# Asthma, atopy and lung function at 11 years of age after bronchiolitis in infancy

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*In bronchiolitis we must now contend*
*with both the disease and the "now" and the "then";*
*For many such infants a mold has been cast,*
*perhaps by their unborn and unknown past,*
*which destines that they shall in time wheeze again.*
*For them this disease*
*is the distant, boding knell*
*Of vulnerable lungs to a microbe's mystic spell.*
C.B.Hall (1)

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# 1. PREFACE

## 1.1 Acknowledgements

The present work was carried out during the years from 2008 to 2013 in collaboration with the Paediatric Department at Stavanger University Hospital and Haukeland University Hospital. I became a PhD candidate at the University of Bergen in January 2011. The work has been performed as part of everyday clinical work and with financial support from the Paediatric Department and the Department of research at Stavanger University Hospital.

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*Stavanger, March 2014*
*Ingvild Bruun Mikalsen*

## 1.2 Summary of thesis

**Background**

Bronchiolitis is a frequently occurring respiratory disorder in young children, and also an established risk factor for subsequent recurrent wheeze and asthma. The association between bronchiolitis and later asthma is complex and not fully understood, and probably related to interactions between viral aetiology, various host factors and environmental mechanisms. Few long-term follow-up studies have included children hospitalized for bronchiolitis below 12 months of age with Respiratory syncytial virus (RSV) positive as well as RSV negative disease.

**Aim**

The overall aim of this thesis was to study the prevalence of asthma and atopy, and the respiratory function at 11 years of age after hospitalization for bronchiolitis in infancy, in order to contribute to the understanding of the pathophysiological and clinical outcomes after bronchiolitis.

**Methods**

A prospective observational and partly controlled cohort design was applied. One hundred and thirty one children hospitalized for bronchiolitis during their first year of life were enrolled. Markers of eosinophilic airway inflammatory were measured in urine and blood in 105 children at hospitalization. At two years of age parents of 101 children filled in questionnaires, and a skin prick test (SPT) was performed. The second follow-up at 11 years of age included 121 children from the post-bronchiolitis group and a control group of 141 children. All parents answered a questionnaire regarding respiratory symptoms of the child, and assessment of lung function, exhaled nitric oxide (FeNO), bronchial hyperresponsiveness (BHR) and a SPT were performed.

**Results**

Children hospitalized with bronchiolitis in infancy had an increased risk of subsequent asthma, reduced lung function and higher BHR, but not an increased risk

of atopy at 11 years of age. After stratifying for viral aetiology (RSV+/RSV-) and gender, higher prevalence of asthma and an obstructive lung function pattern was only present in children with a history of RSV negative bronchiolitis. Higher BHR was confined to boys, irrespective of viral aetiology.

Blood eosinophil counts measured during bronchiolitis in infancy were higher in children with current asthma than those without asthma at 11 years of age. Blood eosinophil counts during bronchiolitis were associated with reduced lung function and higher BHR at 11 years of age.

FeNO did not differ between the post-bronchiolitis and control group at 11 years of age. FeNO was associated with atopy, but not asthma in children hospitalized for bronchiolitis as well as in the control group.

Prediction of asthma at 11 years of age based on a model including clinical parameters available at two years of age proved to be challenging, but was as good as more complex models including invasive tests. However, the model did predict the absence of asthma reasonably well, with low negative post-test probabilities.

**Conclusions**

Severe bronchiolitis in infancy was associated with long-term pulmonary features such as asthma, airway obstruction and BHR. These associations seemed to be modulated by viral aetiology and possibly also by aspects of the inflammatory response pattern during the acute episode. Clinical parameters available at two years of age were better to predict the absence than the presence of asthma at 11 years. The understanding of the pathophysiology and the long-term outcomes of bronchiolitis is still fragmented, and further studies including large cohorts of subjects preferably representing entire geographic regions should ideally be followed from birth to adulthood, including well selected control subjects.

## 1.3 List of papers

I. Mikalsen IB, Halvorsen T, Øymar K.
**The outcome after severe bronchiolitis is related to gender and virus.**
Pediatr Allergy Immunol. 2012; 23: 391-98

II. Mikalsen IB, Halvorsen T, Øymar K.
**Blood eosinophil counts during bronchiolitis are related to bronchial hyper-responsiveness and lung function in early adolescence.**
Acta Paediatr. 2014; 103: 86-92

III. Mikalsen IB, Halvorsen T, Øymar K.
**Exhaled nitric oxide is related to atopy, but not asthma in adolescents with bronchiolitis in infancy.**
BMC Pulm Med. 2013; 13: 66

IV. Mikalsen IB, Halvorsen T, Eide GE, Øymar K.
**Severe bronchiolitis in infancy: Can asthma in adolescence be predicted?**
Pediatr Pulmonol. 2013; 48: 538-44

## 1.4 Abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| AD | Atopic dermatitis |
| API | Asthma Predictive Index |
| BHR | Bronchial hyperresponsiveness |
| BPO | Bronchopulmonary obstruction |
| CPAP | Continuous positive airway pressure |
| CI | Confidence interval |
| DC | Dendritic cell |
| DRS | Dose response slope |
| ECA | Environment and Childhood Asthma |
| EAACI | European Academy of Asthma and Allergy in Childhood |
| EPO | Eosinophil peroxidase |
| EPX | Eosinophil protein X |
| ERS | European Respiratory Society |
| $FEF_{25-75}$% | Forced expiratory flow between 25-75% of the forced vital capacity |
| $FEV_1$ | Forced expiratory volume in first second |
| FVC | Forced vital capacity |
| FeNO | Fractional exhaled nitric oxide |
| GINA | Global Initiative for Asthma |
| GLI | Global Lung Initiative |
| GM-CSF | Granulocyte macrophage colony stimulation factor |
| ICS | Inhaled corticosteroid |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| iNOS | Inducible Nitric oxide synthase |
| ISAAC | International Study of Asthma and Allergy in Childhood |
| IQR | Inter-quartile range |
| kUa | Kilo international unit |
| LR | Likelihood ratio |
| Ln | Natural logarithm |

| | |
|---|---|
| LT | Leukotriene |
| MAS | Multicentre allergy study |
| MPT | Methacholine provocation test |
| OR | Odds ratio |
| PCR | Polymerase chain reaction |
| PG | Prostaglandin |
| PPV | Positive predictive value |
| RQ | Research question |
| RSV | Respiratory syncytial virus |
| RV | Rhinovirus |
| SPT | Skin prick test |
| TLR | Toll like receptor |
| Th | T-helper |
| T-reg | T -regulatory |

# 2. GENERAL INTRODUCTION

The earliest medical description of acute bronchiolitis was published by Eberle in his book from 1857, describing a ‘‘congestive catarrhal fever” of infants younger than 12 months of age with pulmonary congestion, cough and wheeze that could “resemble a violent attach of asthma” (2). However, the first virus involved in this scenario was not isolated until 1935 (3). Respiratory Syncytial Virus (RSV) has since been established as the most common respiratory virus related to acute bronchiolitis in children. The virus was first isolated in 1955 from young chimpanzees with respiratory symptoms and was given the name Chimpanzee coryza agent. The same virus was the following, year isolated from wheezing infants and renamed RSV (4).

The word asthma derived from Greek means a short drawn breath or panting, and the condition was described on Egyptian medical papyri already in 1550 B.C. (5). Asthma was described by Sir William Osler in 1892 as a neurotic condition characterised by vagus induced contraction of the bronchi. Osler was the first to describe asthma as episodes of breathing attacks characterized by contraction of smooth muscles of the bronchi (6). Cells later named as eosinophils were first observed in the sputum of asthmatics by Henry Hyde Salter in 1868, and in 1879 defined as eosinophils by Paul Ehrlich. The concept of asthma as a possible allergic phenomenon was introduced by Meltzer in 1910 (5).

An association between bronchiolitis and later asthma was already described in a study by Wittig and Glaser published in 1959 (7). Several studies have confirmed their results and some of these are summarised in the review from Piippo-Savolainen and Korppi (8).

A viral pathogen can be isolated in up to 90% of children with wheezing episodes during the first three years of life (9). Still, the debate about a possible link between viral respiratory tract infections in young children and subsequent asthma is on-going (10). Asthma after bronchiolitis is less related to atopy and probably represents a different inflammatory phenotype than atopic asthma (10-12). Associations between inflammatory responses during acute bronchiolitis and subsequent asthma, lung function and bronchial hyperresponsiveness (BHR) are less well studied.

## 2.1 Viral bronchiolitis

### 2.1.1 Definition

The term bronchiolitis refers to inflammation of the bronchioles and is mostly used for children with a first sign of viral respiratory tract infection and respiratory distress (13). The definition of bronchiolitis varies between countries and clinical studies. Clinical trials during the last years have used an upper age limit ranging from 3 to 24 months, 50% focused only on the first episode and 41% included only children with an RSV infection (14). The discrepancies between studies of bronchiolitis regarding a range of findings, including risk factors for later asthma, are probably related to relatively vague definitions, both regarding clinical criteria and the lack of an exact upper age limit.

A subcommittee of the American Academy of Pediatrics with support from the European Respiratory Society (ERS) underlines that bronchiolitis is a clinical diagnosis and often recognized as a "a constellation of clinical symptoms and signs including a viral upper respiratory prodrome followed by increased respiratory effort and wheezing in children less than two years of age" (15). This is consistent with the definition from the Scottish Intercollegiate Guidelines Network (16). However, studies from North America seem to emphasize the presence of wheezing, while crackles and crepitations seem to be important parts of the definition in studies from Europe (17, 18). Regarding age, several studies of bronchiolitis from Europe and USA have only included children up to 12 months of age (19-22).

### 2.1.2 Epidemiology

Approximately 20% of children develop bronchiolitis during their first year of life (23). Male gender is both a risk factor for bronchiolitis and for a more severe clinical course (23, 24).

Studies from USA have found increasing rates of bronchiolitis (188/1000 infants in 1996/97 compared to 265/1000 in 2002/03) in children below 12 months of age (23) and increasing rates of hospitalization for bronchiolitis in children below 24 months of age (3.3% in 2002 compared to 5.5% in 2007) (24).

The hospitalization rate for bronchiolitis in Norway is varying according to age. In one Norwegian study the mean annual hospitalization incidence for RSV bronchiolitis was 21.7 per 1000 for children below 12 months and 6.8 per 1000 children from 12 to 24 months (25). In the Norwegian Mother and Child Cohort study which included 3011 mother-child pairs, 5.4% of the mothers reported hospitalization for lower respiratory tract infection in their children before 6 months of age (26). In the region covered by Stavanger University Hospital, the yearly hospitalization rate for bronchiolitis in children below 12 months of age was 3.4% during four years from 2008 to 2012 (Øymar K et al., submitted).

Bronchiolitis is generally seasonal, most frequent during the winter months and appears in epidemics (26, 27). For RSV the same seasonal pattern is observed particularly in countries with temperate climate, and most infections occur from October until May (27, 28). The increased prevalence of RSV infections during the winter months is probably due to indoor crowding and/or seasonal changes in immune function (28). Adults with chronic obstructive lung disease and immunocompromised patients may have RSV infection throughout the year and thereby represent a reservoir of the virus (28). In addition, high RSV infectious burden prior to birth is associated with a decreased risk of lower respiratory tract infections in infants; this may be due to high maternal RSV antibody concentration (26).

Bronchiolitis is a disease with high morbidity, but low mortality. Death from respiratory failure in bronchiolitis is rare with an incidence ranging from 2.9 (UK) to 5.3 (USA) per 100 000 children below 12 months of age infected with RSV (29, 30). A study from the UK underline that the mortality rate for bronchiolitis in children below 12 months is low and fell from 21.5 to 1.8 per 100 000 children below 12 months of age from 1979 to 2000 (31).

### 2.1.3 Viral aetiology

RSV is the most common virus involved in bronchiolitis, irrespective of age, but most common in younger children. A study from Finland including children hospitalized for acute wheezing, reported a prevalence for RSV in children with bronchiolitis of 80, 60 and 40% in children below 6, 12 and 24 months of age,

respectively (14). The prevalence varies slightly between studies. Midulla et al. found a prevalence of 75% for RSV in children below 12 months of age, but could only detect a virus in 57% of the infants (27, 32). In children below 12 months of age, Rhinovirus (RV) is the second most common virus (14-30%), thereafter human bocavirus (14-15%), human metapneumovirus (3-12%), entero-, adeno-, corona and influenza viruses (1-8%). More than one virus has been reported in 20-30% of children below 12 months of age (14, 27, 32).

For children hospitalized for bronchiolitis before two years of age, RV is found in 25-35%, enterovirus in 5-17%, human metapneumovirus in 6-7% and more than one virus is reported in 20-30% of children (14, 33). RV bronchiolitis is more common in older children and associated with atopy and eosinophilia (14, 34). A more severe clinical picture has been described in children having co-infections with RSV and RV (27), but these observations are not consistent (35). Concurrent bacterial infections are not common, but have been reported in patients with serious symptoms (15, 17, 24).

### 2.1.4 Pathophysiology

The infection starts in the upper respiratory tract, spreading to the lower airways within few days. The inflammation in bronchiolitis is characterized by a peribronchial infiltration of white blood cells, mostly mononuclear cells, and oedema of the sub mucosa and adventitia (13). Damage may occur by a direct viral injury to the respiratory airway epithelium, or indirectly by activating immune responses (17). RSV can destroy epithelial cells, and viral replication in the epithelial cells results in secretion of multiple cytokines, chemokines and adhesion molecules with secondary recruitment of inflammatory cells such as neutrophils and eosinophils (36).
The severity of bronchiolitis may be associated with an impairment of the immune system. Reduced interferon gamma (IFN-γ) response in cord blood has been inversely associated with the severity of the viral airway disease (37). Further, deficiencies in Toll like receptor (TLR)-4, surfactant proteins and Interleukin (IL)-8 production have been related to a more severe RSV disease (38). Down regulation of leukocyte cell–surface receptors has been found in Norwegian children developing RSV disease which may cause an impaired immune response to RSV (39). As summarised in

several reviews, findings may indicate that a weak T-helper (Th)-1 response to a virus infection may contribute to the severity of the disease (40, 41). However, studies on RSV bronchiolitis, underline that these findings may be an oversimplification, as IFN-γ may induce wheezing in RSV bronchiolitis possible by induction of leukotriene release (42). In one study, children with acute bronchiolitis caused by RSV had higher Th-1 cytokine response in nasopharyngeal secretions than children with bronchiolitis without identification of a virus (43). A Finish study has indicated that children with wheezing caused by RV may have a predisposition towards an immune response dominated by Th-2 cytokines (44).

A neutrophilic inflammation has been found in children with bronchiolitis, which is different from the inflammation in older children with atopic asthma, and the neutrophil cells may contribute to the damage of the respiratory epithelium (38, 45, 46). However, also eosinophils and markers of eosinophilic inflammation have been observed, particularly in younger children with bronchiolitis (47). The role of eosinophils as provokers of lung pathology, bronchial inflammation and BHR is still not fully understood, and these characteristics probably involve several types of inflammatory cells (47). Eosinophils have also been suggested to be important in the defence against viral infections (47).

Clinically, the damage of the airway epithelium with necrosis may cause partial or total airflow obstruction, distal air trapping, atelectasis and a ventilation perfusion mismatch leading to hypoxemia. Smooth-muscle constriction seems to play a minor role in the pathologic process of bronchiolitis (13).

The increased risk of bronchiolitis in boys has been related to the relatively smaller airways in males compared to females (48, 49) with lower levels of airflow rates observed in male lungs (50). Other mechanisms explaining this gender difference are the immunosuppressive effect of androgens (51) and IL-9 genetic polymorphisms (52). In the Coast study, gender difference of atopic disease in early childhood, was accompanied by sex-specific differences in immune response profiles with higher response of IFN-γ, IL-5 and IL-13 and increased rates of sensitization in boys (53).

### 2.1.5 Clinical characteristics and treatment

Bronchiolitis often starts with symptoms of an upper respiratory tract infection with rhinorrhoea and gradually increasing signs of a lower respiratory tract infection including tachypnoea, wheezing and cough. The children may have fever or a history of fever, but high fever is uncommon (16). Feeding problems are common. Very young children, particularly those with a history of prematurity, may present with apnoea as the major symptom (17, 36).

The treatment for bronchiolitis remains supportive, with maintenance of oxygenation, fluids and nutritional status (15). The effect of inhalations with bronchodilators and saline is debatable and not recommended for routine treatment (15, 54, 55). Continuous positive airway pressure (CPAP) is frequently used to relieve respiratory distress and improve oxygenation. However, the evidence that CPAP reduces $PCO_2$ or the need of endotracheal intubation is low, as summarised in the review from Donlan et al. (56). In a multicentre study from the USA, approximately 3% of children hospitalized for bronchiolitis below 24 months of age were in need of intubation (57), whereas in Stavanger during four seasons, only 0.4% of infants hospitalized for bronchiolitis before the age of one, were in need of mechanical ventilation (Øymar K, et al., submitted). The risk factors for endotracheal intubation are low age, low birth weight, bronchopulmonary dysplasia, neurological disease, maternal smoking during pregnancy, apnoea and inadequate oral hydration (57).

Monoclonal antibody against RSV (Palivizumab) is recommended as prophylaxis for RSV in high risk children below 24 months of age (15). Palivizumab has reduced the hospitalization rate, but not influenced the mortality rate (15), and the evidence of effect on severe RSV bronchiolitis is limited (58). In addition, several economic reports have failed to show any overall savings in health costs by this treatment (15, 59).

The median length of hospitalization in a study including children below 24 months of age was two days (33), another study found a significant longer median length of hospitalization for children with RSV positive bronchiolitis (three days) than RSV negative bronchiolitis (two days) (24). In studies from Norway, the median length of stay was four days for children below 24 months of age hospitalized for RSV

bronchiolitis (25), others have reported a mean length of stay of 80 hours for children hospitalized for bronchiolitis below 12 months of age (55). A study from South-Africa, including children with bronchiolitis who were treated as outpatients, found that resolution of symptoms took more than 14 days in 40% of the children, and approximately 10% still had symptoms after four weeks (60).

The risk factors for bronchiolitis include male gender, a history of prematurity, young age, timing of birth in relation to the RSV season, pre-existing disease such as bronchopulmonary dysplasia, underlying chronic lung disease or congenital heart disease, exposure to environmental tobacco smoke as well as high parity, young maternal age, no/short duration of breastfeeding, maternal asthma and poor socioeconomic factors (17, 48, 49, 61).

The majority of children hospitalized for bronchiolitis have no such underlying conditions. However, one study including 4800 children hospitalized for bronchiolitis, suggests that the proportion of children with underlying conditions is higher among children with RSV negative bronchiolitis than RSV positive bronchiolitis (24).

## 2.2 Asthma in children

### 2.2.1 Definition

In an updated report from 2012, the Global Initiative for Asthma (GINA) gave the following definition of asthma:

*"Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment"* (62).

This definition includes pathological features, lung function measurements, and clinical characteristics, and emphasizes the role of inflammation. However, assessment of inflammation requires the use of methods that, particularly in small children, are

difficult, less acceptable and time-consuming to carry out, such as bronchoscopy and induced sputum. To some extent, methodological challenges also hamper regular use of lung function measurements in everyday clinical settings. In light of these methodological difficulties, the GINA guidelines for children five years and younger were developed. These guidelines include the same definition as above, in addition to a more descriptive approach to symptoms, according to the various phenotypes of asthma in small children (63).

In 2008, both the European Academy of Allergy and Clinical Immunology (EAACI) and the ERS developed guidelines for diagnosis and treatment of asthma in children (64, 65). These guidelines underline that similar symptoms still may represent different phenotypes and pathophysiology, with differences regarding treatment and prognosis.

The PRACTALL consensus report from the EAACI describes asthma as "*repeated attacks of airway obstruction and intermittent symptoms of increased airway responsiveness to triggering factors, such as exercise, allergen exposure and viral infections*" (64). Further, the consensus underlines the role of phenotypes and the natural history and emphasize that age is the strongest determinant of asthma phenotype in childhood. The consensus report describes four patterns of wheeze in children, but underlines that pattern 1 and 2 only can be discriminated retrospectively:

1. Transient wheezing
   Wheezing during the first 2-3 years of life, but not after the age of three years.
2. Non-atopic wheezing
   Wheezing related to viral infections, and tends to remit later in childhood.
3. Persistent asthma
   Wheezing in children associated with clinical manifestations of atopy (atopic dermatitis, rhinitis or food allergy), atopic sensitization or a parental history of asthma.
4. Severe intermittent wheezing

Infrequent wheezing associated with atopy, but minimal morbidity outside the time of respiratory tract illness.

The ERS guidelines are developed for children below five years of age (65). In order to be useful in a clinical setting, these guidelines use temporal patterns of wheeze and define two distinct phenotypes:

1. Episodic viral wheeze
   Wheeze during discrete time periods often associated with respiratory viral infections, but absence of wheeze between episodes.
2. Multitrigger wheeze
   Wheezing that shows discrete exacerbations, but also symptoms between episodes.

However, when children are defined retrospectively by questionnaires according to the ERS guidelines, their classification may even change within a one-year period (66).

The guidelines from the ERS and EAACI both underline the complexity of asthma, particularly for children, and recommend that asthma not only should be defined as a specific disease, but more as a syndrome of symptoms with different phenotypes. Recently, reports also describe endotypes of asthma, defined as subtypes of a disease by an intrinsically distinct pathogenic mechanism with specific underlying molecular causes and/or distinct treatment responses (67). Understanding of endotypes might in the future facilitate use of specific biomarkers in order to classify the different phenotypes of asthma.

### 2.2.2 Epidemiology

Epidemiological data on asthma are challenging to compare, as methodologies and definitions differ between studies (68). A report from GINA has estimated that 300 million people in the world currently have asthma, thereby being one of the most common chronic diseases (68, 69). This report is based on questionnaires and reports previously published through the International Study of Asthma and Allergies in

Childhood (ISAAC) and the European Community Respiratory Health Survey. The GINA report shows varying prevalence of clinical asthma between countries and age groups, ranging from 1-18% in complete (68). The prevalence of clinical asthma is particularly high (> 10%) in developed countries (69).

For children in developed countries, the prevalence of those reporting asthma symptoms has increased during the last 50 years from 4-5% in 1955 to approximately 15% in 2010 (70). The prevalence of clinical asthma is most striking for children in English speaking countries reporting prevalence rates of >30% (69).

The phase III study from ISAAC also found a slight increase globally in the total number of children who reported to have had "asthma ever" over a period from 5-10 years, estimated to 0.28% per year to 13.8% (13-14 year age group) and 0.18% per year to 10.8% (6-7 year age group) (71). This study also underlined that the worldwide differences in asthma symptom prevalence are decreasing, with decreasing prevalence in Western Europe and increasing prevalence in countries where the prevalence was low.

For adults, data from the World Health Organization estimated the global prevalence of doctor diagnosed asthma to be 4.3%, but this report also found variation among countries ranging from 0.2% in China to 21% in Australia (72). The GINA report avoided use of the term "doctor diagnosed asthma", due to the variation of diagnostic labelling and treatment by doctors between populations (68).

The lifetime prevalence of asthma in Norway also seems to be increasing and does not show the same pattern of flattening out as in other developed countries. A study from school children in Oslo found a lifetime prevalence of physician diagnosed asthma of 3.4% in 1981 increasing to 9.3% in 1994 using the same questionnaire in the two surveys (73). This increasing rate of asthma seems to continue. The Environment and Childhood Asthma (ECA) Study in Oslo reported a lifetime prevalence of asthma in 10 year old children of 20.2% and a current asthma prevalence of 11.1%, representing the highest reported prevalence ever in Scandinavia (74). The prevalence of current asthma and wheeze ever, but not asthma or doctor diagnosed asthma, was higher in boys than girls in the ECA study. Similar prevalence rates and increasing

rates, particularly for “asthma ever”, from 1985 to 2008 were found in a recent report from the north of Norway (75).

The rate of asthma is increasing as more communities adopt a western lifestyle and become urbanized. The increasing prevalence is not explained by more knowledge of asthma and its recognition, but has been associated with an increase in atopic sensitization and change of environmental influences (68, 69). Epidemiological studies have also suggested that children do not seem to “outgrow” their asthma. After a non-symptomatic period during late childhood the symptoms can reappear during adolescence (69).

During the 1960s and 1980s, there was a peak in the mortality of asthma, probably related to side effects of newly introduced drugs and/or poor assessment and inappropriate treatment (70, 76). The asthma admission rate reached a peak in the 1990s, but both the mortality and admission rates for asthma are now declining (70).

The discrepancies between asthma prevalence, hospitalization rates and mortality are usually attributed to more effective asthma education and treatment resulting in a less severe disease presentation (70).

The prevalence of asthma is dependent on gender. Boys are more likely to have asthma and wheeze in early life and through early school years, while there seems to be a female dominance during and after puberty (77). Lately, some studies have observed a decreased prevalence of current wheeze in boys, suggesting that these overall gender differences may diminish (77, 78). The reasons for these gender differences are not entirely understood, but are suggested to be linked to the smaller airway size and more atopic sensitization observed in boys during childhood (77). Hormonal changes and differences in environmental exposures may explain the increased risk in the prevalence of asthma among females observed during and after puberty (77). Underdiagnosing of asthma among girls during childhood and adolescence has also been suggested (79, 80).

### 2.2.3 Pathophysiology

**Inflammation**

The pathophysiology of asthma is complex and involves a variety of inflammatory cells, mediators and local airway cells and requires an interaction between genes and environment (81). Asthma is characterised by airway inflammation, reversible airway obstruction and BHR and is largely restricted to the conducting airways, but may spread proximally and also distally to include smaller airways (81). Asthma develops after environmental exposure to allergens, infectious agents (mainly viruses) and air pollutants. These triggers may cause an immune response characterised mainly by the appearance of Th-2 lymphocytes, Immunoglobulin (Ig) E secretion, eosinophils, dendritic cells (DC), mast cells, basophils, neutrophils and structural cells. The Th-2 lymphocytes represent a subpopulation of the CD 4+ T-lymphocytes, and are characterised by the production of specific Th-2 cytokines. The results of this inflammatory response are increased mucus production, mucosal oedema, reversible airway obstruction, BHR and eventually remodelling of the airways (81, 82) (Figure1). Remodelling includes smooth muscle hypertrophy, thickening of basement membrane, deposition of proteins like collagen fibres and proliferation of micro vessels and vascular leakage (81). Remodelling may also be present in children with severe asthma (83).

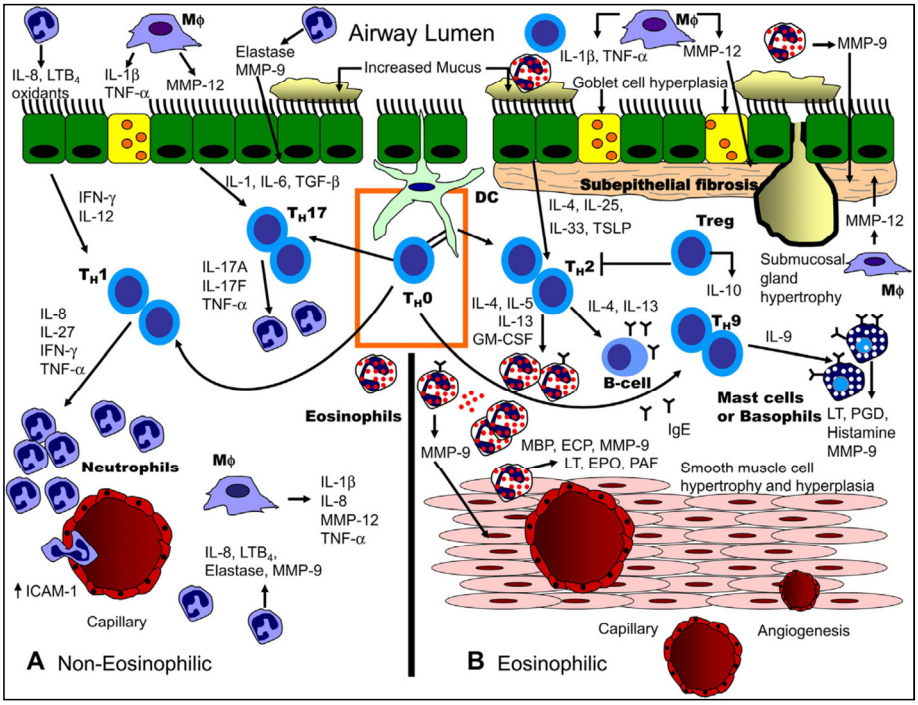


**Figure 1**. Airway inflammation in the setting of asthma. The orange box represents activities in the lymph node. The dendritic cell (DC) processes antigens, migrates to the lymph nodes, and associates with TH0, which then differentiates and migrates back to the airway. A, noneosinophilic/neutrophilic asthma, B, Eosinophilic asthma. Remodelling occurs in patients with all forms of asthma but is only shown in Fig 1, B.
EPO, Eosinophil peroxidase; ICAM-1, intercellular adhesion molecule 1; LTB4, leukotriene B4; MF, macrophage; MBP, major basic protein; MMP, matrix metalloproteinase; PAF, platelet-activating factor; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin.



Bronchial biopsies from asthmatics have shown damage of the airway epithelium such as epithelium metaplasia and damage and thickening of sub-epithelial basal lamina (81). Similar findings have been observed in asthmatic children, and there are suggestions that the epithelium in asthmatics is chronically injured and unable to repair properly (81).

The loss of barrier function with incomplete formation of tight junctions between the epithelial cells will facilitate the penetration of allergens through the

epithelial barrier. This breakdown of barrier function is also under genetic regulation (84). Damage or irritations to the respiratory epithelium by microbes, allergens and/or irritants, stimulate the maturation and movement of DCs from the bone marrow to the respiratory airway epithelium. Further breakdown and exposure to environmental stimuli triggers the DCs which move back to the T-cell area in the lymph nodes and become antigen presenting cells. This will initiate Th-2 responses, both by stimulating B-cell follicles to switch from IgM to IgE production and by stimulating the production of the Th-2 cytokines (Figure 1) (84, 85).

The most important Th-2 cytokines encoded on chromosome 5q 31-33, are IL-3, IL-4, IL-5, IL-9, IL-13 and macrophage colony stimulating factor (GM-CSF) (84). The main responsibilities of the cytokines in the allergic cascade that occurs in asthma are as followed (84):

- IL-3 is responsible for recruitment and maturation of eosinophils, mast cells and basophils
- IL-4 promotes IgE production and is responsible for Th-2 survival
- IL-5 promotes the differentiation and maturation of eosinophils in the bone marrow
- IL-9 promotes mast cell differentiation and maturation
- IL-13 promotes activation of mast cells, IgE production, increased mucus production by epithelial cells in the lungs and directly causes BHR by binding to IL-13 receptors on airway smooth muscle cells
- GM-CSF is promoting eosinophil and basophil recruitment

Mast cells play an important role in the inflammatory and allergic reactions. IgE binds to the FcεR1 on mast cells resulting in the release of preformed mediators (histamines, tryptases, chemokines, and cytokines) and newly formed arachidonic acid (AA) metabolites. The enzymes cyclooxygenase and lipoxygenase are responsible for the production of prostaglandins (such as Prostaglandin $D_2$) and cysteinyl leukotrienes (such as Leukotriene $E_4$) from AA, respectively (Figure 2). These mediators act on the vasculature, smooth muscle, connective tissue, mucus glands and inflammatory cells resulting in oedema, mucus production and airway obstruction (86). Human mast cells

also produce different cytokines such as IL-4, IL-5 and IL-6, which are stimulating the proliferation and differentiation of activated B-cells (86). The mediators (cytokines and chemokines) are secreted over a period of 72 hours and may contribute both to the immediate allergic reaction as well as partly contribute to the late phase allergic response (81).

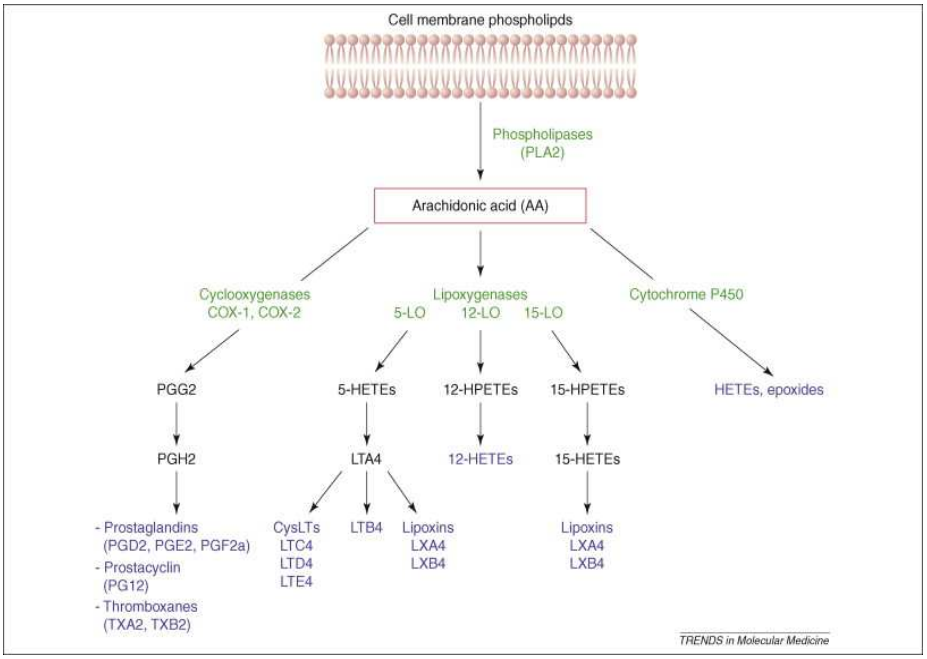


**Figure 2**. Eicosanoid biosynthesis from Arachidonic acid (AA). In response to a variety of non-specific activating stimuli, including cytokines, hormones and stress, AA is released from membrane phospholipids by phospholipases, especially cytosolic phospholipase A2 (cPLA2). Free AA can be converted to bioactive eicosanoids through the cyclooxygenase (COX), lipoxygenase (LOX) or P-450 epoxygenase pathways. LOX enzymes (5-LO, 12-LO, 15-LO) catalyse the formation of LTs, 12(S) hydroperoxyeicosatetraenoic acids and lipoxins (LXs), respectively. COX isozymes (constitutive COX-1 and inducible COX-2) catalyse the formation of PGH2, which is converted by cell-specific PG synthases to biologically active products, including PGE2, PGF2α, PGI2 and TXA2, known collectively as prostanoids. The P-450 epoxygenase pathway catalyses the formation of hydroxyeicosatetraenoic acids (HETEs) and epoxides.



The eosinophils were early described in asthmatics and have long been recognised as one of the most important cells in asthmatic inflammation (87). The

eosinophil granulocytes are recruited from the bone marrow following the release of prostaglandins, leukotrienes, chemokines and cytokines from the asthmatic airways (81). GM-CSF, IL-3 and particularly IL-5 are important for the differentiation, proliferation and maturation of eosinophils from the bone marrow (88). The eosinophils pass from the bone marrow through the circulation and into the airway wall. The cells secrete a variety of mediators such as eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil protein X (EPX-also named eosinophil-derived neurotoxin) and also have the capacity to generate eicosanoids, cysteinyl leukotrienes and other cytokines and chemokines (81). These mediators result in degranulation of mast cells and basophils with further cellular cytotoxicity, mucus production and bronchoconstriction (87, 89). Eosinophils may also activate T-cells by serving as antigen presenting cells (89). Reduced numbers of eosinophils in the asthmatic airways along with clinical improvement are found after treating asthmatic patients with inhaled or systemic corticosteroids. Although eosinophils have been related to the severity of asthma and thereby BHR (90), some studies suggest that eosinophils may not be necessary for the induction of BHR (47).

As described in this thesis, there are forms of asthma that appear to be independent of atopy, and the neutrophilic cell seems to dominate the inflammation in children with non-atopic asthma (82, 91). A neutrophilic driven inflammation has also been described in patients with severe asthma, particularly during viral induced exacerbations (81) and in small children with viral induced bronchiolitis (11). For older children a paucigranulocytic asthmatic phenotype, with normal values of neutrophils and eosinophils, is most commonly described followed by the eosinophilic phenotype (92). However, this picture is further complicated as the inflammatory phenotype in an individual child is not stable and may change over time (93).

The immunological response in asthma is controlled by the regulatory T (T reg) cells. T reg cells express the surface proteins CD4 and CD25 and the forkhead box protein 3, which is a transcription factor that is important for the development and function of T reg cells. T reg cells may influence the allergic pathways by secreting cytokines (IL-10 and Transforming growth factor-β) that supress the DCs, inhibit Th-1, Th-2 and Th-17 cells and induce $IgG_4$. This will in turn supress IgE, inhibit mast

cells, basophils and eosinophils and prevent migration of effector T cell into the target tissue (84).

Lately, also other T cells have been discovered and linked to asthma. Both Th-9, a subpopulation of Th-2, and Th-17 cells seem to be important (84). Th-17 cells do not share development pathways with Th-1 or Th-2 cells. Th-17 cells produce cytokines such as IL-17, and regulate neutrophilic airway inflammation, although the exact roles of Th-17 cells in asthma are not entirely known (84).

**Bronchial hyperresponsiveness**

BHR is central in the pathogenesis of asthma and included in the definition of asthma (62, 64). BHR is defined as an excessive narrowing of the airways in response to chemical and physical stimuli that have no similar effect on healthy individuals (94, 95). BHR is measured by challenge tests that cause variable obstruction of the airways (96). BHR may be present in subjects without asthma, but is usually related to the severity of asthma (96, 97).

Usually these challenges are performed by exposing the subjects to increasing dose of a bronchoconstrictor while measuring lung function. The outcome of the challenge is the dose-response slope which is characterised by [1)] The position (sensitivity), [2)] The slope (reactivity) and [3)] The plateau (maximal response) (Figure 3) (95, 98).

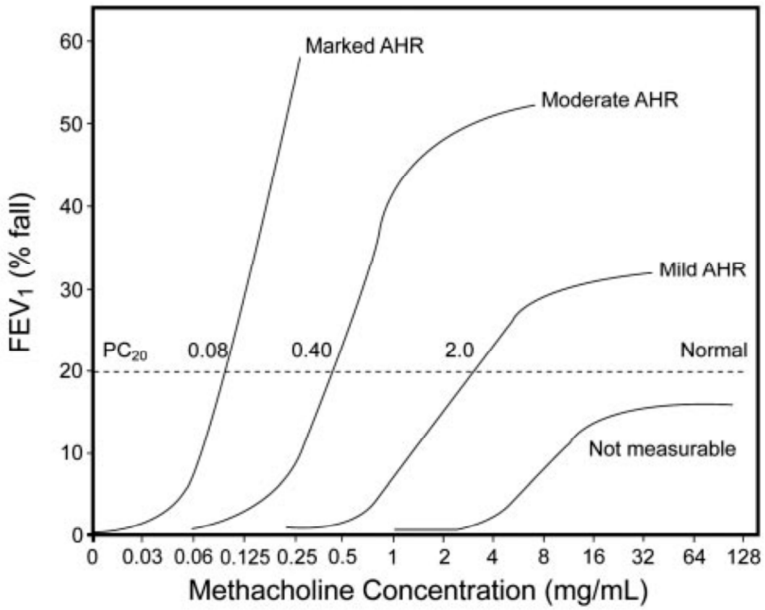


**Figure 3.** Hypothetical methacholine dose-response curves for four individuals: one with normal airway responsiveness, and one each with mild, moderate, and marked airway hyper-responsiveness (AHR). These four curves demonstrate hyper-responsiveness both in the increase in magnitude of the response and the ease of the response, the latter identified by the leftward shift of the curve and the smaller PC20 (provocation concentration causing a 20% fall in FEV1). The PC20 values decrease from non-measurable in the normal curve to 2.0 mg/mL in a subject with mild AHR, 0.40 mg/mL in a subject with moderate AHR, and 0.08 mg/mL in a subject with marked AHR. One important caveat is that mild, moderate, and marked AHR do not equate with mild, moderate, and severe asthma or necessarily with differences in asthma severity and the degree of asthma control.



Both a fixed persistent and a variable inducible form of BHR has been observed (97). The persistent form is present in chronic asthmatics and probably reflects structural alterations of the airways and airway remodelling. The variable form reflects acute airway inflammation such as airway infections, allergen exposure and the response to treatment. However, this classification may be an oversimplification, and BHR is probably a result of overlapping mechanisms, each contributing to a specific part of the BHR (99). Activation of sensory nerve fibres with secondary bronchoconstriction may also contribute to BHR (99).

Being aware of the complexity of factors involved in BHR, the fixed persistent form seems to be less related to eosinophilia and Th-2 inflammation than the variable and inducible form of BHR, as summarised in the reviews from Busse (99) and Cockcroft et al. (100). The stimuli used to detect BHR are divided into direct and indirect stimuli. The direct stimuli (methacholine, histamine) act directly on airway smooth muscle and probably reflect the persistent form of BHR. The indirect stimuli (exercise, cold air, mannitol) act via release of mediators from inflammatory cells and reflect the variable and inducible form of BHR, the latter suggested as more clinically relevant (97). These different methods of measuring BHR are important to consider, particularly when comparing the results of different studies.

## 2.3 Atopy

### 2.3.1 Definition

The World Allergy Organization has defined atopy, hypersensitivity and allergy as follows:

"*The terms 'atopy' and 'atopic' should be reserved to describe the genetic predisposition to become IgE-sensitized to allergens commonly occurring in the environment and to which everyone is exposed but to which the majority do not produce a prolonged IgE antibody response".*

This means that the term atopy is used to describe an immunological reaction and cannot be used until an IgE sensitization has been documented by IgE antibodies in serum or by a positive SPT (101).

*"The term hypersensitivity should be used to describe objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons" (101).*

The term allergy is defined as:

"*A hypersensitivity reaction initiated by specific immunologic mechanisms" (101).*

### 2.3.2 Test methods

Atopic sensitization is usually measured *in vivo* by SPT or *in vitro* by specific IgE in serum (102, 103). Although, studies show correlation between SPT and specific IgE (104), disagreements are found both in young children (105, 106) and adults (107), underlining that these tests should be used complementary and not assumed as equivalent.

The Phadebas radioallergosorbent test (RAST; Pharmacia, Uppsala, Sweden) was the first assay reported for the detection of the allergen-specific IgE antibody. This was a qualitative test, and the results were regarded as positive or negative. Over the last years, different quantitative IgE-antibody detection methods have been developed, and ImmunoCAP is the assay most studied (103, 108). The results of most test systems are reported in arbitrary mass units (kilo international units of allergen specific antibody per unit volume of sample [kUa/L]), ranging from <0.1 to >100 kUa/L (103, 108). A 0.35kU/L cut-off point criterion to define the presence of atopy is often used in clinical studies, and the clinical relevance of lower values has not been determined (109). Recently, the use of a combined sum of specific IgE antibodies and also the importance of regarding atopy as phenotypes and not only as a dichotomous variable has been highlighted (110). The atopic phenotypes described by Simpson and Custovic, are defined according to the age of the subject at sensitization and whether the subject is sensitized to a single or several allergens (110).

Atopy measured and monitored both by SPTs and specific IgE may be classified as binary or linear variables. Quantitative measures such as specific IgE or numbers of positive SPTs are probably more robust assessments of atopy than binary classification such as sensitized or not sensitized (111).

In a large study from Switzerland, SPT had higher positive predictive values (PPV) to diagnose atopic respiratory diseases than positive Phadiatop ® (which detects the presence of specific serum IgE against common inhalant allergens) (112). Higher

PPV for SPT compared to specific IgE was also found when diagnosing food allergy in children with atopic dermatitis (AD) (113).

### 2.3.3 Epidemiology

Worldwide the prevalence of atopic sensitization is variable. The prevalence of atopy in 8-12 year old children measured by SPT in the ISAAC phase II study was varying from 1.7% (Ghana) to 45.3% (China) and measured by specific IgE 16.7% (Estonia) to 48.5% (Spain) (114).

In Norway, the prevalence of atopic sensitization measured by SPT was higher in 10 year old children living in Troms and Finnmark (30.8%) than children living in Oslo (23.9%) (115). The prevalence of atopic sensitization measured by SPT in 10 year old children participating in the ECA from Oslo was 29.3%. In the ECA study, atopy was more frequent reported in boys (74). In Stavanger, a prevalence of 34% of atopy (defined as specific IgE > 0.35 kU/l), was reported among 12 year old children (Kristine Byberg, personal communication).

Boys have a higher risk of atopy than girls (116). Although, these differences seem to be less pronounced in adolescents and adults, the same pattern has also been reported among adults (117).

There has been extensive research regarding the pathogenesis of atopy and allergic disease. The pathogenesis is complex and involves genetic and dietary factors as well as exposure to allergens, tobacco smoke, air pollution and infections (118). There is evidence that growing up on a farm with early life exposure to endotoxins is protective against sensitization and allergic diseases in childhood (119). Similarly, a higher prevalence of atopy has been observed among children living in urban than rural areas (120). The observations that early exposures to certain infectious agents, gastrointestinal bacteria and endotoxins may have a protective role in relation to later development of asthma, has led to the so-called hygiene hypothesis (121).

### 2.3.4 Asthma and atopy

Allergy involves immunological reactions, and allergic asthma is therefore asthma due to an immunological reaction. When initiated by IgE antibodies, the proper term is IgE mediated allergic asthma (64, 101). Atopic asthma is defined as asthma in

a subject with concomitant atopic sensitization (114, 122), irrespective of whether allergens are obvious triggers of disease activity.

The majority of children with asthma are atopic, and atopy is associated with asthma at all ages, but the fraction of children with asthma who are atopic increases with age (64, 123).

The association between atopy and asthma/wheeze is strongly documented (9, 74, 114, 124). The link between atopic sensitization and asthma symptoms differs between populations and increases with increasing economic development (114). In the ISAAC phase II study in affluent countries, an odds ratio (OR) of 4.0 (95% Confidence Interval (CI): 3.5, 4.6) and 3.5 (2.9, 4.2) for current wheeze was observed in 8-10 year old children with atopic sensitization measured by SPT and specific IgE, respectively (114). The association was weaker in non-affluent countries. The overall fraction of asthma symptoms attributed to atopy was 30% in adults in the European Community Respiratory Health Survey, but varied widely between centres (125). Recently, the association between asthma and atopy seems to be most pronounced for individuals with a multiple early atopic phenotype (110).

## 2.4 Markers of inflammation

Bronchiolitis and asthma are both inflammatory disorders. The diseases are characterized by different inflammatory patterns with various inflammatory cells. Inflammatory cells, inflammatory mediators and inflammatory markers can be measured in urine, blood, nasopharyngeal aspirate, sputum, bronchoalveolar lavage or exhaled air (126-128), and may provide valuable knowledge about the inflammatory pattern present during these airway diseases. Sputum and bronchoalveolar lavage are less accepted test methods by children. Inflammatory cells and markers of airway inflammation that can be measured in blood, urine and exhaled air were therefore the main focus of this thesis.

### 2.4.1 Eosinophils and eosinophil granule proteins

As described in chapter 2.1.4 and 2.2.3, the eosinophilic cell is present in the airways in children with bronchiolitis and asthma (47, 81), both in atopic and non-atopic asthma (87, 129). However, in children with bronchiolitis, the role of

eosinophils as provokers of the airway disease and/or as an important defence against the viral infection has not been resolved (47). Cough and recurrent wheeze in children below two years of age with reversible airway obstruction, may also be independent of eosinophilic inflammation (130).

Several studies have assessed eosinophils and eosinophil granule proteins as markers of airway inflammation in children with bronchiolitis and asthma (131-136). These mediators can be measured in blood, urine and sputum (126, 137). Serum ECP and blood eosinophil counts correlate with eosinophilic airway inflammation in children with current wheezing (138). U-EPX correlates with blood and bronchoalveolar eosinophil cell counts in patients with various hyper-eosinophilia diseases (139).

Studies have observed associations between eosinophil activity during acute wheezing and persistent asthma later in life (133, 136, 140, 141), and eosinophilia has been included in algorithms for the diagnosis of asthma in preschool children with recurrent wheeze (8, 135). The study of Karakoc et al., pointed out that the association between eosinophils and asthma is independent of atopy (134). In children with viral bronchiolitis, particularly in children with RSV positive bronchiolitis, markers of eosinophil activity are associated with the severity of the disease (47, 136). However, these findings are not overall consistent. A study including 110 children at one year of age, observed higher U-EPX in those with AD, but not in children with respiratory symptoms (142).

Still, eosinophils are only one of several inflammatory cells involved in the pathogenesis of bronchiolitis and asthma. Eosinophilic inflammation is present also in subjects without asthma and in other allergic diseases such as AD as mentioned above. The heterogeneity regarding inflammation and the involvement of eosinophils also in other allergic diseases, limit the role of blood eosinophils and eosinophil granule proteins in the diagnosis of asthma, as summarised in the reviews from Wolthers (137) and Wennergren (126).

### 2.4.2 Leukotrienes and prostaglandins

As indicated in chapter 2.2.3, leukotrienes and prostaglandins are metabolites from AA and are central in the pathogenesis of asthma. These mediators are bronchoconstrictors and increase micro vascular permeability (81).

Leukotriene $E_4$ ($LTE_4$) is the end metabolite of cysteinyl leukotrienes and can be measured in urine (143). Increased levels of U-$LTE_4$ have been found during acute episodes of bronchiolitis in infancy (144), and in older children with preschool viral wheeze (141, 145, 146), although these findings are not entirely consistent (142). U-$LTE_4$ correlate with lower levels of lung function and increased levels are found during acute asthma exacerbations and allergen challenges as summarised in the review from Wennergren (126). U-$LTE_4$ is also a sensitive biomarker of aspirin induced asthma (147) and has also been suggested as a marker of leukotriene receptor response in asthmatics (148).

Prostaglandin $D_2$ ($PGD_2$) is a major product from COX catalysed reactions in a variety of tissues and cells, including mast cells and macrophages (149). $PGD_2$ is regarded as a marker of mast cell activation (150) and a marker of allergic asthma (151). There are few studies on the role of prostaglandins as predictors of asthma after bronchiolitis. $PGD_2$ consists of at least two isoforms; the hematopoietic form is present in mast cells, macrophages and dendritic cells and found in asthmatic airways. However, the role in the pathophysiology of asthma is less clear as $PGD_2$ can act both pro-and anti-inflammatory (149). Measuring U-$PGF_2$ has been reported to be a sensitive and specific marker of the $PGD_2$ pathway and thereby a marker of mast cell activation in children (150, 152).

Many studies analysing differences in U-$LTE_4$ and U-$PGF_2$ between asthmatic and non-asthmatic subjects have included only a few numbers of subjects and report varying results (153). One study including 168 adults with asthma and 175 controls without asthma, observed a great variation of both U-$PGF_2$ and U-$LTE_4$ and no overall difference in urinary eicosanoid concentrations between the groups. However, U-$PGF_2$ was negatively correlated with asthma severity and lung function (153).

### 2.4.3 Exhaled nitric oxide (FeNO)

Endogenous Nitric oxide (NO) is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). Three isoforms of the enzyme have been identified (154). NOS is expressed in different cells in the respiratory tract and the inducible form of NOS (iNOS) has been found in the bronchial epithelium of asthmatics (155). iNOS can be induced by pro-inflammatory cytokines and exogenous factors (allergens, bacteria, virus, oxidants ) through an up-regulation of the gene transcription and translation of iNOS mRNA, and NO induced by iNOS has been considered as a pro-inflammatory mediator (154). One study has also found that iNOS mRNA is up-regulated by IL-13 (156).

Increased fraction of exhaled nitric oxide (FeNO) was first observed in asthmatics in the 1990s (154). FeNO has been associated with eosinophilic airway inflammation and BHR, and raised values may predict steroid responsiveness in patients with non-specific respiratory symptoms (128). However, conflicting results have been reported regarding the association between FeNO and eosinophil counts in airway biopsies both in children and adults (157). FeNO has been associated with atopy, and there are suggestions that FeNO reflects atopy rather than airway inflammation, although these findings are equivocal (128, 157).

Measuring FeNO is easy, non-invasive and acceptable for children. However, there is a need for better defined reference values and also “normal” values for asthmatics (128). Associations between FeNO and BHR, but not necessarily respiratory symptoms, have been described in atopic children (158). In children below two years of age, FeNO has been shown to discriminate between various airway diseases, probably reflecting different inflammatory patterns (159). FeNO may distinguish between different phenotypes of wheezing in small children, but more studies are required to standardise this method (160, 161). Few studies have assessed the role of FeNO in children with bronchiolitis and in children with asthma after bronchiolitis.

## 2.5 Outcomes after bronchiolitis

### 2.5.1 Asthma

**Epidemiology**

The Tucson Children's Respiratory Study is probably the most cited follow-up study of children with early viral lower respiratory tract infections. In this study, 888 of the 1246 children enrolled, were followed for the first three years of life with outpatient medical examination and virus tests whenever signs of lower of respiratory tract infections were reported (162, 163). RSV was the most common respiratory virus, and RSV lower respiratory tract infection was a three to four fold independent risk factor for subsequent wheeze up to the age of six years. After the age of six years, the prevalence of wheeze decreased and did not differ compared to controls at the age of 13 years. The children were classified into four different phenotypes according to their history of wheezing including a group (50%) with no wheezing symptoms up to the age of six years.

Other studies including children hospitalized for RSV bronchiolitis below 12 months of age have found increased risk of subsequent wheezing during early school years (20, 164, 165). However, the risk of asthma after hospitalization for RSV bronchiolitis seems to decrease by age, as recently published in the meta-analysis from Règnier and Huels (166).

Studies from Scandinavia also including children hospitalized for bronchiolitis, have observed a similar tendency of decreasing wheeze over time, but still 15-30% have asthma during early school years compared to the 4-10% prevalence of asthma among school children in Scandinavian population-based studies (8) (Figure 4). Apart from the studies from Sigurs et al., these Scandinavian post-bronchiolitis studies have included children hospitalized with bronchiolitis below 24 months of age. After a temporary period with minor symptoms during early adulthood, long-term follow-up studies have shown relapsing symptoms in late adulthood and early adult life (8) (Figure 4). In a Swedish follow-up study at 17-20 years of age (Wennergren-Figure 4) the increased risk of asthma was particularly evident in females, while there was a decreasing frequency of asthmatic symptoms in males during childhood (167).

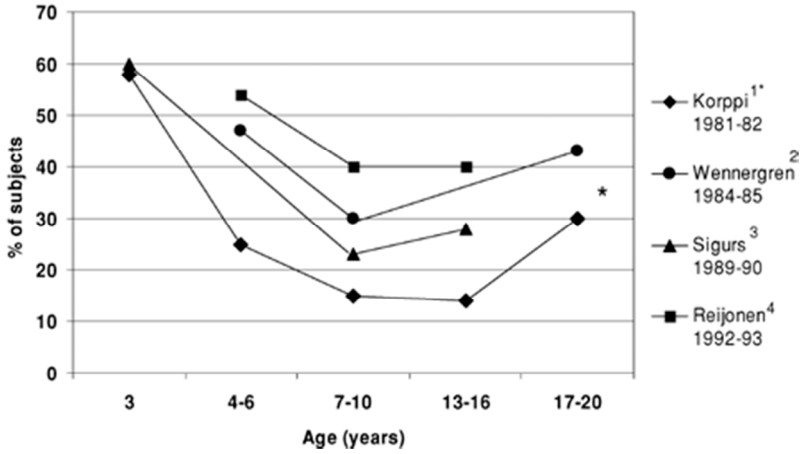


**Figure 4**. Subsequent asthma until school age after hospitalization for bronchiolitis in early life. Results from four cohorts prospectively followed until teenage or adulthood.



In a Finish study (Reijonen-Figure 4) the authors underline that the risk of subsequent wheezing/asthma seem to depend on the viral aetiology, and an increased prevalence of asthma was observed after RV bronchiolitis (168). In this study, an increased risk of teenage asthma was found in those with a history of AD and atopic sensitization before the age of two years.

Backman and Korppi et al. (Korppi 1981-82, Figure 4) recently published follow-up data from adults. They found a higher prevalence of doctor diagnosed asthma (31.3% vs. 10.9%; adjusted $p = 0.002$) and self-reported asthma (35.4% vs. 14.5%; $p = 0.003$) 30 years after hospitalization for bronchiolitis compared to controls (169) .

Sigurs et al. found a 28% prevalence of current asthma by the age of 13 years in children with previous hospitalization for RSV bronchiolitis < 12 months of age (170). At 18 years of age the prevalence of current asthma was 33% (19).

Subsequent wheezing has also been found after bronchiolitis caused by human metapneumovirus, similar to children with a history of RSV bronchiolitis (171).

The Coast study included 289 children with at least one parent with respiratory allergies or asthma. This study found that among outpatient viral wheezing illnesses, episodes due to RV before three years of age were the most significant predictors of asthma at the age of six years, and associated with a near 10-fold increase in the risk of asthma (9). Wheezing and atopic sensitization in early childhood were particularly increased in boys (53).

The study from Perth (Australia) by Kusel et al., also included children at high risk of atopy and observed an increased risk of persistent wheeze by the age of five years after wheezing with RV or RSV in the first year of life, but only for those with atopic sensitization before the age of two years (22).

Together, these studies show that the risk of asthma after bronchiolitis and early viral respiratory tract infections depends on the viral aetiology, atopy, gender and the age when the child has bronchiolitis. Age is an important risk factor, but difficult to study separately from the viral aetiology and atopic status as these factors vary between different age groups; the prevalence of children with atopic sensitization and non-RSV bronchiolitis is increasing with age (14). Several of these long-term follow-up studies have included children with an upper age-limit of 24 or 36 months during bronchiolitis (9, 162, 168). However, studies including older children probably also include subjects with different wheezing phenotypes or with an early manifestation of asthma. This is important to be aware of when comparing results from different studies (172).

Comparing results from different studies is also challenging because of the heterogeneity of symptoms. Viral wheezing up to the age of three years not requiring hospitalization differs substantially from bronchiolitis in infancy requiring hospitalization. These groups of children may have different pre morbidity before the viral respiratory tract infection, with different impact on the risk of subsequent asthma.

Because of major climatic and environmental differences, comparing the results from studies done in different parts of the world may be difficult. As an example, the dry desert conditions, housing and environmental exposures in Tucson Arizona are very different from those in Scandinavia. Alternaria is the major triggering allergen for asthma in children in Arizona (173), whereas pollen, animal dander and house dust

mite may be more important in Scandinavia (174, 175). The importance of climate for the development of asthma was underlined in a study including 57 centres from 12 countries in the Western-Europe. In this study, altitude, the annual variation of temperature and the relative outdoor humidity were negatively associated with asthma symptoms (176).

**Pathophysiology**

Asthma after bronchiolitis in infancy has been associated with a variety of pathophysiological mechanisms. There is still a debate whether this risk of asthma is related to premorbid innate characteristics of the child, or if the bronchiolitis alters the development in otherwise healthy children. Environmental factors may have impact both through epigenetic mechanisms and more directly both during foetal life and early childhood. The viral infection may contribute to asthma both through direct viral damage of the airway epithelium or indirectly by alterations of the immune response (177). The pathophysiological pathways are probably overlapping and dependent on virus vs. host interactions (10, 178).

*Environmental factors*

Exposure to tobacco smoke is probably the most important environmental factor. An increased risk for developing bronchiolitis and a more severe form of the disease have been found in children with exposure to smoking (40). As for other chronic obstructive lung diseases, active and passive smoking are also a risk factor for subsequent asthma after bronchiolitis up to adult life (179, 180).

*Premorbid factors*

A Danish twin study observed no difference according to asthma and atopy in monozygotic twins discordant for hospitalization to RSV in infancy. The authors suggest that the risk of asthma is not linked directly to the RSV virus infection, but rather a premorbid genetic susceptibility (181). However, as described in the next section these findings are not entirely consistent.

Until now, no major "asthma gene" has been found, although there is evidence that several genetic predispositions may contribute to the development of asthma, both through congenital defects in lung function, BHR and altered immunological response towards viral infections (182). Again with regard to bronchiolitis, few candidate gene relations have been reported, and there are few genetic studies on asthma after bronchiolitis (40). There are studies reporting associations between single nucleotide polymorphisms in the promoter genes of Th-2 cytokines, variations in the innate immunity genes and the risk of severe RSV infection, but most of these associations have not been confirmed (177). A variant of the promoter gene of IL-8 was more often transmitted in children with wheezing after RSV positive bronchiolitis, suggesting a genetic predisposition to wheeze after RSV bronchiolitis (183). Down-regulation of different genes and a pathway involved in the regulation of the actin cytoskeleton in cord blood from infants hospitalized for RSV infection have been observed in a Norwegian study (184). A recent published study found that variants at the 17q21 locus were associated with asthma in children with HRV wheezing illnesses in early life (185).

Structural alterations of the airway calibre and narrow airways are suggested as premorbid disposing factors in children developing bronchiolitis and subsequent wheezing and asthma (186). The same level of reduced lung function has been reported both before and after viral wheeze, indicating that low premorbid lung function predisposes to bronchiolitis and later asthma (187, 188).

*Virus and immunological factors*

Both immunological and non-immunological mechanisms may be involved in the pathogenesis of viral bronchiolitis; these mechanisms may also have impact on the long-term outcome. An overview of some of the most important pathways contributing to RSV disease and recurrent wheeze is given in Figure 5 (42).

The risk of pre-school asthma for children born more than 121 days before the winter virus peak is increased, particularly for those with a previous history of bronchiolitis in infancy, suggesting that "winter viruses" are involved in the causal pathways leading to asthma after bronchiolitis (189). In line with this, children born

prematurely, treated with anti-RSV immunoglobulin (palivizumab) and not hospitalized for RSV bronchiolitis, had less chance of subsequent recurrent wheezing than those without this treatment (190). Similarly, in a recently published paper from the Netherlands, preterm healthy infants treated with palivizumab had a significant reduction in wheezing days during their first year of life (191).

The ability of the virus to cause long-term pulmonary sequel has been studied in mouse models. RSV-infected mice developed chronic airway disease characterized by BHR and persistent airway inflammation months after the initial RSV infection (192). Further, the administration of anti-RSV immunoglobulin in a murine model was associated with reductions in long-term airway morbidity such as inflammation and BHR (193). These studies support that the virus itself may cause long-term lung-sequel.

The risk of asthma after bronchiolitis may be related to the inflammatory response to the virus on the respiratory epithelium. As described in 2.1.4, a neutrophilic inflammation has been found during acute bronchiolitis, which is different from that observed in older children with atopic asthma (45, 46), although during RSV bronchiolitis also an eosinophilic inflammation has been described (47). These inconsistent observations indicate that the exact pathophysiology regarding inflammation both during the acute bronchiolitis and in children developing asthma after bronchiolitis is not entirely known.

Alterations of the primary immune response have been suggested as one explanation for subsequent asthma after bronchiolitis. The foetal and infant immune system is dominated by a Th-2 response with diminished capacity of the infant T-cells to produces IFN-γ (194). Differences in cytokine profiles have been observed between those groups developing and not developing asthma after bronchiolitis. Both RSV and other respiratory viruses may induce Th-2 like responses (36, 195). A positive correlation between serum IL-13 and number of wheezing episodes have been reported (196). In addition, positive associations between monocyte IL-10 responses and the risk of wheezing one year after RSV positive bronchiolitis have been found, indicating that several virus induced changes in cytokine responses are involved (197).

Particularly for RVs, but also for RSV, the risk of severe bronchiolitis and subsequent asthma has been linked to the "double hit" hypothesis. This hypothesis indicates that children infected by a viral infection in a vulnerable period of life and who also have a susceptibility towards a Th-2 immune response, are particularly at risk of developing asthma later in life (10). The mechanisms may be linked to reduced IFN-γ response towards airway infections and early atopic sensitization (40), although these findings may be contradictory (42). Interferons are important in the defence mechanisms against viral infections, and bronchial biopsies from asthmatic airways have shown reduced ability of the epithelial cells to generate IFNs (81). The increased risk of asthma in atopic children with early viral infections may be linked by a common down-regulation of the immune system in producing Th-1 cytokines (IFN-γ) and/or an up-regulated activity towards the airway mucosal dendritic cells and further activating of the pre-existing Th-2 immune response observed in atopic children (111).

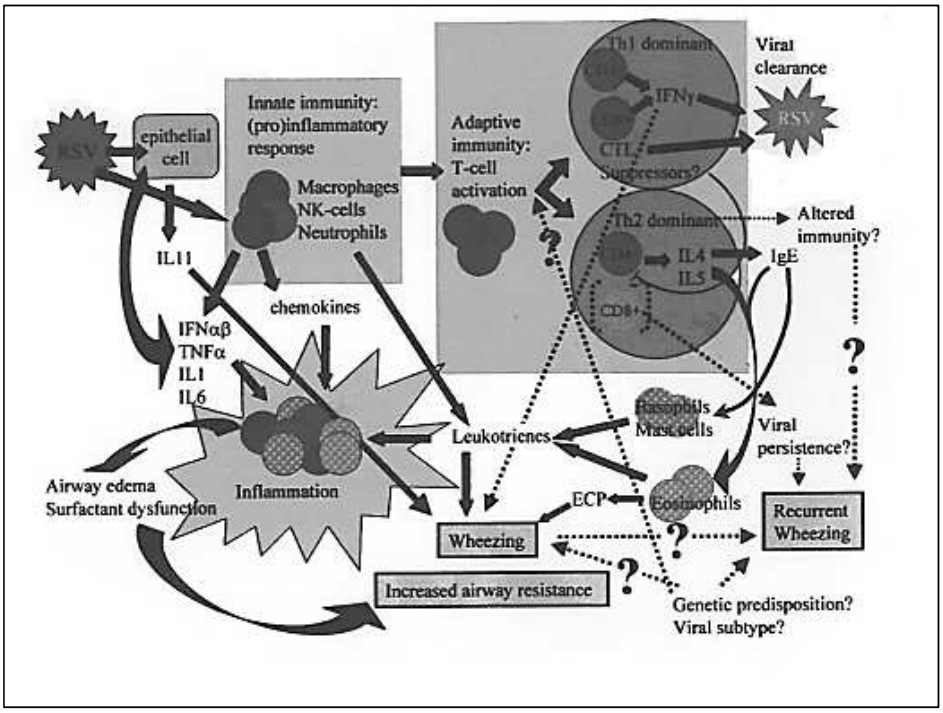


**Figure 5**. Immune activation during acute bronchiolitis and recurrent wheezing.

In conclusion, as summarised in Figure 5, several premorbid, immunological and environmental factors are related to the outcome after bronchiolitis. The exact pathophysiology is overlapping, and it is still difficult to determine whether premorbid characteristics of the child or factors directly related to the bronchiolitis are of most importance.

### 2.5.2 Atopy

Higher prevalence of atopic sensitization up to18 years of age, in children hospitalized for RSV bronchiolitis during their first year of life was observed in the study by Sigurs et al. (19, 170). As summarized in the reviews by Wennergren (36) and Stein (10), these findings have not been confirmed by others. However, several studies have found an increased risk of asthma after bronchiolitis/viral wheezing in children with concomitant early atopic sensitization (9, 21, 168, 198). The increased risk of viral wheezing during the first six years of life after early atopic sensitization to allergens, was particularly observed in children with wheezing due to RV (199). However, there is little data describing the causal pathways (10), and there is less evidence that wheezing with RV increases the risk of subsequent atopic sensitization (199).

### 2.5.3 Lung function and bronchial hyperresponsiveness

Longitudinal studies up to early adult life (17-20 years) have revealed reduced lung function after severe bronchiolitis in early childhood (19, 200-202) and also in children with mild wheezing in early life (203). Both persistent bronchial obstruction (19, 201) and a restrictive lung function pattern have been observed (200). Persistent low lung function has also been found years after cessation of wheezing (203). As discussed above, these changes in lung function could be present already prior to the viral infection, more as a risk factor for bronchiolitis than as a result of the bronchiolitis (187, 188). Such tracking of lung function has also been shown for healthy infants included in the Tucson birth cohort study (204).

The Tucson study defined three main phenotypes of early childhood wheezing described as follows with percentages in each group (163, 203, 205): Transient wheeze

(19.9%), persistent wheeze (atopic wheeze) (13.7%) and late onset wheeze (non-atopic wheeze) (15.0%). The children with transient wheeze had reduced lung function from birth which improved, but remained subnormal up to the age of 16 years, despite diminishing airway symptoms. The children with persistent wheeze had normal lung function at birth, but reduced lung function at the age of 16 years and increased BHR. The children with late onset wheeze had similar lung function compared to those who never wheezed and they had no BHR.

The results from the Tucson study underline that the children with transient early wheeze enter adult life with deficits in lung function and may therefore have an increased risk of developing chronic obstructive pulmonary lung disease during late adult life (186). However, this birth cohort study is not entirely comparable to the other post-bronchiolitis studies, as the children had viral wheezing up to 36 months of age when included and were not hospitalized for bronchiolitis. Similarly to the post-bronchiolitis studies, the results underline that in certain subgroups of children with early viral wheezing, permanent changes of lung function are tracking through life. The exact reason behind these changes of lung function is still not known, and probably involves inborn, immunological and environmental factors (186).

Both the Tucson birth cohort study and other post-bronchiolitis studies have revealed that children or subgroups of children with early viral wheezing and/or bronchiolitis, have an increased risk of on-going BHR later in life (200, 205), although the underlying mechanisms are not well understood. Some studies have highlighted associations between atopic sensitization/AD and BHR (200) as well as an association between prenatal smoke exposure and BHR (179, 200). As mentioned in chapter 2.5.1, results from murine studies have found evidence that RSV may induce long-term BHR persistent for several months after the viral infection (192).

BHR in preschool years have has also been found to be an independent risk factor for asthma in early adult life, underlining that latent alterations of airway response has long-term impact on the risk of future airway disease (206).

## 2.6 Clinical prediction of asthma after bronchiolitis

The prevalence of wheeze and asthma after bronchiolitis decrease throughout childhood, but still 15-30% of children will be diagnosed with asthma during early school years (8). The symptoms of children with persistent asthma or wheeze may be indistinguishable from those with transient symptoms. Prediction of asthma after wheezing in early childhood is difficult, but important in order to select "high risk" children in need of later follow-up and provide proper treatment.

Although different prediction algorithms have been suggested, many of these models are better to predict the absence than the presence of subsequent asthma (111). The overall predictive power of these models is often poor (207). Various clinical characteristics, environmental exposures, allergens and viruses of preschool wheezing have been included (8, 135, 207-209). The Asthma predictive index (API) developed by using data from the Tucson study, has been suggested to be a good clinical tool for the prediction of persistent asthma in preschool wheezers. The API includes simple clinical markers with the major criteria being parental asthma and AD in the child and the minor criteria being allergic rhinitis, wheezing apart from colds and the invasive assessment of blood eosinophilia (135). A revised version also including parental smoking and food allergy as major criteria, has been suggested as more suitable for children hospitalized for bronchiolitis (8).

Most studies have enrolled a variety of risk factors in their prediction models, including the number of respiratory infections with or without hospitalization (207, 209); although a clear statement underlining the risk factors with greatest impact is often missing. In the ECA study from Oslo, a high asthma severity score at two years of age, calculated by the frequency, persistence and hospitalization for bronchial obstruction, was a strong risk factor for asthma at 10 years of age, whereas allergic skin sensitization was not (208).

### 2.6.1 Atopy

As described, a Th-2 response has been observed both in subjects with RSV positive and RSV negative bronchiolitis, and blood eosinophils have been found during acute episodes of viral wheezing (47, 111). The risk of asthma after viral

induced wheeze and bronchiolitis is higher in children with atopic sensitization (9), particularly if the sensitization manifests in very early childhood (194). Phenotypes of atopy including quantification may be appropriate when studying the association between atopy and asthma. In the study from Simpson, Custovic et al., only a multiple early atopic phenotype could predict asthma (110).

### 2.6.2 Atopic dermatitis

Studies from Europe and USA report a prevalence of AD ranging from 15-20% in the childhood population, and the disease is often considered to be the first step of the atopic march (210). Approximately half of the children with AD develop asthma and 2/3 of children with AD develop allergic rhinitis, as summarised in the review from Spergel et al. (210). The association between asthma and AD is probably related to AD and concomitant atopic sensitization particularly to hens' egg, and not to AD without sensitization (111). A study from Norway observed a 16.5% prevalence of AD among two year old children. Of those, 71.8% had atopic sensitization towards at least one allergen (211).

### 2.6.3 Family history of atopy and asthma

A family history of asthma or atopy has been studied as risk factors for asthma in children, although, as listed below, contradictory results have been reported. A meta-analysis has shown that maternal asthma represents the greatest risk factor for asthma, hay fever or "AD ever" for the offspring (212). In the COAST study, maternal or paternal asthma were not associated with asthma at age six years (9). A prospective birth cohort study from the Isle of Wight underlined that a combination of a family history of asthma, early life atopy and recurrent chest infections in children were risk factors for persistent wheeze from 4-10 years of age (209). A large Swedish study found an equal impact of maternal and paternal asthma on the development of asthma in the child. This study also observed a more important role of parental asthma than parental atopy (213), although these findings are contradictory (207). In the German Multicentre Allergy Study (MAS), parental atopy and atopic sensitization as well as elevated total IgE and high allergen exposure in children with wheezing before the age of three years, were the most important risk factors for wheezing at the age of 13

years. However, in this study, parental atopy was more widely defined than usual, and the associations were stronger in children with early life wheezing (123).

## 2.7 Summary of introduction

Through this introduction I have focused on important aspects of viral bronchiolitis and the outcome after bronchiolitis. The definition of bronchiolitis is heterogenic, and different age limits are used in the inclusion processes of the studies. Several post-bronchiolitis studies have included children up to 24 months of age. By including children younger than 12 months of age, the study population will be more homogenous both according to the pathophysiological mechanisms and the clinical outcome of the disease.

Inborn structural or functional alterations of the airways, such as premorbid reduced airway size and increased BHR, have been reported in relation to bronchiolitis as well as to later asthma. Tracking of lung function from birth is observed in several studies up to adult life. Reduced lung function after bronchiolitis could be the result of inborn and/or early alterations in airway size. This may further predispose individuals for both bronchiolitis and later asthma. Similar inborn alterations have been suggested also for BHR.

The pathophysiology in bronchiolitis and asthma involves several mechanisms. Both a neutrophilic and an eosinophilic inflammation have been found in children with bronchiolitis and asthma. For children with RSV bronchiolitis, an eosinophilic inflammation without a concomitant increased risk of atopic sensitization has been observed. However, for children with RV bronchiolitis, the risk of subsequent asthma seems to be increased in subjects with an early atopic sensitization. These differences may indicate that different inflammatory mechanisms are involved in subsequent asthma after RSV positive and RSV negative bronchiolitis. These inflammatory patterns are probably the result of genetic and immunological mechanisms, but may also be related to the virus induced inflammation per se. The inflammatory pattern during bronchiolitis may have impact on the long-term outcome after bronchiolitis.

FeNO have been suggested as a marker of asthma and eosinophil inflammation, but also a marker of atopy. FeNO may differentiate between various airway diseases in

young children. Whether the role of FeNO is different in children with subsequent asthma after bronchiolitis and other children with asthma is not properly studied.

The “prediction studies” highlight various important aspects and risk factors for subsequent asthma after early childhood wheezing. It is important to be aware that differences in study design may influence the results. Some of the proposed prediction algorithms are designed for use in a general population and not in a high-risk population, such as the one studied in this thesis. Clinical prediction models should be easy to perform and not include too many steps and calculations, in order to be useful in an everyday clinical setting.

# 3. AIMS OF THE STUDY

*The overall aim of this thesis was to study the prevalence of asthma and atopy, and the respiratory function at 11 years of age after hospitalization for bronchiolitis in infancy, in order to contribute to the understanding of the pathophysiological and clinical outcomes after bronchiolitis.*

*The main hypotheses of this thesis were:*

1. The prevalence of asthma and atopy is increased, lung function is decreased and bronchial hyperresponsiveness is increased in 11 year old children with a history of hospitalization for bronchiolitis in infancy compared to 11 year old children without previous hospitalization for bronchiolitis.
2. Markers of inflammation during bronchiolitis in infancy are related to the prevalence of asthma and atopy, and to lung function and bronchial hyperresponsiveness at 11 years of age.

*The following specific research questions (RQs) were established:*

1. Is the prevalence of asthma and atopy, and lung function and bronchial hyperresponsiveness at 11 years of age different in children hospitalized for bronchiolitis during their first year of life compared to children without previous hospitalization for bronchiolitis?

2. Is the prevalence of asthma and atopy, and lung function and bronchial hyperresponsiveness at 11 years of age in children hospitalized for bronchiolitis during their first year of life related to gender and the viral aetiology, i.e. RSV positive vs. RSV negative bronchiolitis?

3. Are the inflammatory markers U-$PGF_2$, U-$LTE_4$, U-EPX and eosinophil counts in blood measured in children during acute bronchiolitis related to lung function,

bronchial hyperresponsiveness and the prevalence of asthma and atopy at 11 years of age?

4. Is exhaled nitric oxide at 11 years of age different in children hospitalized for bronchiolitis during their first year of life compared to children without previous hospitalization for bronchiolitis, and is the role of exhaled nitric oxide as a marker of asthma, atopy or BHR different in these two groups of children?

5. In children hospitalized for bronchiolitis during their first year of life, can non-invasive clinical parameters at two years of age predict asthma at 11 years of age?

# 4. SUBJECTS AND METHODS

The present thesis reports results from a longitudinal prospective follow-up study of children hospitalized for bronchiolitis. Children below 12 months of age hospitalized for RSV positive and negative bronchiolitis at the university hospitals in Stavanger and Bergen (Norway) during the winter seasons 1997 and 1998 were invited to participate. Inflammatory markers were measured in urine and blood during the primary hospitalization.

The children were invited to a first follow-up at two years of age. At this follow-up, respiratory symptoms and signs of AD of the child, the parental history of asthma and atopy and parental smoking habits were recorded.

The results described in this thesis are mainly based on data collected in 2008 and 2009 at the second follow-up at 11 years of age. At this second follow-up an age matched control group was recruited from 3 schools in Stavanger. Diagnoses and symptoms of asthma were recorded and spirometry, methacholine provocation test (MPT) and a SPT test were performed.

## 4.1 Subjects

### 4.1.1 Post-bronchiolitis group

The bronchiolitis group included 131 children hospitalized for bronchiolitis during their first year of life. Of these, 103 (79%) were hospitalized at Stavanger University Hospital and 28 were hospitalized at Haukeland University Hospital. Inflammatory markers were measured in 105 children during hospitalization (Figure 6). One hundred and one children participated in the first follow-up at approximately two years of age, and except for one adopted child, all parents fully completed the questionnaire. All children were invited to the second follow-up at 11 years of age, and 121 children were able to participate and answered the questionnaire. Of these, 90 children (74%) were positive for RSV and 108 children agreed to take SPT and lung function measurements (Figure 6). Assessment of BHR by methacholine provocation test (MPT) was not performed in two children and FeNO was not performed in three children due to technical reasons.

### 4.1.2 Control group

All children born in 1997 attending three different schools in Stavanger (six school classes in total) were invited to participate. Age-matching to the bronchiolitis group was performed by inviting children born in the same year (1997) as the children in the post-bronchiolitis group. The children had no previous history of hospitalization for bronchiolitis, but otherwise there were no other exclusion criteria. In total, 190 children were invited and 141 children (74%) responded positively (Figure 6).

Ninety children agreed to take SPT and lung function measurements. One child was not able to perform neither spirometry, FeNO nor MPT. MPT was not performed in two children; one had $FEV_1\% < 65\%$ and one was not able to cooperate. A table of relevant demographic information available for all included subjects are given in Table 1.

Table 1.
Demographic information of included subjects

| | n | Bronchiolitis | n | Controls | P-values |
|---|---|---|---|---|---|
| **Boys/girls, n (%)** [a] | 121 | 68 (56) | 141 | 85 (60) | 0.504 |
| **Age (months)** [b] **(hospitalization)** | 121 | 4.0 (2.0, 7.0) | | | |
| **Age (months)** [c] **(first follow-up)** | 101 | 20.0 (19.0, 23.0) | | | |
| **Age (years)** [b] **(second follow-up)** | 121 | 11.3 (10.9, 11.8) | 141 | 12.0 (11.4, 12.3) | **<0.001** |
| **Weight (kg)** [d] **(second follow-up)** | 108 | 41.4 (8.7) | 89 | 42.3 (10.9) | 0.747 |
| **Length (cm)** [d] **(second follow-up)** | 108 | 148.7 (7.1) | 89 | 150.9 (8.2) | 0.064 |

[a] Ratios (% of group), [b] median (inter-quartile range), [c] median (range), [d] mean (SD)

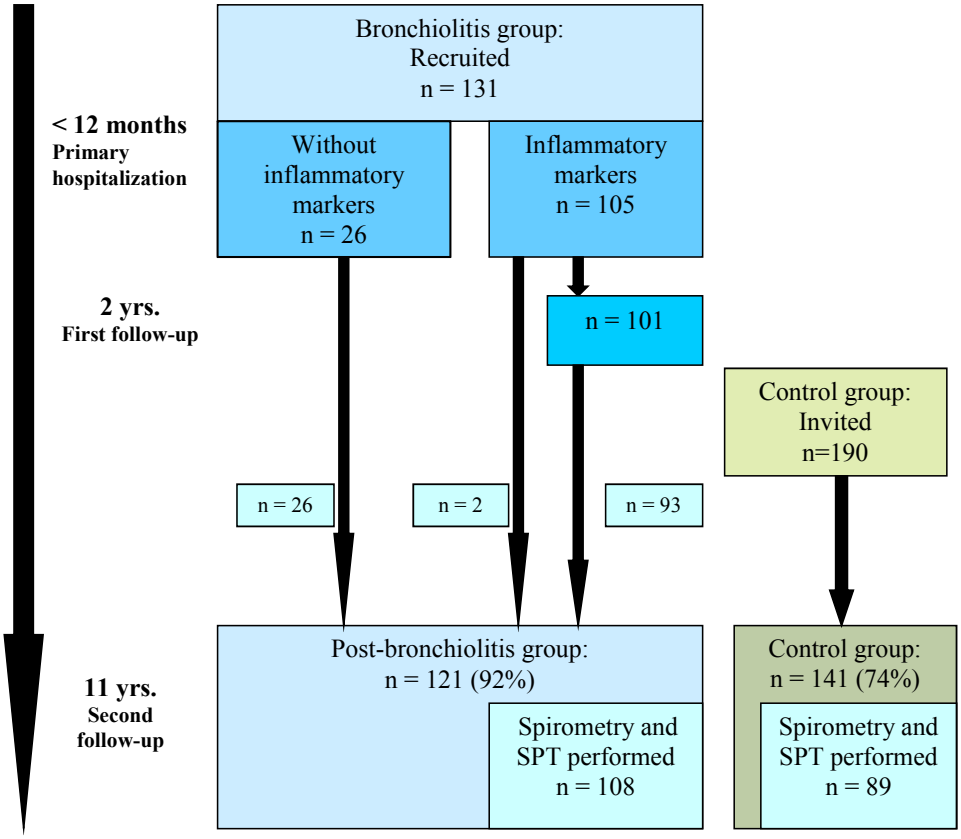


**Figure 6**. Simplified study design with numbers of children (% of invited) in each part of the study.

### 4.2.1 Primary hospitalization

The children had no previous hospitalization for bronchiolitis, but 18 children (18 %; data from 101 children) had previous respiratory symptoms without hospitalization (rhinitis, rattling in the chest or bronchial obstruction). The children with airway symptoms prior to the hospitalization for bronchiolitis were generally older 8.5 months (5.8, 10.3) (median (IQR)) compared to children without such symptoms, 4.0 months (2.0, 7.0) ($p < 0.001$) (unpublished data).

**Analysis of RSV virus and inflammatory markers**

Nasopharyngeal mucus was examined for RSV by direct immunofluorescence in all infants with bronchiolitis during the hospitalization (bioMèrieux, Marcy-l'Ètoile, France).

U-$PGF_2$, U-$LTE_4$, U-EPX and blood eosinophils were measured during the acute hospitalization for bronchiolitis (140, 141). Urine was collected within four hours after admission and kept in a refrigerator until aliquoting and freezing (-20 ºC) within 10 hours. U-$PGF_2$ and U-$LTE_4$ were analysed by a specific enzyme-linked immunoassay (Cayman Chemical, Ann Arbor, MI, USA). The detection limit in the assay was <5 ng/l for U-$PGF_2$ and <10 ng/l for U-$LTE_4$, and the interassay variation was <12%. Levels of urinary eosinophil protein X (U-EPX) were analysed by a specific radioimmunoassay (Pharmacia, Uppsala, Sweden). The detection limit in the assay was <3 µg/l and the coefficient of variation was <5% within the assay and <10% between the assays. Urine creatinine levels were measured by Vitros 250 system (Ortho Clinical Diagnostics, Inc., Rochester, NY, USA). All measurements were carried out in duplicate. U-$PGF_2$, U-$LTE_4$ and U-EPX levels are presented as nanogram per millimole creatinine. Blood was drawn immediately after admission, and eosinophil counts were determined with Technicon H*2/H*3 or Coulter STKS.

### 4.2.2 First follow-up

The first follow-up took place 20 months (19.0, 23.0) (median, range) after initial hospitalization. A questionnaire was used to interview parents and a SPT was performed.

**Questionnaire**

A standardized questionnaire was used to interview parents regarding the health of their child after discharge from the hospitalization for bronchiolitis. Episodes of wheeze and symptoms of AD were recorded, as was the parental history of asthma and atopy (AD and/or allergic rhinitis) and parental smoking habits. In order to identify an episode of wheeze as bronchopulmonary obstruction (BPO), the parents were asked if the child after the hospitalization for bronchiolitis had experienced respiratory

symptoms similar to those experienced when hospitalized with the initial bronchiolitis episode. One or more consecutive days of wheeze preceded and followed by a healthy period lasting at least one week, was counted as one independent episode. Episodes of wheeze reported by parents or by a physician or in relation to hospitalization were recorded and counted. A copy of the questionnaire is enclosed (Appendix I).

**Skin prick test**

A SPT to common inhalant and food allergens was performed with Soluprick ® allergens (ALK Albello, Hørsholm, Denmark). Histamine (10 mg/ml) was used as a positive control and a 0.9% saline solution as a negative control. A wheal diameter ≥ 3 mm larger than the negative control was defined as a positive result. The following allergens were used: *Dermatophagoides pteronyssinus*, dog and cat dander, *Cladosporium herbarium*, birch, timothy, egg white, milk, peanut, codfish, pea, hazelnut and shrimp.

### 4.2.3 Second follow-up

All included children were invited to a second follow-up examination in 2008-2009 at the age of 11 years. For the 11- year follow-up examination, an unselected age matched control group was recruited from three schools in Stavanger. The follow-up included a questionnaire, assessment of lung function, FeNO, BHR and a SPT. At the second follow-up, the children in the control group were older than children in the post-bronchiolitis group (Table 1).

**Questionnaire**

The second follow-up included a questionnaire evaluating symptoms of asthma (ISAAC) (214). The questionnaire recorded both the wheezy episodes ever and during the preceding 12 months, the occurrence of wheeze or respiratory symptoms during physical activity, sleep and talking and recurrent cough at night apart from during airway infections, all during the preceding 12 months and a history of "asthma ever". Use of asthma medication during the preceding 12 months was also recorded

(bronchodilators, inhaled corticosteroid, leukotriene antagonists). A copy of the questionnaire is enclosed (Appendix II).

**Skin prick test**

A SPT to common inhalant and food allergens was performed with Soluprick® allergens (ALK Albello, Hørsholm, Denmark). Histamine (10 mg/ml) was used as a positive control and a 0.9% saline solution as a negative control. A wheal diameter ≥ 3 mm larger than the negative control was defined as a positive result. The following allergens were used: *Dermatophagoides pteronyssinus*, dog and cat dander, *Cladosporium herbarium*, birch, timothy, egg white, milk, peanut, codfish and German cockroach.

**Spirometry**

Spirometry was performed according to established guidelines (215), using a Vmax Encore 229D spirometer (SensorMedics Inc., Anaheim, USA). Forced expiratory volume in first second ($FEV_1$), forced vital capacity (FVC) and forced expiratory flows at 25-75% of FVC ($FEF_{25-75}$) were recorded. Except for the ratio $FEV_1$/FVC, measurements were compared to values predicted by reference equations considered standard at the time of the examinations in 2008, and expressed as percentages of predicted $FEV_1$% (216) and $FEF_{25-75}$% (217).

**Bronchial hyperresponsiveness**

BHR was assessed with MPT, using an inhalation-synchronised, dosimetric nebulizer, Spira Elektra 2® (Spira, Hämeenlinna, Finland). The test was not performed if baseline $FEV_1$% was <65% predicted. Methacholine was administered in doubling doses until a 20% reduction in $FEV_1$ was obtained, or until a final given cumulative dose of 11.54 $\mu$mol had been given. A dose response slope (DRS) was calculated as the ratio between the maximum percentage decline in $FEV_1$ from baseline and the total administered dose of methacholine (%/$\mu$mol) (218).

**Exhaled nitric oxide (FeNO) measurements**

FeNO was measured online by the single breath technique according to published guidelines (219), with an EcoMedics Exhalyzer ® CLD 88sp with DENOX 88 (ECO MEDICS AG, Duernten, Switzerland). NO-free air was inhaled to near total lung capacity, followed immediately by full exhalation at a constant flow of 50 ml/s, and the expiratory pressure was maintained between 5-20 mm Hg to prevent upper airway contamination. FeNO was recorded as mean value from 3 reproducible plateaus within 10% acceptability. FeNO was measured prior to spirometry, SPT and MPT.

## 4.3 Definitions

| Variable | Definition | Paper number |
|---|---|---|
| Bronchiolitis | Acute febrile respiratory illness with tachypnoea, dyspnoea, prolonged expiration and wheeze on auscultation.<br>*Exclusion criteria:*<br>Previous hospitalization for bronchiolitis, previous use of inhaled or systemic corticosteroids, pre-existing lung disease, signs of bacterial infection during hospitalization. | I, II, III, IV |
| Current asthma | Positive answer to the ISAAC question regarding "asthma ever" and a positive answer to at least one of the two questions:<br>1) Wheezing or whistling in the chest or chest tightness during the preceding 12 months.<br>2) Use of asthma medication during the preceding 12 months. | I, II, III, IV |
| Recurrent wheeze | ≥3 episodes of recurrent wheeze before two years of age. | IV |
| Atopy | A positive SPT for at least one allergen. | I, II, III, IV |
| Healthy | No current asthma and no atopy at the 11 year follow-up. | III |
| Current non-atopic asthma | Current asthma without atopy at the 11 year follow-up. | III |
| Current atopic asthma | A combination of current asthma and atopy at the 11 year follow-up. | III |

## 4.4 Statistical analyses

Results and demographic data were presented as means with standard deviations (SDs) / 95% confidence intervals (CI), medians with range / inter-quartile range (IQR) or counts (%). The distribution of the DRS to methacholine and FeNO (designated as ppb) was regarded as natural logarithm (ln) normally distributed. For FeNO the results were presented as back-transformed values given as geometric mean with asymmetric 95% CI. When ln-transforming DRS, non-positive values were set to 0.001.

Categorical variables were compared with Pearson's chi-squared exact test or Fisher`s exact tests. Group comparisons for normally distributed data were performed with Student`s *t*-test. The Mann Whitney *U*-test was used for not normally distributed data. Multiple factors were compared using two-way Analysis of Variance (ANOVA). Dunnett's test was used for post-hoc comparisons between sub-groups if F-test was significant in the overall ANOVA analysis.

P-value < 0.05 was regarded as statistically significant. All statistical tests were two-tailed and data were analysed using the latest version of SPSS statistical package.

**Regression analyses**

Multivariate linear and logistic regression analyses were applied to explore the effects from a set of potential explanatory variables for lung function ($FEV_1$%, $FEV_1$/FVC ratio, $FEF_{25-75}$%), BHR (ln DRS), FeNO (ln FeNO), asthma and atopy, respectively, as the outcome variables. Each explanatory variable was entered separately into a univariate regression model and odds ratios (OR) and regression coefficients (B) with 95% CI were calculated. Explanatory factors with p-values < 0.1 (Paper I and IV) and p-values <0.2 (Paper II and III) by univariate analyses were further analysed in multivariate models. Analyses of interaction terms were used to test if explanatory variables influenced children differently with respect to the outcome variable according to gender, viral status, atopic status and previous hospitalization for bronchiolitis or not. The number of variables included in the linear regression analyses was less than 1/10 of the sample size (220).

**Risk factor analysis** (Paper IV)

The sensitivity, specificity and positive/negative likelihood ratio (LR) and positive/negative post-test probabilities with 95% CI (221) for current asthma at 11 years of age were calculated for all risk factors that were significantly associated with outcome in the univariate regression analyses. In addition, calculations for the outcome variables in the multivariate model in combinations with "and "and "and/or" for the various significant risk factors in the univariate models were done. Generally, the estimated probability of a disease *before* the test result has been established is referred to as the pre-test probability, which is estimated on the basis of the clinician's personal experience, local prevalence data and published reports (222). The pre-test probability for asthma in children with hospitalization for bronchiolitis was defined as the proportion of children with asthma at 11 years of age, equal to the *overall* prevalence of asthma in the present study. The post-test probabilities were defined from the pre-test probability and likelihood ratios using the principles of Bayes theorem (223).

**Priority of statistical analyses**

The statistical analyses of the main hypotheses were primary to the protocol and the statistical analyses of data from subgroups were secondary to the protocol.

## 4.5 Ethical considerations

The study was approved by the Regional Committee on Medical Research Ethics, and signed statements of informed consent were obtained from all parents. The testing of lung function and BHR by MPT are used daily by pulmonary and allergy clinics throughout Norway, and are not considered to represent any risk for the participants.

# 5. RESULTS

In this section, the results from the four papers as well as some supplementary results to clarify the results from the papers are given. The supplementary results are referred to as unpublished data.

## 5.1. Asthma and atopy after bronchiolitis in infancy

(RQ #1–Paper I)

The overall prevalence of current asthma after bronchiolitis was 21% when all children with a history of bronchiolitis were included. The prevalence of current asthma was higher in the post-bronchiolitis than in the control group (21% vs. 9%; p = 0.009).

Girls with RSV positive bronchiolitis had less atopy than controls; otherwise there was no difference in the occurrence of atopy between the groups.

## 5.2 Lung function and BHR after bronchiolitis in infancy

### 5.2.1 Lung function

(RQ # 1-Paper I)

Children in the post-bronchiolitis group had lower lung function than children in the control group, findings given as means (95% CIs): $FEV_1$%: 95.7 (93.9, 97.5) vs. 99.1 (96.7, 101.4); p = 0.020, $FEF_{25-75}$%: 88.7 (84.4, 93.0) vs.97.3 (92.6, 101.9); p = 0.008 and $FEV_1$/FVC: 81.6 (80.3, 83.0) vs. 83.8 (82.6, 85.1); p = 0.024.

Apart from lower $FEV_1$/FVC ratio among children with asthma in the control group; asthmatics: 80.3 (76.5, 84.1); non-asthmatics: 84.2 (82.9, 85.6); p = 0.047, there were no differences regarding gender, atopy, lung function or BHR between the asthmatic and non-asthmatic subjects neither in the post-bronchiolitis group nor in the control group.

As described in section 4.2.3, lung function measurements were compared to values predicted by reference equations considered standard at the time of the examinations in 2008, and reported as $FEV_1$ and $FEF_{25-75}$ in percentages of the predicted values. Recently, an ERS Task Force (Global lung Function Initiative (GLI))

produced updated predicted values for spirometry across all ages (224). For the post-bronchiolitis group and the control group predicted values using the GLI 2012 reference equations are shown in Table 2. The same differences for the lung function variables between the post-bronchiolitis and controls groups were found, when using the GLI 2012 equation and the predicted values from Quanjer (216) and Wang (217).

Table 2.
Lung function data according to viral aetiology in 121 children hospitalized for bronchiolitis during their first year of life and in an age matched control group, reported as percentages of predicted using the prediction equations of the Global Lung Initiative (GLI 2012) **(224)**

| | **n** | **Post-bronchiolitis** | | | | **Controls** | **Post-bronchiolitis vs. Controls P-values** | | |
|---|---|---|---|---|---|---|---|---|---|
| **$FEV_1$ %** | 108 | 96.7 (94.9, 98.6) | | | 89 | 100.4 (97.9,102.9) | **0.019** | | |
| **$FEF_{25-75}$%** | 108 | 81.6 (77.5, 85.8) | | | 89 | 88.4 (84.1, 92.7) | **0.026** | | |
| | **n** | **RSV positive** | **n** | **RSV negative** | **n** | **Controls** | **RSV positive vs. RSV negative P-values** | **RSV positive vs. controls P-values** | **RSV negative vs. controls P-values** |
| **$FEV_1$ %** | 83 | 97.0 (94.8, 99.2) | 25 | 95.8 (92.2, 99.4) | 89 | 100.4 (97.9, 102.9) | 0.592 | **0.047** | 0.076 |
| **$FEF_{25-75}$%** | 83 | 83.5 (78.6, 88.5) | 25 | 75.2 (67.9, 82.6) | 89 | 88.4 (84.1, 92.7) | 0.094 | 0.138 | **0.004** |

Results given as means (95% confidence intervals). RSV; respiratory syncytial virus, $FEV_1$ %; forced expiratory volume in first second as percentage of predicted, $FEF_{25-75}$%; forced expiratory flow between 25-75% of the forced vital capacity.

### 5.2.2 Bronchial hyperresponsiveness (BHR)

(RQ # 1-Paper I)

Children in the post-bronchiolitis group had higher BHR measured by methacholine DRS than controls, 5.1 (0.1, 330.0) vs. 2.4 (-0.2, 235.7); p = 0.003 (median (range)).

A stepwise multivariate linear regression model with ln DRS as outcome variable showed positive associations to hospitalization for bronchiolitis (p = 0.011) and negative associations to $FEV_1$% (p = 0.001), but no associations with current asthma, male gender, atopy and $FEF_{25-75}$/FVC ratio. BHR may be stratified in three groups by the cumulative dose of inhaled methacholine required to induce a 20%

reduction in $FEV_1$ (PD20); i.e. severe (≤1 µmol methacholine), moderate (> 1 µmol methacholine ≤8) and mild (> 8 µmol methacholine ≤11.5) (225). There were no associations between the different groups of PD20 and current asthma (unpublished data).

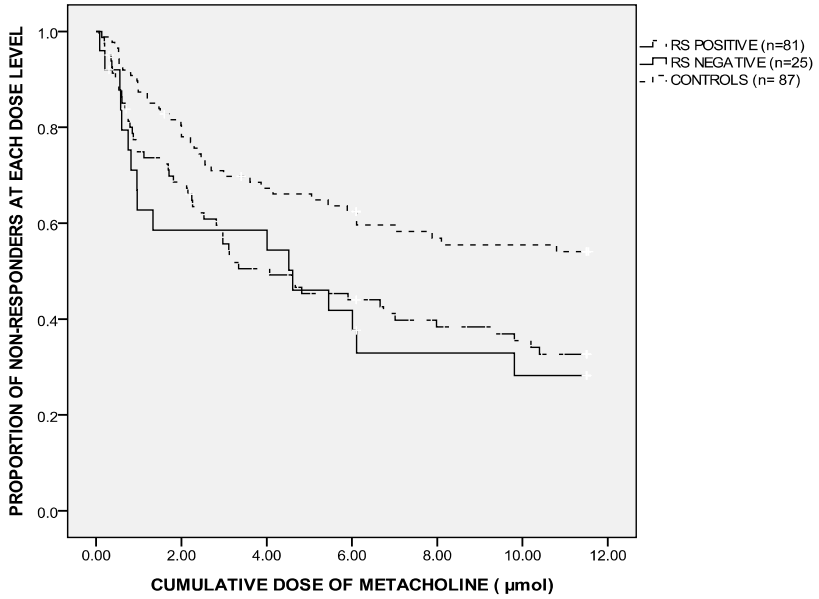


**Figure 7.** Methacholine responsiveness at 11 years of age in 106 children hospitalized for bronchiolitis during their first year of life and in 87 children in an age-matched control group. The x-axis represents the total cumulative dose of methacholine given to each subject, censored at the maximum administered dose of 11.5 $\mu$mol. The y-axis represents the proportion of non-responders at any given dose.



## 5.3 The impact of virus and gender on asthma, atopy, lung function and BHR

### 5.3.1 The impact of virus (RSV negative/RSV positive bronchiolitis)

(RQ # 2-Paper I)

Current asthma was more prevalent after RSV negative bronchiolitis, both when compared to RSV positive bronchiolitis (35% vs. 16%; p = 0.018) and compared to

controls (35% vs. 9%; p = 0.001). There was no difference in the prevalence of current asthma between children with RSV positive bronchiolitis and the control group. The ORs (CIs) for having asthma were as follows (unpublished data):

- RSV positive bronchiolitis vs. controls: 1.81 (0.81, 4.06)
- RSV negative bronchiolitis vs. controls: 5.43 (2.13, 13.74)
- RSV negative bronchiolitis vs. RSV positive bronchiolitis: 2.99 (1.18, 7.57)

The obstructive lung function pattern in the post-bronchiolitis group, with low $FEV_1$%, $FEF_{25-75}$% and $FEV_1$/FVC ratio was only found in the RSV negative group.

By using the GLI 2012 equation for predicted lung function, there was only a tendency for low $FEV_1$% predicted in the RSV negative group compared to the controls, but lower $FEV_1$% predicted was also found in the RSV positive group compared to controls. Lower levels of $FEF_{25-75}$% found in the RSV negative group compared to controls using the equations from Wang (217), was also found when using the GLI 2012 equation. The exact results are given in Table 2 (section 5.2)

### 5.3.2 The impact of gender

(RQ # 2-Paper I)

After stratifying for gender, higher prevalence of current asthma and higher DRS to methacholine in the bronchiolitis group than in the control group was found in boys only.

The impact of gender can also be studied by regression analyses. By univariate logistic regression analyses, male gender was not found to be an independent risk factor for asthma (OR 1.91; 95% CI: 0.90, 4.04; p = 0.091), when all children in the post-bronchiolitis and control groups were analysed together. There was no significant interaction between male gender and hospitalization for bronchiolitis with asthma as outcome variable (unpublished data).

Male gender was not an independent risk factor for BHR (ln DRS) (regression coefficients 0.16; 95% CI: -0.39, 0.70; p = 0.567). There was no significant interaction between male gender and hospitalization for bronchiolitis with ln DRS as outcome variable (unpublished data).

## 5.4 Inflammatory markers during bronchiolitis and outcomes at 11 years of age

(RQ # 3-Paper II)

Higher median values (IQR) of blood eosinophil counts obtained during bronchiolitis were observed in the group with asthma than in the group without asthma (0.27 (0.08, 0.51) vs. 0.09 (0.04, 0.27) $\times 10^9$/litre, respectively; p = 0.048).

Blood eosinophil counts were positively associated with BHR (p = 0.006) and negatively associated with $FEV_1$% (p = 0.025). None of the other inflammatory markers were associated with asthma, lung function, BHR or atopy at 11 years of age.

The levels of inflammatory markers did not differ between the groups of children with or without atopy, and none of the inflammatory markers were risk factors for atopy at 11 years of age by univariate logistic regression analyses.

## 5.5 FeNO at 11years of age after bronchiolitis in infancy

(RQ # 4-Paper III)

FeNO levels did not differ between the bronchiolitis and the control group. However, FeNO levels differed between the four sub-groups (ANOVA analyses including all children). Higher FeNO was found in the children with atopy and atopic asthma, but no difference was found between children with non-atopic asthma compared to healthy.

Separate analyses for the post-bronchiolitis and control group, revealed that FeNO was higher in children with atopy compared to healthy in both groups (Figure 8). Higher FeNO in children with atopic asthma compared to healthy was only observed in the control group.

Backward multivariate regression analyses including all participating children, revealed that atopy, height ($p<0.001$ for both) and BHR (p = 0.034), but not hospitalization for bronchiolitis (p = 0.359) were positively associated with ln FeNO. Asthma was not associated with ln FeNO by univariate regression analysis (p = 0.805). The associations between atopy, BHR, but not asthma and FeNO were similar between the control and the post-bronchiolitis groups.

Separate regression analyses for the post-bronchiolitis group revealed that BHR ($p = 0.018$), height ($p = 0.006$) and $FEV_1$% ($p = 0.012$) were positively associated with ln FeNO. There was a significant interaction effect between RSV negative bronchiolitis and atopy, and atopy was positively associated with ln FeNO only in the RSV negative group.

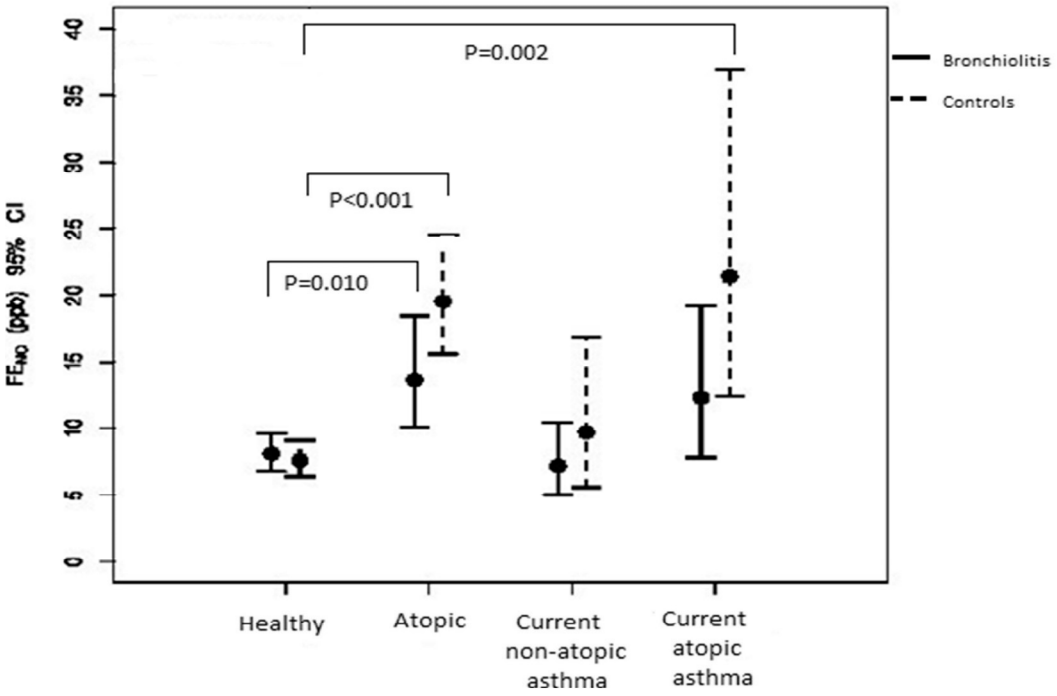


**Figure 8**. FeNO levels in four different sub-groups of children, split by bronchiolitis status in their first year of life. FeNO values are given as geometric mean with 95% confidence intervals.

## 5.6 Prediction of subsequent asthma after bronchiolitis in infancy

(RQ # 5-Paper IV)

Recurrent wheeze before the age of two years was the only significant risk factor for current asthma at 11 years of age in the multivariate regression analysis (OR 7.22; 95% CI: 1.25, 41.63; p = 0.015) and this single risk factor had the highest sensitivity (90.5%; 95% CI: 68.2, 98.3), but a low specificity (58.3%; 46.1, 69.7) for asthma at 11 years of age. The combinations of recurrent wheeze with either parental asthma, AD or parental atopy all had positive LR higher than 3 and specificity above 80%, but with only a moderate sensitivity.

The pre-test probability for asthma in this population was estimated to 22.6%, i.e. set to be equal to the overall prevalence of asthma at 11 years of age. Absence of recurrent wheeze had the lowest negative post-test probability (4.5%; 7.9, 16.7). The combination of recurrent wheeze with parental asthma, AD or parental atopy had a positive post-test probability of ~50%. These combinations were therefore better suited to predict later asthma than either alone.

# 6. DISCUSSION

The four papers included in this thesis report the results from the longest post-bronchiolitis study published in Norway so far. The results show that 11 year old children with a history of hospitalization for bronchiolitis in infancy have a higher risk of asthma, an obstructive lung function pattern, and higher BHR than children in an age matched control group. These findings were modulated by virus (RSV positive / negative bronchiolitis) and associated with blood eosinophils measured during the bronchiolitis.

In this section, the results of each research questions will be discussed individually and also when relevant linked together.

## 6.1 Asthma and atopy after bronchiolitis in infancy

The increased risk of subsequent asthma after bronchiolitis (Paper I) is in line with the results from other studies (19, 25, 168, 226, 227), although differences regarding age at follow-up, make comparisons between studies difficult. Post-bronchiolitis studies from Scandinavia and also the Tucson study show that the prevalence of asthma after bronchiolitis is positively associated with the age at the episode of the bronchiolitis (8, 162). However, the Scandinavian post-bronchiolitis studies referred to in Figure 4 (2.5.1), still show that 15-30 % of children with former bronchiolitis have asthma during early school years. Further, after a symptomatic period during early adolescence the risk of asthma seems to increase in early adulthood (8).

Children with previous hospitalization for bronchiolitis had no increased risk of subsequent atopy. Asthma after bronchiolitis was not related to atopy, neither in the RSV positive nor in the RSV negative group (Paper I). This is in line with other similar studies (20, 162), except the studies from Sigurs et al. (19, 170). As discussed in section 6.8.7, the study did not have sufficient power to detect minor differences in the prevalence of atopy. However, the actual prevalence of atopy was 24 % in the post-bronchiolitis group and 38 % in the control group (Table 2, Paper I). This effect size (-14 % absolute difference in the post-bronchiolitis group) strongly suggests that asthma in the post-bronchiolitis was not positively associated with atopy.

## 6.2. Lung function after bronchiolitis in infancy

In line with others, we found an obstructive lung function pattern with small airway obstruction in children hospitalized for bronchiolitis compared to controls (Paper I) (19, 201, 202). After stratifying for RSV virus positive/negative bronchiolitis, the small airway obstruction was only found in children with former RSV negative bronchiolitis compared to controls (Paper I). In the COAST study, outpatient viral wheezing illnesses due to RV, but not other viruses, were associated with an obstructive lung function pattern at 5-8 years of age (228). In a Finish study, higher $FEV_1$/FVC ratio and lower FVC, consistent with a restrictive lung function pattern, were found in children with former RSV negative compared to children with former RSV positive bronchiolitis (200). However to my knowledge, few other studies have found differences in lung function between these two groups.

Long-term birth cohort studies have observed reduced levels of lung function present already from birth prior to the bronchiolitis and/or early episodes of viral wheezing, and these alterations may represent a risk factor for future airway disease (187, 229). In contrast to this, children with persistent wheeze in the Tucson Children`s Respiratory Study, had normal lung function at birth, but loss of lung function through early childhood up to six years of age (163). The alterations in lung function by age six seemed to persist unto adulthood (203). Other prospective long-term studies following children with early wheezing unto adult life, observed that those with persistent or relapsing wheeze enter adult life with relative deficits in lung function (230).

Together, these studies indicate that alterations in lung function seem to be established during early childhood and continue up to early adult life. This pattern underlines the child`s vulnerability during early childhood and the importance of protecting young children from future airway injuries that may increase these alterations further (230).

The results from the present thesis do not give an answer to whether the lower lung function in children with former bronchiolitis was present already from birth or established during early pre-school years as a result of the bronchiolitis. However, we found a negative association between blood eosinophils measured during bronchiolitis

and $FEV_1$% at 11 years of age (Paper II). This association could possibly suggest that early life factors, particularly the inflammatory response during the bronchiolitis, may have long-term implications on later airway structure. However, studies must include early life factors and the history of respiratory symptoms prior to the bronchiolitis episode in order to reveal the mechanisms behind these associations.

## 6.3 Bronchial hyperresponsiveness after bronchiolitis in infancy

Higher BHR in young adolescents with former bronchiolitis than control subjects was observed also in a study from Finland (231), but in that study the difference did not persist until early adult life (202). High proportions of children with positive provocation test to methacholine are also observed in other post-bronchiolitis studies, but the lack of a control group complicates the interpretation in some of these studies (200, 227).

In our study, atopy and asthma at the age of 11 years were not associated with BHR (Paper I). This may be due to insufficient power and low numbers of children with asthma and atopy. The effect size and confidence intervals are inconclusive and cannot resolve if there was an effect of clinical importance or not. Therefore, whether this was a true negative finding cannot be answered in this thesis. This is further discussed in section 6.8.7. On the other hand, as previously described; MPT is a direct test of BHR and may reflect airway remodelling and structural alterations of the airways as much as airway inflammation (232).

Blood eosinophil counts during bronchiolitis were associated with BHR at 11 years of age (Paper II). BHR may have been present already prior to having bronchiolitis, underlining the possible importance of premorbid constitution and susceptibility (233, 234). The association between eosinophils and BHR could be due to a common genetic susceptibility controlled by chromosome 5q, and may be related to the production of IL-5 (235). Eosinophils and an increased IL-5/IFN-γ ratio in bronchoalveolar lavage have been observed in a subgroup of children with RSV positive bronchiolitis, and this Th-2 dominated immune response could be a risk factor for later asthma and/or BHR (236). In a murine model, IL-5 was essential for the influx of eosinophils into the lungs and the development of BHR (237).

The results from the present thesis, underline the complexity of mechanisms that are involved in children with early life wheezing and subsequent asthma and structural airway alterations. This complexity may be due to coexisting genetic, environmental and inflammatory factors that contribute to the persistent BHR observed in children with former bronchiolitis (238). This may explain the somewhat divergent results regarding BHR in this thesis.

The association between early BHR and later asthma was also observed in the Tucson Children`s Respiratory Study; reporting that BHR measured by cold air at age six was associated with asthma at age 22 (206). Corresponding to this, a recent publication from the ECA study in Oslo observed that BHR at 10 years of age, particularly when measured by MPT, could predict asthma six years later, although the association was modest (225). Long-term post-bronchiolitis studies have observed asthma relapses in adult life after a relatively symptom free period during adolescence (209, 239). The persistent BHR observed in our study could therefore be a risk factor for asthma relapse in adult life, but this can only be confirmed by a later follow-up.

## 6.4 The impact of virus on outcomes after bronchiolitis

Recently, studies have found higher risk of asthma particularly among children with previous wheezing due to infection with RV (9, 22, 168, 240). Several of these studies have included children who were older than 12 months of age during the acute episode, complicating interpretation of the results and comparisons to other studies (9, 168). However, a recent study confirmed that the risk of subsequent asthma was higher after RSV negative than RSV positive bronchiolitis also in children younger than six months of age at inclusion (241).

Unfortunately, the viruses involved in children with RSV negative bronchiolitis in the present study population are not known. RV has been found to be the most common virus in children with RSV negative bronchiolitis and the risk of RV bronchiolitis increases with age (14, 27). The children with RSV negative bronchiolitis were older at hospitalization than those with former RSV positive bronchiolitis (Paper I). This could suggest that the majority of children with RSV negative bronchiolitis were infected by RV.

Recently, particularly RV type C has been found in severe asthma exacerbations in children (242). Both RV type C and RV type A, more than RV type B, are common in preschool children hospitalized for acute respiratory infections and associated with a history of wheezing or asthma, but more seldom reported among infants with lower respiratory tract infections (243). The role of RV type C in young children with bronchiolitis and the association to subsequent asthma is to my knowledge not entirely known.

An increased risk of asthma in children with early wheezing due to other viruses than RSV has been found among children with early atopic sensitization, particularly with sensitization towards aeroallergens (22, 199). These findings may be due to a common underlying susceptibility to both conditions. There is less evidence that the viral infection increases the risk of subsequent atopic sensitization (199). Alterations of immune responses both associated with Th-2 cytokines and IFN-γ seem to be related to the risk of viral wheezing in young children (244). In our study, we did not find an increased risk of atopy among children with former RSV negative bronchiolitis. The same observation has been made in other long-term follow-up studies of children with RSV negative bronchiolitis (245). The discrepancies between studies may be due to different methodological aspects, such as selection of participants and age at inclusion and/or at follow-up. In our study, the low numbers of participants in the RSV negative group and the possible heterogeneity according to the viral aetiology in this group may also influence these results.

This study did not have sufficient statistical power to confirm or reject minor differences in the prevalence of asthma, such as those found for the RSV positive group (16%) vs. the control group (9%). This was a limitation of the study; as an effect size of – 7% absolute difference may also be of clinical interest. Moreover, the ORs and CIs given in the result section, do not preclude that there may be a clinically important difference in the asthma prevalence between these two groups. This is further discussed in section 6.8.7.

As discussed, the risk of asthma after bronchiolitis may be related to underlying premorbid susceptibilities in the child and/or a result of an early viral infection. In the review from Beigelman and Bacharier, the authors underline that both virus-specific

effects and host factors are important when discussing these issues. The authors suggest that RSV might have a more causative role in the pathogenesis of subsequent asthma, whereas wheezing with RV rather is marker of an underlying susceptibility towards asthma (246).

A recent review suggests that the risk of asthma in subjects with former hospitalization for RSV bronchiolitis is sustained until adulthood, and that hospitalization for RSV bronchiolitis is a risk factor also for adult asthma (239); however, this contrasts the results from the Tucson study (162). The different conclusions may be due to different study populations, both according to age at inclusion and the severity of the disease. A publication from Carroll et al. found that the severity of the bronchiolitis was related to the risk of later asthma. In this study, hospitalization for bronchiolitis was a greater risk factor than an ambulatory clinic visit for subsequent asthma (247).

Although, we found no differences in lung function between children with former RSV positive and RSV negative bronchiolitis, there was a trend for lower $FEV_1$/FVC and $FEF_{25-75}$% in children with former RSV negative bronchiolitis (Paper I). The lack of significant results could be due to insufficient power and particularly low numbers of children with former RSV negative bronchiolitis.

## 6.5 The impact of gender on outcomes after bronchiolitis

After stratifying for gender, the increased risk of subsequent asthma after bronchiolitis was only significant for boys (Table 2, Paper I). Higher prevalence of asthmatic wheeze in boys than girls has been observed before puberty and may be related to the higher prevalence of atopy and smaller airway size in boys than girls (248, 249), as also summarized in the GA$^2$LEN review from Almqvist et al. (77). Also in the Tucson study, boys were significantly more likely to experience wheeze than girls, but no gender differences were observed after the age of 16 (80).

After stratifying for gender, higher BHR was only present in boys, while lung function was lower in both boys and girls with former bronchiolitis (Table 3, Paper I). Early structural alterations of the airways may be the reason for the most prominent on-going BHR for boys in early adolescence. Lower flow rates and airway

conductance in males than females have been found shortly after birth, probably due to later expression of surfactant components in males (50).

In summary, a higher prevalence of asthma and higher BHR in the children hospitalized for bronchiolitis compared to the controls were found only in boys. However, in the regression analyses, male gender was not associated with current asthma or BHR respectively. The ORs and CIs in both these analyses, included values that are of clinical interest, and whether these were true negative findings could not be answered in this thesis. There were no significant interaction effects between hospitalization for bronchiolitis and male gender and current asthma and/or BHR respectively as outcome variables. As discussed in section 6.8.7, this study has low statistical power for detecting such an interaction. However, the results according to gender are still in line with results from other studies, as discussed above. Further studies including more participants, is necessary in order to confirm whether the various outcomes after bronchiolitis in infancy differs between boys and girls.

## 6.6 Inflammatory markers during bronchiolitis and outcomes at 11 years of age

**Inflammatory markers in blood and urine**

As previously published, results from the first follow-up showed that the absence of eosinopenia in response to viral infections was found in children with persistent wheezing at 20 months of age (140), in accordance with the results from the Tucson study (250) and others (251). An association between blood eosinophils measured during bronchiolitis and subsequent asthma in school children and adolescents has also been reported by others (226, 252, 253). Eosinopenia is a normal immune response following infections and stress (109). The results from the present thesis could suggest that normal levels or a minor increase in blood eosinophil counts during bronchiolitis could indicate an aberrant immune response in these children with long-term impact on later airway structure and BHR.

The higher levels of eosinophils during bronchiolitis in children with subsequent asthma could be due to activation during the viral infection or related to a premorbid susceptibility towards a Th-2 dominated immune response, although these

questions cannot be answered by the present thesis. Other studies have have found evidence for both of these hypotheses. Although eosinophils are considered as an effector cell in asthma, eosinophils have evolved as part of the innate immune response against parasitic helminthes and are also observed as part of the defence against viral infections (47). RSV has been shown to activate human eosinophils and further induce eosinophilic inflammation in epithelial cells through an up-regulation of adhesion molecules, as summarized in the review by Kato et al. (254). Martinez et al. suggested that blood eosinophils are genetically determined, and found a linkage between markers on chromosome 5q and circulation eosinophils in an unselected population sample (235). Further, high levels of eosinophil secretory proteins at four weeks of age have been associated with two-fold risk of developing wheeze during the first year of life. This might be explained by a Th-2 cytokine pattern present early in life, possibly as a consequence of intrauterine allergen exposure (255). Contrary to this, a recent study reported that markers of eosinophilic inflammation during RSV bronchiolitis were related to IFN-γ and not Th-2 cytokines (256). Thus, these issues are far from settled.

The role of eosinophils in the pathophysiological processes leading from bronchiolitis to subsequent asthma cannot be answered within the frames of this thesis. Whether an aberrant eosinophil response is induced by the bronchiolitis and/or associated with innate traits related to both bronchiolitis and later asthma is a fundamental question. Pre- and post-bronchiolitis assessments and a control group included at hospitalization, are required to answer this issue. Airway symptoms prior to the hospitalization for bronchiolitis were not included as a potential explanatory variable. Children with early airway symptoms were generally older than children without such symptoms, and the analyses were therefor adjusted for age at hospitalization. Also exposure to tobacco smoke and the severity of the bronchiolitis could be important confounders that were not included in the analyses in this thesis.

We did not find any associations between U-$LTE_4$, U-EPX and U-$PGD_2$ and atopy, asthma, BHR or lung function at 11 years of age (Paper II). The lack of association for U-EPX measured during bronchiolitis and the outcome variables at 11 years of age were perhaps most surprising. U-EPX, but not blood eosinophils

measured during early childhood wheezing has been shown to predict persistent atopic wheezing (131).

To my knowledge, few studies have assessed the possible associations between U-$LTE_4$ and U-$PGF_2$ during bronchiolitis in infancy and asthma and lung function in adolescence. There are also few recent studies that have reported associations between U-$PGF_2$ and asthma in children, but studies have observed associations between U-$PGF_2$, U-$LTE_4$ and aspirin-intolerant asthma in adults as described in the review from Higashi et al. (147). Further, increased levels of U-$LTE_4$ have been observed in subjects with atopic asthma after allergen challenge (257), and in smoke exposed children with asthma exacerbations (258). An increase in U-$LTE_4$ has also been shown to correlate with decreases in $FEV_1$ (259). Contrary to this, a study including adult patients observed a weak negative correlation between asthma severity and U-$LTE_4$, and also a negative correlation between U-$PGF_2$ both with asthma severity and $FEV_1$ (153). As already mentioned in chapter 2.4.2, wide variations in the results of prostaglandins and leukotrienes in urine are observed. This may question the role of U-$LTE_4$ and U-$PGF_2$ as reliable markers of airway inflammation, and may be one reason for the lack of associations between these markers and asthma/lung function observed in the present thesis.

However, with a low number of children included in these analyses, only large differences in the inflammatory markers between the children with asthma and no asthma at 11 years of age could be detected. This is further discussed in section 6.8.7.

**Exhaled nitric oxide**

Studies have shown differences between FeNO in children with various wheezing phenotypes (260-262), but we did not observe any differences regarding FeNO between children in the post-bronchiolitis and control group (Paper III). Wheezing in infancy has been regarded as a different inflammatory condition compared to asthma in older children (45). We did not have sufficient information to divide the children in our study into specific wheezing phenotypes. However, the results do not suggest that FeNO can be used to distinguish between asthma after bronchiolitis and asthma in the general population (Paper III).

Although several studies have observed associations between asthma and FeNO (263, 264), we were not able to confirm this (Paper III). The association between FeNO and asthma is related through an eosinophilic airway inflammation, although there is little evidence that eosinophilic airway inflammation directly increase iNOS expression or activity (265). The observed association between FeNO and atopy has also been found by others (266-268). The latest guidelines from the American Thoracic Society (ATS) underline that FeNO can be used in the diagnosis of eosinophilic airway inflammation and to determine the likelihood of steroid responsiveness in individuals with chronic respiratory symptoms, but only weakly recommend that FeNO can be used to support the diagnosis of asthma (265). The ATS guidelines further suggest that only FeNO values >35 ppb are likely to indicate eosinophilic airway inflammation in children, and that values < 20 ppb should be interpreted cautiously (265). These observations are supported by our results, as the mean FeNO values in our study were overall low (Paper III).

Use of inhaled corticosteroids (ICS) during the preceding 12 months was not significantly associated with FeNO in the present study (Table 5 and 6, Paper III). In fact, the regression coefficients were positive, whereas the use of ICS usually decreases the levels of FeNO (265). However, only nine children in the post-bronchiolitis group and eight children in the control group used ICS, suggesting careful interpretation of these results.

The observed association between FeNO and BHR in the present and other studies (158, 269), may suggest that FeNO may be linked to asthma and inflammation. However, the study from Franklin et al. found this association only in atopic children (157), and in a study from the Copenhagen birth cohort, this association was independent of asthma symptoms (269). A Norwegian twin study explained the association between FeNO and BHR by a common genetic effect (270). The relationship between NO metabolism and BHR in asthma is complex (271). The ATS guidelines underline that studies report inconsistent associations and low correlations between FeNO and BHR, and that BHR, airway inflammation and FeNO belong to different domains (265). As reviewed by Brusasco and Pellegrino, also structural

features can influence airway narrowing during methacholine provocation testing (238).

The results from the present thesis confirm that FeNO is a marker of atopy and not asthma also for children with a previous history of bronchiolitis.

## 6.7 Prediction of subsequent asthma after bronchiolitis in infancy

By using simple clinical non-invasive variables available at two years of age, we found a 50% positive post-test probability of having asthma at 11 years of age. The +LR was slightly below 5 (Paper IV). In order to be informative as a diagnostic tool to identify preschool children with later risk of asthma, + LR should at least exceed 5, as higher values generates a larger shift from pre-test to post-test probability (272). Our model had therefore a limited capacity to predict asthma, but was nevertheless comparable to more complex models also including invasive methods like blood test (273).

However, the model did predict the absence of asthma reasonably well, underlined by a negative post-test probability varying from 4.5% to 17.1%, meaning that the risk of asthma was overall low in subjects with negative answers to "*the test*". The absence of recurrent wheeze alone had the lowest negative post-test probability of 4.5%.

The lack of significant risk factors other than recurrent wheeze could be due to a relatively small population sample. Increasing the number of participants would have decreased the CIs, and could possibly also have changed the ORs. In addition, the ORs and CIs for the other clinical variables in the fully adjusted regression model (Table 2, Paper IV), may indicate that also these variables are of clinical importance. Parental atopy with an OR of 2.36 (95% CI: 0.54, 10.31) may be of particular interest. Therefore, in the table with positive and negative post-test probabilities, different combinations of these variables are included (Table 3, Paper IV). The results may suggest that also the non-significant risk factors from the regression analyses are of clinical importance.

All children with previous airway symptoms also had recurrent wheeze at the age of two years. These two variables were therefore completely confounded and only

recurrent wheeze was included in the model. As previously mentioned (section 4.1.1), children with airway symptoms prior to the bronchiolitis were older than children without such symptoms.

As our prediction model is constructed from a population of children with a high risk of asthma, it cannot be used to predict or rule out asthma in the general population. Our model is based on positive answers to recurrent episodes of airway obstruction, AD and parental characteristics. The important role of the frequency of recurrent airway obstructions in young children as a predictive factor for subsequent asthma has been observed by others (208, 274). We did not have an exact count on the total number of episodes with bronchial obstruction, and could therefore not include the frequency of bronchial obstructions in our model. Parental atopy, IgE sensitization, elevated total IgE and exposure to high level of indoor allergens were associated with wheezing at the age of 13 years in the MAS study, but these findings were particularly prominent if the wheezing started before three years of age (123). The predictive roles of parental atopy for asthma have also been shown by others (123, 209, 273), but parental allergic disease alone seem to be insufficient in order to identify high-risk populations at birth (275). In our study, parental asthma and parental atopy was highly associated, and there was an overlap in parents reporting either of them. A Swedish study reported higher impact of parental asthma than parental atopy on asthma in off-springs (213), but we could not confirm this in our study.

On the other hand, a positive SPT was not predictive of asthma, which may be surprising. Others have reported an association between early atopic sensitization measured by specific IgE and subsequent asthma (123), in the ECA study from Oslo only found in boys (276). However, quantitative measures, for instance of specific IgE, in contrast to binary classification of atopy (sensitized/not sensitized), may be more robust as assessment of atopy-related risk of asthma (182). This may be the reason for the lack of associations between atopy and subsequent asthma in our study, as well as the young age of children when tested.

Developing clinical prediction algorithms includes several steps, such as construction, validation and impact analysis, and there are only a few highly validated clinical prediction rules for children in general (277).

The API is an externally validated test, which means that the results of the test are applicable in different populations. The test is often used for comparison with other prediction rules (8, 208, 209, 272, 274). By using the API, a + LR of 7.3 for predicting asthma among 7-8 year old children with ≥ 3 episodes of wheezing/year in early preschool years was reported (273). However the +LR was reduced to 5.0 when predicting the risk of asthma in children aged 11-13 years. As mentioned in chapter 2.6, several other prediction rules have been constructed, but their ability to predict asthma is limited (207, 275, 278). Being aware of different study designs, one of the best predictive models have been reported among a subgroup of children in the German MAS. They reported a PPV of approximately 80% for wheezing at 11-13 years in children with recurrent wheezing and atopic sensitization before three years of age and exposure to high levels of indoor allergens in early life (123).

However, it is important to consider the practical benefits of these predictive indexes. In order to be useful also in a busy out-patient clinic, the variables included should be easy to obtain and not require complicated analyses or measurements.

**The role of other risk factors**

The results from Paper I and Paper II could indicate that gender, viral aetiology (Paper I) and blood eosinophils (Paper II) could be added to the prediction model.

Male gender has been reported as a risk factor for asthma at preschool age and up to the age of 13 years (123, 278). However, gender was not an independent risk factor for asthma after bronchiolitis (Paper IV). After stratifying for gender, higher prevalence of current asthma was found only for boys, when including all children with a previous history of bronchiolitis (Paper I). After stratifying for gender, higher BHR was confined to boys, but BHR was not associated with asthma or atopy (Paper I). The divergent result regarding gender might be a result of insufficient statistical power, and the picture could have been clearer with more participants.

RSV negative bronchiolitis was not an independent risk factor for asthma at 11 years of age (Paper IV). As mentioned earlier, viral aetiology seems to have a high impact on the risk of subsequent asthma after bronchiolitis. The revised version of the API, developed to predict asthma in children with former hospitalization for

bronchiolitis, has included RSV negative bronchiolitis as a minor criterion (8). In our study, the number of children with RSV negative bronchiolitis was lower than the number of children with RSV positive bronchiolitis. In addition, the heterogeneity of virus probably present in the RSV negative group studied in this thesis makes the interpretation of these findings difficult.

Blood eosinophil counts during bronchiolitis seem to be related to subsequent asthma, and associated with BHR and lower lung function in adolescence (Paper II). However, the logistic regression analyses showed only a tendency of association between blood eosinophil counts during bronchiolitis and asthma at 11 years of (Paper II). The previous mentioned API has included eosinophilia as a minor criterion (135). Eosinophils were not included in our prediction model, as the main objective was to create a model without invasive tests and thereby easy to perform in a busy out-patient clinical setting.

Exposure to passive smoking up to the age of two years was not a risk factor for subsequent asthma (Paper IV). The influence from exposure to smoking in utero and in early life on later asthma seems to decrease with increasing age of the offspring (123, 275). An independent association between parental smoking in the child's first four years of life and persistent wheezing at the age of 10 could not be found in the Isle of Wight Cohort (209).

## 6.8 Methodological considerations

### 6.8.1 Study design

This was an observational longitudinal follow-up study of a group of subjects identified during their first year of life due to "exposure" to hospitalization for bronchiolitis. Various outcomes regarding asthma, atopy, lung function and bronchial hyperresponsiveness were compared to similar outcomes in an age matched control group, i.e. "unexposed" to hospitalization for bronchiolitis in their first year of life.

The bronchiolitis group was included during hospitalization for bronchiolitis in their first year of life, while the control group was included at 11 years of age. Thus, those exposed to hospitalization for bronchiolitis were followed prospectively, while the control group was examined once, as in a cross-sectional design. Based on this, the

study is characterised as a prospective follow-up of children hospitalized for bronchiolitis in their first year of life with a historical control cohort.

**The post-bronchiolitis group**

This study has a prospective design with a long-term follow-up and an attendance rate of approximately 90% of those originally included. All children admitted due to bronchiolitis were consecutively considered for inclusion during a given time period. They were *not* selected according to specific risk factors and are therefore considered to be representative for an unselected group of children hospitalized for bronchiolitis. Children hospitalized at the Paediatric Department at Stavanger University Hospital represented the majority (79%) of the post-bronchiolitis group. All children living in South-Rogaland will be admitted to this department, and the children were therefor recruited from both urban and rural areas.

**The control group**

The control group was recruited from three schools in Stavanger. We invited 190 children and 141 (74%) responded positively.

A control group should be similar to the exposed group in all but the lack of exposure (279). From the medical records we know that the children in the control group had no previous hospitalization for bronchiolitis at the University Hospital in Stavanger.

The children were age-matched to the post-bronchiolitis group by being born in the same year as children in the bronchiolitis group. A more optimal matching of the control group could have been done by including children with the same gender born on the same date as children in the study group. The subjects in the control group were slightly older than those in the post-bronchiolitis group at follow-up (12.0 vs. 11.3 median years). This age difference should not influence the predicted values regarding lung function. It could theoretically influence the prevalence of atopy and asthma, but is not likely to influence the overall statistical conclusions regarding asthma and atopy.

As for most control groups, selection bias among those who consented to participate cannot be excluded. It is therefore of particular interest to assess whether or

not the prevalence of asthma and atopy in the control group was similar to the prevalence of asthma and atopy in the general population. The control group was recruited from an urban area. Environmental exposures for pollutants will be different for those living in an urban than those living in a rural area. This could influence the prevalence of asthma and possibly also the risk of atopic sensitization (280). Higher prevalence of atopy has been observed in children living in urban than rural areas (120), although a Swedish study showed that the protective effect of rural areas may disappear with increasing age (281). The prevalence of atopy defined as at least one positive SPT, was 37.8% in the control group. This is neither very different from the 29.3% prevalence of atopy reported among 11 year old children in the ECA study from Oslo (74), nor from the 34 % prevalence of atopy reported among 12 year old children from another study in South-Rogaland (Kristine Byberg, personal communication). The prevalence of current asthma in the control group was 9.2%. This is also comparable to the prevalence of current asthma in the ECA study which was 11.1%, although one must be aware of methodological differences between all these studies.

### 6.8.2 Laboratory tests

**Analysis of virus**

RSV was analysed by direct immunofluorescence (bioMèrieux, Marcy-l'Ètoile, France). This is an antigen based test. The test was performed by trained staff at the microbiological laboratories at Stavanger University Hospital and Haukeland University Hospital. The sensitivity and specificity of direct immunofluorescence for RSV detection is high in both in children and adults, but highly dependent on the quality of the specimen (282). The sensitivity of immunofluorescence for detection of RSV is lower than by using reverse polymerase chain reaction (PCR) and was in one study approximately 70% compared to PCR (283).

For RV there are few available antigen tests, and the diagnostic work-up requires polymerase chain reactions (240), which in 1997 were not available at the hospital laboratories in Stavanger and Bergen.

During recent years, antigen tests also for RSV have been replaced by highly sensitive and specific nucleic acid amplification assays that provide more rapid results. Of these, PCR was the first and is still the most frequently used nucleic acid based assay (282). New PCR techniques recently developed, are able to identify a variety of different respiratory viruses simultaneously, and also allow quantification of viral nucleic acids present in a sample (282). Higher viral load seems to correspond to a more severe disease, and the quantification may help to select the virus that actually cause the infection in samples where multiple viruses are detected (282). As already mentioned in chapter 2.1.3, the role of co-infections are not settled, and when using highly sensitive tests the results must be interpreted with caution and always in the context of the clinical illness (284).

**Inflammatory markers**

At the time of inclusion, U-$PGF_2$, U-$LTE_4$, U-EPX and blood eosinophils were regarded and validated as stable markers of eosinophil and mast cell activity (143, 150, 152, 285-287). Recently, more complex methods of measuring airway inflammation using exhaled breath condensate, induced sputum, bronchoalveolar lavage and bronchial biopsy are available providing a more direct assessment of inflammatory cells, chemokines and cytokines (288). However, blood eosinophils and urinary markers particularly U-$LTE_4$, are still regarded as adequate markers of airway inflammation that are easy to perform also in children (109, 127, 289).

On the other hand, AA metabolites are present in extremely small amounts in biological fluids and mass spectrometry has been recommended as the method of measurements (109) .There are reports suggesting that enzyme-linked immune assay may overestimate U-$LTE_4$ measurements (289). Using mass spectrometry could therefore both have improved the reliability and internal validity of the tests. Reliability means that the test is performed precise (i.e. reproducible or repeatable), whereas internal validity means that the test is performed with accuracy.

It is also important to underline that measurement in urine only indirectly reflect the source of the inflammatory marker.

### 6.8.3 Lung function measurements

A recent paper from Quanjer et al., calculated the prediction bias for reference equations from different authors by comparing the values predicted from these equations to a large set of raw-data from non-smoking healthy boys and girls aged 6-19 years (290). The results showed that the values predicted for $FEV_1$ and FVC using the reference equations from Quanjer (216) were slightly lower than the values actually measured in this large dataset, while the reference equations from Wang (217) seemed to predict higher values than those observed in this dataset. The reference values from GLI 2012 were best fitted to the data-set (290). This means that the values predicted for $FEV_1$ and FVC in this thesis would be higher and the values for $FEF_{25-75}$% lower if the GLI 2012 equation set had been used. This was also what we found when comparing the predicted values for lung function variables in this thesis from Quanjer and Wang to the predicted values from the GLI 2012 (data not shown). However, this has only minor influence on the results, as shown in section 5.2.1 and 5.3.1.

### 6.8.4 The definition of bronchiolitis

The definition of bronchiolitis used in this thesis, is in accordance with the guidelines from the American Academy of Pediatrics (15) and guidelines from Europe (16), except for the upper age limit at inclusion. We chose to include children only below 12 months of age, in order to avoid including children with higher risk of asthma (14, 172), as already discussed in section 2.1.1 and 2.5.1. Previous respiratory symptoms not requiring hospitalization were not exclusion criteria. At inclusion, the diagnosis of bronchiolitis was re-evaluated by a paediatrician, to ensure that the diagnosis was consistent with the definition.

### 6.8.5 Exposure to tobacco smoking

Exposure to tobacco smoke in utero and during childhood is a major risk factor for development of asthma during childhood (291). In the present study, exposure to smoking was only monitored by the questionnaire at the first follow-up. This may be a limitation, and a more detailed questionnaire also including questions about the smoking habits in the family at the second follow-up could have been of value.

However, exposure to tobacco smoking during the first two years was not associated with asthma (Paper IV). Repeated measurements of cotinine in urine could have increased the validity of the assessment of passive smoking (291).

### 6.8.6 The validity of the asthma diagnosis

The definition of asthma in the thesis was based on clinical criteria by analysing answers to the ISAAC questionnaire (Appendix II). This questionnaire is highly validated (292) and used in several international studies both in Norway (293), Sweden (294) and the UK (295).

The main limitation of the questionnaire may be the use of the word "wheeze" which may be difficult to translate to non-English speaking countries and may lead to false positive responses (296). Also in English speaking countries, lack of association between parental reported wheeze and doctor confirmed wheeze has been described, and the parents perception of the word "wheeze" is variable (297). It is therefore important to be aware of the risk of overestimation and underestimation of the true prevalence of wheeze by use of questionnaires (298). In the definition of current asthma, we have combined a positive answer to "asthma ever" with either [1)] wheezing, whistling or chest tightness during the last 12 months or [2)] the use of asthma medication during the last 12 months. By including answers to more than one question in the definition, the risk of errors should be diminished.

It is challenging for parents of 11 year old children to recall a period of nine years for the various items included in this questionnaire. More objective test methods such as home monitoring of peak flow variability or interim-assessments throughout this nine year period could have been included. However, objective recordings over weeks or multiple assessments are time consuming and challenging for families to perform, and could have reduced the attendance rate at the second follow-up.

According to the recently published international consensus on paediatric asthma, the diagnosis of asthma in children is based on recurrent episodes of wheezing triggered by various stimuli such as irritants, viral respiratory infections and exercise (299). The symptoms include episodes of cough, wheeze and chest tightness particularly during night and early morning. As normal lung function tests do not

exclude asthma, reversibility tests with bronchodilator, measurements of BHR or peak expiratory flow are not required, but may be used to support the diagnosis (299). Based on this, objective measurements of lung function or BHR were not included in the definition of asthma in the present study. Assessment of BHR by MPT has low specificity for asthma further complicating its use (300). Studies have shown similar prevalence rates of current asthma by using questionnaires alone as when combined with BHR (249). Other studies have included a positive exercise test in the definition of asthma (74), but this test is also resource intensive and has a low sensitivity of asthma (301).

This discussion underlines the principle of "*not letting the perfect become the enemy of the good*". The level of testing and the number of repeated contact points between the researchers and the participants of clinical studies, represent a delicate balance between what is optimal from the perspective of the research questions and what is feasible and/or ethical from the perspective of the participants.

### 6.8.7 Statistical analyses

**Power**

Calculating the power of a study is important in order to determine the minimum sample size necessary to recognize given group differences or effects as statistically significant. Power is dependent on the chosen cut off for statistical significance, the sample size, the effect size and the variation of the data of the effect size (302). An underpowered study means that there is an increased risk of type II error (false negative results), and that negative results must be interpreted with caution. On the other hand, including too many participants is expensive and may be unethical, because this will include a higher number of participants than strictly necessary (303).

By design, this was a follow-up study with a given number of participants, and the sample size of children in the post-bronchiolitis group could not be changed at the 11-year follow-up. However, we could have increased the power regarding some of the research questions by recruiting more children in the control group.

Power analyses should be done a priori; before the data are collected. Post hoc power analyses using the actually observed data are not recommended as the p-value and power are dependent upon the observed effect size and as the p-value and power are exactly and inversely related. Therefore, calculating power using the observed effect size and variance is simply a way of re-stating the statistical significance of the test (304, 305).

However, if we had performed a power analysis before study start, and included 100 children in the post-bronchiolitis group and 100 children in the control group, the results would have shown that the study had sufficient power to answer the first main hypothesis of the protocol. From previous studies one could predict with reasonable certainty that the prevalence of current asthma in the post-bronchiolitis group would be approximately 25 % (8) and that the prevalence of current asthma in an unselected control group would be approximately 10 % (74). Thus, the study should ideally at least be powered to detect an absolute effect estimate of 15%. By including 100 children in the post-bronchiolitis and control groups, the power of the study would have been 80% power to detect a 15 % absolute difference in the prevalence of asthma. The power would have exceeded 90 % to detect a 5 % difference in $FEV_1$. Furthermore, the power would have been approximately 80 % to detect a 20 % absolute difference in atopic sensitization between the post-bronchiolitis and control groups. An absolute difference of 20 % in atopic sensitization in children with asthma compared to children with no asthma in early adolescence was found in the ECA study from Oslo (74) and similarly between children hospitalized for bronchiolitis and a control group in the study from Sigurs et al. (170). However, for this variable also a lower difference would be of clinical interest.

For the second main hypothesis, this study has only sufficient power to detect large differences between the groups. However, in order to have a major impact on the outcome of asthma or not at the age of 11 years, the differences in inflammation at the time of bronchiolitis should have been substantial. Further, the inflammatory markers were not normally distributed. Power analyses of non-parametric tests are difficult to perform. Further, as discussed in section 2.4., there are wide variations in the results of

prostaglandins and leukotrienes in urine, and it may therefore be difficult to estimate a clinical important effect size of the inflammatory markers

Lower numbers of participants are included in the analyses of subgroups, reducing the statistical power for these analyses. Thus, larger effect sizes are required in order to be recognized as statistically significant. In this study, this applies to the analyses of gender, RSV positive/negative bronchiolitis, and asthma subgroups. This is illustrated by the analyses of asthma prevalence in children with RSV positive and RSV negative bronchiolitis as discussed in 6.4 (Paper I). There was a significant difference in the occurrence of asthma in children with RSV negative bronchiolitis compared to controls because of a large effect size (35% vs. 9%), although the number of children with RSV negative bronchiolitis was small (31 subjects or 26% of the bronchiolitis group). The relatively large difference of asthma prevalence between children with RSV positive bronchiolitis compared to controls (16% vs. 9%) was not statistically significant, probably due to low sample size.

Considering the effect size and the width of the CIs may be helpful in the interpretation of negative results (304). A CI including values that are clinically important, may suggest that the findings are inconclusive and that clinical important effects cannot be ruled out, despite p-values exceeding 0.05. Alternatively, the CIs may be narrow and close to zero, and exclude values that may be of clinical importance. In such cases, the evidence from the data is conclusive as the upper and lower confidence limits do not cross any values that could be regarded as clinically and practically important, even though it could be statistically significant (304). We found no association between current asthma, atopy and BHR (Table 5, Paper I). However, the regression coefficients and wide CIs in both of these analyses include values that may be of clinical interest. The wide CIs underline the uncertainty in the statistical estimate, and make it difficult to conclude that this may be due to no effect although the p-values are above 0.05. For the other negative results, these particular findings have been discussed under the various subheadings in the first part of the discussion section (6.1-6.7).

A small sample size requires large differences between the groups in order to yield a statistically significant result as shown in the power analyses above. However,

for clinical variables such as those included in this thesis, one may argue that minor group differences may not be of clinical relevance in order to predict the risk of for instance asthma in individual subjects. Further, a priori, it may be difficult to estimate the minimal clinical important difference as illustrated in section 6.4. Overall, a larger study sample could have answered the research questions included in this thesis more exact, reduced the variance of the results and improved the reliability and validity.

However, the issue of sample selection and sample size is complex and involves several difficult and ethical aspects (303, 306, 307).

**Multiple testing**

Multiple testing may be a problem when several statistical analyses with various explanatory variables occur simultaneously. On the other hand, correction for multiple testing increases the risk of type II errors (false negative results).

In order to answer the various research questions, the statistical analyses performed in this thesis included a number of targeted and different explanatory variables. Apart from the analyses performed in Paper III, the results in this thesis were therefore not corrected for multiple testing.

It could be argued that the analyses including the four inflammatory markers in Paper II involve simultaneous testing of several null hypotheses i.e.; the inflammatory response measured by different inflammatory markers during bronchiolitis are not related to asthma, lung function and BHR at 11 years of age. However, only the association between the blood eosinophil counts and $FEV_1$% (Table 4, Paper II) and not the association between blood eosinophil counts and ln DRS would change by Bonferroni correction for multiple testing (Table 3, Paper II). On the other hand, as discussed in the introduction, the analyses of the inflammatory markers included targeted variables which in various ways reflect the inflammatory cells present in children with obstructive airways disease.

## 6.9 Clinical implications

The major clinical implications of this thesis are that children hospitalized for bronchiolitis in infancy have higher risk of asthma, reduced lung function and higher

BHR than children without such history. These changes may be modulated by RSV status, meaning that clinicians should pay particular attention to children with a previous history of RSV negative bronchiolitis.

Prediction of children at risk of developing asthma after bronchiolitis is difficult to perform by the age of two years. Assessments of risk factors at this age are best suited to predict the absence of asthma.

The role of exhaled nitric oxide as a marker of eosinophilic airway inflammation has not been finally settled. The association between FeNO and atopy, but not towards asthma is important to consider when using this test in a clinical practice.

## 7. FUTURE PERSPECTIVE

There is still a debate whether the long-term outcomes after bronchiolitis in infancy are related to premorbid factors or to the insult induced by the early viral infection. The present thesis was not designed to answer these important questions. These are issues that must be addressed in future studies, as the answers may have implications for preventive interventions.

Long-term follow-up studies of birth cohorts controlling for immunological and other potential risk factors present from birth are on-going, also including pre-morbid assessment of lung function. However, there are few such large prospective follow-up studies in children *hospitalized* for bronchiolitis.

There is a need for large prospective and preferably population based follow-up studies including children at birth, before they develop respiratory symptoms and before they achieve their first viral infection. Studies should include serial measurements of relevant premorbid features, such as lung function, immunological, inflammatory and genetic/epigenetic factors from birth. It is also essential to investigate the role of maternal, antenatal and postnatal environmental factors. Both children and parents deserve that we dedicate our time and effort to this research.

# 8. CONCLUSION

Based on the results from the studies included in this thesis and by considering the methodological limitations, the following answers can be given to the specific research questions that were raised:

1. Children hospitalized with bronchiolitis during their first year of life have higher prevalence of asthma, lower lung function and higher BHR at 11 years of age than children without previous hospitalization for bronchiolitis.

   There was no difference in the prevalence of atopy between children in the post-bronchiolitis group and children in the control group, and the risk of asthma was not related to atopy.

2. The prevalence of asthma was higher after RSV negative compared to RSV positive bronchiolitis and controls. We did not find a higher prevalence of asthma in children with previous hospitalization for RSV positive bronchiolitis compared to controls, although whether this was a true negative finding could not be answered.

   The obstructive lung pattern in children with bronchiolitis compared to controls was found only in children with RSV negative bronchiolitis.

   Stratified for gender, higher prevalence of asthma and higher BHR in children hospitalized with bronchiolitis in infancy than in controls was only found in boys. However, there was no difference in the prevalence of asthma between boys and girls.

3. Blood eosinophil counts during bronchiolitis were higher in children with asthma than children without asthma at 11 years of age and negatively associated with lung function and positively associated with BHR at 11years of age.

4. FeNO at 11 years of age was not different in children with a history of bronchiolitis in infancy and children without previous hospitalization for bronchiolitis. FeNO was associated with atopy but not asthma, both in children hospitalized for bronchiolitis and in the control group. FeNO does not seem to be an exclusive marker of asthma.

5. Clinical parameters at two years of age after bronchiolitis in infancy were modest predictors of asthma at 11 years of age, although comparable to more complex models including invasive tests. However, early life risk factors for later asthma did predict the absence of asthma reasonably well, with low negative post-test probabilities.

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# 10. ERRATA

**Paper I**

Abstract

- At follow-up, current asthma was more common after RSV negative bronchiolitis compared to controls (35.5% vs. 9.2%; p< 0.001); should be:

  At follow-up, current asthma was more common after RSV negative bronchiolitis compared to controls (35.5% vs. 9.2%; *p = 0.001*)

Table 2

- Age at second follow up–controls; should be *12.0* (10.5, 12.8)

Statistical methods

- Using log DRS as outcome variable; should be:

  Using *ln* DRS as outcome variable
- Table 5

  Log dose-response slope; should be:

  *ln* dose response slope
- Metacholine; should be

  methacholine

**Paper II**

Results

- Parental atopy and parental asthma were highly associated (OR16.1, CI: 3.4, 76.8; p<0.01); should be:

  Parental atopy and parental asthma were highly associated (OR16.1, *95%* CI:3.4, 76.8*; p<0.001*).

*After submission to the evaluation committee, the following errors have been found:*

**I.**

The following references have been updated due to missing data.

26. Birkhaug IM, Inchley CS, Aamodt G, Anestad G, Nystad W, Nakstad B. Infectious Burden of Respiratory Syncytial Virus in Relation to Time of Birth Modifies the Risk of Lower Respiratory Tract Infection in Infancy: The Norwegian Mother and Child Cohort. Pediatr Infect Dis J 2013; 32: 235-41.
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**II:**

Demographic table in the thesis (Table I, page 56 )

Age at first follow-up should be changed from 20 (19-23 ) (median, range) to 25 (22-31) (mean, range).

# 11. APPENDIX

# Appendix I

## Questionnaires from the first follow-up at two years of age

**SPØRRESKJEMA TIL FORELDRE**
**ETTERUNDERSØKELSE AV BARN MED BRONKIOLITT**

Undersøkelses nummer:____

Allergi eller astma hos far:_____ (ja/nei) Hvis ja beskriv:_____

Allergi eller astma hos mor: ____(ja/nei) Hvis ja beskriv:_____

Allergi eller astma hos søsken: ___(ja/nei) Hvis ja beskriv:_____

Hadde mor eller far bronkitter, astmatiske bronkitter eller astma de første leveårene?

Ja/nei:___

Hvis ja, hvem, hvor mye? Beskriv:_____

Hvor mange eldre søsken har barnet: _____

Har dere dyr hjemme: _____

Røyker en eller begge av foreldrene inne? ___(ja/nei)

Har barnet etter innleggelse for bronkiolitt:

Vært innlagt på sykehus med lignende symptomer:___(ja/nei)

Hatt flere episoder med tung pipete pust:___(ja/nei) Hvis ja, hvor mange:___

Hvor mange siste år:___

Hatt flere episoder med langvarig hoste: ___(ja/nei) Hvis ja, hvor mange:___

Fått diagnosen bronkitt av lege:___(ja/nei)

Fått diagnosen astma av lege:___(ja/nei)

Har barnet hatt symptomer på atopisk eksem(barneeksem-ikke bleieutslett):___(ja/nei)

Har barnet atopisk eksem nå:___(ja/nei)

Har barnet tatt allergitest:___(ja/nei) I tilfelle ja, hva var resultatet:_____

Har barnet hatt andre symptomer på allergi: _____

_____ _____ _____

Sted Dato Foresattes underskrift

Pasient nummer:_____

# Appendix II

## Questionnaires from the second follow-up at 11 years of age

## Spørreskjema BRIO studien

**1. Har du <u>noen gang</u> hatt tung pust eller piping/surkling/tetthet i brystet?**

☐ ja
☐ nei
Hvis du har svart nei, gå til spørsmål 31

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**2. Har du hatt tung pust eller piping/surkling/tetthet i brystet i løpet av <u>de siste 12 måneder ?</u>**

☐ ja
☐ nei
Hvis du har svart nei, gå til spørsmål 31

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**3. Hvor mange anfall av tung pust eller piping/surkling/tetthet i brystet har du hatt i løpet av <u>de siste 12 måneder</u>?**

☐ ingen
☐ 1 til 3
☐ 4 til 12
☐ mer enn 12

**4. Hvor ofte har din søvn i gjennomsnitt blitt forstyrret på grunn av tung pust eller piping/surkling/tetthet i brystet <u>de siste 12 måneder</u> ?**

☐ aldri våknet
☐ mindre enn 1 natt pr. uke
☐ 1 eller flere netter pr. uke

**5. Har piping/surkling/tetthet i brystet eller tung pust vært så alvorlig <u>de siste 12 måneder</u> at du har hatt problemer med å snakke slik at du bare kunne si ett eller to ord mellom hvert pust?**

☐ ja
☐ nei

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**6. Har du <u>noen gang</u> hatt astma?**

☐ ja
☐ nei

**7. Har du i løpet av <u>de siste 12 måneder</u> hatt tung pust eller piping/surkling/tetthet i brystet under eller etter fysisk trening, aktiv lek eller mosjonering?**

☐ ja
☐ nei

**8. Har du i løpet av de siste 12 måneder hatt tørr hoste om natten, utenom hoste i forbindelse med en forkjølelse eller andre luftveisinfeksjoner?**

☐ ja
☐ nei

**9. Har du i løpet av de siste 12 måneder noen gang brukt noen av disse astma-medisinene?**

☐ Ventoline, Bricanyl, Oxis, Salbutamol, Atrovent
☐ Pulmicort, Flutide, Becotide, Symbicort, Seretide
☐ Singulair
☐ Andre (skriv navnet): _____