association between FeNO and onset of respiratory symptoms, showing that increased FeNO precedes the onset of respiratory symptoms only in infants of atopic and smoking mothers [48]. These results would strengthen the observation of previous cohort studies [49, 50], which showed that infants of smoking and atopic mothers are more likely to have severe symptoms early in life that persist during childhood, suggesting that eosinophilic bronchial inflammation may be present in those infants with increased risk of later development of asthma. The mechanisms underlying the association between increased FeNO and development of severe and persistent wheezing are still unknown, as there is a lack of prospective studies from early infancy until young adulthood. Indeed, it is tempting to speculate that mediators that induce NO-synthase may be causally related to the development of bronchial inflammation and that increased FeNO represent a risk factor for the development of asthma, rather than a consequence of chronic airways inflammation.

Limitations – Methodological considerations

Some limitations related to the method used to measure FeNO in infants should be considered. We found that FeNO was highly correlated with ambient NO concentrations. Infants inhaled NO-free air only if ambient NO concentrations were higher than 10 ppb. This procedure appeared not sufficient to avoid contamination of FeNO by ambient NO. In order to overcome this issue, ambient NO was included in the multivariable analyses, suggesting that the associations found were not due to methodological issues [51].

A negative correlation between respiratory flow and FeNO has been described in infants [31, 37]. In the studies presented in this thesis, FeNO was measured without simultaneous measurements of respiratory flow. Differences in tidal flows might introduce variability and explain some of the overlap and the relatively small differences between the groups studied in the present thesis. This would be even more important in those infants with abnormal airway mechanics due to underlying disease or to tobacco smoke exposure. However, it is remarkable that most of the findings that we have reported throughout this thesis are in agreement with the results of previous studies, although different methods for the measurement of FeNO have been used [37, 39, 40, 52]. Therefore, the use of more sophisticated methods to measure FeNO controlling for expiratory flow, might be useful to assess differences at individual level, whereas simple methodologies may be sufficient to evaluate differences in FeNO at a group level.

We assessed sensitivity, specificity, positive predictive value and negative predictive value for different cut-off of FeNO values measured at 6 months with respect to wheezing reported in the second year. Our results suggest that if a proper cut-off is selected, FeNO is able to predict with a reasonable degree of accuracy which children with low FeNO will not wheeze during the second year. Reversely, many children with wheezing in the second year had no increased FeNO during infancy. The negative predictive value was high for a range of cut-off values, as the great majority of children who did not wheeze during the second year had low FeNO at 6 months. The relatively low values of sensitivity and specificity suggest that FeNO measurements cannot be used to accurately predict the development of wheezing in individual infants within this age range.

Perhaps the most important limitation of the studies presented in the present thesis is the relatively short follow-up. We limited the analyses to the first 2 years of life, a period that is crucial for the later development of respiratory morbidity

including asthma, although at this age it is not possible to diagnose asthma. Indeed, it seems clear that timing and intensity of environmental exposures occurring in the preschool child may direct the immune system toward an allergic or non-allergic phenotype. Therefore, the prospective and detailed assessment of several potential risk factors for asthma in pregnancy and in the first years of life will allow us to evaluate whether and to what extent such factors affect the risk for the subsequent development of asthma and atopic diseases. Further follow-up of the cohort to school age and adulthood will show whether the associations between respiratory symptoms, ethnic background and FeNO found in the first 2 years of life will be maintained.

8.3 Clinical and epidemiological implications

In chapter 6 we described risk factors for respiratory morbidity in infants with different ethnic background. Identifying ethnicity-specific risk factors for respiratory morbidity in early life might be useful for the implementation of preventive strategies against the development of asthma and atopic diseases. We found that a positive history of URS, respiratory and non-respiratory infections were risk factors for symptoms of the lower respiratory tract in the first 2 years of life. These factors, which were more prevalent in infants with Turkish background as compared their Dutch peers, mediated part of the effect of Turkish ethnicity on lower respiratory symptoms (LRS) both at 12 and 24 months. Therefore, the reduction of the prevalence of infections and URS early in life in Turkish infants could lead to a parallel reduction of the burden of respiratory symptoms up to the levels of their Dutch peers. However, it still has to be shown whether a decrease of the risk of respiratory symptoms in early life corresponds to a decrease of incident cases of asthma later in life.

On the other hand, several other fetal and postnatal exposures suppressed the relationship between ethnicity and LRS, as these variables increased the magnitude of the risk of respiratory symptoms of ethnic minorities as compared to Dutch infants. For example, we showed that higher SES was associated with increased risk of LRS in the first 2 years of life, with a major effect of high income and maternal educational level at 0-12 months and of marital status (mother living alone) at 12-24 months. In particular, SES completely suppressed the increased risk of LRS at 12-24 months found in Antillean infants compared to Dutch and partly suppressed the increased risk of LRS at 0-12 and 12-24 months of Turkish infants. These findings indicate that that the

lower SES of the Turkish and Antillean subgroups may have suppressed their risk of LRS. The same reasoning can be applied to some postnatal exposure variables such as daycare attendance and breastfeeding in the first 6 months of life. Indeed, daycare attendance was a risk factor and breastfeeding a protective factor for LRS. Turkish infants used less daycare and were more likely to be breastfed, suggesting that the lower use of day care and the higher rate of breastfeeding in Turkish infants suppressed part of their increased risk of LRS. Our findings cannot allow to conclude about causality. The low use of daycare seems an important factor that contributes to the reduction of the risk of LRS in non-Dutch ethnic groups. Whether this depends on the reduction of the total burden of infections transmitted by other children in the kindergarten or whether other factors are responsible remains to be shown. Any advantages of environmental avoidance in protecting children against the development of respiratory disease can be better elucidated after a longer follow-up.

In chapters 7-11, we assessed whether FeNO measurements in infants were feasible, reproducible and helpful in differentiating infants with various airways diseases and respiratory symptoms. The identification of early markers of eosinophilic bronchial inflammation would help identify those infants who are at increased risk of developing asthma later in life, thus leading to better management and, possibly, to targeted therapy strategies. Indeed, the development of standardized and reproducible methods to measure FeNO is essential for a better understanding of the role of FeNO in the early phases of asthma development. We present an easy and reproducible method to measure FeNO in infants, which could be easily applied to large-scale epidemiological studies, as shown by the high success rate of over 80% in the first 6 months of life. However, we acknowledged the methodological limitations of FeNO measurements as described in this thesis and suggest that further improvement of the method is needed in order to obtain accurate FeNO values. In particular, recording expiratory flow and breathing pattern during the FeNO measurements could be useful to improve the comparability of FeNO values between centers.

Our findings strengthen the suggestion of recently published guidelines [28] to use NO-free air always when measuring FeNO in infants, in order to reduce contamination with ambient NO. We also showed that 2 breaths of NO-free air might not be sufficient for this purpose and suggest that infants inhale at least 5 breaths of NO-free air prior to have their FeNO measured. However, this might reduce the success rate of the measurements, as infants would start breathing irregularly.

Although the paranasal sinuses are less developed in infants, they could represent an important source of NO production. The most feasible method that could reduce the nasal contamination without altering the breathing pattern of the infants would be the use of a partitioned facemask with a septum that separates the nose from the mouth so that oral and nasal FeNO can be measured separately. Several types of such facemasks are available for use in newborns, infants and toddlers. In order to overcome the problems related to the variable flow during the FeNO measurements, a pneumotachograph attached to the facemask could be used in order to measure tidal flow. If a pneumotachograph is not available (i.e. general hospital settings), we suggest that at least breathing frequency is recorded during each FeNO measurement. In this way, a correlation between FeNO and respiratory rate can be computed and, if necessary, statistical adjustment can be performed in order to improve the reliability of the results.

Finally, there are several factors that are associated with FeNO in infants and they should be accounted for. In particular, gender and anthropometrics have been found to be associated with FeNO. Therefore, it is important to record age, weight and length at the study date in order to investigate any possible association with FeNO and, if necessary, to adjust for their influence on FeNO in the analysis. Other important factors that should be recorded are personal or family history of atopy/atopic diseases and exposure to tobacco smoke. In particular, effects of fetal and/or postnatal smoke exposure on FeNO in infants should be considered and appropriate statistical methods applied depending on whether smoke exposure acts as a confounder or effect modifier in the associations under the study. Finally, as respiratory symptoms are not disease-specific, it would be advisable to record several respiratory symptoms in order to identify those that best correlate to the later development of asthma.

8.4 Future research

The Generation R study offered us the opportunity to investigate prospectively the associations between several fetal and postnatal environmental exposures and respiratory morbidity in infants with various ethnic backgrounds. Future research is warranted in order to assess whether and to what extent environmental exposures associated with symptoms in infancy represent a risk factor for the later development of asthma. As the children of the cohort will turn 5 years, they will all be invited for

detailed assessments at the Sophia Children's Hospital. Then, it will be possible to perform standardized measurements of objective parameters of airways function, atopy and bronchial inflammation. Fetal and early postnatal exposures will be correlated with these markers and with the development of asthma in different ethnic groups in order to better define specific preventive measures that could reduce the prevalence of asthma. Moreover, the questionnaires, which have been developed for school-age children, will be administered to the children participating in the Generation R study at the appropriate age.

FeNO is a promising tool for identifying a specific asthma phenotype (i.e. atopic asthma). Large-scale epidemiological studies are needed in order to confirm our findings in other centers and also in ethnically different populations. In particular, there is a need to identify those factors that might affect FeNO values in infants, in order to meaningfully interpret FeNO values in this age group. Further research within the Generation R study is needed to assess whether and to what extent FeNO measured off-line with uncontrolled flow in infants correlates with the single-breath on-line FeNO measurement performed in the same cohort when children will be again assessed at 5 years of age. As wheezing phenotypes will become evident with time, it will be possible to evaluate whether FeNO measured in the first 6 months of life correlates with the subsequent development of asthma. The early identification of children with underlying eosinophilic bronchial inflammation will allow pediatricians and pediatric pulmonologists to better target steroid therapy to those infants who are more likely to benefit from such treatment.

References

1. Victora CG, Huttly SR, Fuchs SC, Olinto MT. The role of conceptual frameworks in epidemiological analysis: a hierarchical approach. International journal of epidemiology. 1997 Feb;26(1):224-7.

2. MacKinnon DP, Krull JL, Lockwood CM. Equivalence of the mediation, confounding and suppression effect. Prev Sci. 2000 Dec;1(4):173-81.

3. Koopman LP, Wijga A, Smit HA, De Jongste JC, Kerkhof M, Gerritsen J, et al. Early respiratory and skin symptoms in relation to ethnic background: the importance of socioeconomic status; the PIAMA study. Arch Dis Child. 2002 Dec;87(6):482-8.

4. Hjern A, Rasmussen F, Johansson M, Aberg N. Migration and atopic disorder in Swedish conscripts. Pediatr Allergy Immunol. 1999 Aug;10(3):209-15.

5. Kabesch M, Schaal W, Nicolai T, von Mutius E. Lower prevalence of asthma and atopy in Turkish children living in Germany. Eur Respir J. 1999 Mar;13(3):577-82.

6. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet. 2006 Aug 26;368(9537):733-43.

7. Uijen AA, Schermer TR, van den Hoogen HJ, Mulder J, Zantinge EM, Bottema BJ. Prevalence of and health care consumption for asthma and COPD in relation to ethnicity. Ned Tijdschr Geneeskd. 2008 May 17;152(20):1157-63.
8. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Lancet. 1998 Apr 25;351(9111):1225-32.
9. Hjern A, Haglund B, Hedlin G. Ethnicity, childhood environment and atopic disorder. Clin Exp Allergy. 2000 Apr;30(4):521-8.
10. Kuehni CE, Strippoli MP, Low N, Brooke AM, Silverman M. Wheeze and asthma prevalence and related health-service use in white and south Asian pre-schoolchildren in the United Kingdom. Clin Exp Allergy. 2007 Dec;37(12):1738-46.
11. Hunninghake GM, Weiss ST, Celedon JC. Asthma in Hispanics. Am J Respir Crit Care Med. 2006 Jan 15;173(2):143-63.
12. Kuehni CE, Strippoli MP, Low N, Silverman M. Asthma in young south Asian women living in the United Kingdom: the importance of early life. Clin Exp Allergy. 2007 Jan;37(1):47-53.
13. Rose D, Mannino DM, Leaderer BP. Asthma prevalence among US adults, 1998-2000: role of Puerto Rican ethnicity and behavioral and geographic factors. American journal of public health. 2006 May;96(5):880-8.
14. Grant EN, Lyttle CS, Weiss KB. The relation of socioeconomic factors and racial/ethnic differences in US asthma mortality. American journal of public health. 2000 Dec;90(12):1923-5.
15. Joseph CL, Ownby DR, Peterson EL, Johnson CC. Racial differences in physiologic parameters related to asthma among middle-class children. Chest. 2000 May;117(5):1336-44.
16. Shapiro GG, Stout JW. Childhood asthma in the United States: urban issues. Pediatr Pulmonol. 2002 Jan;33(1):47-55.
17. Brim SN, Rudd RA, Funk RH, Callahan DB. Asthma prevalence among US children in underrepresented minority populations: American Indian/Alaska Native, Chinese, Filipino, and Asian Indian. Pediatrics. 2008 Jul;122(1):e217-22.
18. Tedeschi A, Barcella M, Bo GA, Miadonna A. Onset of allergy and asthma symptoms in extra-European immigrants to Milan, Italy: possible role of environmental factors. Clin Exp Allergy. 2003 Apr;33(4):449-54.
19. Center for Research and Statistics, Rotterdam (COS); 2008.www.cos.rotterdam.nl.
20. Jaddoe VW, van Duijn CM, van der Heijden AJ, Mackenbach JP, Moll HA, Steegers EA, et al. The Generation R Study: design and cohort update until the age of 4 years. Eur J Epidemiol. 2008;23(12):801-11.
21. Klebanoff MA, Cole SR. Use of multiple imputation in the epidemiologic literature. American journal of epidemiology. 2008 Aug 15;168(4):355-7.
22. Crawford SL, Tennstedt SL, McKinlay JB. A comparison of anlaytic methods for non-random missingness of outcome data. Journal of clinical epidemiology. 1995 Feb;48(2):209-19.
23. Rubin DB. Multiple Imputation for Nonresponse in Surveys. Wiley & Sons, New York. 1987.
24. Gehring U, Leaderer BP, Heinrich J, Oldenwening M, Giovannangelo MECA, Nordling E, et al. Comparison of parental reports of smoking and resudential air nicotine concentrations in children. Occup Environ Med. 2006(63):766-72.
25. Margolis PA, Keyes LL, Greenberg RA, Bauman KE, LaVange LM. Urinary cotinine and parent history (questionnaire) as indicators of passive smoking and predictors of lower respiratory illness in infants. Pediatr Pulmonol. 1997 Jun;23(6):417-23.
26. Brunekreef B, Leaderer BP, van Strien R, Oldenwening M, Smit HA, Koopman L, et al. Using nicotine measurements and parental reports to assess indoor air: the PIAMA birth cohort study. Prevention and Incidence of Asthma and Mite Allergy. Epidemiology (Cambridge, Mass. 2000 May;11(3):350-2.
27. Strippoli MP, Silverman M, Michel G, Kuehni CE. A parent-completed respiratory questionnaire for 1-year-old children: repeatability. Arch Dis Child. 2007 Oct;92(10):861-5.
28. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med. 2005 Apr 15;171(8):912-30.
29. Baraldi E, de Jongste JC. Measurement of exhaled nitric oxide in children, 2001. Eur Respir J. 2002 Jul;20(1):223-37.
30. Deykin A, Massaro AF, Drazen JM, Israel E. Exhaled nitric oxide as a diagnostic test for asthma: online versus offline techniques and effect of flow rate. Am J Respir Crit Care Med. 2002 Jun 15;165(12):1597-601.
31. Hall GL, Reinmann B, Wildhaber JH, Frey U. Tidal exhaled nitric oxide in healthy, unsedated newborn infants with prenatal tobacco exposure. J Appl Physiol. 2002 Jan;92(1):59-66.
32. Silkoff PE, Wakita S, Chatkin J, Ansarin K, Gutierrez C, Caramori M, et al. Exhaled nitric

oxide after beta2-agonist inhalation and spirometry in asthma. Am J Respir Crit Care Med. 1999 Mar;159(3):940-4.
33. Deykin A, Massaro AF, Coulston E, Drazen JM, Israel E. Exhaled nitric oxide following repeated spirometry or repeated plethysmography in healthy individuals. Am J Respir Crit Care Med. 2000 Apr;161(4 Pt 1):1237-40.
34. Deykin A, Halpern O, Massaro AF, Drazen JM, Israel E. Expired nitric oxide after bronchoprovocation and repeated spirometry in patients with asthma. Am J Respir Crit Care Med. 1998 Mar;157(3 Pt 1):769-75.
35. Barreto M, Villa MP, Montesano M, Rennerova Z, Monti F, Darder MT, et al. Reduced exhaled nitric oxide in children after testing of maximal expiratory pressures. Pediatr Pulmonol. 2006 Feb;41(2):141-5.
36. Gabriele C, Pijnenburg MW, Monti F, Hop W, Bakker ME, de Jongste JC. The effect of spirometry and exercise on exhaled nitric oxide in asthmatic children. Pediatr Allergy Immunol. 2005 May;16(3):243-7.
37. Frey U, Kuehni C, Roiha H, Cernelc M, Reinmann B, Wildhaber JH, et al. Maternal atopic disease modifies effects of prenatal risk factors on exhaled nitric oxide in infants. Am J Respir Crit Care Med. 2004 Aug 1;170(3):260-5.
38. Baraldi E, Dario C, Ongaro R, Scollo M, Azzolin NM, Panza N, et al. Exhaled nitric oxide concentrations during treatment of wheezing exacerbation in infants and young children. Am J Respir Crit Care Med. 1999 Apr;159(4 Pt 1):1284-8.
39. Franklin PJ, Turner SW, Mutch RC, Stick SM. Comparison of single-breath and tidal breathing exhaled nitric oxide levels in infants. Eur Respir J. 2004 Mar;23(3):369-72.
40. Wildhaber JH, Hall GL, Stick SM. Measurements of exhaled nitric oxide with the single-breath technique and positive expiratory pressure in infants. Am J Respir Crit Care Med. 1999 Jan;159(1):74-8.
41. Ho LP, Innes JA, Greening AP. Nitrite levels in breath condensate of patients with cystic fibrosis is elevated in contrast to exhaled nitric oxide. Thorax. 1998 Aug;53(8):680-4.
42. Steagall WK, Elmer HL, Brady KG, Kelley TJ. Cystic fibrosis transmembrane conductance regulator-dependent regulation of epithelial inducible nitric oxide synthase expression. Am J Respir Cell Mol Biol. 2000 Jan;22(1):45-50.
43. Elphick HE, Demoncheaux EA, Ritson S, Higenbottam TW, Everard ML. Exhaled nitric oxide is reduced in infants with cystic fibrosis. Thorax. 2001 Feb;56(2):151-2.
44. Franklin PJ, Hall GL, Moeller A, Horak F, Jr., Brennan S, Stick SM. Exhaled nitric oxide is not reduced in infants with cystic fibrosis. Eur Respir J. 2006 Feb;27(2):350-3.
45. Baraldi E, Bonetto G, Zacchello F, Filippone M. Low exhaled nitric oxide in school-age children with bronchopulmonary dysplasia and airflow limitation. Am J Respir Crit Care Med. 2005 Jan 1;171(1):68-72.
46. Frey U, Suki B. Complexity of chronic asthma and chronic obstructive pulmonary disease: implications for risk assessment, and disease progression and control. Lancet. 2008 Sep 20;372(9643):1088-99.
47. Franklin PJ, Turner SW, Hall GL, Moeller A, Stick SM. Exhaled nitric oxide is reduced in infants with rhinorrhea. Pediatr Pulmonol. 2005 Feb;39(2):117-9.
48. Latzin P, Kuehni CE, Baldwin DN, Roiha HL, Casaulta C, Frey U. Elevated exhaled nitric oxide in newborns of atopic mothers precedes respiratory symptoms. Am J Respir Crit Care Med. 2006 Dec 15;174(12):1292-8.
49. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med. 1995 Jan 19;332(3):133-8.
50. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med. 2003 Oct 9;349(15):1414-22.
51. Latzin P, Frey U. Environmental exposure in relation to exhaled nitric oxide in newborns: is it all about timing? Eur Respir J. 2008 Aug;32(2):252-4.
52. Franklin PJ, Turner S, Mutch R, Stick SM. Parental smoking increases exhaled nitric oxide in young children. Eur Respir J. 2006 Oct;28(4):730-3.

Summary

Asthma is a complex disease with a strong genetic component. However, there is evidence that environmental exposures and events occurring during the prenatal and the early postnatal development and their interaction with the individual genetic susceptibility may influence the onset of respiratory symptoms in infancy and asthma during childhood. Many infants wheeze in the first years of life, but only few of them will develop persistent symptoms. On the other hand, the onset of asthma in early adulthood has its origin during the school years. Therefore, in the last decade the concept of asthma as one unifying disease has disappeared and several distinct asthma phenotypes have been described with specific pathological and immunological mechanisms and inflammatory pathways. Hence, the early identification of risk factors associated with a specific asthma phenotype might be crucial for the development of preventive strategies in the first years of life, when interventions may modify the natural history of the disease.

The study described in the ***first part*** of this thesis *(chapter 2)* was conducted within the framework of the Generation R Study, a multiethnic population-based prospective cohort study from fetal life until young adulthood designed to identify early environmental and genetic causes of growth, development and health from fetal life until young adulthood. We evaluated whether and to what extent environmental exposures in the pre- and early postnatal period were associated with the occurrence of respiratory symptoms in ethnically different infants living in the same urban area. Compared to Dutch, Antillean and Turkish infants had increased risk of lower respiratory symptoms, whereas Moroccan ethnicity was associated with reduced risk of lower respiratory symptoms during the first 2 years of life. In our study, postnatal exposures mediated part of the associations between ethnicity and respiratory symptoms, whereas socioeconomic variables mainly suppressed such associations, hinting that there are ethnicity-specific risk factors for respiratory morbidity in early life. However, the effect of pre- and postnatal environmental exposures could not entirely explain the association between ethnic background and risk of respiratory symptoms in infancy. Possible explanations are that genetic factors and/or hidden way of life/cultural differences, different attitudes towards the use of the medical system could be responsible of the residual association. As the Generation R cohort matures and longer follow-up data will be available, it will be evaluated whether the risk of respiratory symptoms in the first 2 years of life parallels the increased risk of asthma development at older age.

The ***second part*** of this thesis focused on exhaled nitric oxide (FeNO) measurements in infants, describing methodological issues, clinical and epidemiological applications of FeNO measured in the first 2 years of life. FeNO is a marker of eosinophilic bronchial inflammation and several studies in adults and children have shown the utility of FeNO as 'inflammometer' in the management of asthma. As most asthma begins early in life, a bed-side test that could help identify infants who are more likely to develop asthma during childhood, would be of great value. Several factors related to measurement conditions and environmental exposures have been shown to influence FeNO values, but to date no standardized method to measure FeNO in infants is available.

In *chapter 3* the method used to measure FeNO in infants was described and success rate and short-term reproducibility of the FeNO measurement were also assessed. We showed that the off-line FeNO measurement in infants during tidal breathing can be successfully performed by the majority of infants aged 6 weeks and 6 months and that FeNO values are reproducible within infants. Also, we explored other methodological aspects of FeNO measurement, such as the effects of sedation, lung function test, nasal contamination and breastfeeding on FeNO values. Although expiratory flow was not measured during the measurements and we could not adjust FeNO values for respiratory parameters, in *chapter 4* we showed that off-line FeNO measurements with uncontrolled flow could differentiate infants with various airway diseases as well as more sophisticated techniques, suggesting that the technique should be improved in order to evaluate the utility of FeNO measurements at individual level.

In *chapter 5* we evaluated whether FeNO could be used to discriminate between infants with and without cow's milk allergy who underwent a food challenge. In particular, we assessed whether FeNO changed during a cow's milk challenge test, thus reflecting underlying immune responses that would allow us to differentiate between tolerant and allergic infants. However, our results do not support a clinical usefulness of FeNO in differentiating allergic form tolerant infants, as FeNO values were not predictive and not related to the occurrence of a positive reaction during a cow's milk challenge in infants. FeNO is a marker of bronchial inflammation and none of the infants developed respiratory reactions, but rather skin and gastrointestinal symptoms. Therefore, the absence of eosinophilic infiltration in the lower airway

mucosa of infants undergoing the food challenge might have been responsible of our findings.

The associations of pre- and early post-natal exposures and respiratory symptoms with FeNO values measured at 6 months were evaluated in *chapter 6*. In a large sample of children participating to the Generation R Focus Study we showed that the effects of smoke exposure on FeNO largely depend on the timing and intensity of the exposure, with lower FeNO values in infants exposed to smoke both in utero and after birth as compared to unexposed infants. Furthermore, we demonstrated that severe respiratory symptoms in the first 2 months of life were associated with lower FeNO. These findings are apparently in contrast with most previous studies in schoolchildren and adults. However, the reduced FeNO in infants with respiratory symptoms could well be explained by an effect of viral infections, which are a predominant cause of respiratory symptoms in the first months of life and have been previously associated with lower FeNO as a result of neutrophilic rather than eosinophilic bronchial inflammation.

Prospective data of the Generation R cohort were used to assess the association between FeNO at 6 months and symptoms of the lower respiratory tract in the second year of life. The results reported in *chapter 7* showed that infants with high FeNO at 6 months were more likely to report wheezing and persistent symptoms during the first 2 years. These findings would strengthen the observation that the first 2 years of life represent a window of time where intervention could modify the natural course of allergic diseases.

Finally, in the general discussion *(chapter 8)* the main findings of this thesis are integrated and interpreted. Also, implication of the results and directions of future research are outlined.

Samenvatting

Astma is een complexe ziekte met een sterke genetische component. Ook blootstelling aan omgevingsfactoren en invloeden tijdens de prenatale en vroege postnatale ontwikkeling hebben in interactie met de individuele genetische vatbaarheid invloed op het ontstaan van luchtwegsymptomen op kleuterleeftijd en astma in de jeugd. Veel kinderen hebben gedurende hun eerste levensjaren last van piepen op de borst, maar slechts een minderheid van deze kinderen zal persisterende klachten ontwikkelen. Aan de andere kant heeft astma in de vroege volwassenheid meestal zijn oorsprong in de eerste levensjaren.

Gedurende het laatste decennium is het concept van astma als één overkoepelende ziekte verdwenen en zijn diverse verschillende astmafenotypes beschreven met specifieke pathologische en immunologische kenmerken. Daarom is het van belang om in een vroeg stadium risicofactoren te identificeren die geassocieerd zijn met een specifiek astmafenotype. Dit kan bepalend zijn voor de ontwikkeling van preventieve strategieën in de eerste levensjaren, als interventies de natuurlijke ziektegeschiedenis nog kunnen beïnvloeden.

De studie die in het eerste gedeelte van dit proefschrift (hoofdstuk 2) wordt beschreven werd uitgevoerd in het kader van de Generation R studie, een multi-etnische prospectieve cohortstudie van de vroege zwangerschap tot de volwassen leeftijd. Generation R is opgezet om vroege omgevings- en genetische determinanten voor groei, ontwikkeling en gezondheid van foetus tot jonge volwassene te onderzoeken. We hebben geëvalueerd of en in welke mate blootstelling aan omgevingsfactoren in de pre- en vroege postnatale periode waren geassocieerd met het vóórkomen van luchtwegklachten in etnisch verschillende kinderen in de stad Rotterdam. Vergeleken met Nederlandse kinderen hadden Antilliaanse en Turkse kinderen een grotere kans op lage luchtwegklachten, terwijl bij de Marokkaanse kinderen een verminderde kans bestond op lage luchtwegklachten gedurende de eerste 2 jaar. Dit kon gedeeltelijk worden verklaard uit postnatale blootstellingen, wat zou kunnen betekenen dat er specifieke aan etniciteit gerelateerde risrisicofactoren bestaan voor luchtwegklachten op jonge leeftijd. Echter, het effect van pre- en postnatale blootstelling aan omgevingsfactoren kon het verband met luchtwegklachten niet helemaal verklaren. Mogelijke verklaringen zijn dat genetische factoren of niet-onderzochte lifestyle factoren of culturele verschillen en verschillende attitudes t.o.v. het gebruik van het medische systeem mede verantwoordelijk zouden kunnen zijn voor het verband. Wanneer het Generation R-cohort volwassen wordt en gegevens van een langere follow-up beschikbaar zullen zijn, zal worden geëvalueerd

of de kans op luchtwegklachten gedurende de eerste 2 jaren voorspellend was voor de verhoogde kans op ontwikkeling van astma op latere leeftijd.

Het tweede gedeelte van dit proefschrift concentreert zich op de meting van stikstof mono-oxide fractie in de uitademingslucht (FeNO) bij kinderen, en op methodologische aspecten, klinische en epidemiologische toepassingen van FeNO in de eerste 2 jaar. FeNO is een marker voor eosinofiele luchtwegontsteking. Verschillende studies bij volwassenen en kinderen hebben het nut van FeNO als "inflammometer" bij de astmabehandeling aangetoond. Omdat astma meestal op jonge leeftijd voor het eerst optreedt, zou een test die snel en gemakkelijk gedaan kan worden waardoor kinderen die meer kans hebben om gedurende hun jeugd astma te ontwikkelen kunnen worden opgespoord van grote waarde zijn. Er is aangetoond dat verschillende factoren bij de meting en in de omgeving FeNO kunnen beïnvloeden, en tot op heden is er geen gestandaardiseerde methode om FeNO bij jonge kinderen te bepalen. In hoofdstuk 3 beschrijven we onze methode om FeNO bij kinderen te meten, en de succeskans en korte-termijn reproduceerbaarheid. We hebben aangetoond dat de off-line FeNO metingen bij kinderen gedurende rustige ademhaling succesvol uitgevoerd kon worden door een meerderheid van de kinderen op een leeftijd van 6 weken en 6 maanden en dat FeNO waarden binnen kinderen reproduceerbaar zijn. We hebben ook andere methodologische aspecten van FeNO metingen onderzocht, zoals het effect van sedatie, longfunctiemetingen, contaminatie vanuit de neus, en het effect van voorafgaande borstvoeding.

In hoofdstuk 4 hebben we aangetoond dat we met de off-line meting van FeNO tijdens rustademhaling verschillen kunnen aantonen tussen groepen kinderen met verschillende luchtwegziekten. Wellicht kan de precisie van de meting nog verbeterd worden, bijvoorbeeld door de uitademingsnelheid te controleren, zodat ook resultaten kunnen worden verkregen die bruikbaar zijn in individuele patiënten.

In hoofdstuk 5 hebben we geëvalueerd of FeNO gebruikt zou kunnen worden bij kinderen met en zonder koemelkallergie die een koemelkprovocatietest ondergaan. We hebben bepaald of FeNO veranderde gedurende een koelmelkprovocatie, tengevolge van de onderliggende immuunrespons. Op deze manier zou in een vroeg stadium verschil kunnen worden gevonden tussen tolerante en allergische kinderen. Onze resultaten lieten zien dat FeNO geen verschil liet zien tussen allergische en tolerante kinderen.

De relatie tussen pre- en vroege postnatale blootstellingen en luchtwegklachten enerzijds en FeNO gemeten op de leeftijd van 6 maanden anderzijds werden

geëvalueerd in hoofdstuk 6. In een streekproef van kinderen die deelnemen aan de Generation R focusstudie hebben we aangetoond dat de effecten op FeNO van blootstelling aan sigarettenrook grotendeels afhangen van de tijd en intensiteit van de blootstelling. Wij vonden lagere FeNO waarden bij kinderen die zowel in utero als na de geboorte waren blootgesteld aan rook dan in kinderen die alleen na de geboorte, of niet aan rook waren blootgesteld. Verder hebben we gevonden dat ernstige luchtwegklachten in de eerste 2 maanden waren geassocieerd met een lagere FeNO. Deze bevindingen staan schijnbaar in contrast met de meeste eerdere studies bij kinderen op de schoolleeftijd en bij volwassenen. De lage FeNO bij kinderen met luchtwegklachten kan worden verklaard door het effect van virale infecties, die de belangrijkste oorzaak zijn van luchtwegklachten in de eerste maanden en waarvoor eerder al een verband is gevonden met lagere FeNO, waarschijnlijk omdat hier neutrofiele in plaats van eosinofiele bronchiale ontsteking bestaat.

In het Generation R cohort hebben we het verband onderzocht tussen FeNO op 6 maanden en symptomen van de lagere luchtwegen in het tweede levensjaar. De resultaten die in hoofdstuk 7 worden gerapporteerd tonen aan dat kinderen die op de leeftijd van 6 maanden een hoge FeNO hebben een grotere kans hebben op piepen en andere chronische luchtwegklachten op de leeftijd van 2 jaar. Deze bevindingen ondersteunen de theorie dat in de eerste 2 jaren het natuurlijke beloop van astma mogelijk nog zou kunnen worden beïnvloed met bepaalde interventies.

Uiteindelijk worden in de discussie (hoofdstuk 8) de belangrijkste bevindingen samengevat en geïnterpreteerd. De implicaties van de resultaten en suggesties voor verder onderzoek worden aangegeven.

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About the author

Carmelo Gabriele was born in Naples, Italy, on December 16th, 1973. He received his M.D. degree at the Second University of Naples with top grades and honors in 1998. In the same year he started his residency at the Department of Pediatrics, Second University of Naples (head Prof. dr. M. Miraglia del Giudice) and he received his degree as specialist in 2003 with top grades and honors.

During the period September-November 2002, he completed a fellowship at the Department of Pediatric Respiratory Medicine, Sophia Children's Hospital – Erasmus University Medical Centre (head Prof. dr. J.C. de Jongste), supported by the Italian Nitric Oxide Club and the Valeas. He conducted a research project on the effect of spirometry and exercise on exhaled nitric oxide in asthmatic children.

In 2003, he started with the research project presented in this thesis, being constantly involved in the research activities of the Departments of Pediatric Respiratory Medicine and of the Generation R Study at the Erasmus University Medical Centre. He obtained a Master of Science degree in Clinical Epidemiology from the Netherlands Institute for Health Sciences (Nihes) in 2005. In 2006, he received a grant from the Stichting Astma Bestrijding for a research project on the effect of air pollution on respiratory health. From December 2005 to July 2006, he worked as neonatologist at the Department of Neonatology of the Loreto Nuovo Hospital in Naples, Italy (head dr. S. Gabriele). Since July 1st, 2009 he has worked as attending physician at the Department of Pediatrics of the Salesi Children's Hospital, Ancona, Italy (head Prof. dr. F.M. de Benedicitis).

Dr. Gabriele is married with dr. Maria Luisa Conte and they have a daughter named Camilla, born on July 18th, 2006.

PhD Portfolio

Name PhD student: C. Gabriele **Erasmus MC Department: The Generation R Study; Pediatric Respiratory Medicine, Sophia Children's Hospital.** **Research School:**	**PhD period: 2003-2008** **Promotors:** **Prof. dr. JC de Jongste** **Prof. dr. A Hofman** **Supervisor: Prof. dr. JC de Jongste**		
1. PhD training			
	Year	**Workload**	
		Hours	**ECTS**
Specific courses			
Post Graduate Course, ATS Conference, San Diego, CA	2005		1
Presentations			
10 presentations at seminars	2003-2009	140	
International conferences			
Poster presentation ERS congress, Vienna, Austria	2003		1
Poster presentation ATS Conference, San Diego, CA	2005		1
Poster presentations ERS congress, Copenhagen, Denmark	2005		2
Oral presentation ERS congress, Munich, Germany	2006		1
Poster presentations ERS congress, Stockholm, Sweden	2007		2
Poster presentations ERS congress, Vienna, Austria	2009		2
Seminars and workshops			
2 workshops	2004, 2007		2
Other			
Oral presentation Italian Society of Children's Respiratory Diseases congress, Taormina, Italy. Awarded as best abstract.	2006		1
Oral presentation Italian Society of Pediatric Immunology and Allergology, Congress, Florence, Italy. Mead Johnson award as best abstract.	2007		1
Note			
Master of Science in Clinical Epidemiology	2003-2005	652	
2. Teaching activities			
	Year	**Workload**	
		Hours	**ECTS**
Supervising practicals and excursions			
Nihes courses	2005, 2006	40	
Supervising Master's theses	2006-2007	56	
Total		888	14