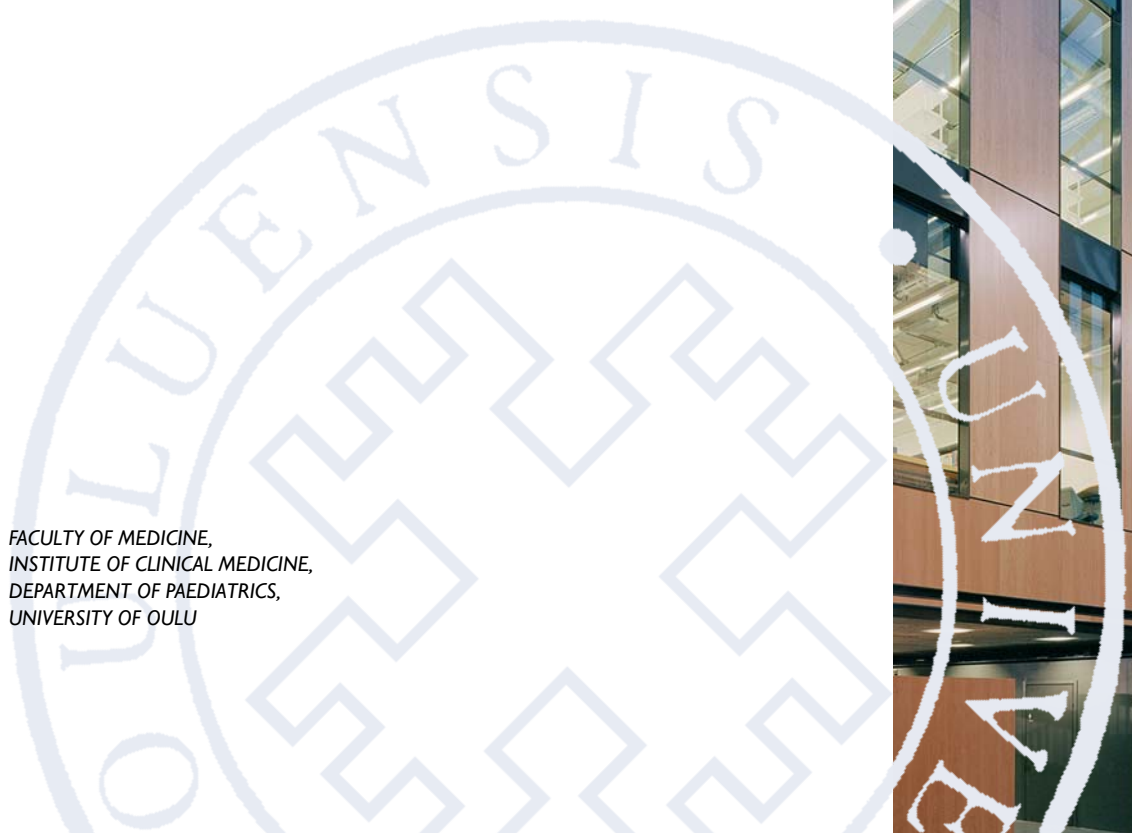


Hanna Juntti

ASSOCIATION OF
RESPIRATORY SYNCYTIAL
VIRUS INFECTION
WITH ASTHMA AND
ATOPIC ALLERGY

FACULTY OF MEDICINE,
INSTITUTE OF CLINICAL MEDICINE,
DEPARTMENT OF PAEDIATRICS,
UNIVERSITY OF OULU



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HANNA JUNTTI

**ASSOCIATION OF RESPIRATORY
SYNCYTIAL VIRUS INFECTION
WITH ASTHMA AND ATOPIC
ALLERGY**

Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium 12 of the Department of Paediatrics, on June 13th, 2008, at 12 noon.

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Abstract

Respiratory syncytial virus (RSV) infection may be associated with the development of asthma and atopy. The aim of the present study was to investigate this association and the related immunological mechanisms.

Seventy-six children admitted to Oulu University Hospital in 1991–1994 for an RSV infection at an age of less than 12 months and healthy controls were called for a visit at the age of 6–10 years. Twenty subjects (26%) had asthma compared with 12 controls (16%) (difference 11%, 95% confidence interval (CI) –3% to 24%). Asthma had been diagnosed significantly earlier in the subjects. Eight per cent of the subjects had at least one positive skin prick test as compared with 43% of the controls (difference –35%, 95% CI –50% to –19%). Serum concentrations of interferon- γ and soluble intercellular adhesion molecule-1 were significantly higher among the subjects than among the controls and among the subjects with asthma or current wheezing than among the corresponding controls.

All children born in Finland in 1986–1995 were arranged in birth cohorts by month and year of birth and grouped by exposure to an RSV epidemic at age 0–6 months, resulting in 97 exposed and 23 unexposed cohorts. The proportions of children taking asthma medication or receiving special reimbursement for asthma medication in 1995–2002 were similar in the unexposed and exposed cohorts.

Altogether 47 children born between August and November 2001 with a cord blood sample taken were admitted to hospital ($n = 26$) or seen in an outpatient department ($n = 21$) for RSV infection before the age of six months. Twenty-eight children had some other respiratory viral infection and 84 children formed a group of healthy controls. High scores on a factor combining the cord blood interleukin-6 and interleukin-8 responses (as derived by factor analysis) were shown in logistic regression analysis to predict hospitalization for RSV infection by comparison with the healthy controls (odds ratio 2.29, 95% CI 1.21 to 4.33).

We suggest that RSV does not induce asthma but inborn features of immunity affect the severity of RSV infection and the postinfectious development of asthma.

Keywords: asthma, cord blood, cytokines, factor analysis, immediate hypersensitivity, intercellular adhesion molecule-1, respiratory syncytial virus infections

To my family

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Oulu, May 2008

Hanna Juntti

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. Article III is also included in the dissertation of Teija Dunder, MD.

- I Juntti H, Kokkonen J, Dunder T, Renko M, Niinimäki A & Uhari M (2003) Association of an early respiratory syncytial virus infection and atopic allergy. *Allergy* 58: 878–884
- II Juntti H, Kokkonen J, Dunder T, Renko M, Karttunen R & Uhari M (2005) Serum concentrations of interferon- γ and soluble intercellular adhesion molecule-1 eight years after an early respiratory syncytial virus infection. *Clin Exp Allergy* 35: 59–63
- III Dunder T, Juntti H, Renko M, Kokkonen J, Waris M & Uhari M (2007) Consumption of asthma medication after RS-virus epidemic – A population based survey. *Pediatr Allergy Immunol* 18: 105–109
- IV Juntti H, Österlund P, Kokkonen J, Dunder T, Renko M, Pokka T, Julkunen I & Uhari M (2008) Cytokine responses in cord blood leukocytes predict the severity of later respiratory syncytial virus infection. Manuscript.

In addition, some unpublished data are presented.

Contents

Abstract	
Acknowledgements	7
List of original articles	11
Contents	13
1 Introduction	17
2 Review of the literature	19
2.1 Aspects of allergology and immunology	19
2.1.1 Wheezing and asthma in children.....	19
2.1.2 Other atopic manifestations.....	21
2.1.3 Cytokines in innate and adaptive immunity	22
2.1.4 Cytokine profiles in asthma and atopic allergy	25
2.1.5 Summary of cytokine responses in asthma and allergy.....	29
2.1.6 Intercellular adhesion molecule-1 and CD14	30
2.2 Epidemiology and clinical features of RSV infection in infants.....	31
2.3 Immune responses in RSV infection.....	32
2.3.1 Local cytokine responses in infants during RSV infection.....	33
2.3.2 Peripheral blood cytokine responses in infants during RSV infection.....	34
2.3.3 Roles of ICAM-1 and CD14 in RSV infection.....	35
2.4 Association of RSV infection with asthma and atopic allergy.....	35
2.4.1 Evidence from clinical studies.....	35
2.4.2 Evidence from immunological studies	41
3 Aims of the research	43
4 Subjects	45
4.1 Asthma, atopy and cytokine production eight years after hospitalization for RSV infection in infancy (I and II)	45
4.2 Consumption of asthma medication after exposure to an RSV epidemic in infancy (III)	46
4.3 Cord blood cytokines predicting RSV infection (IV)	50
5 Methods	53
5.1 Use of a questionnaire (I and II)	53
5.2 Definitions (I, II and III) of asthma, atopic diseases and asthma medication.....	53
5.3 Lung function tests (I).....	54
5.4 Skin prick tests and blood samples (I and II)	54
	13

5.5	Cytokine assay (IV).....	54
5.6	Statistical analysis	55
5.7	Ethical considerations	56
6	Results	57
6.1	Asthma, atopy and cytokine production eight years after hospitalization for RSV infection in infancy (I and II)	57
6.2	Consumption of asthma medication after exposure to an RSV epidemic in infancy (III)	61
6.3	Cord blood cytokines predicting RSV infection (IV).....	63
7	Discussion	67
7.1	Development of asthma and atopic allergy at the age of 7–9 years after an RSV infection in infancy (I)	67
7.2	Relation of cytokine production after an RSV infection in infancy to the development of asthma and atopic allergy (II).....	68
7.2.1	Cytokine responses in children with previous RSV infection in relation to asthma and atopic allergy	68
7.2.2	ICAM-1 and CD14 as a link between RSV infection and asthma.....	70
7.3	Development of asthma by the age of 16 years as defined by the consumption of asthma medication (III)	71
7.4	Cytokine responses in cord blood, predicting susceptibility to RSV infection during infancy and the severity of the infection (IV).....	72
7.4.1	Relation of the findings to cord blood cytokines as predictors of infections in general and of RSV infection	73
7.4.2	Association of the findings with immune responses measured during RSV infection	73
7.4.3	Relation of the findings to immune responses in asthma	74
7.5	Methodological aspects.....	75
7.5.1	Strengths of the study	75
7.5.2	Shortcomings of the study	77
8	Conclusions	81
8.1	Does RSV infection during infancy have a positive association with the development of asthma and atopy during childhood?.....	81
8.2	Do children with an RSV infection in infancy differ in their immunological parameters after the infection from those who	

remain healthy, and do these differences relate to the development of asthma and atopic allergy?	81
8.3 Does RSV infection affect the occurrence of asthma to such a degree that it could be seen in terms of the consumption of asthma medication at the population level?	82
8.4 Can susceptibility to RSV infection or its severity be predicted from immune responses measured at birth?	82
9 Summary	83
References	85
Original articles	105

1 Introduction

Respiratory syncytial virus (RSV) is one of the most common causes of respiratory infections among infants that lead to hospitalization. There is accumulating, though partly controversial, evidence of an association between a severe RSV infection in infancy and the subsequent development of asthma and allergy (Sims *et al.* 1978, Pullan & Hey 1982, Sigurs *et al.* 1995, Stein *et al.* 1999, Sigurs *et al.* 2000, Kneyber *et al.* 2000, Korppi *et al.* 2004b, Sigurs *et al.* 2005, Perez-Yarza *et al.* 2007). More recently, rhinovirus has also been suggested as having similar or even stronger effects on the appearance of asthma (Kotaniemi-Syrjänen *et al.* 2003b, Lemanske, Jr. *et al.* 2005, Lehtinen *et al.* 2007). The mechanisms behind these sequelae are largely unknown.

Host innate and adaptive immune responses towards a virus may be responsible for a large proportion of the consequences of an infection. It has been suggested that the role of the immune response in the severity of the infection might be more important during infections in which the virus is known to induce only minor tissue destruction than in those caused by more destructive viruses (Gern *et al.* 2002). The inborn capacity to produce cytokines, for example, might affect both the susceptibility to infections and the development of post-infectious diseases. The concept of gene-environment interaction also includes the idea of the capability of infections (or other environmental exposures) during early childhood for altering the type of immune responses that develop. According to the hygiene hypothesis, infections in general induce a Th1-type immune response which downregulates the allergic Th2-type responses and thus provides protection from allergies.

We set out to study a number of aspects of RSV infection in infancy: factors affecting the susceptibility and severity of the infection or the development of asthma and atopic allergy after the infection and related immunological mechanisms.

2 Review of the literature

2.1 Aspects of allergology and immunology

The main task of the immune system is to protect the host from infectious microbes, i.e. to control and eliminate these organisms (Chaplin 2003). These mechanisms are based on detecting structures in the pathogens that distinguish them from host cells. This host-pathogen discrimination is essential to enable the host to eliminate the pathogen without damage to its own tissues.

Allergy is defined as a hypersensitivity reaction initiated by specific immunological mechanisms (Johansson *et al.* 2004). Atopy is a tendency to produce immunoglobulin (Ig) E antibodies in response to exposure to allergens, leading to symptoms of asthma, rhinoconjunctivitis, eczema or food allergy. Most cases of allergic diseases are initiated by IgE antibodies, but similar symptoms can also be evoked by non-allergic mechanisms, which are less well defined and probably include a great variety of mechanisms.

2.1.1 Wheezing and asthma in children

Asthma is defined as a chronic inflammatory disorder of the airway mucosa associated with airway hyperresponsiveness and consequently recurrent episodes of wheezing, breathlessness, chest tightness and coughing (Global Initiative for Asthma (GINA) 2007). The diagnosis of asthma is based on symptoms and the measurement of lung function. In small children lung function tests may be difficult or impossible to perform due to limited co-operation and the diagnosis must mainly be based on symptoms and clinical findings.

About 30% of children have at least one acute episode of wheezing verified by a physician by the age of three years, most of these episodes being related to viral infections (Martinez *et al.* 1995). Some of these children develop asthma, while the majority grow out of the tendency to wheeze. Attempts have been made to find risk factors predicting asthma among these early wheezers, and certain factors characteristic of different types of wheezing have been identified in birth cohort studies (Martinez *et al.* 1995, Martinez 2002, Reed 2006). Transient early wheezing has been found to be associated with maternal smoking, normal serum IgE levels and diminished lung function before any wheezing illness develops. Atopy and high levels of serum IgE but normal lung function during infancy are

typical of children who continue wheezing throughout childhood. Non-atopic children with wheezing as toddlers and at preschool age form a third group. A clinical index for predicting the persistence of asthma among children with early wheezing symptoms has been derived from these findings (Castro-Rodriguez *et al.* 2000). Children with frequent wheezing at less than three years of age and a positive index have an increased risk of being asthmatic at school age. Correspondingly, the severity of school-age asthma has been found to predict the severity during adulthood (Reed 2006).

The prevalence of asthma symptoms among children aged 13–14 years in Finland is 13–20%, while the prevalence of physician-diagnosed asthma at the age of 11–12 years in a Swedish study was 7.7% (Pekkanen *et al.* 1997, Bjerg-Bäcklund *et al.* 2006). The incidence of asthma at 12 years of age was found to have increased from 4.2% in 1973 to 15.4% in 2003 in South Wales (Burr *et al.* 2006). Changes in asthma diagnostics, the availability of new effective treatments and the increase in public awareness of the disease make reliable comparisons of results from different points in time problematic.

The proportion of IgE-mediated asthma differs between populations (Weinmayr *et al.* 2007). The occurrence of atopic wheezing at the age of ten years in the Isle of Wight birth cohort study was 10.9% and that of non-atopic wheezing 9.7%, the median onset of symptoms being 2.0 years in both groups (Kurukulaaratchy *et al.* 2004). In a large sample of U.S. children aged 6 to 16 years the prevalence of doctor-diagnosed atopic asthma was 4.8% and that of non-atopic asthma 1.9% (Kelley *et al.* 2005).

Airway hyperreactivity, allergic sensitization, wheezing during early childhood, allergic rhinitis and dermatitis are independent risk factors for the development of asthma (Wright *et al.* 1994, Bachert *et al.* 2002, Kotaniemi-Syrjänen *et al.* 2003a, Porsbjerg *et al.* 2006, Bjerg-Bäcklund *et al.* 2006). Several other risk factors have also been suggested, namely asthmatic heredity, passive smoke exposure and recurrent respiratory infections (Nafstad *et al.* 2005, Arshad *et al.* 2005, Ramsey *et al.* 2007). The effect of passive smoking in infancy seems to be mediated via increased bronchial hyperreactivity and is related to the subjects' own smoking later in life (Goksör *et al.* 2006). Day care attendance during early life has been associated with either an increased or decreased risk of asthma depending on whether there is a maternal history of asthma (Celedon *et al.* 2003). Hygiene intervention in day care centres had no effect on the development of asthma and atopic diseases in a survey with a 12-year-follow-up despite a reduction in respiratory infections (Dunder *et al.* 2007). Breastfeeding

has been found to prevent asthma in children with a positive family history of atopy, while no such effect, or even a promoting effect, has been found among those with a negative history or in populations with a low occurrence of asthma and atopic allergy (Gdalevich *et al.* 2001, Siltanen *et al.* 2003, Kramer *et al.* 2007). Increased body mass index has been shown to increase the risk of non-atopic asthma (Kelley *et al.* 2005).

2.1.2 Other atopic manifestations

The order of appearance of atopic diseases seems to follow a certain pattern referred to as ‘the atopic march’ (Johansson *et al.* 2004). The most common atopic manifestations during the first years are gastrointestinal and eczema symptoms, often related to food allergens, while asthma and rhinitis develop later along with aeroallergen sensitization. Atopic eczema is a chronic, relapsing, pruritic, inflammatory skin disease, the nomenclature for which has varied considerably, so that the term ‘atopic’ has often been used without any evidence of IgE-mediated disease (Leung 2000, Johansson *et al.* 2004, Bardana, Jr. 2004). Allergic rhinitis is defined as a symptomatic disorder of the nose which is induced by an IgE-mediated inflammation of the nasal membranes after allergen exposure (Bachert *et al.* 2002).

The prevalence of symptoms of allergic rhinitis was from 8 to 15% in the ISAAC (International Study of Asthma and Allergies in Childhood) Phase III study, with a slight worldwide increase during the follow-up of seven years (Björkstén *et al.* 2007). In a study from South Wales the prevalence of allergic rhinitis rose from 16.0% in 1988 to 19.4% in 2003 among children aged 12 years (Burr *et al.* 2006) In the Tucson Children’s Respiratory Study the prevalence of doctor-diagnosed allergic rhinitis at the age of six years was as high as 42% (though only 50% of these children had positive skin prick tests) (Wright *et al.* 1994). The prevalence of flexural eczema in ISAAC Phase II varied from 0.4% in Ghana to 14.2% in Sweden (Flohr *et al.* 2007).

In the Isle of Wight birth cohort study 16.5% of the children were positive in skin prick tests at the ages of four and ten years, 4.3% were positive only at four years, 11.2% only at ten years and 68% were negative at both four and ten years (Kurukulaaratchy *et al.* 2005). Croup and recurrent acute otitis media have been shown to have a negative association with atopic sensitization as defined by positive skin prick tests (Ramsey *et al.* 2007). The effect of breastfeeding seems to be complicated (Mandhane *et al.* 2007).

2.1.3 Cytokines in innate and adaptive immunity

Cytokines are polypeptide mediators generated in and secreted from a variety of cells (DeFranco *et al.* 2007d). Their main function is to regulate immune responses by acting either on neighbouring cells (paracrine action) or on the producing cell itself (autocrine action), but they have systemic functions as well. Interleukins (IL) are cytokines first discovered as signalling molecules produced by and acting on leukocytes, although they can also be secreted by non-immune cells or act on non-haematopoietic cells.

There may be as many as 100 cytokines. Most of them act in a proinflammatory, anti-inflammatory, macrophage activating, B and/or T cell activating or eosinophil and/or mast-cell activating capacity or as inhibitors of virus replication. Many cytokines act on several types of cell, and cells may respond in a similar way to different cytokines. Cytokines work as cascades in complicated networks. Depending on the cytokines produced, the immune response may be cytotoxic, humoral, cell-mediated or allergic (Borish & Steinke 2003). Classification of cytokines relevant to the study is represented in table 1.

Table 1. Classification of cytokines.

Type of immunity	Cytokine
Innate	tumour necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12 ¹
Adaptive	
Th1	TNF- α , IFN- γ , IL-2, IL-10
Th2	TNF- α , IL-2, IL-4, IL-5, IL-10

¹link between innate and adaptive immunity

Innate immunity, involving barrier mechanisms, soluble proteins (including cytokines) and cell surface receptors, constitutes the initial rapid host response to pathogens (Chaplin 2003). The proinflammatory cytokines of innate immunity predominantly produced by monocytes include TNF- α , the interleukins IL-1, IL-6, IL-8, IL-12, IL-15, IL-18 and IL-23, and IFN- γ , primarily produced by natural killer cells (Borish & Steinke 2003, DeFranco *et al.* 2007c). The main functions of these cytokines are to increase vascular permeability and endothelial cell adhesiveness, to recruit immune cells such as neutrophils and monocytes to the site of inflammation and to activate phagocytes and natural killer cells. The

anti-inflammatory cytokines of innate immunity are IL-10 and transforming growth factor (TGF)- β , produced by monocytes (Borish & Steinke 2003).

The second set of immune responses constitutes adaptive immunity, which is based on antigen-specific receptors expressed on the surfaces of T and B lymphocytes (Chaplin 2003). This manifests itself slowly as compared with innate immunity but produces long-living cells that can function rapidly when re-exposed to an antigen. T lymphocytes are responsible for adaptive immunity against intracellular pathogens and are also important in activating B cell responses to extracellular pathogens (DeFranco *et al.* 2007b). Dendritic cells are professional antigen-presenting cells which after uptake of the antigen migrate to lymphoid tissues to interact with T cells. To initiate the adaptive immune response, dendritic cells activate naïve T cells to produce IL-2, which acts in an autocrine and paracrine manner, further promoting the activation of T cells. T cell subset differentiation is influenced both by cytokines released from dendritic cells and by other immune cells. The major classes of T cells are CD4 helper T (Th) cells and CD8 cytotoxic T (Tc) cells, which can be further divided into subsets of Th1, Th2, Th17, T regulatory cells and Tc1, Tc2 and Tc regulatory cells (DeFranco *et al.* 2007a). IL-12 is the main cytokine promoting differentiation to Th1 cells, while the mediators of the initiation of Th2 cell differentiation are poorly understood, though the role of IL-4 has been found to be important. Th1 cells produce IFN- γ , which mediates the activation of phagocytic cells leading to the destruction of microorganisms and also promotes the maturation of dendritic cells and enhances the production of IL-12 (Table 1) (Tournoy *et al.* 2002). Th2 cells produce IL-4, IL-5, IL-9 and IL-25, the major functions of which are to recruit, activate and promote the survival of eosinophils, basophils and mast cells. With the exception of IL-5, they act on B cells leading to the production of IgE. Both Th1 and Th2 cells produce TNF- α , IL-2, IL-13 and IL-10 (Borish & Steinke 2003, DeFranco *et al.* 2007a). IL-13 inhibits the production of proinflammatory cytokines from monocytes and suppresses IL-12 production, thus downregulating Th1 cell differentiation (Williams *et al.* 2000). It has been suggested that the Th1- and Th2-type cytokines are capable to inhibit each others' production. (Maggi *et al.* 1992) However, e.g. in allergic inflammation they may act in similar ways (Borish & Steinke 2003).

Development of cytokine responses during early childhood

The functional capacity of the immune system is reduced at birth compared with later life (Holt *et al.* 2005). There is a modest reduction in the number of circulating dendritic cells, and many of them appear to be relatively immature. T cells from neonates initially have higher proliferation rates in response to *in vitro* stimulation than those of adults, but they are unable to sustain the responses. Furthermore, the capacity for activation through the T cell receptor is reduced.

It has been suggested that the neonate's immune responses may be Th2-skewed due to downregulation of the Th1 responses necessary to protect the foetus from rejection during pregnancy (Lin *et al.* 1993, Koch & Platt 2007). Further studies have shown the responses at the foeto-maternal interface to be more complicated (Chaouat *et al.* 2004). Healthy infants born at term have been shown to have low IL-4 responses both at birth and at the age of three months. The IL-6, IL-8 and TNF- α responses are higher at birth than those of adults, the IL-6 and TNF- α responses decreasing by the age of three months (Dembinski *et al.* 2002, Keski-Nisula *et al.* 2003, Keski-Nisula *et al.* 2004). IFN- γ and IL-10 responses are of the same magnitude in newborns as in their mothers or other adults, with no specific change in IFN- γ responses by the age of three months (Karlsson *et al.* 2002, Keski-Nisula *et al.* 2003). Some authors have suggested that the capacity for producing IL-12 is low at birth and still below that of adults at the ages of 5 and 12 years, while others report levels similar to those in adults (Lee *et al.* 1996, Karlsson *et al.* 2002, Upham *et al.* 2002, Itazawa *et al.* 2003). In a cohort of children with a family history of asthma and atopy, IL-5 responses were low at birth but increased greatly by the age of one year (Neaville *et al.* 2003). Levels of IFN- γ were moderate and those of IL-13 high, both decreasing significantly during the first year of life.

There is great individual variation in both cytokine responses at the time of birth and the development of the responses throughout infancy. Factors thought to affect the production of cytokines at birth include a family history of asthma and atopy, maternal age, smoking during pregnancy, gestational age, birth order, mode of delivery and the duration of labour (Gabrielsson *et al.* 2001, Macaubas *et al.* 2003, Brown *et al.* 2003, Keski-Nisula *et al.* 2003, Dembinski *et al.* 2003, Keski-Nisula *et al.* 2004, Amoudruz *et al.* 2005, Malamitsi-Puchner *et al.* 2005, Ly *et al.* 2006).

Cytokine responses have been shown *in vitro* to vary according to the stimulating bacterial strains. It has been suggested that the type of bacteria

inhabiting the neonate's gastrointestinal mucosa shortly after birth could have a profound effect on the developing immune system (Kalliomäki *et al.* 2001, Karlsson *et al.* 2002, Noverr & Huffnagle 2005). Moreover, infections during infancy and exposure to environmental antigens, e.g. lipopolysaccharide (LPS), have been thought to modify subsequent immune responses (Martinez & Holt 1999).

2.1.4 Cytokine profiles in asthma and atopic allergy

The initial programming of the immunological memory against allergens typically occurs during early childhood (Yabuhara *et al.* 1997). In atopic children and adults in general, a Th2-type cytokine response to allergens predominates (Tang *et al.* 1993, Yabuhara *et al.* 1997, Macaubas *et al.* 2003). IL-4 is the main cytokine involved in the pathogenesis of allergic responses. IL-5 is the primary cytokine involved in the production and activation of eosinophils, the key effector cells of allergic inflammation (Kips 2001, Renauld 2001, Tournoy *et al.* 2002).

Biopsy studies have shown the presence of a complex cytokine network in the asthmatic airways (Kips 2001). IL-4 and IL-5 seem to be sequentially involved in the pathogenesis of airway changes in allergic asthma, and IL-13 has very similar activities to IL-4, but its role is more important during secondary allergen challenge. The role of IL-9 is less clear. Expression of the Th1-type cytokine IL-12 has been shown to be reduced in bronchial biopsy samples from asthmatic patients. It is known to reduce Th2 cell development if administered during primary sensitization and to inhibit eosinophilia and airway responsiveness during secondary exposure by increasing the production of IFN- γ (Kips 2001). IL-10 can downregulate both Th1- and Th2-type responses, but its role in allergic inflammation is unclear. Increased expression of proinflammatory cytokines such as IL-1 β , IL-6 and TNF- α and of chemokines has been found in the lungs of asthmatics (Kips 2001). TNF- α recruits neutrophils, which have been found in the sputum and bronchoalveolar lavage (BAL) fluid and biopsy samples of patients with asthma.

There are more similarities in cytokine production than differences between atopic and non-atopic asthma (Bettiol *et al.* 2000, Renauld 2001). A Th2-type response has been found in both types, though with variation in its magnitude. The expression of IL-4, IL-5 and IL-13 and amount of eosinophils in induced sputum have been shown to be higher in atopic than non-atopic asthmatics, while IFN- γ expression was higher in non-atopic asthmatics and showed a positive

correlation with the severity of asthma (Truyen *et al.* 2006). IL-4-responses in peripheral blood were highest among atopic asthmatics, while both high and low IFN- γ responses have been reported compared with non-atopic asthmatics (Tang *et al.* 1995, Bettiol *et al.* 2000). Children with atopic asthma have been shown to have elevated responses of IL-5 and IL-13 in stimulated peripheral blood mononuclear cells compared with non-atopic children with bronchial hyperresponsiveness. Elevated levels of IL-10, TNF- α and IFN- γ have been found in the non-atopic children (Heaton *et al.* 2005). Overproduction of IL-6 is common in both atopic and non-atopic asthma, a fact which has been related to downregulation of the T regulatory cells (Bettiol *et al.* 2000, Seroogy & Gern 2005). IL-8 has been found in excess in the bronchoalveolar lavage fluid of patients with atopic asthma (Folkard *et al.* 1997). Patients with neutrophilic asthma have been shown to have higher expression of IL-8 than those with eosinophilic asthma (Simpson *et al.* 2007). A predominance of either eosinophils or neutrophils in the lungs of patients with asthma seems to be independent of atopy, though one study suggested an association of neutrophilic asthma with a non-atopic phenotype (Bettiol *et al.* 2000, Renauld 2001, Karakoc *et al.* 2002, Green *et al.* 2002, Simpson *et al.* 2007).

Increased numbers of Th2-type cells have been found in the skin lesions of patients with acute atopic eczema, whereas chronic inflammation is associated with increased local IL-5, IL-12 and IFN- γ expression (Leung 2000). Children aged 6–18 months with atopic dermatitis had reduced production of IFN- γ , TNF- α and IL-10 in PBMC, with the exception of house dust mite stimulation, which caused enhanced production of these cytokines and of IL-13 and IL-5 (Dunstan *et al.* 2005). An exaggerated Th2-type response to allergens in PBMC has been found in allergic rhinitis, while IFN- γ expression has either been decreased, increased or comparable to that of healthy controls (Imada *et al.* 1995, Laaksonen *et al.* 2003, Sun *et al.* 2007).

Cytokine responses in infancy as predictors of asthma and wheezing

Low IL-13 and IFN- γ responses at birth or at the age of three months have been shown to predict wheezing, with the least changes in responses occurring among those with wheezing infections by one year of age (Guerra *et al.* 2004, Gern *et al.* 2006). Children with a detectable amount of IFN- γ or IL-4 in their cord blood serum have been shown to run a lower risk of asthma at the age of six years (Macaubas *et al.* 2003). IFN- γ production at age nine months has been found to

be inversely related to wheezing either before or before and after the age of six years (Stern *et al.* 2007).

Cord blood cytokines as predictors of atopic allergy

Children with a positive family history of asthma and atopy have been shown to have lower IL-12, IL-13 and IFN- γ responses at birth and a slower development of IFN- γ responses than children with a negative history (Holt *et al.* 1992, Tang *et al.* 1994, Liao *et al.* 1996, Prescott *et al.* 1998, Gabrielsson *et al.* 2001). Considerable variation in cord blood cytokine responses as predictors of atopic allergy has been found in different studies (Table 2). For IL-4, decreased responses have been found to be associated with sensitization or clinical atopic disease (Prescott *et al.* 1999, Macaubas *et al.* 2003, Nilsson *et al.* 2004). IL-5 responses have been shown to be similar in children with and without later atopic diseases or sensitization (Prescott *et al.* 1999, Neville *et al.* 2003, Prescott *et al.* 2003a, Rowe *et al.* 2004). For IL-6, both high and low responses have been associated with an atopic predisposition (Liao *et al.* 1996, Prescott *et al.* 1999). Low IL-6 responses were found to be associated with a positive family history combined with clinical atopic disease at the age of two years, but no longer at the age of six (Prescott *et al.* 1999, Prescott *et al.* 2003a). The same was true of IL-10 responses in two studies, while in others no association was seen between IL-10 responses and atopic diseases or sensitization either in infancy or at pre-school age (Prescott *et al.* 1999, Neville *et al.* 2003, Macaubas *et al.* 2003, Lange *et al.* 2003, Prescott *et al.* 2003a, Rowe *et al.* 2004). Low IL-12 responses were found to predict sensitization at the age of two years but not at the age of six years (Macaubas *et al.* 2003, Prescott *et al.* 2003b, Nilsson *et al.* 2004). The responses of IL-13 show great diversity and no conclusion can be reached regarding the role of an early capacity to produce IL-13 in the subsequent development of atopic diseases (Prescott *et al.* 1999, Williams *et al.* 2000, Neville *et al.* 2003, Macaubas *et al.* 2003, Lange *et al.* 2003, Prescott *et al.* 2003a, Rowe *et al.* 2004). Low IFN- γ responses at birth or after the age of six months seem to be predictive of atopic diseases, though this has not been confirmed in all studies (Prescott *et al.* 1999, Neville *et al.* 2003, Macaubas *et al.* 2003, Lange *et al.* 2003, Prescott *et al.* 2003a, Rowe *et al.* 2004, Nilsson *et al.* 2004, Rowe *et al.* 2007).

Table 2. Cord blood cytokine responses as predictors of atopic diseases and positive skin prick tests (only statistically significant results presented).

Author (Year of publication)	Number of patients	Family history of asthma and atopy	Site of sample collection	Duration of follow-up	Cytokine measured	Association of cytokine responses to atopy
Nilsson <i>et al.</i> (2004)	82	+ / -	CBMC	2 years	IL-12 IFN- γ	Lower among children with atopic sensitization Lower among children with atopic dermatitis
Macaubas <i>et al.</i> (2003)	407	+ / -	Cord blood serum	6 years	IL-4 IFN- γ TNF- α	Lower among children with atopic sensitization Low concentrations associated with increased risk of positive skin prick tests
Prescott <i>et al.</i> (1999)	31	+ / -	CBMC	2 years	IL-4 IL-6 IL-10 IL-13 IFN- γ	Lower among children with a positive family history and atopic disease than in non-atopic children with a negative history
Rowe <i>et al.</i> (2004)	175	+	CBMC	2 years	IFN- γ IL-13	High responses predictive of positive skin prick tests
Lange <i>et al.</i> (2003)	40	+ / -	CBMC	3 years	IL-13	Higher among children with atopic dermatitis
Williams <i>et al.</i> (2000)	43	+ / -	CBMC	3 years	IL-13	Lower among children with a positive family history and atopic disease than in those with negative family history
Neaville <i>et al.</i> (2003)	285	+	CBMC	1 year	IL-10	Low responses associated with sensitization to egg

2.1.5 Summary of cytokine responses in asthma and allergy

The cord blood cytokine responses and responses in asthma and allergy are summarized in Table 3.

Table 3. Cytokine responses in cord blood and in asthmatic and atopic patients¹

Type of immunity/ Cytokine	Cord blood responses vs. adults	Cord blood responses as predictors for asthma/ wheezing or atopy ²	Cytokine responses in asthma	Cytokine responses in atopy ²
Innate				
IL-6	High	Low responses predictive for atopy ³	Increased in lungs in both atopic and non-atopic	
IL-8	High		Increased in lungs in both atopic and non-atopic, increased in neutrophilic vs. eosinophilic asthma	
IL-12	Low or similar	Low responses predictive for atopy ³	Decreased in lungs	Increased locally in chronic AD ⁴
Th1 IFN- γ	Similar	Low responses or undetectable serum concentrations predictive for asthma or wheezing, both low and high responses predictive for atopy	Decreased in lungs of atopic vs. non-atopic Increased/decreased in PBMC of atopic vs. non-atopic	Increased locally in chronic AD ⁴ Decreased in PBMC in AD ⁴ Incr./decr. in PBMC in AR ⁵
Th2				
IL-4	Low	Undetectable serum concentrations predictive for asthma or wheezing, low responses predictive for atopy	Increased in lungs Higher in atopic vs. non-atopic	Increased locally in acute AD ⁴ Increased in PBMC in AR ⁵
IL-5	Low	Similar responses in infants later becoming atopic or non-atopic	Increased in lungs Higher in atopic vs. non-atopic	Increased locally in acute and chronic AD ⁴

¹Only cytokines relevant for the study presented; for references see the text in chapters 2.1.3. And 2.1.4.

²Atopy defined as sensitization and/or atopic disease

³Up to the age of two years, but not at the age of 6 years

⁴AD = atopic dermatitis

⁵AR = allergic rhinitis

2.1.6 Intercellular adhesion molecule-1 and CD14

The adhesion of inflammatory cells to the endothelial cells of blood veins is one of the main steps in their migration to tissues (Bloemen *et al.* 1993). This adhesion is mediated by endothelial ligands, one of which, intercellular adhesion molecule-1 (ICAM-1), is constitutively expressed in many types of cells and further upregulated in certain situations (Yonekawa & Harlan 2005). It is found in both a membrane-bound and a soluble form. TNF, IL-1, IFN- γ and IL-18 have an important role in promoting the expression of ICAM-1, which may act as a co-stimulatory molecule for T cell activation and as a receptor for rhinoviruses (Bloemen *et al.* 1993, Bella & Rossmann 1999, Lebedeva *et al.* 2005).

Because of its importance in eosinophil and neutrophil adhesion, it has been suggested that soluble ICAM-1 (sICAM-1) may be pivotal for the pathogenesis of airway hyperresponsiveness and asthma (Wegner *et al.* 1990). The concentration of sICAM-1 in the bronchoalveolar lavage fluid and serum of asthmatic children has been shown to be higher than in controls, the levels being correlated with disease severity (Marguet *et al.* 2000, Tang *et al.* 2002). ICAM-1 was found more often in the bronchial biopsies of patients with non-atopic asthma than in controls, but no such difference was observed between patients with atopic asthma and controls (Bentley *et al.* 1993). Serum sICAM-1 levels have shown to be higher among children with atopic dermatitis than in healthy controls (Kojima *et al.* 1994).

CD14 is expressed on the surfaces of macrophages and monocytes and is present as a soluble form (sCD14) as well. It has been shown to act as a receptor for several bacteria, LPS and RSV (Kurt-Jones *et al.* 2000, Karlsson *et al.* 2002, Baldini *et al.* 2002). Interaction between LPS and CD14 enhances IL-12 production from dendritic cells, promoting Th1 cell differentiation. Promoter polymorphism in the CD14 gene has been found to be associated with atopy and IgE production, and also with the serum concentration of soluble CD14 (Baldini *et al.* 1999). Low serum levels of sCD14 at birth were found to be indicative of wheezing during the first year of life, but the levels measured at three months of age had no association with subsequent wheezing (Guerra *et al.* 2004).

2.2 Epidemiology and clinical features of RSV infection in infants

RSV is one of the most common causes of lower respiratory tract infections in children under the age of 12 months and the most common cause of wheezing respiratory infections requiring hospitalization in this age group. The role of other respiratory viruses such as rhinovirus, influenza A, parainfluenza viruses and human metapneumovirus becomes more important with increasing age (Jartti *et al.* 2004, Bosis *et al.* 2005, Nicholson *et al.* 2006, Manoha *et al.* 2007). The picture of agents causing respiratory infections changes rapidly as new methods for detection of viruses are developed and new viruses (like recently bocavirus) are discovered (Allander *et al.* 2007). Furthermore, the epidemiology of respiratory infections varies between countries and different timepoints.

About 70% of children catch their first RSV infection before the age of one year and 1–3% need to be hospitalized (Glezen *et al.* 1986, Ruuskanen & Ogra 1993, Domachowske & Rosenberg 1999, Openshaw & Tregoning 2005). The hospitalization rates associated with influenza, parainfluenza and metapneumovirus are much lower, although a rate similar to RSV-associated hospitalization has been suggested for rhinovirus in one study (Iwane *et al.* 2004, Lemanske, Jr. *et al.* 2005, Miller *et al.* 2007). RSV infection is most common in infants between 2 and 7 months of age, but hospitalization rates are highest in the age range 1 to 4 months (Hoffman *et al.* 2004). In some countries such as Finland, RSV epidemics follow a 2-year cycle, beginning with a minor peak in the spring of an uneven year followed by a major peak during the next winter, while in several other countries the epidemics occur at approximately same time every year (Waris 1991, Duppenhaler *et al.* 2003, Stensballe *et al.* 2003, Alonso *et al.* 2007, Panozzo *et al.* 2007).

Premature birth, congenital heart disease, immunodeficiency and chronic lung diseases are known risk factors for a severe RSV infection (Hall *et al.* 1986, Weisman 2003, Welliver 2003). In children who are otherwise healthy and were born at term the major risk factors for contracting RSV during the first year of life include age less than six months, male sex, exposure to tobacco smoke, a number of siblings and day care attendance (Carlsen *et al.* 1987, Simoes 2003, Nielsen *et al.* 2003, Bradley *et al.* 2005, Stensballe *et al.* 2006). The possible protective role of breastfeeding is controversial (Simoes 2003).

The incubation period for RSV infection is estimated to be 2–8 days (Ruuskanen & Ogra 1993, Domachowske & Rosenberg 1999). At the beginning of the illness the virus replicates in the nasopharynx, and it can spread from cell

to cell by inducing cell fusion and syncytium formation (forming multinucleated giant cells). In the case of a lower respiratory tract infection the virus damages the epithelial cells in the lungs, leading to destruction and loss of ciliary motility. Submucosal oedema, mucus secretion and peribronchiolar mononuclear cell infiltrate formation lead to bronchiolar obstruction (Ruuskanen & Ogra 1993, Openshaw & Tregoning 2005). This results in the trapping of air and hyperinflation or collapse of the distal lung tissue.

Primary RSV infection typically presents as an upper respiratory infection followed by lower respiratory tract symptoms in about 40% of cases (Openshaw & Tregoning 2005). The clinical picture varies according to age, and reinfections in older children are usually less severe (Ruuskanen & Ogra 1993). Common symptoms of the upper respiratory tract infection are rhinorrhoea, nasal congestion and cough, while the lower respiratory tract manifestations include bronchiolitis and pneumonia, the signs of which are tachypnoea, hyperinflation, retractions, crackles and wheezing together with feeding difficulties. These two illnesses are difficult to differentiate without a chest radiograph and may actually represent two stages of the same illness. Acute otitis media is more common than in respiratory infections caused by some other respiratory virus, even after adjustment for age (Uhari *et al.* 1995). RSV infection is rare in the first four weeks of life, but when appearing, apnoea may be the only symptom of the infection, particularly in infants born prematurely (McNamara & Smyth 2002).

2.3 Immune responses in RSV infection

The respiratory epithelial cells are the primary target for RSV at the initiation of the infection. After entering the cell, it upregulates several factors, e.g. the STAT and NF- κ B pathways leading to the production of cytokines and chemokines, which recruit neutrophils, monocytes, T cells and eosinophils (Smith *et al.* 2001). Neutrophils are the main immune cells found in the BAL samples of patients with a severe RSV infection (Hansbro *et al.* 2008). RSV infects alveolar macrophages and monocytes in the airways. Cytokines are produced from a variety of cells, including the infected cells and cells recruited to the site of the infection. Due to their non-specific cytotoxic actions, neutrophils and eosinophils destroy both the RSV-infected cells and healthy bystander cells, thus affecting the severity of the infection. As part of the innate immune response, natural killer cells are also activated (Garofalo & Haeberle 2000, Openshaw & Tregoning 2005, van Drunen Littel-van den Hurk *et al.* 2007). Increased neutrophil and decreased lymphocyte

counts have been found in peripheral blood during RSV infection (O'Donnell & Carrington 2002). In immunocompromised children profound lymphopenia seems to predict lower respiratory tract involvement (El Saleeby *et al.* 2008). The factors that affect the spread of the infection in the lower respiratory tract include the viral load, the RSV subgroup, pre-existing RSV-neutralizing antibodies, mechanical barriers in the airways and host factors affecting the type of immune response (DeVincenzo 2005).

Dendritic cells initiate the adaptive immune response by activating T cells, and substantial numbers of CD8 cytotoxic T cells are recruited in the airways during a severe RSV infection (Heidema *et al.* 2007). The generation of this CD8 T cell response eliminates RSV, but the more widespread the infection is at that time, the greater the immune-mediated lung injury will be (DeVincenzo 2005). In one study, though, in cases of fatal RSV pneumonia there was lack of pulmonary CD8 effector cells. (Welliver *et al.* 2007) CD4 T helper cells have been shown both to inhibit virus replication and to enhance the RSV-induced pathology (Ruuskanen & Ogra 1993, Openshaw & Tregoning 2005, van Drunen Littel-van den Hurk *et al.* 2007). The use of formalin-inactivated RSV vaccine resulted later in a severe natural infection among the vaccine recipients, which was found to be related to an exaggerated Th2-response (Graham 1996, Domachowske & Rosenberg 1999).

2.3.1 Local cytokine responses in infants during RSV infection

RSV infection causes upregulation of the production of several cytokines. In most studies IL-6 and IL-8 expression have been shown to be higher in airway samples of children with severe RSV bronchiolitis than in samples from controls (Sheeran *et al.* 1999, Smyth *et al.* 2002, McNamara *et al.* 2004, Bennett *et al.* 2007). In one study the children hospitalized for an RSV infection did not differ from those treated as outpatients (Laham *et al.* 2004). IL-8 levels in nasal wash samples from children with respiratory infections not requiring hospitalization have been found to be higher than in those from healthy controls regardless of the causative virus. The levels were highest in children with an RSV infection, but they did not correlate with symptom severity scores (Gern *et al.* 2002).

Children with severe RSV bronchiolitis have higher TNF- α and IL-1 β expression in BAL samples and nasal wash samples than controls with non-RSV bronchiolitis or without any respiratory disease. No significant difference was found between children hospitalized for an RSV infection and children treated as

outpatients (McNamara *et al.* 2004, Laham *et al.* 2004, Bennett *et al.* 2007). The levels of IL-10 and IL-12 in nasal wash samples did not differ among children with RSV infection in relation to the need for hospitalization or the severity of the disease among those who were hospitalized (Bont *et al.* 2001, Legg *et al.* 2003, Laham *et al.* 2004).

IL-4 and IFN- γ levels in nasopharyngeal secretions are increased during RSV and non-RSV viral infections and are higher in those with wheezing than in those with an upper respiratory infection alone (van Schaik *et al.* 1999, Kristjansson *et al.* 2005). Influenza A and rhinovirus have been shown to cause significantly higher levels of IL-4 and IFN- γ than either RSV or human metapneumovirus, although one study showed higher IFN- γ levels during RSV bronchiolitis than non-RSV bronchiolitis or in a healthy state (Joshi *et al.* 2003, Bennett *et al.* 2007, Melendi *et al.* 2007, Welliver *et al.* 2007). In children with RSV infection, however, the IFN- γ levels have shown an inverse correlation with the severity of the disease (Bont *et al.* 2001, Legg *et al.* 2003, Semple *et al.* 2007). IL-5 levels were shown to be similar in children with RSV infection and healthy controls, but they were significantly higher in children with influenza A or parainfluenza infection (Kristjansson *et al.* 2005). In one study children with influenza A infection showed higher levels of most cytokines measured (IL-1 β , IL-4, IL-6, IL-12, TNF α and IFN- γ) than those with RSV infection (Welliver *et al.* 2007).

2.3.2 Peripheral blood cytokine responses in infants during RSV infection

Plasma levels of IL-6 and IL-8 measured during RSV infection have been found to be higher in severely ill infants than in those with a milder infection or in healthy controls (Biswas *et al.* 1995, Bont *et al.* 1999, Brandenburg *et al.* 2000). Serum IL-12 and IL-10 levels were significantly increased during RSV bronchiolitis, but whole blood IL-10 responses and plasma levels were found to be comparable to those of healthy controls (Blanco-Quiros *et al.* 1999, Bont *et al.* 2000b, Fernandez *et al.* 2005). IL-12 production correlated inversely with the duration of ventilation during a severe infection and increased levels of IL-2 and IL-13 were found during RSV infection, (Bont *et al.* 2000b, Tripp *et al.* 2002, de Waal *et al.* 2003). Both increased and decreased levels have been found for IFN- γ and IL-4 (Roman *et al.* 1997, Bont *et al.* 1999, Tripp *et al.* 2002, Chen *et al.* 2002, de Waal *et al.* 2003). Studies of IFN- γ responses have showed levels to be similar in children with the most severe RSV infections and in healthy controls, with

increased levels in children with moderate RSV infection (though severe enough to require hospitalization). The highest levels seen during RSV infection still fall behind the levels induced in children with rhinovirus or adenovirus infection (Aberle *et al.* 1999, Aberle *et al.* 2004, Fernandez *et al.* 2005).

2.3.3 Roles of ICAM-1 and CD14 in RSV infection

At the initiation of RSV infection the fusion protein of the virus binds a receptor complex that includes CD14 and Toll-like receptor 4 on the epithelial cell surface upon entering the cell (Harris & Werling 2003). This complex is involved in the innate immune response to RSV (Kurt-Jones *et al.* 2000, Openshaw & Tregoning 2005). A CD14 gene polymorphism related to high sCD14 concentrations has been thought to be associated with RSV bronchiolitis (Inoue *et al.* 2007).

Increased production of ICAM-1 has been shown in airway epithelial cells, peripheral blood neutrophils and serum among infants with an RSV infection (Smyth *et al.* 1997, Wang & Forsyth 2000, Wang *et al.* 2000). RSV can induce ICAM-1 production directly (Wegner *et al.* 1990). Upregulation of ICAM-1 in the airway epithelial cells during an acute paramyxoviral infection has been shown to be associated with inflammation and hyperreactivity in a mouse model (Stark *et al.* 1996, Walter *et al.* 2002).

2.4 Association of RSV infection with asthma and atopic allergy

2.4.1 Evidence from clinical studies

The known clinical studies on the association of RSV infection during early childhood with asthma or wheezing in relation to the situation in healthy children are summarized in Table 4. The exclusion criteria used include major neurological illnesses, congenital heart disease, chronic respiratory diseases, neonatal respiratory distress and in some studies premature birth, and additional exclusion criteria, mainly hospitalization for respiratory symptoms in infancy, have been used for the controls. The definition of wheezing as an outcome has mainly been based on parental reports and has varied from any wheezing during the follow-up to frequent or recurrent wheezing, i.e. at least three episodes of wheezing either during the whole follow-up or in the past year. Asthma has usually been defined in terms of either parentally reported doctor-diagnosed asthma (with ongoing

maintenance medication in some studies) or recurrent wheezing verified by a doctor.

A positive association between RSV infection and asthma or wheezing has been found in most studies (Jartti *et al.* 2005). Children with an early infection have had a 2–9-fold risk of recurrent wheezing as compared with healthy children, while the risk of asthma has been as great as 22-fold at its highest. In several studies, however, either no association has been found or the positive association has been lost during follow-up. In the Tucson Children’s Respiratory Study it was found that the children with previous RSV infection were no more likely to have infrequent or frequent wheezing at the age of 13 than the controls (Stein *et al.* 1999). In a quantitative review of ten controlled studies, recurrent wheezing was seen to have occurred significantly more often among children previously hospitalized for RSV infection than among healthy controls, but the significance was lost after five years of age (Kneyber *et al.* 2000). Also in another review of twelve selected studies (of which three were included in that of Kneyber *et al.*) it was concluded that there was a positive association between RSV infection and asthma (Perez-Yarza *et al.* 2007).

Table 4. Clinical studies of the association of RSV infection with wheezing/asthma employing healthy controls.

Author (Year of publication)	Number of RSV patients/controls followed up	Age at the time of RSV infection	Hospitalized/ outpatients	Age at the end of follow- up	Outcome	Odds Ratio (OR) / Relative Risk (RR) and 95% confidence intervals (CI) or differences in proportions
Sims <i>et al.</i> (1978)	35/35	Infancy	Hospitalized	8 years	Any wheezing	51.4% vs. 2.9%, diff. 0.49 (0.30 to 0.65)
Pullan & Hey (1982)	130/111	< 1 year	Hospitalized	10 years	Wheezing at any age	42.3% vs. 18.9%, diff. 0.23 (0.12 to 0.34)
Mok & Simpson (1982)	100/200	< 1 year	Hospitalized	7 years	Wheezing in last two years	22.3% vs. 12.6%, diff. 0.10 (-0.0 to 0.19)
Murray <i>et al.</i> (1992)	42/73	< 1 year	Hospitalized	5.5 years	Asthma	6.0% vs. 2.5%, diff. 0.04 (-0.01 to 0.10)
Stein <i>et al.</i> (1999)	207/369	< 3 years	Outpatients	6 years	Wheezing in previous year	39.5% vs. 15.1%, diff. 0.24 (0.07 to 0.40)
				8 years	Frequent wheezing	OR 4.3 (2.2 to 8.7)
				8 years	Infrequent wheezing	OR 2.5 (1.5 to 4.3)
				11 years	Frequent wheezing	OR 1.9 (0.9 to 4.2)
				11 years	Infrequent wheezing	OR 1.7 (1.0 to 2.9)
				13 years	Frequent wheezing	OR 2.4 (1.3 to 4.6)
				13 years	Infrequent wheezing	OR 1.4 (0.7 to 2.7)
				13 years	Frequent wheezing	OR 1.4 (0.7 to 2.6)
Schauer <i>et al.</i> (2002)	42/84	< 1 year	Hospitalized	1 year	Recurrent wheezing	OR 8.9 (1.4 to 55.9)
Singleton <i>et al.</i> (2003)	95/113	< 2 years	Hospitalized	5-8 years	Current wheezing	RR 1.2 (0.9 to 1.7)
				5-8 years	Asthma	RR 3.1 (1.6 to 6.1)
Korppi <i>et al.</i> (2004b)	36/45	< 2 years	Hospitalized	18-20 years	Previous asthma	OR 2.1 (0.6 to 7.6)
				18-20 years	Current asthma	OR 1.4 (0.4 to 5.4)

Author (Year of publication)	Number of RSV patients/controls followed up	Age at the time of RSV infection	Hospitalized/ outpatients up	Age at the end of follow- up	Outcome	Odds Ratio (OR) / Relative Risk (RR) and 95% confidence intervals (CI) or differences in proportions
Henderson <i>et al.</i> (2005)	96/9826	< 1 year	Hospitalized	30–42 months	Reported wheezing	OR 2.3 (1.3 to 3.9)
	62/7216			69–81 months	Reported wheezing	OR 3.5 (1.8 to 6.6)
	73/8039			91 months	Asthma	OR 2.5 (1.4 to 4.3)
Sigurs <i>et al.</i> (1995, 2000, 2005)	47/93	< 1 year	Hospitalized	3 years	Recurrent wheezing	RR 1.8 (0.8 to 3.9)
					Asthma	RR 21.8 (2.9 to 163.6)
				7.5 years	Any wheezing	RR 1.9 (1.3 to 2.7)
					Asthma	RR 12.7 (3.4 to 47.1)
Fjaerli <i>et al.</i> (2005)	35/64	< 1 year	Hospitalized	7 years	Any wheezing	RR 5.3 (2.2 to 12.5)
					Current asthma	OR 10.1 (3.4 to 29.8)
					Current asthma or recurrent wheezing	OR 9.3 (3.6 to 24.5)
					Wheezing	48.6% vs. 14.1%, diff. 0.35 (0.16 to 0.52)
Garcia-Garcia <i>et al.</i> (2007)	32/38	< 2 years	Hospitalized	4 years	Current follow-up for asthma by a doctor	60.0% vs. 7.8%, diff. 0.52 (0.34 to 0.68)
					Current asthma medication	34.3% vs. 7.8%, diff. 0.26 (0.11 to 0.44)
					Asthma	OR 10.1 (2.5 to 40.1)
					Recurrent wheezing	75.0% vs. 40.0%, diff. 0.35 (0.10 to 0.56)

Infections caused by other viruses as well as RSV have been found to be associated with asthma (Mok & Simpson 1982, Murray *et al.* 1992, Piippo-Savolainen *et al.* 2007). Hospitalization for wheezing caused by non-RSVviruses, especially rhinovirus, was found to have an even stronger association with the later development of asthma and wheezing than hospitalization for RSV-induced wheezing. (Reijonen *et al.* 2000, Lemanske, Jr. *et al.* 2005, Piippo-Savolainen *et al.* 2007). Among the children with a non-RSV infection, a history of wheezing, atopic dermatitis, skin prick test positivity and age more than 12 months were significant predictors of asthma three years after hospitalization (Reijonen *et al.* 2000). Children requiring hospitalization for wheezing during rhinovirus infection have atopic dermatitis and blood eosinophilia more often than those with RSV infection and they are older (Korppi *et al.* 2004a).

The association of RSV infection with subsequent atopy remains controversial. In many studies the term atopy has been used solely to indicate positive findings of sensitization, e.g. in skin prick tests. Both studies with a positive association and some with a negative one have been published, while in most studies no association has been found (Table 5). Atopy does not seem to play a significant role in the development of asthma after RSV infection, with the exception of the findings reported by Sigurs *et al.* (Sigurs *et al.* 1995, Sigurs *et al.* 2000, Sigurs *et al.* 2005).

Atopic manifestations after RSV infection other than asthma or atopic sensitization have seldom been assessed in clinical studies. In one study an increased occurrence of cumulative and current allergic rhinoconjunctivitis was found at the ages of both 7.5 and 13 years after an early RSV infection, while in another study no such difference was found. No difference has been found in the occurrence of atopic dermatitis (Sigurs *et al.* 2000, Schauer *et al.* 2002, Sigurs *et al.* 2005, Garcia-Garcia *et al.* 2007).

Table 5. Clinical studies of the association of RSV infection with atopic sensitization employing healthy controls.

Author (Year of publication)	Number of RSV patients/ controls followed up	Age at the time of RSV infection	Hospitalized/ outpatients	Age at the end of follow-up	Sensitization defined by	Association of RSV infection with sensitization; OR/RR (95% CI) or difference in proportions
Sims <i>et al.</i> (1978)	32/26	infancy	Hospitalized	8 years	Skin prick tests	20.0% vs. 17.1% difference 0.03 (-0.16 to 0.22)
Pullan & Hey (1982)	130/111	< 1 year	Hospitalized	10 years	Skin prick tests	15.0% vs. 27.5% difference -0.12 (-0.24 to -0.01)
Stein <i>et al.</i> (1999)	207/369	< 3 years	Outpatients	6 years	Skin prick tests	37.4% vs. 39.7% difference -0.02 (-0.12 to 0.07)
Schauer <i>et al.</i> (2002)	42/84	< 1 year	Hospitalized	1 year	Serum IgE antibodies	59.3% vs. 58.9% difference 0.00 (-0.10 to 0.10)
Korppi <i>et al.</i> (2004b)	36/45	< 2 years	Hospitalized	18-20 years	Skin prick tests	OR 20.7 (3.5 to 120.8) 60.0% vs. 47.7 % difference 0.12 (-0.10 to 0.33)
Henderson <i>et al.</i> (2005)	48/6377	< 1 year	Hospitalized	7 years	Skin prick tests	OR 0.7 (0.2 to 1.7)
Garcia-Garcia <i>et al.</i> (2007)	32/38	< 2 years	Hospitalized	4 years	Skin prick tests	25.8% vs. 37.5% difference -0.12 (-0.40 to 0.15)
Sigurs <i>et al.</i> (1995, 2000, 2005)	47/93 47/93	< 1 year	Hospitalized	3 years 7.5 years	Skin prick tests or serum IgE antibodies	RR 3.6 (1.6 to 8.0) RR 2.4 (1.1 to 5.5)
	46/92			13 years	Animal dander	OR 5.6 (2.2 to 14.4)

2.4.2 Evidence from immunological studies

Murine models

The state of immunological maturation at the time of primary RSV infection seems to have an impact on subsequent immune responses. Mice with a primary RSV infection as newborns developed enhanced airway hyperresponsiveness during reinfection or allergen sensitization and challenge as compared with mice infected at weaning (Dakhama *et al.* 2005, You *et al.* 2006). The newborn mice also had increased IL-13 levels in the lung tissue.

The timing of RSV infection relative to allergen sensitization has been shown to be important in determining the type of immune response induced. RSV infection of allergic adult mice during allergen challenge resulted in airway hyperresponsiveness and similar or increased IL-4 and IL-5 levels as compared with those with either RSV infection or sensitization only (Peebles, Jr. *et al.* 2001b, Barends *et al.* 2002, Mäkelä *et al.* 2003, Becnel *et al.* 2005, Liu & Kimura 2007). IL-13 and IFN- γ concentrations in the lungs were reduced. When allergen sensitization and challenge is preceded by RSV infection, both increased and reduced airway hyperresponsiveness and IL-4 and IL-13 levels have been found during subsequent challenge as compared with the levels in those only sensitized (Schwarze *et al.* 1997, Lukacs *et al.* 2001, Peebles, Jr. *et al.* 2001a, Becnel *et al.* 2005, Liu & Kimura 2007). IL-5 levels in the lung tissue have been reduced or similar relative to those in individuals that are only sensitized.

Cytokine responses during RSV infection and subsequent asthma and atopic allergy

Atopic children have lower IFN- γ responses in PBMC during RSV infection than non-atopic children, but no differences are found in IL-4 responses (Kaneko *et al.* 2006). Low IFN- γ responses during bronchiolitis (53% RSV positive) have been shown to be associated with an increased risk of wheezing after the infection and possible or probable asthma at the age of two years (Renzi *et al.* 1997, Renzi *et al.* 1999). High IL-4 production predicted postbronchiolitic wheezing but not asthma. Increased serum levels of IL-12 and IL-10 have been found during bronchiolitis (23/28 patients RSV positive), but they were not associated with recurrent wheezing afterwards (Blanco-Quiros *et al.* 1999). Table 6 summarizes cytokine responses related to RSV infection and subsequent development of asthma and wheezing.

Table 6. Cytokine responses related to RSV infection and subsequent asthma and wheezing¹.

Type of immunity/ Cytokine	Early responses as predictors for RSV infection	Cytokine responses during RSV infection		Responses during RSV infection as predictors for asthma or wheezing	Responses measured after RSV infection as predictors for asthma or wheezing
		Local (nasal wash or nasopharyngeal secretion)	Peripheral blood		
Innate					
IL-6		Increased vs. influenza A	Increased plasma levels in severe infection		
IL-8		Increased vs. other viruses or healthy	infection or healthy		
IL-12	Low cord blood serum concentration	No correlation with severity, decreased vs. influenza A	Serum levels increased, inverse correlation with severity	No association	High convalescent responses predictive for recurrent wheezing at 3 years of age
Th1 IFN- γ	Slow maturation related to susceptibility to infection	Increased vs. healthy, decreased vs. rhinovirus or influenza A, inverse correlation with severity	Increased/decreased, similar in severe and healthy, increased in mild infection, decreased vs. rhino- or adenovirus	Low responses predictive for wheezing	Convalescent responses not predictive
Th2					
IL-4		Increased vs. healthy, decreased vs. rhino or influenza A	Increased/decreased	High responses predictive for wheezing, not asthma	Convalescent responses not predictive, high responses 5 months after bronchiolitis and at 7.5 years predictive for wheezing or asthma
IL-5		Similar than in healthy, decreased vs. parainfluenza or influenza A			

¹ Only cytokines relevant for the study presented

3 Aims of the research

The aims of the research were to find answers to the following questions:

1. Does RSV infection during infancy have a positive association with the development of asthma and atopy during childhood?
2. Do children with an RSV infection in infancy differ in their immunological parameters after the infection from those who remain healthy, and do these differences relate to the development of asthma and atopic allergy?
3. Does RSV infection affect the occurrence of asthma to such a degree that it could be seen in terms of the consumption of asthma medication at the population level?
4. Can susceptibility to RSV infection or its severity be predicted from immune responses measured at birth?

4 Subjects

4.1 Asthma, atopy and cytokine production eight years after hospitalization for RSV infection in infancy (I and II)

The study population comprised 117 children who had been admitted to Oulu University Hospital during the years 1991 to 1994 because of an RSV infection in their first year of life. There were two major and two minor RSV epidemics during that period. The mean age at the time of admission for RSV infection was 4.0 months (range 0.7–11.9). The diagnosis was based on antigen detection performed on nasopharyngeal secretions using either a rapid diagnostic kit (Directigen®, Becton Dickinson, Sparks, MD, USA) or an in-house laboratory method of EIA (Hietala *et al.* 1988). Thirty children were excluded, on the following criteria: premature birth less than 37 gestational weeks ($n = 22$), need for respiratory assistance more than 6 hours during the neonatal period ($n = 2$) or a concomitant disease such as congenital heart disease ($n = 2$), chromosome abnormality ($n = 3$) or severe neurological disease ($n = 1$). Ten children could not be reached and one refused to participate. The resulting 76 subjects who met the inclusion criteria (47 boys, 29 girls) were called for a follow-up visit during the autumn and winter of 2000–2001, when they were 6–10 years old. Fifty-one of these subjects visited the outpatient department, and the parents of the remaining 25 completed an interview questionnaire by phone. The study profile is presented in Figure 1.

Controls matched with the subjects for sex and date of birth were chosen from the National Population Register. Ten controls were excluded on the same criteria as for the subjects and three more because of hospitalization for a respiratory infection during the main RSV seasons in 1991 to 1994. Oulu University Hospital is the only hospital treating children with respiratory infections in the area. If the first family contacted refused to participate, we contacted the second ($n = 12$) or third ($n = 4$) in order. Of those contacted first 78% agreed to participate. They were either invited for a visit or asked to fill in the questionnaire, according to the behaviour of the subjects with whom they were matched. The subjects and controls were considered to have had a similar risk of RSV infection because of their identical date of birth. Due to the duration of the selection process, the controls were two months older than the subjects when they visited the outpatient department.

The subjects had significantly more siblings than the controls (4.11 (range 0-16) vs. 2.79 (0-14), difference 1.32, 95% CI 0.28 to 2.36) and their birth order was significantly higher (3.57 (1-16) vs. 2.72 (1-13), difference 0.84, 95% CI 0.02 to 1.66). A family history of asthma as a whole was more common among them (32/76 vs 15/76, OR 2.99, 95% CI 1.45 to 6.21) There were no other significant differences to be found in the background data. Similarly, there were no significant differences in the background factors between the subjects who visited the hospital and those who did not.

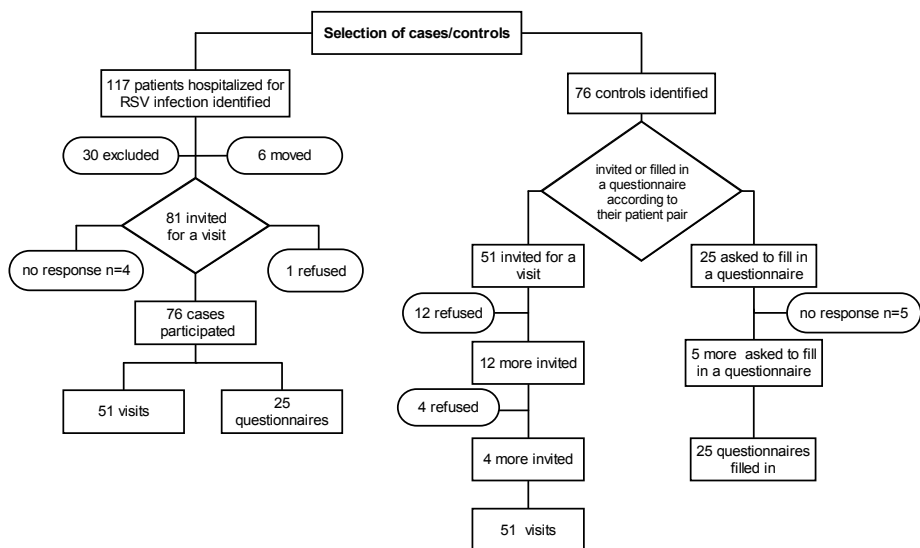


Fig. 1. Study profile depicted the recruitment of the children followed up for eight years after hospitalization for RSV infection in infancy and their matched controls.

4.2 Consumption of asthma medication after exposure to an RSV epidemic in infancy (III)

Altogether 637 922 children were born in Finland in 1986–1995 (data provided by the Population Register at Statistics Finland). These children were sorted into a total of 120 birth cohorts according to the month and year of birth. The cohorts were grouped according to their exposure to an RSV epidemic at age 0–6 months by reference to the known timing of the epidemics, resulting in 97 exposed cohorts and 23 unexposed ones. To control the month of birth in the study

population, we included 23 pairs of cohorts in the final analysis (Figure 2). These 46 cohorts included 126 303 children who had been exposed to RSV and 127 555 who had not been exposed (Figure 3).

Monthly data on RSV epidemics are available from 1979 onwards from the Department of Virology, University of Turku, which supplies laboratory services throughout the country (Waris 1991). Out of a total of 29 998 respiratory tract specimens tested by antigen detection between January 1986 and December 1995, 3 396 specimens from 3 249 patients were positive for RSV (Figure 2).

Data on purchases of asthma medicines and the granting of reimbursements for asthma medication for each individual were obtained from the Social Insurance Institution. Prescription-only asthma medicines were purchased for 133 119 patients older than 3 years of age between 1995 and 2002 (data available from 1995). Of these, 12 293 were entitled to a special reimbursement.

The proportions of children for whom asthma medication had been purchased in 1995–2002 were calculated. We chose to make the comparison by calendar months (January vs. January etc.) because of the known monthly variation in the purchase of asthma medicines. We did not include the consumption of asthma medication under the age of 3 years, in order to exclude the medication used to treat possible transient wheezing in early childhood.

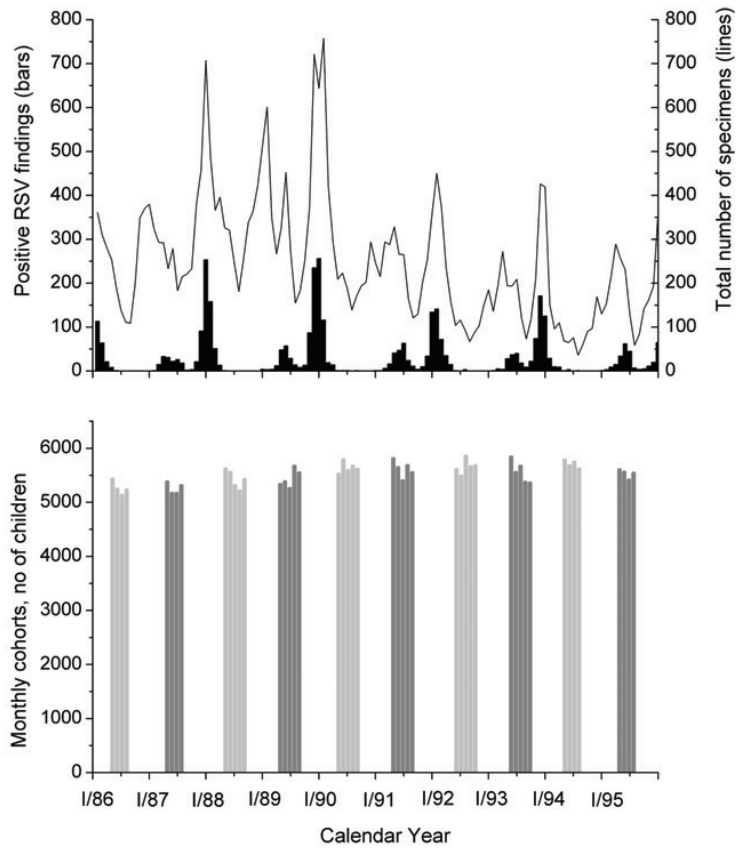


Fig. 2. RSV epidemics in Finland between 1986 and 1995, as defined by number of specimens shown by antigen detection to be RSV-positive. Monthly birth cohorts grouped by exposure to an RSV epidemic at the age 0–6 months (no. of unexposed children presented in light grey bars and no. of corresponding exposed children in dark grey bars).

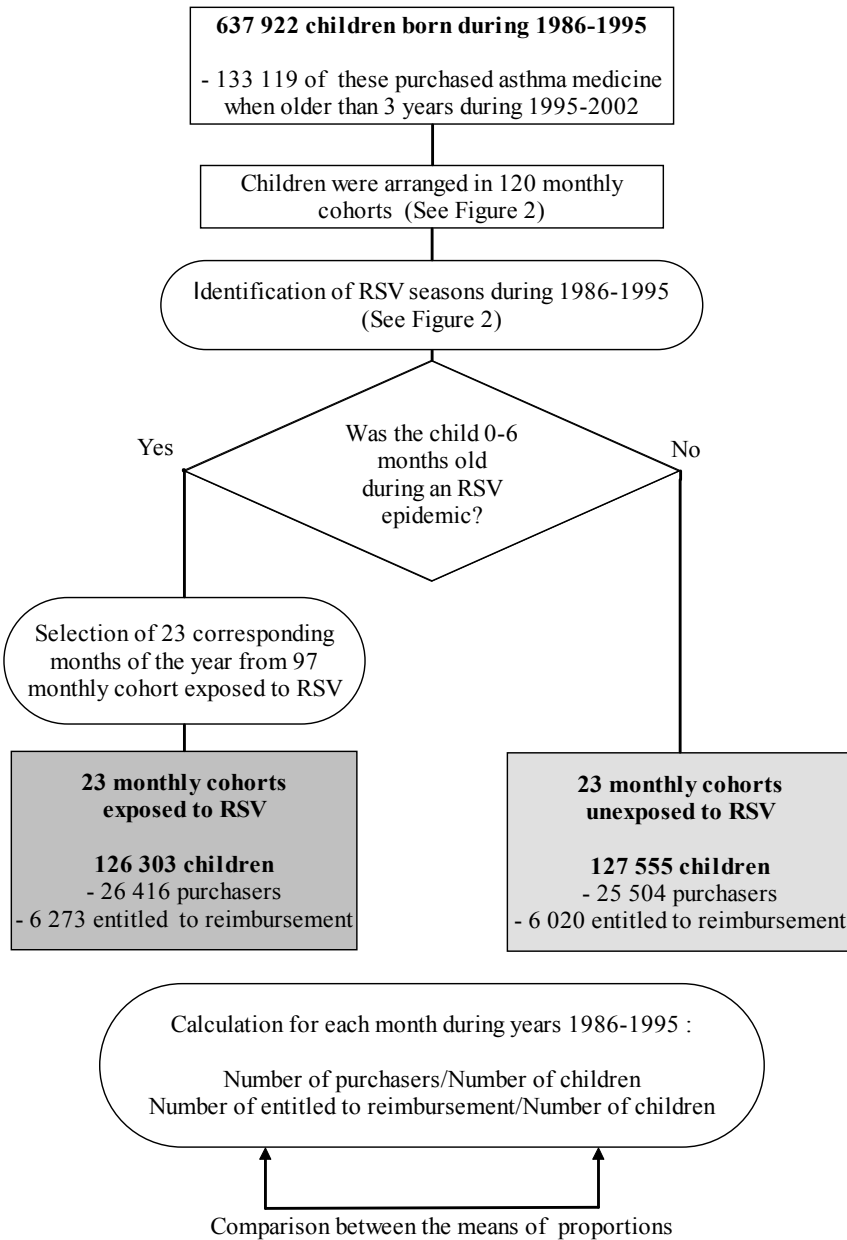


Fig. 3. Study profile depicting determination of the consumption of asthma medicine and the number of children entitled to reimbursement in relation to exposure to an RSV epidemic at age under 6 months.

4.3 Cord blood cytokines predicting RSV infection (IV)

A cord blood sample was collected from each infant of at least 37 gestational weeks born at Oulu University Hospital from August to November 2001 whose parents gave an informed consent (n = 1 084; Figure 4). The parents were requested to contact us if their infant caught a respiratory tract infection before the age of four months and the parents were interested in taking part in a study of the association between respiratory infections and asthma and atopy. When the parents contacted us, a visit was arranged within 24 hours to examine the child and interview the parents. The aetiology of the viral infection was determined from a nasopharyngeal secretion sample by antigen detection using an in-house method of EIA and a polymerase chain reaction (for rhinovirus detection) (Hietala *et al.* 1988, Hyypiä *et al.* 1989). The decision regarding hospitalization was based mainly on the severity of the symptoms (breathing difficulty and/or feeding problems), but it was also possible to hospitalize an infant for a short observation period to see how the infection would proceed. Subjects were also recruited from children hospitalized for a lower respiratory tract infection without any primary contact with the authors.

The children with a clinical respiratory infection and a positive result for RSV were enrolled in the study and those with a negative result for RSV formed one control group. A second group of healthy controls with no respiratory infections before the age of six months and matched with the above for date of birth and sex was selected from those children whose cord blood specimens were available. The children were seen each time they caught a respiratory infection up to the age of six months. Children in a control group who caught either an RSV infection or some other lower respiratory tract infection before the age of six months were analysed according to the aetiology of the infection.

Altogether 48 children (28 males) suffered from RSV infection and were either admitted to hospital (n = 27, 14 males) or seen in an outpatient department (n = 21, 14 males). The mean age at the time of admission for RSV infection was 3.0 months (range 3 weeks to 5.5 months). One hospitalized subject was found after the RSV infection to have a chromosome abnormality and was excluded from the final analysis, leaving 47 subjects. One child had an RSV infection twice during the epidemic but did not require hospitalization. The 28 children with another respiratory viral infection (7 with rhinovirus, 3 with enterovirus, 18 with virus not detectable, including 14 males altogether) had a mean age of 3.1 months at the time of the infection (range 3 weeks to 5.7 months) and three were

hospitalized. The group of healthy controls consisted of 84 children (48 males). None of the subjects or controls had required respiratory assistance during the neonatal period or had a concomitant disease such as a congenital heart disease or a severe neurological disease. None of the infants hospitalized needed intensive care. To avoid the possible confounding effect of the month of birth on RSV-induced immune responses all the children in the analysis had been born outside the main RSV season (Legg *et al.* 2002, Gern *et al.* 2006).

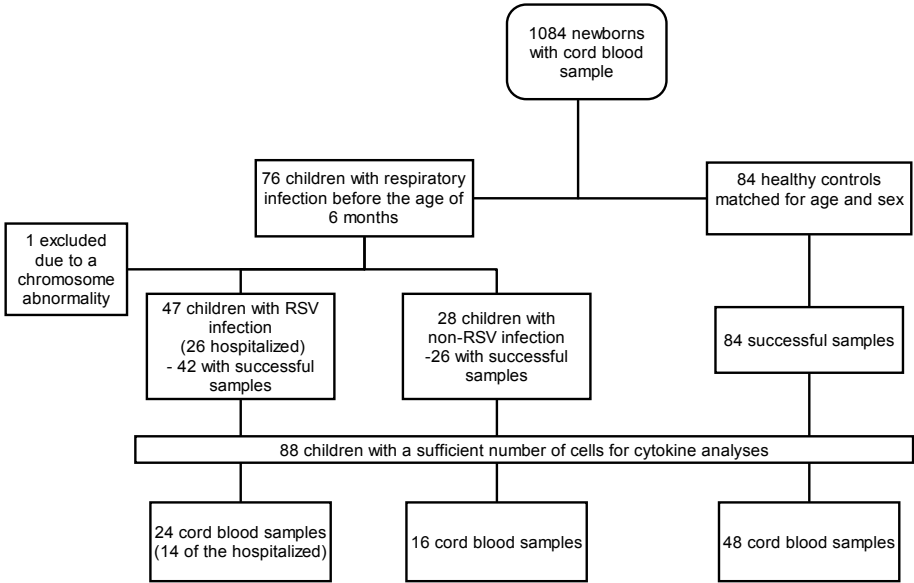


Fig. 4. Study profile depicting the distribution of children with a cord blood sample taken at birth who were followed up until the age of six months.

The birth order was highest among the children with some other respiratory infection (3.6, range 1–11) followed by the children with RSV infection (2.6, 1–10) and the healthy controls (2.0, 1–9). The difference was significant for the difference between RSV and the healthy controls ($P = 0.008$) and for the difference between controls with some other infection and healthy controls ($P < 0.001$). No other differences were found in the background data between the RSV subjects as a whole and the control groups.

A larger proportion of the mothers among the infants hospitalized for RSV infection had ceased breastfeeding before the time of the infection than among

those treated as outpatients (16/26 vs. 20/21, $P = 0.007$), the controls with some other viral infection (16/26 vs. 26/28, $P = 0.005$) or the healthy controls at a corresponding age (16/26 vs. 53/65, $P = 0.04$). Smoking during pregnancy had been significantly more common among the mothers of the infants hospitalized for RSV infection than among those of the healthy controls (7/26 vs. 9/84, $P = 0.03$), and smoking in the family at the time of the infection was more common than among the children suffering from some other viral infection (13/26 vs. 6/28, $P = 0.03$). No statistically significant differences were found in the other background variables. Birth weight, birth order, smoking during pregnancy and breastfeeding status at the time of the infection or corresponding age were controlled for as confounding factors in the logistic regression analyses. In addition, smoking habits in the family were treated as a confounding factor when comparing the children hospitalized for RSV infection with the controls having some other viral infection, as was age at the time of the infection when comparing the subjects with the controls having some other viral infection.

The children hospitalized for RSV infection had significantly lower amounts of eosinophils in their blood at the time of the infection than did the controls with some other viral respiratory infection ($0.25 \times 10^9/L$ (SD 0.39) vs. $0.35 \times 10^9/L$ (SD 0.35), $P = 0.003$).

A sufficient number of cells for the analyses of unstimulated and LPS-stimulated cytokine production were found in about a half of the cord blood samples, while the number of samples for other stimulations varied from 29 to 46 out of the total of 159 children. The birth weight was significantly higher among the healthy controls with a successful analysis of unstimulated responses than among those without (3 656 g (SD 549) vs. 3 384 g (412), $P = 0.032$). No other differences in background factors were found between those with successful analyses and those without.

5 Methods

5.1 Use of a questionnaire (I and II)

The parents were asked before the visit to fill in a questionnaire asking about asthma and allergic symptoms and whether any atopic disorders had been diagnosed by a physician. We used the same questionnaire as in the ISAAC study with some additional questions about the type of day-care, breastfeeding, number of siblings, occurrence of otitis media, food allergy, family history of asthma and atopy, pets and smoking at home (Asher *et al.* 1995). The answers were checked during the visit to avoid misunderstandings. If the parents were not willing to participate in the clinical examinations, we interviewed them by phone to gather the same information.

5.2 Definitions (I, II and III) of asthma, atopic diseases and asthma medication

Atopic diseases and symptoms were defined in articles I and II according to the ISAAC questions concerning wheezing at any time, wheezing during the last year, physician-diagnosed asthma, nose symptoms, diagnosis of allergic rhinitis and atopic eczema (Asher *et al.* 1995). Several children reported to have had either wheezing or dry cough at night for more than three weeks without symptoms of infection in the 12 months before the study. They were further examined by an independent paediatric allergologist outside the research group. Those diagnosed as having asthma were classified accordingly. Since hay fever is often understood as rhinitis merely caused by hay allergy, we added an item on allergic rhinitis of some other type to the questionnaire. A family history of asthma was defined in terms of physician-diagnosed asthma in first degree relatives. Physician-diagnosed allergic rhinitis and atopic eczema in the family were also recorded.

Asthma medication was defined in article III as medicines with the Anatomic Therapeutic Chemical code R03 (drugs for obstructive airway diseases) in the register of Finnish National Agency for Medicines.

5.3 Lung function tests (I)

The lung function tests (spirometry and oscillometry with a bronchodilator test, Master Screen IOS, Jaeger, Würzburg, Germany) were performed by a trained nurse blinded to the group to which the child belonged. The spirometry results were expressed as percentages of the national reference values (Koillinen *et al.* 1998). In the oscillometry test the best of three impulse oscillometer measurements of Rrs at 5, 20 and 35 Hz and Xrs at 5 Hz were accepted for the analysis and the results were compared with the national reference values (Malmberg *et al.* 2001).

5.4 Skin prick tests and blood samples (I and II)

Skin prick testing was done on the volar aspect of the forearm with Soluprick® allergens (ALK; Allergologisk Laboratorium A/S, Horsholm, Denmark) including birch, mugwort, meadow grass, milk, egg, wheat, soy, hazelnut, peanut, banana, *Dermatophagoides pteronyssinus*, *D. farinae*, dander of dog and cat and latex (Stallergenes A.S., France). A 1-mm, one-peak lancet with a shoulder to prevent deeper penetration was used. 10 mg/ml of histamine dihydrochloride was used as a positive control and Soluprick® control solution as a negative control. The reactions were read at 15 minutes, and those with a mean diameter of the weal minus the diameter of the negative control ≥ 3 mm were regarded as positive. The reactions were analysed by trained nurses without knowing to which group the child belonged.

Immunoglobulin E concentrations were determined by a chemiluminescence method and those above 130 kU/l for children less than eight years old and above 320 kU/l for children over eight years old were considered elevated according to our laboratory reference values.

Serum samples stored at -20°C were measured for IFN- γ , IL-5, sCD14 and sICAM-1 with commercial EIA tests (IFN- γ , R&D, sCD14 and sICAM-1; HyCult biotechnology). The lower detection limits were 120 pg/ml for IFN- γ , 1 $\mu\text{g}/\text{ml}$ for sCD14 and 63 ng/ml for sICAM-1.

5.5 Cytokine assay (IV)

After thawing, the cord blood mononuclear cells were inoculated onto culture plates at 2×10^6 viable cells/ml in RPMI 1640 medium supplemented with 0.6

mg/ml of penicillin, 60 mg/ml streptomycin, 2mM L-glutamine, 20 mM HEPES and 5% foetal calf serum (FCS) (Integro BV, Dieren, The Netherlands) and stimulated with human influenza A virus (A/Beijing/353/89 H3N2, National Institute of Medical Research, London, U.K.), RSV (RSV Long strain, RSV-A, ATCC VR-26), lipopolysaccharide (LPS) from *E. coli* (HB101, Sigma, St. Louis, Mo, at 100ng/ml), *Lactobacillus rhamnosus GG* (ATCC 53103, from Valio R&D, Helsinki, Finland) and *Streptococcus pyogenes* (serotype T1M1, IH32030, from the National Public Health Institute, Helsinki, Finland). The viruses were used at dilutions that resulted in a 50% infection rate. The influenza A virus was cultured in embryonated hen eggs and stored at -70°C and the RS virus in a HEp-2-cell line (ATCC CCL-23) until a 80% cytopathic effect (CPE) was achieved, harvested in the culture medium and stored at -70°C . The bacteria were stored in skimmed milk at -70°C and passaged three times prior to stimulation of mononuclear cells at a bacteria:CBMC ratio of 5:1 as previously described (Miettinen *et al.* 1996). The mononuclear cells were stimulated for 24h at $+37^{\circ}\text{C}$ in 5% CO_2 and the cell culture supernatants harvested and stored at -20°C for cytokine determination. Approximately half of the cord blood samples had a sufficient number of cells for the control and LPS stimulation experiments, while the number of samples for the other microbial stimulation experiments varied from 29 to 46 out of the total of 159 children (Figure 4).

Cytokine levels in the cell-culture supernatants were detected using a commercial human Th1/Th2 10plex Kit II (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The detection limits for cytokines were as follows: TNF- α 7.9 pg/ml, IFN- γ 7.0 pg/ml, IL-1 β 4.5 pg/ml, IL-2 8.9 pg/ml, IL-4 6.4 pg/ml, IL-5 5.3 pg/ml, IL-6 4.7 pg/ml, IL-8 6.4 pg/ml, IL-10 6.9 pg/ml and IL-12p70 9.7 pg/ml. The results of different runs were equalized by comparing them with the standard curves and are expressed as pg/ml. The responses presented are those evoked by the medium alone and those produced by LPS or microbial stimulation after subtraction of the cytokine levels observed in unstimulated cultures.

5.6 Statistical analysis

The data were analysed with SPSS for Windows, versions 10.0–14.0 (SPSS Inc, Chicago, IL, USA). To calculate the significance of differences between the groups we used χ^2 -test, Fisher's exact test and the test for a difference in proportions for categorical variables and the Mann-Whitney U-test and Student's

t-test for continuous variables. Odds ratios and 95% confidence intervals were calculated. In article III the analysis of covariance adjusted for time was used to compare the means of the proportions of children receiving reimbursement or consuming asthma medication and the adjusted mean number of packages of asthma medication purchased per child between the exposed and unexposed cohorts. The data were adjusted for time because of the tendency for an annual increase in the use of asthma medication.

Logistic regression analysis was used in articles I and II to assess the influence of risk factors on RSV infection while adjusting for confounding factors. The factors were decided upon on the grounds of biological plausibility, and all the variables were entered into the model.

Factor analysis was used in article IV to combine the closely correlated cytokine responses in order to simulate the cascade nature of cytokine actions. The analysis was performed for unstimulated and LPS-stimulated cytokine responses only because of the small number of samples in the analyses with other stimulants. Factor-specific loadings for each cytokine assay were estimated in the healthy controls in order to describe a cytokine profile for healthy children, the loadings indicating the degree of correlation between each factor and the raw data for each cytokine assay. Standardized factor score variables were derived from the loadings and interpreted as summaries of those variables with high factor loadings associated with the factor from which the scores were derived. Factors with an eigenvalue > 1 were used in further analysis to compare factor scores between the groups. The factor scores were used as continuous variables in Student's t-test to compare the mean scores between the groups and in the logistic regression analyses. Logistic regression analysis was used to analyse the cytokine responses and factor scores derived from the factor analysis as predictors of susceptibility to RSV infection and of its severity while adjusting for confounding factors.

The sample size in article I was based on a 20% difference in the cumulative incidence of asthma between the RSV subjects and controls (Sigurs *et al.* 1995). A sample size of 75 subjects and controls was needed to detect a difference of 20% with a power of 80% and an alpha error of 0.05.

5.7 Ethical considerations

This research was found acceptable by the Ethical Committee of the Northern Ostrobothnia Hospital District. All the parents gave their written informed consent.

6 Results

6.1 Asthma, atopy and cytokine production eight years after hospitalization for RSV infection in infancy (I and II)

There were 20 subjects and 12 controls with physician-diagnosed asthma (difference 11%, 95% CI -3% to 24%) (Table 7). Asthma had been diagnosed significantly earlier in the subjects than in the controls, the mean ages at diagnosis being 3.0 years (SD 2.6) and 5.6 years (SD 3.0), respectively ($P = 0.02$). Of the 20 RSV subjects with asthma 70% had had wheezing only during respiratory infections, as compared with 25% of the 12 controls with asthma. 46% of the subjects with asthma diagnosed before the age of three years had had wheezing during the last 12 months, as compared with one of the two controls with asthma. Age at the time of diagnosis among those with current asthma was 3.4 years (SD 2.6) among the subjects and 6.1 years (2.7) among the controls (difference 2.6, 95% CI 0.46 to 4.83, $P = 0.02$).

The subjects reported significantly less sneezing and/or a runny or blocked nose without a concomitant infection than did the controls, but the occurrence of allergic rhinitis and atopic dermatitis was similar in both groups. There were significantly less subjects with serum IgE above the reference values than controls, though the difference in the mean concentration was not significant. No significant differences were found in the lung function tests as measured by spirometry and oscillometry.

Table 7. Asthma and atopy among the subjects and controls; symptoms and diagnoses, no. (%).

Findings	Subjects (n = 76) ¹	Controls (n = 76) ¹	P-value ²	Odds ratio	95% CI
Wheezing at any time	47 (62)	36 (48)	0.09	1.76	0.92 to 3.36
Asthma at any time	20 (26)	12 (16)	0.11	1.91	0.86 to 4.24
Asthma diagnosed at age < 3 years	11 (15)	2 (3)	0.02	6.21	1.32 to 29.41
Current asthma ³	15 (20)	11 (15)	0.39	1.45	0.62 to 3.41
Symptoms of sneezing, or a runny, or blocked nose without a cold	21 (28)	37 (49)	0.008	0.40	0.21 to 0.79
Allergic rhinitis	7 (9)	10 (13)	0.44	0.67	0.24 to 1.86
Atopic eczema at any time	22 (29)	24 (32)	0.72	0.88	0.44 to 1.76
At least one positive SPT	4 (8)	22 (43)	< 0.001	0.11	0.04 to 0.36
Serum IgE above reference values	11 (22)	22 (43)	0.02	0.36	0.15 to 0.86

¹n = 75 among controls for wheezing at any time due to missing information; n = 20 among subjects and 12 among controls for age at the time of asthma diagnosis, n = 51 among subjects and controls for at least two positive SPTs and serum IgE above reference values

²by χ^2 -test (Fisher's exact test, when n < 5)

³ diagnosis of asthma at any time with wheezing and/or use of inhaled corticosteroids during the last 12 months

Eight per cent of the subjects had at least one positive skin-prick test as compared with 43% of the controls (difference -35%, 95% CI -50% to -19%, P < 0.001) (Table 7, Figure 5). Significant differences were found both in sensitization to animal dander and pollen (4% vs. 28%, OR 0.11, 95% CI 0.02 to 0.50, and 6% vs. 30%, OR 0.14, 95% CI 0.04 to 0.54, respectively). All those with asthma diagnosed at an age of less than 3 years had negative skin-prick tests as well as all the asthmatics without current symptoms.

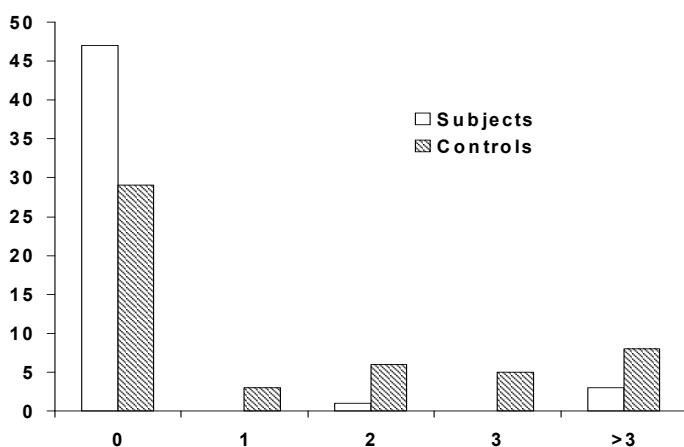


Fig. 5. Numbers of subjects and controls with 0, 1, 2, 3 or > 3 positive skin-prick tests (P = 0.001, χ^2 -test).

A logistic regression analysis adjusted for duration of breastfeeding, number of siblings and family history of asthma and/or atopy showed RSV infection to be associated with negative skin-prick tests and a shorter duration of breastfeeding (in months) (Table 8).

Table 8. Logistic regression analysis of associations with RSV infection in infancy.

Risk factor	Odds Ratio	95% CI	P
No. of positive skin-prick tests at follow-up	0.60	0.42 to 0.87	0.007
Duration of breastfeeding, months	0.89	0.80 to 0.99	0.036
No. of siblings	1.14	0.99 to 1.31	0.066
Family history of asthma and/or atopy, yes/no	0.80	0.27 to 2.32	0.679

The mean serum concentrations of IFN- γ and sICAM-1 were significantly higher among the RSV subjects than among the controls (Table 9). No differences were found between the groups in sCD14 or IgE concentrations, and IL-5 remained undetectable in all the samples.

Table 9. Concentrations of S-IgE, IFN- γ , soluble CD14 and ICAM1 in the serum of the RSV subjects and controls.

Substance	RSV subjects (n = 51)	Controls (n = 51)	P ¹
	mean (SD)	mean (SD)	
S-IgE kU/l	119.2 (176.7)	244.1 (343.2)	0.17
IFN- γ pg/ml	224.9 (271.3)	187.1 (372.9)	0.05
sCD14 μ g/ml	1.84 (0.42)	1.83 (0.40)	0.8
sICAM1 ng/ml	170.0 (63)	148.0 (57)	0.04

¹by Mann-Whitney U-test

IFN- γ concentrations in the subjects and the controls with asthma or atopy are presented in Table 10. IFN- γ was detectable in 5/15 RSV subjects with asthma (P = 0.03 vs. 0/11 controls) and 6/13 subjects with wheezing during the previous 12 months (P = 0.008 vs. 0/13 controls). No significant difference was found in the concentration of IFN- γ in the RSV subjects with or without asthma, wheezing during the previous 12 months or atopic dermatitis. IFN- γ was undetectable in all the samples from the subjects with at least one positive skin-prick test, in 19 out of the 22 samples from the corresponding controls and in all the samples from the children with allergic rhinitis.

Table 10. Concentrations of IFN- γ in the serum of RSV subjects and controls with asthma or atopy.

Asthma or atopy	IFN- γ (pg/ml)		P ²
	RSV subjects ¹	Controls ¹	
	mean (SD)	mean (SD)	
Asthma	174.7 (101.1)	< 120 (0)	0.04
Wheezing during the previous 12 months	335.1 (445.7)	< 120 (0)	0.007
Atopic dermatitis	320.0 (435.9)	133.2 (44.2)	0.08
At least one positive skin prick test	< 120 (0)	131.4 (48.9)	0.4

¹n = 15 for RSV subjects and n = 11 for controls with asthma, n = 13 for RSV subjects and controls with wheezing during the previous 12 months, n = 15 for RSV subjects and n = 18 for controls with atopic dermatitis, n = 4 for RSV subjects and n = 22 for controls with at least one positive skin prick test

²by Mann-Whitney U-test

Soluble ICAM-1 concentrations were significantly higher in the RSV subjects with wheezing during the previous 12 months than in the corresponding controls (difference 71.3 ng/ml, 95% CI 9.6 to 133.0 ng/ml, P = 0.03, Figure 6). The difference was most pronounced among the children with wheezing and negative skin-prick tests (difference 95 ng/ml, 95% CI 16.5 to 174.2 ng/ml, P = 0.007).

The RSV subjects with atopic dermatitis had significantly higher sICAM-1 concentrations than the corresponding controls (difference 42.3 ng/ml 95% CI 9.3 to 75.4 ng/ml, $P = 0.04$). The concentrations did not differ between the RSV subjects and controls with asthma, allergic rhinitis or positive skin prick tests. The mean sICAM-1 concentration in the RSV subjects with or without asthma or wheezing during the previous 12 months did not differ significantly. No significant differences in sCD14 or IgE concentrations were found between the RSV subjects and controls with any atopic diseases.

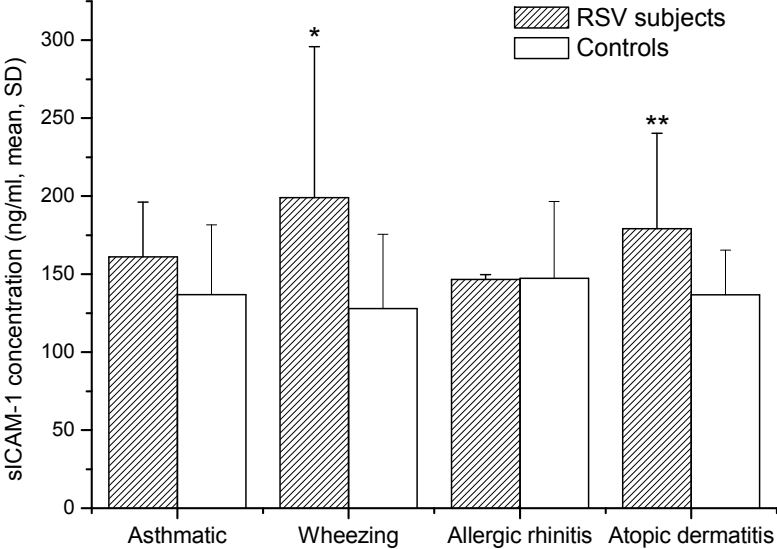


Fig. 6. sICAM-1 concentrations in RSV subjects and controls with atopic diseases, * $P = 0.03$, ** $P = 0.04$.

6.2 Consumption of asthma medication after exposure to an RSV epidemic in infancy (III)

The 46 cohorts included 126 303 children who had been exposed to RSV in infancy (6 273 entitled to reimbursement for asthma medication costs) and 127 555 who had not been exposed (6 020 entitled to reimbursement). The adjusted means of the proportions of children taking asthma medication were similar among the unexposed and exposed cohorts (20.5% vs 20.3%; difference 0.2%, 95% CI -1.2 to 1.5, $P = 0.8$) (Figure 7).

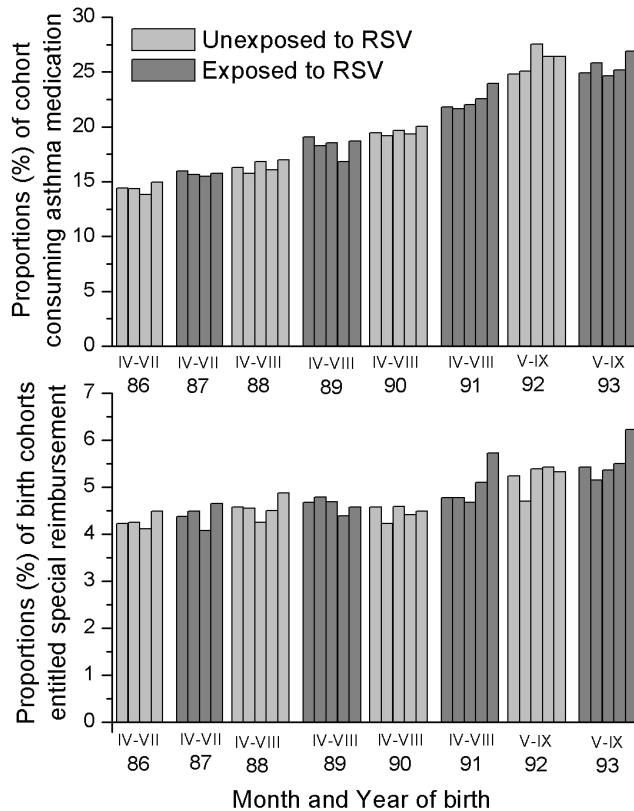


Fig. 7. Proportions of the paired cohorts consuming asthma medication (upper) or entitled to special reimbursement for asthma medication costs (lower), grouped into those exposed or unexposed to RSV epidemics in the years in question. Roman numerals on the bars refer to months of the year (i.e. I = January etc).

The adjusted mean number of packages of inhaled corticosteroids purchased per child was 12.7 in the unexposed group vs. 12.4 in the exposed group (difference 0.2, 95% CI -0.4 to 0.9, $P = 0.5$) and the corresponding figures for inhaled β_2 -agonists were 4.6 vs 4.5 (difference 0.1, 95% CI -0.1 to 0.4, $P = 0.4$). No significant difference were found in the adjusted mean proportions of unexposed and exposed children who received special reimbursement for asthma medication (4.8% vs. 4.9%, difference -0.1% 95% CI -0.3 to 0.1, $P = 0.2$). Purchases of asthma medicines increased approximately 1.5-fold overall from 1995 to 2002.

6.3 Cord blood cytokines predicting RSV infection (IV)

The production of IL-6 and IL-12 from unstimulated CBMC was significantly lower in the subjects than in the healthy controls (3.5 pg/ml (SD 9.8) vs. 7.4 pg/ml (10.1), $P = 0.003$, and 5.6 pg/ml (17.7) vs. 11.7 pg/ml (18.1), $P = 0.005$, respectively). The difference was most pronounced between the children hospitalized for an RSV infection and the healthy controls (for IL-6 0.8 pg/ml (1.9) vs. 7.4 pg/ml (10.1), $P = 0.002$ and for IL-12 0.7 pg/ml (2.6) vs. 11.7 (18.1), $P = 0.003$) (Figure 8). The children subsequently hospitalized for an RSV infection produced higher amounts of IFN- γ and IL-4 than did the healthy controls (for IFN- γ 14.0 pg/ml (30.2) vs. 0.4 pg/ml (1.9), $P = 0.03$ and for IL-4 11.7 pg/ml (11.7) vs. 5.7 pg/ml (17.9), $P = 0.01$). The difference between the hospitalized children and those with another viral infection was significant for IL-4 (11.7 pg/ml (11.7) vs. 1.9 pg/ml (4.2), $P = 0.03$). No significant differences were found in IL-1 β , IL-2, IL-5, IL-8 or TNF- α responses, while the IL-10 responses were undetectable in all the groups except for two of the healthy controls.

Four mutually uncorrelated factors were produced by factor analysis of the unstimulated cytokine responses among healthy controls (Table 11). No differences were found in the comparison of factor scores derived from the loadings within each factor analysed by Student's t-test or logistic regression analysis.

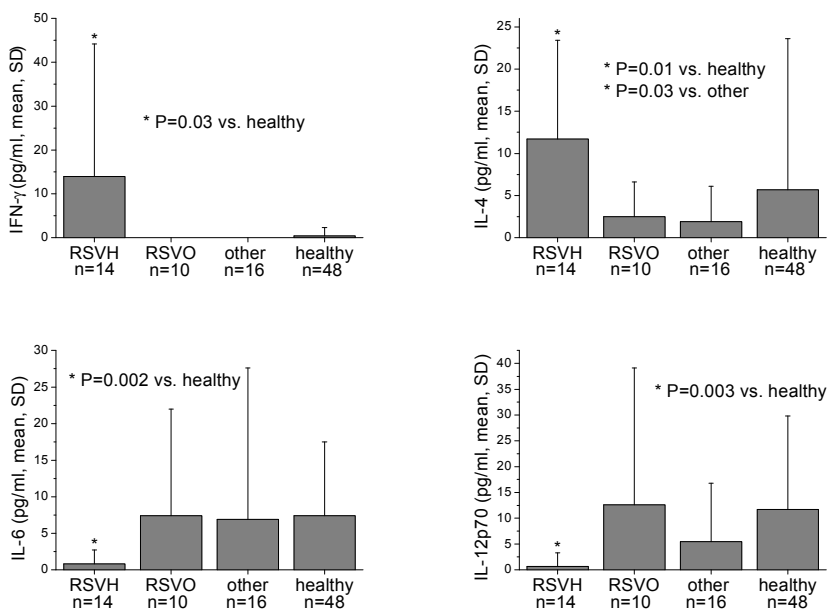


Fig. 8. Cytokine responses in unstimulated cord blood mononuclear cells. Cytokine levels in the supernatant collected at 24h from cord blood mononuclear cell cultures obtained from children who contracted RSV or some other type of respiratory viral infection before 6 months of age and cells from uninfected control children. Results are shown as pg/ml (means \pm 1 SD unit). RSVH = subjects hospitalized for an RSV infection; RSVO = subjects treated as outpatients for an RSV infection; other = controls with some other respiratory viral infection; healthy = controls without any respiratory infection.

Table 11. Summary of a factor analysis including factor loadings from a model of cytokine responses in the unstimulated cord blood mononuclear cells of healthy control individuals¹.

Factor 1		Factor 2		Factor 3		Factor 4	
Cytokine	Loading	Cytokine	Loading	Cytokine	Loading	Cytokine	Loading
IL-6	0.903	IL-2	0.831	IFN- γ	0.802	IL-1 β	0.942
IL-12	0.969	IL-4	0.948	IL-10	0.922	IL-8	0.985
TNF- α	0.908	IL-5	0.655				
Total variance explained (%)							
28.1		21.0		20.9		20.0	

¹Standardized factor score variables derived from the loadings were used in the logistic regression analyses.

LPS-stimulated IL-6 responses were significantly higher among the children hospitalized for an RSV infection (53.6 pg/ml (67.7)) than in the healthy controls (8.8 pg/ml (20.1), $P = 0.001$) or the controls with another viral infection (1.1 pg/ml (8.0, $P < 0.001$)). Those treated as outpatients for a milder RSV infection had significantly reduced responses (-0.6 pg/ml (4.7), $P < 0.001$) (Figure 9). As a whole, the subjects had higher levels of LPS-stimulated IL-6 responses than the children with another respiratory viral infection (31.0 pg/ml (57.8) vs. 1.1 pg/ml (8.0), $P = 0.009$). The LPS-stimulated IFN- γ responses were most suppressed among the children hospitalized for an RSV infection as compared with the healthy controls (-14.0 pg/ml (30.2) vs. -0.2 pg/ml (1.2), $P = 0.009$). LPS-stimulated IL-5 responses were significantly lower among the subjects than in the healthy controls (-1.3 pg/ml (3.1) vs. -0.5 pg/ml (8.9), $P = 0.028$). No significant differences were found in the other cytokines, and the IL-10 responses were undetectable in all the groups.

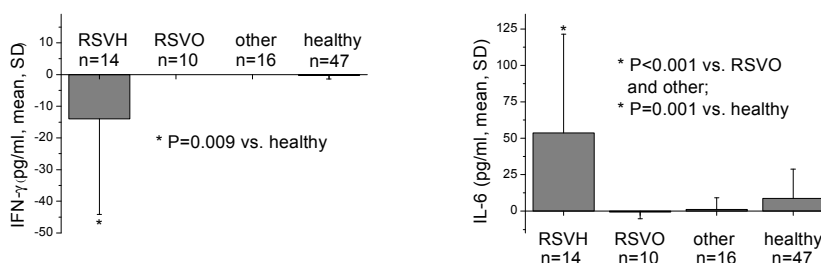


Fig. 9. Cytokine responses in lipopolysaccharide-induced of cord blood mononuclear cell cultures. RSVH = subjects hospitalized for an RSV infection; RSVO = subjects treated as outpatients for an RSV infection; other = controls with some other respiratory viral infection; healthy = controls without any respiratory infection.

Four uncorrelated factors were formed by factor analysis after LPS stimulation among the healthy controls, (Table 12). The children hospitalized for an RSV infection had higher scores on a factor combining the IL-6 and IL-8 responses than did the healthy controls ($P = 0.015$) or the children treated as outpatients for RSV infection ($P = 0.005$). In the logistic regression analysis high scores of this factor predicted hospitalization for RSV infection by comparison with the healthy controls (OR 2.29 (95% CI 1.21 to 4.33), $P = 0.011$). The children hospitalized for an RSV infection had lower scores on a factor combining five cytokines, IL-1 β , IL-2, IL-4, IL-5 and IL-10, than those treated as outpatients ($P = 0.022$). In the logistic regression analysis using the factor scores as continuous explanatory

variables this factor was not significantly associated with either susceptibility to RSV infection or its severity. The other factors formed, the IFN- γ response alone and the combined responses of IL-12 and TNF- α , were not significantly associated with RSV infection.

Table 12. Summary of the factor analysis including loadings from a model of cytokine responses in the LPS-stimulated cord blood mononuclear cells of healthy controls.¹

Factor 1		Factor 2		Factor 3		Factor 4	
Cytokine	Loading	Cytokine	Loading	Cytokine	Loading	Cytokine	Loading
IL-1 β	0.775	IL-6	0.831	IL-12	0.898	IFN- γ	0.975
IL-2	0.789	IL-8	0.824	TNF- α	0.682		
IL-4	0.922						
IL-5	0.791						
IL-10	0.909						
Total variance explained (%)							
37.4		15.4		13.7		10.5	

¹Standardized factor score variables derived from the loadings were used in the logistic regression analyses.

No significant differences in cytokine responses were found after either RSV, *Lactobacillus rhamnosus*, *Streptococcus pyogenes* or influenza A stimulation, and no further analyses were performed with these stimulants.

7 Discussion

7.1 Development of asthma and atopic allergy at the age of 7–9 years after an RSV infection in infancy (I)

We found that children hospitalized for RSV infection under the age of 12 months did not differ significantly in their prevalence of asthma at the age of 7–9 years from that of healthy children. The children with asthma after RSV infection had received the diagnosis at an age significantly lower than that of the controls with asthma. About half of the subjects and the controls with asthma diagnosed at an age of less than three years still had symptoms of wheezing at the time of the study, suggesting that in a great proportion of infants the tendency to wheeze is transient.

Our results are in line with previous demonstrations of a positive association of early RSV infection with asthma and a diminution of this effect with age (Pullan & Hey 1982, Stein *et al.* 1999, Kneyber *et al.* 2000, Korppi *et al.* 2004b). Wheezing after an RSV infection has been shown to decrease even during the first year, but the loss of a significant association seems to occur somewhere around 7–8 years of age (Bont *et al.* 2004). The groups used for assessing asthma and atopy after RSV infection in various studies differ in a number of ways. Most studies exclude children with diseases known to predispose them to a severe RSV infection, with the exception that prematurely born children are usually enrolled. We excluded the latter from both the subjects and the controls, because it is likely that the mechanism of asthma development after RSV infection among them differs from that among children born at term. Another aspect that differs between studies is the age of the subjects at the time of infection, which has often been restricted to below 12 months but in some cases has been extended to three years. The importance of an infection for the development and persistence of wheezing may depend on age at the time of the primary infection. Since follow-up times differ, it is not possible to analyse the impact of this age difference on the loss of association, for example. Furthermore, the severity of RSV infection is likely to be important for the development of asthma and wheezing. Even those treated as outpatients had increased wheezing up to the age of 11 years, but in most studies only those with an infection severe enough to necessitate hospitalization have been included (Stein *et al.* 1999). The severity of infection most probably plays a role in the development of asthma and wheezing, because the infection is

common and there is evidence for that mere positive serological evidence of RSV infection is not associated with the development of asthma (Glezen *et al.* 1986, Kotaniemi-Syrjänen *et al.* 2005). The need for hospitalization is a somewhat arbitrary definition of the severity of the disease for use in research settings, because the criteria for hospitalization differ between countries. Taking into account the retrospective identification of the subjects in our study, however, we found hospitalization to be a more useful selection criterion than the definition of bronchiolitis, which may be even more problematic.

We found a significant negative association between RSV infection and atopic sensitization, although there have been remarkable discrepancies in the findings reported in previous studies. It is still not clear whether RSV infection has an impact on the development of atopy, but what does seem clear is that the additional risk for the development of asthma after RSV infection is not related to the development of atopy. None of our children with asthma diagnosed at an early age had a positive skin prick test at the time of the examination. Actually, skin prick test positivity was exceptionally low among our subjects, while it was approximately the same among the controls as in a previous study of schoolchildren in Finland (Remes & Korppi 1996). The figures quoted in assessments of atopic sensitization after RSV infection have ranged from 15 to 40% among RSV subjects and 17 to 40% among healthy controls at the age of 6 to 10 years (Sims *et al.* 1978, Pullan & Hey 1982, Stein *et al.* 1999, Sigurs *et al.* 2000, Henderson *et al.* 2005).

7.2 Relation of cytokine production after an RSV infection in infancy to the development of asthma and atopic allergy (II)

7.2.1 Cytokine responses in children with previous RSV infection in relation to asthma and atopic allergy

We found serum concentrations of IFN- γ to be significantly higher among the RSV subjects than among the controls 6–8 years after the infection, whereas IL-5 remained undetectable in all the samples. The results for IFN- γ are consistent with a study showing greater PBMC IFN- γ responses at one year of age among those with verified RSV infection during the first year of life than among those not infected (Gern *et al.* 2006). In another study, though, the frequency of IFN- γ -producing cells in RSV-infected cultures of monocytes was reported to be highest

in the group with previous mild RSV infection as opposed to those with either RSV bronchiolitis or no RSV infection by the age of one year (Schauer *et al.* 2004).

We found that IFN- γ was more often detectable in the RSV subjects with asthma or wheezing than in the controls with asthma or wheezing, but there were no significant differences between the RSV subjects with or without asthma or wheezing. IFN- γ was undetectable in nearly all the samples from subjects and controls with at least one positive skin prick test. In contrast to our findings, atopic children examined during the convalescent phase of RSV infection are reported to have shown higher IFN- γ responses than non-atopic children, although the situation was the opposite during the infection (Kaneko *et al.* 2006). Low IFN- γ responses at 5 months after bronchiolitis have been associated with an increased risk of asthma at the age of two years (Renzi *et al.* 1999). IFN- γ is the main Th1-type cytokine, and Th1-type responses are associated with a non-atopic phenotype. Our results therefore reflect the clinical findings in our population, namely the lower frequency of atopic sensitization among the RSV subjects than among the controls. Furthermore, our children with asthma after RSV infection were less atopic than the controls with asthma, suggesting different types of asthma in these groups. Since the production of IFN- γ was similar in children with and without asthma, we suggest that this cytokine is not the principal mediator of asthma development, though Th1 cells have been shown to induce airway hyperreactivity (Cui *et al.* 2005).

Studies performed on cytokines other than those that we measured have suggested that IL-4 production 5 months after bronchiolitis may be associated with subsequent wheezing, though in one case IL-4 and IFN- γ responses at the convalescent phase did not predict wheezing during the next six years (Renzi *et al.* 1997, Ermers *et al.* 2007). Children with RSV bronchiolitis in infancy showed significantly higher frequencies of RSV-specific cells producing IL-4 than controls at the age of 7.5 years (Pala *et al.* 2002). High frequencies were also associated with a risk of asthma and wheezing. These results, like ours, reflect the incidence of atopic sensitization in the population concerned (Sigurs *et al.* 2000). High IL-10 and IL-12 responses in peripheral blood monocytes during the convalescent period after RSV bronchiolitis have been shown to predict recurrent wheezing up to three years of follow-up, but no longer at the age of six years (Bont *et al.* 2000a, Ermers *et al.* 2007). These results suggest differences between those with a subsiding tendency to wheeze and those developing asthma after the infection. No difference in IL-13 responses at the age of one year was found

between those with wheezing during a previous RSV infection, no wheezing during the infection or no RSV infection (Gern *et al.* 2006).

7.2.2 ICAM-1 and CD14 as a link between RSV infection and asthma

We found significantly higher serum concentrations of sICAM-1 among the RSV subjects than among the controls, but no differences in sCD14 or IgE concentrations. sICAM-1 concentrations were higher in the RSV subjects with wheezing during the previous 12 months than in the corresponding controls, suggesting differences in the mechanisms of wheezing between these children. No differences were found between the RSV subjects and controls with an asthma diagnosis, a group that also included those who had become asymptomatic during the follow-up. It has been suggested that sICAM-1 may be essential for the development of airway hyperresponsiveness and asthma (Wegner *et al.* 1990). sICAM-1 concentrations were similar in our RSV subjects with or without asthma or wheezing during the previous 12 months, suggesting additional mechanisms lying behind wheezing among these children. They were significantly higher in the RSV subjects with wheezing and negative skin-prick tests than in the corresponding controls. Bronchial biopsy studies have linked ICAM-1 production with non-atopic asthma in particular (Bentley *et al.* 1993). We are not aware of any other assessments of sICAM-1 production after RSV infection, but no association has been found between ICAM-1 polymorphisms and the severity of RSV infection (Krueger *et al.* 2006).

CD14 has been shown to act as a receptor for several bacteria, LPS and RSV (Kurt-Jones *et al.* 2000, Karlsson *et al.* 2002, Baldini *et al.* 2002). Increased serum levels of sCD14 during RSV bronchiolitis may provide protection from recurrent wheezing after the infection by shifting the balance of the Th cells towards Th1 cells (Soferman *et al.* 2004). Polymorphism of the CD14 gene has been shown not to be associated with susceptibility to RSV infection (Puthothu *et al.* 2006a). We were unable to find any differences in sCD14 or IgE concentrations between the groups at 7–9 years of age.

7.3 Development of asthma by the age of 16 years as defined by the consumption of asthma medication (III)

We found no significant differences in the proportions of children receiving asthma medication or special reimbursement for asthma medication between the cohorts of children unexposed or exposed to an RSV epidemic in infancy. The numbers of packages of inhaled corticosteroids and β_2 -agonists purchased per child were also similar.

Since RSV infection is common in infancy, its possible causative effect on the development of asthma should have been seen at the population level in the consumption of asthma medication after exposure to the disease in infancy (Glezen *et al.* 1986). Since virtually all asthmatics in Finland requiring continuous medication are included in the special reimbursement data, the effect of RSV exposure on subsequent asthma development should have been seen in these figures as well.

An association of RSV infection with the development of asthma has nevertheless been found in several studies, so that we must ask how the lack of association in our study can be accounted for. There are two possible explanations. The first is that RSV does not have a causative role in the development of asthma, but is capable of identifying children with a tendency for recurrent wheezing leading to asthma. There could have been an identical number of children with such a tendency among the unexposed children forming the present control group, children who would develop asthma despite the lack of RSV exposure in infancy. This idea is in line with our clinical findings of the children with RSV infection in infancy having more asthma at an age of less than three years with the controls catching up with them by the age of 7–9 years. The second possibility is that only the most severe infection leads to modulation of asthma development. During the first year of life 1–3% of children need to be hospitalized for RSV infection. If we assume that only a subgroup of these children develop asthma, the resulting increase in the overall occurrence of asthma is unlikely to have any marked impact on the consumption of asthma medication at the population level. (Ruuskanen & Ogra 1993, Domachowske & Rosenberg 1999, Iwane *et al.* 2004) It has, however, been shown that an increased frequency of recurrent wheezing can be found up to 11 years of age among children treated for RSV infection as outpatients (Stein *et al.* 1999).

The controls not exposed to RSV under the age of six months were mainly (with the exception of a couple of endemic cases) unable to catch a severe RSV

infection during early infancy, and the first epidemic they faced was only a minor one. Thus the majority of them could be expected to catch their first RSV infection after the age of 12 months. They formed an exceptional group as compared with children in countries with annual RSV epidemics. We suggest that the controls included both children with a susceptibility to severe infection and children without any such susceptibility, while control groups are usually formed from those children who are left over after a subgroup has caught the RSV infection. The control group used here thus serves better to represent the general population.

7.4 Cytokine responses in cord blood, predicting susceptibility to RSV infection during infancy and the severity of the infection (IV)

We found that the children hospitalized for RSV infection at the age of less than six months had an LPS-stimulated cytokine response profile at birth that was different from that of the children treated for RSV infection as outpatients and the healthy children. As in earlier studies of risk factors for RSV infection, higher birth order, a shorter duration of breastfeeding and greater exposure to smoking were all found here to be risk factors of RSV infection (Carlsen *et al.* 1987, Simoes 2003, Nielsen *et al.* 2003, Bradley *et al.* 2005). The high combined IL-6 and IL-8 responses derived by factor analysis remained as a significant predictor of RSV infection requiring hospitalization after adjusting for these confounding factors in the logistic regression analysis. The children hospitalized for RSV infection had lower scores on a factor consisting of IL-1 β , IL-2, IL-4, IL-5 and IL-10 responses than those who were treated as outpatients, although this factor was not found to predict hospitalization in the logistic regression analysis.

No significant differences in cytokine responses were found after stimulation with RSV, *Lactobacillus rhamnosus*, *Streptococcus pyogenes* or influenza A, and no further analyses were performed with these stimulants. Only about a half of the cord blood samples had a sufficient number of cells for the analyses of unstimulated and LPS-stimulated cytokine production, while the number of samples for the other stimulations were even lower.

7.4.1 Relation of the findings to cord blood cytokines as predictors of infections in general and of RSV infection

Cord blood mononuclear cells have in general been shown to produce high amounts of IL-6 in response to LPS and RSV stimulation, but only low amounts of IFN- γ (Tsutsumi *et al.* 1996, Langrish *et al.* 2002, Angelone *et al.* 2006). Little is known about cord blood responses as predictors of RSV infection, and virtually no other studies have been performed on the role of IL-6 and IL-8 responses in this respect. The genes of innate immunity have been shown to have the strongest association with RSV bronchiolitis as compared with genes affecting other types of immune responses (Janssen *et al.* 2007).

Among children with a family history of allergies and/or asthma, IFN- γ responses in cord blood were inversely related to the frequency of viral respiratory infections. Detectable RSV-induced IFN- γ responses at birth provided protection from wheezing during infections before the age of one year (Copenhaver *et al.* 2004, Gern *et al.* 2006). Slow maturation of the IFN- γ responses has also been related to susceptibility to RSV infection (Rowe *et al.* 2001). In line with these findings, the LPS-stimulated IFN- γ responses in our study were most clearly suppressed among the children hospitalized for RSV infection. A low IL-12 concentration in cord blood serum has been found to predict hospitalization for bronchiolitis during infancy as compared with healthy controls (Blanco-Quiros *et al.* 1999). This is consistent with our findings of lower spontaneous IL-12 responses among hospitalized children than in healthy controls. It has been reported among children with a family history of allergies and/or asthma that IL-13 responses at birth were lowest among those with wheezing during RSV infection by the age of one year and more pronounced in those with no RSV infection (Gern *et al.* 2006). Those with a non-wheezing RSV infection had levels similar to those with no RSV infection.

7.4.2 Association of the findings with immune responses measured during RSV infection

An exaggerated immune response plays a role in the pathogenesis of an infection as is seen in seen e.g. in avian influenza, while moderate levels of inflammatory cytokines enable the elimination of pathogens (Cinatl, Jr. *et al.* 2007, Lee *et al.* 2007). Among the cytokines produced as part of the innate immune response, IL-6 has several functions with both pro-inflammatory and anti-inflammatory

properties, while IL-8 recruits and activates neutrophils (Harada *et al.* 1994, Jones 2005). Both of them are secreted by several types of cell. BAL samples taken during RSV infection have shown over 80% of the cells in the samples to be neutrophils (McNamara *et al.* 2004). Plasma levels of IL-6 and IL-8 during RSV infection have been found to be higher in severely ill infants than in those with a milder infection or in healthy controls (Biswas *et al.* 1995, Bont *et al.* 1999, Brandenburg *et al.* 2000). RSV has been shown to upregulate the expression of the Toll-like receptor-4 on airway epithelial cells, sensitizing them to LPS (Monick *et al.* 2003). This sensitization leads to increased production of IL-8.

IL-1 β , which is also produced by monocytes as part of the innate immune response to microbes, participates in the activation of naïve T cells, which in turn secrete IL-2 in an autocrine manner, further enhancing their proliferation. Increased levels of IL-2 have been found during RSV infection (Tripp *et al.* 2002, de Waal *et al.* 2003). The Th2-type cytokines IL-4 and IL-5 promote antibody-mediated immune responses. An exaggerated Th2 response during RSV infection has been suggested, but many studies have reported a mixed Th1 and Th2 response during acute infection (Roman *et al.* 1997, Garofalo *et al.* 2001, Tripp *et al.* 2002, de Waal *et al.* 2003, Kristjansson *et al.* 2005, Lee *et al.* 2007). IL-10 is produced by phagocytic cells, T cells and natural killer cells, for example. Increased IL-12 and IL-10 levels in serum have been found during RSV bronchiolitis, but whole blood IL-10 responses and plasma levels have been similar to those in healthy controls (Blanco-Quiros *et al.* 1999, Bont *et al.* 2000b, Fernandez *et al.* 2005). The factor consisting of IL-1 β , IL-2, IL-4, IL-5 and IL-10 responses represents a cascade of cytokines leading from innate immunity to a Th2-type adaptive response. The present children hospitalized for an RSV infection had lower scores on this factor than those treated as outpatients, suggesting a Th1-type immune response profile among them, but the logistic regression analysis revealed a more important role for innate immunity in determining the severity of the infection.

7.4.3 Relation of the findings to immune responses in asthma

Similar to our findings, overproduction of IL-6 has been shown to be characteristic of both atopic and non-atopic asthma. The main distinctive features of atopic as opposed to non-atopic asthma are an increase in IL-4 production and a decrease of IFN- γ production, cytokine responses which in our study did not predict RSV infection (Bettioli *et al.* 2000). IL-8 gene polymorphism has been

related both to the severity of RSV infection and to wheezing following the infection (Hull *et al.* 2000, Goetghebuer *et al.* 2004). A positive association of an IL-8 polymorphism with asthma has been found but no association with RSV infection (Puthothu *et al.* 2006b). An IL-13 polymorphism has been found to be associated with late wheezing after severe RSV infection but not with early postbronchiolitic wheezing suggesting that early and late wheezing after RSV infection are not related to each other (Ermer *et al.* 2007). In that study polymorphisms for IL-4, IL-8, IL-9, IL-10 and TNF α did not show any association with late wheezing.

Increased amounts of eosinophils and mast cells have been found in BAL samples from patients with atopic asthma as opposed to predominantly neutrophilic types of cell among those with virus-induced wheezing (Townshend *et al.* 2007). Patients with neutrophilic asthma have been shown to have higher expression of IL-8 than those with eosinophilic asthma, and neutrophilic asthma has been shown to respond less well to corticosteroids (Green *et al.* 2002, Simpson *et al.* 2007). This distinction seems to bear several similarities to those found between RSV-induced and rhinovirus-induced wheezing, in that children with rhinovirus-induced wheezing have more blood eosinophilia, a better response to prednisolone and are more often atopic than those with RSV-induced wheezing (Korppi *et al.* 2004a, Jartti *et al.* 2006, Jartti *et al.* 2007). We are not aware of any other studies apart from ours that have assessed the differences in cellular or cytokine responses between those with asthma after RSV infection and other asthmatics.

7.5 Methodological aspects

7.5.1 Strengths of the study

The main strength of the study lay in the examination of RSV infection in infancy and its association with the development of asthma and atopy from several perspectives. We studied patterns of immune responses existing before the infection as being related to susceptibility to the infection and its severity. Further, we studied the long-term clinical development of asthma and atopy, immunological parameters after hospitalization for RSV infection in infancy and the impact of RSV exposure on the development of asthma at the population level.

The subjects for the follow-up (articles I and II) were identified by diagnosis of an RSV infection reliably verified by antigen detection. Data on asthma and atopic diseases were obtained from up to 87% of those eligible for the study. In addition, 68% of the subjects and controls attended for a clinical examination, lung function tests, skin prick tests and blood samples. The exclusion criteria included known risk factors for severe RSV infection (which might have affected the decision regarding hospitalization as well). The controls were matched for sex and the date of birth. The questionnaire had previously been shown to be valid in a large population-based study.

The two-year-pattern of RSV epidemics in Finland provides a unique opportunity to compare children born at the same time of year but exposed or unexposed to an RSV epidemic at an early age (article III). Timing of the exposure to RSV was defined from a virological database covering the whole country (40% of specimens from outside the region served by Turku University Hospital). Nearly all asthmatics in Finland requiring continuous medication are included in the special reimbursement data of the Social Insurance Institution, which also records the purchases of asthma medicines. These databases make it possible to compare the usage of asthma medication reliably with RSV exposure at the level of the whole population.

Lack of information on potential individual differences preceding RSV infection is one of the main problems when studying the association of RSV infection with subsequent asthma and atopy. This information is crucial for resolving the question of whether RSV is capable of affecting the development of asthma by modulating immune responses, for example, or of identifying infants with a susceptibility to infection and possible post-infectious sequelae. Studying cord blood immune responses is an excellent way of answering this question, as we showed by indicating differences in innate immunity capacity between those hospitalized and those remaining healthy during an RSV epidemic (article IV). Furthermore, we made comparisons with infants treated as outpatients for RSV infection, infants with a respiratory infection caused by some other virus and healthy infants.

The viral diagnoses were verified by antigen detection or polymerase chain reaction, and the cytokine assay covered the most important cytokines involved in innate and adaptive immune responses. The fact that cytokines act in complex networks means that no cytokine alone can answer for the variation between individuals in susceptibility to infection or the severity of an infection. Statistical analyses of cytokines may be complicated by their interactions and overlapping

effects, resulting in biologically unrelated statistical associations. We used factor analysis to combine responses that were closely correlated with each other and were thus able to form new variables that could be used in the analyses. Factor analysis has seldom been used so far to evaluate the association of cytokine responses with outcome variables (Koukkunen *et al.* 2001, Booth *et al.* 2004).

7.5.2 Shortcomings of the study

The study design in articles I and II was retrospective. The diagnosis had been based on a rapid diagnostic test in 13 cases and on an in-house laboratory method of EIA in the remaining 63. Taking the reverse-transcription-polymerase chain reaction as a reference, the sensitivity of the rapid test used has been shown to be about 80%, with a specificity of about 90% (Aslanzadeh *et al.* 2008). The laboratory methods for antigen detection have shown sensitivity and specificity rates of up to 95 and 100 %, respectively (Waris *et al.* 2007). It is common in clinical practice to take a new sample for laboratory analysis if the result of a rapid test on a symptomatic patient is negative. This is likely to reduce the number of patients with false-negative findings. We were unable to track the number of possible subjects with a negative result in the rapid test and a positive result in the laboratory test. Further, we may have missed some false-negative subjects, but we consider reaching the true-positive subjects more important. We also excluded all potential controls who had been hospitalized during RSV epidemics at an age of under 12 months regardless of whether RSV or other viruses had been identified during hospitalization or not.

The retrospective study setting may have caused a recall bias with regard to symptoms of wheezing and atopic diseases, but is not likely to affect the data on physician-diagnosed diseases. We were able to reach 87% of the subjects eligible, which makes the comparisons reliable. The low amount of skin prick test positivity among the subjects attending the clinical examination might reflect a selection bias, as children who have already been extensively examined are less likely to be interested in participating. We did evaluate the occurrence of atopic diseases by means of the questionnaire among those subjects not participating in the clinical study, however, and found no differences among those refusing and those participating. The considerable diversity in the occurrence of asthma and atopic sensitization also makes selection bias unlikely.

Some kind of a selection bias among the controls is likely to have occurred, since parents of children suspected of having atopic diseases are more likely to

participate. Since about 78% of the controls contacted at first agreed to participate, we regard a considerable selection bias as improbable. The overall number of participants was relatively low, but still high enough for significant differences to be found in atopic sensitization. The sample size was calculated according to the findings of Sigurs *et al.* on the occurrence of asthma, but they found a much greater difference between the subjects and controls than in the present work (Sigurs *et al.* 2000).

The serum IFN- γ and sICAM-1 concentrations reflect production at a certain point in time and not the general capacity of an individual to produce cytokines, for example. The production measured here was probably susceptible to uncontrolled confounding factors resulting from ongoing processes in the host. These factors are unlikely to explain the differences found between the subjects and controls, which are in line with the clinical findings.

We examined exposure to an RSV epidemic in article III and not a verified infection among infants. Since RSV infection is so common, it is likely that a substantial proportion of infants will catch the disease during an epidemic. On the other hand, only a few cases of RSV infection are found outside epidemics, implying that the vast majority of those unexposed to an epidemic remain healthy. These cohorts of children thus represent infected and uninfected infants well enough to show the impact of RSV infection on asthma medication, if any. The severity of the infection might affect the findings, though, as the percentage of children hospitalized for a primary infection is generally low. If only the most severe infection had an effect on the development of asthma, this would not be noticed in the population level. In the Tucson Children's Respiratory Study it was found, that those with a mild infection had an increased frequency of wheezing afterwards (Stein *et al.* 1999).

We were unfortunate in having a sufficient number of cord blood cells only in about a half of the cases (article IV). For cytokine analyses other than unstimulated or LPS-stimulated responses the numbers were even lower making reliable comparisons between the groups impossible. Significant differences were found for LPS-stimulated responses, however. *In vitro* studies of stimulated cytokine responses are naturally not capable of simulating the complex immunological processes taking place in the human body perfectly, but we suggest that our measurements represent a better way of evaluating differences in individual cytokine production capacity than *in vivo* studies on cytokines measured during RSV infection.

We probably did not catch all potential subjects with a mild RSV infection, because entering the study was in those cases solely based on parental interest in contacting us. This may have caused a selection bias as compared with those with a more severe infection and thus a need to seek for medical help. Further, the healthy controls were invited in the study and the decision of participating among them was based on aspects other than among the other groups. It is unclear how this selection bias might have affected the findings.

In article IV the diagnosis was based on an in-house laboratory EIA method. As previously mentioned, the laboratory methods for antigen detection have shown high sensitivity and specificity relative to the reverse-transcription-polymerase chain reaction (Waris *et al.* 2007). Their sensitivity is lower, however, if the volume of the sample is small, as might be the case among subjects with mild symptoms and little sputum formation. Our control group of 28 children with a non-RSV infection included 18 with no virus identified by antigen detection or polymerase chain reaction, two of which were hospitalized. It is possible that some of these children were misclassified and actually should have been in the group treated as outpatients for an RSV infection. This kind of misclassification would have affected the comparisons between the groups, but not those between the hospitalized children and the healthy controls.

We have not studied the relation of cord blood cytokine responses to the development of asthma and atopy. Thereby no firm conclusions can be reached as to whether the same cytokine pattern can affect both the severity of RSV infection and the post-infectious sequelae. This is a subject that would call for further investigation.

8 Conclusions

8.1 Does RSV infection during infancy have a positive association with the development of asthma and atopy during childhood?

We found a positive, though non-significant association of RSV infection in infancy with the development of asthma, while the association with atopic sensitization was negative and no association was found between RSV infection and atopic diseases. The mean age of the RSV subjects at asthma diagnosis was significantly lower than that of the controls. In the light of previous studies we suggest a positive association of RSV infection with the development of mainly wheezing rather than persistent asthma, with a loss of the effect occurring with increasing age. We agree with other authors that the later development of asthmatic symptoms among children hospitalized for RSV infection in infancy is mainly non-atopic or independent of atopy.

8.2 Do children with an RSV infection in infancy differ in their immunological parameters after the infection from those who remain healthy, and do these differences relate to the development of asthma and atopic allergy?

We found significantly higher serum concentrations of IFN- γ and sICAM-1 among children of age 7–9 years hospitalized for an RSV infection in infancy than in the controls. IFN- γ was more often detectable in the RSV subjects with asthma or wheezing than in the controls with asthma or wheezing, while no significant differences in the concentration of IFN- γ were found between the RSV subjects with or without asthma or wheezing. The sICAM-1 concentration during the previous 12 months was higher in the RSV subjects with wheezing than in the corresponding controls, but no differences were found between the RSV subjects and the controls with an asthma diagnosis. We suggest that RSV infection may be able to identify children with a pre-existing tendency to produce high amounts of IFN- γ and sICAM-1 and thus having a tendency to wheeze during viral respiratory infections. Another explanation for our findings could be that an early RSV infection induces long-term immunomodulatory changes, as seen in the higher production of IFN- γ and sICAM-1.

8.3 Does RSV infection affect the occurrence of asthma to such a degree that it could be seen in terms of the consumption of asthma medication at the population level?

The consumption of asthma medication was similar among children aged 3–16 years irrespective of exposure to an RSV epidemic at age under six months. We suggest that RSV infection during infancy identifies infants with susceptibility to both the infection and the development of asthma and does not cause immunomodulation leading to asthma development.

8.4 Can susceptibility to RSV infection or its severity be predicted from immune responses measured at birth?

We found that children hospitalized for RSV infection at the age of less than six months had had a different LPS-stimulated cytokine response profile at birth from children with no respiratory infections. The innate immunity cytokines IL-6 and IL-8 were found to correlate with each other in factor analysis, and this combination was found in logistic regression analysis to predict hospitalization for RSV infection. No combination of cytokines formed in the factor analysis predicted the susceptibility to RSV infection in general. We suggest that a predisposition to develop exaggerated innate immune responses may be a factor affecting the severity of RSV infection in infancy. The role of an atopic Th2-type response does not seem to be important in this respect.

9 Summary

We suggest a positive but subsiding association between RSV infection and subsequent asthma and a negative association with atopic sensitization as demonstrated in articles I and II. Likewise, children who had had an early RSV infection were found to differ from their healthy controls in their immune parameters 7–10 years later. The study design does not make it possible to answer the question of causality. According to the findings presented in article III, however, we suggest that RSV does not cause asthma, since a causal effect should have been seen in an increase in the consumption of asthma medication, although this is to some extent a matter of severity of the infection. The findings in article IV show definite differences in innate immune responses at birth between children with subsequent RSV infection severe enough to necessitate hospitalization and those with a similar exposure who remained healthy. No differences in immune responses suggestive of atopy were found in logistic regression analysis, which is consistent with previous findings of no (or a negative) association of RSV infection with atopy. In conclusion, we suggest that RSV does not induce asthma but inborn features of immunity affect the severity of RSV infection and the postinfectious development of asthma, though not atopy.

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- I Juntti H, Kokkonen J, Dunder T, Renko M, Niinimäki A & Uhari M (2003) Association of an early respiratory syncytial virus infection and atopic allergy. *Allergy* 58: 878–884
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