Respiratory syncytial virus induced recurrent wheeze: genetic determinants & preventive glucocorticosteroid treatment

Marieke Ermers

Printing: Optima Grafische Communicatie, Rotterdam
ISBN: 978-90-8559-532-8

Address of Correspondence:
Marieke JJ Ermers, Wilhelmina Children's Hospital, University Medical Center Utrecht,
Department of Pediatric Infectious Diseases, PO Box 85090, 3508 AB Utrecht,
The Netherlands.
E-mail: marieke@ermers.net.

Respiratory syncytial virus induced recurrent wheeze: genetic determinants & preventive glucocorticosteroid treatment

Recidiverend piepen na respiratoir syncytieel virus lagere luchtweg infectie: genetische determinanten & preventieve behandeling met glucocorticosteroïden
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op vrijdag 5 juni 2009 des middags te 2.30 uur

door

Maria Johanna Jacoba Ermers

geboren op 12 februari 1975 te Nijmegen

Promotor:	Prof.dr. J.L.L. Kimpen
Co-promotoren:	Dr. L.J. Bont Dr. M.M. Rovers

The studies presented in this thesis were funded by the Dutch Asthma Foundation (Grant nr. 3.2.03.22). Publication of this thesis was kindly supported by Abbott BV; Dutch Asthma Foundation; J.E. Jurriaanse Stichting; Merck BV; Nycomed BV; Stichting Astma Bestrijding; Stichting WKZ Fonds & Teva Nederland.

CONTENTS

General Introduction

RSV LRTI

Respiratory syncytial virus (RSV) is a single-stranded enveloped RNA virus within the family of paramyxoviridae. During yearly winter epidemics, RSV circulates within the community. (1) RSV infects more than 50% of all infants in their first year of life and at age two 90% of infants have been infected.(2;3) Inflammation and mucous obstruction of the small airways following RSV infection lead to lower respiratory tract infection (LRTI). About 1% of the population is hospitalized for RSV LRTI during the first year of life.(4)

Both direct virus-induced airway damage and RSV-induced inflammation may contribute to LRTI in RSV-infected infants. This indicates that, at the time of RSV infection, a complex series of events takes place in which both the virus and the host play a role. Since the association between viral load and disease severity is controversial,(5;6) the common belief is that the RSV-induced inflammatory reaction of the host has a major impact on disease severity.(7;8)

RECURRENT WHEEZE

Follow-up studies consistently show that approximately half of the infants with RSV LRTI go on to have recurrent wheeze episodes during childhood.(9-11) The mechanisms by which RSV LRTI results in long-term airway morbidity are poorly understood. It is still a matter of debate whether recurrent wheeze following RSV LRTI is mainly a nonallergic condition with a good long-term prognosis or the early onset of allergic asthma. Sigurs *et al.* (12) reported a strong association between RSV LRTI in the first year of life and asthma, clinical allergy, and allergic sensitization up to adolescence. Other studies could not confirm the association between RSV LRTI and subsequent allergic sensitization and showed a transient wheezing pattern diminishing with age.(10;11) Early wheeze during the first years following RSV LRTI has a high prevalence,(13) influences quality of life(14) and generates substantial health-care costs.(15) In this thesis we compare characteristics of early and late wheeze following RSV LRTI. Current research suggests that both genetic and environmental factors determine the type of immune response to the acute RSV infection and that the type of response, in turn, may affect the development of early wheeze.(16;17) In this thesis we investigate the associations of genetic polymorphisms and early wheeze following RSV LRTI. In addition we investigate the role of RSV reinfection in the development of early wheeze following RSV LRTI (Table 1; Figure 1).

Table 1. Aims and hypotheses.

Aims
• To evaluate the effectiveness of early-initiated high-dose inhaled glucocorticosteroids to prevent early wheeze following RSV LRTI
• To increase knowledge about pathofysiological mechanisms underlying early wheeze following RSV LRTI
Hypotheses
• Early and late wheeze following RSV LRTI are distinct entities
• Early-initiated high-dose inhaled corticosteroids prevent the occurrence of early wheeze following RSV LRTI
• RSV reinfection contributes to early wheeze following RSV LRTI
• The occurrence of wheeze following RSV LRTI is influenced by genetic variance

TREATMENT

No effective treatment for RSV LRTI or subsequent recurrent wheeze is currently available for the general population. Research focused on the development of agents aimed to prevent RSV LRTI. Vaccination and passive administration of neutralizing antibodies and antiviral and anti-inflammatory medication have been investigated.(18-22) Vaccination experienced a major setback in the 1960s when the use of formalin-inactivated virus in young babies resulted in increased disease severity and death following subsequent virus infection.(23) Palivizumab, an anti-RSV monoclonal antibody, reduces hospitalization and subsequent recurrent wheeze in high-risk preterm infants. Due to its high costs palivizumab is not available for the general population.(24)

Inhaled Glucocorticosteroids To Prevent Recurrent Wheeze Following RSV LRTI

The infection and concomitant inflammatory reaction in the acute phase of RSV LRTI might lead to early wheeze. Therefore, immune modulation during the acute phase of RSV LRTI might influence subsequent early wheeze. Although evidence regarding the effectiveness of early anti-inflammatory therapy in the prevention of early wheeze is

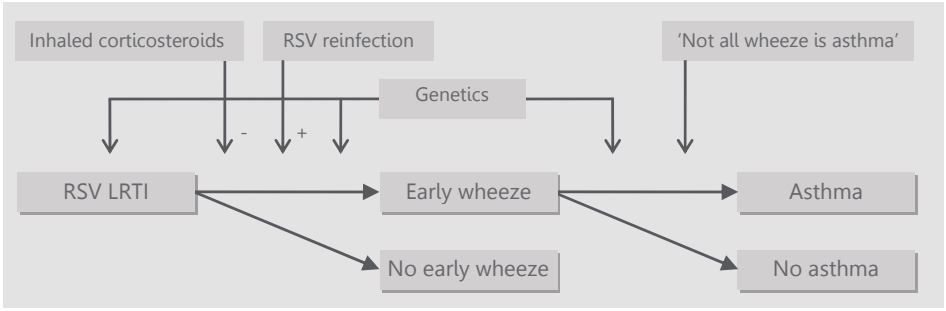

Figure 1. The hypotheses assessed in this thesis.

conflicting,(25-27) use of inhaled glucocorticosteroids is very common.(28) In this thesis we investigate the effectiveness of early-initiated high-dose hydrofluoroalkane extrafine beclomethasone dipropionate to prevent early wheeze following RSV LRTI. A methodologically sound large placebo controlled randomized trial into the effect of inhaled glucocorticosteroids on recurrent wheeze following RSV LRTI might help in formulating final recommendations regarding the use of glucocorticosteroids in infants hospitalised for RSV LRTI.(21)

OUTLINE OF STUDIES

Chapter 1 provides a general introduction to the field of viral infections and childhood asthma. In Chapter 2, clinical, genetic and immunological determinants of early and late wheeze following RSV LRTI are investigated. Chapter 3 reports the results of a systematic Cochrane Review to evaluate the evidence for corticosteroids to prevent post-bronchiolitis wheeze. Chapter 4 and 5 report on the placebo controlled randomized trial that we conducted to evaluate the effectiveness of early-initiated high-dose inhaled hydrofluoroalkane extrafine beclomethasone dipropionate to prevent early wheezing following RSV LRTI. Chapter 6 reports on potential associations between genetic polymorphisms and severe RSV LRTI, and Chapter 7 reports on genetic polymorphisms and recurrent wheeze following RSV LRTI. In Chapter 8 the role of RSV-reinfection during episodes of airway symptoms following initial RSV LRTI is evaluated. The thesis ends with a brief discussion and a summary of all findings.

REFERENCE LIST

(1) Ogra PL. Respiratory syncytial virus: the virus, the disease and the immune response. Paediatr Respir Rev 2004; 5 Suppl A:S119-S126.

(2) Glezen P, Denny FW. Epidemiology of acute lower respiratory disease in children. N Engl J Med 1973; 288(10):498-505.

(3) Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. Am J Dis Child 1986; 140(6):543-546.

(4) Iwane MK, Edwards KM, Szilagyi PG, Walker FJ, Griffin MR, Weinberg GA et al. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. Pediatrics 2004; 113(6):1758-1764.

(5) Buckingham SC, Bush AJ, DeVincenzo JP. Nasal quantity of respiratory syncytical virus correlates with disease severity in hospitalized infants. Pediatr Infect Dis J 2000; 19(2):113-117.

(6) Wright PF, Gruber WC, Peters M, Reed G, Zhu Y, Robinson F et al. Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. J Infect Dis 2002; 185(8):1011-1018.

(7) Meissner HC. Selected populations at increased risk from respiratory syncytial virus infection. Pediatr Infect Dis J 2003; 22(2 Suppl):S40-S44.

(8) Simoes EA, Carbonell-Estrany X. Impact of severe disease caused by respiratory syncytial virus in children living in developed countries. Pediatr Infect Dis J 2003; 22(2 Suppl):S13-S18.

(9) Sigurs N. A cohort of children hospitalised with acute RSV bronchiolitis: impact on later respiratory disease. Paediatr Respir Rev 2002; 3(3):177-183.

(10) Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 1999; 354(9178):541-545.

(11) Bont L, Steijn M, van Aalderen WM, Brus F, Th Draaisma JM, Diemen-Steenvoorde RA et al. Seasonality of long term wheezing following respiratory syncytial virus lower respiratory tract infection. Thorax 2004; 59(6):512-516.

(12) Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F et al. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med 2005; 171(2):137-141.

(13) Kuehni CE, Davis A, Brooke AM, Silverman M. Are all wheezing disorders in very young (preschool) children increasing in prevalence? Lancet 2001; 357(9271):1821-1825.

(14) Bont L, Steijn M, van Aalderen WM, Kimpen JL. Impact of wheezing after respiratory syncytial virus infection on health-related quality of life. Pediatr Infect Dis J 2004; 23(5):414-417.

(15) Stevens CA, Turner D, Kuehni CE, Couriel JM, Silverman M. The economic impact of preschool asthma and wheeze. Eur Respir J 2003; 21(6):1000-1006.

(16) Singh AM, Moore PE, Gern JE, Lemanske RF, Jr., Hartert TV. Bronchiolitis to asthma: a review and call for studies of gene-virus interactions in asthma causation. Am J Respir Crit Care Med 2007; 175(2):108-119.

(17) Stensballe LG, Simonsen JB, Thomsen SF, Larsen AM, Lysdal SH, Aaby P et al. The causal direction in the association between respiratory syncytial virus hospitalization and asthma. J Allergy Clin Immunol 2009; 123(1):131-137.

(18) Fuller H, Del Mar C. Immunoglobulin treatment for respiratory syncytial virus infection. Cochrane Database Syst Rev 2006;(4):CD004883.

(19) Ventre K, Randolph AG. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. Cochrane Database Syst Rev 2007;(1):CD000181.

(20) Patel H, Platt R, Lozano JM, Wang EE. Glucocorticoids for acute viral bronchiolitis in infants and young children. Cochrane Database Syst Rev 2004;(3):CD004878.

(21) Blom D, Ermers M, Bont L, van AW, van WJ. Inhaled corticosteroids during acute bronchiolitis in the prevention of post-bronchiolitic wheezing. Cochrane Database Syst Rev 2007;(1):CD004881.

(22) Chavez-Bueno S, Mejias A, Welliver RC. Respiratory syncytial virus bronchiolitis : current and future strategies for treatment and prophylaxis. Treat Respir Med 2006; 5(6):483-494.

(23) Kim SG, Card JW, Brandt C. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 89, 422-434. 1969.

(24) Simoes EA, Groothuis JR, Carbonell-Estrany X, Rieger CH, Mitchell I, Fredrick LM et al. Palivizumab prophylaxis, respiratory syncytial virus, and subsequent recurrent wheezing. J Pediatr 2007; 151(1):34-42, 42.

(25) Reijonen T, Korppi M, Kuikka L, Remes K. Anti-inflammatory therapy reduces wheezing after bronchiolitis. Archives of Pediatrics & Adolescent Medicine 1996; 150(5):512-517.

(26) Carlsen KH, Leegaard J, Larsen S, Orstavik I. Nebulised beclomethasone dipropionate in recurrent obstructive episodes after acute bronchiolitis. Arch Dis Child 1988; 63(12):1428-1433.

(27) Kajosaari M, Syvanen P, Forars M, Juntunen-Backman K. Inhaled corticosteroids during and after respiratory syncytial virus-bronchiolitis may decrease subsequent asthma. Pediatric Allergy and Immunology 2000; 11(3):198-202.

(28) Chauliac ES, Silverman M, Zwahlen M, Strippoli MP, Brooke AM, Kuehni AC. The therapy of pre-school wheeze: appropriate and fair? Pediatr Pulmonol 2006; 41(9):829-838.

Chapter 1

Viral Infections And Childhood Asthma

Marieke JJ Ermers

Louis J Bont

The Microbe-Host Interface In Respiratory Tract Infections. Edited by Jan LL Kimpen and Octavio Ramilo. Horizon Bioscience 2005; 253-69.

ABSTRACT

Viral respiratory tract infections (VRTI) have a strong epidemiological relationship with wheezing disorders. At least three different types of relationships between VRTI and wheezing disorders have been described. First, VRTI during early childhood can induce recurrent wheezing. For example, respiratory syncytial virus (RSV) infection is followed by post-bronchiolitis wheezing in about 50% of cases. Pre-existent diminished lung function is probably an important mechanism of virus-induced wheezing. It is debated whether viral infections can change immunological status or induce allergy. Second, viral infections are the most important triggers of asthma exacerbations in atopic patients. In 85% of asthma exacerbations in children VRTI are found in airways. In the majority of cases rhinovirus is the infectious agent. Third, exposure to respiratory viruses during infancy might prevent allergy development. The mechanism of virus-induced prevention of asthma is by skewing the immune system towards a Th1-phenotype. Different types of relationships between VRTI and wheezing can co-exist in one individual. For example, common colds during infancy may induce both increased risk of recurrent wheezing during childhood and decreased risk of allergic asthma later in life. The temporal relationship between viral infection and allergy development, as well as the interplay between genetic factors and viral infection, appear the keys to understand the intriguing relationship between viruses and asthma.

INTRODUCTION

Viral infections during childhood are strongly related to wheezing and asthma. Different types of relationships can be considered. First, acute viral lower respiratory tract infections (LRTI) can lead to airway obstruction and wheezing, often referred to as bronchiolitis. Respiratory syncytial virus (RSV) is the single most important cause of bronchiolitis. In 50% of hospitalized infants, bronchiolitis is followed by recurrent episodes of wheezing. Second, upper respiratory tract infections may contribute to asthma exacerbations and may facilitate allergic responses. In fact, up to 80% of exacerbations of asthma in children coincide with viral infections, most of which are attributable to rhinovirus. By apparent contrast, recent epidemiological studies suggest the possibility of a third potential relationship between viral infection and development of asthma. It is hypothesized that decreased exposure to common viral infections during early childhood is associated with increased risk of asthma and allergic diseases (hygiene hypothesis). This chapter provides an overview of co-existing relationships between viral infections and childhood asthma and emphasizes the importance of timing in the interplay between infection and allergen sensitization.

WHEEZING DURING CHILDHOOD

The Epidemic Of Childhood Asthma

Asthma can be defined as an inflammatory disease of the airways, characterized by intermittent airway narrowing and variable symptoms of chest tightness, wheeze and shortness of breath. It is now a leading cause of chronic illness in childhood. The asthma complex comprises non-atopic, e.g. viral-associated wheezing, and atopic asthma. As many as 10-15% of boys and 7-10% of girls may have some form of asthma at some time during childhood. Before puberty, approximately twice as many boys as girls are affected; thereafter the sex prevalence is equal. During the last three decades both prevalence and mortality rate of asthma have increased.(1) Asthma is most common in industrialized nations with a 'Western' life style with a prevalence up to twenty times higher compared to undeveloped countries. The explanation for these differences is not clear. It has been suggested that improved hygiene in the Western world results in decreased exposure to infectious diseases.(2) Decreased exposure to common infections during early childhood would then result in increased tendency to atopic responses and therefore leads to increased incidence of atopic asthma.

Different Wheezing Phenotypes

During the last decades wheezing illness has become one of the most frequent causes of consultation in pediatric practice. Wheezing can accompany viral respiratory tract infection and is a well-known sign of childhood asthma. Wheezy respiration is a nonspecific sign associated with restriction of airflow through narrowed airways and is believed to be generated by turbulent airflow causing oscillation of the bronchial wall. Wheezing during infancy seems to reflect a heterogeneous condition comprising different phenotypes and different underlying mechanisms. It is related to both atopic and non-atopic status. For example, RSV bronchiolitis is associated with recurrent non-atopic wheezing up to the age of ten. This relationship is transient, since a history of RSV LRTI is no longer associated with wheezing at the age of thirteen. To identify characteristics of children who will persist in wheezing beyond infancy, Martinez and colleagues attempted to distinguish different 'wheezing patterns'. They observed the clinical 'wheezing behavior' and related this to several population risk factors and lung function outcomes. Based on these outcomes wheezers were divided in 'transient' and 'persistent' wheezers (Table 1). 'Transient wheezers' experience wheezing episodes during the first three years of life, but do not wheeze anymore at age six. Transient wheezing is associated with reduced lung function during infancy but not with atopic status. 'Persistent wheezers' experience wheezing episodes during early childhood and persist in wheezing during school age. Some of them do not wheeze as infant but start to experience wheezing later on. Lung function during infancy is within normal ranges. Children with a family predisposition for allergies or increased serum IgE levels during the first year of life are at increased risk of persistent wheezing later in life.(3)

In general, transient wheezing subsides during school age, and is non-atopic in origin. Persistent wheezing in school-age children appears to be associated with atopic status

Table 1. Classification of wheezing illness during childhood[1,2]

	Lung function			Association with atopic status
	0 years - FRC	6 years - FRC	11 years - PEFvar /BHR	
No wheezing	normal	normal	normal	no
Transient wheezing	decreased	decreased	normal	no
Persistent wheezing	normal	normal/ decreased	increased	yes

Note. FRC, functional residual capacity (mean flow); PEFvar, peak expiratory flow variability; BHR, bronchial hyperreactivity to metacholine.

[1]Martinez FD, Wright AL, Taussig LM et al. Asthma and Wheezing in the First 6 Years of Life. New England Journal of Medicine 1995; 332(3):133-138.

[2]Stein RT, Holberg CJ, Morgan WJ et al. Peak flow variability, methacholine responsiveness and atopy as markers for detecting different wheezing phenotypes in childhood. Thorax 1997; 52(11):946-952.

and in this group, wheezing experienced in early life is the first manifestation of allergic asthma.

Risk Factors For Childhood Asthma

Several risk factors are associated with development of asthma (Table 2).(1;4) The complex of genetic, immunologic and environmental influences determines the development of asthma. Atopic status and asthma are strongly familial and have a genetic basis, which will be discussed later in this chapter. Immunological events during early life play a role in the development of asthma. In both atopic and non-atopic children, IFNγ production is low at birth (Th2 skewing). Low IFNγ production at birth is thought to be exaggerated in atopic children. Moreover, transition from Th2-type to Th1-type immunity is thought to be delayed in these children. The initial persistence of Th2-type immunity in atopic children reveals the potential for a critical period in the development of the immune system. In this critical period atopic children may be more vulnerable to environmental interactions with aeroallergens or viruses that may produce adverse consequences within the developing airway. Environmental influences, such as exposure to cigarette smoke, diesel,(5) allergens and cold, have been extensively studied in relation to childhood asthma and post-LRTI wheezing. Several known risk factors for allergic asthma could not be associated with post-LRTI wheezing. It now becomes clear that different patterns of childhood asthma have their own pathogenesis and therefore, their own risk factors.

RSV Bronchiolitis And Recurrent Wheezing

During the first three years of life, RSV is the predominant cause of wheezing LRTI. RSV occurs in winter epidemics and infects almost all children before the age of two. RSV in-

Table 2. Potential risk factors for childhood asthma.

Genetics	Atopy
	Parental history of asthma (especially maternal)
	Male sex
Immunology	IFNy production
	Th2 skewing
Environment	Socio-economic status
	Western lifestyle
	Diet
	Passive exposuse to cigarette smoke
Infections	Respiratory syncytial virus infections
	Other agents
Others	Prematurity
	Lung function abnormalities

fection causes upper respiratory tract symptoms and (less often) lower respiratory tract symptoms. Typically, infants present with a runny nose, cough, dyspnoe and wheeze. When signs of airflow limitation accompany RSV LRTI, it is referred to as RSV bronchiolitis. Approximately ten percent of children hospitalized with RSV bronchiolitis require intensive care because of severe respiratory distress. Risk factors for severe disease are neonatal status, prematurity with or without chronic lung disease, congenital cardiac disease and immune deficiencies. Prospective studies have investigated the incidence of recurrent wheezing following RSV bronchiolitis and the association with the allergic phenotype. About half of infants with RSV bronchiolitis develop recurrent wheezing, usually associated with common colds. Wheezing occurs five to six times more frequent following RSV bronchiolitis than in children who do not experience RSV bronchiolitis. Stein *et al.* studied the occurrence of LRTI and subsequent wheezing in the general population.(6) RSV was detected in 43.9% of all children with LRTI symptoms in the first three years of life. In 14.4% of children para-influenza was detected and in another 14.4% of children other agents (adenovirus, influenza, chlamydia, cytomegalovirus, rhinovirus, bacteria and mixed infections) were detected. In 27.3% of children no viruses were isolated. Compared to children with no LRTI, those with RSV LRTI are 3.2 times more likely to have infrequent wheeze and 4.3 times more likely to have frequent wheeze at the age of six. Similar but less obvious trends were observed for LRTI due to other viruses and where no viruses were isolated. No significant association between a history of LRTI and positive skin-prick reactivity to allergens was found. Similarly, allergen-specific serum IgE concentrations and a history of LRTI were not related. Post bronchiolitic wheezing thus appears to occur independent of atopic sensitization. In line with this study Bont *et al*. showed that besides allergen-driven Th2 cytokines, virus-induced changes in cytokine responses may lead to asthmatic symptoms like wheezing.(7) It was shown in a prospective study in 50 hospitalized children that IL10 levels, measured during the convalescent phase of RSV infection, are significantly higher in patients who develop recurrent wheezing during the year following RSV bronchiolitis than in patients without recurrent episodes of wheezing. Although these data may suggest that the subsequent development of wheezing following LRTI is not caused by an increase of allergic sensitization, other data suggest the opposite. Sigurs *et al*. found that RSV bronchiolitis during the first year of life forms an important risk factor for the development of asthma and sensitization to common allergens.(8) A positive test for IgE antibodies was noted in 32% of RSV children and in 9% of children in the control group. The apparent differences in results may be explained by different study methodologies.

A large number of studies have attempted to identify clinical risk factors that determine the development of wheezing following RSV LRTI. In particular, known risk factors for allergic asthma have been evaluated. Age at onset of disease, sex, disease severity, and cigarette smoke exposure could not be associated with post-bronchiolitis wheezing.

Recently, the predictive value of signs of airflow limitation during RSV LRTI subsequent wheezing was evaluated.(9) The incidence of recurrent wheezing during follow-up was significantly lower for infants without signs of airflow limitation during the acute RSV infection.

In summary, RSV is the major cause of LRTI in infants and about half of infants will go on to experience recurrent wheezing. There is ongoing debate whether RSV bronchiolitis leads to allergic sensitization; results from prospective studies are inconclusive. Thus far, airflow limitation during primary infection is the only available predictor of wheezing following RSV LRTI.

INCEPTION OF ASTHMA

Asthma Pathogenesis

Asthma is a complex disorder involving autonomic, immunologic, infectious, endocrine and psychologic factors in varying degrees in different individuals. Manifestations of airway obstruction are due to bronchoconstriction, hypersecretion of mucus, mucosal edema, cellular infiltration and desquamation of epithelial and inflammatory cells. Various stimuli initiate bronchoconstriction and inflammatory responses in the presence of hyperreactive airways. These stimuli include exposure to environmental allergens, especially animals, moulds, pollens and mites, cold, exercise, air pollution and drugs. Many clinicians consider asthma virtually synonymous with allergic asthma. Allergy results from inappropriate IgE response to common allergens in genetically susceptible individuals.

Th2 cells are thought to have a pivotal role in orchestrating the recruitment and activation of effector cells of the allergic response. The theoretical model concerns the equilibrium between Th1 and Th2 cells which are defined by their own cytokine profiles. Th1 cells are defined by the production of Th1-type cytokines, such as IFNγ and IL2. Th1 cells promote cell-mediated defense by activating cytotoxic T-cells and macrophages. Th2 cells are defined by the production of Th2 cytokines and provide help to B cells required for humoral immunity. IL4 is thought to be a prerequisite for Th2 responses, including IgE production. IL5 is required for attraction and activation of eosinophils. An imbalance of the Th1-Th2 cytokine balance in favor of Th2 is thought to be the immunopathological substrate for allergic asthma.

The pathogenesis of non-atopic wheezing illness is less well understood. Important determinants appear structural airway changes, lung function abnormalities and recurrent RTI. Non-allergic immunological mechanisms in this type of wheezing disease are largely to be defined. As discussed below, animal studies have suggested that IL10 production,

independent from Th2-type responses, plays an important role in the pathogenesis of non-atopic asthma.

Genetics Of Asthma

Asthma and the atopic state are strongly familial and have a genetic background. Studies of twins show that concordance rates for asthma are significantly higher in monozygotic twins than dizygotic twins, and that heritability of asthma is as high as 75%.(10) In contrary, airway morbidity following RSV is not associated with parental history of asthma.(9)
Genetic studies have provided compelling evidence that as many as 20 genes are involved in conferring increased risk of allergic asthma. Polymorphisms of Th2-type genes, including *IL4*, *IL4R*, *IL13* and *CD14* polymorphisms, are the base of familial predisposition for allergy related diseases. *IL4*, *IL4R* and *IL13* are associated with upregulation of IgE, the immunoglobulin typically involved in allergic reactions. The challenge for current and future studies is to understand how environmental factors, in particular viral infections, interact with these genes to reveal allergic disease.

Diminished Lung Function At Birth

It has been argued that children with a history of LRTI during infancy have a poorer lung function and a higher prevalence of bronchial hyperresponsiveness than subjects without such a history. This leads to the hypothesis that LRTI during childhood may alter normal lung development. LRTI may produce sequelae that predispose patients to chronic airflow obstruction in later life. Alternatively the reported associations can be explained by a host factor predisposing to both LRTI and abnormalities in lung function. Martinez *et al.* assessed lung function in a group of healthy infants less than six months of age before any LRTI developed and subsequently observed the incidence of LRTI during the first year of life.(11) No differences in pre-existent lung function between infants with LRTI without wheezing and infants with no LRTI at all were found, where children with wheezing LRTI had pre-existent reduced lung function. This suggests that pre-existent lower levels of lung function do not increase susceptibility to infection itself, but increase susceptibility to occurrence of clinically relevant airway obstruction. Interestingly, a further division could be made between different wheezing phenotypes (Table 1). 'Transient wheezers' had diminished pre-existent lung function where 'persistent wheezers' had pre-existent lung function levels within normal values.(12)
Taken together, diminished lung function during infancy is a risk factor for transient wheezing in childhood. It remains to be elucidated whether this reflects a causal relationship or whether other endogenous or acquired factors underlie to both reduced lung function and wheezing illnesses.

Virus-induced Wheezing Illness

It has long been recognized that children who develop wheezing LRTI are at increased risk of recurrent wheezing and possibly allergic asthma. In a birth cohort including 499 children with risk factors for atopy (elevated cord blood IgE or at least two atopic family members) and 815 children without these risk factors, the German MAS (multicentre allergy study) group investigated the association between different types of early childhood infections and subsequent development of asthma.(13) It was shown that repeated LRTI are strongly associated with subsequent wheezing and a doctor's diagnosis of asthma. This association is even stronger in children with a family history of asthma. Unfortunately the subsequent development of allergic sensitization after LRTI was not measured.

RSV is the respiratory virus most often associated with subsequent asthma and has been proposed as a cause of the inception of (allergic) asthma. An increase in prevalence of childhood asthma and hospitalizations due to RSV infection coincided over the past two decades. Sigurs *et al.* reported that infants with wheezing RSV LRTI requiring hospitalization are more likely to have allergen-specific IgE and asthma by the age of three and seven than prospectively identified control subjects.(8) It was shown that RSV LRTI forms a risk factor for asthma development and allergic sensitization even without a family history of asthma. Other studies have failed to confirm this relationship between RSV infection and allergic asthma. Stein *et al.* prospectively assessed recurrent wheezing, atopy status, and lung function in children who had confirmed viral LRTI during the first three years of life.(6) They showed that RSV LRTI is an independent risk factor for subsequent development of wheezing during the first ten years. However, no association between incidence of RSV LRTI in early life and subsequent development of allergic sensitization was found.

A number of clinical studies have related cytokine profiles during RSV LRTI to long-term airway morbidity with conflicting results. For example, Bendelja *et al.* showed by flow cytometry increased IL4 and decreased IFNγ production by peripheral blood T cells of 30 RSV infected children as compared to 10 healthy controls.(14) By contrast, Bont *et al.* found normal IFNγ/IL4 ratio during RSV LRTI and no association with subsequent wheezing.(7) In this study monocyte IL10 production during the convalescent phase of RSV LRTI correlated with long-term airway morbidity, which is in line with a mouse study showing that IL10 production independent from atopy is required for airway hyperresponsiveness (AHR).(15) These studies demonstrate that immunological mechanisms distinct from Th2-type responses can underlie the occurrence of virus-induced AHR.

Schwarze *et al.* showed in the Balb/c mouse that primary RSV infection results in AHR during the acute phase and -subsequently- facilitates ovalbumin-induced AHR. During acute RSV infection Th1-type cytokines are produced whereas in subsequently sensitized

mice (11-21 days later) responses to allergen stimulation are mainly of the Th2 type. In this model, however, it is puzzling that RSV infection enhanced OVA-induced AHR is not paralleled by increased production of allergen-specific IgE.(16)
In summary, RSV infection is strongly associated with subsequent airway disease. Most clinical studies cannot confirm that allergy induction is the underlying mechanism of RSV-associated wheezing.

Viral Infections Other Than RSV

The role of RSV in virus-induced wheezing illness may be overemphasized. RSV may not have critical characteristics that are different from other respiratory viruses. From a number of epidemiologic observations, it appears that viral infections other than RSV during early childhood are associated with chronic LRTI symptoms, including asthma. The individual contribution of specific viruses to long-term airway morbidity is not well defined. Measurements of lung function before the onset of infection indicate that children with reduced lung function in infancy are at increased risk for chronic LRT sequelae after most viral infections. The most frequently isolated infectious agents causing LRTI are RSV, adenovirus, influenza virus, para-influenza virus and Bordetella pertussis. In relation to subsequent wheezing, LRTI due to other viruses than RSV show similar trends as those caused by RSV LRTI, although less marked and consistent.(6)
Experimental parainfluenza 3 infection in a guinea pig model has shown that this virus can potentiate OVA-induced AHR.(17) Interestingly, similar mechanisms were found for RSV-induced aggravation of allergy induction. Virus-induced increase in production of neuroimmune mediators, such as bradykinin and substance P, cause increased susceptibility to allergy induction with OVA in both parainfluenza 3 and RSV.
Recently a new respiratory virus called human metapneumovirus (hMPV) has been identified in the Netherlands. The clinical symptoms following hMPV infection are similar to those caused by RSV infection and range from upper respiratory tract disease to severe bronchitis and pneumonia. By the age of five, virtually all children in the Netherlands have been exposed to hMPV.(18) Greensill *et al.* studied coinfection with hMPV in infants with RSV LRTI sufficiently severe to require admission to the pediatric intensive-care unit for ventilatory support. Bronchoalveolar lavage samples from 30 infants were collected and detection of hMPV using PCR techniques showed co-infection in 70% of the infants.(19) Co-infection with RSV and hMPV might be another determinant of RSV disease severity and of subsequent LRT symptoms. Evaluation of a role for hMPV will require longitudinal studies in hospitalized and non-hospitalized populations.

Protective Effects Of Early Exposure To Infection

Because viral LRTI contribute to the inception of asthma, the concept that viral infections play a role in preventing the development of allergic asthma appears counterintuitive. Initial momentum for this line of thinking arose in 1989 with the introduction of the "hygiene hypothesis" by Strachan. To explain the increasing prevalence of allergic diseases in many parts of the world, this theory submits that the recent epidemic of atopic disease and asthma may have occurred as a consequence of a decline in certain childhood infections or a more general lack of exposure to a broad range of infectious agents in the first years of life.(20) At birth, cytokine profiling of cord blood suggests that the newborn's mononuclear cell response is skewed toward a Th2-like phenotype. It is hypothesized that continuous infectious stimuli are necessary to provoke the immune system with a Th1 boost to depolarize the immune response. This is facilitated by the presence of older siblings, attendance of daycare centers, a farming environment, helminthic infections and exposure to microbial products. Deprivation of infectious stimuli will induce delayed Th1 development of the immune system, which may result in allergy development in susceptible individuals. Absence of Th1 stimuli results in a tendency to react on several stimuli with a Th2-like, or allergic, response. The Tucson Children's Respiratory Study Group provides support for the hygiene hypothesis. In a longitudinal birth cohort, determinants of asthma were studied.(21) Incidence of asthma and prevalence of subsequent frequent wheezing is related to number of siblings at home and day care attendance during the first six months of life, representing infectious load. Exposure to older siblings or day care attendance augments the frequency of wheezing at the age of two. In contrary, exposed children are less likely to experience frequent wheezing from the age of six through thirteen as compared to controls. They are protected against asthma as diagnosed by physicians and have a lower prevalence of high serum IgE concentrations and skin-test reactivities to allergens.

The Multicentre Allergy Study (MAS) group investigated the association between early infectious stimuli and subsequent development of asthma in another way. In a longitudinal birth cohort including 1314 children, asthma and asthma symptoms were assessed and related to different types of childhood infections in early life.(13) Repeated viral infections, other than LRTI, are inversely related to development of asthma. Particularly 'runny nose' upper RTI and viral infections of the herpes type are associated with reduced development of asthma and respiratory symptoms. Repeated viral infections early in life, other than LRTI, may stimulate the immature immune system towards the Th1 phenotype. Observed effects are strongest for infections in the first year of life. In line with the proposed immature immune system being most susceptible to the influence of infections in the first year of life, a window of vulnerability may exist. The protective function of viruses in the induction of asthma is depicted in Figure 1.

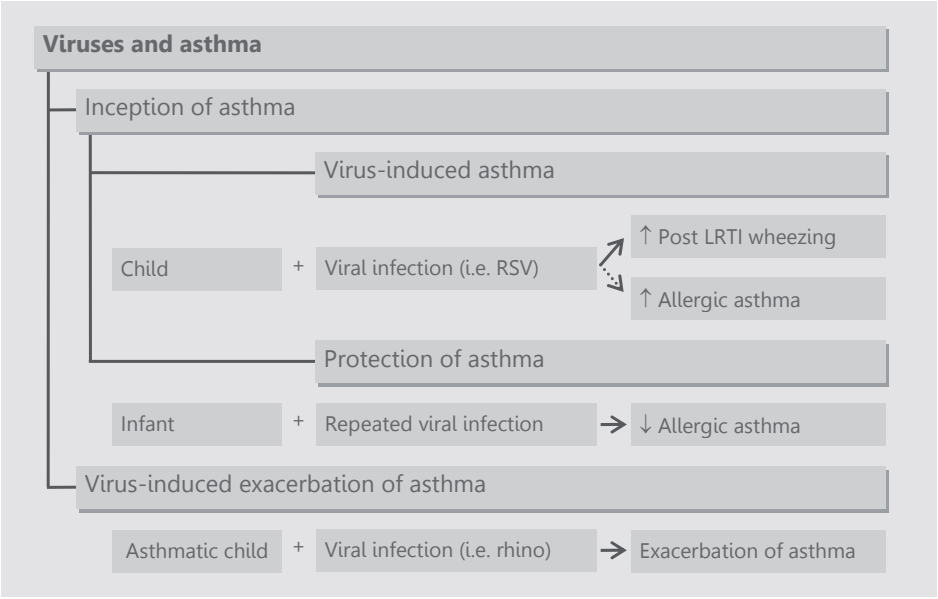

Figure 1 Possible relationships between viral infections, wheeze and asthma.

Peebles *et al.* were the first to show in a murine model that RSV infection before allergic sensitization can protect against the development of an allergic phenotype.(22) In a Balb/c mouse model they show that RSV infection prevents subsequent ovalbumin-induced AHR and eosinophilic inflammation in the lung. However, RSV infection after allergic sensitization leads to significantly increased AHR. Unfortunately OVA-specific IgE production following RSV infection and sensitization was not measured. The model shows that allergen sensitization prior to infection augments the AHR response to RSV infection, but also that infection prior to allergen sensitization protects against induction of allergy. This suggests that timing of RSV infection in relationship to allergic sensitization is a key factor in development of allergic airway disease. The protective effects of infection before allergic sensitization were also found in an influenza model,(23) in line with the strong Th1 inducing potency of influenza.

Summarized, epidemiological data provide evidence for the hygiene hypothesis. Future studies will have to dissect which specific infectious agents protect against allergy development. Moreover, the relevance of timing of infection in relation to allergy development will have to be clarified.

EXACERBATIONS OF ASTHMA

The Epidemic Link Between Viruses And Exacerbations Of Asthma

The link between respiratory infections and asthma exacerbations is well established. In the 1950's this association was wrongly attributed to bacterial allergy, but nowadays it is clear that the majority of exacerbations are associated with viral infections. Several studies have observed a temporal relation between viral infections and asthma exacerbations, while other ones have found associations between severity of wheezing illness and rate of viral detection. In early epidemiological studies the role of viral infections may have been underestimated due to difficulties in both isolation and identification of viruses. Introduction of molecular biology techniques, such as polymerase chain reaction (PCR) gave new impulse to epidemiological studies. Johnston *et al*. studied the association between upper and lower respiratory viral infections and acute exacerbations of asthma in schoolchildren. 108 children aged nine to eleven who reported wheeze or cough on a questionnaire were included. Using PCR-based assays upper respiratory viruses were detected in 80-85% of the cases of asthma exacerbations. Picornaviruses, mostly rhinovirus, accounted for two thirds of the viral infections. Coronavirus was the next commonest but caused less severe asthma exacerbations than other viruses.(24) Adenoviruses, enteroviruses and coronaviruses are detected less frequently and influenza is only found during annual epidemics. The rate of detection of viruses when individuals are asymptomatic, between asthma exacerbations, is only in the order of 3-12%.(25)

Rhinovirus Infection And Exacerbations Of Asthma

Whereas other respiratory viruses (such as influenza, para-influenza, RSV and adenovirus) are well recognized causes of LRTI such as pneumonia and bronchiolitis and are capable of replication in the lower airways, there has been uncertainty as to whether rhinovirus infection can reach the lower airways. Most rhinoviruses preferentially grow at temperatures from thirty-three degrees to thirty-five degrees, and this temperature level has been cited as a potential barrier to grow in the lower airway. There is evidence, however, that temperature may not be an absolute barrier to rhinovirus replication in lower airways. Lower airway temperatures have been directly mapped using a bronchoscope equipped with a thermometer. During quiet breathing of air at room temperature, airway temperatures are conducive to rhinovirus replication down to second generation bronchi and in fact, exceed thirty-five degrees only in peripheral airways. In line with these measurements, rhinovirus has now been detected in lower airway clinical specimens such as sputum, tracheal brushings and BAL by both PCR and culture methods. These data confirm that rhinovirus infection of the lower airways does occur.(26)

Epidemiological data show that rhinovirus produces common cold-like symptoms in non-asthmatic persons. It also produces significant alterations in lower airway physiology in asthmatic patients. It is postulated that people with asthma are more susceptible to rhinovirus infection than healthy individuals. Corne *et al.* compared the susceptibility of these groups.(27) They recruited 76 cohabiting couples, adults aged 18 to 50, of which one person had atopic asthma and the other one was healthy. Nasal aspirates, taken every two weeks, were examined for rhinovirus. It was found that people with atopic asthma were not at greater risk of rhinovirus infection than healthy individuals but that they suffered from lower respiratory tract symptoms more often, to a higher degree and longer. The effect of rhinovirus infection on the frequency of upper respiratory tract clinical illness was independent of atopic asthma.

To determine the causality of rhinovirus infections accompanying asthma exacerbations, effects of experimental inoculated rhinovirus infection were measured in adults. Grünberg *et al.* examined the effect of experimental rhinovirus 16 (RV 16) infection on home recordings of FEV1, common cold and asthma symptoms, lung function and airway hyperresponsiveness.(28) In a placebo-controlled study in adult subjects with atopic asthma they showed that experimental RV 16 infection induces the clinical and functional features of a mild asthma exacerbation. Experimental RV 16 infection leads to a transient fall in daily home recordings of FEV1 in asthmatic individuals. RV 16 infection is related to cold and asthma symptoms and increases the airway hyperresponsiveness to histamine. The exacerbation after RV 16 inoculation resembles exacerbations observed after natural rhinovirus infection.

In summary, asthmatic persons are prone to experience more severe symptoms accompanying rhinovirus infection and frequently experience asthma exacerbations during rhinovirus infection. Studies involving experimental RV 16 infection show that rhinovirus infections are causally related to asthma exacerbations. The relationship between viruses and exacerbation of asthma are depicted in Figure 1.

Mechanisms Of Virus-induced Exacerbations Of Asthma

Mechanisms of virus-induced wheezing and asthma in man have been studied with the aid of experimental virus infection in mildly asthmatic adults, usually with specific serotypes of rhinovirus. These data are supplemented by animal studies and by models of in vitro infection of epithelial cell culture systems. The relevance of these data for virus-induced wheezing in infants and children may, however, be limited. Immunological mechanisms underlying the interaction between viral infection and asthma exacerbation suggest that virus-induced exacerbations of asthma occur because of a combination of interactions.

First, in an asthmatic individual, exacerbation of asthma may occur because of functional interaction between viral pathology and asthmatic pathology or by sharing the same pathogenetic mechanism in an additive or synergistic way. Neutrophils, eosinophils and their activation products contribute to airway obstruction, virus-induced wheezing and exacerbations of asthma.

Second, pre-existing asthmatic inflammation may interfere with effective antiviral response and may allow the virus to cause increased airway damage. Recent reports indicate a distinctive immuno-inflammatory response in asthmatic patients. Parry *et al.* e.g. showed that allergic subjects have a reduced production of Th1 type cytokines (IL12 and IFNγ) after experimental infection.(29) Volunteers with strong IFNγ responses to virus, shed less virus during the peak of the cold. Moreover, an evaluation of cytokine patterns during acute illness revealed that volunteers with a stronger Th1 response tend to have reduced symptom scores and more rapid viral clearing.

Third, pre-existing asthmatic inflammation may increase the vulnerability of asthmatic persons for viral infections. A possible mechanism for increased viral entry and replication in asthmatic persons is the tendency of increased expression of intracellular adhesion molecule 1 (ICAM1) on airway epithelial cells (the cell surface receptor for the major group of rhinoviruses) in patients with asthma.(30)

Fourth, virus infection may increase the sensitivity of asthmatic airways to allergic responses. In the airways, the dominant neural control is provided by cholinergic fibers in the vagus nerves. Viral infections increase vagally mediated reflex bronchoconstriction. Increased cholinergic reflex bronchoconstriction is demonstrated in humans with natural occurring viral infections.(31) Viral infections also potentate inflammatory responses and bronchoconstriction towards subsequent inhaled allergens. Experimentally induced rhinovirus infections in allergic patients can alter the pattern of lower airway response to allergen challenge by significantly enhancing the persons' propensity to develop late asthmatic responses.(32) Another mechanism by which Th1-type inflammation enhances allergy is via an effect of recruitment. Stephens *et al.* showed in a murine model that Ag-specific activation of Th1 cells enhances the recruitment potential of the lung leading to recruitment and activation of Th2 cells.(33) This implies that circulating Th2 cells in allergic individuals can enter the lungs in response to infection or inflammation and become activated to trigger allergy. As noted before, the timing of both allergic sensitization and viral infection may determine whether airway inflammation and AHR occur after allergen exposure.

In summary, the full cascade of events leading from virus infection to exacerbation of allergic inflammation in human asthmatics remains only partially understood. It is clear, however, that viral RTI facilitate subsequent allergic responses in asthmatics and vice versa and that asthmatic individuals are more susceptible to severe viral infection of the lower airways.

IMPLICATIONS FOR THERAPY

Vaccinations

The success of vaccination to prevent respiratory virus infections has been limited by significant variation within the major virus types causing disease. There are more than 100 serotypes of rhinovirus and no effective vaccine has been introduced so far. Since they are all immunologically distinct, antibodies to a very wide range of antigens would be required to confer immunity to all rhinoviruses. The influenza viruses display antigenic shift and drift, so new vaccines must be developed every two or three years to cover the strains prevalent at the time. Still, vaccination against influenza has proven to be cost-effective. No vaccines are available for para-influenza and corona virus. RSV is a prime target for vaccine development because of the considerable morbidity and mortality associated with RSV infections. Vaccination against RSV experienced a major setback in the 1960s when the use of formalin-inactivated virus in young babies resulted in increased disease severity following subsequent virus infection. Of vaccinated children, 80% required hospitalization when subsequently infected with RSV, as compared to 5% of controls.(34) A successful RSV vaccine would need to provide more effective protection than natural infection, which is itself frequently followed by reinfection. Such a vaccine should be administered early in infancy to have an effect on infant bronchiolitis. With the hygiene hypothesis in mind one should realize that vaccination replaces recovery from infections with a rather different type of immunological stimulus. Therefore, novel vaccines should be designed not only to prevent infections, but also to replace infections with an equivalent education stimulus to the immune system.

In addition to active vaccination, passive immunization strategies were developed. Both donor-derived RSV intravenous immune globulin (RSV IGIV) and RSV-specific monoclonal antibodies (palivuzimab) have been proven to be safe and effective in reducing hospitalization of high-risk infants with a RSV infection. Passive immunization with RSV IGIV is effective as long as the neutralizing antibody titer is adequate. The greatest benefit of RSV IGIV was observed in preterm infants. Drawbacks associated with the use of RSV IGIV are the long duration of intravenous administration, the considerable volume needed, possibly interference with normal vaccinations, high costs and limited availability. Palivuzimab is an IgG1 humanized monoclonal antibody that is able to neutralize RSV and inhibits its fusion activity. Currently, no data are available on the use of palivuzimab for treatment or prevention of bronchiolitis or recurrent wheezing. The advantages of palivuzimab are the easy administration and lack of interference with normal vaccinations. Its major disadvantage is its high cost. In spite of that, palivuzimab is nowadays used more commonly than RSV IGIV. Other IgG monoclonal antibodies are being studied

that may be future candidates for phase III clinical trials of prevention of RSV infection in high-risk infants.(35)

Anti-viral Medication

Conceptually, antiviral agents could be used to treat viral infections early in the course of the illness to prevent wheezing and pulmonary complications. A limitation with this approach is that, once viral respiratory infections are recognized, much of the viral replication has already occurred and, consequently, there is likely to be a limited potential for antiviral agents to affect the course of the illness. In fact, antivirals may be most effective when given prophylactically, as demonstrated by a recent trial on prophylactic zanamivir in which 79% fewer cases of influenza occurred in family members exposed to influenza who received zanamivir compared with placebo.(36) For influenza, this approach has no advantage over the less costly alternative of immunization, but preventive use of an antiviral may be a viable strategy against agents for which vaccines are not available, e.g., rhinovirus. Several compounds with activity against rhinovirus have been tested in clinical trials. These compounds include molecules such as inhibitors of rhinovirus-3C-protease, soluble intracellular adhesion molecule (s-ICAM1) and capsid-binding agents, which either hinder rhinovirus binding to cellular receptors or inhibit uncoating of the virus to release RNA inside the cell. As yet, no effective agent is available for clinical use. For RSV, ribavarin inhibits replication during the active replication phase. Any beneficial effects of ribavirin, such as a reduction in duration of mechanical ventilation or hospitalization, are unproven, and routine use in high-risk children with RSV infections is no longer warranted.(35)

Anti-inflammatory Medication

An alternative approach to moderate viral infections is to treat virus-induced inflammation. Inhaled and systemic corticosteroids play a key-role in prevention and treatment of asthma attacks. The effects of corticosteroids are the result of actions at many points in various inflammatory cascades. The use of inhaled corticosteroids is the most important tool to control allergic asthma. Systemically used corticosteroids are highly effective to treat acute asthma attacks. Systemic administration of corticosteroids reduces acute airway obstruction and reduces the risk of hospitalization in virus-induced exacerbations of asthma.(36;36) However, systemic steroids may interfere with the antiviral immune response resulting in reduced viral clearance. The available data fail to show that corticosteroid therapy during or after RSV bronchiolitis effectively prevents recurrent wheezing. Although there are indications that corticosteroids may alter course of disease in severely infected RSV patients, there is a need for longer-term studies, especially those

that include pulmonary function. It is challenging to develop more focused inhibition of specific components of virus-induced inflammation, such as pro-inflammatory cytokines or mediators, which might be successful in reducing the severity of viral respiratory infections or exacerbations of asthma.

DISCUSSION

Viral infections, wheezing and asthma are strongly interrelated. Different wheezing phenotypes can be distinguished which have their own interaction with viral RTI. Possible influences of viruses on childhood asthma are depicted in Figure 2. Different types of interactions between viral infections and asthma can co-exist within one individual. First, viral infections can induce transient wheezing illness. In addition, animal models have shown that virus-induced aggravation of allergy-induction is possible. A positive effect of viral RTI on allergy development has not been convincingly confirmed in the human. In contrary, viral infections in early life can diminish the risk to develop atopic asthma (hygiene hypothesis). Here, repeated exposure to viral pathogens during early life is particularly important to prevent allergy development. This suggests the existence of a window of susceptibility for viruses to prevent allergic asthma. Animal models confirm the critical importance of timing for the relationship between viral infection and prior al-

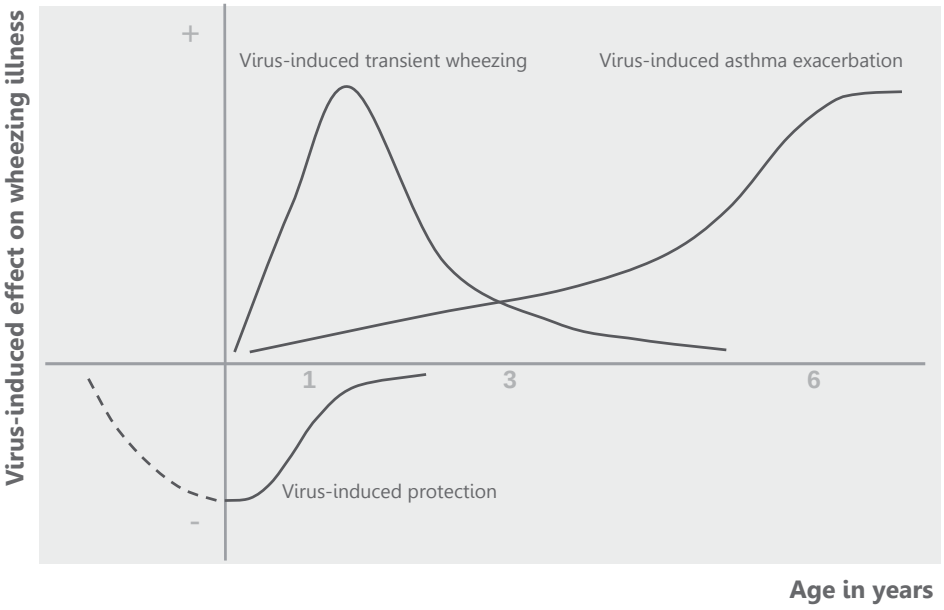

Figure 2 Windows of vulnerability. Schematic representation of age-related virus-induced effects on wheezing illness.

lergen sensitization. Finally, in asthmatic children viral infections are strongly associated with exacerbations of asthma.

The different relationships between wheezing, viral RTI and asthma depend on genetic background and on timing of infection in relation to allergen sensitization. The precise underlying mechanisms are not yet fully understood and form key topics of future research.

REFERENCE LIST

(1) Sly RM. asthma. In: Behrman RE, Kliegman RM, Jenson HB, editors. Nelson Textbook of Pediatrics. 2000: 664-680.

(2) Wills-Karp M, Santeliz J, Karp C, Aquilina AT. The germless theory of allergic disease: revisiting the hygiene hypothesis. Nature reviews 2001;(1):69-75.

(3) Martinez FD, Helms PJ. Types of asthma and wheezing. Eur Resp Journal 1998; 12(Supp 27):3S-8S.

(4) Lemanske RF. Inflammation in childhood asthma and other wheezing disorders. Pediatrics 2002; 109(2):368-372.

(5) Pandya RJ, Solomon G, Kinner A, Balmes JR. Diesel exhaust and asthma: hypotheses and molecular mechanisms of action. Environ Health Perspect 2002; 110 Suppl 1:103-112.

(6) Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 1999; 354:541-545.

(7) Bont L, Heijnen CJ, Kavelaars A, Aalderen van WMC, Brus F, Draaisma JTM et al. Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one year follow-up study. Am J Respir Crit Care Med 2000; 161:1518-1523.

(8) Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B, Bjorksten B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. Pediatrics 1995; 95(4):500-505.

(9) Bont L, Aalderen van WMC, Versteegh J, Brus F, Draaisma JTM, Pekelharing-Berghuis M et al. Airflow limitation during respiratory syncytial virus lower respiratory tract infection predicts current wheezing. Pediatr Infect Dis J 2001; 20:277-282.

(10) Cookson W. The alliance of genes and environment in asthma and allergy. Nature 2003; 402(6760 Suppl):B5-B11.

(11) Martinez FD, Wayne MJ, Wright AL, Holberg CJ, Lynn M, Taussig LM et al. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. N Engl J Med 1988; 319(17):1112-1117.

(12) Martinez FD, Wright AL, Taussig LM Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing during the first six years of life. N Engl J Med 1995; 332:133-138.

(13) Illi s, Mutius von E, Lau S, Bergmann R, Niggemann B, Sommerfeld C et al. Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. BMJ 2001; 322:390-395.

(14) Bendelja K, Gagro A, Bace A, Lokar-Kolbas R, Krsulovic-Hresic V, Drazenovic V et al. Predominant type-2 response in infants with respiratory syncytial virus (RSV) infection demonstrated by cytokine flow cytometry. Clin Exp Immunol 2003; 121(2):332-338.

(15) Makela MJ, Kanehiro A, Borish L, Dakhama A, Loader J, Joetham A et al. IL-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. Proc Natl Acad Sci 2000; 97(11):6007-6012.

(16) Schwarze J, Hamelmann E, Bradley KL, Takeda K, Gelfand EW. Respiratory Syncytial Virus Infection Results in Airway Hyperresponsiveness and Enhanced Airway Sensitization to Allergen. J Clin Invest 1997; 100(1):226-233.

(17) Folkerts G, Vlieger JW, de Vries A, van Der Linde H, Engels F, de Jong JC et al. Virus- and bradykinin-induced airway hyperresponsiveness in guinea pigs. Am J Respir Crit Care Med 2000; 161(5):1666-1671.

(18) Hoogen van den BG, Jong de JC, Groen J, Kuiken T, Groot de R, Fouchier RAM et al. A newly discovered human pneumovirus isolated from young children with respiratory disease. Nature Medicine 2001; 7(6):719-724.

(19) Greensill J, McNamara PS, Dove W, Flanagan B, Smyth RL, Hart CA. Human metapneumovirus in severe respiratory syncitial virus bronchiolitis. Emerging Infectious Diseases 2003; 9(3):372-375.

(20) Strachan DP. Hay fever, hygiene, and household size. British Medical Journal 1989; 299:1259-1260.

(21) Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, Day-Care Attendance, and the Risk of Asthma and Wheezing during Childhood. N Engl J Med 2000; 343(8):538.

(22) Peebles RS, Hashimoti K, Collins RD, Jarzecka K, Furlong J, Mitchell DB et al. Immune Interaction between Respiratory Syncytial Virus Infection and Allergen Sensitizations Critically Depends on Timing of Challenges. J Inf Dis 2001; 184:1374-1379.

(23) Tsitoura DC, Tsitoura DC, Kim S, Dabbagh K, Berry G, Lewis DB et al. Respiratory infection with influenza A virus interferes with the induction of tolerance to aeroallergens. J Immunol 2000; 165(6):3484-3491.

(24) Johnston SL, Pattemore PK, Sandersson G, Smith S, Lampe F, Josephs L et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. BMJ 1995; 310(6989):1225.

(25) Johnston SL. Viruses and asthma. Allergy 1998; 53:922-932.

(26) Gern JE. Rhinovirus respiratory infections and asthma. Am J Med 2002; 112(6A):19S-27S.

(27) Corne JM, Marshall C, Smith S, Schreiber J, Sanderson G, Holgate ST et al. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. Lancet 2002; 359:831-834.

(28) Grunberg K, Timmers MC, Klerk de EPA, Dick EC, Sterk PJ. Experimental Rhinovirus 16 infection causes variable airway obstruction in subjects with atopic asthma. Am J Respir Crit Care Med 1999; 160:1375-1380.

(29) Parry DE, Busse WW, Sukow KA, Dick CR, Swenson C, Gern JE. Rhinovirus-induced PBMC responses and outcome of experimental infection in allergic subjects. J Allergy Clin Immunol 2000; 105:692-698.

(30) Bentley AM, Durham SR, Robinson DS, Menz G, Storz C, Cromwell O. Expression of endothelial and leukocyte adhesion molecules intracellular adhesion molecule-1, E-selectin, and vascular cell ashesion milecule-1 in the bronchial mucosa in steady-stat and allergen-induced asthma. J Allergy Clin Immunol 1993; 92:857-868.

(31) Aquilina AT, Hall WJ, Douglas RG, Utell MJ. Airway reactivity in subjects with viral upper respiratory tract infections: the effects of exercise and cold air. Am Rev Resp Dis 1980; 122:3-10.

(32) Lemanske RF, Dick EC, Swenson CA, Vrtis RF, Busse WW. Rhinovirus upper respiratory infection increases airway hyperreactivity and late asthmatic symptoms. J Clin Invest 1989; 83:1-10.

(33) Stephens R, Randolph DA, Huang G, Holtzmann MJ, Chaplin DD. Antigen-nonspecific recruitment of Th2 cells to the lung as a mechanism for viral infection-induced allergic asthma. J Immunol 2002; 169:5458-5467.

(34) Kim HW, Canchola JG, Brandt CD. Respiratory Syncytial virus disease in infants despite prior asministration of antigenic inactivated vaccine. Am J Epidemiol 1969; 89:422-434.

(35) Kimpen JLL. Prevention and treatment of respiratory synsytial virus bronchiolitis and post-bronchiolitic wheezing. Respir Res 2002; 3(suppl 1):S40-S45.

(36) Hayden FG, Gubareva LV, Monto AS, Klein TC, Elliot MJ, Hammond JM. Inhaled zanamivir for the prevention of influenza in families. N Engl J Med 2000; 343:1282-1289.

Chapter 2

IL13 Genetic Polymorphism Identifies Children With Late Wheezing After Respiratory Syncytial Virus Infection

Marieke JJ Ermers

Barbara Hoebee

Hennie M Hodemaekers

Tjeerd G Kimman

Jan LL Kimpen

Louis J Bont

Journal of Allergy and Clinical Immunology 2007; 119:1086-91.

ABSTRACT

Background

The nature of wheezing following respiratory syncytial virus lower respiratory tract infection (RSV LRTI) is usually transient. However, some children will go on to develop persistent or late wheezing.

Objective

We hypothesized that early and late post-bronchiolitis wheezing are determined by distinct clinical, immunological and genetic variables. For the first time, genetic data are related to late wheezing following RSV LRTI in a large longitudinal follow-up study.

Methods

A cohort of 101 children hospitalized for RSV LRTI was prospectively followed for 6 years. During RSV LRTI cytokine studies were performed and genetic polymorphisms were determined. Parents performed daily log registration of respiratory symptoms during the first 3 years of follow-up and again at age six during the winter season.

Results

Distinctive associations for early and late post-bronchiolitis wheezing were found. We previously showed that airflow limitation during RSV LRTI as well as convalescent monocyte IL10 production are associated with early wheezing. These variables were not associated with late wheezing. On the other hand, atopic family history was not associated with early wheezing, but was associated with late wheezing. Most importantly, the *IL13* Gln allele was associated with late wheezing (OR 3.23, 95%CI 1.27-8.25), but was not associated with early wheezing.

Conclusion

This study revealed distinct clinical, immunological and genetic determinants of early and late wheezing following RSV LRTI, indicating distinct pathophysiological mechanisms. We conclude that late wheezing at age six, but not early post-bronchiolitis wheezing, is an asthmatic phenomenon and genetically related to a functional *IL13* polymorphism.

INTRODUCTION

Numerous epidemiological studies have demonstrated increased risk for recurrent episodes of wheezing following acute RSV LRTI. Recurrent wheezing is found in 42-71% of patients.(1) Whether post-bronchiolitis wheezing is mainly a transient allergic or non-allergic condition with a good long-term prognosis, or early onset of allergic asthma is still a matter of debate. Sigurs *et al.* reported a strong association between RSV LRTI in the first year of life and asthma, clinical allergy and allergic sensitization up to early adolescence.(2) Other studies could not confirm the association between RSV LRTI and subsequent allergic sensitization and showed a transient wheezing pattern diminishing with age.(3;4)

Our previous work focused on the prediction of post-bronchiolitis wheezing. Simple clinical variables, but not allergic risk factors, were associated with the development of post-bronchiolitis wheezing during the first 3 years following the initial episode.(5) In addition, we showed a significant decrease in airway symptoms during the first 12 months following RSV LRTI.(4)

In this 6-year prospective follow-up study we compared genetic and non-genetic risk factors of early and late wheezing in order to learn about the relationship between these clinically different wheezing patterns. We hypothesized that early and late post-bronchiolitis wheezing are associated with distinct clinical, immunological and genetic variables.

METHODS

Cohort Description

A cohort of 140 children younger than 13 months of age and hospitalized because of RSV LRTI was recruited during the 1997-1998 and 1998-1999 winter seasons (Figure 1).(4) Infants with wheezing illness prior to RSV LRTI were not included. 133 children (95%) were successfully traced. Parents of 101 of these children (76%) agreed to participate in this study and recorded daily respiratory symptoms during the winter season at age 6 years. Parents of 32 children (24%) did not participate for various reasons. Informed consent was obtained from parents. The study was approved by the Ethics Review Committee, University Medical Center Utrecht.

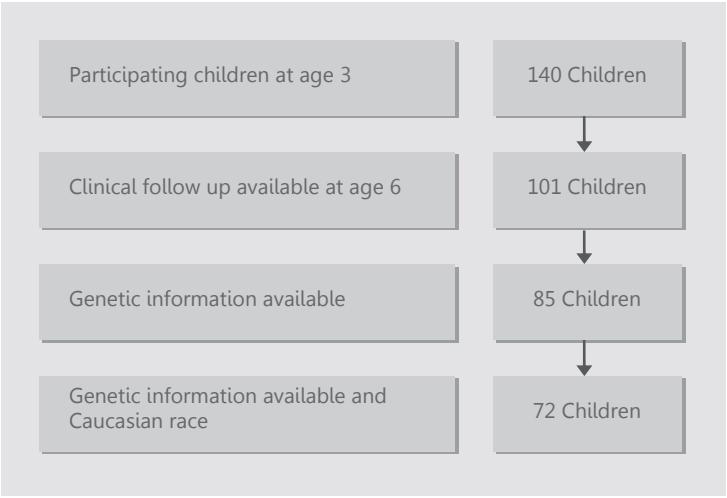

Figure 1 Diagram showing the flow of participating children.

Log Information

During the first 3 years and during the winter season (from January till March) at age 6 years parents recorded respiratory symptoms in a log as previously described.(5) Numbers of days of wheezing were counted. The primary outcome variable of this study was 5 or more wheezing days during the winter season at age 6 years.
Extended Dutch versions of the standardized questionnaire of the British Medical Council and the European Community Respiratory Health Survey questionnaires were used to obtain data on allergy, parental smoking habits and atopic symptoms among parents. Parental symptoms of atopy were eczema, hay fever, bronchitis, asthma and food allergy.(6) A semi-quantitative parental atopy score was arbitrarily defined. One point was added to the parental atopy score for the presence of each atopic symptom (thus the maximum score was 10).

Immunologic Measurements

Immunologic parameters during primary RSV LRTI (as described before in relation to the first year of follow up) (7;8) were related to wheezing behavior at age 6 years. Cytokine profiles were determined during the acute and convalescence phase of RSV LRTI as described previously.(7) Measurements included in vitro monocyte IL10 and IL12 production after 48 hours lipopolysaccharide (LPS) + IFNγ stimulation and IFNγ/IL4 ratios after 48 hours phytoheamagglutinin (PHA) stimulation. Total and specific IgE against common food and inhalation allergens was measured at age 3 years.

DNA Isolation And Genotyping

DNA isolation from blood samples and buccal swabs, the genotyping of *IL4 C-590T* (RefSNP rs2243250), *IL4Rα* I50V (RefSNP rs1805010), *IL4Rα* Q551R (RefSNP rs1801275), *IL10* C-592A (RefSNP rs1800872), *IL9* A-345G (RefSNPrs1799962) and *TNFα* G-308A (RefSNP rs1800629) polymorphisms as well as the description of the control population (447 persons randomly taken from a large Dutch population health examination study) were described before.(9;10)
The *IL10* A-1082G, *IL8* A-251T and C-781T, *IL13* C-1112T and Arg130Gln, *SP-D* Met11Thr and Thr160Ala polymorphisms were genotyped by polymerase chain reaction (PCR) and restriction fragment-length polymorphism (RFLP) or by pyrosequencing using the experimental conditions listed in Table 1. Internal control samples (representing the 3 genotypes and a buffer sample) were included on each plate.

Statistical Analysis

Days of wheezing according to log registration were recorded. Chi square test and Mann-Whitney U test were used to compare proportions of baseline characteristics of children. Parental atopy scores are expressed as median (25-75% interquartile range). Genotype and allele distribution were analyzed using the Chi square or Mantel-Haenszel trend test, respectively. Logistic regression analysis was used to determine the predictive value of different variables in one model to predict late wheezing. The explained variance of the model was expressed as Nagelkerke R2. All tests of significance were two-sided. *P*-values under 0.05 were considered statistically significant. All data were analyzed using SPSS (SPSS for Windows, Release 12.0.1; SPSS Inc., Chicago, IL).

RESULTS

Wheezing After RSV

A comparison between participating and non-participating children at age 6 years revealed that mothers of participating children smoked less frequently before the birth of the child (26% vs 50%, *P*=0.01). Other characteristics did not differ between participants and non-participants.
We compared wheezing illness during the first 3 years after RSV LRTI hospitalization with wheezing illness at age 6 years. Seventy (69%) children experienced wheezing at any time during follow up. Wheezing during RSV LRTI was not considered wheezing during follow-up. Patterns of wheezing are shown in Table 2.

Table 1. Primers, PCR conditions, and restriction enzymes used to determine genetic variation at the studied polymorphic sites.

	Primers(5′-3′), Restriction enzyme, DNA fragments, bp	PCR conditions	References
RFLP, *IL8*, A-251T	Forward, CTTGTTCTAACACCTGCCACTC; Reverse, GGCAAACCTGAGTCATCACA; Restriction enzyme MfeI; DNA fragments, bp, A allele 141/85; T allele 222	45 s at 94°C, 45 s at 56°C and 60 s at 72°C	RefSNP rs4073
Pyrosequencing, *IL8*, C781T	Forward, TGCTGGAGAGTCTTAGCTTGC; Reverse with universal tail, AGCGCTGCTCCGGTTCATAGATTTCCTAGCCCTTGACCTCAGT; Biotin labeled universal tail, 5′-biotin- GCTGCTCCGGTTCATAGATT; Sequence primer forward strand, ACTCTTTATATAGGAAGT	45 s at 94°C, 45 s at 57°C and 60 s at 72°C	RefSNP rs2227306
Pyrosequencing, *IL10*, A-1082G	Forward, GGCTCCCCTTACCTTCTACA; Reverse with universal tail, AGCGCTGCTCCGGTTCATAGATTGATTCCATGGAGGCTGGATA; Biotin labeled universal tail, 5′-biotin- GCTGCTCCGGTTCATAGATT; Sequence primer forward strand, ACTAAGGCTTCTTTGGGA	45 s at 94°C, 45 s at 57°C and 60 s at 72°C	RefSNP rs1800896
Pyrosequencing, *IL13*, C-1112T	Forward, AGGAAGTGGGTAGGGGAGAA; Reverse, 5′-biotin- CTACAGCCATGTCGCCTTTT; Sequence primer forward strand, TTCTGGAGGACTTCTAGG	45 s at 94°C, 45 s at 55°C and 60 s at 72°C	RefSNP rs1800925
Pyrosequencing, *IL13*, Arg130Gln	Forward, 5′-biotin-AGCAGTTTTCCAGCTTGCAT; Reverse, TCAGGTCCTGTCTCTGCAAAT; Sequence primer reverse strand, TTTCGAAGTTTCAGTTGAAC	45 s at 94°C, 45 s at 55°C and 60 s at 72°C	RefSNP rs20541
Pyrosequencing, *SP-D* Met11Thr	Forward, TCACCTCTAGAAGCTGAGCCAAGCC; Reverse, 5′-biotin-ACAAAGTACCCAGAGTTGCTG; Sequence primer forward strand, CCTACTCCCACAGAACA	45 s at 94°C, 45 s at 57°C and 60 s at 72°C	RefSNP rs721917
Pyrosequencing, *SP-D* Thr160Ala	Forward, GCAGGCCCTAAGGGAGAG; Reverse with universal tail, AGCGCTGCTCCGGTTCATAGATTCAGGCACAGGAGAACTGGAC; Biotin labeled universal tail, 5′-biotin- GCTGCTCCGGTTCATAGATT; Sequence primer forward strand, GTGGAGTCCCTGGAA	45 s at 94°C, 45 s at 57°C and 60 s at 72°C	RefSNP rs17885900

Note. RFLP, restriction fragment length polymorphism; bp, base pairs; PCR, polymerase chain reaction; s, seconds.

Remarkably, proportions of children with early post-bronchiolitis wheezing were similar in children with and without wheezing at age 6 years (77% vs 65%, not significant). Analyses of log registration showed that practically all episodes of wheezing were accompanied by upper airway complaints. We subsequently compared characteristics of children with and without wheezing at age 6 years. Median parental atopy score of children with wheezing at age 6 years was 2.5 (25^{th}-75^{th} percentile 0.25-3.75), vs 1 (25^{th}-75^{th} percentile 0-2) in children without wheezing at this age (P=0.036). A borderline signifi-

cant higher proportion of children with wheezing at age 6 years received bottle-feeding only (without complementary breastfeeding) in their first months (67% vs 37%, *P*=0.048). Other characteristics, including signs of airflow limitation during RSV LRTI, did not differ between children with and without wheezing at age 6 years (Table 3).

Table 2. Wheezing patterns. Relationship between wheeze during the first 3 years and wheeze at age 6 years. Wheezing at RSV LRTI was not considered wheezing during follow-up. Values express absolute numbers of children (%).

	Wheeze during first 3 yrs		No wheeze during first 3 yrs	
Wheeze at 6 yr	10 (10%)	persistent wheeze	3 (3%)	late onset wheeze
No wheeze at 6 yr	57 (56%)	transient wheeze	31 (31%)	no wheeze

Table 3. Baseline clinical characteristics of children with and without wheeze at age 6 years.

	No wheeze at age 6	Wheeze at age 6	OR, CI 95%, *P*
Mean age at bronchiolitis in months (range)	3.37 (0-12) (N=88)	3.15 (0-12) (N=13)	NS
Male sex (%)	48/88 (54.5)	8/13 (61.5)	0.8 (0.2-2.5), NS
Early wheezing (%)	57/87 (65,5)	10/13 (76.9)	1.8 (0.5-6.9), NS
Median parental atopy score (25th-75th percentile)	1 (0-2) (N=76)	2.5 (0.25-3.75) (N=12)	– , 0.036
Bottle feeding only (%)	32/87 (36.8)	8/12 (66.7)	3.4 (1.0-12.3), 0.048
Prematurity (%)	25/86 (29.1)	6/12 (50.0)	0.4 (0.1-1.4), NS
Admitted to intensive care unit (%)	25/88 (28.4)	2/13 (15.4)	0.5 (0.1-2.2), NS
Maternal smoking before birth (%)	23/87 (26.4)	2/12 (16.7)	0.6 (0.1-2.7), NS

Note. OR, odds ratio; CI, confidence interval; NS, Not significant.

Immunological Characteristics

We previously described monocyte and lymphocyte cytokine profiles in the blood during RSV LRTI and we showed that early post-bronchiolitis wheezing is associated with convalescent monocyte IL10 production during the first year of follow-up.(7) For the current study, follow-up information was expanded compared to previous reports. Wheezing during the first 3 years of follow-up was associated with convalescent monocyte IL10 and convalescent monocyte IL12 production. We found no relationship between cytokine profiles and wheezing at age 6 years (Table 4). Convalescent monocyte IL10 and monocyte IL12 production was similar for children with and without wheezing at age 6 years (IL10: 53,0 vs 53.5 pg/ml, not significant; IL12: 75,2 vs 77,5 pg/ml, not significant). Geometric mean of IgE measured at age 3 years was similar for children with and without wheezing in the first 3 years and at age 6 years (8 vs 13 IU/ml, not significant). Further-

Table 4. Relationships between measured cytokines and wheezing behavior.

Cytokine measurements were performed during the convalescence phase of RSV LRTI. Data were normally distributed after log transformation. Geometric means and 95% CI's on the geometric mean are presented.

Cytokine	Post-bronchiolitis wheeze, first 3 years					Wheeze at age 6				
	N	Mean value wheezers (95% CI)	N	Mean value non-wheezers (95% CI)	*P*	N	Mean value wheezers (95% CI)	N	Mean value non-wheezers (95% CI)	*P*
IL10	69	71.5 (55.1-92.8)	34	44.7 (32.1-62.2)	0.04[1]	13	53.0 (28.2-99.5)	82	53.5 (42.1-67.4)	0.98
IL12	72	66.7 (49.9-88.2)	34	102.5 (74.4-139.8)	0.05[1]	13	75.2 (40.4-139.8)	85	77.5 (60.3-99.5)	0.93
IFNγ	72	862.6 (639.1-1164.4)	34	837.1 (572.2-1236.5)	0.93	13	1224.1 (550.0-2724.4)	84	828.8 (639.1-1074.9)	0.29
IL4	69	8.17 (7.0-9.5)	34	9.6 (7.5-12.2)	0.25	13	9.9 (7.1-13.7)	82	8.4 (7.4-9.7)	0.37
IFNγ/IL4 ratio	69	106.7 (78.3-144.0)	34	87.4 (60.3-129.0)	0.46	13	124.0 (46.5-327.0)	81	99.5 (76.7-127.8)	0.54

Note. CI, confidence interval.

[1]Cytokines for which the associated *P*-value ≤ .05

more there were no differences in the prevalence of reactivity to specific allergens in children with and without wheeze in the first three years and at age 6.

Genetic Polymorphisms

Seventy-three children of native Dutch origin participated in the genetic studies. 13 non-native Dutch children were genotyped but were not included in the current analysis. Parents of non-participating children refused for various reasons. Baseline characteristics of children participating or not participating in the genetic studies were not different (data not shown). The results of the genetic studies are summarized in Table 5. No significant genetic association related to wheezing during the first three years of follow-up was found (data not shown). For example, similar *IL13* Arg130Gln allele frequencies were found in children with and without early post-bronchiolitis wheezing. In contrast a significant genetic association was found related to wheezing at age 6 years for the *IL13* Arg130Gln polymorphism. The *IL13* 130 Gln-allele is more common among children with wheezing at age 6 years (*P*=0.007, OR 3.27 (95% CI1.32 – 8.06)). Genotype analysis confirmed the association between the IL13 Arg130Gln polymorphism and late wheezing (Figure 2). The *IL4Rα* Ile-allele frequency showed borderline significant association with late wheezing. However, genotype analysis did not reveal an association between the *IL4Rα* Ile50Val polymorphism and wheezing at age 6 years (data not shown). No associa-

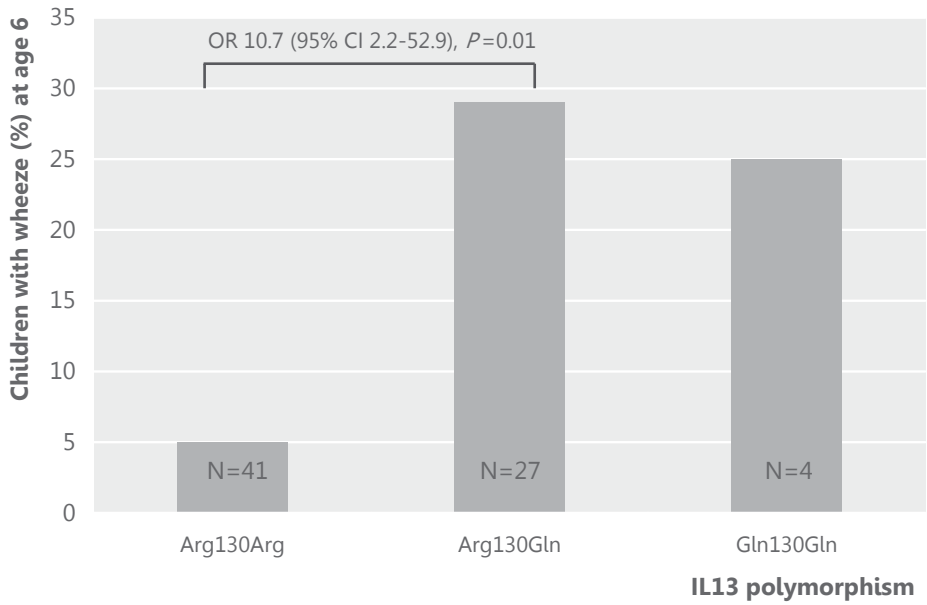

Figure 2 Relationship between *IL13* Arg130Gln genotypes and the presence of wheeze at age 6 years.

Table 5. Distribution of genetic polymorphisms in children with and without wheeze at age 6 years following RSV LRTI hospitalization and in controls.

Polymorphism	Allele	Allele frequency in children with wheeze at age 6 (n=11)	Allele frequency in children without wheeze at age 6 (n=62)	Allele frequency in controls (n=447)	OR and 95% CI wheeze at age 6 vs. controls	OR and 95% CI no wheeze at age 6 vs. controls	OR and 95% CI wheeze at age 6 vs. no wheeze at age 6
IL4 C-590T	T	13.6	17.4	13.0	1.05 (0.32 - 3.48)	1.39 (0.86 - 2.23)	0.73 (0.20-2.65)
IL4Rα Ile50Val	Val	63.6	40.9	46.2	2.02 (0.84 – 4.85)	0.81 (0.56 – 1.17)	2.54 (1.00-6.44)[1]
IL4Rα Gln551Arg	Arg	27.3	18.2	22.1	1.33 (0.50 – 3.52)	0.77 (0.48 – 1.25)	1.74 (0.62-4.88)
IL10 C-592A	A	27.3	22.0	23.9	1.19 (0.46 – 3.07)	0.90 (0.58 – 1.39)	1.37 (0.50-3.77)
IL10 A-1082G	G	59.1	54.0	50.2	1.41 (0.61 – 3.27)	1.15 (0.80 – 1.66)	1.19 (0.48-2.95)
IL13 C-1112T	T	31.8	19.4	19.1	1.96 (0.78 – 4.88)	1.01 (0.63 – 1.62)	1.91 (0.71-5.13)
IL13 Arg130Gln	Gln	45.5	20.2	21.7	3.27 (1.32 – 8.06)[1]	0.91 (0.57 – 1.46)	3.23 (1.27-8.25)[1]
IL9 A-345G	A	95.5	96.2	93.2	1.52 (0.21 – 11.26)	1.84 (0.73 – 4.63)	1.50 (0.15-13.72)
TNFα G-308A	A	22.7	16.7	15.9	1.56 (0.57 – 4.27)	1.06 (0.65 – 1.74)	1.53 (0.51-4.53)
IL8 A-251T	T	59.1	58.1	52.0	1.34 (0.56 – 3.21)	1.28 (0.87 - 1.88)	1.07 (0.43-2.67)
IL8 C781T	C	68.2	62.9	57.9	1.55 (0.63 – 3.80)	1.23 (0.84 – 1.81)	1.30 (0.11-16.00)
SP-D Met11Thr	Thr	45.5	48.5	41.9	1.14 (0.50 – 2.60)	1.28 (0.89 – 1.85)	0.86 (0.35-2.12)
SP-D Thr160Ala	Thr	63.6	62.9	61.3	1.11 (0.46 – 2.67)	1.07 (0.73 - 1.58)	1.01 (0.40-2.57)

Note. OR, odds ratio; CI, confidence interval.

[1]Polymorphisms for which the associated *P* value ≤ .05

tion was found between the other studied polymorphisms and late wheezing. Logistic regression analysis to predict wheezing at age 6 years was performed. Family history of atopy, bottle-feeding only and *IL13* polymorphisms were independent predictors (R2 0.41). No other independent predictor was identified.

DISCUSSION

Early and late post-bronchiolitis wheezing following RSV LRTI are important pediatric diseases. The relationship between these wheezing disorders is not fully understood.
Early post-bronchiolitis wheezing is common in children with a history of RSV LRTI. Children suffer from asthma-like airway symptoms during the first years and often need medication. In addition to long-term airway symptoms, these children sleep and eat less well.(11) Fortunately, post-bronchiolitis wheezing decreases and most children are relieved from symptoms by the age of 3 years.(4) Late or persistent wheezing following RSV LRTI occurs less frequently but is probably a more serious condition. Predicting which children are at risk for late asthma could identify children who will benefit from early-targeted interventions.
In this cohort of hospitalized RSV LRTI patients, we compared risk factors of early and late wheezing following RSV LRTI. For the first time distinctive risk factors of early and late post-bronchiolitis wheezing were found in one large longitudinal follow-up study (Table 6).
Contrary to the concept that post-bronchiolitis wheezing is the first manifestation of late wheezing or asthma, we showed similar proportions of early post-bronchiolitis wheezing in groups of children with and without late wheezing. The prevalence of late wheezing in our RSV hospitalized cohort (13%) equaled the prevalence of asthma at this age in the total population,(12) suggesting that RSV LRTI hospitalization and subsequent post-bronchiolitis wheezing are not related to late wheezing or allergic asthma in later life. Furthermore, previously published risk factors of post-bronchiolitis wheezing (airflow limitation during RSV LRTI and monocyte IL10 and monocyte IL12 production dur-

Table 6. Distinctive clinical, immunologic and genetic characteristics in early and late post-bronchiolitis wheeze.

Characteristics		Early wheeze	Late wheeze[1]
Clinical	Airflow limitation during RSV LRTI	+	-
	Atopic family burden	-	+
Immunological	Monocyte IL10 production during RSV LRTI	+	-
	Monocyte IL12 production during RSV LRTI	+	-
Genetic	*IL13* gene polymorphism	-	+

[1]Late wheeze was defined as 5 or more wheezing days during the winter season at age 6 years.

ing convalescence) (5;7) were not associated with late wheezing. Atopic family history was associated with late wheezing but was not associated with early post-bronchiolitis wheezing. In conclusion, early and late post-bronchiolitis wheezing have distinctive predictors suggesting a distinctive etiology.

Recent genetic studies have shown an association between cytokine genes, including *IL4*, *IL4R*, *IL8* and *IL10*, and the risk of severe RSV LRTI.(9;13;14) In our study, these genetic polymorphisms were not related to either early or late wheezing following RSV LRTI. This is in apparent contrast with findings by Hull *et al.*, who showed an *IL8* polymorphism to be associated to the risk for severe RSV LRTI as well as for subsequent wheezing.(15) Hull *et al.* used a single measurement with a validated questionnaire, which could possibly introduce recall bias,(4) whereas our study analyzed data derived from continuous airway symptoms. Furthermore our study might be underpowered to detect differences in for example *IL8*. In our study genetic polymorphisms related to onset and severity of RSV LRTI could be distinguished from genetic polymorphisms related to long-term outcome. These results support the concept that mechanisms underlying the development of severe RSV LRTI are to be studied separately from the pathophysiology of wheezing illness.

We found a significant overrepresentation of the *IL13* Gln-allele (*IL13* Arg130Gln/Gln130Gln) polymorphisms in children with late wheezing. Studies in both humans and mice clearly show that *IL13* is a central regulator of asthmatic inflammation.(16) Recent animal data suggest that *IL13* promotes asthma by stimulation of bronchial epithelial mucus secretion and smooth muscle hyper-reactivity.(17;18) Numerous single nucleotide polymorphisms (SNPs) have been identified in the *IL13* gene and have been found to be associated with allergic and/or asthmatic phenotypes in different populations throughout the world. In the *IL13* Gln polymorphism (also described as: Arg164Gln,(19) Gln110Arg,(20) +2044 NIaIV RFLP),(21) the amino-acid change probably changes the affinity for the IL13Rα1 chain, part of the multimeric IL4/IL13 receptor. In our study, the *IL13* Gln polymorphism was strongly related to late wheezing but did not relate to early post-bronchiolitis wheezing or severity of disease. The distinctive predictive value of the *IL13* Gln polymorphism for persistent wheeze is in line with Heinzmann's recently published suggestion that RSV bronchiolitis and asthma have (at least some) different genetic predisposing factors.(22)

Recent studies show associations between the *IL13* Gln polymorphism and IgE levels in three distinct populations from the US and Europe.(20) Vladich *et al.* unveiled the functional relevance of the polymorphism by demonstrating increased activity of the *IL13* Gln polymorphism as compared to the *IL13* Arg variant. This structural polymorphism determines resistance to neutralization by sIL-13R α2.(23) By contrast, Arima *et al.* showed no difference in IgE synthesis between the *IL13* Arg and *IL13* Gln recombinant forms.(24) It was concluded that the *IL13* Gln variant may be a functional genetic factor in bronchial

asthma, whereas an effect on IgE regulation was ruled out. In our cohort, no relationship between *IL13* polymorphisms and IgE levels was found either (data not shown). Further studies are warranted to better understand the role that *IL13* polymorphisms may play in the pathogenesis of allergic inflammation and IgE synthesis.

The *IL4* and *IL4Rα* pathways play an important role in the development of atopic diseases. We found a borderline significant overrepresentation of the *IL4Rα* Ile50Val polymorphism in children with late wheezing. No overrepresentation of the *IL4* C-590 T polymorphism in children with late wheeze was found. This could be due to lack of power of our study to detect small differences between children with and without wheezing at age 6 years. A combined analysis of polymorphisms in the IL4/IL13 pathway may prove valuable to predict late wheeze in children.(25)

In conclusion, distinctive clinical and immunological characteristics for early and late post-bronchiolitis wheezing were found. Furthermore, the *IL13* Gln variant was strongly associated with late wheezing but was not associated with early post-bronchiolitis wheezing. Early post-bronchiolitis wheezing and wheezing later in childhood appear distinct pathophysiological entities.

Early post-bronchiolitis wheezing is not atopic in origin and does not predict the development of wheezing later in childhood. In contrast to early post-bronchiolitis airway morbidity, wheezing at age 6 is associated with an atopic backgound and a functional *IL13* genetic polymorphism.

REFERENCE LIST

(1) Bont L, Aalderen WM, Kimpen JL. Long-term consequences of respiratory syncytial virus (RSV) bronchiolitis. Paediatr Respir Rev 2000; 1(3):221-227.

(2) Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F et al. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med 2005; 171(2):137-141.

(3) Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 1999; 354(9178):541-545.

(4) Bont L, Steijn M, van Aalderen WM, Brus F, Th Draaisma JM, Diemen-Steenvoorde RA et al. Seasonality of long term wheezing following respiratory syncytial virus lower respiratory tract infection. Thorax 2004; 59(6):512-516.

(5) Bont L, van Aalderen WM, Versteegh J, Brus F, Draaisma JT, Pekelharing-Berghuis M et al. Airflow limitation during respiratory syncytial virus lower respiratory tract infection predicts recurrent wheezing. Pediatr Infect Dis J 2001; 20(3):277-282.

(6) van Woensel JB, Kimpen JL, Sprikkelman AB, Ouwehand A, van Aalderen WM. Long-term effects of prednisolone in the acute phase of bronchiolitis caused by respiratory syncytial virus. Pediatr Pulmonol 2000; 30(2):92-96.

(7) Bont L, Heijnen CJ, Kavelaars A, van Aalderen WMC, Brus F, Draaisma JTh et al. Monocyte IL-10 Production during Respiratory Syncytial Virus Bronchiolitis Is Associated with Recurrent Wheezing in a One-Year Follow-up Study. Am J Respir Crit Care Med 2000; 161(5):1518-1523.

(8) Bont L, Heijnen CJ, Kavelaars A, van Aalderen WM, Brus F, Draaisma JM et al. Local interferon-gamma levels during respiratory syncytial virus lower respiratory tract infection are associated with disease severity. J Infect Dis 2001; 184(3):355-358.

(9) Hoebee B, Rietveld E, Bont L, Oosten M, Hodemaekers HM, Nagelkerke NJ et al. Association of severe respiratory syncytial virus bronchiolitis with interleukin-4 and interleukin-4 receptor alpha polymorphisms. J Infect Dis 2003; 187(1):2-11.

(10) Hoebee B, Bont L, Rietveld E, van Oosten M, Hodemaekers HM, Nagelkerke NJ et al. Influence of promoter variants of interleukin-10, interleukin-9, and tumor necrosis factor-alpha genes on respiratory syncytial virus bronchiolitis. J Infect Dis 2004; 189(2):239-247.

(11) Bont L, Steijn M, van Aalderen WM, Kimpen JL. Impact of wheezing after respiratory syncytial virus infection on health-related quality of life. Pediatr Infect Dis J 2004; 23(5):414-417.

(12) Pattemore PK, Ellison-Loschmann L, Asher MI, Barry DM, Clayton TO, Crane J et al. Asthma prevalence in European, Maori, and Pacific children in New Zealand: ISAAC study. Pediatr Pulmonol 2004; 37(5):433-442.

(13) Hull J, Thomson A, Kwiatkowski D. Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. Thorax 2000; 55(12):1023-1027.

(14) Choi EH, Lee HJ, Yoo T, Chanock SJ. A common haplotype of interleukin-4 gene is associated with severe respiratory syncytial virus disease in Korean children. J Infect Dis 2002; 186(9):1207-1211.

(15) Goetghebuer T, Isles K, Moore C, Thomson A, Kwiatkowski D, Hull J. Genetic predisposition to wheeze following respiratory syncytial virus bronchiolitis. Clin Exp Allergy 2004; 34(5):801-803.

(16) Wills-Karp M, Chiaramonte M. Interleukin-13 in asthma. Curr Opin Pulm Med 2003; 9(1):21-27.

(17) Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999; 103(6):779-788.

(18) Bancroft AJ, McKenzie AN, Grencis RK. A critical role for IL-13 in resistance to intestinal nematode infection. J Immunol 1998; 160(7):3453-3461.

(19) Howard TD, Whittaker PA, Zaiman AL, Koppelman GH, Xu J, Hanley MT et al. Identification and association of polymorphisms in the interleukin-13 gene with asthma and atopy in a Dutch population. Am J Respir Cell Mol Biol 2001; 25(3):377-384.

(20) Heinzmann A, Mao XQ, Akaiwa M, Kreomer RT, Gao PS, Ohshima K et al. Genetic variants of IL-13 signalling and human asthma and atopy. Hum Mol Genet 2000; 9(4):549-559.

(21) Graves PE, Kabesch M, Halonen M, Holberg CJ, Baldini M, Fritzsch C et al. A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children. J Allergy Clin Immunol 2000; 105(3):506-513.

(22) Heinzmann A, Ahlert I, Kurz T, Berner R, Deichmann KA. Association study suggests opposite effects of polymorphisms within IL8 on bronchial asthma and respiratory syncytial virus bronchiolitis. J Allergy Clin Immunol 2004; 114(3):671-676.

(23) Vladich FD, Brazille SM, Stern D, Peck ML, Ghittoni R, Vercelli D. IL-13 R130Q, a common variant associated with allergy and asthma, enhances effector mechanisms essential for human allergic inflammation. J Clin Invest 2005; 115(3):747-754.

(24) Arima K, Umeshita-Suyama R, Sakata Y, Akaiwa M, Mao XQ, Enomoto T et al. Upregulation of IL-13 concentration in vivo by the IL13 variant associated with bronchial asthma. J Allergy Clin Immunol 2002; 109(6):980-987.

(25) Kabesch M, Schedel M, Carr D, Woitsch B, Fritzsch C, Weiland SK et al. IL-4/IL-13 pathway genetics strongly influence serum IgE levels and childhood asthma. J Allergy Clin Immunol 2006; 117(2):269-274.

Chapter 3

Inhaled Corticosteroids During Acute Bronchiolitis In The Prevention Of Post-Bronchiolitic Wheezing

Daniel Blom

Marieke JJ Ermers

Louis J Bont

Wim MC van Aalderen

Job BM van Woensel

Cochrane Database of Systematic Reviews 2007; 1:CD004881.

ABSTRACT

Background

Acute bronchiolitis in infants and young children is associated with long-term airway disease also known as post-bronchiolitic wheezing. Two major hypotheses have been proposed to explain the association between bronchiolitis and post-bronchiolitic wheezing. The first hypothesis considers bronchiolitis to be the first manifestation of recurrent wheezing in infants and children who are susceptible to obstructive airway disease. The second hypothesis suggests that the infection and concomitant inflammatory reaction in the acute phase leads to airway epithelium injury resulting in long-term obstructive airway disease. In line with the latter hypothesis, corticosteroids may have a beneficial effect on the prevention of post-bronchiolitic wheezing.

Objectives

The objective of this review was to evaluate the effect of inhaled corticosteroids, started during the acute phase of bronchiolitis, on the prevention of post-bronchiolitic wheezing.

Search Strategy

We searched the Cochrane Central Register of Controlled Trials (CENTRAL) (*The Cochrane Library* 2006, issue 3) which contains the Cochrane Acute Respiratory Infections Group's trials register, MEDLINE (1966 to September 2006), EMBASE (1980 to September 2006) and Current Contents (September 2006). Abstracts and reports of congresses (ERS 1999 to September 2005, ATS 1999 to September 2005) were obtained. We contacted experts in the field and pharmaceutical companies for ongoing or unpublished studies.

Selection Criteria

Randomized placebo-controlled trials studying the effect of inhaled corticosteroids in children younger than two years of age with the clinical diagnosis of acute bronchiolitis were included.

Data Collection And Analysis

Two authors independently extracted data and assessed trial quality using the Jadad 5-point scale.

Main Results

Five studies matched the inclusion criteria, with a median Jadad score of 4 (Inter Quartile Range 3 to 4), involving 374 infants. Pooling of the data was limited, due to the clinical diversity of the studies. However, no effect of inhaled corticosteroids in the prevention of wheezing (diary records or GP diagnosed), hospital re-admissions or use of corticosteroids or bronchodilators could be demonstrated. Duration of therapy, length of follow up or causative agent (respiratory syncytial virus or not) did not influence the pooled effect. In the three studies that also evaluated the adverse events, none were reported.

Authors' Conclusions

This review does not demonstrate an effect of inhaled corticosteroids given during the acute phase of bronchiolitis in the prevention of post-bronchiolitic wheezing. The small number of included participants and the inability to pool all clinical outcomes precludes us from making strong recommendations.

BACKGROUND

Description Of The Condition

Bronchiolitis is a very common lower respiratory tract infection in infancy.(1) In 1996 the hospitalization rate in the US for bronchiolitis among children younger than one year of age was 31.2 per 1000.(2) A variety of pathogens, almost exclusively viruses, can cause bronchiolitis in early childhood, among which are (para)influenza virus, adenovirus and respiratory syncytial virus (RSV). RSV is worldwide by far the most common cause.(1) Apart from the airway morbidity in the acute phase, bronchiolitis is also associated with long-term wheezing airway disease, also known as post-bronchiolitic wheezing (PBW). Although it may be difficult to distinguish PBW from asthma, PBW is transient in the majority of the cases. Wheezing episodes have been reported in up to 75% of children in the first two years after hospitalization for bronchiolitis, decreasing to 40% by the age of five and 20% by the age of 10.(3-5) Stein *et al.* found no increased prevalence of wheeze at the age of 13.(6) The impact of long-term airway morbidity after bronchiolitis may be substantial. It leads to increased use of medication, respiratory tract related hospital re-admissions(7) and lower health related quality of life.(8)

Description Of The Intervention

Two major hypotheses have been proposed to explain the association between bronchiolitis and PBW. The first hypothesis considers bronchiolitis as the first manifestation of recurrent wheezing in infants and children who are susceptible to obstructive airway disease. The second hypothesis suggests that the infection and concomitant inflammatory reaction in the acute phase leads to airway epithelium injury resulting in long-term obstructive airway disease.(9) In line with the latter hypothesis, it has been examined to see if immune modulation with corticosteroids during the acute phase may prevent PBW. Two small randomized controlled trials (RCTs) could not show a beneficial effect in preventing PBW with systemic corticosteroids given during the acute phase.(10;11) Inhaled corticosteroids in the acute phase of bronchiolitis seemed effective in the prevention of wheezing in one observational study (12) although this could not be confirmed in an RCT conducted by Cade *et al.*.(7)

Why It Is Important To Do This Review

Inhaled corticosteroids are relatively safe when given for a short period of time. The risk for adverse effects on growth, adrenal function and bone mineralisation is low.(13) The incentive for investigating the effect of inhaled corticosteroids in the prevention of wheezing episodes after bronchiolitis is based on the high prevalence of PBW, its implications on the participants' well-being as well as the low risk for adverse effects.

OBJECTIVES

The objective of this review was to systematically identify the effect of inhaled corticosteroids given during the acute phase of bronchiolitis in preventing post-bronchiolitic wheezing in young children and to identify areas of uncertainty for future research.

CRITERIA FOR CONSIDERING STUDIES FOR THIS REVIEW

Types Of Studies

Only randomized controlled trials (RCTs) or quasi-randomized controlled trials (for example, based on date of birth or day of the week) comparing inhaled corticosteroid vs placebo were included.

Types Of Participants

Studies that included children younger than two years of age with acute bronchiolitis, irrespective of the causative agent, were considered for inclusion. If children older than two years of age had been included, we would only have collected data of those children younger than two years of age. The clinical diagnosis of bronchiolitis was defined as the first period of cough, tachypnoea, chest recessions, hyperinflation and crepitations with or without wheezing. Children with a history of wheezing (bronchiolitis, bronchial obstruction or asthma) needed to be excluded. Studies performed in outpatient and inpatient settings were included.

Types Of Interventions

Trials comparing inhaled corticosteroid vs placebo were included. Therapy had to be started during the acute phase of the infection.

Types Of Outcome Measures

The primary outcome measure was the incidence of physician-diagnosed wheezing episodes. Secondary outcomes were hospital admissions for bronchial obstructions, symptomatic days recorded by parents and use of bronchodilator and/or corticosteroid treatment during follow up. The duration of the follow up period had to have been at least three months.

SEARCH METHODS FOR IDENTIFICATION OF STUDIES

We searched the Cochrane Central Register of Controlled Trials (CENTRAL) (*The Cochrane Library* 2006, issue 3) which contains the Cochrane Acute Respiratory Infections Group's trials register, MEDLINE (1966 to August 2006), EMBASE (1980 to June 2006) and Current Contents (September 2006).
MEDLINE was searched using the terms in conjunction with the highly sensitive search strategy designed by the Cochrane Collaboration for identifying randomized controlled trials.(14) The same strategy was used to search CENTRAL and adapted to search EMBASE and Current Contents.

Medline (OVID)

1 exp Bronchiolitis/
2 bronchiol$.mp.
3 or/1-2
4 exp Glucocorticoids/
5 (glucocortic$ or androstadienes or steroid$).mp.
6 exp Adrenal Cortex Hormones/
7 corticosteroid$.mp.
8 exp Budesonide/
9 (fluticasone or mometasone or flunisolide or triamcinolone acetonide).mp.
10 exp Beclomethasone/
11 or/4-10
12 exp Administration, Inhalation/
13 exp "Nebulizers and Vaporizers"/
14 exp Aerosols/
15 (inhal$ or spacer$ or nebuli$ or aerosol$).mp.
16 or/12-15
17 3 and 11 and 16

We obtained abstracts and reports of congresses (ERS 1999 to 2005, ATS 1999 to 2005) and consulted trial registers. Reference lists of the studies that were considered for inclusion were also critically checked for potential studies. Experts in the field and pharmaceutical companies were contacted for ongoing or unpublished studies. There were no language restrictions.

METHODS OF THE REVIEW

Study Identification

From the list of references, two review authors independently selected studies as being potentially relevant based on titles and, when available, abstracts. We obtained full text versions of the selected trials and ambiguous articles. The same review authors independently assessed the quality of each study. Discrepancies were resolved by discussion and consensus or by consulting a third review author.

Data Extraction

Two review authors independently extracted data using a standard form that contained the following data; study characteristics (design, methods of randomization, withdrawals/dropouts), participants, intervention and control characteristics (duration of therapy), follow up, outcome measures and results. We attempted to contact the trial authors of included studies if there was missing data or insufficient information about randomization or blinding but we did not obtain any additional data or methodological information.

Quality Assessment Of Trials

The same two review authors independently performed quality assessment using the Jadad 5-point scale, a validated scale used to assess randomization, double-blinding and the handling of withdrawals and dropouts.(15) Furthermore, we attempted to find out if sponsorship by a pharmaceutical company or any other institution could have caused a conflict of interest.

Data Analysis

We used Review Manager (RevMan) software from the Cochrane Collaboration for statistical calculations. Heterogeneity was assessed by visual inspection of forest plots and the I-squared statistic. Due to the small number of included studies the chi-squared test

Table 1. Characteristics of excluded studies.

Study	Reason for exclusion
Callen Beclua 2000	No placebo controlled trial
Kajosaari 2000	No placebo controlled trial
Reijonen 1996	No placebo controlled trial

to detect heterogeneity was not appropriate. We used fixed-effect models for outcomes without heterogeneity and random-effects models for outcomes with heterogeneity.
For dichotomous outcomes the relative risk (RR) was used as measure of treatment effect, and for continuous outcomes the mean difference was to be used. In case of sufficient data, outcomes were to be determined at 3, 6, 9 and 12 months follow up. Subgroup analyses were made on the duration of inhaled corticosteroid therapy (1 to 2 weeks, 3 to 8 weeks, and 9 to 16 weeks).

DESCRIPTION OF STUDIES

The original list of references contained 68 articles from which 8 studies were identified as being potentially relevant.(7;16-22) Three of these studies were of lesser quality (Jadad score 2; no placebo) and were excluded (Table 1).(17;19;20) The remaining 5 studies that were included were published in English and of parallel design (Table 2). All studies included hospitalised infants, less than 1 year of age. Bentur included infants aged 3 to 12 months.(16)
All studies excluded children with congenital heart disease, chronic respiratory disease (bronchopulmonary dysplasia) and immune deficiency. In addition, 2 studies also excluded premature born infants.(21;22) Two studies (18;22) excluded mechanically ventilated children and 3 studies (7;16;22) excluded infants with prolonged exposure to corticosteroids.
All studies described the clinical diagnosis of bronchiolitis, which met the description in the inclusion criteria. Two studies only included children with a proven RSV infection. (7;16) In the remaining 3 studies, the proportion of children with a proven RSV infection ranged from 41.6% to 82.5% and was equal in both treatment arms. Nebulised budesonide was evaluated in 2 studies,(7;21) budesonide by dose inhaler was evaluated in 1 study,(18) fluticasone by dose inhaler was evaluated in 1 study (22) and nebulised dexamethasone was evaluated in 1 study.(16) In 1 study the trial medication (both dexamethasone and placebo) was nebulised with epinephrine.(16)
Medication was started on admission (7;16;21) or at hospital discharge.(18;22) The duration of therapy ranged from 2 to 13 days (16) up to 3 months.(22) One study treated the patients only during hospitalization.(16)

Table 2. Characteristics of included studies.

Study	Methods	Participants	Interventions	Outcomes	Notes	Allocation concealment
Bentur 2005	Randomised, placebo controlled trial	Sixty-one infants aged 3 to 12 months, hospitalised for RSV bronchiolitis	Nebulised dexamethasone 0.25 mg with epinephrine 1 ml 4 times daily or placebo with epinephrine.	Clinical scores. Duration of oxygen therapy. Duration of IV fluids. Length of hospital stay. Number of patients with hospital re-admissions. Number of patients with recurrent wheeze.	Jadad 3. No information about follow up. Commercial funding unknown	Unclear
Cade 2000	Randomised, placebo controlled trial	One hundred and sixty-one infants younger than 12 months of age, hospitalised for RSV positive bronchiolitis	Nebulised budesonide BID 1 mg or placebo. Started within 12 hours after admission. Continued until 14 days after discharge (maximum 21 days).	Episodes of cough or wheezing. Re-admission for respiratory symptoms. GP visits for respiratory symptoms. Type and amount of additional medication taken for respiratory symptoms. Follow up 12 months	Jadad 4. Three infants in the placebo group and one infant in the budesonide group excluded from analysis. No differences between groups except for furry pets (placebo 36% versus bufenolide 21%) Funding by Astra Foundation	Unclear
Fox 1999	Randomised, placebo controlled trial	Sixty infants younger than 12 months of age, hospitalised for viral bronchiolitis	Aerosol budesonide BID 200 mcg or placebo. Started at discharge for eight weeks.	Number patients with symptoms (cough and wheeze). Re-admission for respiratory symptoms. Median symptom episodes. Median symptom days. Follow up 12 months	Jadad 4. Eleven lost to follow up. No significant differences between groups, although there were more boys in the budesonide group. A logistic regression analysis was performed to assess whether budesonide predicted the incidence of wheeze and cough independent of male gender, which was not the case (P value = 0.051). No commercial funding	Unclear

Table 2 Continued

Study	Methods	Participants	Interventions	Outcomes	Notes	Allocation concealment
Richter 1998	Randomised, placebo controlled trial	Forty infants younger than 12 months of age, hospitalised for bronchiolitis	Nebulised budesonide BID 1 mg for 5 days, followed by BID 0.5 mg for the rest of six weeks. Started soon after admission	Prevalence wheezing. Median cough and wheezing. Median wheezing alone. Number of infants given oral or inhaled corticosteroids or bronchodilators. Number of infants readmitted for respiratory problems. Follow up for six months.	Jadad 4. One lost to follow up. No differences between groups Funding by Astra Clinical Research Unit	Unclear
Wong 2000	Randomised, placebo controlled trial	Forty-eight infants aged between 2 weeks and 12 months, hospitalised for bronchiolitis	Aerosol fluticasone TDS 50 mcg or placebo for three months. Started on discharge	Cough events recorded on tape during night. Cough episodes recorded on tape during night. Median symptom days. Follow up 12 months	Jadad 3. Seven lost to follow up. No differences between groups Funding by Glaxo Wellcome	Unclear

Note. BID, twice daily; IV, intravenous; TDS, three times a day.

Most studies reported results of data collected at the end of the follow-up period that varied from 3,(16) 6,(21) 9 (22) to 12 months respectively.(7;18) Wong *et al.* also presented results at the additional time point of 3 months follow up.(22) Fox *et al.* gave additional data at a time of follow up of 1 and 2 months on one of their outcomes (number of infants with symptoms).(18)
In 4 studies, parents were asked to keep diary records.(7;18;21;22) Data on symptom days, additional medication taken, general practitioner (GP) visits or hospital re-admissions were collected in all studies on a regular basis (at least 3 times during follow up) either during outpatient clinic visits (16;18;21) or home visits.(7;22) Cade also checked GP notes after 12 months.(7)

METHODOLOGICAL QUALITY

The median Jadad score of the 5 included studies was 4 (inter-quartile range 3 to 4). Although all trials described their study as randomized, a sufficiently detailed description of the randomization procedure was not provided in any of them. Therefore we were unable to score the item 'concealment of allocation' in any of the included studies.
All trials were described as double-blinded. In 2 trials (7;21) the blinding could be considered as appropriate. In the other 3 trials, the method of blinding was not described in detail. In 1 study it was unclear whether follow up was complete.(16) All other studies described their loss to follow up. Sponsorship was reported in 4 trials. Sponsorship by a pharmaceutical company was reported in 3 trials (7;21;22) and by a charity institution in 2 trials.(18;21)

RESULTS

Comparisons

In the comparison of any inhaled corticosteroid vs placebo, 5 studies (involving 374 infants) were included with complete follow up of 358 infants. Data on the number of infants with wheeze, hospital re-admissions and the use of corticosteroids and bronchodilators could be pooled (Table 3; Figure 1-7). Other outcomes were clinically too heterogeneous to be pooled or were presented as medians. The relative risk (RR) on wheeze, recorded in a diary or diagnosed by a physician was 1.15 (95% confidence interval (CI) 0.80 to 1.65). Visual inspection of the forest plots and the I-squared statistic showed statistical heterogeneity in this outcome. This could be caused by the study of Fox *et al.*, who favoured control.(18) Exclusion of this study from the analysis however,

Table 3. Comparison of any inhaled corticosteroid versus placebo.

	Outcome title	No. of studies	No. of participants	Statistical method	Effect size
01	Number of infants given or prescribed steroids	2	200	Relative Risk (Fixed) 95% CI	0.85 [0.64, 1.12]
02	Number of infants given or prescribed bronchodilators	2	200	Relative Risk (Fixed) 95% CI	0.95 [0.76, 1.17]
03	Hospital re-admissions - duration of therapy	4	309	Relative Risk (Fixed) 95% CI	1.14 [0.76, 1.72]
04	Number of infants with wheeze (GP or diary)	3	149	Relative Risk (Random) 95% CI	1.15 [0.80, 1.65]
05	Hospital re-admissions in a follow up period of 12 months - RSV positive	5	358	Relative Risk (Fixed) 95% CI	1.09 [0.74, 1.59]
06	Hospital re-admissions – follow up	5	358	Relative Risk (Fixed) 95% CI	1.09 [0.74, 1.59]
07	Hospital re-admissions	5	358	Relative Risk (Fixed) 95% CI	1.09 [0.74, 1.59]

Note. GP, general practitioner; CI, confidence interval.

did not alter the outcome effect. The RR to be re-admitted to hospital for respiratory problems was 1.09 (95% CI 0.74 to 1.59) and the use of steroids or bronchodilators (either given by careers or prescribed by a physician) was 0.85 (95% CI 0.64 to 1.12) and 0.95 (95% CI 0.76 to 1.17) respectively. Although the I-squared statistic did not suggest important heterogeneity, visual inspection of the forest plots on the outcome of hospital re-admissions revealed 1 study(21) that favoured a control group. Exclusion of this study from the analysis did not alter the pooled outcome effect.

Although the data of the other endpoints could not be pooled, none of the included individual studies showed a significant beneficial effect of corticosteroids on symptom days of cough and/or wheeze. One study mentioned a decrease in the number of infants who received bronchodilators or steroids, or both after three months in the treatment group (*P* value 0.07).(22) However this effect could not be demonstrated after 9 months of follow up.

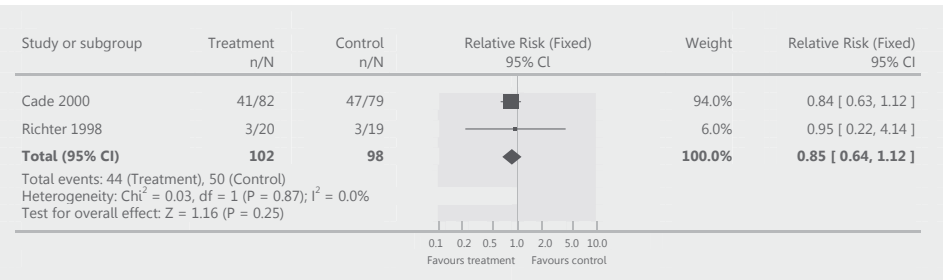

Figure 1 Outcome 01. Number of infants given or prescribed steroids.

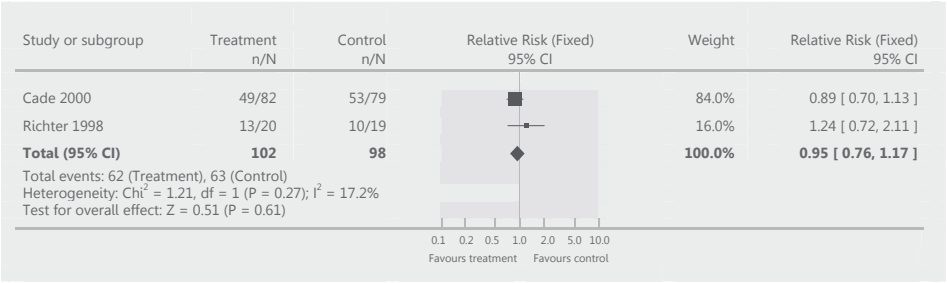

Figure 2 Outcome 02. Number of infants given or prescribed bronchodilators.

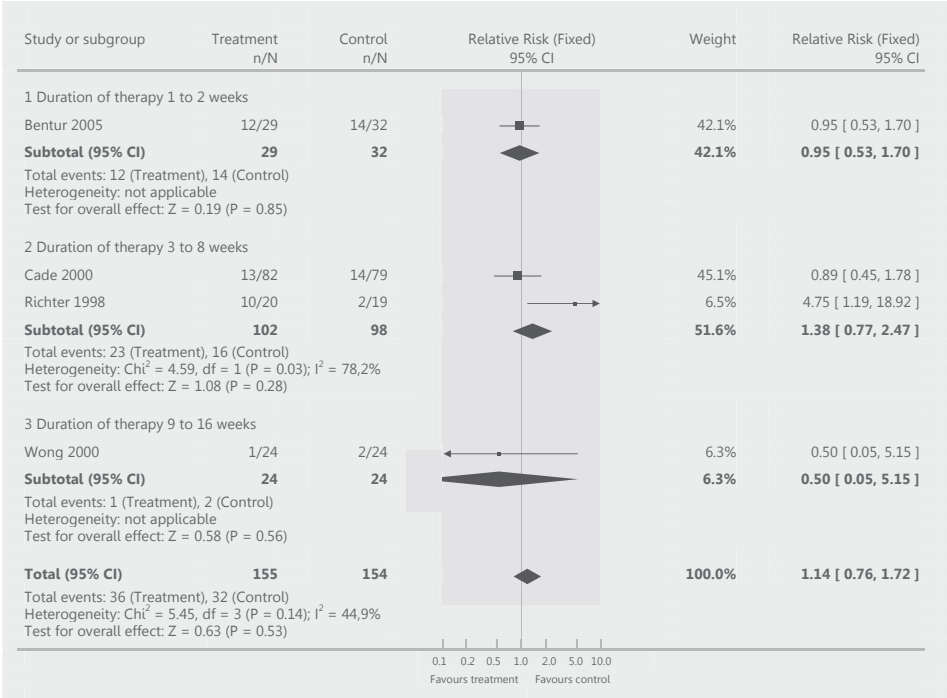

Figure 3 Outcome 03. Hospital re-admissions – duration of therapy.

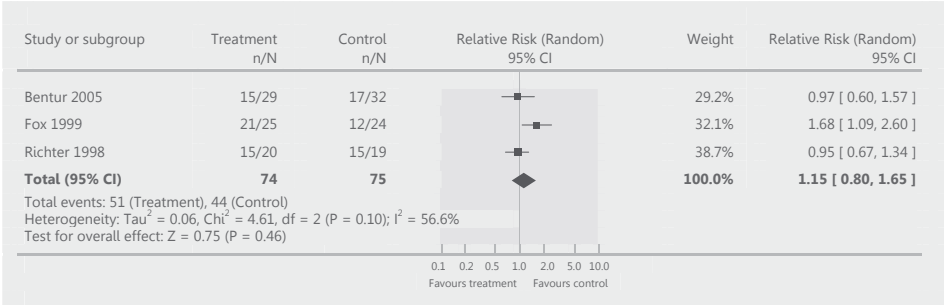

Figure 4 Outcome 04. Number of infants with wheeze (GP or diary).

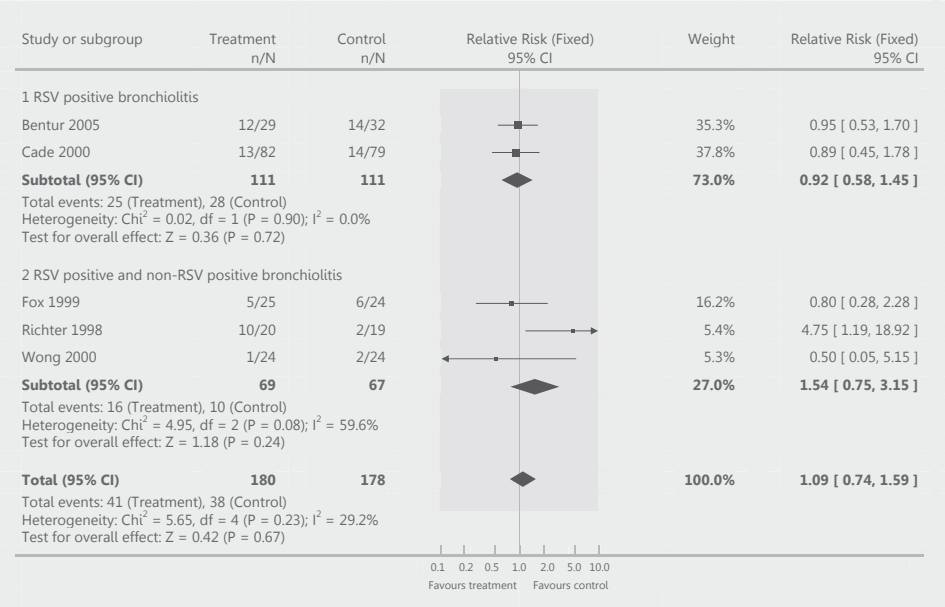

Figure 5 Outcome 05. Hospital re-admissions in a follow up period of 12 months –RSV positive.

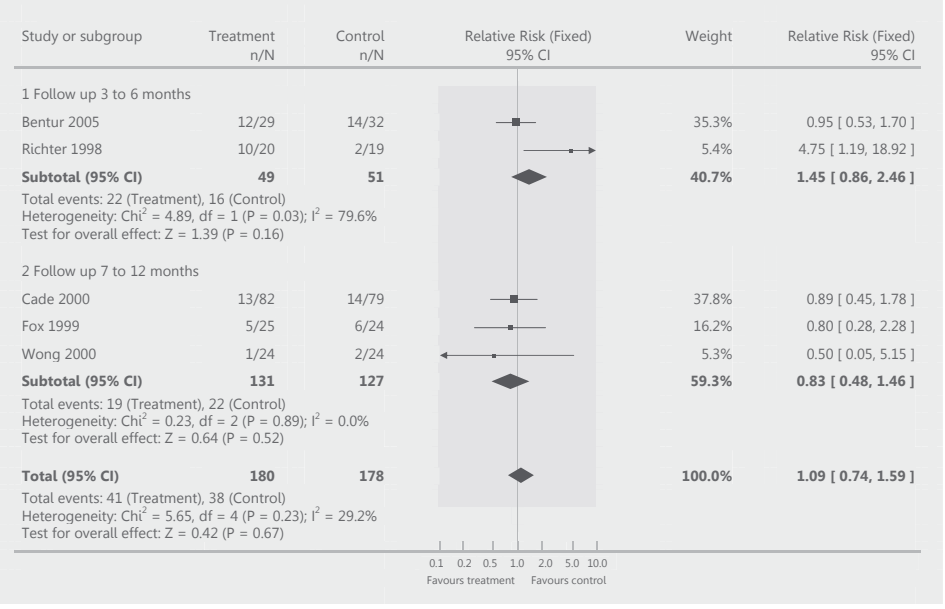

Figure 6 Outcome 06. Hospital re-admissions – follow up.

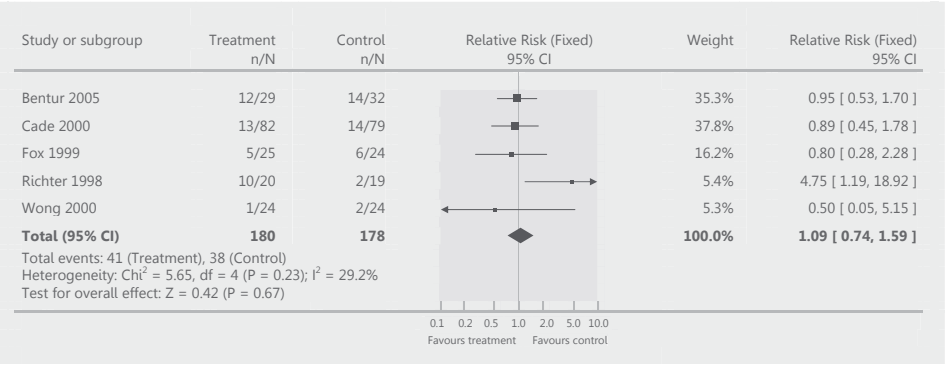

Figure 7 Outcome 07. Hospital re-admissions.

Subgroup And Sensitivity Analysis

Subgroup analysis did not suggest any influence of duration of therapy or length of follow up on the outcome effect. RSV as a cause of bronchiolitis did not influence the outcome effect in a post-hoc analysis. A post-hoc analysis on type of corticosteroid was not performed due to the variability of the corticosteroids used.

Publication Bias

Considering the negative results of all included studies, publication bias was less likely.

DISCUSSION

We found a limited number of RCTs that addressed the effect of inhaled corticosteroids on the prevention of post-bronchiolitic wheezing. Moreover, pooling of the data was complicated by the use of different outcomes and effect measures in the various trials. Therefore we were unable to evaluate the effect on the incidence of physician-diagnosed wheezing episodes, which was the primary outcome measure of this review. If we considered this more widely and also included wheeze reported by parents in a diary,(21) we found no effect on the incidence of wheeze. We found no effect of inhaled corticosteroids on the prevention of post-bronchiolitic wheezing, associated re-hospitalization, or the use of bronchodilators or steroids. Data on symptom days could not be pooled.

None of the individual included trials demonstrated a beneficial effect on any of the outcomes, except for 1 (the use of bronchodilators or steroids at 3 months follow up (*P* value 0.07).(22) Only 2 studies (16;18) had enough power. In 2 studies, one (21) or two (18) of the outcomes favoured the control group. However, in the study of Richter

et al. no significant difference between the study groups was found on all the other four outcomes (prevalence of wheeze, scores for wheeze and cough, scores for wheeze only, number of infants given steroids or number of infants given bronchodilators).(21) It remains unclear why infants in the treatment group were hospitalized more often for respiratory problems. In the study by Fox *et al.* more infants in the treatment group experienced symptoms of cough and wheeze and the median of symptom episodes was also higher in the treatment group. (18) No differences were found on the other three outcomes (number of infants re-hospitalized, number of infants with more than 3 episodes and median of symptom days). However, the authors found an unequal gender distribution between the two study arms, since fewer boys were lost to follow up in the treatment group. After correction for this confounding factor, the detrimental effect of inhaled steroids in comparison to placebo disappeared.

Three of the 5 included studies evaluated adverse event. Wong *et al.* reported 2 infants with oral candidiasis in the treatment group, but no significant difference in length, weight and systolic blood pressure between the treatment and placebo group during 6 months of follow up.(22) Fox *et al.* only reported 2 children who needed to be hospitalized with adverse events.(18) One child with viral gastroenteritis and another with mild cough and wheezing. It is not clear why this latter child has been reported as experiencing an adverse event instead of a negative outcome effect. Finally Richter *et al.* found no difference in growth rates between the steroid and placebo group during 6 weeks of study treatment, although this period may have been too short to draw firm conclusions. (21)

Safety of inhaled corticosteroids has been studied in children with asthma. However, in general in these studies infants are underrepresented. In an RCT, Berger *et al.* found no difference in adrenal function in infants aged 6 to 12 months of age treated with budesonide or placebo inhalation for 12 weeks.(10) To the best of our knowledge, there are no data on the effects of inhaled corticosteroids on lung growth or neuro-development.

It has been shown that RSV is an independent risk factor for post-bronchiolitic wheezing.(6) The vast majority of patients included in the studies of this review suffered from RSV bronchiolitis. In a post-hoc analysis we were unable to show a beneficial effect of inhaled steroids on hospital re-admissions in patients who suffered from bronchiolitis caused by RSV.

AUTHORS' CONCLUSIONS

Implications For Practice

There is limited data on the effect of inhaled corticosteroids on the prevention of post-bronchiolitic wheezing. The available data, however, does not support its clinical use.

Implications For Research

Large RCTs are needed to investigate the effect of inhaled corticosteroids. Future studies should consider stratification to the causative agents of bronchiolitis, in particular RSV, and uniform outcomes need to be established.

POTENTIAL CONFLICT OF INTEREST

No (financial) conflict of interest.

ACKNOWLEDGEMENTS

The review authors wish to thank the following people for commenting on the review draft: Amy Zelmer, Michelle Garrison, Jonathan Mansbach, Max Bulsara and George Swingler.

SOURCES OF SUPPORT

External sources of support
No sources of support supplied.

Internal sources of support
Emma Children's Hospital / AMC Amsterdam Netherlands

REFERENCE LIST

(1) Wright AL, Taussig LM, Ray CG, Harrison HR, Holberg CJ. The Tucson Childrens Respiratory Study. Lower Respiratory-Tract Illness in the 1St Year of Life. American Journal of Epidemiology 1989; 129(6):1232-1246.

(2) Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. Journal of the American Medical Association 1999; 282(15):1440-1446.

(3) Pullan CR, Hey EN. Wheezing, asthma, and pulmonary dysfunction 10 years after infection with respiratory syncytial virus in infancy. Br Med J (Clin Res Ed) 1982; 284(6330):1665-1669.

(4) Henry RL, Hodges IGC, Milner AD, Stokes GM. Respiratory problems 2 years after acute bronchiolitis in infancy. Archives of Disease in Childhood 58, 713-716. 1983.

(5) Murray M, Webb MSC, Ocallaghan C, Swarbrick AS, Milner AD. Respiratory Status and Allergy After Bronchiolitis. Archives of Disease in Childhood 1992; 67(4):482-487.

(6) Stein RT, Sherrill D, Morgan WJ et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 1999; 354(9178):541-545.

(7) Cade A, Brownlee KG, Conway SP et al. Randomised placebo controlled trial of nebulised corticosteroids in acute respiratory syncytial viral bronchiolitis. Archives of Disease in Childhood 2000; 82(2):126-130.

(8) Bont L, Steijn M, van Aalderen WM, Kimpen JL. Impact of wheezing after respiratory syncytial virus infection on health-related quality of life. Pediatr Infect Dis J 2004; 23(5):414-417.

(9) Long CE, McBride JT, Hall CB. Sequelae of respiratory syncytial virus infections. A role for intervention studies. Am J Respir Crit Care Med 1995; 151(5):1678-1680.

(10) Berger WE, Qaqundah PY, Blake K et al. Safety of budesonide inhalation suspension in infants aged six to twelve months with mild to moderate persistent asthma or recurrent wheeze. J Pediatr 2005; 146(1):91-95.

(11) van Woensel JB, Kimpen JL, Sprikkelman AB, Ouwehand A, van Aalderen WM. Long-term effects of prednisolone in the acute phase of bronchiolitis caused by respiratory syncytial virus. Pediatr Pulmonol 2000; 30(2):92-96.

(12) Hesselmar B, Adolfsson S. Inhalation of corticosteroids after hospital care for respiratory syncytial virus infection diminishes development of asthma in infants. Acta Paediatr 2001; 90(3):260-263.

(13) Allen DB. Safety of inhaled corticosteroids in children. Pediatric Pulmonology 2002; 33(3):208-220.

(14) Dickersin K, Scherer R, Lefebvre C. Systematic Reviews - Identifying Relevant Studies for Systematic Reviews. British Medical Journal 1994; 309(6964):1286-1291.

(15) Jadad AR, Moore RA, Carroll D et al. Assessing the quality of reports of randomized clinical trials: Is blinding necessary? Controlled Clinical Trials 1996; 17(1):1-12.

(16) Bentur L, Shoseyov D, Feigenbaum D, Gorichovsky Y, Bibi H. Dexamethasone inhalations in RSV bronchiolitis: a double-blind, placebo-controlled study. Acta Paediatr 2005; 94(7):866-871.

(17) Callen Beclua M, Aizpurua Galdeano P, Ozcoidi Erro I, Mancisidor Aguinagalde L, Guedea Adiego C, Busselo Ortega E. Inhaled corticosteroids and wheezing post-bronchiolitis. Anales Espanoles de Pediatria 52[4], 351-355. 2000.

(18) Fox GF, Everard ML, Marsh MJ, Milner AD. Randomised controlled trial of budesonide for the prevention of post-bronchiolitis wheezing. Arch Dis Child 1999; 80(4):343-347.

(19) Kajosaari M, Syvanen P, Forars M, Juntunen-Backman K. Inhaled corticosteroids during and after respiratory syncytial virus-bronchiolitis may decrease subsequent asthma. Pediatric Allergy and Immunology 2000; 11(3):198-202.

(20) Reijonen T, Korppi M, Kuikka L, Remes K. Anti-inflammatory therapy reduces wheezing after bronchiolitis. Archives of Pediatrics & Adolescent Medicine 1996; 150(5):512-517.

(21) Richter H, Seddon P. Early nebulized budesonide in the treatment of bronchiolitis and the prevention of postbronchiolitic wheezing. Journal of Pediatrics 1998; 132(5):849-853.

(22) Wong JY, Moon S, Beardsmore C, O'Callaghan C, Simpson H. No objective benefit from steroids inhaled via a spacer in infants recovering from bronchiolitis. Eur Respir J 2000; 15(2):388-394.

Chapter 4

RSV Corticosteroid Study: Design, Materials & Methods

ABSTRACT

The RSV Corticosteroid Study is an investigator-initiated and -conducted multicenter, double-blind, randomized, placebo-controlled, parallel-group comparison of high-dose hydrofluoroalkane extrafine beclomethasone dipropionate to placebo in infants under thirteen months of age and hospitalized for RSV LRTI. The RSV Corticosteroid Study aimed to assess whether high-dose inhaled glucocorticosteroids during the first three months following admission for RSV LRTI prevent the occurrence and severity of long-term airway morbidity. The primary outcome variable was the number of days with wheeze during the observation year following the intervention. Secondary outcome variables were the number of coughing days, the number of days with airway medication use, duration of hospitalization, health-related quality of life, lung function, potential adverse effects of treatment on growth, and thrush incidence. Poisson regression analysis was used to study potential differences in number of wheezing days. All analyses were conducted according to the 'intention-to-treat' principle.

An educational 'Videotape Performance Program' (VPP) was incorporated to monitor and maximize competence and adherence. Three weeks following hospital discharge parental performance was videotaped and scored according to modified criteria of the Dutch Asthma Foundation. Sixty-two percent of parents performed all essential steps correctly and another 33% performed 5 out of 6 steps correctly. The most frequent error in case of inadequate performance was the absence of visible spacer valve movements. Parental adherence to the treatment regimen was determined by weighing returned canisters. Sixty-six percent of parents administered the prescribed medication twice daily during at least nine full weeks of the prescribed three months.

DESIGN

The RSV Corticosteroid Study is a multicenter, double-blind, randomized, placebo-controlled, parallel-group comparison of high-dose hydrofluoroalkane extrafine beclomethasone dipropionate to placebo in infants under thirteen months of age and hospitalized for RSV LRTI. Infants were randomized to one of two treatment groups, one receiving active treatment and the other placebo. Study medication was administered for a period of three months, followed by an observation period of twelve months during which the main outcomes were assessed (Figure 1). According to Good Clinical Practice the protocol (Attachment 1) was registrated on Current Controlled Trials (ISRCTN12352714). It was conducted in accordance with the principles of the Declaration of Helsinki and has been approved by the Ethics Review Committee, University Medical Center Utrecht and by the Ethics Review Committees at all participating centers.

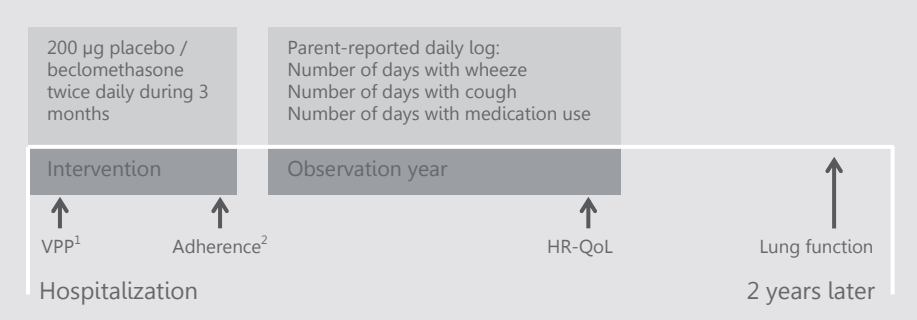

Figure 1. Design of the RSV Corticosteroid Study.

Note. VPP, video performance program; HR-QoL, Health related Quality of Life.

[1]Competence was determined using the Video Performance Program (VPP)

[2]Adherence was determined by weighing returned canisters

OBJECTIVES

The primary objective of the RSV Corticosteroid Study was to assess whether high-dose inhaled glucocorticosteroids during the first three months following admission for RSV LRTI prevent the occurrence and severity of long-term airway morbidity. The primary outcome of the study was the counted number of days with parent-reported wheeze during the observation year following the intervention. Secondary outcomes included counted days of cough during the observation year, use of airway medication during the observation year, health-related quality of life during the next winter season and lung function at the age of 2 years.

STUDY COHORT

Recruitment took place during the winter seasons from November 2004 to February 2006. 243 infants were included and randomized. The intervention period of the study ended in May 2006 and the observation period for the primary outcome ended in May 2007. Patients were recruited in pediatric departments of 19 clinical centers in the Netherlands. Selection criteria were designed to enroll previously healthy children with a first episode of RSV LRTI and to exclude infants with a history of wheezing or cardiac or pulmonary disease (Table 1).

Table 1. In- and exclusion criteria for participation.

Inclusion criteria	Infants under 13 months of age
	Hospital admission for LRTI
	Positive immunofluorescence for RSV infection in epithelial cells from nasopharyngeal aspirates
Exclusion criteria	Previous use of steroids
	History of cardiac or pulmonary disease
	Wheezing illness prior to RSV LRTI

RANDOMIZATION AND BLINDING

Consecutive infants were assigned randomly to placebo or active medication. Randomization was by means of a computer-generated list of six numbers in each block and fixed blocks within each hospital. Blinding was accomplished by use of active and placebo medication of identical shape and taste. Randomization codes could distinguish active and placebo medication. Randomization codes were secured for physicians, nurses, parents and investigators until data entry was complete (July 2007).

STUDY MEDICATION

Hydrofluoroalkane extrafine beclomethasone dipropionate (HFA BDP) (Qvar, kindly provided by 3M Nederland BV, Leiden, the Netherlands. Currently manufactured by Teva-Pharma, Haarlem, The Netherlands), a dose of 200 microgram twice daily, or matching placebo was administered by pressurized metered-dose inhaler and a spacer (Aerochamber, kindly provided by Trudell International Europe Ltd., Nottingham, United Kingdom). Hydrofluoroalkane-based beclomethasone dipropionate was used to obtain a maximal effect of immune modulation. This extrafine beclomethasone dipropionate formulation contains 1.1 µm particles that are smaller than the conventional chlorofluorocarbon (CPC)-based beclomethasone dipropionate particles (3,5-4,0µm). Extrafine hydrofluoro-

alkane aerosols have better access to the distal lung, with less oropharyngeal deposition and are described to be more effective without inducing more toxicity.(1)
No rescue therapy was predefined. Prescribed and used other medication, i.e. bronchodilation medication, was notified in the daily log. In case of an absolute need to use glucocorticosteroid therapy, study medication was discontinued and prescribed glucocorticosteroid medication started.

INHALATION TECHNIQUES, COMPETENCE AND ADHERENCE

Competence

Correct use of the Aerochamber is a prerequisite for succesful drug treatment.(2;3) An educational 'Videotape Performance Program' (VPP) was incorporated in the current trial to monitor and maximize competence and adherence. Asthma nurses who were related to the participating centers uniformed their inhalation technique instructions. Each center was provided with educational materials and with a camcorder, kindly provided by JVC (JVC Nederland, Leiden, the Netherlands). Following randomization, parents received individual education from the asthma nurses. During hospitalization parents administered the study-medication to their infant under supervision of educated nurses. Before discharge parental performance was videotaped. Asthma nurses gave reinstructions if necessary. Three weeks after discharge parental performance was videotaped and scored according to modified criteria of the Dutch Asthma Foundation (shaking of the inhaler, correct assembly of spacer and canister, activation of the canister, no more than 3 puffs at the same time, inhale at least 10 seconds, moving spacer valve).(2) Competence was classified as adequate (6 points) or inadequate (0-5 points). Two independent researchers scored one hundred seventy-seven parental performances (median infant age 3.4 months). Inter-observer correlations were high (Spearman's rho 0.7 (summed score), *P*-value < 0,001). One hundred nine (62%) parents performed all essential steps correctly and another 58 parents (33%) performed 5 out of 6 steps correctly. Absence of visible spacer valve movements was the most frequent error in case of inadequate performance (Table 2).

Adherence

In a subset of infants (infants included during the second recruitment season), parental adherence to the treatment regimen was determined by weighing returned canisters. A pilot study showed a linear relation between number of dispensed actuations and weight of canisters in both placebo and active medication canisters (Figure 2). Weights

Table 2. Parental competence.

Percentages of correct performances on scored competence criteria (modified criteria of the Dutch Asthma Foundation)

Shake inhaler	92%
Correct assembly	99%
Activation of canister	100%
No more than two puffs	100%
Inhale at least ten seconds	92%
Moving valve	72%

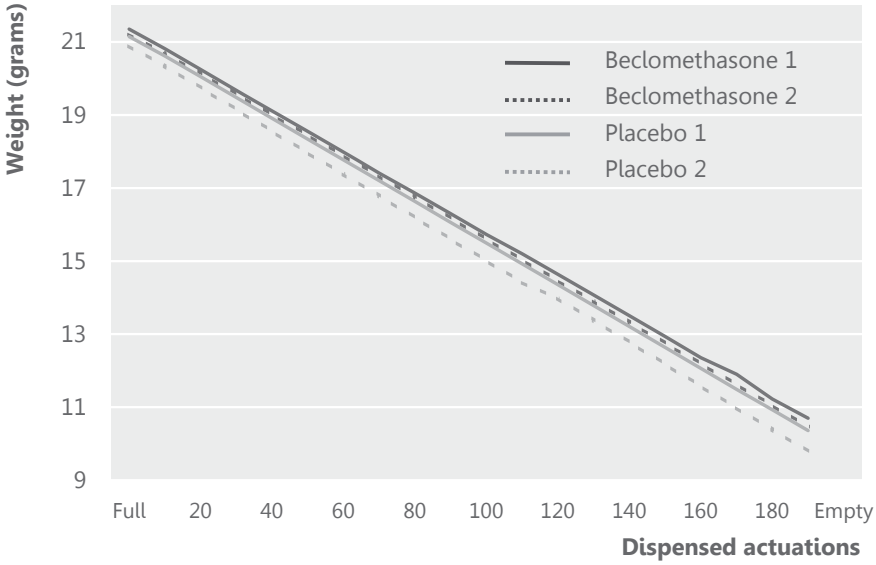

Figure 2. Pilot study.

Weight of canisters versus experimental emptying of canisters.

of canisters of 121 infants were determined. Eighty parents (66%) administered the prescribed medication twice daily during at least nine full weeks of the prescribed three months (Figure 3). Age, sex, family history of asthma and social background did not relate to competence and adherence (data not shown).

OUTCOMES AND CLINICAL ASSESSMENTS

Primary Outcome: Wheeze During Follow Up

Following hospital discharge parents noted respiratory symptoms in a previously described daily log.(4) Recorded respiratory symptoms were "runny nose", "coughing",

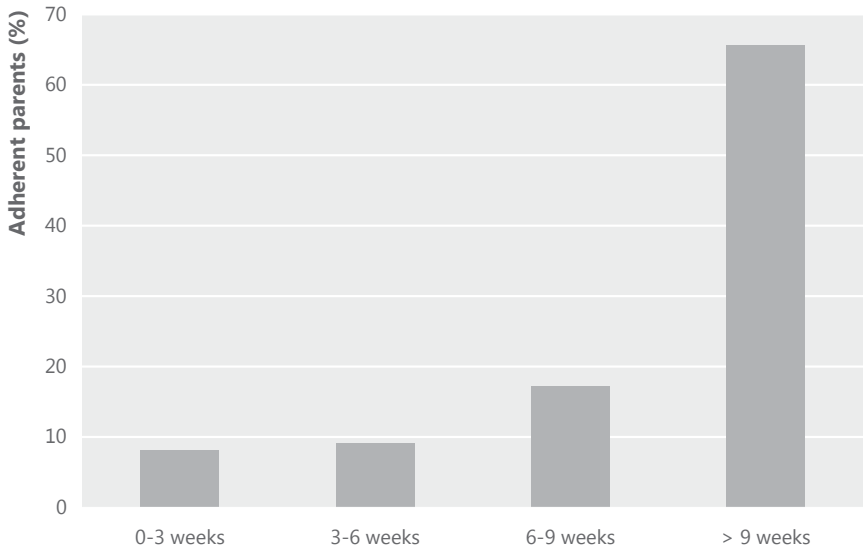

Figure 3. Parental adherence as calculated by weighing canisters.

"wheezing" "snoring" and "shortness of breath". Symptoms were graded in severity on a scale from 0-3 (absent, moderate, severe, very severe) (Figure 4). A single investigator (ME) instructed parents. Telephone calls for motivational purposes were scheduled every three months. Logs were kept for 15 months from start of the intervention. The primary outcome of the RSV Corticosteroid Study was the number of days with wheeze (score 1-3) during the observation year following the intervention.
The response rate of returned logs was 81% during complete follow-up. The occurrence of recurrent wheeze was remarkably similar in placebo-treated infants of the current cohort and infants of the 'natural course' cohort of L.Bont recruited in 1997-1998 (Figure 5).

Secondary Outcomes

Secondary outcome variables were the number of coughing days, the number of days with airway medication use, duration of hospitalization, health-related quality of life, lung function, potential adverse effects of treatment on growth, and thrush incidence.

Health-related Quality Of Life & Questionnaires

Recurrent wheeze following RSV LRTI has broad implications for long-term well-being of children.(5) Health-related quality of life was assessed using the TAPQOL (TNO-AZL Pre-school children Quality of Life) questionnaire, a generic instrument for assessing health-related quality of life of pre-school children.(6) Health-related quality of life was assessed during the winter season one year after hospitalization. In addition an extended

2005, **Week 40**

• When your child had no respiratory symptoms at all during this week, you can mark it here ☐

• When symptoms have been observed, then symptom scores for every day of the week have to be completed.

0 = not
1 = mild
2 = moderate
3 = severe

	Runny nose (1-3)	Coughing (1-3)	Wheezing (1-3)	Snoring (1-3)	Shortness of breath (1-3)	Use of medication (y/n)	Doctor's visit (y/n)
Monday							
Tuesday							
Wednesday							
Thursday							
Friday							
Saturday							
Sunday							

Figure 4. Daily log as used in the RSV Corticosteroid Study.

Dutch version of the British Medical Council standardized questionnaire and the Dutch version of the European Community Respiratory Health Survey questionnaire were used to obtain data on respiratory symptoms, allergy, parental smoking habits, and allergy symptoms among first-degree family members. Follow up health-related quality of life assessment and physician-diagnosed asthma at age 6 years will be considered.

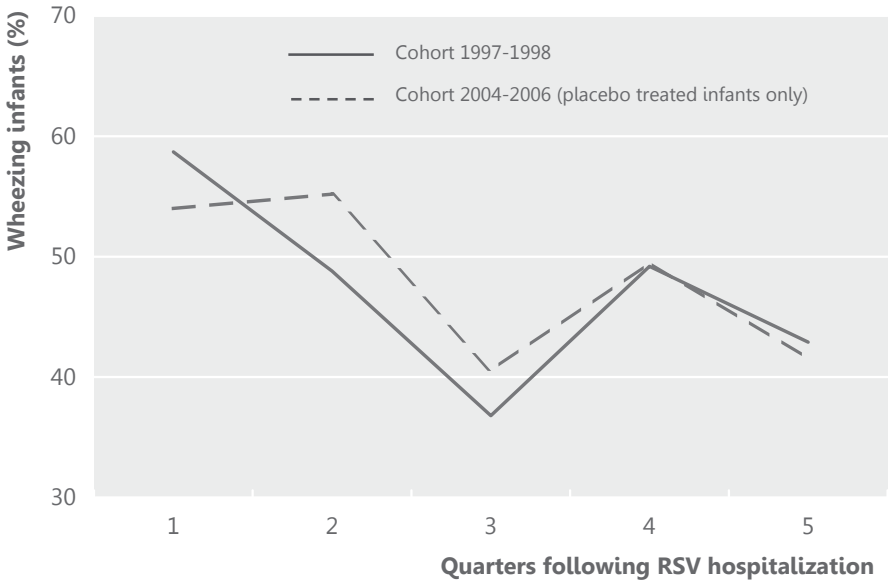

Figure 5. Recurrent wheeze during follow up in two distinct cohorts.

Lung Function

Lung function and bronchial responsiveness were tested by measuring interrupter resistance using MicroRint® at the age of 2 years.(7) The MicroRint® is a small portable data recording airway resistance meter. It measures airway resistance during quiet breathing, requires minimal subject co-operation and can be used during spontaneous breathing and without sedation. Alveolar pressure and mouth pressure equilibrate within a few milliseconds during brief airflow interruption. Changes in mouth pressure are measured after a brief airflow interruption at the mouth. Airway resistance can be calculated from the ratio of mouth pressure to airflow at the mouth just prior to occlusion and was measured before and after administration of 400 microgram of inhaled salbutamol with the use of a spacer. Follow up lung function at age 6 years will be considered.

Adverse Effects

Height was measured at age 2 to evaluate the effect of inhaled glucocorticosteroids on growth. Parents were questioned about side-effects of anti-inflammatory treatment, i.e. oral fungal infections, growth and development.

ANALYSES

Power Calculation

The sample size was based on prevalence data for the primary outcome obtained from our previous RSV cohort study.(4) In that cohort, 80% of infants wheezed at any time, with a mean number of wheezing days during 4-15 months following RSV LRTI of 34.2 (95% CI 26.2-42.3 days). The predefined target of 250 patients provided at least 90% power to detect a difference of 14 wheezing days between both arms.

Intention To Treat

All analyses were conducted according to the 'intention-to-treat' principle, i.e. including data from all randomized infants and with infants analyzed in the group to which they were assigned at randomization, irrespective of whether study treatment was given or taken. No interim analyses were conducted.

Statistical Analyses

Effects of treatment with beclomethasone on the occurrence of any wheeze, cough, airway medication use, and thrush during follow up were analyzed by calculating percent-

ages and associated 95% confidence intervals of infants suffering any symptoms. Poisson regression analysis was used to study potential differences in number of wheezing and coughing days and in days of airway medication use. Duration of hospitalization (in days), health-related quality of life domain scores, interrupter resistance measurements (in kPA/L/s), and height (in cm) were expressed as medians and means. χ^2 tests, Student's t tests, and Mann Whitney U test were used to evaluate differences in percentages, mean values, and median values between the groups.

Subgroup Analyses

Two pre-defined subgroup-analyses were performed. Subgroup-analyses were performed for children with and without need for mechanical ventilation. Because signs of airflow limitation during acute RSV LRTI (i.e. physician-diagnosed wheeze) are associated with the development of recurrent wheeze,(8) subgroup-analyses were performed for children with and without signs of airflow limitation during initial RSV LRTI.

Missing values

To decrease bias and to increase statistical efficiency, missing data were imputed for health-related quality of life domain scores, interrupter resistance measurements, duration of hospitalization, height and presence of thrush. Imputation was done using the linear regression method (Missing Value Analyses) available in SPSS (SPSS for Windows, version 13.0, SPSS Inc.) software.(9;10) Such imputation is based on the correlation between each variable with missing values and all other variables as estimated from the set of complete subjects. In addition to intention-to-treat analyses, complete case analyses were performed using cases with complete log data.

STUDY ORGANISATION

The RSV Corticosteroid Study is an investigator-initiated and –conducted trial financially supported by the Dutch Asthma Foundation (grant nr. 3.2.03.22). Local investigators of the nineteen participating centers were responsible for recruiting subjects, providing study medication and educating parents to administer medication (Table 3). The RSV research group of the University Medical Center Utrecht was responsible for follow-up and quality control for all aspects of data collection and analysis.

Table 3. RSV Corticosteroid Study Group (in alphabetic order).

Maartje ten Berge-Kuipers	St. Antonius Hospital, Nieuwegein
Joost de Bie	Hofpoort Hospital, Woerden
Grada van Bleek	University Medical Center, Utrecht
Daniel Blom	Academical Medical Center, Amsterdam
Louis Bont	University Medical Center, Utrecht
Machtelt Bouwman	Gelderse Vallei Hospital, Ede
Reitze Bruinsma	Gelre Hospital, Apeldoorn
Frank Brus	Rijnstate Hospital, Arnhem
Max Colombijn	Beatrix Hospital, Gorinchem
Gert van Enk	Gelderse Vallei Hospital, Ede
Kors van de Ent	University Medical Center, Utrecht
Marieke Ermers	University Medical Center, Utrecht
Rene van Gent	Maxima Medical Center, Veldhoven
Hennie Hodemaekers	National Institute of Public Health and the Environment, Bilthoven
Barbara Hoebee	National Institute of Public Health and the Environment, Bilthoven
Mark Hoetjer	Meander Medical Center, Amersfoort
Saskia van Hoogdalem	University Medical Center, Utrecht
Riny Janssen	National Institute of Public Health and the Environment, Bilthoven
Johan van der Kaaden	Hilversum Hospital, Hilversum
Jan Kimpen	University Medical Center, Utrecht
Ted Klok	Gelderse Vallei Hospital, Ede
Ed van Leer	Groene Hart Hospital, Gouda
Ton van Loon	University Medical Center, Utrecht
Erik Mooiweer	University Medical Center, Utrecht
Ronald de Moor	Twee Steden Hospital, Tilburg
Anne Nieuwenhuis	Jeroen Bosch Hospital, 's-Hertogenbosch
Charlotte Onland-Moret	University Medical Center, Utrecht
Martha Pekelharing	Diakonessen Hospital, Utrecht
John Rossen	University Medical Center, Utrecht & Elisabeth Hospital, Tilburg
Maroeska Rovers	University Medical Center, Utrecht
Annemieke Schuurhof	University Medical Center, Utrecht
Carien Smeets	Elisabeth Hospital, Tilburg
Jossi Tijdink	University Medical Center, Utrecht
Esther de Vries	Jeroen Bosch Hospital, 's-Hertogenbosch
Annemarie van Wermeskerken	Flevo Hospital, Almere
Mieke Westra	University Medical Center, Utrecht
Floor van Woerkum	University Medical Center, Utrecht
Job van Woensel	Academical Medical Center, Amsterdam
Diana van de Zwet	Mesos Medical Center, Utrecht

REFERENCE LIST

(1) Vanden Burgt JA, Busse WW, Martin RJ, Szefler SJ, Donnell D. Efficacy and safety overview of a new inhaled corticosteroid, QVAR (hydrofluoroalkane-beclomethasone extrafine inhalation aerosol), in asthma. Journal of Allergy and Clinical Immunology 2000; 106(6):1210-1226.

(2) Kamps AW, van Ewijk B, Roorda RJ, Brand PL. Poor inhalation technique, even after inhalation instructions, in children with asthma. Pediatr Pulmonol 2000; 29(1):39-42.

(3) Kamps AW, Brand PL, Roorda RJ. Determinants of correct inhalation technique in children attending a hospital-based asthma clinic. Acta Paediatr 2002; 91(2):159-163.

(4) Bont L, Heijnen CJ, Kavelaars A, van Aalderen WMC, Brus F, Draaisma JTh et al. Monocyte IL-10 Production during Respiratory Syncytial Virus Bronchiolitis Is Associated with Recurrent Wheezing in a One-Year Follow-up Study. Am J Respir Crit Care Med 2000; 161(5):1518-1523.

(5) Bont L, Steijn M, van Aalderen WM, Kimpen JL. Impact of wheezing after respiratory syncytial virus infection on health-related quality of life. Pediatr Infect Dis J 2004; 23(5):414-417.

(6) Bunge EM, Essink-Bot ML, Kobussen MP, Suijlekom-Smit LW, Moll HA, Raat H. Reliability and validity of health status measurement by the TAPQOL. Arch Dis Child 2005; 90(4):351-358.

(7) Arets HG, Brackel HJ, van der Ent CK. Applicability of interrupter resistance measurements using the MicroRint in daily practice. Respir Med 2003; 97(4):366-374.

(8) Bont L, van Aalderen WM, Versteegh J, Brus F, Draaisma JT, Pekelharing-Berghuis M et al. Airflow limitation during respiratory syncytial virus lower respiratory tract infection predicts recurrent wheezing. Pediatr Infect Dis J 2001; 20(3):277-282.

(9) Greenland S, Finkle WD. A critical look at methods for handling missing covariates in epidemiologic regression analyses. Am J Epidemiol 1995; 142(12):1255-1264.

(10) Harrell FE, Jr., Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med 1996; 15(4):361-387.

ATTACHMENT 1. RSV CORTICOSTEROID STUDY PROTOCOL

RSV Corticosteroid Study Protocol.

Official title	Local anti-inflammatory treatment in the prevention of long-term airway morbidity following hospitalisation for respiratory syncytial virus infection: clinical efficacy
Condition targeted	Recurrent wheeze following respiratory syncytial virus infection
Intervention	Hydrofluoroalkane extrafine beclomethasone dipropionate (Qvar)
Study design	Randomized double-blind placebo-controlled trial
Phase	Phase 4
Primary outcome	Number of wheezing days during the observation year (3-15 months following hospitalization for RSV LRTI)
Secondary outcomes	Number of coughing days, number of days of airway medication use, duration of hospitalization, health related quality of life, lung function (interrupter resistance measurements), and length
Inclusion criteria	Infants under 13 months of age Hospital admission for RSV LRTI Positive immunofluorescence for RSV infection of epithelial cells in nasopharyngeal aspirates
Exclusion criteria	Previous use of corticosteroids History of cardiac or pulmonary disease Wheezing illness prior to RSV LRTI
Enrollment status	Completed
Principal investigators	Louis J. Bont, MD PhD, Principal Investigator Marieke JJ. Ermers, MD, Study Coordinator Affiliation: University Medical Centre Utrecht, Department of Pediatric Infectious Diseases
Overall contact	Louis J. Bont, MD PhD, Phone +31-88-7554555, Email: l.bont@umcutrecht.nl
Sponsored by	Dutch Asthma Foundation (grant nr. 3.2.03.22)

Detailed Description

Background

Respiratory syncytial virus lower respiratory tract infection (RSV LRTI) is the most prevalent acute wheezing disorder in infants and is associated with recurrent wheeze during early childhood. At the moment, no effective intervention preventing recurrent wheeze following RSV LRTI is available.
Study Protocol

Hypothesis

This study protocol is designed to evaluate the hypothesis that treatment with high-dose hydrofluoroalkane extrafine beclomethasone dipropionate for 3 months following RSV LRTI prevents the occurrence and severity of recurrent wheeze in hospitalized infants.

Patients

Pediatricians in the participating hospitals recruit infants during winter seasons, starting November 2004. Based on power calculations (90% power to detect a difference of 14 wheezing days) we aim to include 250 infants in 2 consecutive winter seasons (2004-2005 and 2005-2006). In eligible infants, pediatricians check the in- and exclusion criteria and inform the parents about the study. Written informed consent has to be obtained before enrollment.

Randomization

Consecutive infants are assigned randomly to placebo or active medication (consecutive boxes of study medication). Randomization is by means of a computer-generated list of six numbers in each block and fixed blocks within each hospital. Blinding is accomplished by using visually identical active and placebo medications. The participants, treating paediatricians, nurses and study coordinator remain masked to the treatment assignment as long as the observation period lasts (until July 2007).

Study Intervention

Treatment has to be started within 24 hours following RSV detection and continues for 3 months. Hydrofluoroalkane extrafine beclomethasone dipropionate (Qvar, kindly provided by 3M, TevaPharma NL, Haarlem, the Netherlands), at a dose of 200 microgram twice daily, or matching placebo is administered by pressurized metered-dose inhaler and a spacer (Aerochamber, kindly provided by Trudell International Europe Ltd., Nottingham, United Kingdom). Asthma nurses educate the patients' parents correct inhaler techniques.

FOLLOW UP

Log Information

Parents record airway symptoms and the use of airway medication in a daily log. Logs are kept for 15 months, from start of the intervention. A single investigator (Marieke Ermers, study coordinator University Medical Center Utrecht) instructs the parents about the log and telephone calls are scheduled for motivational purposes.

Questionnaires

Three weeks following discharge a study visit is planned. A single investigator (Marieke Ermers, study coordinator University Medical Centre Utrecht) evaluates the parental performance of administering the study medication. Parents fill an extended Dutch version of the standardized questionnaire of the British Medical Council and the Dutch version of the European Community Respiratory Health Survey questionnaire to obtain data on

respiratory symptoms, parental smoking habits, allergy, and allergy symptoms among first-degree family members. One year after hospitalization in the winter season, health-related quality of life is assessed with the TAPQOL questionnaire.

Lung function

Lung function and bronchial responsiveness are tested by measuring interrupter resistance using MicroRint® at the age of 2 years in the summer season. Interrupter resistance is measured before and after administration of 400 microgram of inhaled salbutamol with the use of a spacer.

Length

Length is measured at the age of 2 years. This is combined with the visit for lung function.

Chapter 5

The Effect Of High-Dose Inhaled Corticosteroids On Wheeze Following Respiratory Syncytial Virus Infection: A Randomized Double-Blind Placebo-Controlled Trial

Marieke JJ Ermers

Maroeska M Rovers

Job B van Woensel

Jan LL Kimpen

Louis J Bont

on behalf of the RSV Corticosteroid Study Group

British Medical Journal 2009;338:b897

ABSTRACT

Objectives

To determine whether early-initiated anti-inflammatory therapy with prolonged high-dose inhaled glucocorticosteroids influences the occurrence and severity of recurrent wheeze following respiratory syncytial virus lower respiratory tract infection.

Design

Randomized double-blind placebo-controlled trial.

Setting

Pediatric departments of 19 Dutch clinical centers.

Participants

243 infants (126 boys, 117 girls) hospitalized because of respiratory syncytial virus lower respiratory tract infection. Infants were younger than 13 months and previously healthy. Infants with a history of cardiac or pulmonary disease were excluded.

Interventions

Hydrofluoroalkane extrafine beclomethasone at a dose of 200 microgram twice daily, or matching placebo was administered by pressurized metered-dose inhaler and a spacer during the first 3 months following hospitalization.

Main Outcome Measures

The primary outcome was the number of wheezing days in the year following the intervention.

Results

Of the 243 randomized infants, 119 were assigned to receive hydrofluoroalkane extrafine beclomethasone. There was no significant difference in the number of wheezing days between the two groups (1761/33568 total days in the hydrofluoroalkane extrafine beclomethasone group vs 2301/36556 total days in the placebo group, P=0.31) and the proportion of infants with wheeze did not differ between the two groups (61% in the hydrofluoroalkane extrafine beclomethasone group vs 62% in the placebo group, P=0.90). In the pre-defined subgroup of infants who did not need mechanical ventilation (n=221), hydrofluoroalkane extrafine beclomethasone reduced the number of wheezing days by 32% (relative reduction, 1315/30405 total days in the hydrofluoroalkane extrafine beclomethasone group vs 2120/33149 total days in the placebo group, P=0.046). This reduction was most pronounced during the first 6 months of the year following the intervention. The proportion of infants with wheeze did not differ between the two groups (59% in the hydrofluoroalkane extrafine beclomethasone group vs 60% in the placebo group, P=0.89).

Conclusions

Early-initiated high-dose hydrofluoroalkane extrafine beclomethasone during the first 3 months after admission for respiratory syncytial virus lower respiratory tract infection has no major effect on the development of recurrent wheeze. The general use of

high-dose hydrofluoroalkane extrafine beclomethasone during respiratory syncytial virus lower respiratory tract infection should not be advocated.

Trial registration

Current Controlled Trials. Identifier: ISRCTN12352714.

INTRODUCTION

Respiratory syncytial virus lower respiratory tract infection is the most common cause of infant hospitalization during the winter season and is frequently followed by recurrent episodes of wheeze of transient nature.(1-3) Recurrent wheeze following respiratory syncytial virus lower respiratory tract infection has a high prevalence,(4) influences quality of life(5) and generates substantial health-care costs.(6)

It has been hypothesized that the infection and concomitant inflammatory reaction in the acute phase of respiratory syncytial virus lower respiratory tract infection lead to recurrent wheeze.(7) In line with this hypothesis, immune modulation during the acute phase might influence recurrent wheeze. Evidence regarding the effectiveness of early anti-inflammatory therapy in the prevention of recurrent wheeze is conflicting,(8-10) and the use of inhaled glucocorticosteroids varies greatly among countries.(11-15) Thirty percent of Swiss pediatricians reported that they always start inhaled glucocorticosteroids in hospitalized patients whereas 11% of Irish pediatricians selected the treatment of inhaled glucocorticosteroids in the case of a 3-month-old moderately severe diseased infant. (14;15) Thus far no large trials have studied the effect of inhaled glucocorticosteroids on recurrent wheeze following respiratory syncytial virus lower respiratory tract infection,(16) impeding strong recommendations regarding inhaled glucocorticosteroid use. To investigate whether early-initiated high-dose hydrofluoroalkane extrafine beclomethasone for 3 months following hospitalization for respiratory syncytial virus lower respiratory tract infection prevents the subsequent occurrence of recurrent wheeze, we performed a large randomized double-blind placebo-controlled trial.

METHODS

Patients

The 'RSV Corticosteroid Study' is a multicenter, randomized double-blind placebo-controlled trial comparing early-initiated high-dose hydrofluoroalkane extrafine beclomethasone with placebo in infants younger than 13 months and hospitalized for respiratory syncytial virus lower respiratory tract infection. Selection criteria are summarized

in Table 1. Recruitment took place during the winter seasons from November 2004 to February 2006 in pediatric departments of 19 clinical centers in the Netherlands. The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Review Committee, University Medical Center Utrecht and by the Ethics Review Committees at all participating centers. Before enrollment, written informed consent was obtained from the infants' parents. Good Clinical Practice guidelines were adhered to and the protocol was registered with Current Controlled Trials (ISRCTN12352714). Data are fully owned and controlled by the principal investigator.

Table 1. In- and exclusion criteria for participation.

Inclusion criteria	Infants under 13 months of age
	Hospital admission for LRTI
	Positive immunofluorescence for RSV infection in epithelial cells from nasopharyngeal aspirates
Exclusion criteria	Previous use of steroids
	History of cardiac or pulmonary disease
	Wheezing illness prior to RSV LRTI

Randomization

Infants whose parents gave informed consent were randomly assigned to treatment with either hydrofluoroalkane extrafine beclomethasone or placebo. Randomization was by means of a computer-generated list of six numbers in each block and fixed blocks within each hospital. Local investigators at the pediatric departments enrolled participating infants and provided subsequent, previously randomised medication boxes on enrolment. Blinding was accomplished by using active and placebo medications of identical shape and taste. Randomization codes were secured for physicians, nurses, parents and investigators until data entry was complete.

Study Intervention

Hydrofluoroalkane extrafine beclomethasone dipropionate (Qvar, kindly provided by 3M, TevaPharma NL, Haarlem, the Netherlands), at a dose of 200 microgram twice daily, or matching placebo was administered by pressurized metered-dose inhaler and a spacer (Aerochamber, kindly provided by Trudell International Europe Ltd., Nottingham, United Kingdom). This extrafine beclomethasone dipropionate formulation has smaller particles than conventional inhaled glucocorticocosteroids and is possibly more effective.(17) Treatment was started within 24 hours following respiratory syncytial virus detection and continued for 3 months. In severely diseased infants who needed mechanical ventilation, treatment was started within 24 hours following extubation. Professional asthma nurses

taught the patients' parents correct inhaler techniques. Parental competence was evaluated three weeks following hospital discharge in 177 infants. Performance was scored on 6 items according to modified criteria of the Dutch Asthma Foundation (18) (shaking of the inhaler, correct assembly of spacer and canister, activation of the canister, no more than 3 puffs at the same time, inhalation during at least 10 seconds, moving spacer valve). Median parental performance scores in the placebo and intervention groups were 6 (placebo: 6, 25th-75th percentile 5-6; hydrofluoroalkane extrafine beclomethasone: 6, 25th-75th percentile 5-6).

Infants were followed up for 1 year following treatment completion, during which time the primary outcome measure (i.e. the number of wheezing days) was determined. At the discretion of the pediatricians at the clinical centers, open-label bronchodilator medication could be added to the study treatment if required. If steroid therapy was necessary, study treatment was discontinued.

Primary And Secondary Outcome Measures

Parents recorded airway symptoms and the use of airway medication in a previously described daily log.(19) From start of the intervention logs were kept for 15 months. Logs were returned to the investigators every three months. Parents were instructed by a single investigator and telephone calls were scheduled every three months for motivational purposes.

The primary outcome variable was the number of wheezing days. Secondary outcome variables were the number of coughing days, the number of days with airway medication use, duration of hospitalization, health-related quality of life, lung function, potential adverse effects of treatment on growth, and thrush incidence. Health-related quality of life was assessed with the TNO-AZL Preschool Children Quality of Life questionnaire (20) 1 year after hospitalization. Lung function and bronchial responsiveness were tested by measuring interrupter resistance using MicroRint at the age of 2 years. The interrupter technique measures changes in mouth pressure after a brief airflow interruption at the mouth. It is based on the principle that during brief airflow interruption alveolar pressure and mouth pressure equilibrate within a few milliseconds. Airway resistance can be calculated from the ratio of mouth pressure to airflow at the mouth just prior to occlusion(21) and was measured before and after administration of 400 microgram of inhaled salbutamol with the use of a spacer. Growth was evaluated by measuring height at the age of 2 years.

An extended Dutch version of the standardized questionnaire of the British Medical Council and the Dutch version of the European Community Respiratory Health Survey questionnaire were used to obtain data on respiratory symptoms, allergy, parental smoking habits, and allergy symptoms among first-degree family members.(22;23)

Statistical Analysis

The sample size was based on prevalence data for the primary outcome obtained from our previous respiratory syncytial virus follow-up study.(19) In that cohort, 80% of infants wheezed at any time, with a mean number of wheezing days during 4 to 15 months following respiratory syncytial virus lower respiratory tract infection of 34.2 (95% CI 26.2 to 42.3 days). The predefined target of 250 patients provided at least 90% power to detect a difference of 14 wheezing days between both arms.

We analyzed the effects of treatment with hydrofluoroalkane extrafine beclomethasone on the occurrence of any wheeze, cough, airway medication use, and thrush during follow up. Percentages and associated 95% confidence intervals of infants suffering any symptoms were calculated. We counted the number of days with symptoms and / or medication use to evaluate the effect of treatment on the severity of wheeze, cough and airway medication use. A typical Poisson probability distribution arose and therefore Poisson regression analysis was used to study potential differences in number of wheezing and coughing days and in days of airway medication use. Duration of hospitalization (in days), quality of life domain scores, interrupter resistance measurements (in kPA/L/s), and height (in cm) are expressed as medians and means. Chi square test, Student's t test, and Mann Whitney U test were used to evaluate differences in percentages, mean values, and median values between the groups. To detect possible effect modification, two pre-specified subgroup analyses were performed, namely, need for mechanical ventilation (yes vs no) and presence of signs of airflow limitation during initial respiratory syncytial virus lower respiratory tract infection, i.e. physician diagnosed wheezing by auscultation (yes vs no)(24).

To decrease bias and to increase statistical efficiency, missing data were imputed for quality of life domain scores, interrupter resistance measurements, duration of hospitalization, height and presence of thrush. Imputation was done using the linear regression method (Missing Value Analyses) available in SPSS (SPSS for Windows, version 13.0, SPSS Inc.) software.(25;26) Such imputation is based on the correlation between each variable with missing values and all other variables as estimated from the set of complete subjects. All analyses were performed on an intention to treat basis, implying that no adjustments were made for the need for steroid therapy during the intervention period. In addition, complete case analyses were performed using cases with complete log data.

Conflict Of Interest

This study was fully supported by the Dutch Asthma Foundation (grant number 3.2.03.22). No financial support was obtained from pharmaceutical companies. As indicated in the methods, study drugs and inhalers were provided unconditionally by the manufacturers.

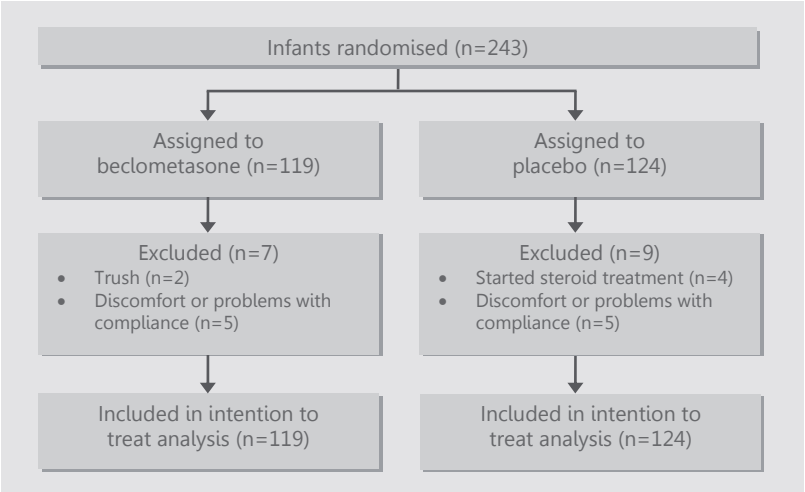

Figure 1. Diagram showing the flow of participating children.

RESULTS

A total of 323 parents were approached to participate in this intervention study and 78 did not participate for various reasons (previous use of steroids, cardiac history, not willing to participate). Two hundred forty-three infants were randomly assigned to treatment with either hydrofluoroalkane extrafine beclomethasone or placebo (Figure 1). Baseline characteristics of both groups are shown in Table 2. During the observation year airway symptoms were recorded for 2305 months (79% of total months) and the median dura-

Table 2. Baseline characteristics.

Characteristic	Placebo (N=124)	Beclomethasone (N=119)
Male sex (%)	51.6	52.1
Caucasian race (%)	83.9	85.3
Duration of illness before hospitalization in days (median, 25th-75th pct)	2 (1-4)	3 (1-4)
Age at hospitalization in months (median, 25th-75th pct)	2 (1-5)	2 (1-4)
Admitted to intensive care unit, needing mechanical ventilation (%)	8.9	9.2
Gestational age in weeks (median, 25th-75th pct)	39.5 (37.5-40.6)	39.5 (37.5-40.6)
Breast-fed for at least 1 month (%)	58.1	66.3
Signs of airflow limitation during initial RSV LRTI(%)	50.8	59.8
Maternal smoking before birth (%)	10.2	18.3
Parental atopy score[1] (median, 25th-75th pct)	1 (0-3)	1 (0-3)

[1]A semiquantitative parental atopy score was defined as previously described. One point was added to the parental atopy score for the presence of each atopic symptom (eczema, hay fever, bronchitis, asthma and food allergy)[2]

[2]Ermers MJ, Hoebee B, Hodemaekers HM, Kimman TG, Kimpen JL, Bont L. IL-13 genetic polymorphism identifies children with late wheezing after respiratory syncytial virus infection. J Allergy Clin Immunol 2007; 119(5):1086-1091.

tion of follow-up for both the hydrofluoroalkane extrafine beclomethasone and placebo groups was 15 months (hydrofluoroalkane extrafine beclomethasone 15 months, 25th-75th percentile 12-15 months; placebo 15 months, 25th-75th percentile 12-15 months).

Airway Symptoms / Medication Use During Observation Year

The number of days with symptoms and / or medication use during the entire observation year is given in Table 3. In the total group and in the pre-defined subgroup of infants with signs of airway limitation during respiratory syncytial virus lower respiratory tract infection, the number of wheezing days during the entire observation year was similar

Table 3. Airway symptoms / medication use during follow up.
Total days with airway symptoms / medication use versus total log days in treatment groups.

		Beclomethasone	Placebo	*P*[3]	Rate ratio
Total group (n=243)	Wheeze	1761/33568	2301/36556	0.31	0.83
	Cough	7341/33568	9585/36556	0.03[4]	0.83
	Steroid use	2578/33568	3105/36556	0.69	0.90
	Bronchodilator use	2066/33568	2749/36556	0.39	0.82
Subgroup 1 (n=221)[1]	Wheeze	1315/30405	2120/33149	0.05[4]	0.68
	Cough	6681/30405	8724/33149	0.05[4]	0.83
	Steroid use	2055/30405	2916/33149	0.34	0.77
	Bronchodilator use	1725/30405	2332/33149	0.39	0.81
Subgroup 2 (n=133)[2]	Wheeze	1148/19290	916/18465	0.41	1.20
	Cough	4084/19290	4377/18465	0.37	0.89
	Steroid use	1739/19290	1028/18465	0.16	1.62
	Bronchodilator use	1252/19290	1152/18465	0.90	1.04

[1]Subgroup 1: Children without need for mechanical ventilation

[2]Subgroup 2: Children with signs of airflow limitation during acute RSV LRTI

[3]By poisson analyses.

[4]Analyses for which the associated *P* value ≤ .05

in the two treatment groups. However, the number of wheezing days was lower in the major subgroup of non-ventilated infants treated with hydrofluoroalkane extrafine beclomethasone (N=221). An absolute reduction in wheezing days of 2.1% was observed (1315/30405 total days (4.3%) in the hydrofluoroalkane extrafine beclomethasone group vs 2120/33149 total days (6.4%) in the placebo group, *P*=0.046), this is a relative reduction of 32%. Hydrofluoroalkane extrafine beclomethasone treated infants also had fewer coughing days. No other differences were observed. Complete case analyses revealed similar results.

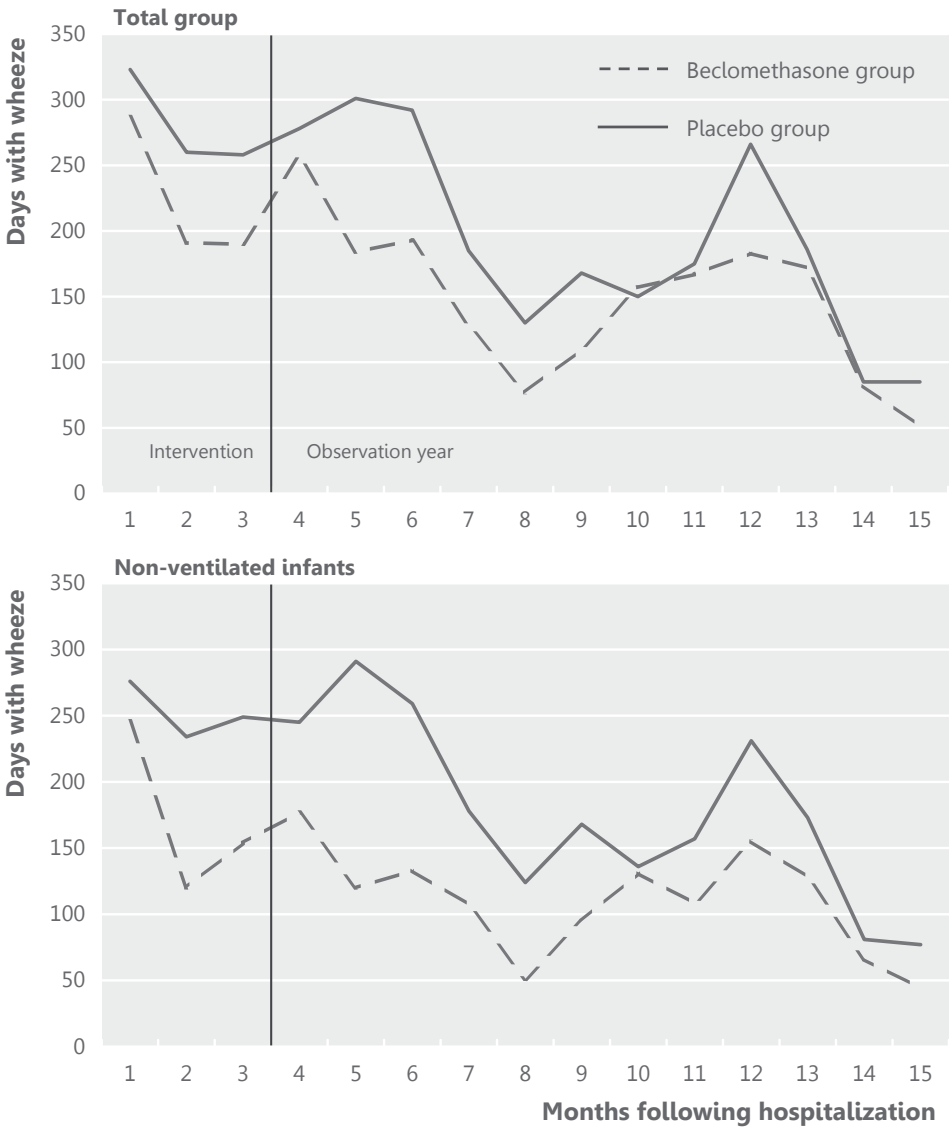

Figure 2. Wheeze during follow up.

Counted days with wheeze in the total group of infants (*P* (observation year) = 0.31) and counted days with wheeze in the subgroup of non-ventilated infants (*P* (observation year) = 0.05).

The distribution of wheezing days during follow-up in the two treatment groups is shown in Figure 2. To gain insight in short term effects of the intervention we conducted Poisson analyses for the first and second six months of the observation year separately. These post hoc Poisson analyses showed a more pronounced reduction in the number of wheezing days during the first 6 months than during the second 6 months of the

observation period (total group: 1354/19048 total days (7.1%) in the placebo group vs 949/17664 total days (5.4%) in the hydrofluoroalkane extrafine beclomethasone group, *P*=0.18; subgroup of non-ventilated infants: 1265/17215 total days (7.3%) in placebo group vs 683/16043 total days (4.3%) in the hydrofluoroalkane extrafine beclomethasone group, *P*=0.02) No differences were observed in the subgroup of infants with signs of airflow limitation during respiratory syncytial virus lower respiratory tract infection (569/9752 total days (5.8%) in the placebo group vs 599/10116 total days (5.9%) in the hydrofluoroalkane extrafine beclomethasone group, *P*=0.96).
The proportion of infants with wheeze, cough, and airway medication use did not differ between the placebo and hydrofluoroalkane extrafine beclomethasone groups during the observation year (wheeze 62 vs 61%, respectively; cough 86 vs 83%; bronchodilator use 42 vs 40%; steroid use 26 vs 25%). Comparable results were obtained in the predefined subgroups.

Secondary Outcomes And Adverse Events

The median duration of hospitalization was similar in the two groups (4 days (25^{th}-75^{th} percentile 3-8 days) in the placebo group vs 5 days (25^{th}-75^{th} percentile 4-8 days) in the hydrofluoroalkane extrafine beclomethasone group, *P*=0.07). The TNO-AZL Preschool Children Quality of Life questionnaire, which was completed 1 year after hospitalization, was returned by 191 parents (79%). Comparison of the domain scores for stomach problems, skin problems, lung problems, sleeping, appetite, liveliness, positive mood, problem behavior, anxiety, social functioning, motor functioning, and communication showed that infants in the hydrofluoroalkane extrafine beclomethasone group had lower scores for appetite (placebo median 83 (25^{th}-75^{th} percentile 50-100) vs hydrofluoroalkane extrafine beclomethasone median 67 (25^{th}-75^{th} percentile 50-100), *P*=0.03). There were no differences in weight at the end of the study between hydrofluoroalkane extrafine beclomethasone-treated and placebo-treated infants.
Measurement of airway resistance during the summer season at the age of 2 years was attempted in 163 children and successfully performed in 91 (56%) children. No differences in percentage of successful measurements, in baseline interrupter resistance, and in interrupter resistance after salbutamol inhalation were found between the two treatment groups (mean baseline interrupter resistance 1.15 kPA/L/s (95% CI 1.10 to 1.19 kPA/L/s) in the placebo group vs 1.10 kPA/L/s (95% CI 1.03 to 1.17 kPA/L/s) in the hydrofluoroalkane extrafine beclomethasone group, *P*=0.9).
No severe adverse events were reported. Two hydrofluoroalkane extrafine beclomethasone-treated infants stopped treatment prematurely because of repeated thrush during the intervention period; however, the number of infants with thrush was similar in the two groups (41/124 in the placebo group vs 47/119 in the hydrofluoroalkane extrafine

beclomethasone group, OR=1.3 (95% CI 0.8 to 2.2), *P*=0.3). Height at age 2 years was similar in both groups (placebo group mean 86.8 cm (95% CI 86.2 to 87.5 cm) vs hydrofluoroalkane extrafine beclomethasone group mean 86.9 cm (95% CI 86.2 to 87.7 cm), *P*=0.8).

DISCUSSION

Our results showed that early-initiated high-dose hydrofluoroalkane extrafine beclomethasone for 3 months following respiratory syncytial virus lower respiratory tract infection did not prevent the occurrence of recurrent wheeze. The number of wheezing days in infants that did not require mechanical ventilation during respiratory syncytial virus lower respiratory tract infection showed a modest reduction from 6.4% to 4.3%, that is a relative reduction of 32%. No positive effect of our intervention on health-related quality of life was observed. There was no evidence of side-effects of the intervention.

Comparison With Other Studies

No treatment during respiratory syncytial virus lower respiratory tract infection has been convincingly shown to have a beneficial effect on the course of acute disease or on long-term airway morbidity. A beneficial effect of leukotriene receptor antagonists was suggested,(27) but could not be replicated in a large double-blind study.(28) The current trial was based on the hypothesis that early-initiated high-dose inhaled glucocorticosteroids modify the immune response following respiratory syncytial virus infection resulting in the reduction of subsequent recurrent wheeze. Although studies suggest that approximately a quarter of infants hospitalized with bronchiolitis receive corticosteroids,(29;30) the efficacy of these agents has not been consistently demonstrated. Several studies demonstrated that corticosteroids are not effective in the acute treatment of respiratory syncytial virus lower respiratory tract infection.(31-34). A randomized controlled trial conducted in a birth cohort at risk for asthma found no effect of inhaled glucocorticosteroid therapy on the progression of episodic wheeze into persistent wheeze.(35) In addition, no disease-modifying effect of a two year therapy of inhaled corticosteroids was observed in a third, treatment-free year in preschool children at high risk for asthma.(36) The temporary effects of inhaled steroids in the latter study and our study are remarkably similar. The role of inhaled glucocorticosteroids in the prevention of recurrent wheeze following respiratory syncytial virus lower respiratory tract infection is of particular interest because the pathogenesis of respiratory syncytial virus lower respiratory tract infection is thought to be distinct from that of other wheezing disorders of infancy.(37) It has been shown that episodic high doses of inhaled glucocorticosteroids provided a partially effective strategy

in children with episodic viral wheeze.(38) Previous studies investigating whether inhaled glucocorticosteroids can reduce the occurrence of recurrent wheeze following respiratory syncytial virus lower respiratory tract infection have yielded conflicting data and had major limitations, including small sample size, low dosage of treatment, mean age at inclusion older than 12 months, and lack of accurate tools to measure long-term airway morbidity. (10;16;39-42).

Strengths And Limitations Of Study

The major strength of the current study is that high-dose hydrofluoroalkane extrafine beclomethasone treatment was initiated early, within 24 hours of the diagnosis of respiratory syncytial virus lower respiratory tract infection. Furthermore, the effect on recurrent wheeze was specifically evaluated in the recognizable group of infants hospitalized because of respiratory syncytial virus lower respiratory tract infection. To our knowledge, this is the first randomized controlled trial that was sufficiently powered to evaluate the effect of early high-dose inhaled glucocorticosteroids on the occurrence of recurrent wheeze following respiratory syncytial virus lower respiratory tract infection.

Some of our findings merit further discussion. Firstly, the data suggest that inhaled hydrofluoroalkane extrafine beclomethasone does not prevent or diminish recurrent wheeze following respiratory syncytial virus lower respiratory tract infection in the total group of infants. No decrease in the number of wheezing days and a small decrease in the number of coughing days were observed. Quality of life scores and interrupter resistance did not differ between the two treatment groups. However, the study might be confounded because severely diseased infants who needed mechanical ventilation were analyzed together with more mildly diseased infants who did not need mechanical ventilation. No benefit of inhaled hydrofluoroalkane extrafine beclomethasone was observed in mechanically ventilated infants. The small number of infants in this subgroup impedes a conclusion about the effect of inhaled hydrofluoroalkane extrafine beclomethasone on recurrent wheeze in ventilated infants. However, we speculate that the delayed start of treatment prevented a treatment-induced modification of the disease course. Furthermore it is possible that severely diseased infants did not respond to inhaled hydrofluoroalkane extrafine beclomethasone due to mechanical injury during supportive care or due to enhanced virus-mediated lung damage at the time of the initial process. In the major subgroup of infants (N=221) who did not need mechanical ventilation during respiratory syncytial virus lower respiratory tract infection the number of wheezing days reduced from 6.4% to 4.3%, a relative reduction of 32%. The effect of inhaled hydrofluoroalkane extrafine beclomethasone was most pronounced during the first 6 months following treatment. The beneficial effect of inhaled hydrofluoroalkane extrafine beclomethasone that was observed in the short term might be due to reduced airway reactivity dur-

ing that period. No differences in quality of life scores and interrupter resistance were observed between the two treatment groups, measured at a time when the severity of wheeze was similar in the two groups. Because the TNO-AZL Preschool Children Quality of Life questionnaire measures health-related quality of life over the past 3 months only, the outcome might have been different if measurements were taken earlier.

Secondly, we found hydrofluoroalkane extrafine beclomethasone treatment to be ineffective in infants presenting with signs of airflow limitation during respiratory syncytial virus lower respiratory tract infection, a subgroup reported to be at increased risk of developing recurrent wheeze.(24) This finding appears counter-intuitive. The disease-modifying effect of inhaled hydrofluoroalkane extrafine beclomethasone on recurrent wheeze following respiratory syncytial virus lower respiratory tract infection might be limited to specific phenotypes. The relevance of the presence of signs of airflow limitation during respiratory syncytial virus lower respiratory tract infection to both the development of recurrent wheeze and the effect of treatment needs further research.

Thirdly, inadequate administration of and adherence to the medication might have influenced the results. However, most parents performed all essential administration steps correctly. Moreover, a sensitivity analysis for the infants whose parents performed all steps correctly did not change the results. We determined parental adherence to the treatment regimen in a subset of infants by weighing returned canisters. The vast majority of parents gave the prescribed medication twice daily during at least nine full weeks of the prescribed three months.

Fourthly, this study was powered to detect a difference of 14 days between both treatment arms. The difference of 14 wheezing days was arbitrarily defined and we might have missed a smaller but important effect. The power was based on mean differences whereas we finally used Poisson regression analysis to study differences in numbers of wheezing days, which will detect smaller differences as significant.

Fifthly, no other research group externally validated the log. We used the log in two cohorts and observed that the occurrence of wheeze was virtually identical between the cohorts. This similarity encouraged our trust in the internal validity of the log information.

And lastly, not all parents returned complete log data for the follow-up period, and thus the selective loss of data could have biased the results. However, complete case analysis revealed similar results, the response rate in this trial was high and missing data were equally distributed over both treatment groups.

Conclusions And Policy Implications

A Cochrane review about inhaled corticosteroids in the prevention of recurrent wheeze could not provide strong recommendations because of the small number of included participants and the inability to pool all clinical outcomes.(16) We found that early-ini-

tiated high-dose hydrofluoroalkane extrafine beclomethasone during the first 3 months after admission for respiratory syncytial virus lower respiratory tract infection did not reduce the severity of recurrent wheeze. Only in our a priori specified subgroup of non-ventilated children there was a modest and temporary reduction in wheezing days. Therefore, we conclude that general use of early-initiated high-dose hydrofluoroalkane extrafine beclomethasone should not be advised in infants hospitalized for respiratory syncytial virus lower respiratory tract infection.

REFERENCE LIST

(1) Bont L, Steijn M, van Aalderen WM, Brus F, Th Draaisma JM, Diemen-Steenvoorde RA et al. Seasonality of long term wheezing following respiratory syncytial virus lower respiratory tract infection. Thorax 2004; 59(6):512-516.

(2) Perez-Yarza EG, Moreno A, Lazaro P, Mejias A, Ramilo O. The association between respiratory syncytial virus infection and the development of childhood asthma: a systematic review of the literature. Pediatr Infect Dis J 2007; 26(8):733-739.

(3) Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 1999; 354(9178):541-545.

(4) Kuehni CE, Davis A, Brooke AM, Silverman M. Are all wheezing disorders in very young (preschool) children increasing in prevalence? Lancet 2001; 357(9271):1821-1825.

(5) Bont L, Steijn M, van Aalderen WM, Kimpen JL. Impact of wheezing after respiratory syncytial virus infection on health-related quality of life. Pediatr Infect Dis J 2004; 23(5):414-417.

(6) Stevens CA, Turner D, Kuehni CE, Couriel JM, Silverman M. The economic impact of preschool asthma and wheeze. Eur Respir J 2003; 21(6):1000-1006.

(7) Agertoft L, Pedersen S. Effect of long-term treatment with inhaled budesonide on adult height in children with asthma. N Engl J Med 2000; 343(15):1064-1069.

(8) Reijonen T, Korppi M, Kuikka L, Remes K. Anti-inflammatory therapy reduces wheezing after bronchiolitis. Archives of Pediatrics & Adolescent Medicine 1996; 150(5):512-517.

(9) Carlsen KH, Leegaard J, Larsen S, Orstavik I. Nebulised beclomethasone dipropionate in recurrent obstructive episodes after acute bronchiolitis. Arch Dis Child 1988; 63(12):1428-1433.

(10) Kajosaari M, Syvanen P, Forars M, Juntunen-Backman K. Inhaled corticosteroids during and after respiratory syncytial virus-bronchiolitis may decrease subsequent asthma. Pediatric Allergy and Immunology 2000; 11(3):198-202.

(11) Chauliac ES, Silverman M, Zwahlen M, Strippoli MP, Brooke AM, Kuehni AC. The therapy of pre-school wheeze: appropriate and fair? Pediatr Pulmonol 2006; 41(9):829-838.

(12) Kimpen JL, Schaad UB. Treatment of respiratory syncytial virus bronchiolitis: 1995 poll of members of the European Society for Paediatric Infectious Diseases. Pediatr Infect Dis J 1997; 16(5):479-481.

(13) Plint AC, Johnson DW, Wiebe N, Bulloch B, Pusic M, Joubert G et al. Practice variation among pediatric emergency departments in the treatment of bronchiolitis. Acad Emerg Med 2004; 11(4):353-360.

(14) Cahill P, Finan E, Loftus BG. Management of bronchiolitis: current practices in Ireland. Ir Med J 2002; 95(6):167-169.

(15) Barben J, Hammer J. Current management of acute bronchiolitis in Switzerland. Swiss Med Wkly 2003; 133(1-2):9-15.

(16) Blom D, Ermers M, Bont L, van AW, van WJ. Inhaled corticosteroids during acute bronchiolitis in the prevention of post-bronchiolitic wheezing. Cochrane Database Syst Rev 2007;(1):CD004881.

(17) Janssens HM, de Jongste JC, Hop WC, Tiddens HA. Extra-fine particles improve lung delivery of inhaled steroids in infants: a study in an upper airway model. Chest 2003; 123(6):2083-2088.

(18) Kamps AW, van Ewijk B, Roorda RJ, Brand PL. Poor inhalation technique, even after inhalation instructions, in children with asthma. Pediatr Pulmonol 2000; 29(1):39-42.

(19) Bont L, Heijnen CJ, Kavelaars A, van Aalderen WMC, Brus F, Draaisma JTh et al. Monocyte IL-10 Production during Respiratory Syncytial Virus Bronchiolitis Is Associated with Recur-

rent Wheezing in a One-Year Follow-up Study. Am J Respir Crit Care Med 2000; 161(5):1518-1523.

(20) Bunge EM, Essink-Bot ML, Kobussen MP, Suijlekom-Smit LW, Moll HA, Raat H. Reliability and validity of health status measurement by the TAPQOL. Arch Dis Child 2005; 90(4):351-358.

(21) Arets HG, Brackel HJ, van der Ent CK. Applicability of interrupter resistance measurements using the MicroRint in daily practice. Respir Med 2003; 97(4):366-374.

(22) Burney PG, Luczynska C, Chinn S, Jarvis D. The European Community Respiratory Health Survey. Eur Respir J 1994; 7(5):954-960.

(23) Rijcken B. Het Europees luchtwegonderzoek Nederland. Tijdschr Soc Gezondheidsz 1992;(70):259-260.

(24) Bont L, van Aalderen WM, Versteegh J, Brus F, Draaisma JT, Pekelharing-Berghuis M et al. Airflow limitation during respiratory syncytial virus lower respiratory tract infection predicts recurrent wheezing. Pediatr Infect Dis J 2001; 20(3):277-282.

(25) Greenland S, Finkle WD. A critical look at methods for handling missing covariates in epidemiologic regression analyses. Am J Epidemiol 1995; 142(12):1255-1264.

(26) Harrell FE, Jr., Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med 1996; 15(4):361-387.

(27) Bisgaard H. A randomized trial of montelukast in respiratory syncytial virus postbronchiolitis. Am J Respir Crit Care Med 2003; 167(3):379-383.

(28) Bisgaard H, Flores-Nunez A, Goh A, Azimi P, Halkas A, Malice MP et al. Study Of Montelukast for theTreatment of Respiratory Symptoms of Post-RSV-Bronchiolitis in Children. Am J Respir Crit Care Med 2008; .

(29) Behrendt CE, Decker MD, Burch DJ, Watson PH. International variation in the management of infants hospitalized with respiratory syncytial virus. International RSV Study Group. Eur J Pediatr 1998; 157(3):215-220.

(30) Christakis DA, Cowan CA, Garrison MM, Molteni R, Marcuse E, Zerr DM. Variation in inpatient diagnostic testing and management of bronchiolitis. Pediatrics 2005; 115(4):878-884.

(31) Corneli HM, Zorc JJ, Majahan P, Shaw KN, Holubkov R, Reeves SD et al. A multicenter, randomized, controlled trial of dexamethasone for bronchiolitis. N Engl J Med 2007; 357(4):331-339.

(32) De Boeck K, Van der Aa N, Van Lierde S, Corbeel L, Eeckels R. Respiratory syncytial virus bronchiolitis: a double-blind dexamethasone efficacy study. J Pediatr 1997; 131(6):919-921.

(33) Bulow SM, Nir M, Levin E, Friis B, Thomsen LL, Nielsen JE et al. Prednisolone treatment of respiratory syncytial virus infection: a randomized controlled trial of 147 infants. Pediatrics 1999; 104(6):e77.

(34) Roosevelt G, Sheehan K, Grupp-Phelan J, Tanz RR, Listernick R. Dexamethasone in bronchiolitis: a randomised controlled trial. Lancet 1996; 348(9023):292-295.

(35) Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F. Intermittent inhaled corticosteroids in infants with episodic wheezing. New England Journal of Medicine 2006; 354(19):1998-2005.

(36) Guilbert TW, Morgan WJ, Zeiger RS, Mauger DT, Boehmer SJ, Szefler SJ et al. Long-term inhaled corticosteroids in preschool children at high risk for asthma. New England Journal of Medicine 2006; 354(19):1985-1997.

(37) Bont L, Kimpen JL, Ermers MJ. Inhaled corticosteroids and children. New England Journal of Medicine 2006; 355(6):624-625.

(38) McKean M, Ducharme F. Inhaled steroids for episodic viral wheeze of childhood. Cochrane Database Syst Rev 2000;(2):CD001107.

(39) Richter H, Seddon P. Early nebulized budesonide in the treatment of bronchiolitis and the prevention of postbronchiolitic wheezing. Journal of Pediatrics 1998; 132(5):849-853.

(40) Fox GF, Everard ML, Marsh MJ, Milner AD. Randomised controlled trial of budesonide for the prevention of post-bronchiolitis wheezing. Arch Dis Child 1999; 80(4):343-347.

(41) Lehtinen P, Ruohola A, Vanto T, Vuorinen T, Ruuskanen O, Jartti T. Prednisolone reduces recurrent wheezing after a first wheezing episode associated with rhinovirus infection or eczema. J Allergy Clin Immunol 2007; 119(3):570-575.

(42) Ermers MJ, Hoebee B, Hodemaekers HM, Kimman TG, Kimpen JL, Bont L. IL-13 genetic polymorphism identifies children with late wheezing after respiratory syncytial virus infection. J Allergy Clin Immunol 2007; 119(5):1086-1091.

Chapter 6

Genetic Susceptibility To Respiratory Syncytial Virus Bronchiolitis Is Predominantly Associated With Immune Genes

Riny Janssen

Louis J Bont

Christine LE Siezen

Hennie M Hodemaekers

Marieke JJ Ermers

Gerda Doornbos

Ruben van 't Slot

Ciska Wijmenga

Jelle J Goeman

Jan LL Kimpen

Hans C van Houwelingen

Tjeerd G Kimman

Barbara Hoebee

The Journal of Infectious Diseases 2007;196:826-34.

ABSTRACT

Background

Respiratory syncytial virus (RSV) is a common cause of severe lower respiratory tract infection in infants. Only a proportion of infected children require hospitalization. Because known risk factors for severe disease, like premature birth, cannot fully explain differences in disease severity, genetic factors have been implicated.

Methods

To study the complexity of RSV susceptibility and to identify genes and biological pathways involved, we performed a genetic association study in 470 children hospitalized for RSV bronchiolitis, their parents and 1008 random, population controls. We analyzed 384 SNPs in 220 candidate genes involved in airway mucosal responses, innate immunity, chemotaxis, adaptive immunity, and allergic asthma.

Results

SNPs in innate immune genes, *VDR* (rs10735810, *P*=0.0017)), *JUN* (rs11688, *P*= 0.0093), *IFNA5* (rs10757212, *P*=0.0093), *NOS2* (rs1060826, *P*=0.0031), displayed the strongest association with bronchiolitis. Apart from association at the allele level, these five SNPs also displayed association at the genotype level (*P*=0.0056, 0.0285, 0.0372 and 0.0117 for the SNP in *VDR, JUN, IFNA5* and *NOS2*, respectively). The role of innate immunity as a process was reinforced by association of the whole group of innate immune SNPs when applying the global test (*P*=0.046).

Conclusion

SNPs in innate immune genes are important in determining susceptibility to RSV bronchiolitis.

INTRODUCTION

The severity of RSV infections in young children varies from a sub-clinical or mild symptomatic upper respiratory tract infection to severe lower respiratory tract disease leading to hospitalization and occasionally to death. Some children are more prone to a severe course of disease, like premature infants and children younger than 6 weeks of age.(1;2) However, also in children without any of these risk factors, RSV infection can result in serious disease.

In animal models Th2 responses have been implicated in the disease process. In humans, genetic association studies have been performed to study susceptibility to RSV infection and such studies in children have shown that polymorphisms in *IL4* and the *IL4 receptor* genes are associated with severe RSV disease,(3-5) confirming a role for Th2 responses in severe RSV bronchiolitis in humans. However, also genes involved in various other immune processes have been implicated in determining susceptibility to or severity of RSV infection. For example, polymorphisms in *TLR4*, involved in innate immunity, *IL10*, involved in regulation of adaptive immunity, *IL8*, involved in chemotaxis, and *SP-D*, involved in airway mucosal responses, have been associated with the disease.(6-8) Recent review of the literature revealed that altogether only 13 genes have been studied for association with susceptibility to severe RSV infection.(9) Of these genes, 9 displayed association with severe RSV bronchiolitis in at least one study. Apparently, susceptibility to RSV infection is a complex trait and a broad range of immune-mediated processes play a role.

Both direct virus-induced airway damage and RSV-induced inflammation may contribute to severe disease in RSV-infected children indicating that a complex series of events takes place upon RSV infection in which both the virus and the host have a role. Since correlation of severity of disease with viral load is controversial,(10-12) the common belief is that RSV-induced inflammation has a major contribution to disease severity.(1;2) Consistent with this, ribavirin treatment was shown to be of not much clinical benefit,(13-15) underlining that factors other than viral load play a role. To identify novel genes and biological pathways involved in determining susceptibility to severe RSV infection and to shed light on the complexity of genetic susceptibility to severe RSV bronchiolitis, we performed a large scale genotyping study using a candidate gene approach. Based on data available in the literature, and on our recent gene-expression analyses in a murine model of RSV infection,(16) 384 SNPs in 220 candidate genes, involved in a broad array of immunological processes, were analysed.

METHODS

Study Design

Children (N=480, median age 70 days, 10 children > 12 months) included in the study were hospitalized because of RSV bronchiolitis during the period 1992-2006. Part of the cohort, i.e. 207 children and their parents, has been used in our previous analyses.(4;8) RSV infection was confirmed by direct immunofluorescent assay of nasopharyngeal cells. Children with a history of airway morbidity, airway medication or wheeze were excluded. Blood or buccal swabs were collected from these 480 children and both their parents for DNA isolation. In thirteen cases samples were obtained from the child and one parent only. All parents completed a questionnaire on medical data, pregnancy and ethnic origin. An unselected control population(4;8) of 1030 persons born in the Netherlands (447 have been used in our previous studies(4;8)) was randomly taken form the REGENBOOG study, a large Dutch population health examination survey. All parents gave informed consent and the study was approved by the local ethical committee.

Selection Of Genes And SNPs

220 genes were selected based on literature searches in which the gene was found in the context of RSV infection, or because they were upregulated in our murine model of RSV infection using microarray analysis.(16) The 220 genes were categorized into five processes: (i) the airway mucosal response (ii) innate immunity, (iii) chemotaxis, (iv) adaptive immunity and (v) allergic asthma. These five processes were selected because in each process genetic associations have previously been found. Some genes were categorized in more than one proces. The genes, their role in a process and the five processes are listed in Table 1. Genes in the "innate immunity" category included a group IFN regulated genes since these genes were highly upregulated in the murine lung upon RSV infection.(16) Genes involved in "chemotaxis" were included as a separate category because a large number of chemokines was induced upon RSV infection.(16) All chemokines, their receptors and several other adhesion factors were included in this category.

Where possible, SNPs were selected based on published associations with any disease or functional parameter. Alternatively, promoter- or coding SNPs were selected. This approach was used to increase our chances of selecting SNPs with functional consequences. If such SNPs were not present, HAPMAP-validated SNPs with a frequency > 5% were selected. Our initial list of candidate genes contained more than 220 genes. However, some genes displayed linkage to neighboring candidate genes. In that case one SNP was used to tag more than one gene, for instance in the case of chemokine genes *CXCL9, CXCL10* and *CXCL11*. Genes for which no suitable SNPs could be found were excluded.

Table 1. Candidate genes studied in our single-nucleotide polymorphism (SNP) analysis and genes associated with susceptibility to RSV bronchiolitis.

Process	Role in the process	Genes	Associated *P* <.05
Airway mucosal response	Inflammatory mediator	*HDC, PTGS2, ALOX5, CYSLTR1, HNMT, CHIA*	
	Mucus layer	*MUC1, SCGB1A1, SFTPC, MUC2, MUC4, MUC5AC, MUC5B, MUC7, SFTPB, SFTPD*	
	Bronchoconstriction	*TACR2, NGFB, EDN1, NGFR, TAC1, TACR3, ADRB2, VIP*	
	Radical formation	*NCF2*[1], *NOS2A*[1], *CYBA, NOS1, ARG1*	*NOS2A*[1,2], *NCF2*[1]
Innate immunity	TLR pathway	*CD14, TLR1,TLR10, TLR2, TLR3, TLR4,TLR5, TLR6, TLR7, TLR8, TLR9*	*TLR8*
	IL1 pathway	*IL1R1, IL1R2, IL1RN,*	
	Signal transduction	*IKBKAP, IRAK4, NFKBIA, CHUK, IKBKB, NFKB2, JUN, FOS, VDR, NR3C1*	*JUN*[2], *VDR*[2]
	IFN pathway	*IFNA13, IFNAR2, IFNB1, IRF1, IRF3, JAK1, TYK2, STAT1, IFNAR1, IRF5, ISGF3G, IFNA1, IFNA2, IFNA5, IFNA6, IFNA8, IFNA14*	*IFNA13, STAT1, IFNA5*[2]
	Pro-inflammatory respone	*IL12A, NOS2A*[1], *TNF, IL1B, IL27, 1L6R, TNFRSF1A, TNFRSF1B, CASP1,IL12B, IL18, IL1A, IL6, LTA, CARD15*	*NOS2A*[1,2], *TNF, IL17*
	Cell activation	*IL15, IL15RA, IL17*[1], *NCF2*[1], *LTF, CSF3, CSF3R, CYBA, CSF2*	*IL15, IL17*[1], *NCF2*[1]
	Complement	*C3, C5, MBL2*	
Chemotaxis	Chemokines	*CCL8, CCL19, CCL23, CCL26, CCL28, CCL1, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL2, CCL22, CCL25, CCL27, CCL4, CCL5, CCL7, CX3CL1, CXCL1, CXCL13, CXCL14, CXCL16, CXCL2, CXCL3, CXCL5, CXCL6, CXCL9, IL8, PF4, PPBP, CCL11, CCL24, IL16*	*CCL8*
	Chemokine receptors	*CCR2, CCR3, CCR5, IL8RB, CXCR3*	
	Adhesion factors	*ITGB2, PECAM1, CD209, CDH1, ICAM1,VCAM1, SELL*	*ITGB2, VCAM1*
Adaptive immunity	CD4, general	*CTLA4, CD28, CD80, CD40, CD40LG, CD86, IL2, IL3, IL7, IL11, IL16, IL2RA, IL2RB, IL2RG, SOCS2, SOCS3, STAT3, STAT5A,*	*CD28*
	CD4, Th1	*IL23R,TBX21, IL12RB1, IL18R1, IL12RB2, IL27R, HAVCR2, JAK1, JAK2, STAT1, IFNGR2, STAT4, IFNG*	*STAT1*

Table 1 Continued

Process	Role in the process	Genes	Associated *P* <.05
	CD4, Th2	*GATA3, HAVCR1, IL4, IL5, IL13, IL4R* [1], *IL13RA1, IL9R* [1], *STAT6, IL13RA2, IL5RA, IL9*	*IL4R*[1], *IL9R* [1]
	CD4, Th17	*IL17* [1], *IL17R, IL23R, TGFBR1, TGFB1*	*IL17* [1]
	CD4, Treg	*IL10, IL22, IL20, IL19, IL24, IL26, TGFB1, TGFB2, FOXP3, TGFBR1, IL10RA, IL10RB*	*IL10 2*
	CD8	*PSMB8, TAP1, TAP2, GZMA, GZMB, PRF1, GNLY, IL21*	
Allergic asthma		*ADAM 33, CTLA4, FCER1A, HAVCR1, HDC, IL13, IL13RA1, IL4, IL4R* [1], *IL5, IL9, IL9R* [1], *MS4A2, CCL26, PHF11, CARD15, GPR154, DPP10 FCER2, ARG1, CHIA, CCL11, CCL24, GATA3, IL13RA2, IL5RA, STAT6, HNMT*	*ADAM 33, FCER1A, IL4R*[1], *IL9R* [1], *MS4A2*

Note. IFN, interferon; IL, interleukin; TLR, Toll-like receptor; Treg, regulatory T cells.

[1] Genes involved in more than one immunological pathway.

[2] SNPs for which the associated *P* value < .01

Finally, due to various constraints of the Illumina procedure, specific primer-sets could not be developed for some selected SNPs.

DNA Isolation And Genotyping

DNA was isolated as described before.(4) For all children, parents and controls, SNPs were genotyped using Illumina's Beadarray™ technology on a 384 Sentrix™ array matrix according to Illumina's Goldengate™ protocol. Results of 37 SNPs were excluded due to low signal, overlapping or multiple clusters, or scattering of the clusters. As a result, genotypes could not be accurately deducted from the data. Genotyping failed for 22 controls, 10 children and 15 parents, probably due to poor DNA quality. Identical twins were counted as one case in our analysis. Non-identical twins (n=9) and one sib-pair were counted as separate cases. This resulted in genotype data for 470 children, 459 mothers, 448 fathers and 1008 controls that could be used for analysis. Of the 470 children, 349 were native Dutch (parents and grandparents born in the Netherlands).

Statistical Analysis

The 347 SNPs were all in Hardy-Weinberg Equilibrium (p<0.01). We performed a novel statistical test using Gauss (Aptech Systems Inc., WA, USA). This test takes into account both the case-cohort data using only the Dutch cases and the controls, and the transmis-

sion disequilibrium test (TDT) data, i.e. transmission of alleles from parents to children, using all case-parent trios. Since case-cohort data and TDT data are only partly independent, the test also considers the correlation between the two datasets. The standard TDT-analysis, counting transmitted and non-transmitted alleles, yields only the allele relative risk. In order to obtain genotype relative risks within the TDT analyses we used the pseudo-controls methodology of Clayton and Cordell(18) which constructs matched case-cohort data sets consisting of the case and three pseudo-controls. Together they form the four equally likely genotypes that can arise given the parental genotypes. Under an additive model on the ln (relative risk) scale the pseudo-control methodology yields exactly the same relative risk as the classical TDT analysis. However, there is a slight difference in the way the standard errors are computed. The pseudo-control methodology takes the trio structure into account while the classical TDT ignores that information and only counts transmitted and non-transmitted alleles.

New methodology was developed to combine the relative risk estimates from the case-cohort and the case-parent analysis. The methodology we used previously for combined analysis used all genotypic information assuming random mating and Hardy Weinberg Equilibrium.(19) These assumptions may not always be valid and have been criticized by Epstein *et al*..(20) In our new approach we do not attempt to create a joint model of all data, but instead we estimate the correlation between the two estimates of the same relative risk and use that in our combination procedure to obtain an estimate with minimal standard error as done in meta-analysis. Taken together this methodology enables analysis of all data at the allele level (df=1), under assumption of additive effects on the relative risk, and analysis at the genotype level (df=2), without any such assumptions. In this new test the two estimates of the same relative risk, i.e. the TDT and case-cohort relative risk, are combined to obtain one estimate with minimal standard error as done in meta-analysis (for more details see appendix).

The "global test" for groups of genes was used to evaluate which processes are associated with RSV bronchiolitis. This test was originally developed for analysis of microarray data and generates one *P*-value for the association of a group of genes involved in one process with disease(21) using the R-package *globaltest* (www.bioconductor.org). The global test was performed on case-cohort data at the allele level only, because it compares distribution of all alleles in a process between unmatched cases and controls. In TDT analysis, pseudocontrols are generated based on the parents' genotype and these are directly compared to their respective case in matched case-control analysis. At the moment there is no version of the global test available for matched case-control data.

RESULTS

SNPs Associated With RSV Bronchiolitis

Using the Illumina platform, 384 SNPs were determined in 470 children hospitalized for RSV bronchiolitis, their parents and 1008 Dutch population controls. Genotype determination was successful for 347 of these 384 SNPs. These SNPs are located in 210 of the 220 genes initially analyzed. The complete list of genes for which genotype analysis was successful is shown in Table 1. In total 22 SNPs in 21 genes were associated with severe RSV disease either at the allele- or genotype-level ($P<0.05$) (Table 2). The *P*-value calculated using this test is a measure for the variance in RSV susceptibility in the population that can be explained by this association, and is therefore a measure for the strength of the association.

Associations were found for genes in all processes: 2 out of 47 tested SNPs in genes involved in the "local lung response" displayed association, 11 out of 122 "innate immunity" SNPs, 3 out of 70 "chemotaxis" SNPs, 7 out of 102 "adaptive immunity" SNPs and 6 out of 51 "allergic asthma" SNPs displayed association (Table 1). In one gene, *IL4R*, two SNPs were associated with disease. These SNPs were not in strong linkage disequilibrium suggesting independent effects. The OR for all risk-alleles was between 1.2 and 1.7 and for all protective alleles between 0.5 and 0.8, indicating that all individual SNPs have small effects, which is commonly found for associations in complex genetic diseases.

Closer examination of associated SNPs revealed that they could be divided into three subgroups. The first subgroup comprised five SNPs out of the 22. These SNPs were associated with severe RSV disease both at the allele and genotype level, i.e. a SNP in *VDR, JUN, IFNA5, NOS2A* and *FCER1A* (Table 2). In accordance with this, these associations were among those with the lowest *P*-values and the strongest effects as measured by OR. The first four genes are involved in innate immunity and the last gene in allergic asthma. The second subgroup comprised 12 out of the 22 associated SNPs. For this group associations were only found at the allele level (Table 2), indicating a co-dominant effect. All these associations displayed a *P*-value between 0.01 and 0.05. These genes are involved in innate immunity (*IFNA13, IL15, STAT1, TLR8*), chemotaxis (*CCL8, ITGB2, VCAM1*), adaptive immunity (*CD28, STAT1*) and allergic asthma (*MS4A2, ADAM33, IL4R, IL9R*). *TLR8* is located on the X-chromosome and association was only found in males. The last subgroup comprised five SNPs out of our total of 22. These SNPs only showed an association with RSV disease at the genotype level and were present in genes involved in innate immunity (*TNF* and *NCF2*) and adaptive immunity (*IL10, IL4R* and *IL17)*. Closer examination of the data revealed that for all five SNPs heterozygosity was associated with reduced susceptibility to RSV infection. Only the association with the *IL10* SNP reached a *P*-value<0.01. The other SNPs displayed *P*-values between 0.01 and 0.05. For

Table 2. Single-nucleotide polymorphisms (SNPs) significantly associated with severe RSV bronchiolitis at the allele and genotype level.

				Association at the allele level			Association at the genotype level		
Gene	rs number	SNP	MAF	Allele	OR (95% CI)[1]	*P*[2]	Genotype	OR (95% CI)	*P*[3]
VDR	rs10735810	Thr1Met	0.38	T	1.30 (1.10-1.52)	0.0017[4]	C/C	1.00	0.0056[4]
							C/T	1.23 (0.97-1.57)	
							T/T	1.71 (1.23-2.36)	
JUN	rs11688	c.750G>A	0.05	A	1.51 (1.11-2.07)	0.0093[4]	G/G	1.00	0.0285
							G/A	1.48 (1.07-2.05)	
							A/A	3.45 (0.63-18.9)	
IFNA5	rs10757212	c.453C>T	0.22	T	0.77 (0.63-0.94)	0.0093[4]	C/C	1.00	0.0372
							C/T	0.80 (0.63-1.02)	
							T/T	0.53 (0.30-0.94)	
NOS2A	rs1060826	c.2757G>A	0.40	A	1.27 (1.09-1.49)	0.0031[4]	G/G	1.00	0.0117
							G/A	1.33 (1.04-1.69)	
							A/A	1.60 (1.16-2.22)	
FCER1A	rs2251746	c.-66T>C	0.26	C	1.25 (1.05-1.48)	0.0113	T/T	1.00	0.0238
							T/C	1.17 (0.93-1.47)	
							C/C	1.70 (1.15-2.49)	
MS4A2	rs1441586	c.-109C>T	0.44	T	1.19 (1.02-1.39)	0.0291			
ADAM33	rs574174	c.2241-410A>G	0.21	G	1.24 (1.02-1.50)	0.0308			
IL4R	rs1805011	Glu400Ala	0.12	C	0.76 (0.59-0.98)	0.0324			
IFNA13	rs643070	c.-603C>T	0.34	T	1.21 (1.03-1.43)	0.0201			
IL15	rs2254514	c.667C>T	0.26	T	1.22 (1.02-1.46)	0.0260			
STAT1	rs1914408	c.34753G>A	0.25	A	0.82 (0.68-0.98)	0.0304			
CD28	rs3116494	c.407+309A>G	0.23	G	1.22 (1.02-1.46)	0.0330			
CCL8	rs3138038	Lys69Gln	0.15	C	0.79 (0.64-0.98)	0.0349			

Table 2 Continued

				Association at the allele level			Association at the genotype level		
Gene	rs number	SNP	MAF	Allele	OR (95% CI)[1]	*P*[2]	Genotype	OR (95% CI)	*P*[3]
IL9R	rs3093457	c.27+155T>G	0.30	G	1.19 (1.01-1.40)	0.0406			
ITGB2	rs235326	c.1323C>T	0.34	T	1.18 (1.01-1.39)	0.0422			
TLR8	rs2407992	c.2007C>G	0.40	G	0.73 (0.54-0.99)	0.0433			
VCAM1	rs1041163	c.-1594T>C	0.16	C	0.80 (0.64-1.00)	0.0469			
IL10	rs1800872	c.-592C>A	0.24				C/C	1.00	0.0023[4]
							C/A	0.69 (0.55-0.88)	
							A/A	1.23 (0.80-1.91)	
IL4R	rs2057768	c.-3223C>T	0.31				C/C	1.00	0.0134
							C/T	0.72 (0.57-0.90)	
							T/T	0.92 (0.63-1.33)	
NCF2	rs2274064	Lys181Arg	0.47				A/A	1.00	0.0154
							A/G	0.82 (0.64-1.06)	
							G/G	1.21 (0.89-1.64)	
TNF	rs1799724	c.-857C>T	0.08				C/C	1.00	0.0363
							C/T	0.72 (0.53-0.99)	
							T/T	2.02 (0.78-5.24)	
IL17	rs7747909	c.627G>A	0.23				G/G	1.00	0.0455
							G/A	0.77 (0.61-0.97)	
							A/A	1.13 (0.72-1.79)	

Note. CI, confidence interval; MAF, minor allele frequency; OR, odds ratio.

[1]Reference allele is the major allele

[2]According to Chi-square distribution of 2x2 table on allele frequencies

[3]According to Chi-square distribution of 2x3 table on genotypes

[4]$P<0.01$

these five SNPs there is no evidence of a trend in relative risks for the genotypes, indicating that these effects are not due to co-dominance of the allele, but to a specific effect of the heterozygous genotype.

Group Of Innate Immunity Genes Is Associated With RSV Bronchiolitis.

Like in many genetic studies the strength of the association, as indicated by the *P*-value, was not very high for the SNPs. Therefore, and because of possible spurious association due to multiple testing, our list of associated genes might contain false positive associations. However, based on the number of associated SNPs in a process, two processes, i.e. "innate immunity" and "allergic asthma" were clearly overrepresented. This suggests that out of the 5 processes tested, these two processes are more important and are more likely to contain true associations. Interestingly, this also implies that SNPs in genes involved in chemotaxis, including all tested chemokines, and SNPs in genes involved in local lung responses are less important. However, overrepresentation of associations in a certain process does not take into account the strength of the association. Therefore an independent statistical test was used to evaluate the importance of the 5 selected processes in susceptibility to RSV bronchiolitis. For this purpose, the global test for groups of genes, which was developed for the analysis of microarray data, was used. This test calculates one *P*-value for the association of a group of SNPs. Since only five groups of genes were tested, this reduces the problem of multiple testing and may enable the ranking of pathways and biological processes based on their importance in susceptibility to RSV bronchiolitis. The 5 immunological processes are partly overlapping and therefore certain SNPs were included in more than one immunological proces. Results (Table 3) reveal that only the group of SNPs in genes involved in innate immunity was associated with susceptibility to RSV bronchiolitis ($P<0.05$).

Table 3. Association of the group of genes involved in innate immunity with susceptibility to RSV bronchiolitis.

Process	P^1
Airway mucosal response	0.119
Innate immunity	0.046
Chemotaxis	0.416
Adaptive immunity	0.595
Allergic asthma	0.257

[1]By global test.

DISCUSSION

To identify genes and biological pathways important in determining genetic susceptibility to RSV bronchiolitis we performed a large scale genetic study using a candidate gene approach. In total 22 SNPs in 21 genes displayed significant association with severe RSV bronchiolitis either at the allele- or genotype-level, or at both levels. Associated genes were found in all pathways tested. However, the four SNPs with the strongest association both at the allele- and genotype-level are located in genes involved in innate immunity, i.e. the *VDR, JUN, NOS2A* and *IFNA5* genes, highlighting the importance of this pathway. Indeed, the "global test", testing association of groups of genes, also indicated that the group of innate immune SNPs as a whole is associated with susceptibility to RSV bronchiolitis. SNPs in allergic asthma genes were overrepresented but this category did not reach significance in the global test. This could be a reflection of a possible higher degree of linkage between the SNPs in this category due to the fact that more SNPs were tested per "allergic asthma" gene. In addition, the associations of individual SNPs in this category were clearly less strong than in the category of innate immune genes.
The SNP in the *VDR* (vitamin D receptor) was previously associated with susceptibility to diabetes.(22) Other SNPs in this gene have been associated with susceptibility to tuberculosis and allergic asthma,(23;24) and the VDR has been implicated in down-regulating IL12 and IFNγ production.(25) The SNP in *NOS2A* was previously associated with Parkinson's disease,(26) and iNOS has a role in various airway diseases.(27) The SNPs in the innate immune genes *JUN* and *IFNA5* have, to our knowledge, never been previously associated with susceptibility to disease. The importance of IFNα in RSV infection is however highlighted by the fact that RSV can interfere with IFNα production.(28;29) *JUN* is part of transcription factor AP-1, which is one of the mediators of pro-inflammatory cytokine production.(30) Apparently, *JUN* plays an important role in the host-response to RSV infection. The association with *FCER1A* was also found both at the allele- and at the genotype level, but was less strong (*P*-value between 0.01 and 0.05). This SNP has been previously associated with altered FcεRI expression levels and allergic disease.(31)

The second category of SNPs displayed association at the allele level only. These SNPs are present in genes involved in innate immunity (*IFNA13, IL15, STAT1, TLR8*), chemotaxis (*CCL8, ITGB2, VCAM1*), adaptive immunity (*CD28, STAT1*), and allergic asthma (*MS4A2, ADAM33, IL4R, IL9R*). The SNPs in genes encoding *IL4R*, *IFNA13*, *CD28*, *IL9R* and *TLR8* have, to our knowledge, not been previously associated with disease, although another SNP in *IL4R* has previously been associated with RSV bronchiolitis.(4) For the *IL15* SNP, a haplotype-, but not single SNP interaction, has been found with asthma.(32) For the other SNPs associations have been found with various diseases including severity of fibrosis in Hepatitis-C virus infection, and susceptibility to stroke.(33;34) Interestingly,

this category of SNPs comprises most allergic asthma genes although the associations found are less strong than those found in the first category of SNPs. Using this larger cohort of children we could not confirm our previously described association with a SNP in IL4.(4)

The last category of SNPs only displays association at the genotype level. These SNPs were present in genes encoding *IL10, IL4R, TNF, NCF2* and *IL17.* In all five instances, it appears that the heterozygous genotype has a protective effect on RSV disease as compared to the major and minor homozygous genotype. Interestingly, a heterozygous advantage is seen for SNPs in three cytokine genes and one cytokine receptor gene, i.e. *IL10* (confirming our previous finding),(8) *TNF, IL17* and *IL4R,* which are all involved in determining the extent of inflammation, whereas TNFα and IL17 are inducers of inflammation, IL10 is an inhibitor of Th2 responses and inflammation,(35) and the IL4R is involved in Th2 responses and associated pathology. The apparent heterozygous advantage is not easy to interpret. Firstly, it could be related to the level of local cytokine production or response, which may determine the balance between clearing the virus and the extent of inflammation that occurs. Indeed the *IL4R* SNP has been shown to affect the levels of secreted IL4R that may affect IL4 responses,(36) and the *TNF* SNP has been associated with levels of TNFα production.(37) Interestingly, the latter SNP has also been associated with development of asthma in the Japanese population.(38) Secondly, cell-type specific regulation of expression may be involved in the heterozygous advantage. Since the *TNF, IL10* and *IL4R* SNPs are promoter SNPs and the *IL17* SNP is present in the 3'UTR, they may all affect gene expression. IL10 and TNFα can be produced both by monocytes/macrophages and by T-cells, and it is therefore tempting to speculate that regulation of expression of these cytokines differs in these two cell types. The *TNF* SNP has previously been shown to affect binding of transcription factor OCT1.(39) Tissue-specific effects on expression have previously been described for a variable number of tandem repeats in the *INS-IGF2* gene which affects expression in the thymus and in the pancreas in an opposite manner.(40) Thirdly, the results could be due to linkage. The effects of the *TNF* SNP could for instance be due to linkage of the *TNF* region with the *HLA* region for which a heterozygous advantage has been shown.(41) The *NCF2* SNP results in an amino acid change in this component of the NADPH oxidase and has, to our knowledge, never been associated with any disease. Only the *IL10* SNP displays stronger association with RSV bronchiolitis (*P*-value<0.01). Thus, although we have not established the mechanism, it is clear that heterozygosity for these alleles is associated with reduced susceptibility to RSV infection.

From this work it is clear that RSV bronchiolitis is a genetically complex disease influenced by many host genes, in particular by innate immune genes. One of the limitations of genetic association studies is that for all associations found, it is unclear whether

the associated SNPs are causative, or if associations are found due to linkage of selected SNPs to other causative variants. In addition, our list of associated SNPs might contain some false positive findings. Further genetic analysis, haplotype determination, and functional studies are therefore needed to elucidate the complex pathobiology and genetic nature of RSV disease.

Concluding Remarks

In conclusion our data show that genetic susceptibility to RSV bronchiolitis is a complex trait. Association of several SNPs in allergic asthma genes may support a model in which alleles that confer susceptibility to allergic asthma also confer susceptibility to RSV infection, although the associations with allergic asthma genes were clearly not the strongest in our study. The genes that display the strongest association with RSV bronchiolitis are involved in innate immunity indicating that this process may play a decisive role in determining disease susceptibility, and suggesting that early responses to the virus may not only lead to viral clearance but may also be involved in the development of excessive pathology and disease.

ACKNOWLEDGMENTS

We are grateful for the expert technical assistance of S. Imholz, C. Strien and F. van der Horst. We thank dr. C. Lindemans and dr. J. Heidema for inclusion of patients and dr. H. van Loveren for critically reading the manuscript.

APPENDIX.

STATISTICAL ANALYSIS

In our data we can perform a case-control analysis using the Dutch cases and the controls and a TDT-like analysis using all case-parent trios. The case-control analysis yields genotype relative risks, comparing heterozygotes and homozygote mutations to the homozygote wild types, without any assumption on the disease model or allele relative risks under the assumption of an additive effect of the haplotypes on the ln(relative risk) scale.

To explain the procedure we first consider a one-dimensional parameter such as the ln(allele relative risk). Let $\hat{\beta}_1$ and $\hat{\beta}_2$ be the estimated coefficients in two different analyses with respective standard errors s_1 and s_2. The Z-statistics for testing $\beta=0$ in either analysis are given by $Z_1 = \hat{\beta}_1/s_1$ and $Z_2 = \hat{\beta}_2/s_2$ respectively. Let ρ be the estimated correlation coefficient between the two estimates. Then, the standard error of the difference $\Delta = \hat{\beta}_1 - \hat{\beta}_2$ is given by $se(\Delta) = \sqrt{s_1^2 + s_2^2 - 2\rho s_1 s_2}$. This can be used to test the null hypothesis of no systematic difference ($\beta_1=\beta_2$) between the two analyses. Under the assumption of no systematic difference a weighted mean $\hat{\beta}_{com} = w\hat{\beta}_1 + (1-w)\hat{\beta}_2$ can be computed with standard error $s_{\text{com}} = se(\hat{\beta}_{\text{com}}) = \sqrt{w^2 s_1^2 + (1-w)^2 s_2^2 + 2w(1-w)\rho s_1 s_2}$. The optimal weight is given by $w = (s_1^2 - \rho s_1 s_2)/(s_1^2 = s_2^2 - 2\rho s_1 s_2)$. Using these optimal weights we obtain the Z-statistic for a combined test $Z_{\text{com}} = \hat{\beta}_{\text{com}}/s_{\text{com}}$.

Some insight in the procedure can be obtained if the two analyses give answers with about the same precision: $s_1=s_2=s$, which happens to be the case in our data. In that case $\hat{\beta}_{\text{com}} = (\hat{\beta}_1 + \hat{\beta}_2)/2$ with standard error $s_{\text{com}} = s\sqrt{(1+\rho)/2}$ and $Z_{\text{com}} = \sqrt{[Z_1 + Z_2]^2/[2(1+\rho)]}$. In our data $\rho \approx 0.5$. The implication is that if the two analyses are both borderline significant ($Z_1=Z_2=1.96, P_1=P_2=0.05$), the combined test has $Z_{\text{com}}=2.61$ with $P_{\text{com}}=0.009$, therefore the combined analysis was performed on all SNPs which showed a p-value <0.1 in one of the two individual tests.

Obtaining The Correlation Between The Estimates.

Consider two overlapping data sets, DS_1 and DS_2 with n_1 and n_2 independent observations. In each data set we can fit a multivariate statistical model with multidimensional parameters $\theta_1=(\alpha_1,\beta)$ and $\theta_2=(\alpha_2,\beta)$, respectively. The two models have the k_0-dimensional β-parameter in common and non-shared parameters α_1 and α_2 of dimension k_1 and k_2, respectively. When fitting this models by maximum likelihood we obtain the estimated parameters $\hat{\theta}_1 = (\hat{\alpha}_1,\hat{\beta}_1)$ and $\hat{\theta}_2 = (\hat{\alpha}_2,\hat{\beta}_2)$, the Fisher-information matrices I_1 and I_2 and

the score matrices U_1 and U_2, where, generally $\mathbf{I} = \partial^2 l/\partial\theta^2$ and $U_{ij} = \partial l_i(\hat{\theta})/\partial\theta_j$ is the derivative of the log-likelihood contribution of individual i with respect to parameter θ_j.
The theory of maximum likelihood estimation gives that the asymptotic covariance of the estimator is given by $\hat{\Sigma}_{\text{theor}} = \mathbf{I}^{-1}$. The robust sandwich estimator which is also valid if the model is miss-specified is given by $\hat{\Sigma}_{\text{robust}} = \mathbf{I}^{-1}\mathbf{U}^T\mathbf{U}\mathbf{I}^{-1}$.
Because of the overlap the estimated parameters $\hat{\theta}_1 = (\hat{\alpha}_1,\hat{\beta}_1)$ and $\hat{\theta}_2 = (\hat{\alpha}_2,\hat{\beta}_2)$ are dependent. Their covariance matrix can be estimated by $\text{cov}(\hat{\theta}_1,\hat{\theta}_2) = \mathbf{I}_1^{-1}\mathbf{U}_{1,\text{overlap}}^T\mathbf{U}_{2,\text{overlap}}\mathbf{I}_2^{-1}$ using only the rows of U_1 and U_2 that correspond to the overlapping observations. We used this estimator for the covariance matrix $\text{cov}(\hat{\theta}_1,\hat{\theta}_2)$ and the theoretical one for the separate covariance matrices $\text{cov}(\hat{\theta}_1)$ and $\text{cov}(\hat{\theta}_2)$ to stay closer to the standard output for each analysis separately.
In our genetic problem the first analysis is the logistic regression on the case-control data with genotype as explanatory variable (either taken linear as the number of mutations within a SNP (df=1) or categorical (df=2)) and the second analysis is the matched case-control analysis comparing cases with pseudo controls with separate strata for each case-parent trio. The score and information matrix for these models are well-known. We do not give the details here. From the estimated covariance matrix $\text{cov}(\hat{\theta}_1,\hat{\theta}_2)$ we can obtain the covariance matrix of the common part $\text{cov}(\hat{\beta}_1,\hat{\beta}_2)$. Together with the covariance matrices $\text{cov}(\hat{\beta}_1)$ and $\text{cov}(\hat{\beta}_2)$ we have now the tools to obtain the optimal combination.

Combined Estimate

Let $\hat{\beta}_1$ and $\hat{\beta}_2$ be two correlated estimates of the same k-dimensional parameter vector β with covariance matrices $\text{cov}(\hat{\beta}_1) = C_1$, $\text{cov}(\hat{\beta}_2) = C_2$ and $\text{cov}(\hat{\beta}_1,\hat{\beta}_2) = C_{12}$. Let, $\Delta = \hat{\beta}_1 - \hat{\beta}_2$ then $\text{cov}(\Delta)=C_1+C_2-C_{12}-C_{21}$ with $C_{21}=C_{21}^T$. The hypothesis of no systematic difference can be tested by Hotelling's $T^2_{\text{homo}} = \Delta^T \text{cov}(\Delta)^{-1} \Delta$.
The most efficient estimate of β is given by

$$\hat{\beta}_{\text{com}} = \left[\begin{pmatrix} I_k \\ I_k \end{pmatrix}^T \begin{pmatrix} C_1 & C_{12} \\ C_{21} & C_2 \end{pmatrix}^{-1} \begin{pmatrix} I_k \\ I_k \end{pmatrix}\right]^{-1} \left[\begin{matrix} I_k \\ I_k \end{matrix}\right]^T \left[\begin{matrix} C_1 & C_{12} \\ C_{21} & C_2 \end{matrix}\right]^{-1} \left[\begin{matrix} \hat{\beta}_1 \\ \hat{\beta}_1 \end{matrix}\right]$$

with covariance matrix

$$\text{cov}(\hat{\beta}_{\text{com}}) = \left[\begin{pmatrix} I_k \\ I_k \end{pmatrix}^T \begin{pmatrix} C_1 & C_{12} \\ C_{21} & C_2 \end{pmatrix}^{-1} \begin{pmatrix} I_k \\ I_k \end{pmatrix}\right]^{-1}$$

. The optimal combined test for β=0 is based on another application of Hotelling T^2, namely $T^2_{\text{com}} = \hat{\beta}^T_{\text{com}}\text{cov}(\hat{\beta}_{\text{com}})^{-1}\hat{\beta}_{\text{com}}$.
Simulations (to be reported elsewhere) show that this approach loses very little information when compared to using the full statistical model.

REFERENCE LIST

(1) Meissner HC. Selected populations at increased risk from respiratory syncytial virus infection. Pediatr Infect Dis J 2003; 22(2 Suppl):S40-S44.

(2) Simoes EA, Carbonell-Estrany X. Impact of severe disease caused by respiratory syncytial virus in children living in developed countries. Pediatr Infect Dis J 2003; 22(2 Suppl):S13-S18.

(3) Choi EH, Lee HJ, Yoo T, Chanock SJ. A common haplotype of interleukin-4 gene IL4 is associated with severe respiratory syncytial virus disease in Korean children. J Infect Dis 2002; 186(9):1207-1211.

(4) Hoebee B, Rietveld E, Bont L, Oosten M, Hodemaekers HM, Nagelkerke NJ et al. Association of severe respiratory syncytial virus bronchiolitis with interleukin-4 and interleukin-4 receptor alpha polymorphisms. J Infect Dis 2003; 187(1):2-11.

(5) Puthothu B, Krueger M, Forster J, Heinzmann A. Association between Severe Respiratory Syncytial Virus Infection and IL13/IL4 Haplotypes. J Infect Dis 2006; 193(3):438-441.

(6) Tal G, Mandelberg A, Dalal I, Cesar K, Somekh E, Tal A et al. Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. J Infect Dis 2004; 189(11):2057-2063.

(7) Lahti M, Lofgren J, Marttila R, Renko M, Klaavuniemi T, Haataja R et al. Surfactant protein D gene polymorphism associated with severe respiratory syncytial virus infection. Pediatr Res 2002; 51(6):696-699.

(8) Hoebee B, Bont L, Rietveld E, van Oosten M, Hodemaekers HM, Nagelkerke NJ et al. Influence of promoter variants of interleukin-10, interleukin-9, and tumor necrosis factor-alpha genes on respiratory syncytial virus bronchiolitis. J Infect Dis 2004; 189(2):239-247.

(9) Kimman TG, Janssen R, Hoebee B. Future prospects in respiratory syncytial virus genetics. Future Virology 2006; 1(4):483-492.

(10) Hall CB, Douglas RG, Jr., Geiman JM. Quantitative shedding patterns of respiratory syncytial virus in infants. J Infect Dis 1975; 132(2):151-156.

(11) Buckingham SC, Bush AJ, Devincenzo JP. Nasal quantity of respiratory syncytical virus correlates with disease severity in hospitalized infants. Pediatr Infect Dis J 2000; 19(2):113-117.

(12) Wright PF, Gruber WC, Peters M, Reed G, Zhu Y, Robinson F et al. Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. J Infect Dis 2002; 185(8):1011-1018.

(13) Wheeler JG, Wofford J, Turner RB. Historical cohort evaluation of ribavirin efficacy in respiratory syncytial virus infection. Pediatr Infect Dis J 1993; 12(3):209-213.

(14) Meert KL, Sarnaik AP, Gelmini MJ, Lieh-Lai MW. Aerosolized ribavirin in mechanically ventilated children with respiratory syncytial virus lower respiratory tract disease: a prospective, double-blind, randomized trial. Crit Care Med 1994; 22(4):566-572.

(15) Guerguerian AM, Gauthier M, Lebel MH, Farrell CA, Lacroix J. Ribavirin in ventilated respiratory syncytial virus bronchiolitis. A randomized, placebo-controlled trial. Am J Respir Crit Care Med 1999; 160(3):829-834.

(16) Janssen R, Pennings J, Hodemaekers HM, Buisman A, van Oosten M, de Rond L et al. Host transcription profiles upon primary Respiratory Syncytial Virus infection. J.Virol. in press. 2007.

(17) Viet AL, Van Gils HWV, de Melker H. Risk factors and health in the Netherlands, a survey by municipal health centers; annual report 1998. Centraal Bureau voor de Statistiek (CBS-KPE). GGD Nederland RIBM Rapport 266807003. Available at: http://www.rivm.nl/bibliotheek/rapporten/266807003.html. Accessed 6 August 2007.

(18) Cordell HJ, Barratt BJ, Clayton DG. Case/pseudocontrol analysis in genetic association studies: A unified framework for detection of genotype and haplotype associations, gene-gene

and gene-environment interactions, and parent-of-origin effects. Genet Epidemiol 2004; 26(3):167-185.

(19) Nagelkerke NJ, Hoebee B, Teunis P, Kimman TG. Combining the transmission disequilibrium test and case-control methodology using generalized logistic regression. Eur J Hum Genet 2004; 12(11):964-970.

(20) Epstein MP, Waldman ID, Satten GA. Improved association analyses of disease subtypes in case-parent triads. Genet Epidemiol 2006; 30(3):209-219.

(21) Goeman JJ, van de Geer SA, de Kort F, van Houwelingen HC. A global test for groups of genes: testing association with a clinical outcome. Bioinformatics 2004; 20(1):93-99.

(22) Taverna MJ, Selam JL, Slama G. Association between a protein polymorphism in the start codon of the vitamin D receptor gene and severe diabetic retinopathy in C-peptide-negative type 1 diabetes. J Clin Endocrinol Metab 2005; 90(8):4803-4808.

(23) Wilkinson RJ, Llewelyn M, Toossi Z, Patel P, Pasvol G, Lalvani A et al. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet 2000; 355(9204):618-621.

(24) Poon AH, Laprise C, Lemire M, Montpetit A, Sinnett D, Schurr E et al. Association of vitamin D receptor genetic variants with susceptibility to asthma and atopy. Am J Respir Crit Care Med 2004; 170(9):967-973.

(25) Lemire JM. Immunomodulatory actions of 1,25-dihydroxyvitamin D3. J Steroid Biochem Mol Biol 1995; 53(1-6):599-602.

(26) Levecque C, Elbaz A, Clavel J, Richard F, Vidal JS, Amouyel P et al. Association between Parkinson's disease and polymorphisms in the nNOS and iNOS genes in a community-based case-control study. Hum Mol Genet 2003; 12(1):79-86.

(27) Ricciardolo FL, Sterk PJ, Gaston B, Folkerts G. Nitric oxide in health and disease of the respiratory system. Physiol Rev 2004; 84(3):731-765.

(28) Ramaswamy M, Shi L, Varga SM, Barik S, Behlke MA, Look DC. Respiratory syncytial virus nonstructural protein 2 specifically inhibits type I interferon signal transduction. Virology 2006; 20;344(2):328-339.

(29) Ramaswamy M, Shi L, Monick MM, Hunninghake GW, Look DC. Specific inhibition of type I interferon signal transduction by respiratory syncytial virus. Am J Respir Cell Mol Biol 2004; 30(6):893-900.

(30) Adcock IM, Caramori G. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. Immunol Cell Biol 2001; 79(4):376-384.

(31) Hasegawa M, Nishiyama C, Nishiyama M, Akizawa Y, Mitsuishi K, Ito T et al. A novel -66T/C polymorphism in Fc epsilon RI alpha-chain promoter affecting the transcription activity: possible relationship to allergic diseases. J Immunol 2003; 171(4):1927-1933.

(32) Kurz T, Strauch K, Dietrich H, Braun S, Hierl S, Jerkic SP et al. Multilocus haplotype analyses reveal association between 5 novel IL-15 polymorphisms and asthma. J Allergy Clin Immunol 2004; 113(5):896-901.

(33) Hellier S, Frodsham AJ, Hennig BJ, Klenerman P, Knapp S, Ramaley P et al. Association of genetic variants of the chemokine receptor CCR5 and its ligands, RANTES and MCP-2, with outcome of HCV infection. Hepatology 2003; 38(6):1468-1476.

(34) Zee RY, Bates D, Ridker PM. A prospective evaluation of the CD14 and CD18 gene polymorphisms and risk of stroke. Stroke 2002; 33(4):892-895.

(35) Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity 2006; 24(6):677-688.

(36) Hackstein H, Hecker M, Kruse S, Bohnert A, Ober C, Deichmann KA et al. A novel polymorphism in the 5' promoter region of the human interleukin-4 receptor alpha-chain gene is associated with decreased soluble interleukin-4 receptor protein levels. Immunogenetics 2001; 53(4):264-269.

(37) Higuchi T, Seki N, Kamizono S, Yamada A, Kimura A, Kato H et al. Polymorphism of the 5'-flanking region of the human tumor necrosis factor (TNF)-alpha gene in Japanese. Tissue Antigens 1998; 51(6):605-612.

(38) Noguchi E, Yokouchi Y, Shibasaki M, Inudou M, Nakahara S, Nogami T et al. Association between TNFA polymorphism and the development of asthma in the Japanese population. Am J Respir Crit Care Med 2002; 166(1):43-46.

(39) van Heel DA, Udalova IA, De Silva AP, McGovern DP, Kinouchi Y, Hull J et al. Inflammatory bowel disease is associated with a TNF polymorphism that affects an interaction between the OCT1 and NF(-kappa)B transcription factors. Hum Mol Genet 2002; 11(11):1281-1289.

(40) Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG et al. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. Nat Genet 1997; 15(3):289-292.

(41) Doherty PC, Zinkernagel RM. Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. Nature 1975; 256(5512):50-52.

Chapter 7

IL19 and IL20 Genes Are Associated With Wheeze After Respiratory Syncytial Virus Infection

Marieke JJ Ermers

Riny Janssen

N. Charlotte Onland-Moret

Hennie M Hodemaekers

Maroeska M Rovers

Jan LL Kimpen

Louis LJ Bont

Submitted

ABSTRACT

The aim of the present study was to identify genetic determinants of early wheeze following respiratory syncytial virus lower respiratory tract infection (RSV LRTI). Mechanisms underlying the increased risk of early wheeze following RSV LRTI are unclear and information about genetic determinants is limited.

In a candidate gene association study, 347 polymorphisms were investigated in 166 Dutch infants that had been hospitalized for RSV LRTI. Logistic regression analysis was used to study the association between geno- and haplotypes and early wheeze following RSV LRTI.
Polymorphisms in *IL19, IL20, MUC5AC, TNFRSF1B, C3, CTLA4, CXXL9, IL4R* and *IL7* genes were associated with early wheeze. Two *IL19* SNPs and one *IL20* SNP, both belonging to the *IL10* family genes, were associated with early wheeze, notably in infants without asthmatic parents. Haplotype analysis of the combined *IL19* and *IL20* genotyped polymorphisms demonstrated an inverse association between the TGG haplotype and early wheeze following RSV LRTI (OR 0.43 (95%CI 0.24-0.75) *P*=0.003).

In conclusion, genetic variation in adaptive immunity genes and particularly in *IL19* and *IL20* genes is associated with the development of early wheeze following RSV LRTI, suggesting a central role for these IL10 family members in the etiology of chronic airway disease during infancy.

INTRODUCTION

Respiratory syncytial virus lower respiratory tract infection (RSV LRTI) during infancy is an independent risk factor for subsequent recurrent wheeze, at least within the first years of childhood.(1-3) Mechanisms underlying the increased incidence of wheeze during the first years following RSV LRTI, i.e. early wheeze, are unclear. Disease severity and age at the moment of RSV LRTI were not related to early wheeze(4;5) whereas male sex appeared to be a risk factor in some, but not all studies.(6-8) Early wheeze following RSV LRTI was related to signs of airflow limitation during RSV LRTI(9), eosinophilia during RSV LRTI(10), and monocyte IL10 production during the convalescent phase of RSV LRTI.(11)
To date, only two studies aimed to identify genetic determinants of early wheeze following RSV LRTI. In one association study of 134 RSV hospitalized infants, a variant in the promoter region of the *IL8* gene was related to the development of subsequent wheeze.(12) We previously demonstrated an association between a functional *IL13* polymorphism and wheeze at age six years, whereas no association was found between the *IL13* polymorphism and early wheeze.(13)
The availability of analytic tools to study larger numbers of genes and a larger cohort of RSV LRTI-hospitalized infants in whom recurrent wheeze was evaluated enabled us to extend our previous studies. In this study we will describe the results of 347 genotyped SNPs on 210 genes, including *IL10* family genes.

METHODS

Study Subjects & Study Design

The infants hospitalized for RSV LRTI and included in this study were previously described in other papers.(11;13;14) In brief, they were hospitalized for RSV LRTI during the winter seasons of 1995-1996 or 2004-2006. Infants included in the 2004-2006 seasons received placebo medication in a placebo-controlled trial investigating the role of inhaled beclomethasone to prevent early wheeze following RSV LRTI. For the current study, we included infants who participated in the follow-up program and whose parents prospectively recorded the presence of wheeze in a daily log (9). We selected previously healthy infants of native Dutch origin with a first episode of RSV LRTI under the age of thirteen months. Infants with a history of cardiac or pulmonary disease were excluded. RSV infection was confirmed by a positive immunofluorescence for RSV in nasopharyngeal cells.
The primary outcome of the current study was the presence of wheeze during the first 15 months following RSV LRTI hospitalization. We chose this duration of follow-up to capture the second winter season, which showed high incidence of wheeze following

RSV LRTI in a previous study.(15) Infants who wheezed more than the median counted days with wheeze were (arbitrarily) classified as wheezing infants, i.e. cases, whereas infants who wheezed less than the median counted days with wheeze were classified as non-wheezing infants, i.e. controls. All parents provided written, informed consent. The study was approved by the Ethics Review Committee, University Medical Center Utrecht and by the Ethics Review Committees of the other participating centers.

DNA Isolation, Genotyping And Selection Of Genes And SNPs

A candidate gene approach was followed as described in our previous study focusing on genetic determinants of severe acute RSV LRTI.(14) Briefly, 384 SNPs in 220 genes were selected based on studies in the literature in the context of RSV infection or because they were up-regulated in our murine model of RSV infection by use of microarray analysis.(16) SNPs were classified into five processes, i.e. the airway mucosal response, innate immunity, chemotaxis, adaptive immunity, and allergic asthma. DNA isolation and genotyping of patients and their parents was performed in our previous study.(14) SNPs were genotyped using Illumina's Beadarray™ technology on a 384 Sentrix™ array matrix. To evaluate the accuracy of genotyping, all SNPs were assessed to determine if the observed genotype frequencies reflected the measured allele frequencies with Hardy-Weinberg equilibrium using chi-square tests ($P<0.01$) in control subjects not hospitalized because of RSV LRTI.(14) All SNPs were examined for their minor allele frequency (MAF > 10%) and call rate (call rate ≥ 90%). Thirty-seven SNPs were excluded due to low signal, overlapping of multiple clusters, or scattering of the clusters.

Statistical Analyses

We used logistic regression analysis to estimate the odds ratio (OR) for genotypes associated with early wheeze following RSV LRTI (SPSS for Windows, Release 15.0: SPSS Inc., Chicago Ill). Significance was set at $P < 0.05$. If less than 5 infants in either the cases or the controls were homozygous for the minor allele, we combined this group together with the heterozygous infants. X-linked SNPs were analyzed separately in boys and girls. We performed sensitivity analyses for the observed significant associations in which infants with and without early wheeze were distinguished according to alternative cut-off values (e.g. no wheeze at all vs any wheeze during follow up; the quartile of infants with most frequent wheeze -i.e. more than 50 days during follow up- vs the rest). Because baseline differences existed between infants with and without early wheeze we performed post hoc stratified analyses for groups of infants with and without asthmatic parents (i.e. parental reported physician diagnosed asthma) and for groups of infants with and without signs of airflow limitation during acute RSV LRTI (i.e. physician diagnosed wheezing on

auscultation of the chest). The global test for groups of genes was used to determine whether the groups of genes involved in different immunological processes as pre-classified in our previous study, were associated with early wheeze following RSV LRTI. (14;17). This test was originally developed for analysis of micro-array data, and it generates 1 *P*-value for the association between a group of genes involved in 1 process and disease, by use of the R-package *globaltest* (see the Bioconductor Web page, available at http://www.bioconductor.org/packages/2.0/bioc/html/globaltest.html). The global test compares distributions of all alleles in a process between cases and controls.

Haplotype analysis was performed in regions with moderate to high LD (0.3-0.8) where multiple SNPs were associated with early wheeze. Pairwise linkage disequilibrium (LD) was estimated using Haploview (Haploview, version 4.0, released 21 August 2007, http://www.broad.mit.edu/mpg/haploview)(18) and the extent of LD was expressed in terms of standardized R^2 characteristics. Parental and infant SNP information was used to estimate haplotypes of the infants (Unphased software, version 3.0.7).(19) Haplotypes occurring with a frequency of ≥ 5% were included in haplotype analyses. Logistic regression analysis was used to estimate the odds ratio (OR) for haplotypes associated with early wheeze following RSV LRTI.

RESULTS

One hundred sixty-six infants with a median age of 10 weeks (25^{th}-75^{th} percentile 5-23 weeks) were included in the present study. The median of counted days with wheeze during follow up was 14 days (range 0-279 days). The pattern of wheeze following RSV LRTI in the 1995-1996 and the 2004-2006 cohorts were remarkably similar. Baseline characteristics of infants with and without early wheeze are presented in Table 1. Infants with early wheeze more frequently exhibited signs of airflow limitation during RSV LRTI (63.2% vs 44.2% *P*=0.02) and more parents of infants with early wheeze tended to suffer from asthma (16.9% vs 7.2%, *P*=0.06).

Table 1. Baseline characteristics of participating infants.

	No early wheeze (N=83)	Early wheeze (N=83)	*P*
Sex (% male)	50.6	60.2	0.21
Age at admission in weeks (median, range)	10 (1-51)	9 (1-56)	0.33
Duration of pregnancy in weeks (median, range)	39.2 (25-42.6)	39.0 (27-42)	0.23
Signs of airway limitation during RSV LRTI (%)	44.2	63.2	0.02
Admission to ICU (%)	13.6	14.6	0.87
Parental asthma (%)	7.2	16.9	0.06
Counted days with wheeze (median, range)	2.5 (0-14)	49 (15-279)	NA

Note. ICU, intensive care unit; RSV LRTI, respiratory syncytial virus lower respiratory tract infection; NA, not applicable.

Using the Illumina platform, genotype determination was successful for 347 SNPs. Ten SNPs in 9 genes were associated with early wheeze at the genotype level ($P<0.05$). Results of the case-control association study are presented in Table 2. The strength of most associations was not very high for individual SNPs. The global test for groups of genes was used to evaluate the importance of the selected processes in susceptibility to early wheeze following RSV LRTI. This analysis showed that the group of SNPs in genes involved in adaptive immunity was associated with early wheeze ($P=0.03$) whereas the other processes were not. Six SNPs within the group of SNPs in genes involved in the adaptive immune system were significantly associated with early wheeze following RSV LRTI (*IL4R* SNP rs1805015 (Ser503Pro); *IL7* SNP rs2583762 (c.146+12824 A→T); *CTLA4* SNP rs3087243 (c.1720+236 A→G); *IL19* SNP rs2243188 (c.552+49 C→A) ; *IL19* SNP rs2243191 (Ser213Pro); *IL20* SNP rs2981573 (c.379-152 A→G). The three associated SNPs in the *IL19* and *IL20* genes were in moderate to high LD with each other (Figure 1). To test whether the individual protective effects of *IL19* and *IL20* polymorphisms could be attributed to a specific haplotypic background, haplotype analysis of the *IL19* and *IL20* genes was executed. A combined haplotype analysis was performed with two of the three genotyped SNPs in the *IL19* and *IL20* genes that were associated with early wheeze (*IL19* SNP rs2243191 (Ser213Pro) and *IL20* SNP rs2981573 (c.379-152 A→G)) and with one IL20 SNP that was not associated with early wheeze following RSV LRTI (*IL20* SNP rs2981572 (c.-1053 T→G)). The *IL19* SNP rs2243188 (c.552+49 C→A) was excluded because of high LD (R^2 0.89) with *IL19* SNP rs2243191 (Ser213Pro). Three common haplotypes with a frequency ≥5 % were identified in the total group of infants (Table 3). These

Table 2. Significant associations with early wheeze following RSV LRTI for genotypic analyses.

SNP	Gene	MAF	AB vs AA (OR (95% CI))	BB vs AA (OR (95% CI))	*P*[1]
rs11558499	*MUC5AC*	0.19	2.7 (1.4-5.2)	NA[2]	0.002
rs2243191	*IL19*	0.19	0.4 (0.2-0.7)	NA[2]	0.002
rs1061622	*TNFRSF1B*	0.24	0.4 (0.2-0.8)	NA[2]	0.004
rs2981573	*IL20*	0.20	0.4 (0.2-0.8)	NA[2]	0.004
rs4807893	*C3*	0.43	0.3 (0.1-0.6)	0.5 (0.2-1.2)	0.004
rs3087243	*CTLA4*	0.47	1.7 (0.8-3.7)	4.6 (1.7-12.5)	0.008
rs2276886	*CXCL9*	0.25	2.1 (1.1-4.0)	NA[2]	0.016
rs2243188	*IL19*	0.20	0.5 (0.3-0.9)	NA[2]	0.026
rs1805015	*IL4R*	0.14	0.5 (0.2-1.0)	NA[2]	0.037
rs2583762	*IL7*	0.16	1.9 (1.0-3.8)	NA[2]	0.047

Note. MAF, minor allele frequency; A, major allele; B, minor allele; AA, homozygous for major allele; AB, heterozygous; BB,homozygous for minor allele; NA, not applicable.

[1]According to Chi Square distribution on genotype frequencies.

[2]Homo- and heterozygous infants are grouped together because < 5 infants in one of the cells.

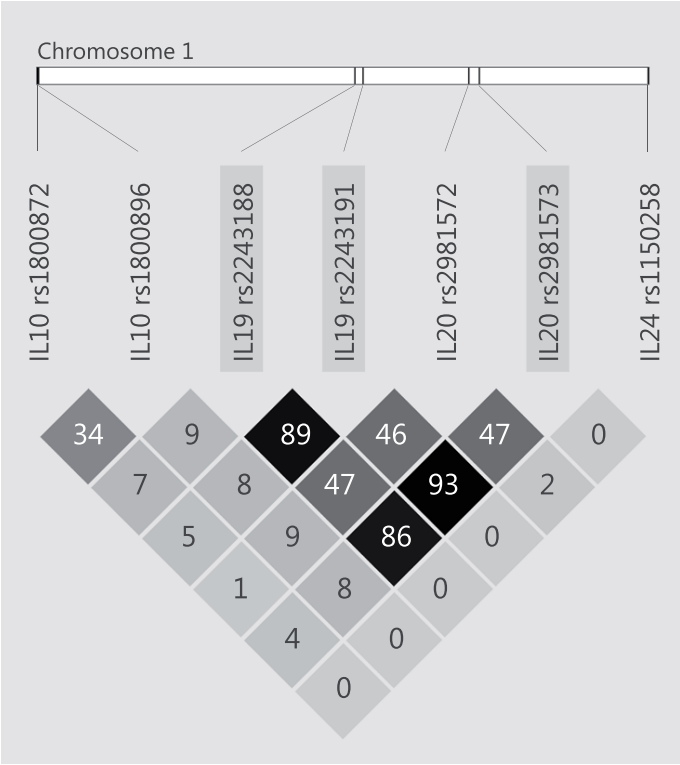

Figure 1. *IL10, IL19, IL20* and *IL24* genes on chromosome 1 and the genotyped SNPs. SNPs rs2243188, rs2243191 and rs2981573 showed significant association with early wheeze following RSV LRTI. Pairwise linkage disequilibrium (LD) between SNPs is characterized in terms of standardized R^2 characteristics. Black blocks indicate high LD between SNPs; dark and light grey blocks indicate moderate to low LD; white blocks indicate that there is little significant LD.

Table 3. Results of combined *IL19-IL20* haplotype analysis in patients with early wheeze following RSV LTRI.

Haplotype	rs2243191	rs2981572	rs2981573	Controls (N=83)	Cases (N=83)	Haplotypic OR (95% CI)[1]	*P*
HT 1	C	T	A	60%	66%	NA	
HT 2	C	G	A	10%	19%	1.72 (0.89-3.22)	0.106
HT 3	T	G	G	29%	13%	0.43 (0.24-0.75)	0.003
Other HT[2]				1%	2%		

Note. HT, haplotype; OR, odds ratio; CI, confidence interval; NA, not appçlicable.

[1]The haplotype combining the most frequent alleles at each site is chosen as the reference haplotype (CTA).

[2]Haplotypes occurring with a frequency of ≤5% were excluded from the haplotype analyses.

common haplotypes comprised 99% of all *IL19/IL20* haplotypes. The combined *IL19/IL20* haplotype TGG had a lower frequency in infants with early wheeze compared to infants without early wheeze (13% vs 29%; OR 0.4 (95% CI 0.2-0.8); *P*=0.003).

Baseline differences in the presence of signs of airflow limitation during RSV LRTI and the presence of parental asthma existed between infants with and without early wheeze (Table 1). To test whether associations between the *IL19* and *IL20* SNPs and early wheeze differed for infants with and without an atopic predisposition and for infants with and without signs of airflow limitation, post hoc stratified analyses were performed. Post-hoc stratification for the presence of signs of airflow limitation did not alter the associations (data not shown). Post-hoc stratification for the presence of parental asthma showed that associations between *IL19* and *IL20* SNPs and early wheeze were limited to the major subgroup of infants without asthmatic parents (N=146; *IL19* SNP rs2243188, OR 0.3 (95% CI 0.2-0.7), *P*=0.003; *IL19* SNP rs2243191, OR 0.2 (95% CI 0.1-0.5), *P*=0.00009; *IL20* SNP rs2981573, OR 0.3 (95% CI 0.1-0.5), *P*=0.0003) (Table 4). No association was observed between the *IL19* and *IL20* SNPs and early wheeze in the minor subgroup of infants with asthmatic parents (N=20; *IL19* SNP rs2243188, OR 1.3 (95% CI 0.2-9.7), *P*=0.83; *IL19* SNP rs2243191, OR 1.3 (95% CI 0.2-9.8), *P*=0.83; *IL20* SNP rs2981573, OR 1.3 (95% CI 0.2-9.8), *P*=0.83). Similar effect sizes were obtained when the analyses were stratified for other atopic features, i.e. infants with and without parents suffering from hayfever and infants with and without parents suffering from eczema (data not shown).

Sensitivity analyses in which infants with and without early wheeze were distinguished according to alternative cut-off values revealed comparable results (data not shown).

Table 4. Results of post-hoc stratified analyses of *IL19-IL20* single nucleotide polymorphisms (SNPs) and early wheeze following RSV LRTI in infants with and without asthmatic parents.

	No asthmatic parent (N=146)		Asthmatic parent (N=20)		Interaction
SNP	OR (95% CI)	*P*[1]	OR (95% CI)	*P*[1]	*P*[2]
rs2243188	0.3 (0.2-0.7)	0.003	1.3 (0.2-9.7)	0.83	0.25
rs2243191	0.2 (0.1-0.5)	0.00009	1.3 (0.2-9.8)	0.83	0.13
rs2981573	0.3 (0.1-0.5)	0.0003	1.3 (0.2-9.8)	0.83	0.16

Note. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval

[1]By logistic regression test.

[2]By interaction test.

DISCUSSION

This study demonstrates that genetic variation in adaptive immunity genes and particularly in *IL19* and *IL20* genes appears to be associated with the occurrence of early wheeze

following RSV LRTI, especially in infants without asthmatic parents. The prevalence of early wheeze was lower in infants with the combined *IL19/IL20* TGG haplotype compared to infants with the CTA haplotype.

Little is known about the role of genetic mechanisms underlying chronic airway disease following RSV LRTI. A previous study of Goetghebuer *et al.* reported an association between the *IL8* -251 C→T polymorphism and recurrent wheeze(12), which we could not confirm in the current study. However, Goetghebuer *et al.* analyzed the occurrence of wheeze in infants with early and/or late wheeze following RSV LRTI whereas our study focused on early wheeze only. These differences in wheezing phenotypes might have influenced the results since we previously showed that early and late wheeze following RSV LRTI are distinct entities with distinct immunological and genetic characteristics.(13)

The role of IL10 in the pathogenesis of RSV LRTI has been debated in literature. IL10 is a pleiotropic anti-inflammatory cytokine known to suppress Th1-like immune responses and promote Th2 responses.(20) IL10 levels measured during acute RSV LRTI related to disease severity in one study,(21) whereas another study showed no association.(11) We previously showed that monocyte IL10 production during the convalescent phase of RSV LRTI is predictive of the subsequent development of early wheeze.(11) Monocyte IL10 levels measured during acute RSV LRTI were not associated with the *IL19/IL20* haplotypes in a subgroup of 40 patients (data not shown). Several studies focused on the role of genetic variation in the *IL10* gene locus in the pathofysiology of acute RSV LRTI, but results are not conclusive.(22-25) Taken together, literature suggests that IL10 influences the course of RSV LRTI, but it's precise role remains to be determined.

IL19 and IL20 are members of the IL10 family that were initially identified during a sequence database search aimed to find potential *IL10* gene homologs.(26;27) *IL19* and *IL20* genes are clustered together with *IL10* and *IL24* genes in a 200 kb region on chromosome 1q31-32 and have similar genomic structures and similar primary and secondary protein structures.(28) Both IL19 and IL20 bind to the IL20 receptor complex, consisting of the IL20R1 and IL20R2 subunits. IL20 also binds to a heterodimeric receptor consisting of IL22R1 and IL20R2.(29). The receptors for IL19 and IL20 are widely expressed, but only lung and skin tissue express both receptors.(30) Both receptors signal through STAT3. (26;29) IL10 family members cross-regulate expression of other IL10 family members. IL19 induces selective expression of IL10 by monocytes and myeloid dendritic cells.(31) IL19 induces IL19 expression by an auto-feedback mechanism, which is not yet fully understood. Control of IL19 expression is provided by IL10, strongly interfering with *IL19* gene transcription.

Little data are available on the role of IL19 and IL20 in the etiology of chronic airway diseases. In asthmatic children, IL19 serum levels are increased, but no human data on levels in bronchoalveolar lavages have been published.(32) In mice and human epithelium, IL19 overexpression enhanced allergic airway inflammation by the induction of Th2 cy-

tokines.(32;33). However, non-allergic mechanisms by which IL19 and IL20 induce airway inflammation have been considered.(34) Adenosine-induced IL19 production by primary bronchial epithelium cells enhanced monocyte TNFα production.(35) In line with these literature data we hypothesize that our findings underscore a central role of bronchial epithelial cells in the pathogenesis of early wheeze following RSV LRTI.

Genotyped SNPs in the current study are located in the intron (*IL19* SNP rs2243188 (c.431+49 C→A) and *IL20* SNP rs2981573 (c.379-152 A→G)), exon (*IL19* SNP rs2243191 (Ser213Pro)) and promoter region (*IL20* SNP rs2981572 (c.-1053 T→G)). Only the *IL19* SNP rs2243191 resulted in an amino acid change, i.e. Ser → Pro. Previous studies showed associations of the genotyped IL19 and IL20 SNPs with Hepatitic C virus clearance(36), psoriasis(37) palmoplantar pustulosis(38) and juvenile idiopathic arthritis(39), suggesting that IL19 and IL20 play a role in the pathology of inflammatory disorders. However, functionality of SNPs in the *IL19* and *IL20* genes is not demonstrated, warranting functional genetic studies.

We found a strong interaction between parental asthma and *IL10* family genes on early wheeze following RSV LRTI. Our results suggest that associations between *IL19* and *IL20* SNPs and early wheeze were limited to infants without an atopic background. This is suggestive of a pathogenetic role of IL19 and IL20 in non-atopic wheeze. We hypothesize that IL10 family members are predominantly involved in virus-induced wheeze. Local production of these cytokines during acute RSV LRTI might modify or skew subsequent immune responses leading to subsequent wheeze. The immune modulating role of IL10 family cytokines seems less consequential in infants with an atopic background, suggesting that early post-bronchiolitis wheeze develops by a different mechanism in infants with and without an atopic background.

In addition to SNPs in *IL10* family genes, other SNPs associated with early wheeze require discussion. None of these SNPs were associated with the risk of hospitalization for RSV LRTI(14). It is emphasized that the association found for these SNPs may result from false discovery. The strongest association was found for *MUC5AC*, the major mucin of the human airways, giving additional support for a role of airway epithelium in the pathogenesis of wheeze following RSV LRTI. Exuberant mucus production is a prominent symptom in children with early wheeze. In humans, no data on the role of this gene in RSV infection or early wheeze have been published. Animal data have shown that RSV induces overexpression of MUC5AC by a CXCR2-dependent mechanism, which is associated with airway hyperresponsiveness(40). There are no reports on the association between the *MUC5AC* SNP and respiratory disease. However, we believe that the association between *MUC5AC* and early wheeze is interesting and requires confirmation by other studies, because of the size of the genetic effect, the clinical plausibility and the link to experimental RSV literature. Tumour necrosis factor (TNF)-α is a pro-inflammatory cytokine which is associated with many asthma-like phenotypes with effects on airway

smooth muscle, mucous production and granulocyte function(41;42). There are no reports on the TNF receptor *TNFRSF1B* polymorphism and airway diseases. The complement *C3* SNP is located in the last exon of the gene and has been associated with adult and childhood asthma in Japan(43;44). The mechanism by which C3 affects the risk of asthma development is not clear, but could be by enhancing IgE production. There are no reports on the association between the *C3* SNP and early wheeze. Associations between *IL7*, *CTLA4* and *IL4R* SNPs and early wheeze further underscored the potential role of the adaptive immune system in the development of early wheeze. The *CTLA4* and *IL4R* SNPs, but not the *IL7* SNP, have previously been associated with asthma(45;46). CXCL9 is a chemokine that binds to the chemokine receptor CXCR3. The *CXCL9* SNP was mildly associated with early wheeze, which is the first report of an association between this SNP and a respiratory disease.

The current study has several limitations. First, although we describe the largest cohort of prospectively followed RSV LRTI hospitalized infants evaluated for the development of recurrent wheeze, this is still a small cohort for genetic association studies. Therefore it had a limited power to detect less strong associations, which could still be relevant to the development of post-bronchiolitis wheeze. SNPs were not selected based on linkage disequilibrium patterns as in genome wide association studies. Therefore, we cannot exclude that important SNPs were overlooked. Second, multiple associations were tested and therefore the presence of false positive results cannot be precluded. Most of the observed associations lost significance after correction for multiple testing.(47) However, two out of three identified associations in the major subgroup of infants without asthmatic parents remained significant after correction for multiple testing. We conclude that a replication cohort is required to confirm the relationship between *IL10* family genes and early wheeze following RSV LRTI.

In conclusion, genetic variation in adaptive immunity genes and particularly in *IL19* and *IL20* genes appears to be associated with the occurrence of early wheeze following RSV LRTI, suggesting a role for IL19 and IL20 cytokines in chronic airway disease. Functional studies are needed to evaluate the pathofysiological mechanism underlying the protective effect of the TGG haplotype on early wheeze following RSV LRTI.

ACKNOWLEDGMENTS

We gratefully acknowledge Jelle Goeman for providing his expertise on the global test for groups of genes, the Dutch Asthma Foundation for financial support (grant nr. 3.2.03.22) and the RSV Corticosteroid Study Group for including patients.

REFERENCE LIST

(1) Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit Care Med 2000; 161(5):1501-1507.

(2) Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 1999; 354(9178):541-545.

(3) Bont L, Aalderen WM, Kimpen JL. Long-term consequences of respiratory syncytial virus (RSV) bronchiolitis. Paediatr Respir Rev 2000; 1(3):221-227.

(4) Pullan CR, Hey EN. Wheezing, asthma, and pulmonary dysfunction 10 years after infection with respiratory syncytial virus in infancy. Br Med J (Clin Res Ed) 1982; 284(6330):1665-1669.

(5) Hall CB, Hall WJ, Gala CL, MaGill FB, Leddy JP. Long-term prospective study in children after respiratory syncytial virus infection. J Pediatr 1984; 105(3):358-364.

(6) McConnochie KM, Roghmann KJ. Bronchiolitis as a possible cause of wheezing in childhood: new evidence. Pediatrics 1984; 74(1):1-10.

(7) Welliver RC, Sun M, Rinaldo D, Ogra PL. Predictive value of respiratory syncytial virus-specific IgE responses for recurrent wheezing following bronchiolitis. J Pediatr 1986; 109(5):776-780.

(8) Sims DG, Gardner PS, Weightman D, Turner MW, Soothill JF. Atopy does not predispose to RSV bronchiolitis or postbronchiolitic wheezing. Br Med J (Clin Res Ed) 1981; 282(6282):2086-2088.

(9) Bont L, van Aalderen WM, Versteegh J, Brus F, Draaisma JT, Pekelharing-Berghuis M et al. Airflow limitation during respiratory syncytial virus lower respiratory tract infection predicts recurrent wheezing. Pediatr Infect Dis J 2001; 20(3):277-282.

(10) Ehlenfield DR, Cameron K, Welliver RC. Eosinophilia at the time of respiratory syncytial virus bronchiolitis predicts childhood reactive airway disease. Pediatrics 2000; 105(1 Pt 1):79-83.

(11) Bont L, Heijnen CJ, Kavelaars A, van Aalderen WMC, Brus F, Draaisma JTh et al. Monocyte IL-10 Production during Respiratory Syncytial Virus Bronchiolitis Is Associated with Recurrent Wheezing in a One-Year Follow-up Study. Am J Respir Crit Care Med 2000; 161(5):1518-1523.

(12) Goetghebuer T, Isles K, Moore C, Thomson A, Kwiatkowski D, Hull J. Genetic predisposition to wheeze following respiratory syncytial virus bronchiolitis. Clin Exp Allergy 2004; 34(5):801-803.

(13) Ermers MJ, Hoebee B, Hodemaekers HM, Kimman TG, Kimpen JL, Bont L. IL-13 genetic polymorphism identifies children with late wheezing after respiratory syncytial virus infection. J Allergy Clin Immunol 2007; 119(5):1086-1091.

(14) Janssen R, Bont L, Siezen CL, Hodemaekers HM, Ermers MJ, Doornbos G et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis is predominantly associated with innate immune genes. J Infect Dis 2007; 196(6):826-834.

(15) Bont L, Steijn M, van Aalderen WM, Brus F, Th Draaisma JM, Diemen-Steenvoorde RA et al. Seasonality of long term wheezing following respiratory syncytial virus lower respiratory tract infection. Thorax 2004; 59(6):512-516.

(16) Janssen R, Pennings J, Hodemaekers H, Buisman A, van OM, de RL et al. Host transcription profiles upon primary respiratory syncytial virus infection. J Virol 2007; 81(11):5958-5967.

(17) Goeman JJ, van de Geer SA, de KF, van Houwelingen HC. A global test for groups of genes: testing association with a clinical outcome. Bioinformatics 2004; 20(1):93-99.

(18) Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005; 21(2):263-265.

(19) Dudbridge F. Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. Hum Hered 2008; 66(2):87-98.

(20) Moore KW, de Waal MR, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001; 19:683-765.:683-765.

(21) Legg JP, Hussain IR, Warner JA, Johnston SL, Warner JO. Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. Am J Respir Crit Care Med 2003; 168(6):633-639.

(22) Helminen M, Nuolivirta K, Virta M, Halkosalo A, Korppi M, Vesikari T et al. IL-10 gene polymorphism at -1082 A/G is associated with severe rhinovirus bronchiolitis in infants. Pediatr Pulmonol 2008; 43(4):391-395.

(23) Hoebee B, Bont L, Rietveld E, van Oosten M, Hodemaekers HM, Nagelkerke NJ et al. Influence of promoter variants of interleukin-10, interleukin-9, and tumor necrosis factor-alpha genes on respiratory syncytial virus bronchiolitis. J Infect Dis 2004; 189(2):239-247.

(24) Wilson J, Rowlands K, Rockett K, Moore C, Lockhart E, Sharland M et al. Genetic variation at the IL10 gene locus is associated with severity of respiratory syncytial virus bronchiolitis. J Infect Dis 2005; 191(10):1705-1709.

(25) Gentile DA, Doyle WJ, Zeevi A, Howe-Adams J, Kapadia S, Trecki J et al. Cytokine gene polymorphisms moderate illness severity in infants with respiratory syncytial virus infection. Hum Immunol 2003; 64(3):338-344.

(26) Blumberg H, Conklin D, Xu WF, Grossmann A, Brender T, Carollo S et al. Interleukin 20: discovery, receptor identification, and role in epidermal function. Cell 2001; 104(1):9-19.

(27) Gallagher G, Dickensheets H, Eskdale J, Izotova LS, Mirochnitchenko OV, Peat JD et al. Cloning, expression and initial characterization of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). Genes Immun 2000; 1(7):442-450.

(28) Fickenscher H, Hor S, Kupers H, Knappe A, Wittmann S, Sticht H. The interleukin-10 family of cytokines. Trends Immunol 2002; 23(2):89-96.

(29) Dumoutier L, Leemans C, Lejeune D, Kotenko SV, Renauld JC. Cutting edge: STAT activation by IL-19, IL-20 and mda-7 through IL-20 receptor complexes of two types. J Immunol 2001; 167(7):3545-3549.

(30) Parrish-Novak J, Xu W, Brender T, Yao L, Jones C, West J et al. Interleukins 19, 20, and 24 signal through two distinct receptor complexes. Differences in receptor-ligand interactions mediate unique biological functions. J Biol Chem 2002; 277(49):47517-47523.

(31) Jordan WJ, Eskdale J, Boniotto M, Lennon GP, Peat J, Campbell JD et al. Human IL-19 regulates immunity through auto-induction of IL-19 and production of IL-10. Eur J Immunol 2005; 35(5):1576-1582.

(32) Liao SC, Cheng YC, Wang YC, Wang CW, Yang SM, Yu CK et al. IL-19 induced Th2 cytokines and was up-regulated in asthma patients. J Immunol 2004; 173(11):6712-6718.

(33) Huang F, Wachi S, Thai P, Loukoianov A, Tan KH, Forteza RM et al. Potentiation of IL-19 expression in airway epithelia by IL-17A and IL-4/IL-13: important implications in asthma. J Allergy Clin Immunol 2008; 121(6):1415-21, 1421.

(34) Sabat R, Wallace E, Endesfelder S, Wolk K. IL-19 and IL-20: two novel cytokines with importance in inflammatory diseases. Expert Opin Ther Targets 2007; 11(5):601-612.

(35) Zhong H, Wu Y, Belardinelli L, Zeng D. A2B adenosine receptors induce IL-19 from bronchial epithelial cells, resulting in TNF-alpha increase. Am J Respir Cell Mol Biol 2006; 35(5):587-592.

(36) Oleksyk TK, Thio CL, Truelove AL, Goedert JJ, Donfield SM, Kirk GD et al. Single nucleotide polymorphisms and haplotypes in the IL10 region associated with HCV clearance. Genes Immun 2005; 6(4):347-357.

(37) Koks S, Kingo K, Ratsep R, Karelson M, Silm H, Vasar E. Combined haplotype analysis of the interleukin-19 and -20 genes: relationship to plaque-type psoriasis. Genes Immun 2004; 5(8):662-667.

(38) Kingo K, Mossner R, Koks S, Ratsep R, Kruger U, Vasar E et al. Association analysis of IL19, IL20 and IL24 genes in palmoplantar pustulosis. Br J Dermatol 2007; 156(4):646-652.

(39) Fife MS, Gutierrez A, Ogilvie EM, Stock CJ, Samuel JM, Thomson W et al. Novel IL10 gene family associations with systemic juvenile idiopathic arthritis. Arthritis Res Ther 2006; 8(5):R148.

(40) Miller AL, Strieter RM, Gruber AD, Ho SB, Lukacs NW. CXCR2 regulates respiratory syncytial virus-induced airway hyperreactivity and mucus overproduction. J Immunol 2003; 170(6):3348-3356.

(41) Brightling C, Berry M, Amrani Y. Targeting TNF-alpha: a novel therapeutic approach for asthma. J Allergy Clin Immunol 2008; 121(1):5-10.

(42) Chung KF. Inflammatory mediators in chronic obstructive pulmonary disease. Curr Drug Targets Inflamm Allergy 2005; 4(6):619-625.

(43) Hasegawa K, Tamari M, Shao C, Shimizu M, Takahashi N, Mao XQ et al. Variations in the C3, C3a receptor, and C5 genes affect susceptibility to bronchial asthma. Hum Genet 2004; 115(4):295-301.

(44) Inoue H, Mashimo Y, Funamizu M, Shimojo N, Hasegawa K, Hirota T et al. Association study of the C3 gene with adult and childhood asthma. J Hum Genet 2008; 53(8):728-738.

(45) Howard TD, Koppelman GH, Xu J, Zheng SL, Postma DS, Meyers DA et al. Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma. Am J Hum Genet 2002; 70(1):230-236.

(46) Munthe-Kaas MC, Carlsen KH, Helms PJ, Gerritsen J, Whyte M, Feijen M et al. CTLA-4 polymorphisms in allergy and asthma and the TH1/ TH2 paradigm. J Allergy Clin Immunol 2004; 114(2):280-287.

(47) Benjamini Y, Hochberg Y. Controlling the False Discovery Rate - A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B-Methodological 1995; 57(1):289-300.

Chapter 8

Post-Bronchiolitis Wheeze Is Not Associated With Respiratory Syncytial Virus Reinfection

Marieke JJ Ermers

Annemieke Schuurhof

Floor van Woerkum

John W Rossen

Anton M van Loon

Jan LL Kimpen

Louis J Bont

ABSTRACT

Introduction

Animal studies suggest that recurrent wheeze following respiratory syncytial virus lower respiratory tract infection (RSV LRTI) relates to RSV reinfection. We studied whether recurrent wheeze during the first year following hospitalization was associated with RSV reinfection in infants previously hospitalized because of RSV LRTI.

Methods

Virologic characteristics of nasopharyngeal aspirates taken during respiratory illness episodes with and without wheeze were evaluated using real-time PCR.

Results

Detection rates of RSV were comparable in aspirates taken during episodes with (2/12) and without (8/37) wheeze.

Conclusion

Presence of wheeze during respiratory illness episodes was not associated with detection of RSV by real-time PCR. This suggests that RSV reinfection does not play a major role in recurrent wheeze.

INTRODUCTION

Respiratory syncytial virus lower respiratory tract infection (RSV LRTI) is the most common cause of infant hospitalization during winter and is clearly associated with the emergence of recurrent wheeze in early childhood.(1) Knowledge on viral and immunologic features of RSV LRTI is increasing but little is known about mechanisms underlying subsequent recurrent wheeze. In the mouse model airway hyperresponsiveness relates to RSV reinfection, presumably due to the host immune response elicited upon reinfection.(2;3)
The contribution of RSV reinfection to recurrent wheeze has not been shown in a prospective human cohort study and little is known about the role of cytokines and chemokines. To address this issue, we used our previously modified nasopharyngeal aspirate (NPA) technique(4) to compare local virologic and immunologic profiles in the mucosa. We hypothesized that recurrent wheeze is associated with RSV reinfection. Furthermore we studied the local immune response during respiratory illness episodes.

METHODS

Study Population

Nasopharyngeal aspirates (NPA) were collected from infants participating in the RSV Corticosteroid Study evaluating long term effects of inhaled corticosteroids (Current Controlled Trials ISRCTN 12352714). Selection criteria were designed to enroll previously healthy children who had been hospitalized with a first episode of RSV LRTI. RSV infection was confirmed by positive immunofluorescence in epithelial cells from nasopharyngeal aspirates. Infants with a history of wheezing or cardiac or pulmonary disease were excluded. Following hospital discharge parents were requested to contact the investigators in case of a new episode of respiratory symptoms. In addition parents were requested to record respiratory symptoms (including the presence of wheeze) on a daily base during the first 6 months following hospital discharge. Instructions were given by a single investigator and telephone calls were scheduled for motivational purposes. A new episode of respiratory illness was defined as the presence of any respiratory symptom (including a runny nose only) preceded by a minimum of two symptom-free days. In case of a new episode NPA sampling was performed within the next 48 hours. Techniques for collection, storage and processing of NPA were described previously.(4) NPA were collected between December and March, during RSV epidemical seasons 2004-2005 and 2005-2006. The study was approved by the Ethics Review Committee, University Medical Center Utrecht. Before enrollment, parental written informed consent was obtained.

Virological Analyses

Nucleic acids were extracted from NPA using the QIAamp DSP Virus kit according to the manufacturer's protocol (Qiagen). Detection of viruses was performed using parallel real-time PCR assays for RSV A and B, influenzavirus A and B, parainfluenzavirus 1–4, rhinoviruses, adenoviruses, coronaviruses OC43, 229E and NL63, and human metapneumovirus as described previously.(5-8)

Immunological Analyses

IFNγ concentrations in NPA were determined using commercial ELISA kits (Sanquin, Amsterdam, the Netherlands). Concentrations of the cytokines and chemokines IL1α, IL1β, IL2, IL4, IL5, IL6, IL8, IL10, IL12, IL13, IL15, IL17, IL18, Eotaxin, IP10, MCP1, MDC, MIF, MIG, MIP1α, OSM, RANTES, sICAM, sVCAM1, Tarc and TNFα were determined in NPA with a custom-made multiplex bioassay using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, USA) as described previously.(9) Cytokines and chemokines in which at least 85% of the samples reached values above detection limits were selected for further analyses.

Statistical Analyses

At the time this study was designed, the incidence of RSV reinfection following RSV LRTI was not known. Our sample size was based on an estimated incidence of RSV reinfection of 20% and an estimated incidence of wheeze during the first respiratory illness episode following RSV LRTI of 25%. The predefined target of 50 sampled patients provided at least 80% power to detect a 3 fold increase of RSV reinfection in infants with recurrent wheeze.

Presence of viruses, i.e. RSV, was compared between infants presenting with and without parent reported wheeze during the sampled illness episode using Chi-square test. In addition, presence of viruses was compared between infants with and without any parent reported wheeze during complete follow up. Cytokine and chemokine levels were compared using Student's T-test. Because the potential drawback of parent reported wheeze, analyses were repeated in a subgroup of infants where physician-diagnosed wheeze was determined. All analyses were performed using SPSS (SPSS for Windows, version 13.0, SPSS Inc.) software. All hypothesis testing was two-sided, with a 5 percent threshold for statistical significance.

RESULTS

Clinical Characteristics

Seventy-three RSV hospitalized infants were included and parents of 49 infants (67%) contacted the investigators because respiratory symptoms emerged. NPA were collected from all these 49 infants, sixty-one percent of them were boys. Sampled infants were hospitalized at a median age of 4.4 months (range 1.2- 12.6 months) and the median interval between hospital discharge and NPA sampling was 26 days (range 11 – 107 days). Thirty (61%) sampled infants wheezed during the initial RSV LRTI and 12 (24%) wheezed at the time of NPA sampling. Parental atopy score was higher in infants presenting with wheeze (median parental atopy score 3 (range 1-5) vs 1 (range 0-4), MWU *P*=0.02. Information gathered via questionnaires, one point was added for the presence of each atopic symptom eczema, hay fever, bronchitis, asthma and food allergy). Sex, age, parental smoking behavior, parental atopy score and presence of wheeze during initial RSV LRTI did not differ between sampled and non-sampled infants.

Virological Results

In 46/49 (94%) NPA at least one virus was detected and in 23/49 (47%) NPA multiple viruses were detected. Numbers of NPA with multiple detected viruses were similar in infants presenting with and without concomitant wheeze (6/12 (50%) vs 17/37 (46%), *P*=0,81). Detection rate of RSV reinfection was similar in infants presenting with and without concomitant wheeze (2/12 (17%) vs 8/37 (22%), *P*=0,71). In addition, no differences in detection rates of other viruses were found between infants presenting with and without concomitant wheeze (Figure 1). Analyses based on a physician-diagnosed definition of wheeze did not alter the results (RSV reinfection 1/4 (25%) vs 5/23 (22%), *P*=0.89). Analyses in which infants were distinguished with and without any wheeze during complete follow up did not show differences in viral detection rates (RSV reinfection 2/9 (22%) vs 7/36 (19%), *P*=0,85).

Immunological Results

Nasopharyngeal levels of IL2, IL4, IL5, IL12, IL13, IL15 and Tarc were not analyzed because less than 85% of the samples reached values above detection limits. All other cytokines were normally distributed after log transformation. Geometric means of analysed cytokines were similar in infants presenting with and without wheeze (Table 1). In particular, IFNγ levels were comparable in infants presenting with and without wheeze

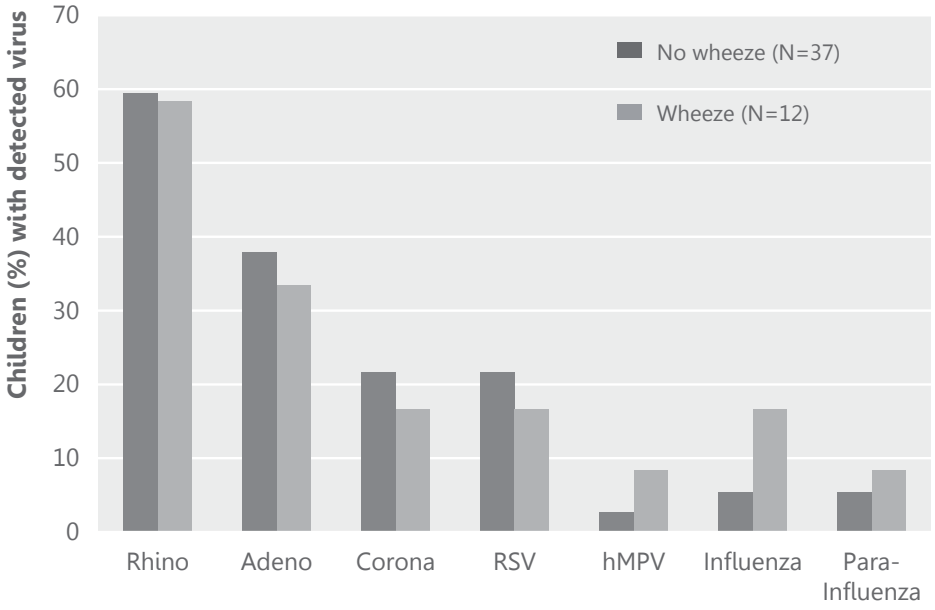

Figure 1. Detected viruses in nasopharyngeal aspirates (NPA) of infants with and without wheeze during the first new episode of respiratory symptoms following RSV-associated hospitalization.

(geometric mean 11.9 pg/ml (95% CI 2.5-56.2 pg/ml) vs 9.4 pg/ml (95% CI 4.6-19.5 pg/ml), *P*=0,76).

DISCUSSION

To our knowledge this is the first clinical study determining the role of RSV reinfection in the development of recurrent wheeze. In this prospective study following children with a history of RSV-associated hospitalization, recurrent wheeze during the first year following hospitalization was not associated with RSV reinfection.

The study is based on the hypothesis derived from animal models that RSV reinfection plays a major role in recurrent wheeze following RSV LRTI.(2;3;10) The major strength of the current study is that it is the first prospective report on clinical, viral and immunologic characteristics of respiratory tract reinfection in infants with a history of RSV-associated hospitalization. Due to a sensitive detection method, viruses were determined in almost all episodes. No difference in RSV detection rate was observed between NPA obtained during respiratory episodes with and without wheezing symptoms.

We furthermore studied the local immune response during respiratory illness episodes following RSV LRTI. Previous studies demonstrated a local Th2-biased immune response

Table 1. Nasopharyngeal cytokine and chemokine levels in children presenting with and without wheeze during the first episode of respiratory tract symptoms following RSV-associated hospitalization.

Cytokine / Chemokine	No Wheeze (N=37)[1]	Wheeze (N=12)[1]
IFNy	9 (5-19)	12 (3-56)
IL1a	254 (112-577)	299 (95-945)
IL1b	341 (165-705)	385 (122-1213)
IL6	1336 (768-2323)	2486 (557-11093)
IL8	2956 (1960-4456)	3979 (1722-9194)
IL10	78 (55-111)	71 (38-133)
IL17	716 (510-1006)	690 (358-1328)
IL18	3165 (2183-4588)	2601 (1191-5682)
Eotaxin	123 (88-171)	137 (91-208)
IP10	13581 (9560-19295)	22347 (11687-42730)
MCP1	649 (452-933)	951 (351-2571)
MDC	59 (22-156)	17 (1-245)
MIF	142608 (114001-178392)	171899 (88121-335324)
MIG	2470 (1558-3915)	6143 (3431-10999)
MIP1a	1140 (416-3121)	1752 (788-3896)
OSM	304 (66-1408)	785 (256-2413)
RANTES	948 (673-1334)	686 (278-1696)
sICAM	39561 (31666-49425)	44396 (24160-81581)
sVCAM1	21918 (13996-34325)	30211 (14477-63043)
TNFa	159 (34-750)	769 (186-3183)

[1] geometric mean and associated 95% confidence interval in pg/ml.

during RSV LRTI.(4;11-14) In a murine RSV model Lee *et al*. recently showed that IFNγ production during primary RSV LRTI is critical in the development of protection against airway hyperresponsiveness upon reinfection.(10) In our current study we did not attempt to measure the immune response during initial RSV LRTI, but in previous studies we were not able to link the IFNγ response during primary RSV LRTI with recurrent wheeze.(15) In the current study respiratory illness episodes with wheeze were not characterized by a local Th2-biased immune response. No differences in cytokine and chemokine profiles were observed between infants presenting with and without wheeze during respiratory illness episodes following RSV-associated hospitalization.

Some of our findings require further discussion. First, evaluations of viral and immunologic characteristics were performed in infants participating in a RCT concerning the effect of inhaled corticosteroids on recurrent wheeze. Some of these infants were treated with inhaled corticosteroids which might have influenced the immunologic profile, the presence of recurrent wheeze following RSV LRTI or even the susceptibility to viral respiratory tract infections. However, analyses in the subgroup of 29 infants who received

placebo did not alter the results (data not shown). Second, recurrent wheeze was defined as the presence of wheeze during the first episode of respiratory symptoms following RSV LTRI. However, not all infants who suffer from recurrent wheeze present with wheeze during each episode of respiratory symptoms. Using the daily logs we evaluated the presence of any wheeze during the first six months following RSV LRTI. Both virologic and immunologic characteristics did not differ in NPA from infants with and without any wheeze during follow up. Third, it is possible that the negative result, i.e. no association of recurrent wheeze with RSV reinfection, is due to a type II error. Based on strong associations of RSV reinfection and airway hyperresponsiveness in animal models, the current study was powered to detect a substantial (threefold) increase of RSV reinfection in infants with recurrent wheeze. A greater number of infants should be studied to detect more subtle differences of RSV reinfection rates in infants with and without recurrent wheeze.

Finally, we were not able to formally distinguish between RSV reinfection and RSV persistence. Recurrent wheeze might partially be explained by RSV persistence, causing chronic inflammation or changing cytokine profiles.(16) We believe that the detection of RSV in this study reflected RSV reinfection, and not viral persistence. The interval between the primary RSV infection and establishment of RSV reinfection was at least 15 days. In a pilot study we were able to show that RSV detection during follow-up was preceded by RSV-negative NPA (data not shown). However, selective persistence of RSV in the lower airways from participants of the current study cannot be excluded.

In summary, recurrent wheeze was not related to RSV reinfection or to a local Th2-biased immune response in infants previously hospitalized for RSV LRTI. Our study suggests that RSV reinfection has modest clinical relevance. The pathogenesis of recurrent wheeze remains intriguing, pre-existent abnormalities of the airways which predispose children to long-term airway morbidity may be the key mechanism. Moreover, airway damage caused by the inflammatory response during primary RSV LRTI may further facilitate the development of post-bronchiolitis wheeze.

ACKNOWLEDGEMENTS

We thank W. de Jager and L. Schuijff for expert technical assistance.

REFERENCE LIST

(1) Perez-Yarza EG, Moreno A, Lazaro P, Mejias A, Ramilo O. The association between respiratory syncytial virus infection and the development of childhood asthma: a systematic review of the literature. Pediatr Infect Dis J 2007; 26(8):733-739.

(2) Dakhama A, Park JW, Taube C, Joetham A, Balhorn A, Miyahara N et al. The enhancement or prevention of airway hyperresponsiveness during reinfection with respiratory syncytial virus is critically dependent on the age at first infection and IL-13 production. Journal of Immunology 2005; 175(3):1876-1883.

(3) Culley FJ, Pollott J, Openshaw PJ. Age at first viral infection determines the pattern of T cell-mediated disease during reinfection in adulthood. J Exp Med 2002; 196(10):1381-1386.

(4) Bont L, Heijnen CJ, Kavelaars A, van Aalderen WM, Brus F, Draaisma JM et al. Local interferon-gamma levels during respiratory syncytial virus lower respiratory tract infection are associated with disease severity. J Infect Dis 2001; 184(3):355-358.

(5) van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK et al. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol 2007; 45(7):2260-2262.

(6) van Elden LJ, van Loon AM, van AF, Hendriksen KA, Hoepelman AI, van Kraaij MG et al. Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcriptase polymerase chain reaction. J Infect Dis 2004; 189(4):652-657.

(7) van Elden LJ, van Loon AM, van der BA, Hendriksen KA, Hoepelman AI, van Kraaij MG et al. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J Clin Microbiol 2003; 41(9):4378-4381.

(8) van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol 2001; 39(1):196-200.

(9) de Jager W, te VH, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003; 10(1):133-139.

(10) Lee YM, Miyahara N, Takeda K, Prpich J, Oh A, Balhorn A et al. IFN-gamma production during initial infection determines the outcome of reinfection with respiratory syncytial virus. Am J Respir Crit Care Med 2008; 177(2):208-218.

(11) Aberle JH, Aberle SW, Rebhandl W, Pracher E, Kundi M, Popow-Kraupp T. Decreased interferon-gamma response in respiratory syncytial virus compared to other respiratory viral infections in infants. Clin Exp Immunol 2004; 137(1):146-150.

(12) Joshi P, Shaw A, Kakakios A, Isaacs D. Interferon-gamma levels in nasopharyngeal secretions of infants with respiratory syncytial virus and other respiratory viral infections. Clin Exp Immunol 2003; 131(1):143-147.

(13) Bendelja K, Gagro A, Bace A, Lokar-Kolbas R, Krsulovic-Hresic V, Drazenovic V et al. Predominant type-2 response in infants with respiratory syncytial virus (RSV) infection demonstrated by cytokine flow cytometry. Clin Exp Immunol 2000; 121(2):332-338.

(14) Roman M, Calhoun WJ, Hinton KL, Avendano LF, Simon V, Escobar AM et al. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am J Respir Crit Care Med 1997; 156(1):190-195.

(15) Ermers MJ, Hoebee B, Hodemaekers HM, Kimman TG, Kimpen JL, Bont L. IL-13 genetic polymorphism identifies children with late wheezing after respiratory syncytial virus infection. J Allergy Clin Immunol 2007; 119(5):1086-1091.

(16) Dakhama A, Vitalis TZ, Hegele RG. Persistence of respiratory syncytial virus (RSV) infection and development of RSV-specific IgG1 response in a guinea-pig model of acute bronchiolitis. Eur Respir J 1997; 10(1):20-26.

General Discussion

GENERAL DISCUSSION

We evaluated the effectiveness of inhaled hydrofluoroalkane extrafine beclomethasone dipropionate to prevent the development of recurrent wheeze following RSV LRTI and we aimed to clarify pathophysiological mechanisms underlying recurrent wheeze following RSV LRTI. Our most important findings are:

- Early and late wheeze following RSV LRTI are distinct entities.(1)
- Early-initiated, high-dosed inhaled hydrofluoroalkane extrafine beclomethasone dipropionate has a moderate and transient beneficial effect on early wheeze following RSV LRTI. This beneficial effect is observed in non-ventilated infants only.(2)
- Early wheeze following RSV LRTI relates to genetic variation in *IL19* and *IL20* genes. (3)
- Early wheeze following RSV LRTI is not related to RSV reinfection.(4)

RSV AND RECURRENT WHEEZE, EPIDEMIOLOGY.

Childhood asthma comprises several distinct disorders, characterised by the common symptom of wheeze.(5) The studies presented in this thesis focus on the occurrence and severity of recurrent wheeze following RSV LRTI hospitalization. Prospective studies have shown that RSV LRTI is associated with recurrent wheeze during childhood.(6) The role of RSV in the induction of allergic asthma has been focus of debate. Sigurs *et al.* demonstrated a relation between RSV LRTI and sensitisation to aeroallergens,(7-9) whereas others did not find an association between RSV and atopy.(1;10-14)
The prevalence of wheeze is most pronounced during the first year following hospitalization and diminishes strongly during subsequent years.(15) We compared determinants of early wheeze (during the first 15 months following RSV LRTI) with determinants of late wheeze (at age 6 following RSV LRTI), and identified distinct clinical, genetic and immunological determinants of early and late wheeze (Table 1).(1) These distinctive characteristics emphasize that early wheeze following RSV LRTI is distinct from allergic asthma and requires to be studied as a separate entity. We therefore investigated possible genetic determinants of RSV LRTI-induced recurrent wheeze. In addition we performed a randomized controlled trial to prevent early recurrent wheeze.

Table 1. Distinct determinants of early and late wheeze following RSV LRTI.

Characteristics	Early wheeze	Late wheeze	Reference
Presence of wheeze during acute RSV LRTI	+	-	(1;2)
Family history of asthma	-	+	(1;2)
Monocyte IL10 production during convalescence	+	-	(1;3)
Genetic variation in IL10 family genes IL19 IL20	+	-	(4)
Genetic variation in IL13 gene	-	+	(1)

(1) Ermers MJ, Hoebee B, Hodemaekers HM, Kimman TG, Kimpen JL, Bont L. IL-13 genetic polymorphism identifies children with late wheezing after respiratory syncytial virus infection. J Allergy Clin Immunol 2007; 119(5):1086-1091.
(2) Bont L, van Aalderen WM, Versteegh J, Brus F, Draaisma JT, Pekelharing-Berghuis M et al. Airflow limitation during respiratory syncytial virus lower respiratory tract infection predicts recurrent wheezing. Pediatr Infect Dis J 2001; 20(3):277-282.
(3) Bont L, Heijnen CJ, Kavelaars A, van Aalderen WMC, Brus F, Draaisma JTh et al. Monocyte IL-10 Production during Respiratory Syncytial Virus Bronchiolitis Is Associated with Recurrent Wheezing in a One-Year Follow-up Study. Am J Respir Crit Care Med 2000; 161(5):1518-1523.
(4) Ermers M, Janssen R, Rovers MM, Onland-Moret C, Kimpen JL, Bont L. IL19-IL20 and recurrent wheeze following RSV LRTI. Submitted.

RSV AND RECURRENT WHEEZE, TREATMENT.

No effective treatment for RSV LRTI or subsequent recurrent wheeze is currently available for the general population. Research focused on the development of agents aimed to prevent RSV LRTI. Vaccination and passive administration of neutralizing antibodies and antiviral and anti-inflammatory medication have been investigated.(16-20) It has been suggested that the inflammatory reaction evoked during acute RSV LRTI has a role in the development of subsequent airway morbidity by skewing future immune responses.(21) Detailed knowledge about underlying mechanisms is lacking, impeding specific immune modulation. General modulation of the immune response might be achieved by using anti-inflammatory agents including corticosteroids and leukotrienes.(19;22;23) The effectiveness of inhaled glucocorticosteroids to prevent or diminish recurrent wheeze has been investigated in birth cohorts and cohorts enriched for atopic diseases. Inhaled glucocorticosteroids reduced wheezing symptoms during treatment but did not prevent long-term airway complaints because the effect waned within months after treatment termination.(24;25) Episodic administration of high-dose inhaled glucocorticosteroids provided a partially effective strategy in children with episodic viral wheeze.(26) The effectiveness of inhaled glucocorticosteroids to prevent recurrent wheeze in the group of infants who wheeze following RSV LRTI has not been investigated extensively. This is of particular interest because the pathogenesis of RSV LRTI is thought to be distinct from that of other wheezing disorders.(27)
In the randomized controlled trial presented in this thesis, we evaluated the effect of early-initiated high-dose hydrofluoroalkane extrafine beclomethasone dipropionate on

recurrent wheeze in infants that were hospitalized because of RSV LRTI. Hydrofluoroalkane extrafine beclomethasone dipropionate was administered for three months and parents prospectively monitored the development of recurrent wheeze in a daily log during the year following the intervention. Hydrofluoroalkane extrafine beclomethasone dipropionate did not prevent the development of early wheeze in the total group of infants (N=243). The differential effects of hydrofluoroalkane extrafine beclomethasone dipropionate in the pre-defined subgroups of infants, i.e. infants with or without wheezing during the acute phase of RSV LRTI and infants with or without need for mechanical ventilation, were remarkable and counter-intuitive. Hydrofluoroalkane extrafine beclomethasone dipropionate relatively reduced early wheeze in the major subgroup of infants that did not require mechanical ventilation (N=221) by 32% and relatively reduced early wheeze in the subgroup of infants that did not wheeze during acute RSV LRTI (N=110) by 43%. No significant effect of hydrofluoroalkane extrafine beclomethasone dipropionate on early wheeze was observed in mechanically ventilated infants and in infants that presented with wheeze during acute RSV LRTI. Definite conclusions are not justified because the pre-defined subgroups were underpowered and observed effects were counter-intuitive. It is nevertheless tempting to speculate about why the beneficial effect of hydrofluoroalkane extrafine beclomethasone dipropionate on early wheeze was limited to the group of non-ventilated infants that did not wheeze during acute RSV LRTI.

First, the window of opportunity to modify the immune response following acute RSV LRTI might be passed when wheeze is present at initial presentation, implying that the presence of wheeze indicates a more advanced and irreversible stage of disease. The lack of an effect of hydrofluoroalkane extrafine beclomethasone dipropionate in mechanically ventilated infants with a delayed start of treatment and the beneficial effect of hydrofluoroalkane extrafine beclomethasone dipropionate in infants presenting without wheeze during acute RSV LRTI are in line with this hypothesis. However, comparable days of preceding illness were reported in infants presenting with and without wheeze during acute RSV LRTI and therefore lapse of time alone cannot sufficiently explain the differential effect of hydrofluoroalkane extrafine beclomethasone dipropionate on early wheeze.

Second, the presence and timing of onset of wheeze during acute RSV LRTI might form a relevant phenotypic feature reflecting underlying airway disease. In some infants RSV might reveal wheeze that developed due to independent pre-existent pathofysiological mechanisms, whereas in other infants RSV might be the causal or essential factor to develop wheeze. The remarkable preventive effect of hydrofluoroalkane extrafine beclomethasone dipropionate on early wheeze in infants that presented without wheeze during acute RSV LRTI suggests that these infants did not suffer from pre-existent disease and that development of early wheeze is predominantly virus-induced. In these infants RSV

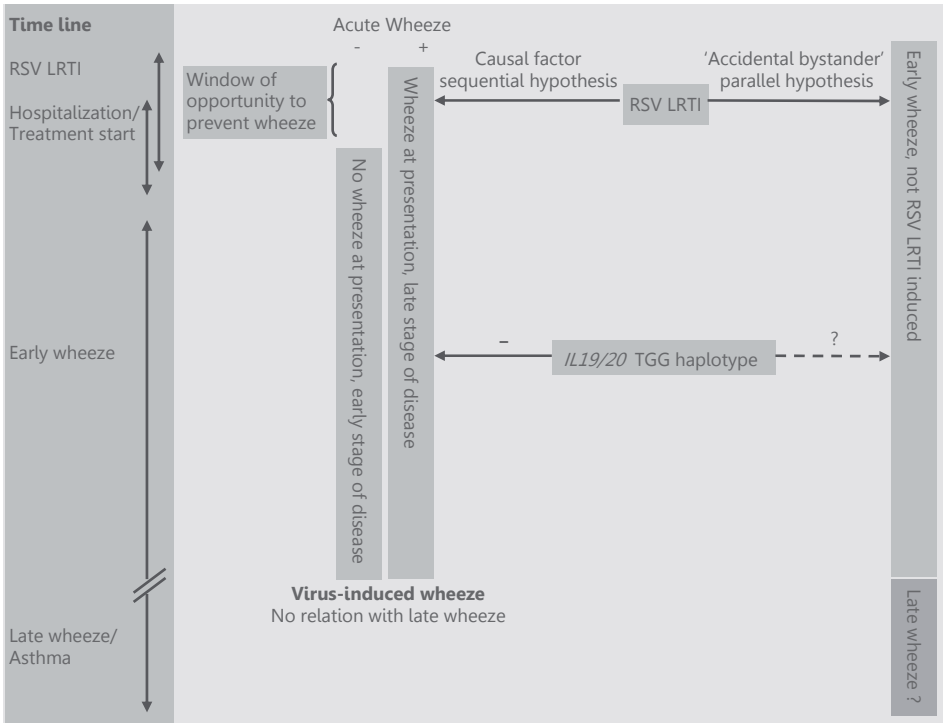

Figure 1. Development of wheeze following RSV LRTI.

Infant wheeze is a heterogeneous disease comprising several co-existing and overlapping phenotypes. The role of RSV LRTI in the development of early wheeze is not established. In a simplified representation of reality, we assume that RSV LRTI can have two distinct roles in the development of subsequent early wheeze. In some infants RSV might reveal wheeze that developed due to pre-existent, independent pathofysiological mechanisms. Here RSV is the "accidental bystander". In other infants RSV might be the causal or essential factor to develop wheeze. This virus-induced wheeze is most pronounced during the 1st year following hospitalisation and quenches over time. Our results suggest that virus-induced wheeze does not relate to late wheeze or asthma.

The presence or absence of virus-induced wheeze during hospital admission for RSV LRTI, i.e. 'acute wheeze', might indicate the stage of disease. The remarkable effect of hydrofluoroalkane extrafine beclomethasone dipropionate in infants that presented without 'acute wheeze' suggests that there is a window of opportunity to prevent the development of recurrent wheeze. This window of opportunity might be passed when wheeze is present at the initial presentation.

We demonstrated that the *IL19/IL20* TGG haplotype was inversely associated with early wheeze following RSV LRTI, in particular in the major subgroup of infants without asthmatic or atopic parents. We hypothesize that the protective effect of the *IL19/IL20* TGG haplotype on the occurrence of early wheeze is limited to virus-induced early wheeze. IL19 and/or IL20 cytokines that are produced during acute RSV LRTI might participate in the causal cascade leading to early wheeze by modifying or skewing subsequent immune responses.

LRTI is the essential factor to develop early wheeze and interventions directed at the virus-induced immune response might be successful (Figure 1).

Third, genetic variation might explain inter-individual response to medication. It was demonstrated in patients with asthma that salmeterol sensitivity is associated with a β2 adrenergic receptor polymorphism(28;29) and that glucocorticoid sensitiviy is associated with a glucocorticoid receptor haplotype.(30) In our trial, we did not observe associations between β2 adrenergic receptor polymorphisms or glucocorticoid receptor polymorphisms and treatment responses (data not shown). However, the absence of such associations does not exclude the existence of a pharmacogenetic effect due to the relative small group of studied infants and the subsequential insufficient power. In addition, development of early wheeze following RSV LRTI might be explained by underlying genetic variation unrelated to treatment response, as will be discussed in the next paragraph.
Summed up, timing, phenotype and genotype may be important in understanding the effect of early-initiated high-dose inhaled hydrofluoroalkane extrafine beclomethasone dipropionate on occurence and severity of early wheeze following RSV LRTI.

RSV-INDUCED RECURRENT WHEEZE, PATHOFYSIOLOGY.

We demonstrated that genetic variation in *IL10* family genes *IL19* and *IL20* is associated with early wheeze following RSV LRTI. The observed association was limited to the major subgroup of infants without asthmatic or atopic parents. Although the group of infants with an atopic family background was insufficiently powered to draw definite conclusions, our results suggest different mechanisms in infants with and without atopic background to develop early wheeze following RSV LRTI. In infants with an atopic family background RSV LRTI might just be an indicator of wheeze that would develop independent from RSV LRTI. In infants without pre-existing pathofysiology, IL10 family members might be involved in the pathogenesis of virus-induced reactive airway diseases. Local production of IL19 and IL20 cytokines during acute RSV LRTI might modify or skew subsequent immune responses leading to subsequent wheeze. Receptors of IL19 and IL20 are widely expressed on bronchial epithelium,(31) making the epithelial cell a potential key cell in the pathogenesis of virus-induced wheeze. The role of IL19 and/or IL20 cytokines in airway diseases and in particular in RSV LRTI-induced early wheeze needs to be confirmed in future studies.

CONCLUSION & FUTURE RESEARCH.

In the presented randomized controlled trial, hydrofluoroalkane extrafine beclomethasone dipropionate during RSV LRTI did not influence the subsequent development of

recurrent wheeze. In the major pre-defined subgroup of infants who did not require mechanical ventilation a beneficial effect of hydrofluoroalkane extrafine beclomethasone dipropionate was observed, quenching over time. We concluded that the use of hydrofluoroalkane extrafine beclomethasone dipropionate should not be advocated in all infants hospitalized for RSV LRTI, but may be considered in non-ventilated infants. The differential effects of hydrofluoroalkane extrafine beclomethasone dipropionate in the pre-specified subgroups emphasized the potential roles of timing, phenotype and genotype in treatment responses. In addition these differential effects contribute to our understanding of underlying pathofysiological mechanisms. RSV LRTI might be the first indicator of an irreversible wheezing phenotype in some children and RSV LRTI might be the causal factor to develop wheeze in other children. Evaluation of genetic modifiers and environmental factors will help to clarify the role of RSV LRTI and the modifying role of hydrofluoroalkane extrafine beclomethasone dipropionate in the development of early wheeze.

IL10 family cytokines might have a central role in virus-induced wheeze following RSV LRTI. Replication studies are needed to confirm the observed associations between genetic variation in *IL19* and *IL20* genes and early wheeze. In addition, functional studies are warranted to clarify specific mechanisms, in particular the role of bronchial epithelial cells. If future studies will confirm the role of IL19 and/or IL20 cytokines in chronic airway diseases, these cytokines may be targeted to prevent the occurrence of early wheeze following RSV LRTI.

Prioritized Future Studies

- Replication study to confirm the association between genetic variation in *IL19* and *IL20* genes and early wheeze following RSV LRTI.
- Functional studies to clarify specific mechanisms underlying the association between genetic variation in *IL19* and *IL20* genes and early wheeze following RSV LRTI, including the role of the bronchial epithelial cell.
- Pharmacogenetic study to evaluate the interaction between genetic background and treatment effect of inhaled glucocorticosteroids on infant wheeze.
- Epidemiological studies to evaluate the relevance of the presence and timing of onset of wheeze during acute RSV LRTI for the understanding of underlying airway disease.

REFERENCE LIST

(1) Ermers MJ, Hoebee B, Hodemaekers HM, Kimman TG, Kimpen JL, Bont L. IL-13 genetic polymorphism identifies children with late wheezing after respiratory syncytial virus infection. J Allergy Clin Immunol 2007; 119(5):1086-1091.

(2) Ermers M, Rovers MM, Woensel, Kimpen JL, Bont. The effect of inhaled glucocorticosteroids on early wheeze following RSV LRTI. BMJ, accepted 2009.

(3) Ermers M, Janssen R, Rovers MM, Onland-Moret C, Kimpen JL, Bont L. IL19-IL20 and recurrent wheeze following RSV LRTI. ERJ, in review 2009.

(4) Ermers M, Schuurhof A, van Woerkum F, Rossen JW, van Loon AM, Kimpen JL et al. Post-bronchiolitis wheeze is not associated with respiratory syncytial virus reinfection. Manuscript in preparation. 2009.

(5) Martinez FD. Development of wheezing disorders and asthma in preschool children. Pediatrics 2002; 109(2 Suppl):362-367.

(6) Bont L, Aalderen WM, Kimpen JL. Long-term consequences of respiratory syncytial virus (RSV) bronchiolitis. Paediatr Respir Rev 2000; 1(3):221-227.

(7) Sigurs N. A cohort of children hospitalised with acute RSV bronchiolitis: impact on later respiratory disease. Paediatr Respir Rev 2002; 3(3):177-183.

(8) Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit Care Med 2000; 161(5):1501-1507.

(9) Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F et al. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med 2005; 171(2):137-141.

(10) Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 1999; 354(9178):541-545.

(11) Sims DG, Gardner PS, Weightman D, Turner MW, Soothill JF. Atopy does not predispose to RSV bronchiolitis or postbronchiolitic wheezing. Br Med J (Clin Res Ed) 1981; 282(6282):2086-2088.

(12) Hall CB, Hall WJ, Gala CL, MaGill FB, Leddy JP. Long-term prospective study in children after respiratory syncytial virus infection. J Pediatr 1984; 105(3):358-364.

(13) Murray M, Webb MS, O'Callaghan C, Swarbrick AS, Milner AD. Respiratory status and allergy after bronchiolitis. Arch Dis Child 1992; 67(4):482-487.

(14) Sly PD, Hibbert ME. Childhood asthma following hospitalization with acute viral bronchiolitis in infancy. Pediatr Pulmonol 1989; 7(3):153-158.

(15) Bont L, Steijn M, van Aalderen WM, Brus F, Th Draaisma JM, Diemen-Steenvoorde RA et al. Seasonality of long term wheezing following respiratory syncytial virus lower respiratory tract infection. Thorax 2004; 59(6):512-516.

(16) Fuller H, Del Mar C. Immunoglobulin treatment for respiratory syncytial virus infection. Cochrane Database Syst Rev 2006;(4):CD004883.

(17) Ventre K, Randolph AG. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. Cochrane Database Syst Rev 2007;(1):CD000181.

(18) Patel H, Platt R, Lozano JM, Wang EE. Glucocorticoids for acute viral bronchiolitis in infants and young children. Cochrane Database Syst Rev 2004;(3):CD004878.

(19) Blom D, Ermers M, Bont L, van AW, van WJ. Inhaled corticosteroids during acute bronchiolitis in the prevention of post-bronchiolitic wheezing. Cochrane Database Syst Rev 2007;(1):CD004881.

(20) Chavez-Bueno S, Mejias A, Welliver RC. Respiratory syncytial virus bronchiolitis : current and future strategies for treatment and prophylaxis. Treat Respir Med 2006; 5(6):483-494.

(21) Silvestri M, Sabatini F, Defilippi AC, Rossi GA. The wheezy infant -- immunological and molecular considerations. Paediatr Respir Rev 2004; 5 Suppl A:S81-S87.

(22) Bisgaard H. A randomized trial of montelukast in respiratory syncytial virus postbronchiolitis. Am J Respir Crit Care Med 2003; 167(3):379-383.

(23) Bisgaard H, Flores-Nunez A, Goh A, Azimi P, Halkas A, Malice MP et al. Study Of Montelukast for theTreatment of Respiratory Symptoms of Post-RSV-Bronchiolitis in Children. Am J Respir Crit Care Med 2008; .

(24) Guilbert TW, Morgan WJ, Zeiger RS, Mauger DT, Boehmer SJ, Szefler SJ et al. Long-term inhaled corticosteroids in preschool children at high risk for asthma. N Engl J Med 2006; 354(19):1985-1997.

(25) Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F. Intermittent inhaled corticosteroids in infants with episodic wheezing. New England Journal of Medicine 2006; 354(19):1998-2005.

(26) McKean M, Ducharme F. Inhaled steroids for episodic viral wheeze of childhood. Cochrane Database Syst Rev 2000;(2):CD001107.

(27) Bont L, Kimpen JL, Ermers MJ. Inhaled corticosteroids and children. N Engl J Med 2006; 355(6):624-625.

(28) Wechsler ME, Lehman E, Lazarus SC, Lemanske RF, Jr., Boushey HA, Deykin A et al. beta-Adrenergic receptor polymorphisms and response to salmeterol. Am J Respir Crit Care Med 2006; 173(5):519-526.

(29) Israel E, Drazen JM, Liggett SB, Boushey HA, Cherniack RM, Chinchilli VM et al. Effect of polymorphism of the beta(2)-adrenergic receptor on response to regular use of albuterol in asthma. Int Arch Allergy Immunol 2001; 124(1-3):183-186.

(30) Stevens A, Ray DW, Zeggini E, John S, Richards HL, Griffiths CE et al. Glucocorticoid sensitivity is determined by a specific glucocorticoid receptor haplotype. J Clin Endocrinol Metab 2004; 89(2):892-897.

(31) Zhong H, Wu Y, Belardinelli L, Zeng D. A2B adenosine receptors induce IL-19 from bronchial epithelial cells, resulting in TNF-alpha increase. Am J Respir Cell Mol Biol 2006; 35(5):587-592.

Summary

SUMMARY

In this thesis we 1) evaluated the effectiveness of early-initiated high-dose inhaled glucocorticosteroids to prevent recurrent wheeze following RSV LRTI; and 2) studied potential pathophysiological mechanisms underlying the development of recurrent wheeze following RSV LRTI.

Chapter 1; Viral infections and childhood asthma.

In this chapter we provided a general overview regarding the relationship between viruses and childhood asthma. Viral respiratory tract infections (VRTI) have a strong epidemiological relationship with wheezing disorders. At least three different types of relationships between VRTI and wheezing disorders have been described. First, VRTI during early childhood can induce recurrent wheeze. RSV LRTI is followed by post-bronchiolitis wheeze in about 50% of cases. It is debated whether viral infections can change immunological status or induce allergy. Second, viral infections are the most important triggers of asthma exacerbations in atopic patients. In 85% of asthma exacerbations in children, VRTI are found in airways, and in the majority of cases rhinovirus appears to be the infectious agent. Third, exposure to respiratory viruses during infancy might prevent allergy development. The mechanism of virus-induced prevention of asthma is by skewing the immune system towards a Th1-phenotype. Different types of relationships between VRTI and wheeze can co-exist in one individual. For example, common colds during infancy may induce both increased risk of recurrent wheeze during childhood and decreased risk of allergic asthma later in life. The temporal relationship between viral infection and allergy development, as well as the interplay between genetic factors and viral infection, appear the keys to understand the intriguing relationship between viruses and asthma.

Chapter 2; *IL13* genetic polymorphism identifies children with late wheezing after respiratory syncytial virus infection.

The nature of wheeze following respiratory syncytial virus lower respiratory tract infection (RSV LRTI) is usually transient. However, some children will go on to develop persistent or late wheeze. In this chapter we aimed to determine clinical, immunological and genetic characteristics of early and late wheeze following RSV LRTI. We hypothesized that early and late post-bronchiolitis wheeze are determined by distinct clinical, immunological and genetic variables. A cohort of 101 children hospitalized for RSV LRTI was prospectively followed for 6 years. During RSV LRTI cytokine studies were performed and genetic polymorphisms were determined. Parents performed daily log registration

of respiratory symptoms during the first 3 years of follow-up and again at age six during the winter season. We observed distinctive associations for early and late post-bronchiolitis wheeze. We previously showed that airflow limitation during RSV LRTI as well as convalescent monocyte IL10 production are associated with early wheezing. These variables were not associated with late wheezing in the current analyses. On the other hand, atopic family history was not associated with early wheeze, but was associated with late wheeze. Most importantly, the *IL13* Gln allele was associated with late wheeze (OR 3.23, 95%CI 1.27-8.25), but was not associated with early wheeze. This study revealed distinct clinical, immunological and genetic determinants of early and late wheeze following RSV LRTI, indicating distinct pathophysiological mechanisms. We concluded that late wheeze at age six, but not early post-bronchiolitis wheeze, is an asthmatic phenomenon and genetically related to a functional *IL13* polymorphism.

Chapter 3; Inhaled corticosteroids during acute bronchiolitis in the prevention of post-bronchiolitic wheezing.

In this chapter we describe the results of a systematic review into the effectiveness of inhaled corticosteroids in the prevention of post-bronchiolitic wheeze in children with acute bronchiolitis. Data were collected from randomized placebo controlled trials investigating the effect of inhaled corticosteroids in the prevention of post-bronchiolitic wheeze in children younger than two years of age with a clinical diagnosis of acute bronchiolitis. The main purpose was to pool data to reach a conclusion about the effect of inhaled corticosteroids. Five studies were found involving 374 infants. Analysis of the pooled data showed that inhaled corticosteroids did not prevent post-bronchiolitic wheeze. No adverse events were observed in the three studies that reported on these adverse events. However, due to the small number of participants and clinical heterogeneity of the available data, no strong conclusions could be made.

Chapter 4; RSV Corticosteroid Study: design, materials & methods.

This chapter reports on the design, materials & methods of the RSV Corticosteroid Study; a multicenter double blind placebo controlled trial into the effectiveness of high-dose hydrofluoroalkane extrafine beclomethasone dipropionate as compared to placebo in infants aged less than thirteen months and hospitalized for RSV LRTI. The RSV Corticosteroid Study aimed to assess whether high-dose inhaled glucocorticosteroids during the first three months following admission for RSV LRTI prevent the occurrence and severity of long-term airway morbidity. The primary outcome variable was the number of days with wheeze during the observation year following the intervention. Secondary outcome variables were the number of coughing days, the number of days with airway medication

use, duration of hospitalization, health-related quality of life, lung function, potential adverse effects of treatment on growth, and thrush incidence.
An educational 'Videotape Performance Program' (VPP) was incorporated to monitor and maximize competence and adherence. Three weeks following hospital discharge parental performance was videotaped and scored according to modified criteria of the Dutch Asthma Foundation. Sixty-two percent of parents performed all essential steps correctly and another 33% performed 5 out of 6 steps correctly. The most frequent error in case of inadequate performance was the absence of visible spacer valve movements. Parental adherence to the treatment regimen was determined by weighing returned canisters. Sixty-six percent of parents administered the prescribed medication twice daily during at least nine full weeks of the prescribed three months.

Chapter 5; The effect of high-dose inhaled corticosteroids on wheeze following respiratory syncytial virus infection: a randomized double-blind placebo-controlled trial.

This chapter describes the randomized double-blind placebo controlled trial into the effectiveness of early-initiated high-dose inhaled glucocorticosteroids on the occurrence of recurrent wheeze. The infants were treated with either high-dose inhaled hydrofluoroalkane extrafine beclomethasone dipropionate (400 microgram per day for 3 months) or placebo. The primary outcome was the number of wheezing days in the year following the intervention. Of the 243 randomized infants, 119 were assigned to receive hydrofluoroalkane extrafine beclomethasone dipropionate. There was no significant difference in the number of wheezing days between the two groups (1761/33568 total days in the beclomethasone group vs 2301/36556 total days in the placebo group, P=0.31). In the pre-defined subgroup of infants who did not need mechanical ventilation (N=221), hydrofluoroalkane extrafine beclomethasone dipropionate relatively reduced the number of wheezing days by 32% (1315/30405 total days vs 2120/33149 total days in the placebo group, P=0.046). This reduction was most pronounced during the first 6 months. We concluded that high-dose inhaled hydrofluoroalkane extrafine beclomethasone dipropionate had a moderate and transient beneficial effect on recurrent wheeze following RSV LRTI. This beneficial effect was observed in non-ventilated infants only.

Chapter 6; Genetic Susceptibility To Respiratory Syncytial Virus Bronchiolitis Is Predominantly Associated With Innate Immune Genes.

In this chapter we report the results of a genetic association study regarding certain genes and hospitalization for RSV LRTI. The study involved 470 children hospitalized for RSV bronchiolitis, their parents, and 1008 random, population controls. We analyzed

347 single-nucleotide polymorphisms (SNPs) in 220 candidate genes involved in airway mucosal responses, innate immunity, chemotaxis, adaptive immunity, and allergic asthma. SNPs in the innate immune genes *VDR*, *JUN*, *IFNA5*, and *NOS2* demonstrated the strongest association with bronchiolitis. Apart from association at the allele level, these 4 SNPs also demonstrated association at the genotype level. The role of innate immunity as a process was reinforced by association of the whole group of innate immune SNPs when the global test for groups of genes was applied. We concluded that SNPs in innate immune genes are important in determining susceptibility to RSV bronchiolitis.

Chapter 7; *IL19* and *IL20* genes are associated with wheeze after respiratory syncytial virus infection.

In this chapter we report the results of a genetic association study regarding certain genes and recurrent wheeze following RSV LRTI. 347 SNPs were investigated in 166 native Dutch infants. Logistic regression analysis was used to study the association between genotypes and early wheeze following RSV LRTI. We observed that 10 SNPs were associated with early wheeze at the genotype level. Three out of four genotyped SNPs in the *IL10* family genes *IL19* and *IL20* were associated with early wheeze. Haplotype analysis of the combined *IL19* and *IL20* genotyped polymorphisms demonstrated an inverse association between the TGG haplotype and early wheeze following RSV LRTI (OR 0.43 (95%CI 0.24-0.75)). We concluded that genetic variation in adaptive immunity genes and particularly in the *IL10* cytokine family members *IL19* and *IL20* influenced the incidence of early wheeze following RSV LRTI. IL19 and IL20 may exert their effects via non-immune cells, emphasizing the potential role of bronchial epithelial cells in the pathogenesis of early wheeze following RSV LRTI. Functional studies are needed to evaluate the mechanism underlying the effect of the *IL19* and *IL20* polymorphisms on early wheeze following RSV LRTI.

Chapter 8; Recurrent wheeze following respiratory syncytial virus lower respiratory tract infection is not associated with respiratory syncytial virus infection.

In this chapter we evaluated whether recurrent wheeze during the first year following RSV LRTI hospitalization is associated with RSV reinfection. We evaluated virologic characteristics of nasopharyngeal aspirates taken during respiratory illness episodes with and without wheeze using real-time PCR. Detection rates of RSV were comparable in aspirates taken during episodes with (2/12) and without (8/37) wheeze. We concluded that the presence of wheeze during respiratory illness episodes was not associated with

detection of RSV by real-time PCR. This suggests that RSV reinfection does not play a major role in recurrent wheeze.

General discussion.

In this chapter we reflect on the results of our studies. Our most important findings are that early and late wheeze following RSV LRTI are distinct entities, that early-initiated inhaled hydrofluoroalkane extrafine beclomethasone dipropionate does not prevent early wheeze following RSV LRTI in all hospitalized infants, that early-initiated inhaled hydrofluoroalkane extrafine beclomethasone dipropionate has a moderate and transient beneficial effect on early wheeze following RSV LRTI in non-ventilated infants, that early wheeze following RSV LRTI relates to genetic variation in *IL19* and *IL20* genes and that early wheeze following RSV LRTI is not related to RSV reinfection.
Hydrofluoralkane extrafine beclomethasone dipropionate during RSV LRTI did not influence the subsequent development of recurrent wheeze. In the major pre-defined subgroup of infants who did not require mechanical ventilation a beneficial effect of hydrofluoroalkane extrafine beclomethasone dipropionate was observed, quenching over time. The differential effect of hydrofluoroalkane extrafine beclomethasone dipropionate in the pre-specified subgroups emphasizes the potential roles of timing, phenotype and genotype in treatment responses and contributes to our understanding of underlying pathophysiological mechanisms.
IL10 family cytokines appear to have a central role in virus-induced wheeze following RSV LRTI. Replication studies are needed to confirm the observed associations and functional studies are warranted to clarify specific underlying mechanisms.

Nederlandse Samenvatting

NEDERLANDSE SAMENVATTING

De onderzoeksprojecten in dit proefschrift richten zich op luchtwegklachten bij jonge kinderen die een ernstige lagere luchtweginfectie (LLWI) doormaakten, veroorzaakt door het respiratoir syncytieel virus (RSV).
RSV veroorzaakt in het winterseizoen frequent luchtweginfecties. De luchtweginfecties zijn veelal onschuldig en leiden vooral tot voorbijgaande klachten van de bovenste luchtwegen. Erg jonge kinderen en bejaarden hebben een verhoogd risico voor uitbreiding van RSV naar de lagere luchtwegen. Dit kan ernstige gevolgen hebben. In het winterseizoen is RSV LLWI de meest voorkomende reden tot ziekenhuisopname van jonge kinderen; in Nederland worden jaarlijks 1500 tot 2000 kinderen onder de leeftijd van 1 jaar opgenomen. 100 tot 150 kinderen worden jaarlijks beademd op een Pediatrische Intensive Care Afdeling; sterfte ten gevolge van een RSV LLWI is nihil.
Na herstel van de RSV LLWI heeft 40-70% van de kinderen in de eerste jaren na de ziekenhuisopname regelmatig last van luchtwegklachten. Vaak is er een piepende ademhaling zoals gezien wordt bij astmapatiënten. Het is onduidelijk welke mechanismen tot deze luchtwegklachten leiden. Mogelijk leidt de RSV LLWI tot een afwijkende ontwikkeling van het afweersysteem. Bij volgende infecties met een luchtwegvirus reageert het afweersysteem dan anders, ondermeer resulterend in terugkerend piepen. De (schadelijke) beïnvloeding van het afweersysteem zou wellicht verminderd of voorkomen kunnen worden door remming van de ontstekingsreactie die ontstaat tijdens de RSV LLWI. Inhalatie-corticosteroïden zijn ontstekingsremmende medicijnen welke ingeademd worden zodat ze voornamelijk de luchtwegen bereiken. Deze medicijnen vormen de standaardbehandeling voor astmatische patiënten. Het is onduidelijk of het gebruik van inhalatie-corticosteroïden het terugkerend piepen na RSV LLWI kan voorkomen.

De onderzoeksprojecten die beschreven zijn in dit proefschrift dienen 2 hoofddoelen:

- Evalueren van het effect van vroeg-geinitieerde hoog-gedoseerde inhalatie-corticosteroïden op het ontstaan van terugkerend piepen na RSV LLWI.
- Vergroten van de kennis over werkingsmechanismen die ten grondslag liggen aan de ontwikkeling van terugkerend piepen na RSV LLWI.

In de afzonderlijke hoofdstukken worden de verschillende onderzoeksprojecten beschreven.

Hoofdstuk 1.

Dit hoofdstuk biedt een algemene inleiding over de relatie tussen virale luchtweginfecties en astma op de kinderleeftijd. Astma op de vroege kinderleeftijd is een niet eenduidige diagnose. Er worden vaak verschillende ziektebeelden mee bedoeld. Hier bedoelen we met 'astma op de kinderleeftijd' alle ziektebeelden bij jonge kinderen (baby's, peuters en kleuters) die gepaard gaan met een piepende ademhaling. De term 'allergisch astma' wordt gebruikt voor atopische[1] patiënten. Bij kinderen onder de leeftijd van 3 jaar kan niet met zekerheid vastgesteld worden of sprake is van allergisch astma of niet-allergisch astma. Dit wordt pas met het verloop van de tijd duidelijk.
Virale luchtweginfecties zijn op verschillende manieren gerelateerd aan astma op de kinderleeftijd. Ten eerste is beschreven dat virale luchtweginfecties de oorzaak zijn van astma op de kinderleeftijd. Een RSV LLWI wordt bijvoorbeeld vaak gevolgd door terugkerend piepen. Ten tweede kan de virale luchtweginfectie een uitlokker zijn van een aanval van allergisch astma. Bij kinderen die allergisch astma hebben wordt in meer dan 85% van de astma-aanvallen een virus gevonden in de luchtwegen, meestal het rhinovirus. Ten derde kan de blootstelling aan luchtwegvirussen op jonge leeftijd juist beschermen tegen de ontwikkeling van allergisch astma. Deze veronderstelling wordt de 'hygiëne hypothese' genoemd. De bescherming tegen allergie door het ondergaan van (virus) infecties op jonge leeftijd ontstaat mogelijk door een verbuiging van het afweersysteem.
De beschreven relaties tussen virale luchtweginfecties en astma op de kinderleeftijd kunnen naast elkaar voorkomen bij hetzelfde kind. Zo kan een RSV LLWI op zeer jonge leeftijd het risico op terugkerend piepen als peuter vergroten, en tegelijkertijd het risico op allergisch astma als schoolgaand kind verkleinen. Sleutelfactoren in de intrigerende relatie tussen virussen en astma op de kinderleeftijd zijn het tijdstip waarop virale luchtweginfecties doorgemaakt worden en het samenspel tussen virale luchtweginfecties, erfelijke achtergrond en omgevingsfactoren.

Hoofdstuk 2.

De periodes met piepende ademhaling (terugkerend piepen) die vaak voorkomen na een RSV LLWI gaan meestal na enkele jaren vanzelf voorbij. Sommige kinderen blijven last houden van terugkerend piepen. In het project beschreven in dit hoofdstuk, vergeleken we kenmerken van kinderen die last hadden van terugkerend piepen in de eerste 3 jaren na de RSV LLWI ('vroeg piepen') met kenmerken van kinderen die last hadden van terugkerend piepen op de leeftijd van 6 jaar ('laat piepen'). 101 kinderen die op een leeftijd jonger dan 13 maanden in het ziekenhuis lagen in verband met RSV LLWI werden gevolgd tot ze 6 jaar oud waren. Tijdens de ziekenhuisopname en tijdens het polikliniek

1. Atopie is een erfelijke gevoeligheid voor allergische ziekten.

bezoek kort na de ziekenhuisopname werd bloed afgenomen voor erfelijkheidsonderzoek en immunologisch onderzoek. Bij erfelijkheidsonderzoek werd bestudeerd of veel voorkomende variaties in erfelijk materiaal wellicht vaker of minder vaak voorkomen bij kinderen die last hebben van terugkerend piepen. Bij immunologisch onderzoek werden eiwitten die belangrijk lijken bij de afweerreactie onderzocht. De ouders van de kinderen hielden gedurende de eerste 3 jaren na de ziekenhuisopname en op de leeftijd van 6 jaar dagelijks dagboekjes bij. Hierin rapporteerden zij de luchtwegklachten van hun kind, inclusief het optreden van een piepende ademhaling.
Dit onderzoek leerde ons dat kinderen met 'vroeg piepen' en 'laat piepen' verschillende kenmerken hebben. Eerder onderzoek toonde al aan dat een piepende ademhaling tijdens de RSV LLWI zelf geassocieerd was met 'vroeg piepen' na RSV LLWI. Ook een verhoging van het IL10 eiwit (een eiwit met een belangrijke rol in ontstekingsprocessen) was geassocieerd met 'vroeg piepen' na RSV LLWI. Deze factoren bleken niet geassocieerd met 'laat piepen' na RSV LLWI. Andersom was het voorkomen van atopische ziekten in de familie wel geassocieerd met 'laat piepen', maar niet met 'vroeg piepen'. Het erfelijkheidsonderzoek liet zien dat een variatie in het *IL13* gen (in andere onderzoeken in verband gebracht met allergisch astma) wel geassocieerd was met 'laat piepen' maar juist niet met 'vroeg piepen'.
Samengevat gingen 'vroeg piepen' en 'laat piepen' na RSV LLWI gepaard met verschillende klinische, immunologische en erfelijke kenmerken. Dit suggereert dat er verschillende onderliggende ziekmakende mechanismen bestaan. We concludeerden dat het terugkerend piepen op de leeftijd van 6 jaar een astmatisch fenomeen lijkt wat genetisch (=erfelijk) geassocieerd is met een functionele[2] variatie in het *IL13* gen. Terugkerend piepen in de eerste 3 jaren na RSV LLWI lijkt een ander ziektebeeld te representeren. Onderzoek naar het specifieke ziektebeeld van terugkerend piepen in de eerste jaren na RSV LLWI is nodig om werkingsmechanismen op te helderen en behandelmogelijkheden te creëren.

Hoofdstuk 3.

Dit hoofdstuk geeft een systematische samenvatting van de literatuur volgens de Cochrane richtlijnen over het gebruik van inhalatie-corticosteroïden tijdens LLWI ter preventie van het daaropvolgende terugkerend piepen.
We doorzochten de literatuur en selecteerden placebo-gecontroleerde gerandomiseerde trials[3] waaraan kinderen deelnamen die jonger dan 2 jaar waren en de diag-

2. Een functionele variatie is een variatie die een meetbaar effect tot gevolg heeft. Variatie in het *IL13* gen is geassocieerd met het IgE gehalte, een eiwit betrokken bij allergisch astma.

3. Placebo-gecontroleerde gerandomiseerde trials zijn studies waarbij een effect van behandeling vergeleken wordt tussen kinderen die behandeld worden met werkzaam medicijn en kinderen die

nose acute bronchiolitis kregen. 'Acute bronchiolitis' was gedefinieerd als een LLWI die gepaard gaat met ademhalingsmoeilijkheden en een piepende ademhaling. De acute bronchiolitis kon door verschillende virussen, inclusief het RS virus, veroorzaakt zijn. Vijf methodologisch goede placebo-gecontroleerde trials werden geselecteerd waaraan in totaal 374 kinderen deelnamen. We beoogden de resultaten van de verschillende trials samen te nemen (poolen van data) om zo een betrouwbare conclusie over het effect van inhalatie-corticosteroïden ter voorkoming of vermindering van terugkerend piepen te geven. Samengenomen werd geen gunstig effect aangetoond. Omdat de studies methodologisch belangrijk verschillend waren konden de resultaten echter maar beperkt samengenomen worden. Omdat ook het totaal aantal geanalyseerde kinderen niet groot was konden geen aanbevelingen geformuleerd worden. We concludeerden dat een grote placebo-gecontroleerde gerandomiseerde trial nodig is om het effect van inhalatie-corticosteroïden op terugkerend piepen na LLWI te kunnen evalueren.

Hoofdstuk 4.

Dit hoofdstuk beschrijft de opzet en de uitwerking van het onderzoek dat we uitvoerden om het effect van inhalatie-corticosteroïden op terugkerend piepen na RSV LLWI te evalueren. We voerden een dubbel-blinde[4] placebo-gecontroleerde gerandomiseerde trial uit bij voorheen gezonde kinderen die op een leeftijd jonger dan 13 maanden in een van de 19 deelnemende ziekenhuizen opgenomen werden met RSV LLWI. Kinderen met een voorgeschiedenis van hartklachten of longklachten (inclusief perioden met piepende ademhaling), deden niet mee aan het onderzoek. Extra fijn hydrofluoroalkaan beclomethason dipropionaat of placebo in een dosering van 200 microgram 2x daags werd gedurende 3 maanden volgend op de ziekenhuisopname toegediend middels een voorzetkamer. Om een mogelijk effect van inhalatie-corticosteroïden zo goed mogelijk zichtbaar te maken werd een hoge dosering medicatie toegediend en werd de medicatie zo vroeg mogelijk in het ziekte-proces toegediend, binnen 24 uur na ziekenhuisopname. We gaven de extra fijn hydrofluoroalkaan variant omdat het werkzame beclomethason dipropionaat bij deze vorm opgelost is in deeltjes met een erg kleine doorsnede. Deze kleine deeltjes bereiken makkelijker de kleine luchtwegen. De ouders dienden deze medicatie met een voorzetkamer toe na instructie door professionele astma-verpleegkundigen. Beoordeling van de medicatietoedieningen door middel van video-observaties leerde dat ouders de medicatie goed toedienden. Weging van teruggestuurde medicatiehulsels

behandeld worden met een placebo medicijn zonder werkzaamheid. Op basis van het toeval (randomisatie) wordt bepaald of kinderen het werkzame medicijn of het placebo medicijn krijgen.

4. Dubbel-blind betekent dat ouders, behandelende dokters en onderzoekers niet weten of het kind het werkzame medicijn of placebo krijgt. Dit wordt gedaan om beïnvloeding van de beoordeling van de uitkomstmaat, door een subjectieve beleving / mening over de werking van het medicijn, te voorkomen.

leerde dat ouders de medicatie trouw toedienden. Vanaf de ziekenhuisopname hielden ouders gedurende 15 maanden een dagboek bij waarin ondermeer dagelijks gerapporteerd werd of het kind een piepende ademhaling had. Ongeveer 80% van de maandelijkse dagboekjes werden teruggestuurd en geëvalueerd. De primaire uitkomstmaat van het onderzoek was het opgetelde aantal dagen met een piepende ademhaling in het observatiejaar volgend op de 3 maanden durende behandelperiode.

Hoofdstuk 5.

Dit hoofdstuk beschrijft de resultaten van de in hoofdstuk 4 toegelichte dubbel-blind placebo-gecontroleerde gerandomiseerde trial naar het effect van vroeg-geiniteerde en hoog-gedoseerde inhalatie-corticosteroïden op terugkerend piepen na RSV LLWI. Van de 243 deelnemende kinderen kregen er 119 beclomethason, de andere 124 kinderen kregen placebo. Het aantal deelnemende kinderen was voldoende groot om betrouwbare resultaten te genereren. We vonden geen statistisch significant verschil[5] in het aantal opgetelde dagen met een piepende ademhaling tussen de twee behandelgroepen. In de vooraf gedefinieerde subgroep van kinderen die geen mechanische beademing op de intensive care nodig hadden (211 kinderen) verminderde het aantal dagen met een piepende ademhaling met 2.1% (relatieve vermindering van 32%) in de groep die behandeld werd met extra fijn hydrofluoroalkaan beclomethason dipropionaat. Dit verschil was statistisch significant. De vermindering was het meest uitgesproken gedurende het eerste half jaar van het observatiejaar dat volgde op de behandelperiode.
We concludeerden dat vroeg-geinitieerd, hoog-gedoseerd extra fijn hydrofluoroalkaan beclomethason dipropionaat, toegediend gedurende de eerste 3 maanden na RSV LLWI ziekenhuisopname een bescheiden en voorbijgaand effect heeft op de ontwikkeling van terugkerend piepen in de groep kinderen die niet mechanisch beademd werd. Het algemene gebruik van extra fijn hydrofluoroalkaan beclomethason dipropionaat gedurende een RSV LLWI moedigen we niet aan.

Hoofdstuk 6.

RSV is een veel voorkomende verwekker van ernstige LLWI. Toch is maar een klein deel van de grote groep kinderen die een RSV infectie krijgt zo ziek dat ziekenhuisopname vereist is. De bekende risicofactoren voor een ernstige RSV LLWI verklaren het verschil in ziekte-ernst onvoldoende, het vermoeden bestaat dat erfelijke aanleg een rol speelt.

5. Een statistisch significant verschil is een verschil dat zo groot is dat het zeer onwaarschijnlijk is –in deze studie een kans van minder dan 5%- dat het verschil toevallig is. Als een verschil in een gerandomiseerde trial statistisch significant is wordt aangenomen dat het verschil veroorzaakt wordt door de interventie, in dit geval de behandeling met beclomethason dipropionaat. Statistische significantie zegt niets over de grootte van het verschil.

Dit hoofdstuk beschrijft de resultaten van een genetische associatie studie waaraan 470 kinderen die in het ziekenhuis opgenomen werden met RSV LLWI, hun ouders en 1008 controlepersonen uit de algemene populatie deelnamen. Een genetische associatie studie kijkt of variatie in het erfelijke materiaal -meer precies de variatie ter plaatse van 1 specifiek aminozuur, 'single nucleotide polymorfisme' of SNP genoemd- samengaat met klinische variatie. We bestudeerden of SNPs geassocieerd zijn met het krijgen van een RSV LLWI leidend tot ziekenhuisopname. We analyseerden 347 SNPs in 220 vooraf geselecteerde genen. De genen werden geselecteerd op basis van hun betrokkenheid bij de luchtweg slijmvlies reactie, de aangeboren afweer, de chemotaxis[6], de specifieke afweer en bij allergisch astma. We observeerden dat SNPs in genen betrokken bij de aangeboren afweer het sterkst geassocieerd zijn met RSV LLWI leidend tot ziekenhuisopname. Ook als we de genen op groepsniveau analyseerden door middel van de 'global test' voor groepen van genen was variatie in deze genen het sterkst geassocieerd met RSV LLWI leidend tot ziekenhuisopname. We concludeerden dat variatie in genen betrokken bij de aangeboren afweer een belangrijke rol speelt in de individuele kwetsbaarheid van een kind om RSV LLWI te krijgen.

Hoofdstuk 7.

Ziekmakende mechanismen die ten grondslag liggen aan terugkerend piepen na RSV LLWI zijn grotendeels onbekend. Eerder onderzoek toonde dat het gehalte van het IL10 eiwit[7] tijdens RSV LLWI geassocieerd is met terugkerend piepen na RSV LLWI. Er is weinig bekend over de rol van erfelijke aanleg bij terugkerend piepen na RSV LLWI. Dit hoofdstuk beschrijft een genetische associatie studie met als doel bepalende factoren in de erfelijke variatie (genetische determinanten) op te sporen die terugkerend piepen na RSV LLWI mede kunnen verklaren. De 347 SNPs zoals beschreven in hoofdstuk 6 werden bestudeerd in 166 kinderen van Nederlandse afkomst die opgenomen werden in het ziekenhuis met een RSV LLWI. De helft van deze kinderen had veel last van terugkerend piepen en de andere helft had hier weinig tot geen last van. Tien SNPs waren significant geassocieerd met terugkerend piepen na RSV LLWI. Opvallend was dat 3 van de 4 SNPs in de *IL19* en *IL20* genen significant geassocieerd waren met terugkerend piepen na RSV LLWI. De *IL19* en *IL20* genen lijken erg op het *IL10* gen qua vorm en functie en spelen een rol in de specifieke afweerreactie. Haplotype[8] analyse waarbij de informatie van verschil-

6. Chemotaxis is de beweging van cellen, bijv. cellen betrokken bij de ontstekingsreactie, in de richting van de ontsteking onder invloed van chemische prikkels.

7. Het IL10 eiwit is een boodschappereiwit betrokken bij de specifieke afweerreactie. Het IL10 eiwit is in meerdere studies geassocieerd met RSV LLWI.

8. Een haplotype is een combinatie van verschillende aminozuren, hier een combinatie van verschillende SNPs.

lende SNPs samengenomen werd liet zien dat het *IL19/IL20* TGG haplotype omgekeerd geassocieerd was met terugkerend piepen na RSV LLWI. We concludeerden dat variatie in genen betrokken bij de specifieke afweerreactie, in het bijzonder variatie in genen van de *IL10* familie, van invloed is op de individuele kwetsbaarheid van een kind om terugkerend piepen na RSV LLWI te ontwikkelen.

Hoofdstuk 8.

Dierstudies suggereerden dat terugkerend piepen na RSV LLWI veroorzaakt wordt door RSV her-infectie. Dit hoofdstuk beschrijft een studie waarin we bij kinderen die in het ziekenhuis opgenomen werden met RSV LLWI evalueerden of terugkerend piepen gepaard gaat met RSV her-infectie. Bij 49 kinderen werd tijdens de 1e verkoudheid na RSV LLWI een neusslijmmonster afgenomen. We rapporteerden of sprake was van een piepende ademhaling en identificeerden de aanwezige virussen met de PCR methode[9]. In nagenoeg alle neusslijmmonsters werden virussen aangetoond. We vergeleken de aanwezige virussen in neusslijmmonsters van kinderen met en zonder last van een piepende ademhaling. RSV werd niet vaker aangetoond in neusslijmmonsters van kinderen met een piepende ademhaling dan in neusslijmmonsters van kinderen zonder een piepende ademhaling. We concludeerden dat de aanwezigheid van RSV tijdens de 1e verkoudheid na de RSV LLWI niet geassocieerd is met de piepende ademhaling. Dit suggereert dat RSV her-infectie geen belangrijke rol speelt in het terugkerend piepen na RSV LLWI.

Algemene Discussie.

In dit hoofdstuk reflecteren we op de resultaten van de afzonderlijke studies. Onze belangrijkste bevindingen zijn:

- 'Vroeg' en 'laat' terugkerend piepen na RSV LLWI representeren verschillende ziektebeelden.
- Hoog-gedoseerde, vroeg-geinitieerde inhalatie-corticosteroiden tijdens RSV LLWI hebben een bescheiden en voorbijgaand effect op het terugkerend piepen na RSV LLWI. Dit effect wordt enkel waargenomen bij kinderen die niet mechanisch beademd worden tijdens de RSV LLWI.
- Terugkerend piepen na RSV LLWI is geassocieerd met variatie in de *IL19* en *IL20* genen.

9. PCR staat voor polymerase chain reaction, een methode om RNA -de erfelijke informatie die virussen bij zich dragen- te vermeerderen en op die manier de aanwezigheid van virussen aan te tonen.

Het verschillende effect van inhalatie-medicatie in de vooraf gedefinieerde subgroepen was opvallend. We observeerden dat extra fijn hydrofluoroalkaan beclomethason dipropionaat het terugkerend piepen na RSV LLWI vooral verminderde in de groep kinderen die niet mechanisch beademd werd en die tijdens de RSV LLWI geen piepende ademhaling had. Mogelijk is het tijdstip in het ziekte-proces waarop de medicatie voor het eerst gegeven wordt van belang. Mogelijk ook representeert de aan- of afwezigheid van een piepende ademhaling tijdens RSV LLWI verschillende fenotypes[10] en vormt de aan- of afwezigheid van een piepende ademhaling tijdens RSV LLWI een onderscheidend kenmerk.

Tot nog toe is onbekend hoe RSV terugkerend piepen veroorzaakt. In sommige kinderen lijken al redenen te bestaan om te gaan piepen en is RSV de 'toevallige voorbijganger' die aan het licht brengt dat het kind last krijgt van terugkerend piepen. In andere kinderen is RSV de veroorzaker van terugkerend piepen. Onze genetische studie toonde dat variatie in *IL10* gelieerde genen geassocieerd is met terugkerend piepen. Omdat de genetische variatie geassocieerd was met terugkerend piepen in kinderen zonder atopische familie verwachten we dat de IL10 gelieerde eiwitten vooral een rol hebben in terugkerend piepen dat veroorzaakt wordt door virussen. Het lijkt zinvol om toekomstig onderzoek naar terugkerend piepen volgend op RSV LLWI te richten op de interactie tussen het individueel genotype, de symptomatologie tijdens RSV LLWI en invloed van omgevingsfactoren.

10. De verschijningsvorm van een ziekte die veroorzaakt wordt door samenwerking van erfelijke informatie (het genotype) en de beïnvloedende omgeving.

Dankwoord

Curriculum Vitae

List of Publications

DANKWOORD

Domburg, 29 maart 2009

Vandaag heb ik zoveel mooie schelpen gezien. Bijna net zoveel mensen zou ik hier willen bedanken. Dit meest gelezen hoofdstuk moet echter wel leesbaar blijven ;-), daarom toch een selectie…

Alle deelnemende kinderen en hun ouders. Voor jullie ontstond deze studie, door jullie staat deze studie! Het blijft me verwonderen dat zo velen belangeloos gemotiveerd waren en bleven, ik heb bakken dagboekjes toegestuurd gekregen. Bladerend door 'het bestand' beleef ik al die puf-filmpjes, ziekenhuisgesprekken en huisbezoeken opnieuw. Duizend maal dank!

Professor Kimpen, beste Jan. Van zeer direct betrokken tot nauw betrokken op afstand. Je heldere visie en duidelijke mening waardeer ik erg. Dank voor de mooie kansen die je me, ook als opleider Kindergeneeskunde, gaf. Evenveel dank voor het respect dat ik kreeg toe ik sommige mogelijkheden achter me liet en andere keuzes maakte.

Doctor Bont, beste Louis. Van gestolen momenten op de cardio-kamer tot gestructureerde onderzoeksbesprekingen en dit proefschrift. Als 1e promovendus ben ik met de wording van jouw onderzoeksgroep meegegroeid. De toenemende drukte beïnvloedde nooit je razendsnelle reactie op toegestuurde stukken en je enthousiasme is zo mogelijk alleen maar toegenomen. Elke dip versloeg jij met stralend optimisme en vertrouwen in de kwaliteit van ons werk. Dank voor al het vertrouwen wat je in mij had.

Doctor Rovers, beste Maroeska. Van externe Nihes begeleider tot co-promotor. Jouw kennis van zaken, duidelijke opvattingen en zeer prettige manier van samenwerken maakte dat je ongemerkt steeds meer deel uitmaakte van 'ons project'. Dat je min of meer co-promotor was voordat je dit zelf wist toont hoe vanzelfsprekend dit liep. Dank voor deze toenemend grote rol, erg fijn dat je je bij ons hebt willen aansluiten.

Charlotte Onland-Moret, Barbara Hoebee, Riny Janssen, Hennie Hodemaekers, Job van Woensel, Daniel Blom, John Rossen, Ton van Loon, Mariska van Dijk, Wilco de Jager, Kors van der Ent, Bert Arets, Ingeborg Prins & Joyce Tersmette. Door jullie was het mogelijk deze klinische trial uit te breiden met genetische, immunologische, virologische en longfunctie studies en kreeg ik de kans mee te werken aan een Cochrane Review. De projecten zijn er beter van geworden, de kennis over wheeze na RSV LRTI is er door toegenomen en ik heb er ontzettend veel van geleerd. Dank voor de fijne samenwerking!

Kinderartsen, apothekers, longfunctie-assistenten en (astma)verpleegkundigen van de deelnemende ziekenhuizen. Met recht was dit een MULTI CENTER studie, de winters in mijn auto al rijdend van ziekenhuis naar ziekenhuis zal ik niet snel vergeten. Door jullie enthousiasme, meedenken en meedoen kreeg dit onderzoek letterlijk power, de inclusie-doelen hebben we gehaald! Ondanks het grote appèl dat ik op jullie deed heb ik me altijd erg welkom gevoeld op jullie afdelingen, dank daarvoor.

Floor, Jossi, Annemieke, Saskia, Eric, Ted, Machtelt, als student of arts-assistent verbonden aan het project. Door jullie werd de one-man show een gedeeld project, ik vind het geweldig dat jullie je zo compleet en all-round inzetten. Het (trachten) jullie de beginselen van wetenschappelijk onderzoek bij te brengen was leuk, gezellig & heeft mij veel geleerd, hopelijk jullie ook een beetje.. Mooi om te zien dat jullie je plek in doktersland aan het vinden zijn! Mieke & Ayleen, als werkstudent hebben jullie zo veel ouders gebeld, dagboekjes geteld en vragenlijsten gescoord. Hoe fijn dat jullie dat wilden doen!

RSV groep; Louis, Jan & Grada, Michiel, Beatrijs, Annemieke, Mirjam, Marije, Maarten, Annemiek, Michael, Eltje, Loes, Pauline en eerder Jojanneke, Caroline en Mariska. Artikel besprekingen, etentjes, eindeloze mail-wisselingen, presentaties, congressen, immunologie.. Goed om deel uit te maken van een ambitieuze groep. Dank voor het delen van kennis, enthousiasme en betrokkenheid.

WKZ collega's, arts-assistenten en kinderartsen, secretaresses. Ik heb hier mooie jaren gehad, zeker ook door de grote collegialiteit en daaraan verbonden gezelligheid. Het WKZ blijft toch een beetje een thuiswedstrijd, juist ook nu ik hier niet meer dagelijks werk. Hoe raar om me te bedenken dat de afronding van dit proefschrift ook een echt afscheid van het WKZ en van de kindergeneeskunde betekent. Ik hoop velen van jullie toch nog af en toe te blijven zien, enneh… gezellig als jullie 5 juni mee komen feesten!
Symfora collega's van de psychiatrie: dank voor een erg leuke en leerzame nieuwe werkplek.

Marieke, Annebeth, Barbara, Bas, Berber, Brita, Caroline, Coralie, Gerben, Jopje, Nienke, Sanne H, Sanne N, Sarah, Stefan. Lieve Flexers! Wat een sub-cultuur creëer je als je mensen maar lang genoeg op een paar vierkante meter samen laat zijn. Alle dagelijkse futiliteiten werden eindeloos besproken, de frustraties gedeeld, de overwinningen gevierd. Af en toe werd er ook nog gewerkt… Ons flex-tijdperk is voorbij, wat zou ik het leuk vinden als we er iets van kunnen blijven behouden. Dank voor het flex-leventje, de slagroom op de taart!

Marieke, Fleur, Maartje, Lotte, Gudule, Maaike, dames 6 van weleer en zoveel anderen.. Door jullie was er altijd een wereld naast 'het werk', hoe druk ik soms ook was. Het doet me zo ontzettend goed dat die wereld weer groter gaat worden! Dank voor zulke mooie vriendschappen.

Para-nimfen, Pieter & Marieke. Pieter, achter de schermen zoveel bijgedragen aan studie en boekje, geweldig dat je nu ook op de voorgrond bijdraagt aan een goede afronding, dank je wel. Mariek, grote vriendin & criticus in de goede zin des woords, ondanks die mega-emigratie erg dichtbij gebleven. Heel fijn!

Papa & Mama, Pieter & Anneke, Jehan, Coby & Feya, Wieteke & Rob. Dank voor al het onvoorwaardelijke en meer, dank voor thuis!

Zo nog even terug naar het strand, genieten van de schelpen. De klok is vannacht teruggedraaid, dus lekker lang licht. Een nieuwe zomertijd, tijd voor nieuwe hoofdstukken & nieuwe kansen...

Marieke

CURRICULUM VITAE

Marieke Ermers werd geboren op 12 februari 1975 te Nijmegen en groeide op in 't Oventje te Zeeland (N.Br.). Na in 1993 haar VWO diploma behaald te hebben aan het Kruisheren Kollege te Uden startte ze met de studie Psychologie aan de Universiteit van Utrecht. In 1995 werd ze ingeloot voor de studie Geneeskunde en startte zij deze studie aan dezelfde universiteit. Tijdens haar studie was zij in 1997-1998 voorzitter van de Medische Studenten Faculteitsvereniging Utrecht 'Sams'. In 2000 verrichtte zij aan de Hebrew University van Jeruzalem haar wetenschapsstage naar chelatie mogelijkheden van niet-transferrine gebonden ijzer bij heamochromatose en thallasemie patiënten onder begeleiding van Prof. Dr. Ioav Cabantchik en Dr. William Breuer. Na haar artsexamen in 2002 werkte zij kortdurend in het Maxima Medisch Centrum te Veldhoven als artsassistent kindergeneeskunde. In 2003 startte zij als assistent geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) met de opleiding Kindergeneeskunde in het Wilhelmina Kinderziekenhuis behorend bij het UMC Utrecht (Opleider Prof. Dr. Jan Kimpen). Het promotie-onderzoek resulterend in dit proefschrift verrichte zij onder supervisie van Prof. Dr. Jan Kimpen, Dr. Louis Bont en Dr. Maroeska Rovers. Ten tijde van het onderzoek heeft zij in 2008 aan het Netherlands Institute for Health Sciences (NIHES) haar Master of Science diploma behaald in de klinische epidemiologie.
Gedurende haar opleiding tot kinderarts was sprake van een toenemende interesse in (kinder)psychiatrische ziektebeelden waarna zij de opleiding tot kinderarts afbrak. In 2006 werkte ze als arts-asssistent kinder- en jeugdpsychiatrie in het UMC Utrecht en Altrecht te Utrecht; dit bevestigde en versterkte het enthousiasme voor deze specialisatie. Na de afronding van het promotie-onderzoek startte zij in 2009 met de opleiding Psychiatrie binnen de Symfora Groep te Amersfoort (Opleider Dr. Peter van Harten).

LIST OF PUBLICATIONS

Marieke JJ Ermers, Maroeska M Rovers, Job B van Woensel, Jan L Kimpen, Louis J Bont, RSV Corticosteroid Study Group. *The effect of high dose inhaled corticosteroids on wheeze in infants after respiratory syncytial virus infection: randomised double blind placebo controlled trial.* BMJ. 2009 Mar 31;338:b897.

Chris L Siezen, Louis J Bont, Hennie M Hodemaekers, Marieke JJ Ermers, Gerda Doornbos, Ruben van 't Slot, Cisca Wijmenga, Hans C van Houwelingen, Tjeerd G Kimman, Barbara Hoebee, Riny Janssen. *Genetic susceptibility to respiratory syncytial virus bronchiolitis in preterm children is associated with airway remodeling genes and innate immune genes.* Pediatr Infect Dis J. 2009 Apr;28(4):333-5.

Riny Janssen, Louis J Bont, Chris L Siezen, Hennie M Hodemaekers, Marieke JJ Ermers, Gerda Doornbos, Ruben van 't Slot, Cisca Wijmenga, Jelle J Goeman, Jan L Kimpen, Hans C van Houwelingen, Tjeerd G Kimman, Barbara Hoebee. *Genetic susceptibility to respiratory syncytial virus bronchiolitis is predominantly associated with innate immune genes.* J Infect Dis. 2007 Sep 15;196(6):826-34.

Marieke JJ Ermers, Barbara Hoebee, Hennie M Hodemaekers, Tjeerd G Kimman, Jan L Kimpen, Louis J Bont. *IL13 genetic polymorphism identifies children with late wheezing after respiratory syncytial virus infection.* J Allergy Clin Immunol. 2007 May;119(5):1086-91.

Daniel Blom, Marieke JJ Ermers, Louis J Bont, Wim M van Aalderen, Job B van Woensel. *Inhaled corticosteroids during acute bronchiolitis in the prevention of post-bronchiolitic wheezing.* Cochrane Database Syst Rev. 2007 Jan 24(1):CD004881. Review.

Louis J Bont, Jan L Kimpen, Marieke JJ Ermers. *Inhaled corticosteroids and children.* N Engl J Med. 2006 Aug 10:355(6):624-5; author reply.

Marieke JJ Ermers, Maarten O Hoekstra. *De rol van bacteriële producten in de primaire preventie van allergische ziekten.* Nederlands Tijdschrift voor Allergie. 2003;3:114-8.

William Breuer, Marieke JJ Ermers, Pensri Pootrakul, Ayala Abramov, Chaim Hershko, Z. Ioav Cabantchik. *Desferrioxamine-chelatable iron, a component of serum non-transferrin-bound iron, used for assessing chelation therapy.* Blood. 2001 Feb 1;97(3):792-8.