INFLAMMATORY MARKERS IN

AND

THE TREATMENT OF

ACUTE PRE-SCHOOL VIRAL WHEEZE

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By

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Specific input into thesis

- Katie Peck and Teresa McNally approached families, discussed the trial, obtained consent and conducted the audit.

- Ramesh Patel performed the flow cytometry analysis, and I collected samples and analysed the data.

- Rachel Hodge performed the soluble L-selectin assay.

- Paul Lambert did the statistical analysis of the clinical trial.

- I discussed the trial with parents, confirmed their consent, ensured that the children recruited were appropriate, obtained blood samples and supervised the daily activity of the clinical trial.

- I prepared the blood and urine samples and performed the radio-immunoassay of eosinophil proteins and the assay of leukotrienes.

- I set up the databases, collated data and analysed them.

to

my wife Ann

and

our children Sneha and Sasha

Inflammatory markers in and the treatment of acute preschool viral wheeze

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Asthma in preschool children is characterised by recurrent viral cold triggered wheezy episodes (PVW), and in the majority may be phenotypically distinct from atopic asthma. In order to address this, I performed four interrelated studies on children (1-5 years) presenting to hospital with acute PVW, and 'normal' and 'atopic' controls.
The study on eosinophil activation performed by analysing urinary (u) Eosinophil Protein X (EPX), showed that uEPX was increased in acute PVW compared to both controls, with no association between uEPX and IgE. uEPX fell on convalescence, but was not predictive of asthma symptoms two years later. The investigation on cysteinyl leukotrienes, using urinary (u) Leukotriene E_4 (LTE_4), showed for the first time, its relationship with wheeze and atopy. Elevated $uLTE_4$ in acute PVW fell rapidly in recovery. $uLTE_4$ correlated with IgE, and the fall in $uLTE_4$ at recuperation was seen only in the high IgE subgroup. The neutrophil activation study was novel, and examined the expression of adhesion molecule L-selectin. In acute PVW, this was reduced on systemic neutrophils and the soluble L-selectin in serum was increased compared to controls.
Children included in the clinical trial were allocated to a high- and low-primed stratum by measuring serum Eosinophil Cationic Protein and EPX, and randomised to receive parent-initiated prednisolone or placebo for the *next* PVW. 108 children were randomised to placebo and 109 to prednisolone. No difference in mean day- and night-time symptom score, salbutamol usage and need for hospitalisation was noted. Sub-analysis within either eosinophil strata also was similar.
These findings demonstrate that inflammatory markers in blood and urine in PVW can provide insights into its mechanisms and signpost new potential treatments. As there is no clear benefit of a short course of parent-initiated oral prednisolone, this current strategy may need re-evaluation.

Chapter 1. Introduction

1.1 Introduction to preschool wheeze (PVW)

Viral infections and wheezy attacks in children have been recognised for decades under different terms such as wheezy bronchitis, wheezy baby and infantile asthma[1].

The International Consensus Report describes asthma as 'a chronic inflammatory disorder of the airways in susceptible individuals, inflammatory symptoms are usually associated with widespread but variable airflow obstruction and an increase in airway response to a variety of stimuli. Obstruction is often reversible, either spontaneously or with treatment'[2]. As a definitive diagnosis of asthma can be difficult to obtain in children, especially preschool children, the diagnosis is made against a clinical background of cough, wheeze (ideally heard by a health professional), breathlessness and noisy breathing and careful exclusion of alternative diagnosis. A preschool wheezy child's symptoms can be analysed 'cross-sectionally' to ascertain their day to day symptoms and 'longitudinally' to analyse the course of symptoms over time. Therefore the clinical and epidemiological phenotype of PVW will vary depending on the process of analysis.

Wheezy episodes in preschool children are common worldwide. In the United Kingdom (UK), a postal questionnaire showed that 29% of all preschool children had wheezed[3], and an Australian study showed that nearly 50% of all children wheezed in the first 2 years[4]. Prospective, longitudinal birth cohort studies show that nearly 40% of all children wheeze within the first 6 years of life[5]. Studies from Australia showed that that nearly 25% of all children wheezed in the first year, 18% of children had current asthma at 6 years and 40% had atopy using stringent criteria[6]. In a large cross sectional study of seven year old children, nearly 17% of them had wheezed, the majority before the age of 2 years[7]. The worldwide prevalence of asthma in older children also varies considerably, with the highest rates noted in the UK[8].

There has been an increase in the prevalence of wheezing and diagnosed asthma over the last few decades and this has been shown in repeated studies within the same geographical area. The prevalence of wheeze in London had increased by 16% in 7 year old children[9], and reported wheeze in 12 year old children in Wales had also increased over the preceding 15 years[10]. In Aberdeen school children, there was a doubling in prevalence of reported wheeze between 1989 and 1994 which appears to have stabilised in the last survey in 1999[11]. Preschool children in Leicestershire were studied for 'current wheeze' and 'wheeze ever' and there was a doubling of the reported prevalence of wheeze over 8 years[3]. This change in prevalence has not been associated with a similar increase in

atopy. Hence the underlying mechanism for a true increase in PVW is unclear[12]. It is possible that the prevalence of PVW has now peaked, and may next be declining as has been demonstrated in Dutch children[13]. In this series of repeated surveys the prevalence of wheeze fell in boys alongwith an increase in treatment rates[13] and in childhood asthma in Italy[14]. Further there is a gradual decrease in the new episodes of asthma presenting to general practitioners in the UK[15].

1.1.2. Epidemiological phenotypes

Data obtained from large birth cohort studies show that about half of all preschool children wheeze at some time and a large proportion of them wheeze repeatedly. When these children are longitudinally followed up over time, it appears that their symptoms alter as they grow older and in some children symptoms resolve, thus demonstrating separate clinical characteristics.

Studies from Tuscon show that there are at least three separate phenotypes[5 16].

Children labeled 'transient early wheezers' typically have recurrent episodes of acute wheezy attacks triggered by viral colds, which start in early infancy and resolve by 6 years of age. They are neither associated with atopic sensitization nor have a family history of asthma[7] and they are more likely to have a history of maternal smoking during pregnancy[17 18]. These children have lower levels of pulmonary function after birth which persists at 11 years[19 20].

Some children continued to wheeze and were labeled 'non atopic persistent wheezers'. These children characteristically had Respiratory Syncytial Virus (RSV) bronchiolitis in the first year of life. They then developed recurrent wheezy attacks before 3 years of age, continued to wheeze at 6 years[5 21], but were no longer wheezing at 13 years. They had lower levels of lung function at 11 years, increased responsiveness to bronchodilators[21] irrespective of current symptoms, but no evidence of atopic sensitisation. These findings would suggest

that an alteration in airway tone might be the cause of wheeze with viral colds, in these children[21].

A small subgroup of children develop 'persistent atopic wheezing' and the majority of them had symptoms from an early age with a smaller group developing symptoms only after 3 years. They have recurrent wheezy episodes, significant symptoms in between episodes, and the majority is diagnosed 'asthmatic' early. They are more likely to have a maternal history of asthma. They have normal cord blood IgE, but high RSV-IgE, and total IgE by the age of 1 year. At 3 years these children do not show skin test reaction against aeroallergens but develop features of atopic sensitisation later in life, suggesting that there is an inherent tendency in these children to become atopic and this predisposes to persistent atopic asthma. Eosinophilia in the first year was a significant variable for chronic asthma and atopy later in life [22] and similarly, increased levels of Eosinophil Cationic Protein (ECP) were seen during viral infection triggered wheezy episodes[23 24]. The lung functions studied early in life, in the future atopic asthmatics are similar to the non wheezers, but become significantly lesser than their healthy counterparts by 6 years of age[5 25] and the 'early atopic wheezers' showed lowest lung functions and highest levels of IgE levels at both 6 and 11 years[16].

The above clinical phenotypes appear to fit the model described and may only be demonstrating a pattern of risk factors. As the assessments were conducted only

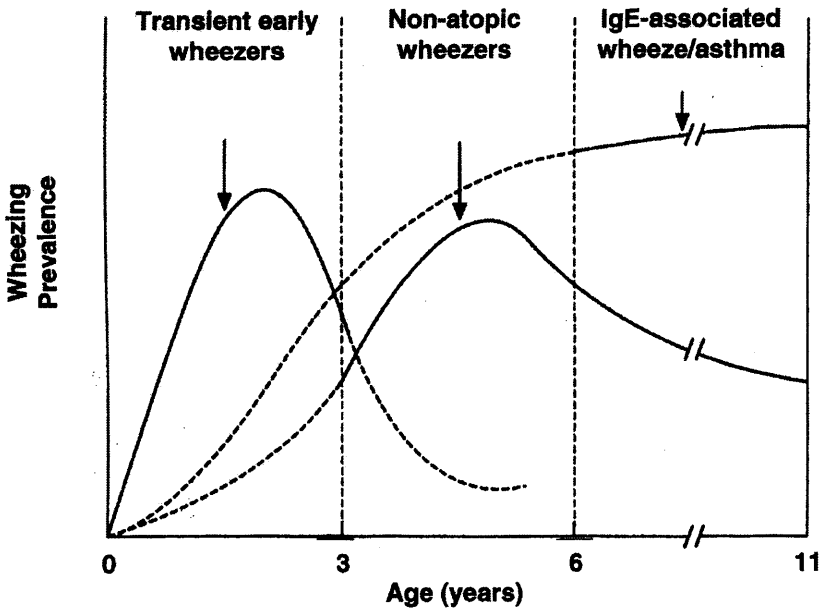
at certain points in the child's life, the pattern shown may be an estimate of the profile of the clinical characteristics. Although children were prospectively followed up, analysis was made retrospectively and the diagnosis of wheeze did not follow stringent criteria. They were enrolled only at birth and nearly a third of all children considered for the study were excluded from further analysis.

Rusconi conducted a retrospective analysis of a large population of Italian school children, used symptoms at 2 years to categorise children and identified similar phenotypes of children with wheezing[7]. The ALSPAC longitudinal study reviewed children at 3 years of age and showed that the majority of children with 'early wheezing' (within the first 6 months) had become asymptomatic, and children who developed wheeze after 6 months were more likely to have atopy. Oddy followed up a large group of children recruited in gestation with a questionnaire at 1 year and assessment at 6 years. In the first year, upto 3 upper respiratory tract infections (URTI) were shown to be protective, but more than 3 URTIs or lower respiratory tract infection (LRTI) with or without wheezing, increased the risk for current asthma at 6 years[6].

Young performed repeated lung functions in the first and second years of life in a group of infants and categorized them into early, persistent and non-wheezers. Lung functions at 1 month of age were lower in the wheezy children compared to non-wheezers. They also showed that persistent wheezers at two years had lower

lung functions than the early wheezers whose symptoms resolved at two years of age[4].

Thus the pattern of symptoms evolve over time and depending on the time when assessments are conducted the risk factors for wheezing are different and separate epidemiological phenotypes of PVW are seen. The following graph demonstrates one pattern of evolution of symptoms over time[19].



1.1.3. Clinical phenotypes

In practice, the majority of preschool children present to a clinician with an acute wheezy episode, the severity of which varies from a mild self limiting disease to severe symptoms requiring hospitalisation and sometimes intensive treatment. When the background history and symptoms in between acute episodes is explored, these children appear to fall broadly into three categories. The majority of children have repeated acute wheezy episodes with no interval symptoms. Some children have in addition, mild interval symptoms in the form of a nocturnal cough or exercise induced cough or wheeze that occasionally requires inhaled bronchodilators. A small group of children have acute wheezy episodes and severe chronic interval symptoms necessitating regular treatment[1]. A vexed issue in this group of severely affected children is the difficulty in distinguishing between an acute exacerbation and loss of asthma control. The association with viral colds or specific triggers and the course of events over a longer period may help but is not conclusive.

1.1.4. Outcome

Studies on children with asthma followed into their adult life showed that, many children categorised as having asthma symptoms at 7 years continued to have symptoms at 35 years. Further, children who had severe asthma initially, continued to have severe persistent asthma as adults thus supporting 'tracking' of

asthma symptoms with the chronic and severe asthmatics having the lowest lung functions and a combination of severe symptoms and atopy increasing the risk for severe asthma[26 27]. The lung function in these children however was preserved with no further significant loss after the age of 7 years. Children with significant wheezy symptoms at 5 years continued to have them at 10 years[28] and children with wheezy bronchitis before 7 years had an increased prevalence of chronic cough at 23 years of age[29].

Castro-Rodriguez *et al* have reported a clinical index, that calculates risk of developing asthma in young children with recurrent wheezing[30]. By using this index, preschool children with one "major" risk factor (parental history of asthma or eczema) or two of three "minor" risk factors (blood eosinophilia, wheezing without colds, and allergic rhinitis) are 9.8 times more likely to be diagnosed as active asthmatics at 6 to 13 yrs[30].

1.1.5. Virus and wheezing

Upper respiratory tract viral infections are the commonest illness in humans and viruses precipitate asthma attacks in both adults and children [31 32].

Viruses are thought to cause an alteration in the neural control of airways, increase the sensitivity of lower airways to nonspecific stimuli and cause airway inflammation. Asthma and viral colds have been epidemiologically associated, and a temporal relation between viral infections and exacerbations of asthma is seen in adults with asthma attacks[33] and in children[34]. They have also been implicated in the development of the asthma phenotype[35] and may even have a protective effect in preventing asthma[36]. Oddy showed that upto three URTIs in the first year may be protective against wheezing but more frequent URTIs increase the risk for asthma later[6]. Viral infections occurred more frequently in asthmatic children compared to their non-asthmatic siblings[37]. Preschool children when followed prospectively had frequent viral colds with a mean duration of three days and the majority of them were associated with wheezing which occurred within two days of onset of the viral cold[38]. A seasonal effect with a marked increase in acute asthma in preschool children in autumn[39] occurs and this is associated with a simultaneous threefold increase in admissions in July and August, for respiratory tract infections[40 41]. In school age children a correlation between the pattern of URTI and hospital admissions for asthma was found and these were commoner during periods of school attendance[34].

Rhinovirus, a common cause of cold, has a peak incidence in autumn and is a

common cause of asthma attacks[42 43] in young adults and school children, whereas RSV infections are commoner in infants[44]. Upper respiratory viruses such as rhinovirus, coronavirus, influenza, parainfluenza and RSV, were detected in nearly 80% of school children in the community with exacerbations of asthma[34]. Severe wheezy attacks in children, requiring hospital visits are also associated with viral colds[41 45].

1.1.6. Summary

Wheezy episodes in preschool children are a common clinical condition and the majority of these attacks are triggered by viral colds. The prevalence of these attacks in the community has increased over the last few years and is now a major cause of hospital admissions. It has now been shown that PVW is a heterogeneous condition with at least three groups of children with different clinical characteristics. Most preschool children with recurrent wheeze are not atopic and their risk factors and prognosis is different from children with atopy. The immune mechanisms and the inflammatory cells involved in the pathogenesis of wheeze are likely to be different in the various sub populations of preschool wheezers. While paediatric research lacks the detail seen in adult biopsy studies, markers of pulmonary inflammation in this age group provide indirect, but important information about the inflammatory substrate of pure viral-wheeze.

1.2. Role of eosinophil granule proteins in PVW

1.2.1. Introduction

The eosinophil was first associated with asthma in 1889[46] initially in the blood and later as eosinophil infiltration in the bronchial mucosa. It exerts its action through three classes of mediators namely, lipid derived mediators, cytotoxic granules and cytokines. The eosinophil is the critical cell involved in the pathological changes of mucosal oedema and bronchial hyperreactivity seen in asthma. Airway hyper reactivity has been related to both blood[47] and pulmonary eosinophilia[48] in adults with asthma. The critical step in the development of airway hyperreactivity is not the mere presence of eosinophil, but its activation and degranulation[48]. The intensity of bronchial reactivity[49] and severity of asthma[50] correlates with both the eosinophils and levels of eosinophil granule proteins in BAL fluid[49].

In natural viral colds, lower airway inflammation in bronchial biopsies occurs in both atopic and nonatopic adults, with activated eosinophils predominant in the atopic subgroup[51]. Induced sputum studies during natural viral colds, showed a significant neutrophil and eosinophil response in asthmatic and healthy individuals[52]. In experimental colds in adults, infiltration of bronchial mucosa with lymphocytes and eosinophils occurs, but this persisted in asthmatics after recovery[53] but no exacerbations occurred here[54].

The eosinophil produces these pathological abnormalities by the release of granular proteins, such as Major Basic Protein (MBP), Eosinophil Cationic Protein (ECP), Eosinophil protein X (EPX) or Eosinophil derived Neurotoxin (EDN) and Eosinophil Peroxidase (EPO). ECP is localized to the matrix of the eosinophil granule. It is a member of the RNase gene superfamily and has similarities to both EPX and human pancreatic RNase[55]. It appears to damage target cells through the formation of pores on transmembrane channels[56]. EPX or EDN is another granular protein, initially shown to produce neurotoxic reactions in experimental animals, which was described as the Gordon phenomenon[57]. EPX is localized to the matrix and is homologous to ECP and both are similar to pancreatic RNase. EPX has a hydrophobic part identical to human urinary RNase and hence is excreted in urine.

1.2.2. Eosinophil proteins in childhood asthma

Eosinophils play a significant role in childhood asthma. In a group of asthmatic children, eosinophils, ECP and EPX were increased in bronchial washings, Bronchoalveolar Lavage (BAL) and in the serum when compared to controls[58]. Bronchial hyperresponsiveness is also associated with increased eosinophils in the bronchial airway[59]. BAL studies on asthmatic children when relatively asymptomatic, showed that ECP levels were highest in atopic asthmatics[60] and similar BAL studies in asthmatic children showed that ECP levels correlated

with severity and was higher in persistent, compared to intermittent asthma[61]. Nasal fluid (NALF) ECP is elevated in asthmatic children, but there was no correlation with serum ECP[62]. BAL studies on pre-school children are limited. In a study on children investigated for stridor, BAL has been shown to be safe and effective process to explore the lower airways[63]. In a population of infants and toddlers with recurrent wheeze, studied when asymptomatic, ECP levels in BAL were increased compared to controls[64]. There was great variation in ECP levels and it was not associated with atopy. ECP levels in this study appeared to correlate with neutrophil counts. Induced sputum in preschool children showed increased levels of ECP in 'asthmatic', compared to children with first episode of wheeze or recurrent wheeze[65].

1.2.2.a. Serum ECP (sECP)

Median sECP levels in healthy controls varied from 6.5μg/L[66] to 11.2μg/L[67]. Some studies have shown no correlation between sECP and age[66 68], whereas studies conducted in the community showed an inverse correlation with age[69]. sECP and serum EPX (sEPX) is increased in asthmatic children and further elevated in symptomatic asthmatics[67]. In a cohort of asthmatic children, sECP was increased in acute asthma and its levels fell as symptoms improved[70]. There however was no correlation with lung function tests done during this period[71]. Atopy appears to further enhance sECP levels in asthmatic children[67 71-73].

The relationship between sECP levels and severity of asthma is tenuous. Symptomatic asthmatic children showed raised sECP levels[71] compared to asymptomatic children and these improved with treatment[73]. Ferguson however showed that sECP did not correlate with symptom scores, or severity as assessed by lung functions[74]. In a longitudinal study over weeks, Robinson found a weak correlation between sECP and lung function[58]. In asthmatic children, corticosteroids down-regulate eosinophil activation[75], and a time lag of several hours between oral steroid therapy and its systemic effects has been shown. Atopic asthmatic children treated with oral steroids[75 76]or inhaled steroids[77] show a fall in sECP levels that mirrors clinical improvement but they still remain higher than healthy controls suggesting ongoing eosinophil driven inflammation in these children[75] even when relatively asymptomatic.

Acutely wheezy preschool children, had raised sECP levels similar to older asthmatic children when compared to controls[71]. sECP levels in children under 5 years with symptomatic asthma were higher in the atopic compared to nonatopic subgroups[77]. High levels of sECP in infants with RSV bronchiolitis was predictive of persistent wheezing five years later[24]. A follow up study over four months, also showed an association between persistent wheeze and elevated serum and nasal ECP[23].

1.2.2.b. Serum EPX (sEPX) and urine EPX (uEPX)

Children with atopic asthma showed increased levels of sEPX[62 67]and uEPX compared to controls, with a further elevation in the subgroup of symptomatic asthmatics[62]. sEPX also correlated with atopy[67] and eczema[78 79]. In preschool children, sEPX levels in symptomatic asthma were higher in the atopic compared to nonatopic subgroups[77]. Although sEPX and uEPX are increased in children with asthma, uEPX appears to correlate better with severity of disease[70 80] and uEPX decreased significantly when symptoms improved with treatment[70]. uEPX is thus a particularly attractive investigative tool in young children.

uEPX studies in children showed a significant diurnal variation with the highest levels seen in the morning. This variation was noted both in asthmatic and healthy children and hence thought, most likely to be physiological[81], probably secondary to high nocturnal eosinophil counts[82]. There is a large degree of overlap in uEPX levels in epidemiological studies on asthmatic and healthy children and this limits its usefulness in the community[83]. uEPX when monitored longitudinally over a period of time has been shown to mirror clinical improvement in chronic asthmatic children. High levels of uEPX seen in acute atopic asthma, fell into the normal range three months later[81]. It has also been shown that uEPX levels in children with acute asthma were significantly raised compared to children with chronic asthma[84]. Further evidence of correlation of uEPX with lung functions and hence severity was shown by Hoekstra[80] and over

a period of time by Lugosi[85]. However in a six month trial, uEPX did not predict exacerbations in asthmatic children[86]. The relationship between uEPX and atopy is variable with higher levels of uEPX in atopic asthmatics[84] contrasted with no effect of atopy on uEPX[72 85] in asthmatic children.

uEPX levels in a group of preschool children with a clinical upper respiratory tract infection was not increased, compared to groups of healthy children with or without a family history of atopy[87]. Preschool children between 1 and 3 years, hospitalised with acute asthma had increased levels of uEPX compared to controls[88]. High levels of uEPX in a group of preschool children with a history of at least three previous episodes of wheeze was predictive of persistent asthma two years later [88] but this was not demonstrated in infants[89].

1.2.3. Background to the study

Viral-colds are an important trigger in acute asthma attacks in school-age children, and also attacks of wheeze in preschool children [34 45]. However, epidemiological studies suggest that some children with preschool viral-wheeze (PVW) do not have atopic asthma [90]. First, 60% of children with PVW will become asymptomatic by 6 yrs of age[16], whereas in only 40%, wheeze will continue [5]. Second, the transient pattern of wheeze is not associated with increased serum IgE at birth, or increased atopic sensitisation at 6 years of age [5]. It has been speculated that these different phenotypes of PVW are associated

with different patterns of pulmonary inflammation [91], but to date, this remains unclear.

In assessing whether eosinophil activation occurs in PVW, urinary markers have an advantage over blood samples, as they can be repeatedly obtained and normal controls are readily available. Increased levels of uEPX occurred in acutely wheezy preschool children with history of recurrent wheeze and when these children were followed up and reviewed two years later, children with higher levels of uEPX were more likely to be diagnosed with atopic asthma[88]. However there have been no studies in preschool children when uEPX were repeatedly analysed during wheeze and convalescence. Therefore, the relationship between acute PVW and eosinophil activation for the majority of children with PVW remains unclear.

The model to explore the inflammatory profile in a cohort of preschool children, would be to study them, when they develop a clinically defined attack of acute wheeze, triggered by a viral cold and compare their profile to well defined controls and to their own profile when their symptoms resolve.

1.3. Role of Leukotrienes (LT) in PVW

1.3.1. Introduction

The cysteinyl containing leukotrienes (cystLT) are mediators of bronchial asthma and are produced by inflammatory cells seen in the airway such as, eosinophils, basophils, mast cells and macrophages. The cystLT are fatty acid metabolites synthesized from arachidonic acid by the activity of lipoxygenase with a cofactor 5-lipoxygenase-activating protein (FLAP)[92 93]. This synthesis leads initially to the formation of an unstable leukotriene A_4 (LTA_4), which is converted to the chemotaxin leukotriene B_4, (LTB_4), or to leukotriene C_4, (LTC_4), the predominant mediator released in the lung. LTC_4 converts to leukotriene D_4 (LTD_4), and is finally converted to leukotrieneE$_4$ (LTE_4), in tissues and in circulation[92] which is then excreted in the urine [94]. *In vivo* studies have shown that LTE_4 is the end metabolite of cystLT that is administered to humans and is excreted in the urine as urinary LTE_4 ($uLTE_4$) in a constant proportion of about 5%[95]. All the three cysLTs, LTC_4, LTD_4 and LTE_4, produce intense bronchoconstriction in asthmatic airways[93].

CystLTs are also potent stimulators of mucus secretion and they increase microvascular permeability and plasma exudation. LTD_4 is a potent chemo-attractant for eosinophils [95]. Inhaled LTE_4 in atopic asthmatics caused increased eosinophils in the sputum and in bronchial biopsies [96] and a dose dependant

excretion of $uLTE_4$ occurred after cystLT inhalation suggesting that this may be a marker of its concentration in the asthmatic lung [97].

Leukotriene studies in pre-school children are limited. Leukotriene C_4 (LTC_4) in nasal secretions of infants with recurrent virus induced wheeze was higher than in infants with a common cold [98], whereas in preschool children with a viral URTI nasopharyngeal LTC_4 was significantly associated with viral disease[99], and was even higher in infants with bronchiolitis, but there were no healthy children included in this study[100]. BAL studies in preschool children with history of recurrent wheeze, when they were relatively asymptomatic, showed there was ongoing significant lower airway inflammation and increased LTE_4 and LTB_4 when compared to healthy children [101].

1.3.2. Urinary leukotrienes

Measurement of intact leukotrienes such as LTE_4 in urine ($uLTE_4$) is considered to be a good target for the analysis of *in vivo* production of cystLT. A urine sample has further advantages such as being non invasive and hence particularly attractive in young children. When cystLT are administered intravenously or by inhalation, this is metabolised to LTE_4 and excreted in urine[102]. $uLTE_4$ has thus been used to monitor endogenous leukotriene synthesis with its excretion measured over the course of a day, not showing any diurnal variation in healthy or asthmatic adults[103].

When adults with aspirin sensitive asthma were challenged with aspirin, urinary leukotriene B_4 ($uLTB_4$), but not $uLTE_4$ increased with development of symptoms. However as $uLTB_4$ is measured by High-Performance liquid Chromatography, it cannot be performed on unprocessed urine[104].

A variety of methods have been used to measure $uLTE_4$, initially by immunofiltration and later by immunoaffinity purification in both adults and healthy children and a good correlation was demonstrated in these measurements[105]. As these analyses are technically difficult, Kumlin attempted a simple enzyme immunoassay (EIA) on crude urine samples and showed good correlation of $uLTE_4$ values when they were compared to measurements

achieved after purification[106]. This methodology to measure $uLTE_4$ has since gained wide acceptance.

In atopic asthmatic adults $uLTE_4$ was elevated in both the early and late asthmatic reactions[107] but there was no correlation with the severity of the asthma attack[108]. Acute asthma attack in both atopic and non-atopic adults showed similarly raised levels of $uLTE_4$[109]. $uLTE_4$ levels were also found to be elevated in adults with history of aspirin sensitive asthma[110] and nocturnal asthma[111] and LT receptor antagonist (LTRA) blocked airway obstruction that occurred secondary to aspirin challenge[112].

In asthmatic children with exercise induced asthma, $uLTE_4$ which was similar to controls initially, increased significantly after exercise, but this did not correlate with spirometry[113]. Atopic children with chronic asthma showed significantly higher levels of $uLTE_4$ compared to controls and significant association between $uLTE_4$ and degree of airway obstruction[114]. In asthmatic children, high levels of $uLTE_4$ seen during acute wheeze, fell significantly in convalescence, but both these levels were higher than seen in controls that were not atopic. This suggests that despite normal lung functions in convalescence, ongoing high $uLTE_4$ excretion reflected chronic airway inflammation[115].

$uLTE_4$ levels in a group of preschool children with a clinical upper respiratory tract infection was not increased, compared to groups of healthy children with or without a family history of atopy[87]. Balfour-Lynn found no difference in $uLTE_4$ levels in wheezy infants admitted to hospital, during an acute wheezy illness and at follow up. However, the median age of these infants was six months and bronchiolitis was not excluded[116].

1.3.3. Background to the study

Viral colds trigger attacks of wheeze in both preschool children[45], and asthmatic school-age children[34]. Birth cohort studies suggest that most children with PVW have a phenotype separate from that of classical atopic asthma, and do not have an increased prevalence of markers for atopic sensitisation[5]. In contrast, increased serum IgE levels were found in the few children with PVW who continued to wheeze to school age. These data suggest that the inflammatory substrate of PVW is different from that of classical atopic asthma[91].

An understanding of the pattern and timing of the pulmonary inflammation in PVW is important since it may identify subgroups that are responsive to specific therapies. In atopic asthma, where pulmonary cystLT production is increased, LTRA is a therapeutic option [117]. By contrast, there is no consistent evidence for increased pulmonary cystLT production in acute PVW, with studies by van Schaik[98] and Balfour-Lynn[116] demonstrating conflicting results. One explanation for these conflicting results may be that increased cystLT production is limited to a subgroup of preschool children with specific risk factors. Markers of increased risk for atopic sensitisation have been shown to affect the clinical severity of wheeze. For example, a high IgE increases the risk of emergency care for rhinovirus-triggered wheeze in children 2 to 16 yrs of age[45].

Although $uLTB_4$ is seen in urine, studies did not show a consistent signal and it could not be measured in unprocessed urine. We hence decided not to analyse $uLTB_4$ in our study.

To date there is no data to confirm that increased levels of $uLTE_4$ occur in a cohort of preschool children, with PVW, and whether their levels in convalescence remain persistently high or resolve completely. Identification of a subgroup of children with increased cysLT production would enable targeted LTRA therapy.

1.4. Role of the neutrophil in PVW

1.4.1. Introduction

Viral infections are a predominant cause of acute wheezy exacerbations in asthma. The inflammatory mechanisms in this condition are poorly understood and the role of neutrophils (PMN) in airway inflammation remains relatively obscure compared to eosinophils[118]. In experimental colds with rhinovirus and influenza in healthy adults, systemic neutrophilia occurs and this is associated with mild lymphopaenia which develops typically on the second day[119]. The atopic status of healthy volunteers was initially identified, and when these subjects developed a natural cold they underwent BAL. Lower airway neutrophil inflammation was present in all subjects, but atopic subjects were in addition more likely to have activated eosinophils[51]. BAL studies in children with current symptoms of a natural cold showed increased neutrophils when compared to healthy controls[120].

1.4.2. Neutrophils and childhood asthma

In adults with allergic asthma, experimentally inoculated rhinovirus, produced increased circulating neutrophils and there was a parallel increase in granulocyte-colony stimulating factor (G-CSF) and nasal Interleukin-8 (IL8). BAL at four days, showed an increase in neutrophils. As this was not apparent at two days post inoculation, an important role for nasal mediators in the development of

airway neutrophilia has been postulated[121]. Induced sputum in adults with a natural cold showed increased neutrophilic inflammation and IL8, in asthmatics but not in healthy subjects[52]. Adults with virus induced acute asthma, had increased neutrophils and neutrophil elastase compared to non infective acute asthma where the proportion of eosinophils were higher[122]. Induced sputum in adults with acute asthma showed predominant airway neutrophilia which was further increased in adults with severe disease[123] and in subjects thought to have a viral trigger for their asthma[124].

Viruses are the commonest trigger for acute wheezy episodes in school age children[34], with RSV and rhinovirus the predominant pathogens in younger and older children respectively[45]. In asthmatic children presenting to emergency department with an acute exacerbation, induced sputum showed airway inflammation with a heterogeneous inflammatory profile of both neutrophils and eosinophils and increased IL8, which decreased with resolution of wheeze[125]. IL8 in nasal fluids were increased in children with proven virus induced asthma attacks and this appeared to correlate with severity of upper respiratory symptoms[126].

Recent attention on the role of neutrophils in preschool wheeze, has shown that they may contribute to its pathophysiology. BAL studies in a highly selected group of infantile wheezers showed significant airway neutrophilia[127] when

compared to asthmatic children and children with chronic cough. There was a higher cell count and neutrophilia the BAL in preschool children with recurrent wheeze when compared with non wheezy pulmonary diseases[128]. The eosinophil counts in these children were similar to controls and significantly lower than asthmatics[127]. Stevenson described different pathological patterns in atopic asthma and viral infection associated wheeze (VAW). Total cell numbers were increased in children with VAW and a predominance of eosinophils was seen in children with asthma[129].

1.4.3. Neutrophil adhesion molecules.

Neutrophil trafficking between the systemic circulation and the lung requires a highly co-ordinated regulation of adhesion molecules, including members of the selectin and β2 integrin families[130]. L-selectin (CD62L) is a member of the selectin family and is expressed on leukocytes. It mediates the initial step of 'rolling' of the leukocyte along the postcapillary venular endothelium of the systemic circulation[131]. The second step is firm 'adhesion' of the neutrophil on the vessel wall, mediated by the integrins receptors. Mac-1 (CD11b), a member of the integrin family is upregulated on neutrophils and this precedes the next step of transmigration of neutrophils from the systemic circulation into tissues seen in cystic fibrosis[132]. In the lung, L-selectin aids in the rolling of leukocytes within the pulmonary venules. The leukocytes then marginate in the vessels, and both L-selectin and integrins play a role in the sequestration and finally

emigration of neutrophils into the lung parenchyma[131]. When neutrophils are activated there is L selectin shedding, and hence a decrease in L-selectin expression with a simultaneous increase in soluble L selectin (sL-selectin)[133], and an upregulation of Mac-1 expression[134].

Neutrophils in nasopharyngeal aspirates (NPA) in infants with bronchiolitis, showed reduced expression of L-selectin and increased expression of Mac-1, when compared with its expression on peripheral blood neutrophils[130]. In asthmatic adults sputum eosinphils showed lower levels of L-selectin and raised Mac-1 expression compared to blood eosinophils[135].

1.4.4. Soluble L-selectin

Once neutrophils are activated, they rapidly shed L-selectin molecules from its surface into the circulation as sL-selectin[136]. Although the functional significance of sL-selectin is unclear, it may serve as a buffer to prevent leukocyte rolling at sites of sub-acute inflammation[137]. In acute asthma in atopic children, sL-selectin was elevated during stable periods in the asthmatics, but was not elevated during acute attacks[138]. sE-selectin has been found to be raised in acute asthma in adults[139]. Similarly sE-selectin in asthmatic children was significantly higher than in controls[140]. To date, sL-selectin has not been studied in acute preschool viral-wheeze.

1.4.5. Background to the study

The neutrophil may be important in the pathogenesis of attacks of atopic asthma triggered by colds. Neutrophils predominate in the sputum of asthmatic adults with viral colds[124]. Furthermore, increased levels of IL8, a potent neutrophil chemoattractant, are present in the lower airway of children with viral triggered deterioration of atopic asthma[125]. Viral colds are also an important trigger for attacks of wheeze in preschool children (PVW), since an upper respiratory tract virus can be detected in more than 80% of preschool children presenting to hospital with acute wheeze[45]. Despite the common trigger factor, epidemiological studies suggest that PVW is a separate phenotype from atopic asthma.

Marguet and colleagues performed BAL in a highly selected population of preschool children with a history of three successive episodes of viral wheeze and failure of standard therapy. Whilst no increase in airway eosinophils was found, this group had an increased percentage of neutrophils in their BAL fluid[127].

Sampling of airway cells during acute PVW is technically and ethically difficult, but evidence for the initial "activation" step in the development of pulmonary neutrophilia can be sought less invasively by the measurement of adhesion molecules, L-selectin and Mac-1, combined with analysis of sL-selectin.

1.5. Treatment of PVW

1.5.1. Bronchodilators

Short acting bronchodilators given by inhalation are the first line of treatment of acutely wheezy children and are thought to work by relaxing airway smooth muscles[141]. The route, dose and device to administer bronchodilators have gradually evolved from prescribing it as required oral medications to regular intense inhaled treatment. Infants under two years with acute wheeze were treated with either nebulised or inhaled terbutaline via a spacer and assessed. There was a similar significant improvement in clinical scores after treatment with both inhaled and nebulised medications, with no adverse effects[142]. Toddlers younger than 2 years, with history of recurrent wheezy episodes were treated with either two doses of nebulised albuterol or saline when they presented to hospital with severe wheeze. An initial tachycardia was noted in the albuterol group, but 30 minutes after treatment, there was an improvement in respiratory symptom scores and oxygen saturations[143]. Children older than 3 years hospitalized with acute asthma tolerated inhaled salbutamol through a spacer well (pMDI), and showed a similar improvement in clinical scores to nebulised salbutamol[144]. In a similar study on older asthmatic children with mild acute asthma, lower doses of inhaled salbutamol (pMDI) with a spacer were equally effective to nebulised treatement[145]. A comparison between inhaled salbutamol (pMDI) with a spacer and nebulised salbutamol, in toddlers under two years with

moderate to severe acute wheeze, showed that in nearly 90% of all children treatment was successful in the pMDI group[146].

Lung function studies, which are difficult to conduct in infants, demonstrated an improvement with inhaled albuterol in wheezy infants[147], whereas nebulised salbutamol did not reduce airway resistance in acutely wheezy young infants. In older toddlers however an improvement in lung function was seen [148]. In children with acute severe asthma, higher doses of nebulised salbutamol produced a more significant improvement in lung functions compared to lower doses[149 150]. In infants with a history of recurrent wheeze, but who were currently asymptomatic, inhaled salbutamol did not produce an improvement in lung function[151]. A Cochrane review comparing these studies reflected the heterogeneity of this population and difficulty in comparing data. It concluded that although the evidence was conflicting, there was no clear benefit of using salbutamol in the first 2 years of life[152]. Inhaled salbutamol however is well tolerated in preschool children with varying severity of wheeze. A Cochrane review confirmed this efficacy and showed that they were as effective as nebulisers and hence more advantageous in young children[153] and the current BTS recommendations reflect this finding[2].

1.5.2. Systemic steroids

The management of wheezy preschool children is difficult and the effectiveness of various interventions is controversial. The aims of treatment are to prevent acute deteriorations, manage them effectively by minimizing the disruption of normal activities and prevent hospital admissions. Some children have significant symptoms in the intervening period between wheezy attacks. Systemic steroids are currently widely used in the management of acute wheezy attacks in both preschool and older asthmatic children and can be administered by different routes[154]. The role of corticosteroids in acute asthma was controversial in the seventies with some authors suggesting the use of steroids as a 'last resort'. During this period, steroids were thought, not to have an additive effect above the improvement seen with bronchodilators in adults[155] and school children[156] with acute asthma.

Over the next decade, there appeared to be a beneficial effect of steroids in adults with severe asthma[157]. Children with status asthmaticus treated with steroids showed an improvement in hypoxaemia [158] and in children with persistent asthma symptoms, short term methylprednisolone improved lung functions and rapidly resolved the lung function reversibility noted before treatment[159]. Consensus statements produced during this period, noted a reduction in hospital stay, and with the possibility of preventing hospitalisation advised an earlier use of steroids[160 161].

In a group of asthmatic children, that included preschool wheezers, a one year cross over trial was conducted with prednisolone or placebo initiated by parents at the onset of a wheezy attack. There was no difference in the number of attacks or proportion of attacks in which treatment was given. There was however an increase in outpatient visits for acute asthma and more attacks resulted in emergency department visits in children administered oral prednisolone. There was no parental preference for prednisolone [162]. Scarfone studied children between 1 and 17 years, presenting to the emergency department with acute asthma. They were treated with frequent doses of nebulised salbutamol and a single dose of oral prednisolone or placebo. The hospitalisation rate at 4 hours was similar in both groups. The cause of exacerbation of asthma was not explored nor was any sub-analysis of preschool children done[163]. Gleeson's study on asthmatic children treated with nebulised bronchodilators and oral prednisolone did not demonstrate an improvement in peak flow rates or reduction in duration of hospital stay or relapses[164]. Hospitalised asthmatic children were treated with two regimens of nebulised salbutamol and additional prednisolone or a placebo. The effect of steroids on symptom improvement was minimal, but more children were fit to be discharged at the end of treatment in the steroid group[165]. A similar population was treated with a single dose of salbutamol and prednisolone or placebo. Significantly higher proportion of children on prednisolone, were discharged at first review, but a similar finding was not evident in infants under two years of age[166]. Asthmatic children over the age of

five years were initially seen in hospital and treated at home with a short course of oral prednisolone and bronchodilators. At the end of treatment symptom scores improved in both groups of children, but the prednisolone group had a more favorable outcome[167]. Lower doses of prednisolone, compared to standard doses have also been shown to be equally effective in asthmatic children (1 to 15 years) with acute asthma[168]. Studies comparing different routes of administration of steroids have shown a similar effect on duration of hospital stay, between oral and intravenous prednisolone in asthmatic children[169], and nebulised dexamethasone was as effective as oral steroids in preventing hospitalisation [170].

The evidence that steroids are effective in acute preschool wheeze is not well documented, primarily because preshool wheezers are a heterogeneous population and second, the clinical characteristics of the population of children studied varied considerably. The majority of earlier clinical trials studied both older asthmatic children and toddlers together, with varying proportions of toddlers and sub-analysis of them. Webb studied infants under 18 months of age who presented with 'wheezy bronchitis' probably similar to PVW, who had a history of at least two previous wheezy episodes. Treatment with oral steroids made no difference to their symptom scores of cough, wheeze or breathlessness or parental preference, either in the whole group or in the subgroup 12-18 months of age[171]. Brunette followed two groups of preschool children with history of recurrent wheeze for 2 years. In an open label study, oral bronchodilators alone

was given in the first year, and oral steroids were given in addition, at the start of a viral cold to one group of children in the second year of the study. A reduction in acute wheezy episodes and frequency of hospital admissions during this year was noted[172]. Preschool children between the ages of 7 months and five years presenting to hospital with acute asthma were treated with intramuscular in addition to inhaled salbutamol. The proportion of children admitted at the end of treatment was significantly lesser in the steroid group, among younger toddlers with a similar trend in older preschool children. An improvement in symptom scores was also noted in the steroid group[173]. Csonka studied the efficacy of a short course of oral prednisolone in preschool children 6 to 35 months, presenting to hospital with acute wheezy attack and a viral cold was analysed. In addition to inhaled bronchodilators they received either prednisolone or placebo. There was a 'less need' for additional asthma medication in the prednisolone treated group. There was no difference in hospitalisation and no side effects were recorded[174].

A counter-productive effect of oral steroids has also been demonstrated. In a double blind crossover study that included preschool and older asthmatic children, there was no reduction in the number of attacks and an increase in out-patient visits in children treated with oral steroids [162]. Kayani demonstrated behavioral changes such as anxiety and increased aggression in a group of

asthmatic children treated with high doses of oral steroids for acute attacks when compared to children on lower doses[175].

The above studies indicate that the evidence for routine use of oral steroids in acute preschool wheeze is conflicting and its side effects may not be insignificant.

1.5.3. Inhaled steroids

Preschool children, with a history of recurrent wheezy episodes were treated with inhaled budesonide by their parents at the start of a viral cold in addition to their inhaled bronchodilators. In the study by Svedmyr *et al*, symptom scores of cough and noisy breathing was reduced, but there was no reduction in the use of oral steroids or hospitalization[176]. In a similar study by Connett *et al*, there was reduction in mean day time and night time scores in the budesonide group[176 177]. High dose inhaled steroids initiated by parents for acute wheeze in preschool children reduced symptom scores but not hospital admissions or the need for further courses of oral steroids[178], whereas inhaled steroids in asthmatic children, that included preschoolers did not show a difference in symptom scores[179].

The role of prophylactic inhaled steroids for prolonged periods has been explored. Nebulised budesonide in a group of infants (6 to 30 months) with severe asthma produced a reduction in daily symptom scores and a trend in the

reduction of exacerbations which did not reach statistical significance[180]. A similar study on severe asthmatics (1 to 3 years), showed a significant improvement in cough scores[181]. In a group of recurrent wheezy children (11 to 36 months), inhaled budesonide over three months improved wheezing but not cough symptom scores. The number of exacerbations as assessed by oral steroid requirement was also reduced[182]. In preschool children with episodic wheeze, treated daily with inhaled budesonide for four months, there was no reduction in symptom scores, or mean scores per episode[183] nor was an effect on symptom scores in a group of asthmatic children[184]. Prophylactic nasal steroids did neither reduce the number of colds, nor prevent acute wheezy attacks[185]. Inhaled steroids were less effective than oral steroids in improving lung functions and preventing hospital admissions in older children[186]. Hence although inhaled steroids may have an effect on symptom scores they are thought to be less effective than oral steroids in acute asthma[187] and a recent Cochrane review concluded that regular inhaled steroids does not prevent acute wheezy episodes in children with episodic wheeze[188].

Recent studies looking into the role of inhaled steroids in modifying the progression of preschool wheeze have convincingly shown a lack of effect. Bisgard *et al* treated episodes of wheezing in high risk infants with two weeks of inhaled budesonide during the first three years of life. There was no effect in the progression of the illness to persistent wheezing and the mean duration of

wheezy episodes[189]. In the PEAK trial, 285 children two to three years of age were treated with inhaled fluticasone for 2 years. In the third treatment free year there was no effect on asthma related outcomes or lung functions. During the treatment period there was a reduction in the proportion of episode free days in the inhaled steroid group[190]. In the IFWIN study, children who were prospectively followed up were randomized to receive inhaled fluticasone after the initial wheezy episode and followed up 3 monthly and medication titrated according to response. There was no difference in wheeze, asthma or current asthma medications when the children were analysed at 5 years of age[191].

1.5.4. Background to the study

Episodes of viral-triggered wheezing are common in children aged 1 to 5 years and in this respect, PVW is similar to atopic asthma in school-age children, where clinical colds are associated with worsening of symptom control. However, cohort studies suggest that PVW and atopic asthma are different pathological conditions. The diagnosis of "asthma" includes different phenotypes of wheeze associated with different risk factors, long term outcomes, underlying inflammation, and responses to therapy[192-194]. Asthma in school age children is typically the classic atopic variant; a condition characterised by widespread airflow obstruction, pulmonary eosinophilia, and a propensity of systemic eosinophils *in vitro* to release ECP and EPX[2 195 196]. By contrast, asthma in children between 1 to 5 years of age is characterised by recurrent, transient

episodes of wheeze triggered by viral-colds[3 38]; a phenotype previously labeled as wheezy bronchitis[38], and now as preschool viral-wheeze[185].

There is indirect evidence that the inflammatory substrate of PVW is separate from atopic asthma. The majority of children with PVW do not have the risk factors for atopic sensitization[7 197]. They present with early wheezing and have IgE levels that are similar to non wheezing children[197]. The majority of them become asymptomatic by 6 years of age[5]. In the minority of children in whom wheezing persists beyond 6 years, the characteristic risk factors for atopic asthma[197], such as family history of asthma and increased systemic eosinophil priming[23 24] is seen. Increased serum ECP in preschool children with episodic wheeze, has been shown to be predictive for diagnosis of current asthma at a two year follow up[198].

Acute PVW is a transient condition triggered by a viral cold and is treated by inhaled bronchodilators[141]. An additional strategy, is to start a short course of systemic corticosteroids at the first sign of PVW, with the aim of attenuating lung inflammation, and thereby preventing progression to severe wheeze[199]. A Cochrane review on the effectiveness of early use of corticosteroids in acute asthma in adults and older children concluded that there was a significant reduction in the need for hospital admissions[200]. The current consensus in the UK supports early use of oral steroids for treatment of PVW[2]. However, the evidence

that corticosteroids started by parents in the community, during the early stages of PVW improve clinical outcome, is conflicting. Two placebo-controlled studies of parent-initiated therapy, that probably included children with PVW, found that a 5-day course of oral prednisolone did not reduce respiratory symptoms[171 201]. On the other hand, an open-label trial found that oral prednisolone initiated by parents at the first sign of a cold resulted in a 90% reduction in hospitalisations in a group of preschool children with a history of recurrent severe attacks[172].

The effectiveness of oral steroids in a well-defined group of PVW, already receiving adequate treatment with inhaled bronchodilators, remains unanswered and the possibility of a subgroup of children who may respond to steroids requires further exploration.

Chapter 2. Hypothesis and aims of the thesis

2.1. The conclusions from the background review suggest that, wheezing is a common symptom in early childhood with the majority of attacks are triggered by viral colds. There is now evidence that PVW is a heterogeneous condition, which includes atopic asthma and has different underlying pathological processes ultimately resulting in airway disease. This heterogeneity of PVW makes the management of these children, often difficult and most treatments have poor efficacy, with few randomised controlled trials on its treatment.

The hypothesis generated for this thesis were twofold

> The inflammatory profile seen in acute preschool wheeze is not limited to eosinophilic inflammation
>
> Oral steroids by being most effective against eosinophilic inflammation may not be effective in acute preschool wheeze.

2.2. Sections of thesis and specific aims

In the first part of this study, the activation of inflammatory cells during an acute wheezy attack was explored. This was performed by analysing the mediators released by inflammatory cells into the blood and urine in wheezy children and

comparing them with controls. In a subgroup of wheezy children, further analysis of urinary inflammatory mediators was done at convalescence.

The second part of this study was to test the hypothesis that oral steroids are not effective in acute viral wheeze. A randomised controlled trial to test the effectiveness of a short course of oral prednisolone initiated by parents was performed when these children developed the *next* attack of PVW.

Specific aims

a. To measure the eosinophil mediator in urine (uEPX) during and after acute PVW, and explore their relationship with IgE, and their correlation with clinical symptoms.

b. To measure urinary leukotriene E_4 during and after acute PVW, and analyse their correlation with IgE and clinical symptoms.

c. To explore neutrophil activation in acute PVW by analysing the shedding of adhesion molecule L-Selectin from neutrophils and measuring sL-selectin in serum.

d. To test the effectiveness of a short course of oral steroids in acute PVW by a double blind randomised controlled trial. The children were stratified based on their systemic eosinophil primed status to identify a subgroup of children who may respond to this treatment.

2.3. Study subjects

All the children in this study presented to the University Hospitals of Leicester NHS Trust, between June 1, 1999, and June 30, 2002. Children between the age of one and five years who presented to hospital with an acute wheezy episode secondary to a viral cold were included in this study.

Two categories of 'healthy' controls were studied.

'Normal controls' were recruited from a random selection of children undergoing elective ear, nose and throat surgery or ophthalmic surgery, who had no symptoms of atopy.

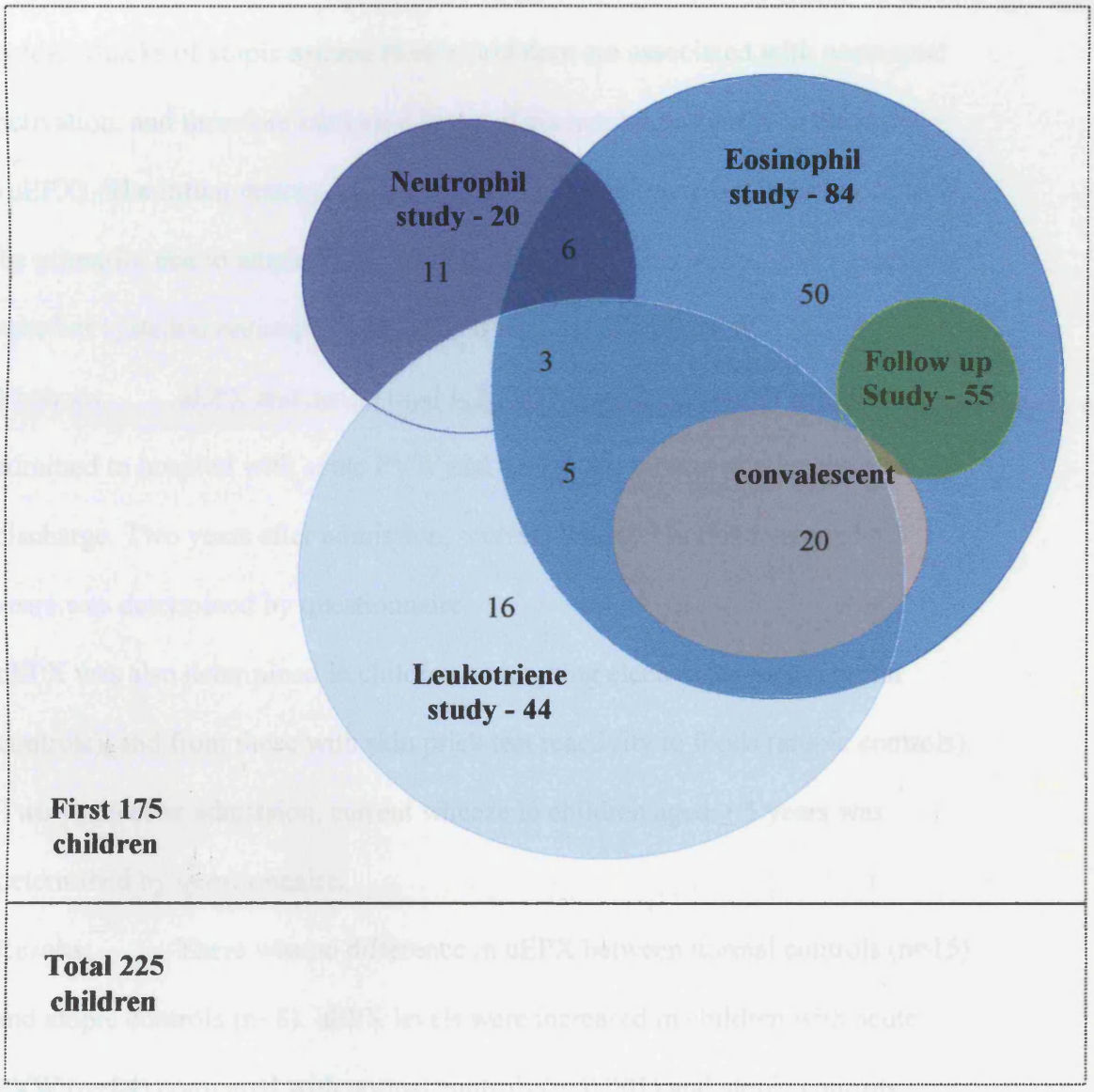
'Atopic controls' were recruited from a group of children who had a history of allergic reaction to a food and at least one positive skin prick test to a food antigen and who were attending hospital for a food challenge.

2.4. Ethics approval

The clinical trial was funded by the grant AM2/01/008 from the NHS R&D Programme on Asthma management.

The study received ethics approval from Leicestershire Health Authority Research Ethics Committee and Ethics Committee of the University Hospitals of Leicester NHS Trust. The study on 'healthy' controls was approved by the Ethics committee of the University Hospitals of Leicester NHS Trust.

2.5. Overview of children recruited to various studies within the thesis



Chapter 3. Eosinophil activation in Preschool viral wheeze

3.1. Summary

Background: Most attacks of wheeze in the preschool period are triggered by colds. Attacks of atopic asthma in older children are associated with eosinophil activation, and therefore increased levels of eosinophil protein X in the urine (uEPX). The inflammatory substrate for PVW is unknown, but is not thought to be primarily due to atopic inflammation. The aim of this study was to ascertain whether systemic eosinophil activation is associated with PVW.

Methods: uEPX and serum total IgE (IgE) were measured in children admitted to hospital with acute PVW and uEPX was measured 6 weeks after discharge. Two years after admission, 'current wheeze' in children aged $\geq$ 5 years was determined by questionnaire.

uEPX was also determined in children undergoing elective surgery (normal controls), and from those with skin prick test reactivity to foods (atopic controls). Two years after admission, current wheeze in children aged $\geq$ 5 years was determined by questionnaire.

Results: There was no difference in uEPX between normal controls (n=15) and atopic controls (n=8). uEPX levels were increased in children with acute PVW (n=84) compared with normal controls ($p<0.001$) and atopic controls ($p<.01$). uEPX levels fell on convalescence (n=20, 95% CI –217 to –31 μg/mmol creatinine $p<0.05$), to similar values seen in controls. In children with acute

PVW, there was no association between uEPX and serum IgE levels or markers of clinical severity. Two-year follow up data was obtained from a respiratory questionnaire. 25 children of the subgroup of 55 eligible children who had reached $\geq$ 5 years responded. There was no difference in uEPX levels during acute PVW when stratified by either 'current wheeze' (n=18) or 'no wheeze' (n=7) two years after the hospital admission.

Conclusions: Systemic eosinophil activation is associated with acute PVW and is an acute process which resolves with normalisation of symptoms. uEPX is not associated with serum IgE, the clinical severity, or persistent wheeze into early school age.

3.2. Background to study and aims

The background to this study is described in chapter 1.2.

Viral colds trigger the majority of attacks of wheeze in both preschool and school aged children[45]. Birth cohort studies suggest that most children with PVW have a phenotype separate from that of classical atopic asthma. Pulmonary eosinophil activation is in turn, associated with increased serum levels of EPX[58]. Since circulating EPX is excreted unchanged in the urine , urinary (u) EPX levels are increased in atopic asthmatics, and increase further during acute attacks [62 81]. This study was to explore the relationship between eosinophil activation and PVW.

The specific aims of this study, therefore were as follows

1. To seek evidence for eosinophil activation in PVW.
2. To find whether uEPX was elevated compared with healthy controls during acute wheeze, and whether levels fell when wheezing resolved.
3. To explore whether elevated uEPX would be associated with the subgroup of children with high serum IgE, i.e. the subgroup at most risk for atopic sensitisation and persistence of wheeze into early school age.

3.3. Methods

3.3.1 Patients

Children (1-5 years) were recruited from those presenting to the admissions unit of the Leicester Royal Infirmary Children's Hospital with PVW. They were recruited, and blood and urine samples were obtained, as part of a randomised double blind trial of oral steroids for a subsequent attack of PVW.

PVW was defined as an acute episode of wheeze that occurred within two days of the onset of coryzal upper respiratory tract symptoms. Children were excluded if there was i) history of chronic lung disease ii) evidence of a gross upper respiratory tract structural abnormality iii) significant non-respiratory tract pathology requiring ongoing systemic pharmacological treatment iv) clinical suspicion of active systemic bacterial infection v) history of prematurity or neonatal respiratory distress vi) a history of chronic rhinitis with no clear pattern of acute episodes and vii) if the child had received oral prednisolone either more than a day prior to the illness or within the preceding fortnight of the admission. Parents were approached within 24 hours of admission and a written information sheet explaining the study was given to them. The presence of wheeze was confirmed by auscultation and clinical evidence of rhinitis was sought. After obtaining informed consent, a blood sample for IgE and eosinophil count and a urine sample was obtained within 36 hours for uEPX when the children were clinically wheezy. On admission, to hospital, all children with PVW received a single dose of oral steroids, and 'as required' nebulised salbutamol. The number

of nebulised bronchodilators during the admission, and the total duration of illness were recorded. After discharge, children were visited at home within 6 weeks. If children were wheeze free, and produced a urine specimen during the visit, a 'convalescent' sample for uEPX was obtained.

'Normal' controls were recruited from a random selection of children undergoing elective ear nose and throat surgery, or ophthalmic surgery. None had clinical evidence of active infection, and their skin prick reactivity to allergens was unknown. Urine was obtained before surgery, and a blood sample for serum IgE obtained soon after induction of anaesthesia. Urine from 'atopic' controls were obtained from children with suspected food sensitivity, who attended hospital for a food challenge. All had history of a suspected allergic reaction to a food, and at least one positive skin prick test to food antigens. Serum IgE was not obtained from atopic normal controls because of ethical restrictions. Controls with a history of chronic respiratory disease, or previous attacks of wheezing, or symptoms of a respiratory tract infection in the preceding week, were excluded.

3.3.2 Follow up

To establish whether increased uEPX during acute PVW was associated with symptoms consistent with a diagnosis of asthma[202], parents of children who had a 2 year interval from the original admission and reached 5 years of age or more were sent a respiratory questionnaire. Children of school age were categorised

either as having "no wheeze" (in the last six months) or "current wheeze" (at least one episode of wheeze in the last 6 months).

3.3.3 Sample collection and analysis

5-10ml urine samples were collected using a sterile potty. Urinary infection was excluded using the 'Multistix 10SG' dipstick (Bayer, UK) screening test. Urine samples were initially stored in a refrigerator, then aliquoted and transferred to -70^{0}C within 12 hours. uEPX concentration was measured in unprocessed urine samples using a specific EPX radioimmunoassay kit (Pharmacia, Uppsala, Sweden). Urine was defrosted at room temperature, and 500 μL used in the assay. The samples were diluted 11 times in a phosphate buffer containing 0.15%NaCl, 1% bovine serum albumin, 0.1%Tween 20, 10nmol/L EDTA and 0.2% N-cetyl-trimethylammonium-bromide as previously described[62]. The assay was done in duplicate and the mean values were taken. The detection limit of the assay was <3μg/L, the within assay coefficient of variation was <5%, and the between assay coefficient variation was <10%. Urinary creatinine was measured in mmol/L by the Jaffe reaction with the Dade Behring Dimension Analyser (Dade Behring, USA) and uEPX levels were expressed as μg/mmol creatinine.

For serum IgE, a 2 ml venous blood sample was collected and allowed to clot at room temperature for 60 minutes. Serum was separated by centrifugation before storage at -20^{0}C. IgE was measured using the UniCAP Analyser machine

(Pharmacia, Sweden) and expressed as kU/L. An absolute eosinophil count was done on a 0.5 mL heparinised sample using routine hospital techniques.

3.4 Statistics and sample size

When this study was conceived and planned there was no available data on uEPX in PVW. In order to prove the concept we planned a pilot study. We decided to recruit children with PVW in whom we could obtain both a blood and urine sample and adequate number of healthy controls. The recruitment of healthy controls was difficult and we discontinued recruitment when we had achieved enough healthy controls and adequate paired urine samples.

Data are presented as medians and interquartile range (IQR). Unpaired data were compared using the Mann-Whitney U test. Paired data were compared using the Wilcoxon signed rank test and expressed as the estimated median difference and 95% confidence interval (CI). Correlations were determined by Spearman rank correlation (r_s). Statistical analyses were performed using SPSS for windows version 10 (SPSS Inc, Chicago, IL, USA). Statistical significance was defined as a p value <0.05.

3.5 Results

Eighty four children with acute PVW were studied. A convalescent urine sample was obtained from 20 children, 6 weeks after discharge. Convalescent samples were not obtained from children who were not potty trained or who refused to produce a sample during the visit (n=63). One child was excluded because of a readmission with PVW.

Serum IgE levels in the normal controls (n=15) were within the range reported for non-atopic children[203]. Blood was not sampled from atopic controls. Serum IgE levels was increased in children with acute PVW (n=73; $p<0.01$ *v* normal controls, table 3.1). Blood eosinophil counts in both children with PVW and normal controls were within the normal range but were higher in normal controls ($p<0.05$ *v* acute PVW, table 3.1). There was no difference in uEPX levels between normal and atopic controls (n=8; table 3.1; figure 3.1) with similar confidence intervals. Both control groups were slightly older than the children with PVW (table 3.1), but there was no association between age and uEPX levels in any group.

uEPX levels were increased during acute PVW ($p<0.001$ *v* normal controls, $p<0.01$ *v* atopic controls, table 3.1; figure 3.1). uEPX levels during acute PVW were not associated with (1) serum IgE (r_s =0.17, p= NS; figure 3.2), (2) the interval between oral steroid treatment and urine sampling (r_s =0.1, p= NS; figure

3.3), and (3) the number of salbutamol nebulisations required during the attack (r_s =0.1, p= NS). Children were categorised into three groups: i) first attack of wheeze ii) previous history of exclusive viral wheeze iii) viral wheeze with interval symptoms. uEPX levels were similar in these groups (figure 3.4). Furthermore there was no significant difference in uEPX levels during acute PVW when categorised by family history of atopy (n=66/84), previous dry cough or shortness of breath without colds (n=14/84), and eczema (n=27/84).

uEPX levels fell between the acute and convalescent phases of PVW (median – 107 μg/mmol creatinine, 95% CI -217 to –31, n=20, p<0.05, figure 3.5). Convalescent uEPX levels were similar to those of normal and atopic controls (p=NS). When convalescent uEPX levels were categorised by the presence of eczema (n=7/20), there was no difference between children with and without eczema and no difference between children with eczema and controls.

Follow up questionnaire were returned for 25 (45%) out of 55 children who had reached school age in 2003 and who were at least 2 years post admission. Eighteen children continued to have parent reported wheeze in the preceding 6 months (table 3.2); all had been prescribed inhaled salbutamol, 11 were receiving regular inhaled steroids. Seven children had no wheeze over the preceding 6 months; none were prescribed inhaled therapy. There was no difference in uEPX

levels when acute PVW was categorised by the presence or absence of wheeze at the two year follow up (table 3.2).

Table 3.1 Demographic, serum and urine parameters in children with preschool viral wheeze (PVW) and controls

	PVW (n=84)	**Normal controls (n=15)**	**Atopic controls (n=8)**
Age (months)	31 (20 to 41)	59 (41 to 66)**	87 (58 to101)**
uEPX (μg/mmol creatinine)	214 (144 to 383)	82 (35 to 211)**	84 (51 to 182)**
Serum total IgE (kU/L)	56 (9 to 209)(n=73)	12 (7 to 25)*	Not done
Absolute eosinophil count ($x10^9$/L)	0.1 (0-0.2)(n=73)	0.25 (0.2-0.3)*	Not done

Data are presented as median (interquartile range).

* $p<0.05$ *vs* PVW; ** $p<0.01$ *vs* PVW; Mann Whitney U test. There was no difference in age between normal and atopic controls.

Table 3.2. Two year follow up of children with preschool viral wheeze (PVW) who had reached 5 years of age in 2003.

	Wheeze (n=18)*	**No wheeze (n=7)***
Age at PVW episode (months)	37 (31 to 43)	34 (25 to 46)
Age at follow up (months)	77 (74 to 86)	83 (73 to 95)
IgE during PVW episode (kU/L)	141 (57 to 235)	31 (10 to 294)
uEPX during PVW episode (μg/mmol creatinine)	245 (166 to 387)	310 (203 to 451)

Data are presented as median (IQR). There were no differences between the two outcome groups (p=NS, Mann Whitney U test).

*parental reported symptoms over the previous 6 months

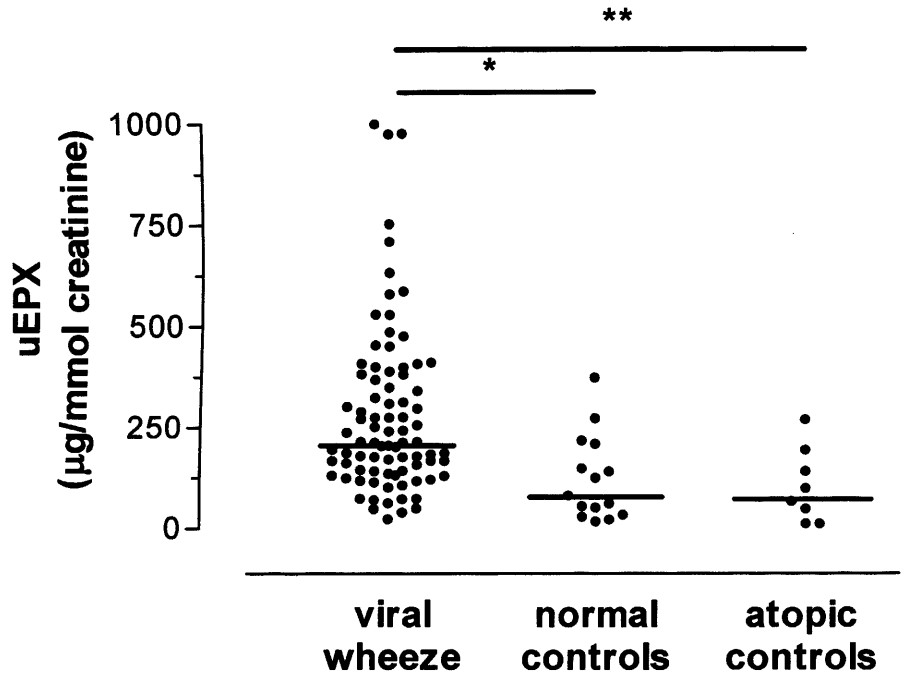


Figure 3.1 Dotplot of urinary EPX (uEPX) levels in children with acute PVW (n=84), normal controls in whom skin prick reactivity was unknown (n=15), and atopic controls (n=8). uEPX levels were higher in the children with acute PVW (* $p<0.001$ *v* normal controls; **$p<0.01$ *v* atopic controls, Mann Whitney U test). Horizontal bars represent medians.

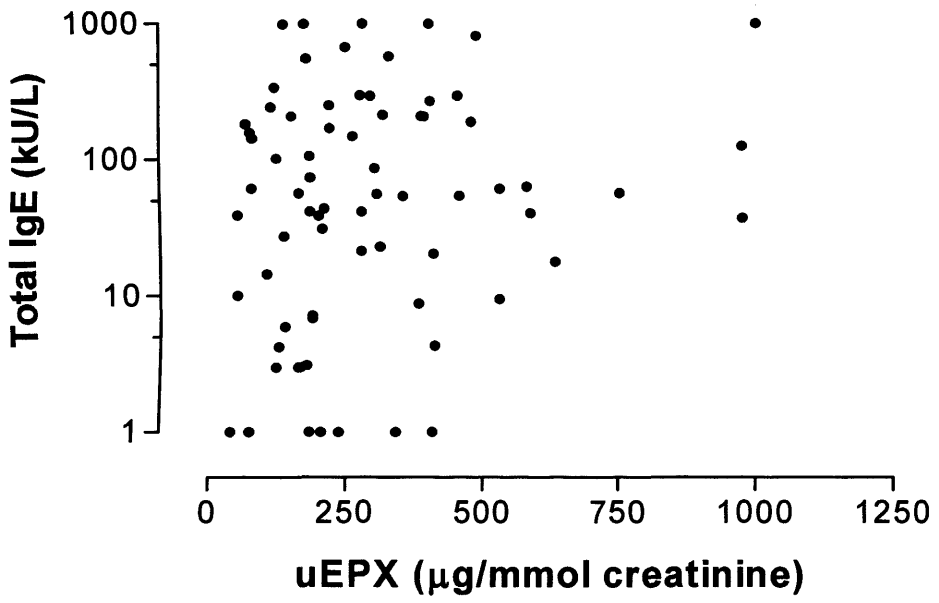


Figure 3.2 Scatterplot of the association between uEPX and serum IgE levels during acute PVW which shows no correlation (r_s=0.17, p=NS; spearman rank)

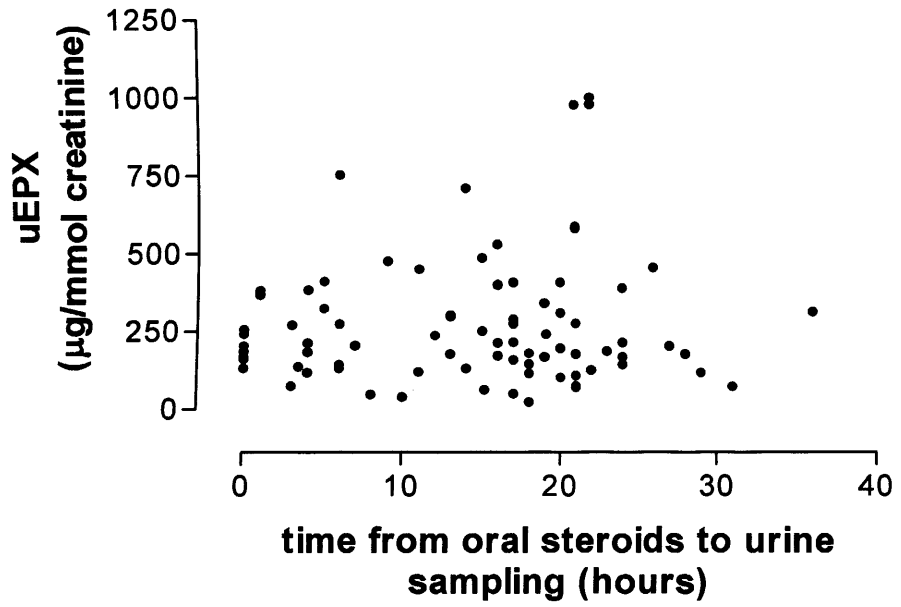


Figure 3.3 Scatterplot of the association between uEPX levels during acute PVW and time of urine sample from oral prednisolone therapy showing no correlation (r_s=0.1, p=NS; spearman rank)

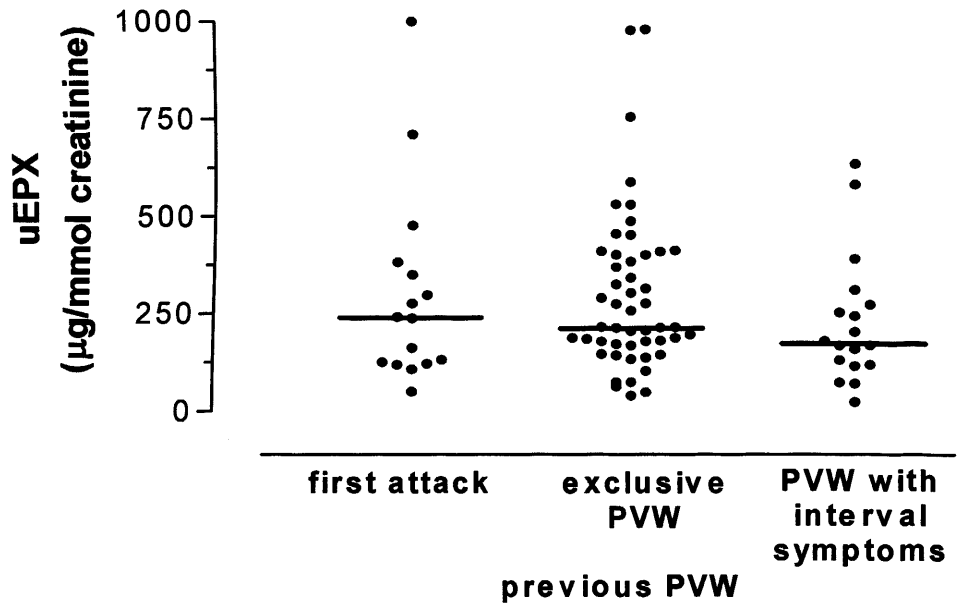


Figure 3.4 Dotplot of uEPX levels in children with i) first attack of wheeze ii) previous history of exclusive PVW iii) PVW with interval symptoms; uEPX levels were similar in all three groups

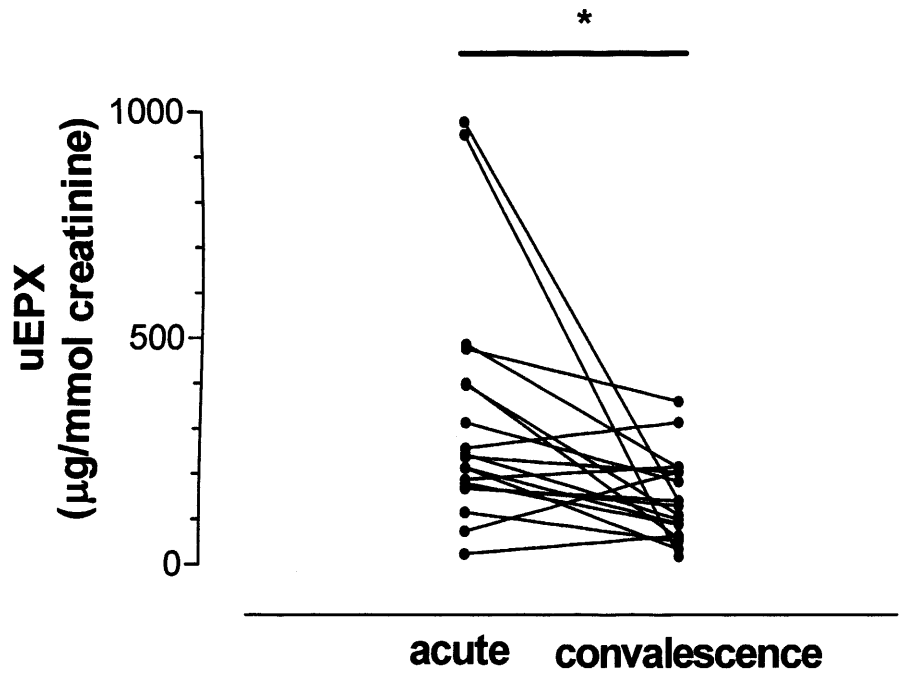


Figure 3.5 Change in urinary eosinophil protein X (uEPX) levels between the acute and convalescent phase of PVW. * $p<0.05$, 95% CI –217 to –31 µg/mmol creatinine, n=20, (Wilcoxon signed rank test)

3.6 Discussion

The main finding of this study is that uEPX levels are increased in acute PVW, and fall when wheezing resolves. The findings in acute PVW are similar to Oymar et al who reported increased uEPX in a group of preschool children with at least 3 previous wheezy episodes[88].

The cause of the higher levels of uEPX was further explored. There was a large degree of overlap between uEPX levels in acute PVW and normal controls suggesting that systemic eosinophil activation may not be a significant pathological process in some children with PVW. This is compatible with epidemiological studies suggesting that PVW is not a single phenotype but may be a heterogeneous population[5].

The significant factors in this group of children included the viral cold, acute wheeze, and severity of symptoms as they needed hospitalisation, the treatment of bronchodilators and oral prednisolone which they received for a period of time, and their background profile.

It was initially speculated that increased uEPX, would be limited to children at risk of atopic sensitisation. The children with PVW in this study had increased IgE compared to controls and normal values[203]. However, no correlation was found between uEPX and serum IgE levels during acute PVW. There was no correlation between the clinical patterns of wheeze, or demographic parameters and children with increased eosinophil activation in PVW.

This is the first study where convalescent data in acute PVW was explored and compared with appropriate controls. uEPX normalised on convalescence, implying that eosinophil activation is an acute process which rapidly resolves and not a chronic process. This finding is similar to the study of Stevenson *et al*[129] who assessed airway inflammation by non bronchoscopic lavage in children with no family history of atopic disease, and who wheezed only with winter colds (viral-wheezers). BAL performed when the viral wheezers were asymptomatic, showed no evidence of airway eosinophilia, in contrast to children with atopic asthma with prolonged asymptomatic periods who had evidence of airway eosinophilia.

Increased levels of uEPX during acute PVW did not predict persistence of wheeze into the early school age years. These data would appear to be different from those reported by Oymar [88], who found increased uEPX in a group of wheezy preschool children with at least 3 previous wheezy episodes who were subsequently diagnosed with atopic asthma. In this study, previous wheeze was not a criterion, and skin prick tests to test atopic status was not done at follow up. Thus a similar association between uEPX and subsequent diagnosis of atopic asthma cannot be excluded.

There are limitations to the findings in this study. The lack of information on uEPX during trivial colds is a significant limitation. A control group of children

with a simple URTI was not studied and hence the effect of viral colds on eosinophil activation and production of uEPX could not be studied. It has however been shown that uEPX levels were not increased in young children with trivial upper respiratory tract illnesses [87]. An effect of atopy *per se* on uEPX is unlikely since no increase in uEPX in the atopic controls was found.

All children with PVW were treated with oral prednisolone and it is therefore possible that steroids may have disrupted an association between eosinophil activation and IgE. Corticosteroids down-regulate eosinophil activation[75], although there is a time lag of several hours between oral steroid therapy and its systemic effects. However *in vitro* studies show that eosinophil degranulation is not a direct target of steroids[204]. Further, uEPX levels showed no correlation between duration of treatment with steroids and uEPX, a relationship that would be expected if steroids had a major role in suppressing uEPX. Eczema is another potential confounder, since active dermatitis is associated with increased uEPX levels [205] and a significant minority (32%) of children with PVW had mild eczema. However eczema was not associated with increased uEPX levels, either during the acute or the convalescent phase of PVW.

The potential explanations for the heterogeneity in eosinophil activation in PVW are that eosinophil activation may have no role in the pathogenesis of viral wheeze, with symptoms driven by other inflammatory cells. Although a "bystander" role for eosinophil activation has not been excluded, since the

changes in uEPX during trivial colds have not been defined, the lack of correlation between uEPX and markers of PVW severity cannot be interpreted as support for a bystander role, since the relationship between uEPX and lung function is inconsistent in the eosinophil-driven symptoms of atopic asthma[86]. A second explanation is that eosinophils are important in the pathogenesis on PVW, but there may be an inflammatory phenotypes in PWV that are driven by other inflammatory cells such as neutrophils, or that neutrophils may initiate eosinophil degranulation without involvement of the allergic pathway[206].

As this study demonstrated a significant difference in the levels of eosinophil activation, a similar sample size would be adequate to explore the relationship between eosinophil activation and PVW.

Summary

This study reinforces the evidence that there is increased urinary excretion of EPX in severe pre-school viral wheeze and confirms that this returns to normal with resolution of symptoms. Pre-school children with viral wheeze are a heterogeneous group and eosinophil activation as measured by uEPX is independent of serum IgE, clinical presentation or outcome two years later.

Chapter 4. Cysteinyl-leukotriene excretion in preschool wheeze

4.1. Summary

Background: Cysteinyl-leukotrienes (cystLTs) are important mediators of wheeze in atopic asthma, but their role in preschool viral-wheeze (PVW) is unclear. This study therefore sought evidence for increased cystLT production in PVW.

Methods: Urinary (u) Leukotriene E_4 (LTE_4) and serum total IgE, were measured in children (1 to 5 yrs) with PVW during the acute (n=44), and convalescent phases (n=19), and compared with levels from normal controls (n=15). The potential confounding effect of atopic sensitisation was assessed in atopic controls (n=6), in whom only $uLTE_4$ was measured.

Results: $uLTE_4$ levels were similar in normal and atopic controls. $uLTE_4$ was increased in acute PVW compared with normal controls (median (IQR); 165 (101,285) *versus* 125 (82,163) ng/mmol creatinine, $p<0.05$). Stratifying children by IgE, showed that the subgroup of acute PVW with IgE >95th centile (n=23) had increased $uLTE_4$ (median 211 (118,312) ng/mmol creatinine, $p<0.05$ *versus* controls), whereas $uLTE_4$ was not increased in acute PVW with IgE $\leq$95th centile. During the convalescent phase, $uLTE_4$ fell into the 'normal' range in the subgroup with high IgE, but remained the same in the low IgE subgroup.

Conclusion: In acute PVW there is heterogeneity of cystLT production. Increased levels of $uLTE_4$ during the acute phase normalised with resolution of symptoms. An association between $uLTE_4$ and elevated serum IgE was identified.

4.2. Background to study and aims

The background to this study is described in chapter 1.3.

Increased production of cysteinyl (cyst) leukotrienes (LTs) within the lung causing bronchoconstriction is seen in atopic asthma[96]. Leukotriene E_4 (LTE_4), a potent bronchoconstricting cystLT[96], is the end product of pulmonary cystLT metabolism and is excreted in the urine as $uLTE_4$. Thus $uLTE_4$ can reflect disease severity in atopic asthma[114], and $uLTE_4$ which is increased during acute attacks of atopic asthma, falls in the convalescent phase[115]. Viral-colds are an important trigger for acute asthma attacks in attacks of wheeze in preschool children[45]. However, epidemiological studies suggest that some children PVW do not have atopic asthma[90]. It has been speculated that different phenotypes of PVW are associated with different patterns of pulmonary inflammation[91].

There is no consistent evidence for increased pulmonary cystLT production in acute PVW. Increased levels of LTC_4 in upper respiratory tract secretions occur in infants during episodes of recurrent wheezing[98]. However, Balfour-Lynn *et al*[116] found no increase in $uLTE_4$ in hospitalised infants with acute PVW. It is possible that, increased cystLT production may be limited to a subgroup of preschool children with specific risk factors and an understanding of the pattern and timing of its production may identify subgroups that are responsive to specific therapies. There is also no data on whether increased levels of IgE influences cystLT production in PVW.

The aims of this study were

1. To determine whether pulmonary cystLT production is increased in PVW severe enough to warrant admission to hospital, by using $uLTE_4$ as a marker for pulmonary cystLT production.
2. To explore the relationship between $uLTE_4$ and IgE, in order to determine levels in the subgroup of children at increased risk of atopic sensitisation.

4.3. Methods

4.3.1. Patients

Children that were included in this study were recruited from those presenting to the admissions unit of the Leicester Royal Infirmary Children's Hospital with acute PVW. The study inclusion and exclusion criteria were as described in Chapter 3. On admission each child received a single dose of oral prednisolone, and 'as required' nebulised salbutamol. The presence of wheeze was confirmed by a clinician, a clinical history obtained from the parents, and clinical evidence of a viral-cold (i.e. rhinitis) sought. A urine sample was then collected for $uLTE_4$ analysis within 36 hours of admission, and a blood sample was obtained for IgE. After discharge, some children were visited at home within 6 weeks. If there had been no respiratory symptoms in the preceding 7 days, a 'convalescent' urine sample was obtained.

Two controls groups were obtained as described in Chapter 3. The study required written parental consent and was approved by the Leicestershire Research Ethics Committee.

4.3.2. Sample collection and analysis.

Urine samples were obtained using a bag or sterile potty. Urinary infection was excluded using the 'Multistix 10SG' dipstick (Bayer, UK) screening test. Urine samples were initially stored in a refrigerator, then transferred to -70^{0}C within 12 hours. $uLTE_4$ concentration was measured in unprocessed samples by ELISA kit (ACE ^{TM} LTE_4 Enzyme Immunoassay kit, Cayman Chemicals, Michigan, USA), that has been previously validated against $uLTE_4$ concentrations measured after high performance liquid chromatography extraction[106]. Urine was initially defrosted at room temperature, and 500μL was used in the immunoassay without further processing. The assay detection limit was 25pg LTE_4/mL. Urinary creatinine was measured using the Dade Behring Dimension Analyser (Dade Behring, USA), and $uLTE_4$ levels were expressed as ng/mmol creatinine. Serum for IgE was obtained from a venous blood sample that had clotted for 60 min at room temperature. Serum was separated by centrifugation and stored at -20^{0}C. IgE was measured using the UniCAP Analyser machine (Pharmacia, Sweden) and expressed as kU/L. Further details of analysis are noted in the appendix.

4.4. Statistics and sample size

When this study was conceived and planned there was no available data on paired data of $uLTE_4$ in PVW. We planned a pilot study to prove the concept that leukotrines had a role in PVW. We recruited children with PVW who entered the clinical trial and in whom we could obtain a blood sample for IgE and a urine sample and adequate number of healthy controls. The recruitment of healthy controls was difficult and we discontinued recruitment when we had achieved enough healthy controls and adequate paired urine samples.

Data was summarised as median and interquartile range (IQR), and comparisons were performed using the Mann-Whitney U test and Chi-squared test. Correlations were determined by Spearman rank correlation (r_s). Paired data were analysed using the paired *t* test. Analysis was performed using a package for microcomputers (SPSS for Windows version 10, SPSS Inc, Chicago, IL). p-values <0.05 were considered statistically significant.

4.5. Results

Forty-four children (32 boys, 12 girls) with acute PVW, 15 normal controls (9 boys, 6 girls), and 6 atopic controls (3 boys, 3 girls) were studied. Children with PVW were slightly younger than normal controls (median age 3.0 yr (1.8, 3.6) *versus* 4.5 yr (3.4, 5.5), $p<0.01$), and had higher IgE (118 (23, 293) kU/L *versus* 15 (7, 26) kU/L, $p<0.01$). The median duration between a viral cold and onset of wheezing was 24 hours (12, 48). In 8 children this was their first attack, 26 children had a previous history of wheezing exclusively with colds (median previous attacks n = 4 (2, 6). Ten children with PVW had a history of additional interval respiratory symptoms, including moderate nocturnal cough and wheeze after playing (median previous PVW attacks n=7 (4-13)). In 19 children with acute PVW, a paired acute and convalescent urine sample was obtained.

Serum IgE of normal controls were within the 95th centile reported for non atopic children[203], and there was no difference in $uLTE_4$ between the normal controls and the atopic controls (figure 4.1). Data from children with PVW were therefore compared with the normal controls only. When considered as a single group, children with acute PVW had higher $uLTE_4$ compared to normal controls (median; 165 (101,285) *versus* 125 (82,163) ng/mmol creatinine, $p< 0.05$, figure 4.1). The $uLTE_4$ of the whole group fell during the convalescent phase to lie within the range of the normal controls (mean fall (95% CI), 70 (28,112) ng/mmol creatinine, $p<0.05$, n=19 (figure 4.2).

There was a moderate correlation between $uLTE_4$ and IgE during acute PVW (Spearman rank, $r_s = 0.35$, $p<0.05$, figure 4.3). Stratification based on the 95th centile for IgE[203] therefore resulted in different patterns of $uLTE_4$ in the two groups. The subgroup with high IgE (n = 23) had elevated $uLTE_4$ during the acute phase ($p<0.05$ *versus* normal controls, table 4.1). The individual data plot (figure 4.4) shows that there was an association between IgE and levels of $uLTE_4$ in acute PVW is apparent in children with the highest levels (>250 kU/L). On convalescence, $uLTE_4$ fell in the high IgE subgroup ($p<0.01$ *versus* acute, n=14, table 4.1), and was not different from normal controls. In contrast, $uLTE_4$ in children with PVW and low IgE (n = 21) was not elevated during the acute phase (*versus* normal controls), and did not change on convalescence (n = 5, table 4.1).

Clinical parameters in children with PVW were similar when stratified into high and low IgE groups (table 4.1). Furthermore there was no difference in IgE, or $uLTE_4$, when children were stratified into those with a previous history of exclusive PVW, and previous PVW with interval symptoms (figure 4.5a, 4.5b). There was no correlation between $uLTE_4$ and time period between administration of oral steroids and urine sampling (figure 4.6). $uLTE_4$ in the six children with acute PVW, in whom urine was obtained before steroid therapy, was not increased compared with those (n=38) who were studied after steroid treatment (figure 4.6)

Table 4.1 Comparison between children with preschool viral wheeze (PVW) after stratification for serum total Immunoglobulin (Ig)E during the acute attack.

	Serum total IgE	
	$\leq 95^{th}$ centile	$>95^{th}$ centile
N	21	23
Age (months)	29 (17,38)	38 (31,47)
Male: female	13: 8	19: 4
Eczema present (n)	9	8
Parental history of asthma (n)	19	13
First attack of PVW (n)	4	4
Previous PVW and no interval symptoms (n)	12	14
Previous PVW with interval symptoms (n)	5	5
Number of previous attacks of PVW (n)	3 (2,7)	5 (2,7)
Urinary LTE_4 during acute PVW (ng/mmol. creatinine)	150 (77,217)	211 (118,312)*
Fall in urinary LTE_4 on convalescence# (ng/mmol. creatinine)	34 (-93,160) n=5	84 (36,131)** n=14

Data are presented as median (interquartile range), except #mean (95% Confidence Interval). * $p<0.05$ *versus* normal controls by Mann-Whitney U test. ** $p<0.05$ *versus* acute levels by paired *t* test. The 95^{th} centile of IgE is based on[203] (<3 years 74 kU/L, 3-5 years 87 kU/L).

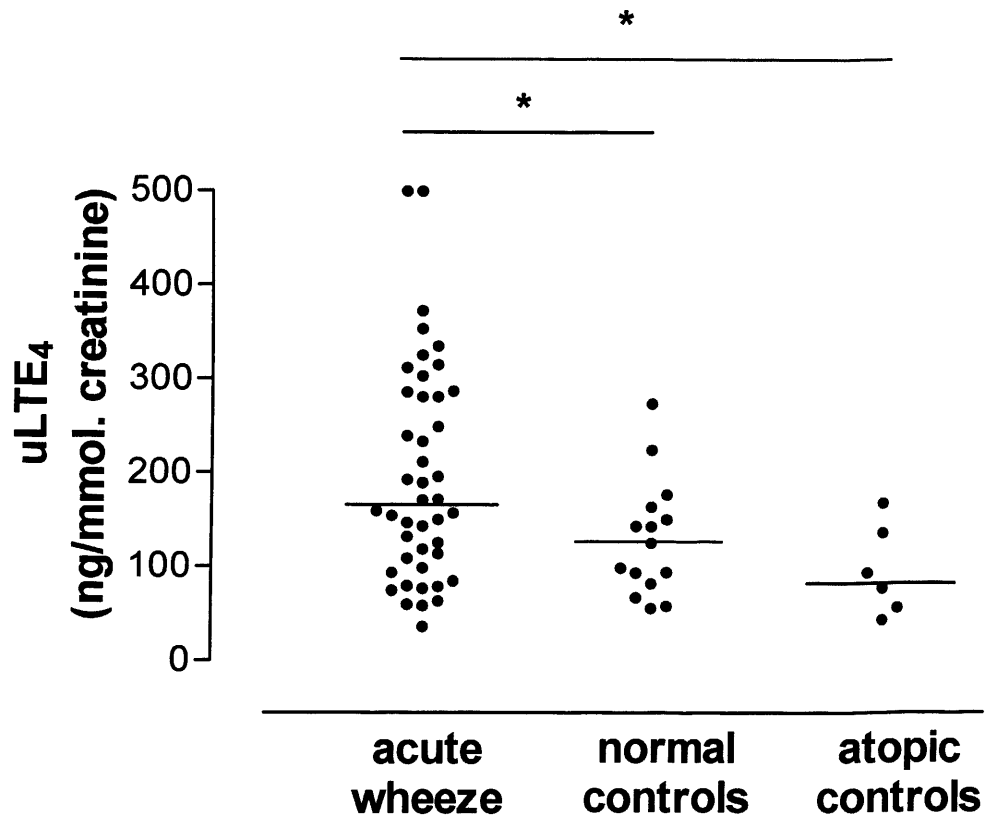


Figure 4.1 Dotplot of urinary Leukotriene E_4 ($uLTE_4$) concentrations, corrected for creatinine, in preschool children with acute viral-wheeze (n=44), normal controls (n=15) and atopic controls (n=6). $uLTE_4$ levels are significantly higher in the acute preschool viral-wheeze group (* $p<0.05$ *versus* normal, and *versus* atopic controls). Horizontal bars represent medians.

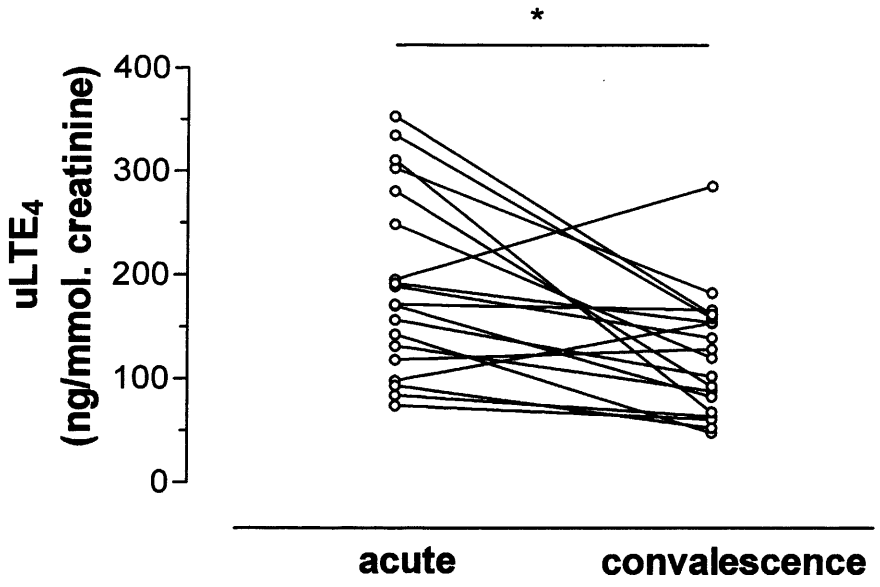


Figure 4.2 Paired change in $uLTE_4$ between the acute and convalescent phase of preschool viral-wheeze. The fall in $uLTE_4$ is significant (* $p<0.05$, n=19, paired *t* test).

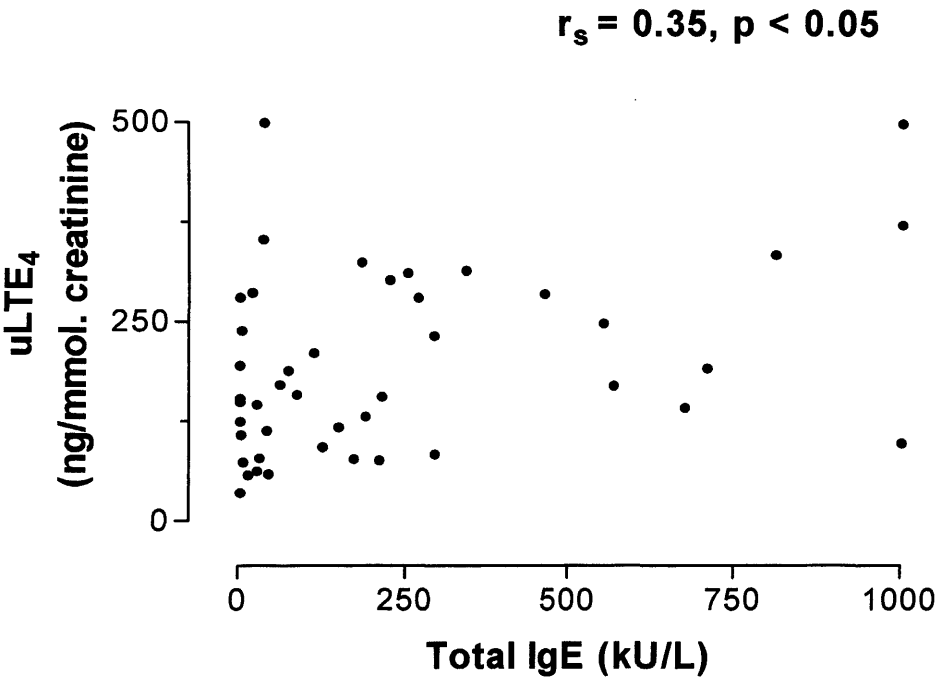


Figure 4.3 Scatterplot of the significant association between $uLTE_4$ and serum IgE levels during acute PVW ($r_s = 0.35$, $p<0.05$; spearman rank)

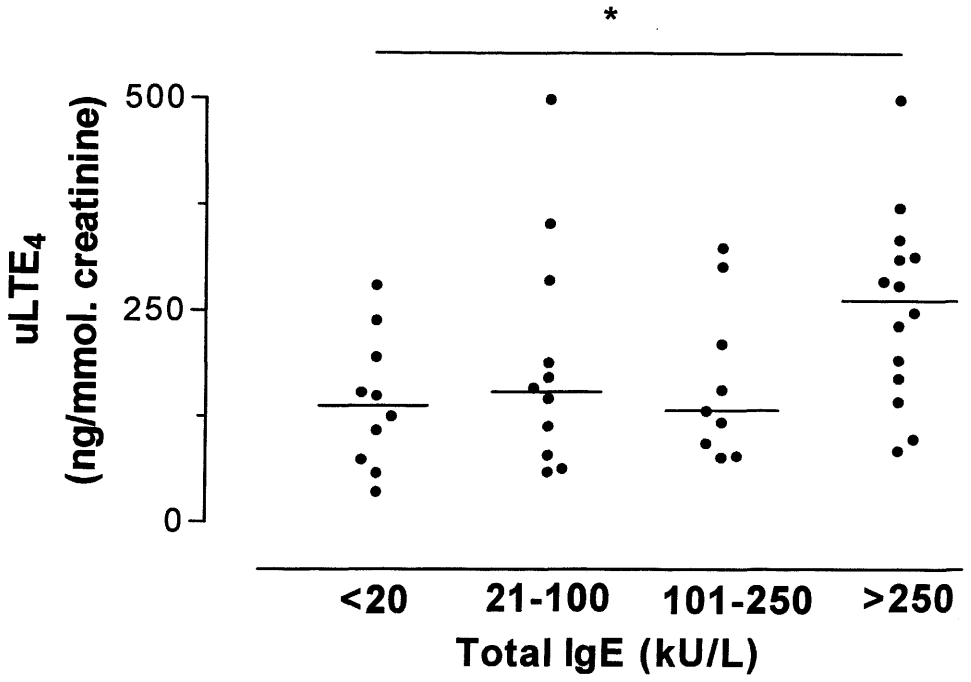


Figure 4.4 $uLTE_4$ of children with acute preschool viral-wheeze categorised by serum total IgE. (*$p<0.05$, Mann-Whitney U Test). Horizontal bars represent medians.

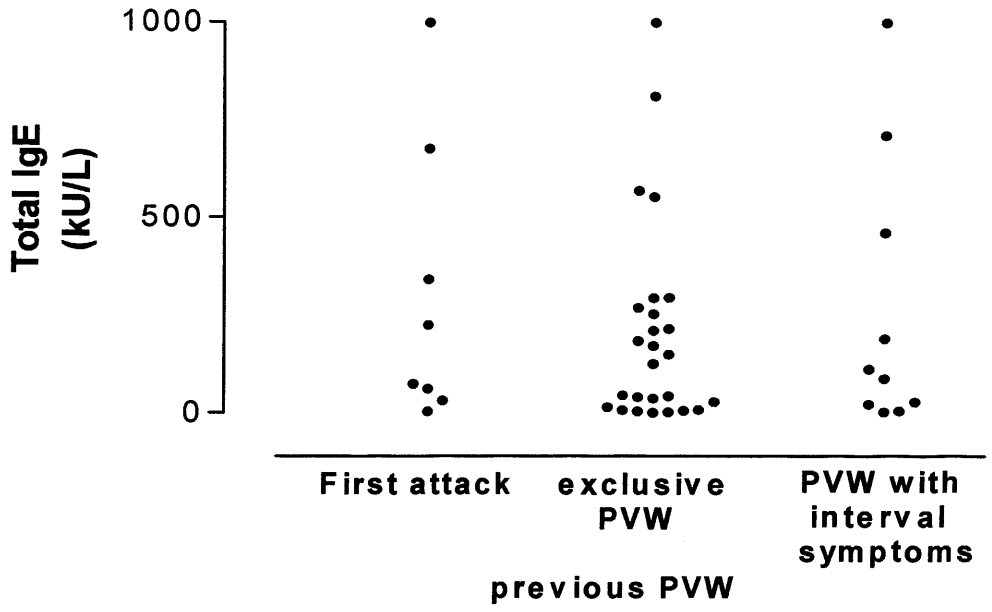


Figure 4.5.a Serum total IgE in acute PVW were similar in children who were categorised as having i) first attack of wheeze ii) previous history of exclusive PVW iii) PVW with interval symptoms.

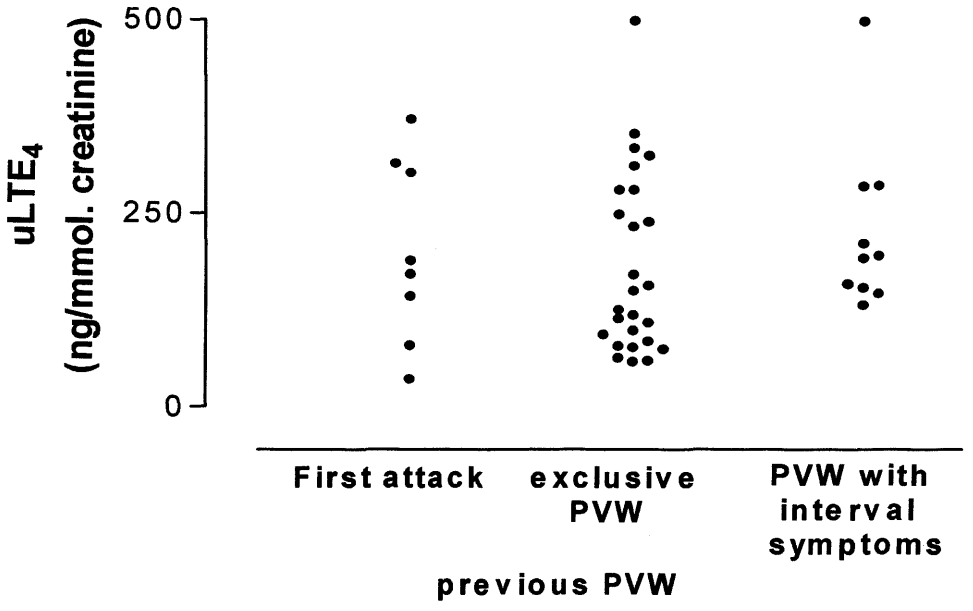


Figure 4.5.b uLTE$_4$ in acute PVW were similar in children who were categorised as having i) first attack of wheeze ii) previous history of exclusive PVW iii) PVW with interval symptoms.

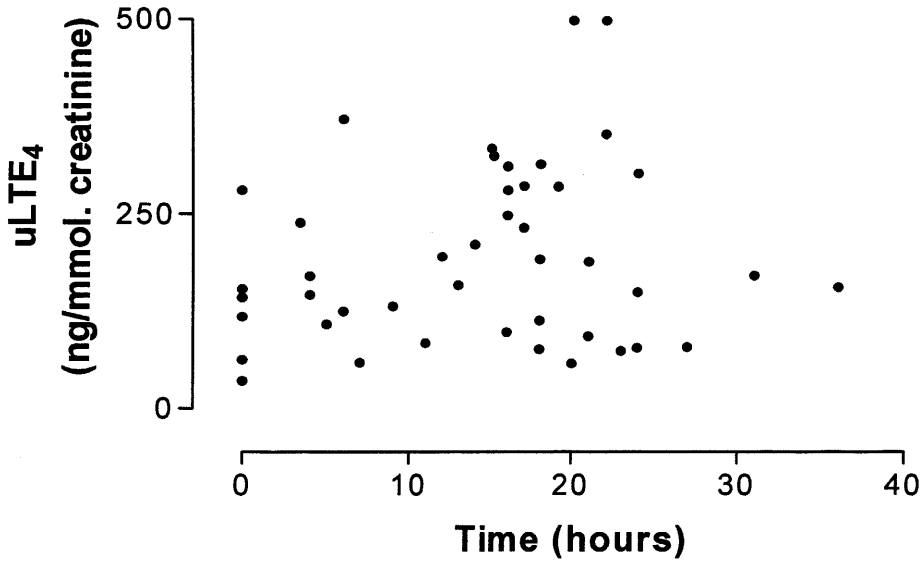


Figure 4.6 Scatter plot of the correlation between $uLTE_4$ and the duration between steroid administration and urine sampling in acute preschool viral wheeze showing no correlation (Spearman rank correlation, p=NS). The zero time point represents children sampled before oral steroid therapy.

4.6 Discussion

The above data shows that children admitted to hospital with acute PVW have elevated $uLTE_4$, and this falls into the normal range with resolution of symptoms. However, not all symptomatic children have evidence of increased cystLT production, since $uLTE_4$ in the acute phase of PVW was not elevated in the subgroup with low serum IgE suggesting that cystLT is unlikely to be a major mediator in this subgroup. Increased $uLTE_4$ was found for the whole group of hospitalised children with PVW in the present study, because children were skewed towards an increased IgE. The differences seen in this data, may be due to differences in serum IgE in the children studied with cystLT activation reported in some studies[98], and not present in some children studied by Balfour-Lynn[116].

High IgE appeared to predispose to increased cystLT production during acute PVW. There was no confounding effect of atopic sensitisation *per se* on basal levels of $uLTE_4$ since; i) there was no association between IgE and $uLTE_4$ in the normal controls, and in children with acute PVW during the convalescent phase, ii) $uLTE_4$ fell into the normal range during the convalescent phase of PVW with high IgE, and iii) there was no elevation of $uLTE_4$ in the atopic controls. This suggests that either a high baseline IgE, or a mechanism that acutely increases IgE during PVW, or a variable strongly associated with IgE, such as atopic sensitisation, primes inflammatory cells in the lower respiratory tract to increase

cystLT production in PVW. As skin prick tests on children with PVW were not performed, the atopic status of the high IgE subgroup could not be determined. The effect of high IgE on $uLTE_4$ may reflect an increased prevalence of specific sensitisation in this subgroup.

Could the inflammatory pattern of children with acute PVW and high IgE be similar to a viral-triggered acute attack of atopic asthma? To date there are no data comparing mediators in acute non-viral attacks and viral-triggered attacks of atopic asthma. However, similarities are seen in the pattern of $uLTE_4$ in the subgroup of acute PVW with high IgE, and those reported during acute atopic asthma in older children[115]. In both these groups, $uLTE_4$ is elevated in the acute phase, and falls on convalescence. However, in contrast to atopic asthma, $uLTE_4$ in acute PVW falls into the normal range in convalescence[115]. This data therefore suggests that pulmonary cystLT production rapidly switches off after acute PVW, and is similar to that of Stevenson *et al*[129] who found persistence of airway inflammation in children with intermittent atopic asthma, but not in children with a history of "exclusive" viral-triggered wheeze.

No clinical parameters were found, that distinguished children with PVW, who had either increased IgE, or increased $uLTE_4$. In particular, acute PVW with a history of exclusive viral-wheeze was not associated with a different pattern of $uLTE_4$. Castro-Rodriguez *et al* have reported a clinical index, that calculates risk

of developing asthma in young children with recurrent wheezing[30]. By using this index, preschool children with one "major" risk factor (parental history of asthma or eczema) or two of three "minor" risk factors (blood eosinophilia, wheezing without colds, and allergic rhinitis) are 9.8 times more likely to be diagnosed as active asthmatics at 6 to 13 yrs[30]. It remains to be determined whether this index better defines the subgroup of PVW with elevated $uLTE_4$.

Two potential confounding variables remain to be considered. First, steroids attenuate cystLT release from the asthmatic lung[207], and most children had received a single dose of oral steroids prior to urine sampling. It is therefore possible that the lower levels of $uLTE_4$ in the low IgE subgroup could represent a rapid reduction from previously high levels. However, a confounding effect of steroids is unlikely since we found no association between $uLTE_4$ and duration of the sampling from oral steroids, both for the whole group, and the low IgE subgroups (figure 4.7). Thus a major confounding effect of oral steroids is unlikely. The second limitation is that the effect of a viral-cold in preschool children was not studied. It was not possible to measure $uLTE_4$ and IgE in normal children during trivial viral colds, since it was ethically unacceptable to obtain blood for IgE in conscious children, and blood sampling during anaesthesia was not possible during colds, since surgery was postponed. Van Schaik showed that cystLT in nasal secretions were higher in young children with virus induced wheezing compared to children with viral colds[98]. Since no

increase in $uLTE_4$ was found in children with low IgE, an effect of colds *per se* on $uLTE_4$ is therefore unlikely in this subgroup. Increased $uLTE_4$ caused by colds *per se* in the high IgE group remains a significant limitation. Further studies of $uLTE_4$ in normal children with high IgE are required during viral colds to assess the clinical relevance of an increased $uLTE_4$ seen in this study.

In preschool children leukotriene receptor blocker therapy improved persistent wheezy symptoms when compared to a placebo over a 12 week treatment period[208]. Meyer used the data from studies on montelukast and found that there were no characteristics that predicted the response to treatment[209]. Stelmach showed that higher doses of budesonide and montelukast reduced IgE and lung function in asthmatic children[210]. Montuschi showed that exhaled LTE4 was reduced in atopic children with asthma and suggested that this population may be most responsive to montelukast[211].

As this study demonstrated a significant difference in the levels of $uLTE_4$, a similar sample size would be adequate to explore the relationship between cystLT and PVW.

Summary

This study showed heterogeneity in urinary leukotriene E_4 excretion in acute PVW. Elevated urinary leukotriene E_4 occurred in children with the highest serum total IgE, but not in those with low IgE. In further clinical trials of therapies targeted at cystLT, it may be important to determine IgE or $uLTE_4$, or both in order not to overlook a potential subgroup of responders in subsequent attacks.

Chapter 5 Neutrophil activation in preschool wheeze

5.1. Summary

Background: Attacks of wheeze triggered by colds are common in preschool children and epidemiological data suggest that the majority will not develop atopic asthma. Although the inflammatory substrate for PVW is unknown, a role for the neutrophil has been postulated.

Objective: We sought evidence for systemic neutrophil activation in PVW by measuring the expression of neutrophil L-selectin (CD62L), Mac-1 (CD11b), and serum soluble (s) L-selectin.

Methods: Children between 1 and 5 years admitted to hospital with acute viral-wheeze (n=20) and normal controls (n=18) were studied. Adhesion molecule expression on CD16 positive neutrophils was determined by flow cytometry and expressed as molecules of equivalent fluorochrome (MEF). Serum sL-selectin was analysed by ELISA.

Results: Children with viral-wheeze had reduced neutrophil L-selectin expression compared with controls (median MEF (IQR); 69 (11,96) units *versus* 136 (109,163) units, $p < 0.001$). Serum sL-selectin was higher in children with wheeze (2.8 (2.3,3.1) *versus* 2.4 (2.2,2.6) μg/ml, $p < 0.05$). There was no significant difference in neutrophil Mac-1 expression.

Conclusion: Preschool viral-wheeze is associated with shedding of L-selectin from neutrophils and a concomitant increase in soluble L-selectin in the serum. These data are compatible with a role for the neutrophil in the pathogenesis of preschool viral-wheeze.

5.2. Background and aims

The background to this study is described in detail in Chapter 1.4

The neutrophil may be important in the pathogenesis of attacks of atopic asthma triggered by colds and it predominates in the sputum of asthmatic adults with viral colds[124]. Viral colds are also an important trigger for attacks of wheeze in PVW, since an upper respiratory tract virus can be detected in more than 80% of preschool children presenting to hospital with acute wheeze[45]. Despite the common trigger factor, epidemiological studies suggest that PVW is a separate phenotype from atopic asthma with the majority of children described as 'transient' wheezers. The inflammatory profile in PVW may be different from chronic atopic asthma[91].

The role for non eosinophilic inflammation in PVW has recently been further explored by Marguet et al. They performed fibre-opitc bronchoscopy in clinically well defined groups of children one of which was 'infantile wheezers'. Eosinophils clearly characterised asthma but was rarely seen in infantile wheeze. Neutrophils were the predominant cell in more than half of infantile wheezers and the authors identified two sub-populations of infantile wheezers - a group with very few neutrophils and another with significant neutrophil counts[127].

Interleukin-8 (IL8), a potent neutrophil chemoattractant has been shown to be associated with neutrophil activation. We performed an exploratory study to look

at the role of neutrophils in PVW by measuring IL8 in serum. A subgroup of children with acute PVW was identified and a pilot study conducted measuring IL8 in the serum. When this was compared to healthy controls we were unable to identify any difference in levels and hence the plan to extend this study further was dropped.

We then prepared a study with the Department of Immunology, University Hospitals of Leicester, to explore neutrophil activation by flow cytometry. The initial neutrophil activation step can be sought less invasively in PVW, by the measurement of adhesion molecules, L-selectin, (CD62L), Mac-1 (CD11b) on neutrophil surface and sL-selectin in serum.

The aims of this study were therefore

1. To determine whether systemic neutrophil activation occurs in PVW by measuring L-selectin and Mac-1 on systemic neutrophils, and sL-selectin in serum, in children admitted to hospital with PVW.
2. To compare this with a group of normal controls undergoing elective surgery.

5.3. Methods

5.3.1. Patient details

Children (1-5 years) were recruited from those presenting to the admissions unit of the Leicester Royal Infirmary Children's Hospital with PVW, as part of the randomised double blind trial of oral steroids. Controls were recruited from children undergoing elective ear, nose and throat surgery, or ophthalmic surgery. All the children included in this study had the same inclusion and exclusion criteria that are described in the study on eosinophil proteins (Chapter 3), and had parental consent as described before. Blood samples from children with viral wheeze were obtained as described before, and samples from controls were obtained immediately after induction of general anaesthesia. PVW was treated with a single oral dose of prednisolone and 'as required' nebulised salbutamol.

5.3.2. Sample collection and analysis

One ml venous blood sample was collected into an EDTA bottle soon after recruitment in children with PVW, and within 5 minutes of induction of anaesthesia in controls and transported on ice for flow cytometry. One ml of blood was allowed to clot at room temperature for 60 minutes; the serum was separated by centrifugation before storage at –70°C for analysis of sL-selectin. A full blood count was done on a 0.5 mL heparinised sample using routine hospital techniques.

Flow cytometry was conducted in the Department of Immunology, within 30 minutes of sample collection. The antibodies used were flourescein isothiocyanate (FITC) conjugated monoclonal anti-CD16, phycoerythrin (PE) conjugated anti-CD 62L (L-selectin) and PE conjugated anti-CD11b (Mac-1) (Becton, Dickinson and Company, Oxford, UK). Isotype matched antibodies, PE conjugated IgG_2a and FITC conjugated IgG_1 (Becton, Dickinson and Company, Oxford, UK) were used as controls. A 50 µl aliquot each of EDTA blood was added to three tubes placed at 4°C, which already contained either control antibodies or anti-CD16 and anti-CD62L or anti-CD16 and anti-CD11b. After incubation at 4°C for 10 minutes, 1ml of 0.8% ammonium chloride was added to lyse red cells and the samples were transferred to 37°C. Flow cytometry was performed on the samples 10 minutes later (Ortho Cytoron-Absolute, Orthodiagnostic systems, USA). The extent of the background non-specific fluorescence, as defined by the control antibodies was restricted to less than 1% of the total events acquired. Discrimination frames were placed around the granulocyte cluster, which was identified, based on their forward and side scatter characteristics. Neutrophils within this cluster were identified by CD16 positivity[212]. The expression of CD62L and CD11b of at least 5000 events within the neutrophil population was then recorded. The mean fluorescence intensity (MFI) of the gated cell population was obtained as channel numbers and was transformed to molecules of equivalent fluorochrome (MEF) using fluorescent

beads (DAKO Flourospheres, Dako A/S, Denmark) according to the manufacturer's guidelines.

Serum levels of sL-selectin was analysed by ELISA according to the manufacturer's instructions (Bender Med Systems, Vienna, Austria). Serum samples were diluted 1:200, and the assay was performed in duplicate. The lower limit for sL-selectin detection was 0.3ng/ml and the manufacturer's overall intra-assay coefficient variation was 3.7%.

5.4. Statistics and sample size

When this study was planned there was no available data on the expression of adhesion molecules on neutrophils in PVW. In order to prove this concept we planned a small pilot study in a subgroup of children from the cohort of children recruited into the clinical trial. As this study required close interaction with the Department of Immunology and rapid analysis of the blood samples recruitment was difficult and was limited by the study period of a year.

Data are summarised as median and interquartile range (IQR), and compared using the Mann-Whitney U test. Correlations were determined by Spearman rank correlation. Analysis was performed using a package for microcomputers (SPSS for Windows version 10, SPSS Inc, Chicago, IL, USA). Statistical significance was defined as a p value <0.05.

5.5. Results

Twenty children (12 boys, 8 girls) with acute PVW and 18 controls (12 boys, 6 girls) were studied. Controls underwent a variety of surgical procedures including tonsillectomy and adenoidectomy (n=6), insertion of grommets (n=7), bronchoscopy (normal) n=2, squint (n=1), and other (n=2). Compared with controls, children with PVW were slightly younger (median 32 (IQR 18 to 47) months *v* 54 (26 to 63) months, p=0.04). Seventeen (85%) children with PVW had previous attacks with no interval symptoms (median number: 2). Three children with PVW had a history of either mild nocturnal cough or wheeze between attacks. In the subgroup where blood neutrophil numbers were measured, the majority of children with PVW and controls were within the normal range (1-8.5 x 10^9 neutrophils/L). However, the PVW group (n=17) had a higher absolute neutrophil count 8 x 10^9/L (5 to 11) *v* 4 x 10^9/L (3 to 5) p<0.01 (figure 5.1), and increased percentage of CD16 positive neutrophils (99.5% (98.3 to 99.7) *v* 97.1% (94.2 to 97.7), p<0.001).

Neutrophil L-selectin expression in PVW (n=20) was significantly lower than controls (n=18), (MEF: 69 (11 to 96) units *v* 136 (109 to 163) units, p< 0.001, figure 5.2, 5.3). There was no difference in Mac-1 expression between the two groups (MEF: 934 (722 to 1287) units *v* 730 (571 to 1000) units, figure 5.4, 5.5). Neutrophil L-selectin expression was not associated with age in either PVW or controls (NS by Spearman rank correlation). sL-selectin was detected in the sera

of all children (PVW, n=15; controls n=16), and was higher in PVW (2.8 (2.3 to 3.1) μg/ml *v* 2.4 (2.2 to 2.6) μg/ml, $p< 0.05$, figure 5.6). There was no association between time from oral steroid therapy and L-selectin expression in the PVW group (NS, Spearman rank correlation, figure 5.7).

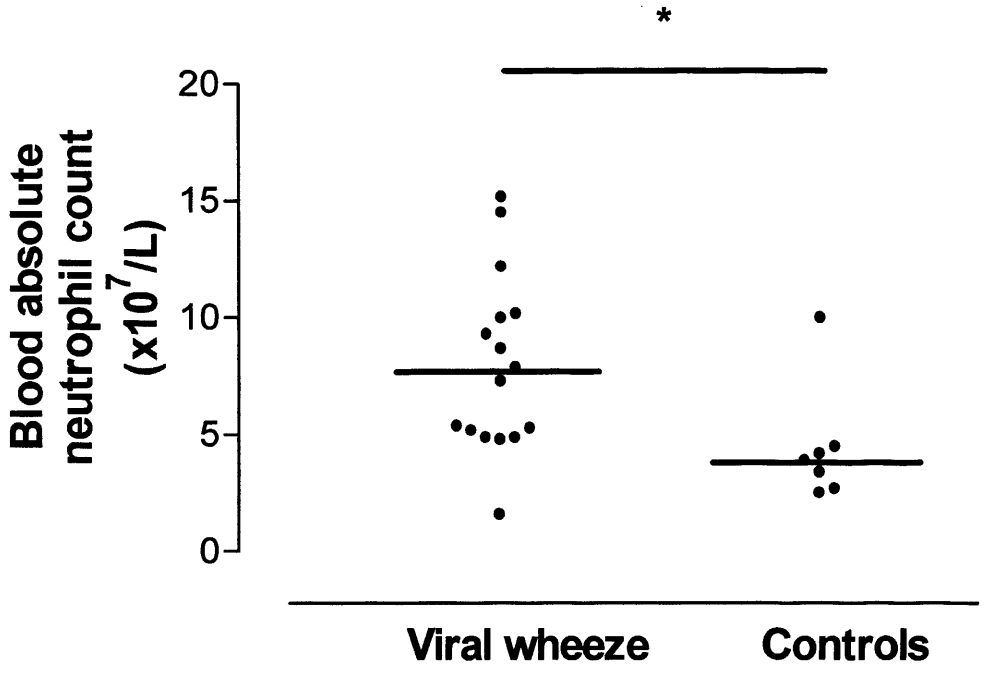


Figure 5.1 The absolute neutrophil count in the blood in acute preschool viral-wheeze and controls. *p<0.01, by Mann-Whitney U test. Bars represent medians.

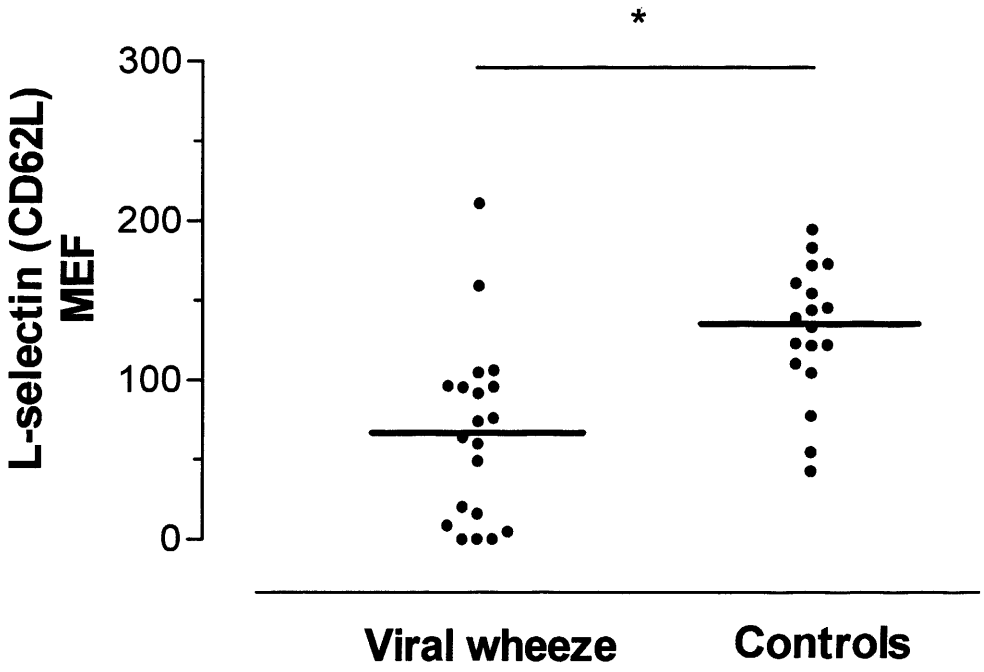
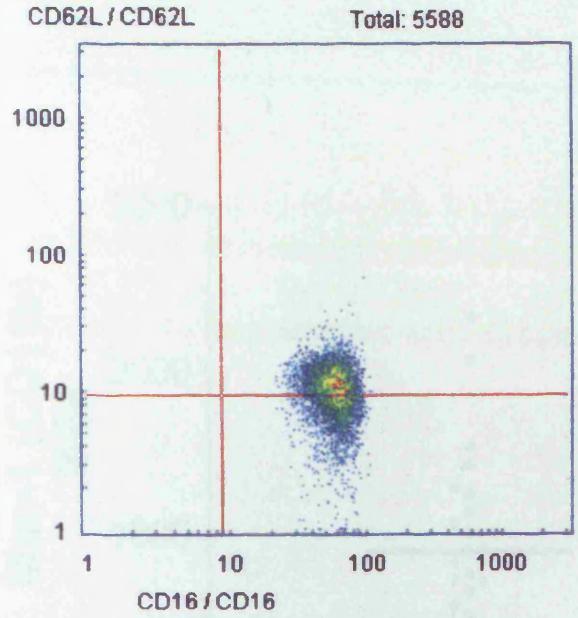
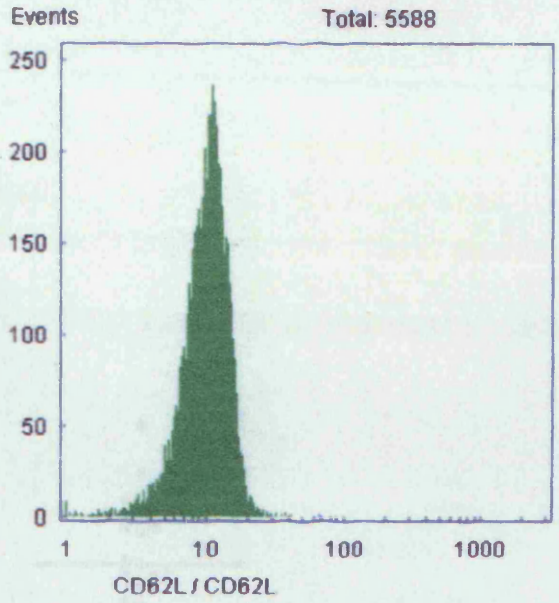


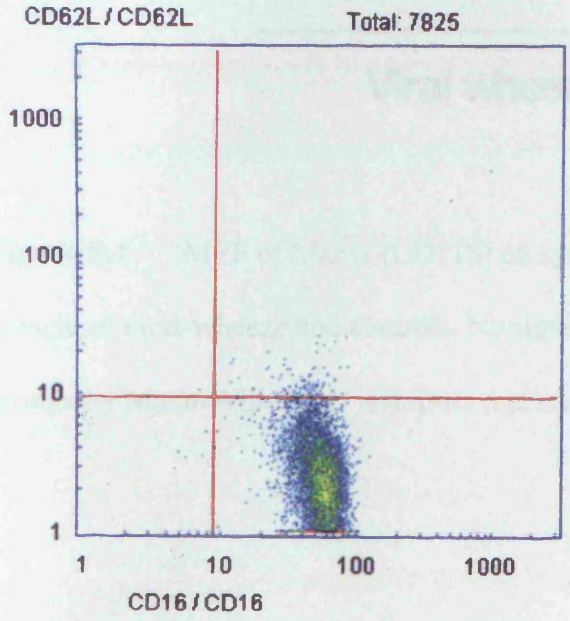
Figure 5.2 Molecules of Equivalent fluorescence (MEF) of L-selectin (CD62L) on systemic neutrophils in acute preschool viral-wheeze and controls. *$p<0.001$, by Mann-Whitney U test. Bars represent medians.



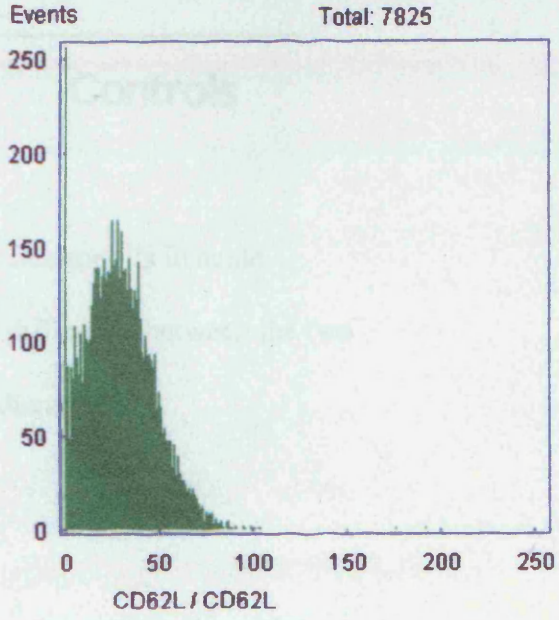
a) CD62L – normal expression in a control child



b) MFI of gated cells – normal in control child



c) CD62L - reduced expression in PVW



d) MFI of gated cells - reduced in PVW

Figure 5.3 Flow cytometry picture of L-selectin (CD62L) expression on CD 16 positive neutrophils; (MFI – mean fluorescence intensity)

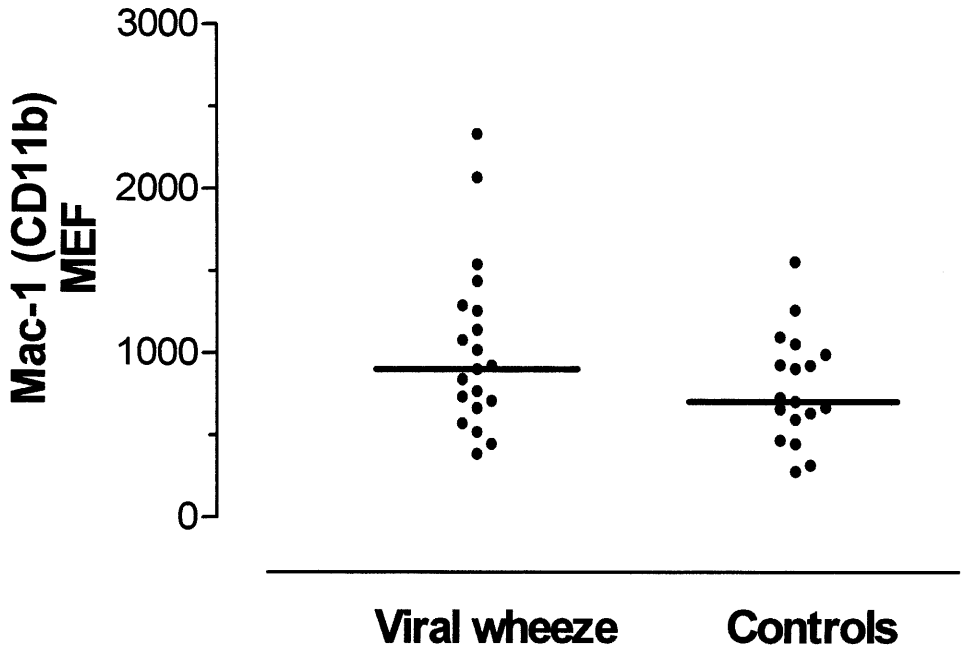
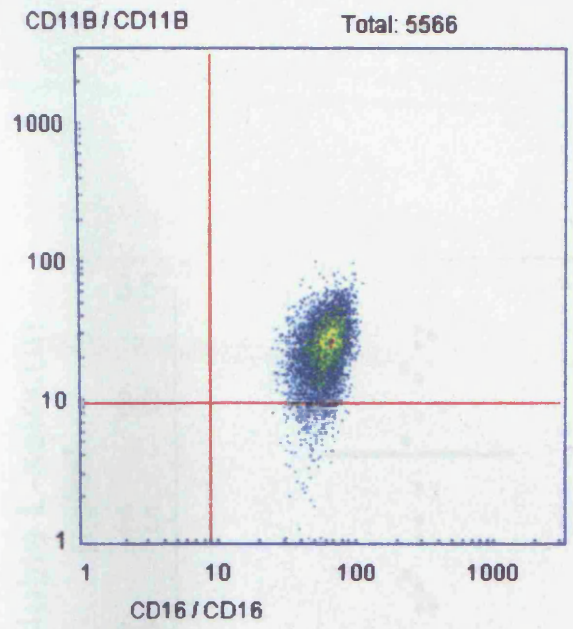
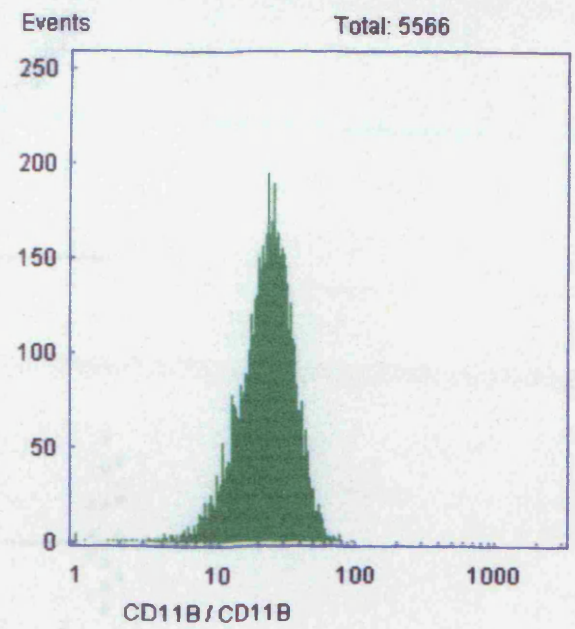


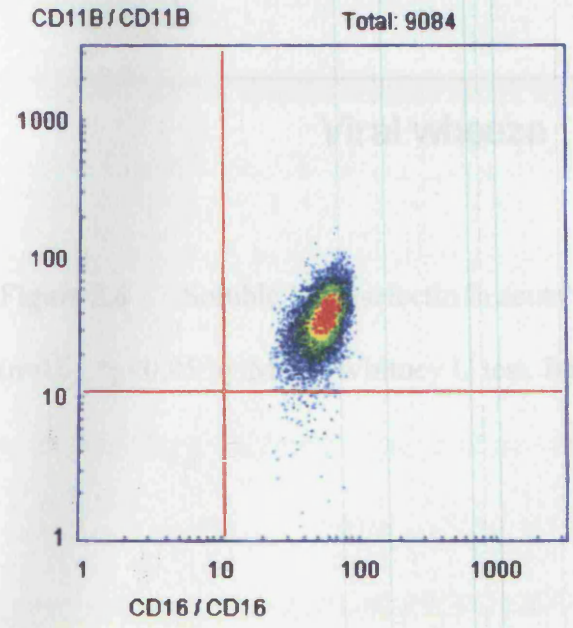
Figure 5.4 MEF of Mac-1 (CD11b) on systemic neutrophils in acute preschool viral-wheeze and controls. No significant difference between the two groups by Mann-Whitney U test. Bars represent medians.



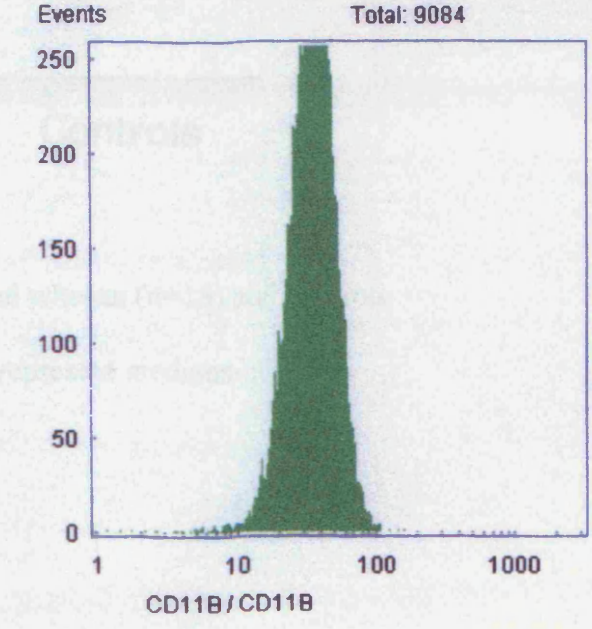
a) CD11b - normal expression in a control child



b) MFI of gated cells – normal in a control child



c) CD11b - expression in PVW



d) MFI of gated cells - in PVW

Figure 5.5 Flow cytometry picture of Mac-1 (CD11b) expression on CD 16 positive neutrophils; (MFI – mean fluorescence intensity)

Figure 5.6 Soluble (s) L-selectin in acute viral wheeze (n=15) and controls (n=16). *p<0.05 by Mann-Whitney U test. Bars represent medians.

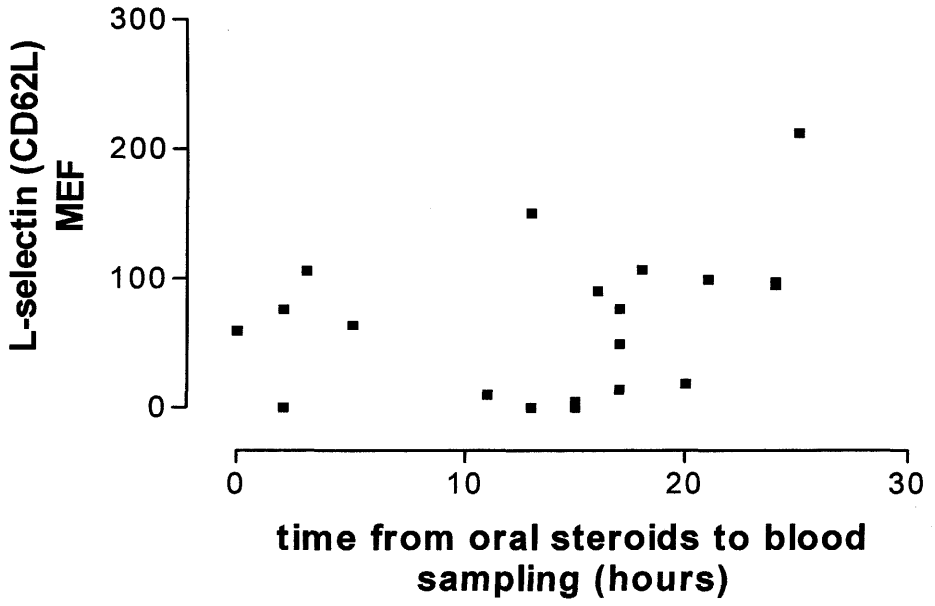


Figure 5.7 Scatterplot of the association between MEF of L-selectin on systemic neutrophils during acute PVW and time of blood sample after oral steroid therapy showing no correlation (r_s=0.4, p=NS; spearman rank)

5.6. Discussion

This study shows that the expression of L-selectin is reduced on systemic neutrophils, and serum sL-selectin is increased in clinically severe PVW. These data are compatible with generalised neutrophil activation and shedding of L-selectin from the neutrophil surface into the systemic circulation[213].

There was no increase in Mac-1 expression. Davey showed that *in vitro* stimulation of neutrophils showed an initial upregulation and later a time dependent apparent loss of the Mac-1 receptor[214]. Further analysis showed that mild stimulation caused upregulation, but strong stimulation caused a subsequent epitope loss into the supernatant solution thereby showing an apparent downregulation[214].

It is possible that the pattern of change in adhesion molecules that is described can occur in both viral triggered atopic asthma in adults and PVW. However, previous studies have reported no association between systemic neutrophil activation and the clinical severity of atopic asthma. For example, in 't Veen and colleagues[135]found no difference in neutrophil L-selectin expression between normal (non-atopic) controls and adults with either mild or severe atopic asthma. Furthermore, Oymar *et al* [138] compared serum sL-selectin in atopic asthmatic children and healthy controls, and found no increase in sL-selectin during an acute wheezy attack.

This study showed a subtle increase in the systemic neutrophil count in PVW similar to experimental colds in asthmatic adults[121]. Whether colds *per se* induce L-selectin shedding is unknown. Although this study shows the first activation step in the development of pulmonary neutrophilia, the direct implication of the neutrophil in the pathogenesis of PVW is not possible. In a study on primary autoimmune neutropaenia, a condition associated with low neutrophil counts, the incidence of asthma was found to be lower than controls[215].

There are several limitations to this study. First, trivial symptoms associated with the common cold may be associated with a similar pattern of neutrophil activation. It was not possible to study the most appropriate control group, healthy children with colds, due to ethical reasons. In a small group of children with rhinovirus colds, BAL fluid neutrophilia[120] has previously been reported, which may indicate that some neutrophil transmigration may occur with trivial colds. However in infants with RSV bronchiolitis, Wang *et al* [130] found no change in L-selectin expression in systemic neutrophils, thus providing indirect evidence that this may not be inevitable in colds. Thus children with PVW may have a propensity to recruit more neutrophils in their airways, and that neutrophil products trigger bronchoconstriction either directly, or by activation of other inflammatory cells, as has been shown in my leukotriene study[216]. Further studies in PVW are required to study the extent of neutrophil activation during colds.

A second potential confounder is the effect of steroids. Steroids decrease the expression of L-selectin on circulating neutrophils[217], an effect of steroids that occurs after a "lag phase" of eight hours. Decreased expression is not caused by shedding from the neutrophil surface, but the release of L-selectin 'low' expressing neutrophils from the bone marrow[218] and as neutrophils are not activated by corticosteroids, serum sL-selectin remains unchanged[217]. Since there was no correlation between L-selectin expression and steroid administration, and sL-selectin levels were raised, it is unlikely that steroid therapy is a major confounder. A confounding effect of atopy is also unlikely, since previous studies have found no affect of atopic sensitisation on neutrophil L-selectin expression or serum sL-selectin[135 138]. There are no data on the effects of anaesthetic agents on paediatric neutrophil L-selectin expression. In the controls, the very short period from induction of anaesthesia to sampling should have minimised any effect. Furthermore in adults, when neutrophils are stimulated by fMLP *in vitro*, ketamine further decreases L-selectin expression[219]. If anaesthesia had activated neutrophils in the control group, this would have reduced rather than increased the difference in L-selectin expression.

Summary

Acute PVW is associated with reduced levels of L-selectin on systemic neutrophils. Elevated levels of sL-Selectin suggest that the L-selectin reduction is in part due to its shedding into the systemic circulation. This data is compatible with a significant role for neutrophils in the pathogenesis of PVW.

Chapter 6. Efficacy of a short course of parent initiated oral prednisolone for acute PVW: randomised controlled trial

6.1. Summary

Background: Episodic wheeze triggered by viral colds is common in children aged between 1 and 5. The majority of affected children are asymptomatic by school age. Persistence of wheeze is associated with increased systemic eosinophil priming. Current guidelines suggest the use of parental-initiated oral prednisolone to be started at the first sign of PVW. However, the evidence to support this therapeutic strategy approach is conflicting. We therefore aimed to assess the efficacy of a short course of oral prednisolone for PVW, with stratification for systemic eosinophil priming.

Methods: Children admitted to hospital with PVW were allocated to a high-primed- and low-primed stratum by serum eosinophil cationic protein and eosinophil protein X, and randomised to receive parent-initiated prednisolone (20 mg once a day for 5 days) or placebo for the next episode of PVW. The primary trial outcome was the mean day-time and night-time respiratory symptom score over 7 days.

Results: 108 children were randomised to placebo and 109 to prednisolone. Outcome data were available for 121 of 153 (79%) children who had a further episode of PVW, of whom 52 received prednisolone and 69 placebo. There was no difference in mean day-time and night-time respiratory symptom score, and

need for hospital admission between the treatment groups. Analysis within the high-primed (n=59) and low-primed (n=62) stratum showed no difference between treatment groups.

Conclusion: There is no clear benefit of a short course of parent-initiated oral prednisolone for PVW. Children with increased eosinophil priming do not constitute a steroid-responsive subgroup.

6.2. Background and aims

The background to this study is described in detail in Chapter 1.4

Episodes of viral-triggered wheezing are common in children aged 1 to 5 years and in this respect, PVW is similar to atopic asthma in the school-age child. However atopic asthma in school age children is typically the classic atopic variant which is characterised by pulmonary eosinophilia, and a propensity of systemic eosinophils *in vitro* to release eosinophil cationic protein (ECP) and eosinophil protein X (EPX)[2 195]. By contrast, asthma in children between 1 to 5 years of age is characterised by recurrent, transient episodes of wheeze trigged by viral-colds, labeled previously as wheezy bronchitis and now as PVW[185]. There is now indirect evidence that the inflammatory substrate of PVW is separate from atopic asthma. The majority of children with PVW who do not have the risk factors for atopic sensitization[7] and have IgE levels that are similar to non wheezing children[197], become asymptomatic by 6 years of age[5]. In the minority of children in whom wheezing persists beyond 6 years, the characteristic risk factors for atopic asthma[197] and increased systemic eosinophil priming[23 24] is seen. Increased serum ECP in preschool children with episodic wheeze, has been shown to be predictive for diagnosis of current asthma at a two year follow up[198].

Acute PVW is a transient condition triggered by a viral cold and is treated by inhaled bronchodilators[220], and an additional strategy, is to start a short course of

systemic corticosteroids at the first sign of PVW, with the aim of preventing progression to severe wheeze[199]. However, the evidence that corticosteroids started by parents in the community, during the early stages of PVW improve clinical outcome, is conflicting.

The aims of this study, therefore were as follows

1. To assess the efficacy of a short course of oral prednisolone, initiated by parents for PVW.
2. To stratify the patient population by systemic eosinophil priming (by measuring serum ECP and serum EPX) and further assess efficacy of oral prednisolone in a subgroup at increased risk for atopic asthma.
3. To explore and compare the clinical profile of children during the acute wheezy episode based on their systemic eosinophil priming status.

6.3. Methods

6.3.1 Patients

Children aged between 1 and 5 years of age, who were admitted with PVW to the University Hospitals of Leicester NHS Trust Children's Hospital between June 1999 and June 2002 were included in this study. PVW was defined as an acute episode of wheeze that occurred within two days of the onset of coryzal upper respiratory tract symptoms. The inclusion and exclusion criteria are as described in Chapter 3. Parents were approached within 24 hours of admission and a written information sheet explaining the study was given to them. The presence of wheeze was confirmed by auscultation and clinical evidence of rhinitis was sought. After allowing for a period of reflection, informed consent was obtained. The supervising paediatrician confirmed that parents understood the trial, and countersigned the consent form. The study had Leicestershire Health Research Ethics Committee approval.

6.3.2. Interpretation of wheeze

Wheeze was defined as a whistling sound from the chest. In preschool children, the assessment and interpretation of wheeze is dependent on parental reporting and associated symptoms of a wet cough and rattle has been shown to have a high sensitivity[221] and a recent review showed that parental understanding of wheeze varies considerably and has an impact on the prevalence of asthma[222].

All the children recruited to this study presented to hospital and were seen by health professionals. All children had symptoms of an URTI and the presence of wheeze was elicited by auscultation by a physician. The symptoms of the viral cold, pattern of cough and wheeze were discussed in detail with the family and they were requested to complete a symptom diary exactly similar to the diary, which was to be used for the future wheezy episode. The research nurse visited the family at home 6 weeks later, and when the diary was collected she once again reiterated symptoms of a an URTI, wheezing. She then dispensed the trial medication and gave parents a new symptom diary. All parents were asked to start the trial medication at the onset of cough and wheeze with the next viral cold and contact the trial centre if they required further clarifications. Although wheeze was not documented at the trial episode of wheeze, parents were suitably trained to pick up the sequence of symptoms of URTI, cough and wheeze.

6.3.3. Procedures

A double-blind, randomised design, placebo controlled design, with stratification for systemic eosinophil priming, was used. Study numbers were assigned sequentially and randomisation within strata was achieved by generating numerical codes in random permuted blocks of 10. Randomisation and packaging of placebo and prednisolone was done by Nova Laboratories Ltd, Leicester, UK. Trial medication was identical capsules containing white powder, and were in identical containers labeled only with the subject number. The

research nurses, clinicians and parents were blinded to treatment allocation at all times. The randomisation envelope was broken only after all the data had been entered into the computer file.

6.3.4. Symptom diary

After consent, a venous blood sample was obtained and parents were provided with a preschool respiratory symptom diary card[178]. Symptom diaries in this group of children are difficult to validate against objective data. We hence used a symptom diary which has been used within our department before, and was noted to be reliable, easy to use by parents and reflected the clinical significance of symptoms[185]. This diary detected symptoms of an URTI and cough and wheeze and further explored the severity of symptoms by analysing the limitation of normal activities, disruption of sleep and use of bronchodilator medications[223]. On a scale of 0 to 3, the severity of night-time symptoms, day-time symptoms and disruption of day-time activity was recorded once daily for 7 days (appendix). Parents chose the score that best described symptom severity, and recorded inhaled medication frequency.

6.3.5. Sample collection and analysis

2 mL venous blood samples were collected in a Vacutainer™ SST tubes and allowed to clot for 60 minutes at 22 °C in a temperature-controlled container. Samples were then centrifuged at 1350g for 10 minutes, the serum was separated

and aliquoted as 120μL samples. The serum was immediately frozen and stored at -70^0C.

Radioimmunoassay

sECP and sEPX was analysed by using a specific double antibody radioimmunoassay (Pharmacia AB Diagnostics, Uppsala, Sweden). The samples were thawed, vortexed and then used for the assay. The assay was done in duplicate and the mean values were taken. The detection limit of the assay was <3μg/L, the within assay coefficient of variation was <5%, and the between assay coefficient variation was <10%. The values were measured as μg/L.

6.3.6. Stratification

After discharge, and before randomisation, children were stratified into a 'high-primed' stratum (serum ECP ≥ 20 μg/L *or* EPX ≥ 40 μg/L, or a combination of both), and a 'low-primed' stratum (serum ECP < 20 μg/L *and* EPX < 40 μg/L). An ECP level of 20 μg/L was chosen since it most accurately predicts wheeze persistence[198 224]. An EPX level of 40 μg/L was chosen since it is equivalent to 20 μg/L ECP by linear regression[67]. Both these eosinophil proteins were used to assess the relationship between serum ECP and EPX and leading from this, to explore the relationship between serum and urine EPX which could have clinical significance in the future.

Within 6 weeks of discharge from hospital, parents were visited by the research nurse and issued with placebo or 20 mg prednisolone capsules, one to be taken orally once a day for 5 days at the start of the next episode of PVW. A further episode of PVW was defined as wheeze occurring within 2 days of the onset of coryzal upper respiratory tract symptoms. Parents were also asked to start 'as required' inhaled salbutamol (to a maximum of 400 μg, 4 hourly, by metered dose inhaler and Volumatic™ spacer), and to record respiratory symptoms in the diary for 7 days. If the child were to become more unwell during the PVW, they were advised to be reviewed by their paediatrician or general practitioner. The trial medication could be substituted with oral prednisolone by the physician, if deemed clinically necessary. If a child was admitted to hospital, the parents were instructed to continue to record symptoms and bronchodilator usage. Children who did not have an episode of PVW within 12 months of randomisation were withdrawn from the trial.

The primary outcome measure was the mean day-time and night-time respiratory tract symptom score over a 7 day period. The secondary outcome measures were mean daily salbutamol actuations, need for hospital admission, and requirement for substitution of the trial medication with oral prednisolone.

6.4. Statistical analysis

When the study was initially planned, power calculation was done without knowledge of symptom scores in the study population. Power was then recalculated from the standard deviation of the mean day-time and night-time symptom score over 7 days from a local population of children with PVW. With 50 children in each treatment arm the detectable difference is 0.34 assuming the standard deviation of mean 7 day-time and night-time symptom score equal to 0.6, at 5% significance level and 80% power. We also wanted to detect a difference in the treatment effect between the high and low-primed strata. Assuming a 5% significance level, power of 80%, a standard deviation of mean day- or night-time symptom score over 7 days of 0.6 and an equal distribution of subjects in the high- and low-primed strata, this leads to a detectable difference of 0.69. Differences between the treatment groups for continuous outcomes were assessed by obtaining the mean difference, with a 95% confidence interval. The mean number of daily salbutamol actuations was log-transformed prior to analysis as the data were positively skewed. The treatment effect thus refers to the ratio of geometric means for this outcome. If there were less than 5 valid observations for the day or night-time symptom score (day or night), or for secondary outcome data, the values were coded as missing.

Differences between treatment groups for categorical data was reported as difference in proportions, with 95% confidence intervals, or Fisher's exact test

when there were small cell frequencies. When investigating if there was a treatment difference between the high- and low-primed strata, a regression model was fitted, and the interaction between treatment groups (prednisolone and placebo) and strata (high- and low-primed) assessed. For continuous outcomes, this was assessed by fitting a linear model with normal errors. For categorical outcomes, this was assessed by fitting a logistic regression model. The primary and secondary end-points between the placebo- and prednisolone-treated groups were first compared followed by within strata analysis.

6.5. Results

708 children were admitted to hospital with acute lower respiratory tact symptoms during the study period and they were screened for eligibility to be entered into the study. The medical notes of these children were reviewed and 345 children were excluded. At review of notes, many children were found not to have PVW as the cause of their lower respiratory tract symptoms. Many children were excluded based on the other study criteria and this included children who presented to hospital during weekends and other periods when research nurse cover for recruitment was not available. The possibility of this population causing bias to the generalisability of the results of the trial was therefore minimal.

The parents of 363 children with PVW were approached by the research nurse and 130 parents did not wish to participate their children in the study. Consent was obtained from the remaining 233 parents. A blood sample could not be obtained from 8 children (figure 6.1), and a total of 225 children with acute PVW were entered into the study; 110 in the high-primed eosinophil stratum, and 115 in the low-primed stratum (figure 6.1).

Eight children were withdrawn prior to randomisation, and 108 were randomised to receive placebo and 109 to receive prednisolone (table 6.1). Baseline characteristics were similar in; i) children allocated to receive placebo and

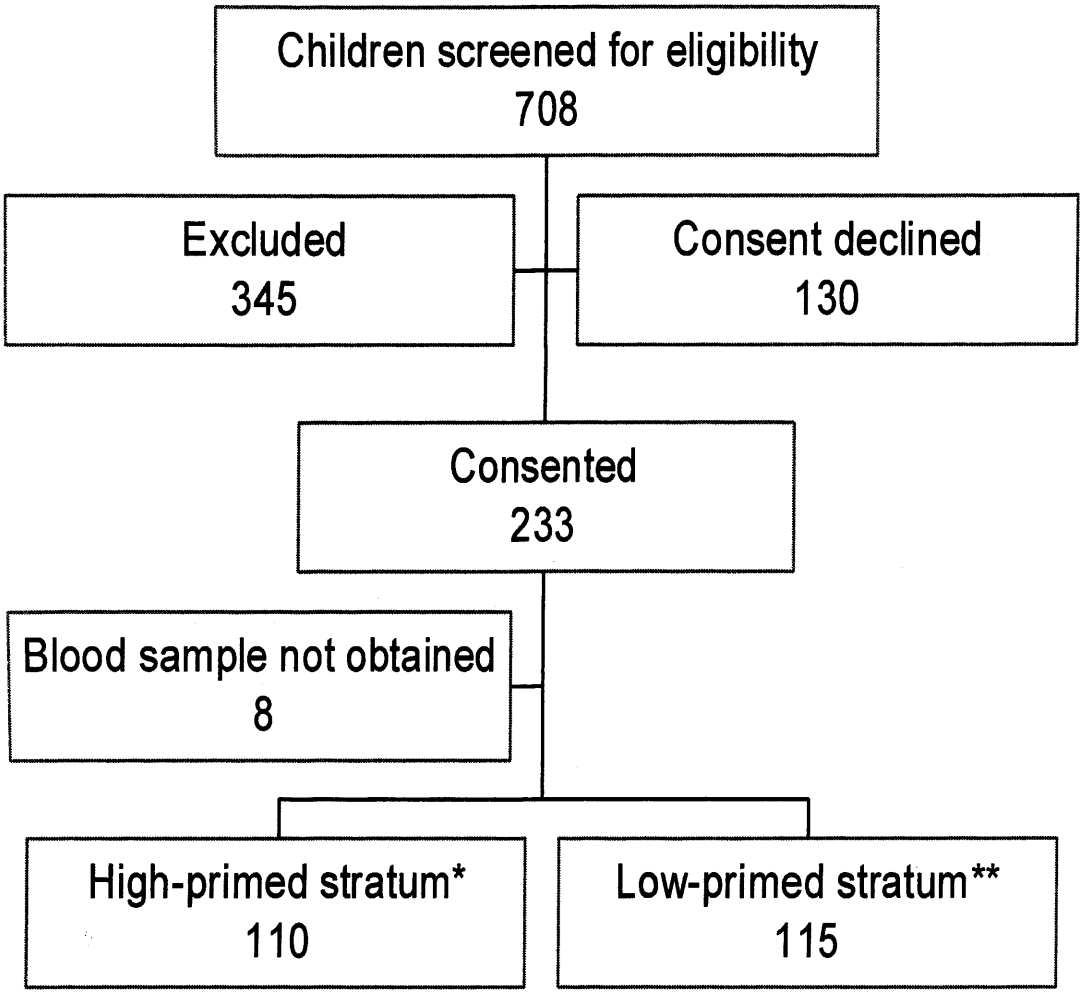
prednisolone (table 6.1), and ii) between children included and not included in the primary outcome analysis (table 6.1). There was a correlation between sECP and sEPX in acute PVW (figure 6.3), and the characteristics of children in the high-primed and low-primed eosinophil strata were similar (table 6.2).

Thirty two parents of 153 (68%) children, who had a further episode of PVW, either did not give the trial medication, or did not fill in the diary (figure 6.2a,b). Secondary outcome data only was available for one child. The mean day-time respiratory symptom score over 7 days was calculated for 120 children, 51 of whom received oral prednisolone, and 69 received placebo. There was no difference in mean day-time symptom score over 7 days between the placebo- and prednisolone-treated groups (difference in means = -0.01, 95% CI; -0.22 to 0.2, table 6.3). The night-time score was completed in 117 children, and no difference was detected between the placebo- and prednisolone-treated groups (difference in means = 0.1, 95%CI -0.12 to 0.32, table 6.4).

For secondary outcomes, the geometric mean number of salbutamol actuations per day was similar for the two treatment groups (table 6.5), as was the need for steroid substitution (table 6.6). There was a trend for increased hospitalisation in the prednisolone-treated group (3 vs 12%, p=0.06, table 6.7), but there was no clear parental preference for placebo or prednisolone, in those who expressed an opinion (table 6.8).

Outcome data were available for 59 children stratified into the high-primed eosinophil strata, and 62 in the low-primed strata (figure 6.2a,b). For primary outcomes, and all secondary outcomes, there were similar treatment effects for the high- and low-primed eosinophil strata, and all formal comparisons of the two treatment groups were not statistically significant (tables 6.3-8).

Figure 6.1. Trial profile to the point of stratification for eosinophil priming



* serum ECP $\geq$ 20 μg/L, or EPX $\geq$ 40μg/L, or a combination of both

** serum ECP < 20μg/L and EPX < 40 μg/L

Figure 6.2a Trial profile in the high-primed eosinophil stratum

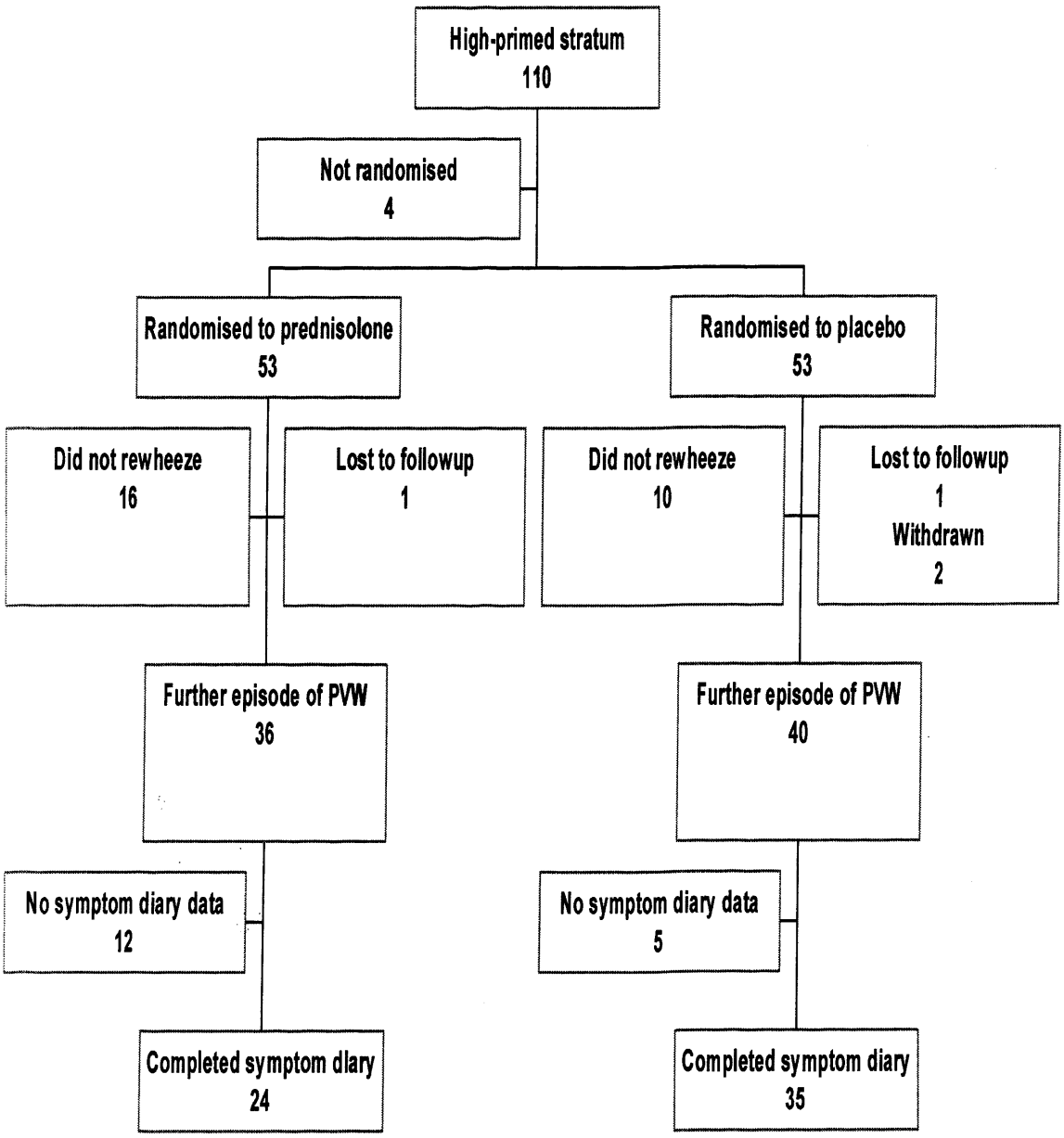


Figure 6.2b Trial profile for the low-primed eosinophil stratum

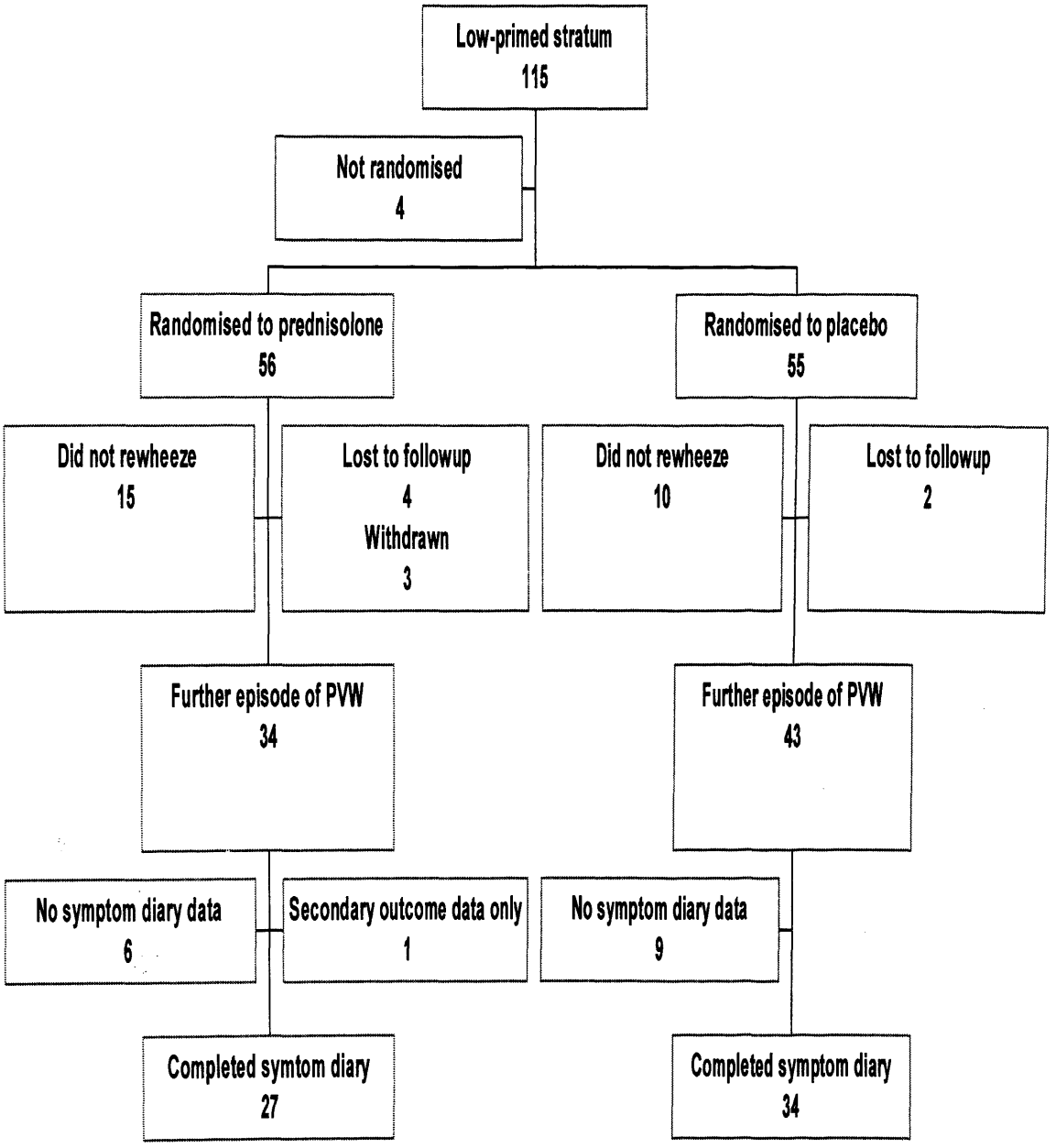


Table 6.1 Characteristics of children randomised to receive the prednisolone and placebo.

	Prednisolone			Placebo		
	all (n=109)	analysed[+] (n=52)	not analysed (n=57)	all (n=108)	analysed (n=69)	not analysed (n=39)
age (mo)	25 (17 to 37)	25	24	27 (19 to 38)	25	30
male (n)	70 (64%)	31	39	77 (71%)	47	30
doctor-diagnosed asthma (n)	35 (32%)	16	19	35 (32%)	25	10
doctor -diagnosed eczema (n)	41 (38%)	17	24	36 (33%)	27	9 *
family history of asthma (n)	84 (77%)	42	42	80 (74%)	51	29
inhaled steroids (n)	30 (28%)	18	12	31 (29%)	23	8
previous wheeze or cough without colds (n)	27 (25%)	10	17	33 (31%)	22	11
age at first wheeze (mo)	12 (6 to 21)	12	12	12 (8 to 23)	12	14
previous PVW episodes (n)	3 (1 to 6)	2	3	3 (1 to 5)	2	3
serum ECP (μg/L)	16 (10 to 25)	15	16	16 (10 to 26)	16	16
serum EPX (μg/L)	29 (19 to 42)	26	32	31 (21 to 42)	32	30

Data are given as median (interquartile range) unless indicated. [+] One child analysed for secondary outcome only *p = 0.05 by χ^2 test *vs* "included in analysis". p=NS for all other comparisons for "included" *vs* "not included". ECP;eosinophil cationic protein. EPX; eosinophil protein-X

Table 6.2 Characteristics of children in the high-primed and low-primed eosinophil strata.

	High-primed* n=110	Low-primed** n=115
age (mo)	29 (20 to 41)	23 (16 to 33)
male (n)	69 (63%)	86 (75%)
doctor-diagnosed asthma (n)	71 (65%)	81 (70%)
doctor-diagnosed eczema (n)	45 41%)	36 (31%)
family history of asthma (n)	85 (77%)	85 (74%)
smoking in the home (n)	47 (43%)	55 (48%)
inhaled steroids (n)	36 (33%)	27 (23%)
Previous history of wheeze or cough without colds (n)	36 (33%)	26 (23%)
age at first wheeze (mo)	15 (8 to 24)	12 (6 to 19)
previous PVW episodes (n)	3 (1,5)	3 (1,6)
serum ECP (μg/L)	26 (1 to 36)	11 (8 to15)
serum EPX (μg/L)	42 (31 to 54)	22 (15 to 30)

*serum ECP $\geq$ 20 μg/L, or EPX $\geq$ 40μg/L, or a combination of both;

** serum ECP < 20μg/L and serum EPX < 40 μg/L.

Data are given as median (interquartile range).

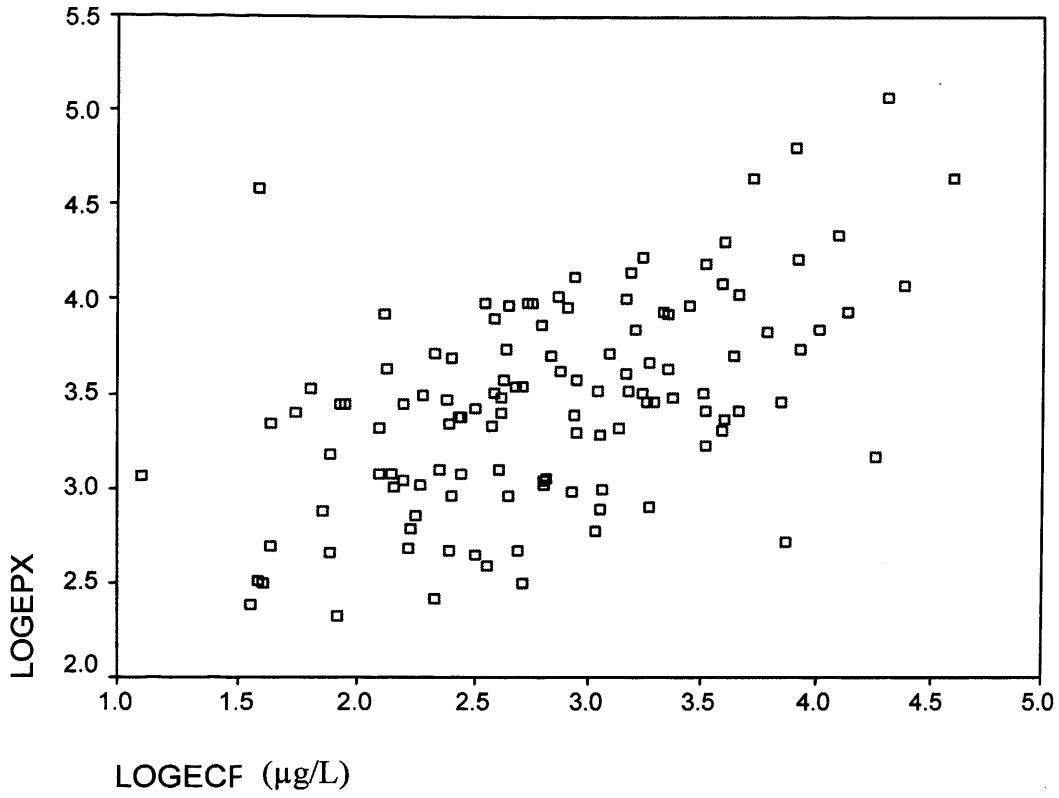


Figure 6.3. Scatterplot showing a moderate correlation between (log transformed) serum ECP (µg/L) and serum EPX (µg/L) in acute PVW (r_s=0.53, Spearman correlation)

Table 6.3 Comparison of day-time symptom score over 7 days.

		n	Mean (SD)	Difference (95% CI)	Test for interaction (P-value)
All	prednisolone	51	0.95 (0.56)	-0.01 (-0.22 to 0.20)	
	placebo	69	0.96 (0.59)		
high-primed	prednisolone	24	0.92 (0.51)	-0.01 (-0.30 to 0.28)	0.96
	placebo	35	0.92 (0.57)		
low-primed	prednisolone	27	0.97 (0.60)	-0.02 (-0.33 to 0.29)	
	placebo	34	0.99 (0.61)		

Data log transformed before analysis; Difference represents the ratio of geometric means. SD; standard deviation. CI; 95% Confidence Interval

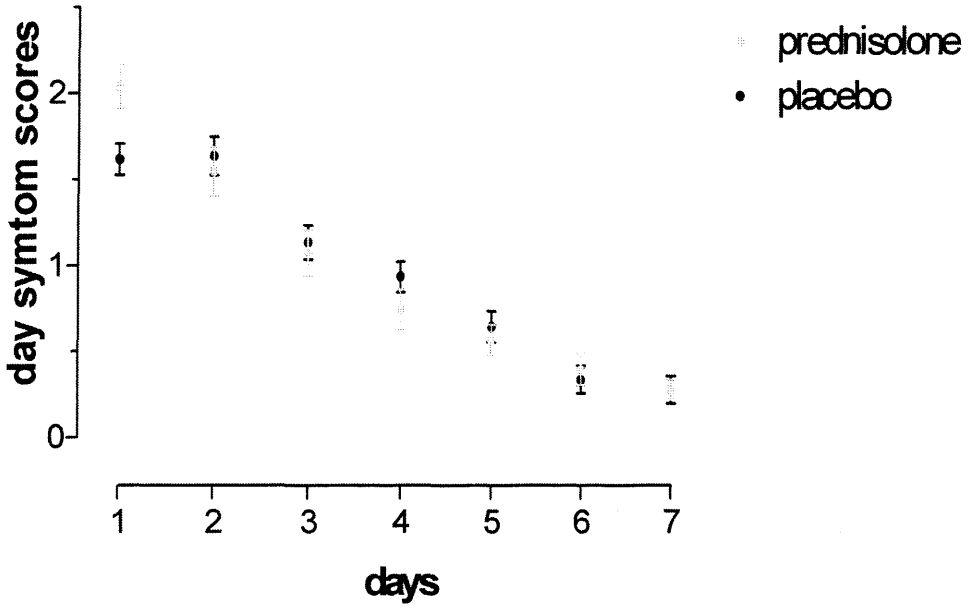


Figure 6.4. Mean day-time respiratory symptom scores for the trial episode of acute PVW, was similar in the prednisolone and placebo groups.

Table 6.4 Comparison of night-time symptom score over 7 days.

		n*	Mean (SD)	Difference (95% CI)	Test for interaction (P-value)
All	prednisolone	50	0.92 (0.58)	0.10 (-0.12 to 0.32)	
	placebo	67	0.82 (0.61)		
high-primed	prednisolone	23	0.96 (0.62)	0.16 (-0.17 to 0.48)	0.63
	placebo	34	0.80 (0.59)		
low-primed	prednisolone	27	0.89 (0.54)	0.05 (-0.26 to 0.36)	
	placebo	33	0.84 (0.63)		

Data log transformed before analysis; Difference represents the ratio of geometric means. SD; standard deviation. CI; 95% Confidence Interval

*night time score not completed; n=3

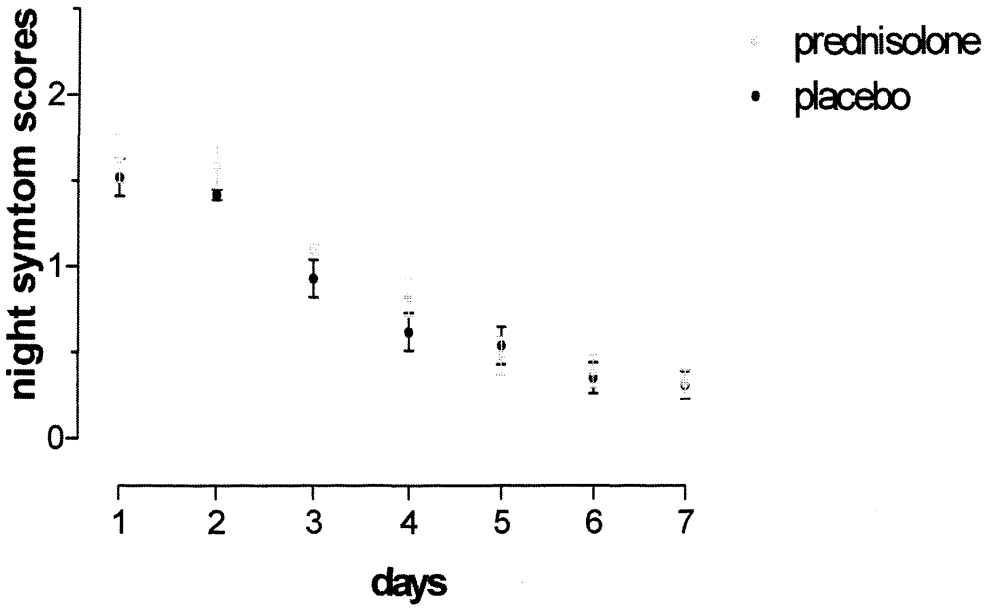


Figure 6.5. Mean night-time respiratory symptom scores for the trial episode of acute PVW, was similar in the prednisolone and placebo groups.

Table 6.5 Comparison of mean number of salbutamol actuations per day.

		n	Log mean (SD)	Ratio of geometric means (95% CI)	Test for interaction (P-value)
All	prednisolone	50	1.59 (0.87)	0.93 (0.65 to 1.32)	
	placebo	67	1.66 (0.99)		
high-primed	prednisolone	23	1.51 (0.75)	0.92 (0.58 to 1.48)	0.99
	placebo	34	1.59 (0.96)		
low-primed	prednisolone	27	1.66 (0.54)	0.92 (0.53 to 1.58)	
	placebo	33	1.75 (1.04)		

Data log transformed before analysis; Difference represents the ratio of geometric means. SD; standard deviation. CI; 95% Confidence Interval

Table 6.6 Comparison of children who required substitution of trial medication with oral prednisolone

	Placebo	Prednisolone	Difference (95%CI)	p-value*	Test for interaction (P value)
All	8/69 (12%)	9/52 (17%)	-5.7% (-18.4% to 7%)	0.37	
high-primed	4/35 (11%)	3/24 (12%)	-1.1% (-18.0% to 15.8%)	0.90	0.57
low-primed	4/34 (12%)	6/28 (21%)	-9.7% (28.3% to 9.0%)	0.30	

*χ^2 test; CI; 95% Confidence Interval

Includes one child in whom the secondary outcome only was recorded

Table 6.7 Comparison of need for hospitalisation

	Placebo	Prednisolone	Difference (95%CI)	p-value*
All	2/69 (3%)	6/52 (12%)	-8.6% (-18.2% to 0.9%)	0.058
high-primed	0/35 (0%)	3/24 (12%)	not done	0.062*
low-primed	2/34 (6%)	3/28 (11%)	not done	0.65*

*Fishers exact test; CI; 95% Confidence Interval

Includes one child in whom the secondary outcome only was recorded

Table 6.8 Comparison of parents who considered the treatment to be effective (of those who expressed an opinion)

	Placebo	Prednisolone	Difference (95%CI)	p-value *	Test for interaction (P value)
All	21/33 (64%)	17/23 (74%)	10.3% (-34.6% to 14.0%)	0.42	
high-primed	11/17 (65%)	9/13 (69%)	-4.5% (-38.4% to 29.3%)	0.79	0.56
low-primed	10/16 (62.%)	8/10 (80%)	-17.5% (-51.8% to 16.8%)	0.35	

*χ^2 test; CI; 95% Confidence Interval

Includes one child in whom the secondary outcome only was recorded

6.6. Discussion

My results show that, in children who had presented with a previous severe episode of PVW, a five day course of oral prednisolone, when initiated by the parents at home at the start of the *next* episode of wheezing, had no effect on the day and night-time lower respiratory tract symptom score, requirement for inhaled salbutamol, or need for hospital admission. The 95% CI would suggest that a significant difference has not been missed. Furthermore, there was no evidence for a beneficial effect of prednisolone in the subgroup of children with increased systemic eosinophil priming.

These results differ from those of Brunette and colleagues[172] who followed a group of children with a history of severe episodes of PVW. Parents at the first sign of a cold gave a single dose of prednisolone, during the 'treatment' year. Although this study was not placebo controlled and used historical controls, compared to the previous year, there was a 65% decrease in the number of wheezing days, and a 90% fall in the need for hospitalisation during the steroid-treated period[172]. In this study, however prednisolone was started by parents at the first sign of wheeze, which may, in part, explain the different conclusions. This data is compatible with the results of Webb and colleagues[171] who prescribed placebo or prednisolone (2 mg/kg/day for 5 days) in an outpatient setting to preschool children and infants with 'wheezy bronchitis', the previous label for PVW. In this cross over study, prednisolone did not reduce the daily

symptom score of cough, wheeze and breathlessness, and there was no evidence of a beneficial effect in children aged between 12 and 18 months.

Stratification for eosinophil priming was included in this study, to ensure that responses in the subgroup of PVW at increased risk of persistence of wheeze could be detected[198 224]. There was no evidence that children with increased systemic eosinophil priming (n=110) had responded to prednisolone. There was no interaction between treatment group and stratification for any primary or secondary outcome measure. However, the ability to detect a difference in symptom score within the two strata was less than for the overall effect of treatment. Hence it is possible that there was a clinically relevant effect of prednisolone in the high-primed strata, but this could not detected.
There was no difference in the clinical profiles between the primed and non-primed eosinophil groups. Whether systemic eosinophil priming accurately reflects pulmonary eosinophilic inflammation in PVW is unknown, but in atopic school age-asthmatics a serum ECP level of >20 μg/L accurately predicted an increased proportion of eosinophils in the lower airways[225] and therefore could help identify a potentially steroid-responsive subgroup. It is possible that although pulmonary eosinophil activation occurs in PVW, it may not be a major pathogenic factor.

The limitations to consider when interpreting this study results are: First, there was a relatively high treatment failure rate. Since parents had witnessed a previous severe episode of PVW, this level of compliance probably reflects the best that can be achieved in the UK. Second, 23% of children who were randomised, did not develop a further episode of PVW. Randomisation at the start of the trial PVW episode would have equalised treatment allocation of the re-wheezers, but we considered that the extra demand placed on parents to contact the research centre would have increased the failure rate. Third, if the trial medication had been substituted by oral steroids at an early stage of the wheezy episode, for a large number of children, the efficacy of prednisolone would not be have been assessed. All children in this study had received at least one dose of trial medication before steroid substitution, and the majority received a full course of trial therapy. The negative result therefore probably reflects both a failure of the treatment strategy, and a lack of efficacy of prednisolone. Fourth, it is possible that the symptom diary may have been too insensitive to detect a beneficial effect, and since wheeze and cough were considered together in the respiratory symptom score, it is theoretically possible that a steroid-induced reduction in wheeze, was masked by an increase in cough. However, before the trial medication could be started, wheeze, but not cough, had to be present.

Could prednisolone be counterproductive in PVW. An unexpected finding was the trend for increased hospitalisation in the prednisolone-treated group. A

similar result was found by Grant and colleagues[162] who reported that a single oral dose of prednisolone, started by parents at home early in an asthma attack, increased physician attendance in asthmatic children aged 2 to 14 years. Kayani described increased behavioral side effects of anxiety and aggression in children treated with higher doses of oral prednisolone therapy[175], during an asthma attack, which may influence the clinical decision to admit to hospital. Increased parental anxiety or changes in the child's behavior may therefore have lowered the threshold for admission to hospital in this study. The present trial was not designed to detect an effect of steroids on behavior, and future studies of oral steroids should include some form of behavioral assessment.

This study does not address the efficacy of oral steroids in children with severe PVW admitted to hospital. Connett found that a single dose of prednisolone produced a minimal objective evidence of improved respiratory symptom score, but more steroid treated children were fit for discharge[165]. Similarly Tal, in a placebo controlled trial of intramuscular methyl prednisolone for wheezy infants and young children, reported a reduction in the need for hospital admission[173]. A recent Cochrane review concluded that systemic steroids produced some improvements for children hospitalised with acute asthma[226].

6.7. Audit results on hospital readmission rates

As part of the recruitment process for the randomised trial on oral steroids in PVW, a database was set up to include all the eligible children for the study, that were admitted to hospital. These childrens' hospital notes were analysed in order to assess disease severity and clinical features and they were prospectively followed up to assess the number of readmissions, and clinical predictors for readmission.

Over a six-month period an audit was carried out on the clinical notes of 208 children who were admitted with PVW. The median duration of hospital stay was twenty-four hours. Four children received mechanical ventilation. 46 (22%) children were readmitted during the following six months after the first admission, some repeatedly with a total of 71 admissions during the study period. Comparison of children who were readmitted and not readmitted did not show any demographic or clinical variable that could distinguish these children, except increased prematurity in the children in the readmitted group.

The conclusion of this audit showed that the hospital stay in PVW is short and admission rates are high in September. There is approximately a 1 in 4 chance that a child with PVW will be readmitted with a similar episode within 6 months, with no clinical features that may identify this subgroup of children.

Summary

A 5-day course of oral prednisolone initiated by parents at the first sign of PVW, does not reduce lower respiratory tract symptom score, or requirement for inhaled bronchodilators. Since the benefits of steroids in children with severe wheeze and in the age range at increased risk of atopic asthma are subtle, it is not surprising that a community-based strategy for treating acute PVW did not show any benefit. These results suggest that the strategy of using steroids in children with PVW^2 may need re-evaluation.

Chapter 7 Contribution to the knowledge of PVW.

7.1. Contribution

In this thesis I have explored and demonstrated important findings in preschool wheeze and thus furthered our current understanding of this challenging group of children.

The clinical trial on preschool wheeze was a large randomised double blind placebo controlled trial on a clinically well-defined population. It clearly demonstrated that a course of oral steroids initiated by parents at the start of an acute attack of PVW, is not effective. These findings have been recorded as practice modifying and has immediately challenged the current guidelines for the management of preschool asthma, which suggests providing parents with a course of oral steroids as part of a management plan[2]. In the light of findings from this study, this strategy may need re-evaluation, since there are no clear benefits to balance any potential risks of steroid administration.

There have been 38 papers to date, where the steroid trial has been quoted. The data has been used in a Cochrane review, and further questions asked about the efficiacy of oral steroids. It has also led to an attempt at understanding and managing different clinical patterns of disease. The lack of effect of steroids has

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led to consideration whether antileukotrienes should be considered for the treatment of acute wheeze.

My chapters on the inflammatory markers has furthered our current knowledge in PVW and signposted possible reasons for the heterogeneity seen in this population in terms of a lack of response to standard treatment, their presentation and likely future modalities of treatment.

The mediators studied in the various sections in this thesis suggest that the inflammatory profile in acute PVW is different from classic acute atopic asthma, in particular that these mediators are not chronically elevated and return to normal after an acute attack. My studies on the role of eosinophil activation show that although eosinophil activation markers are elevated, there is a group of actively wheezy children who may not have significant eosinophil activation. These data would suggest one reason why systemic steroids are not effective in all wheezy children.

My study on cysteinyl leukotriene excretion shows, for the first time, the relationship between leukotriene excretion, acute wheeze and atopy. My finding that elevated leukotriene levels return to normal levels at convalescence strongly supports the hypothesis that leukotriene activation is of clinical significance and raises the prospect of applying other treatment options such as the widely available LTRA therapy.

My observations on markers of neutrophil activation are novel, but in line with recent research in adults that, in some cases of viral-triggered wheeze, neutrophils may trigger bronchoconstriction by activating other inflammatory cells. This in turn suggests a different approach to therapy focused on the neutrophil.

The findings from the series of studies described in the thesis, demonstrate that inflammatory markers in blood and urine can be studied in PVW, and can provide insights into mechanisms and signpost potential treatments. It has particularly highlighted the usefulness of urinary mediators as an attractive, practical and feasible option to explore the pathology of PVW.

In summary, my series of studies provide new insights into the inflammatory mechanisms underlying preschool wheeze, confronts current treatment practice and directly suggests new therapeutic approaches to this widespread problem.

7.2. Limitations of the thesis

I have identified a number of limitations in my thesis. A drawback that runs through most chapters in the thesis was the lack of a group of healthy children with a viral cold but no wheezing for comparison. I had attempted to recruit a small number of healthy children with a cold who presented for surgery but was unable to proceed as their surgical procedure was invariably cancelled. The inclusion of this group of clinically well children with a viral cold and an analysis of their respiratory secretions, blood and urine samples would have considerably strengthened the findings, although this attempt could be difficult to complete in a 'therapy trial' as compared to an 'inflammatory marker study'.

All inflammatory marker studies were performed on groups of children admitted and being treated for an acute wheezy attack. As they were recruited at different time frames in the course of their illness and the treatment they were receiving were not standardised, the effects of these treatments though not detected cannot be completely excluded.

My studies were conducted on blood and urine samples, which are collected from sites that are far from the site of actual disease and hence less sensitive of the disease process. I attempted to strengthen this signal by getting paired urine samples when these children were well, but was unable to gain consent to obtain a second blood sample.

In my attempt to differentiate viral wheeze and atopic asthma, markers of eosinophil activation were utilised. The inability to assess atopic sensitisation in these children was another limitation.

In the clinical trial there was a high rate of 'treatment failures'. Children were recruited into the study soon after the first wheezy attack, and a proportion of them did not wheeze again, thereby reducing the number of children who completed the study. A second factor was that the trial medication was dispensed at follow up and initiating the treatment was primarily the parents' decision. Dispensing the medication at the onset of the next wheezy episode may have reduced the failure rate, but was not logistically possible.

With the benefit of hindsight, in addition to addressing the above limitations, I would have strengthened the thesis by:

Utilising the latest information techniques and video demonstration to educate and train parents especially in the analysis of wheeze and objectively scoring symptom severity.

Including the assessment of atopic status and stringent long term follow up of recruited children.

Thoroughly exploring the side-effects of oral steroids and in future studies an analysis of its behavioral effects will be required.

7.3. Future directions

The clinical profile, inflammatory markers and treatment of preschool viral wheeze suggest that it is a distinct clinical condition and I believe there is substantial knowledge about this condition to be identified within the public domain as individual from 'asthma'.

As PVW is a heterogeneous condition, I feel the most fruitful method of studying these children is by analysing cohorts of clinically defined children (i.e. in the community, hospital and in intensive care unit) with stratification within the study. The data and pattern of analysis from my clinical trial lends itself well to other studies to test efficacy of treatment. Parent initiated high dose inhaled steroids and/or antileukotrienes would be the next logical step. The children could be stratified based on their leukotriene profile.

The data from my clinical trial suggests a satisfactory method of analyzing response to inhaled bronchodilators. Similar methodologically cogent studies on inhaled bronchodilator in these well-defined cohorts are required and with stratification could possibly uncover a group of children in whom bronchodilators may not be effective at standard doses.

Studies on the efficacy of oral steroids in preschool children hospitalised with acute PVW is the next logical step and could be conducted in a similar trial

pattern. This study would require a thorough exploration of the side effects of steroids. There is currently a trial in progress supported by 'Asthma UK' into this clinical scenario.

Bronchoscpic studies on a clearly defined population would help identify the true nature of inflammation in these children. Leukotriene assays and studies of neutrophil activation in the bronchial mucosa would be a future direction especially as the treatment modalities are different.

The profile of inflammatory markers in healthy children and in children with viral colds with simultaneous analysis of the viruses in secretions is urgently required as a baseline to further explore PVW.

Induced sputum in PVW is another attractive and less invasive option of analysing the respiratory tract and with the rapid technological advances seen; this could be a standard investigative tool in the future.

All my studies on inflammatory markers were 'pilot studies' and sample sizes were not calculated. As studies on eosinophil proteins and leukotrienes led to positive findings similar sample sizes would be adequate for future studies. The neutrophil activation study was small and a larger study would be required to explore the role of neutrophils in PVW.

New indications of existing drugs such as Leukotriene Receptor Antagonist (LTRA) in acute PVW and novel drug delivery systems such as sublingual salbutamol will eventually aid the forthcoming preschool wheezy children.

Appendix 1-4. Publications produced from this thesis

1. **A Oommen**, T McNally, J Grigg. Eosinophil activation and preschool viral wheeze. Thorax 2003; 58: 876-879.

2. **A Oommen**, J Grigg. Urinary Leukotriene E_4 in preschool children with acute clinical viral wheeze. European Respiratory Journal 2003; 21: 149-154.

3. **A Oommen**, R Patel, M Browning, J Grigg. Systemic neutrophil activation in acute preschool viral wheeze. Archives of Diseases in Childhood 2003; 88: 529-531.

4. **Abraham Oommen,** Paul C Lambert, Jonathan Grigg. Efficacy of a short course of parent-initiated oral prednisolone for viral wheeze in children aged 1-5 years: randomised controlled trial. Lancet 2003; 362: 1433-38.

PAEDIATRIC LUNG DISEASE

Eosinophil activation and preschool viral wheeze

A Oommen, T McNally, J Grigg

Thorax 2003;**58**:876–879

See end of article for authors' affiliations

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Background: A study was undertaken to ascertain whether systemic eosinophil activation is associated with preschool viral wheeze (PVW).

Methods: Urinary eosinophil protein X (uEPX) and serum total IgE (IgE) levels were measured in children admitted to hospital with PVW, and uEPX was measured 6 weeks after discharge. Two years after admission, current wheeze in children aged ≥5 years was determined by questionnaire. Controls were recruited from children undergoing elective surgery (normal controls) and from those with skin prick test reactivity to foods (atopic controls).

Results: There was no difference in uEPX levels between normal controls (n = 15) and atopic controls (n = 8). uEPX levels were increased in children with acute PVW (n = 84; $p<0.001$ *v* normal controls, $p<0.01$ *v* atopic controls) and fell on convalescence (n = 20, 95% CI −217 to −31 μg/mmol creatinine, $p<0.05$). In children with acute PVW there was no association between uEPX and serum IgE levels or markers of clinical severity. Respiratory questionnaires were returned for 25/55 eligible children. There was no difference in uEPX level during acute PVW when stratified by "current wheeze" (n = 18) or "no wheeze" (n = 7) 2 years later.

Conclusions: Systemic eosinophil activation is associated with PVW but is not associated with serum IgE, clinical severity, or persistence of wheeze into the early school age period.

In preschool children acute exacerbations of wheeze are caused almost exclusively by viral colds (preschool viral wheeze; PVW).[1 2] Birth cohort studies suggest that most children with PVW have a phenotype separate from that of classical atopic asthma. For example, the Tucson Children's Respiratory Study found that 60% of children with PVW were symptom free by 6 years of age,[3] a "transient" pattern that was not associated with markers of an allergic diathesis such as increased serum IgE.[3] In contrast, increased serum IgE levels were found in the few children with PVW who continued to wheeze to school age.[3] From these data we have speculated that the inflammatory substrate in most children with PVW is different from classical atopic asthma.[4] To date, the degree of overlap between PVW and asthmatic inflammation is unknown, a significant deficiency when targeting anti-inflammatory treatment to preschool children.

Between 1999 and 2002 we performed a trial of parent initiated oral steroids for PVW. Children were recruited when admitted to hospital with acute PVW. A blood sample was obtained to stratify them into two groups based on serum levels of eosinophil cationic protein (ECP) and eosinophil protein X (EPX). Prednisolone or placebo was prescribed to be given for the *next* episode of PVW. We used the recruitment phase to gain insights into the inflammatory substrate of PVW. To date, we have reported evidence for generalised systemic neutrophil activation in PVW[5] (a pattern not usually regarded as critical for asthmatic wheeze) and increased urinary leukotriene E4 in the subgroup with the highest serum IgE levels.[6]

One remaining question is whether eosinophil activation is associated with PVW. Inflammation in atopic asthma is, in part, characterised by increased release of ECP and EPX from pulmonary eosinophils.[7] Pulmonary eosinophil activation is, in turn, associated with increased serum levels of EPX.[8] Since serum EPX is excreted unchanged in the urine, urinary (u) EPX levels are increased in atopic asthma and increase further during acute attacks.[9 10] In assessing whether eosinophil activation occurs in PVW, urinary markers have an advantage over blood since samples can be taken when symptoms resolve and normal controls are readily available. To date, increased uEPX levels have been reported in a subgroup of acutely wheezy preschool children who were subsequently diagnosed 2 years later with atopic asthma.[11] However, this study only included children with at least three previous episodes of wheeze, excluded controls with a high probability of atopy, and did not repeat sampling on convalescence.[11] The relationship between acute wheeze and eosinophil activation for the majority of children with PVW therefore remains unclear.

In the present study we sought evidence for eosinophil activation in children admitted to hospital with PVW. Specifically, we wished to determine whether uEPX levels are raised compared with controls, and whether levels fall on resolution of the wheeze. We hypothesised that uEPX levels in acute PVW would be highest in children with increased serum IgE—that is, in those at increased risk of atopic sensitisation and persistence of wheeze into early school age.[3]

METHODS

Patients

Preschool children (1–5 years) with PVW were recruited from those referred by their general practitioner to the admissions unit of the Leicester Royal Infirmary Children's Hospital. Sampling of inflammatory markers in PVW and controls was approved by the ethical committee of the University Hospitals of Leicester NHS trust.

Blood and urine samples from children with acute PVW were obtained if there was a clear symptom history from the parents of a viral cold in the 48 hours preceding the wheeze attack, and physician diagnosed wheeze. Children were excluded if they were premature, had a clinical diagnosis of bacterial infection, and had any other chronic respiratory disease. On admission all children received a single dose of oral prednisolone and nebulised salbutamol as required. Before urine sampling the presence of wheeze and rhinitis was confirmed and a clinical history obtained from the parents. A urine sample was collected within 36 hours of admission for measurement of uEPX, and a simultaneous

blood sample was obtained for serum IgE and blood eosinophil measurement. The number of nebulised bronchodilators during the admission and the total duration of the illness was recorded from the discharge notes. After discharge the children were visited at home within 6 weeks. If they had no current respiratory symptoms and were potty trained, a urine specimen was collected during the visit (convalescent sample).

"Normal" controls were recruited from a random selection of children undergoing elective ear nose and throat surgery or ophthalmic surgery. None had clinical evidence of active infection and their skin prick reactivity to allergens was unknown. Urine for uEPX measurement was obtained before surgery and a blood sample for serum IgE measurement was obtained soon after induction of anaesthesia.

"Atopic normal" controls were recruited from children with suspected food sensitivity who were attending for food challenge. All had history of a suspected allergic reaction to a food and at least one positive skin prick test to food antigens. Serum IgE measurements were not obtained from atopic controls because of ethical restrictions. Controls with a history of chronic respiratory disease, or previous attacks of wheezing, or symptoms of a respiratory tract infection in the preceding week were excluded.

Follow up

To establish whether increased uEPX levels during acute PVW were associated with symptoms consistent with a diagnosis of asthma,[12] parents of children who had (1) a 2 year interval from the original admission and (2) reached 5 or more years of age were sent a respiratory questionnaire. Children of school age were categorised either as having "no wheeze" (in the last 6 months) or "current wheeze" (at least one episode of wheeze in the last 6 months).

Sample collection and analysis

5–10 ml urine samples were collected using a sterile potty. Urinary infection was excluded using the Multistix 10SG dipstick screening test (Bayer, UK). Urine samples were initially stored in a refrigerator, then aliquoted and transferred to −70°C within 12 hours. The uEPX concentration was measured in unprocessed urine samples using a specific EPX radioimmunoassay kit (Pharmacia, Uppsala, Sweden). Briefly, urine was defrosted at room temperature and 500 µl used in the assay. The samples were diluted 11 times in a phosphate buffer containing 0.15% NaCl, 1% bovine serum albumin, 0.1% Tween 20, 10 nmol/l EDTA, and 0.2% N-acetyl-trimethylammonium-bromide as previously described.[9] The assay was done in duplicate and the mean values were taken. The detection limit of the assay was <3 µg/l, the within assay coefficient of variation was <5%, and the between assay coefficient of variation was <10%. Urinary creatinine was measured by the Jaffe reaction with the Dade Behring dimension analyser (Dade Behring, USA) and uEPX levels were expressed as µg/mmol creatinine.

For serum IgE, a 2 ml venous blood sample was collected and allowed to clot at room temperature for 60 minutes. Serum was separated by centrifugation before storage at −20°C. IgE was measured using the UniCAP Analyser machine (Pharmacia, Sweden) and expressed as kU/l. An absolute eosinophil count was performed on a 0.5 ml heparinised blood sample using routine hospital techniques.

Statistics

Data are presented as median and interquartile range (IQR). Unpaired data were compared using the Mann-Whitney U test. Paired data were compared using the Wilcoxon signed rank test and expressed as the estimated median difference and 95% confidence interval (CI). Correlations were determined by Spearman rank correlation (r_s). Statistical analyses were performed using SPSS for Windows Version 10 (SPSS Inc, Chicago, IL, USA) and Minitab release 13.32 (Minitab Inc, PA, USA). A p value <0.05 was considered statistically significant.

RESULTS

Eighty four children with acute PVW were studied. A convalescent urine sample was obtained from 20 children 6 weeks after discharge. Convalescent samples were not obtained from children who were not fully potty trained or refused to produce a sample during the visit (n = 63). One child was excluded because of a readmission with PVW.

Serum IgE levels in the normal controls (n = 15) were within the range reported for non-atopic children[13] (blood was not sampled from atopic controls). Serum IgE levels were increased in children with acute PVW (n = 73; $p<0.01$ *v* normal controls, table 1). Blood eosinophil counts in both children with PVW and normal controls were within the normal range but were higher in normal controls ($p<0.05$ *v* acute PVW, table 1). There was no difference in uEPX levels between normal (n = 15) and atopic controls (n = 8; table 1, fig 1). Both control groups were slightly older than the children with PVW (table 1), but there was no association between age and uEPX levels in any group.

uEPX levels were increased during acute PVW ($p<0.001$ *v* normal controls, $p<0.01$ *v* atopic controls, table 1, fig 1). uEPX levels during acute PVW were not associated with (1) serum IgE ($r_s = 0.17$, p = NS, fig 2), (2) the interval between oral steroid treatment and urine sampling ($r_s = 0.1$, p = NS, fig 3), and (3) the number of salbutamol nebulisations required during the attack ($r_s = 0.1$, p = NS). Furthermore, there was no significant difference in uEPX levels during acute PVW when categorised by family history of atopy (66/84), previous dry cough or shortness of breath without colds (n = 14/84), and eczema (n = 27/84). uEPX levels fell

Table 1 Demographic, serum and urine parameters in children with preschool viral wheeze (PVW) and controls

	PVW (n = 84)	Normal controls (n = 15)	Atopic controls (n = 8)
Age (months)	31 (20 to 41)	59 (41 to 66)**	87 (58 to101)**
uEPX (µg/mmol creatinine)	214 (144 to 383)	82 (35 to 211)**	84 (51 to 182)**
Serum total IgE (kU/l)	56 (9 to 209) (n = 73)	12 (7 to 25)**	Not done
Absolute eosinophil count ($\times 10^9$/l)	0.1 (0 to 0.2) (n = 73)	0.25 (0.2 to 0.3)*	Not done

Data are presented as median (interquartile range).
*$p<0.05$ *v* PVW; **$p<0.01$ *v* PVW (Mann-Whitney U test). There was no difference in age or urinary eosinophil protein X (uEPX) levels between normal and atopic controls.

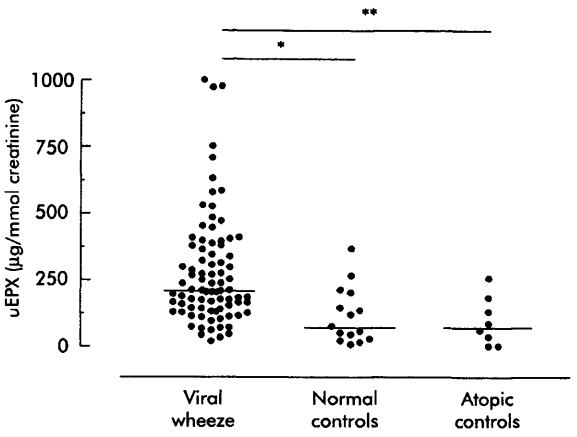

Figure 1 Dotplot of urinary EPX (uEPX) levels in children with acute PVW (n = 84), normal controls in whom skin prick reactivity was unknown (n = 15), and atopic controls (n = 8). uEPX levels were higher in the children with acute PVW (*p<0.001 *v* normal controls; **p<0.01 *v* atopic controls, Mann-Whitney U test). Horizontal bars represent medians.

between the acute and convalescent phases of PVW (median −107 µg/mmol creatinine, 95% CI −217 to −31, n = 20, p<0.05, fig 4). Convalescent uEPX levels were similar to those of normal- and atopic controls (p = NS). When convalescent uEPX levels were categorised by the presence of eczema (7/20), there was no difference between children with and without eczema, and no difference between children with eczema and normal controls.

Follow up questionnaires were returned for 25 (45%) of the 55 children who had reached school age in 2003 and who were at least 2 years post admission. Eighteen children continued to have parent reported wheeze in the preceding 6 months (table 2); all had been prescribed inhaled salbutamol, and 11 were receiving regular inhaled steroids. Seven children had no wheeze over the preceding 6 months; none were prescribed inhaled therapy. There was no difference in EPX levels when acute PVW was categorised by the presence or absence of wheeze at the 2 year follow up (table 2).

DISCUSSION

The main finding of this study is that uEPX levels are increased in acute PVW and fall when wheezing resolves. The large degree of overlap between uEPX levels in acute PVW and normal controls suggests a marked heterogeneity of eosinophil activation and is compatible with epidemiological studies suggesting that PVW is not a single phenotype.[1] We originally hypothesised that the highest uEPX levels would occur in children at increased risk of atopic sensitisation. However, no correlation was found between uEPX and serum IgE levels during acute PVW. Other risk factors for eosinophil activation with PVW could not be identified, since no demographic or clinical parameter at the time of admission was associated with increased uEPX levels.

Increased levels of uEPX during PVW did not predict persistence of wheeze into the early school age years. These data would appear to be different from those reported by Øymar[11] who found increased uEPX in wheezy preschool children who were subsequently diagnosed with atopic asthma. We did not perform skin prick tests at follow up, and an association between acute uEPX and a subsequent diagnosis of "atopic" asthma has not been excluded. However, children who continued to wheeze would be considered as "asthmatic" under the current British Thoracic Society guidelines.[12] Thus, increased uEPX levels do not signify a "pre-asthmatic" state in our population of children with PVW. Furthermore, the lack of association between IgE and uEPX levels suggests that eosinophil activation in PVW is not initiated by atopic mechanisms. A primary role for the neutrophil in initiating viral wheeze has recently been hypothesised for preschool children.[14] Indeed, our previous data showing increased neutrophil activation in a separate group of children with PVW[5] are compatible with a direct role of neutrophils in initiating systemic eosinophil

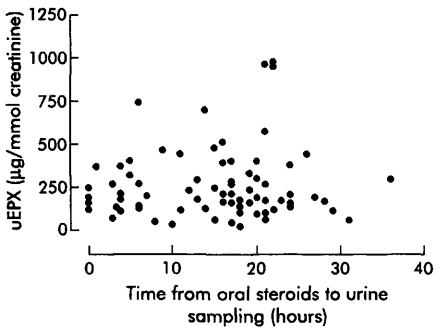

Figure 3 Scatterplot of the association between urinary eosinophil protein X (uEPX) levels during acute PVW and time of urine sample from oral prednisolone therapy ($r_s = 0.1$, p = NS).

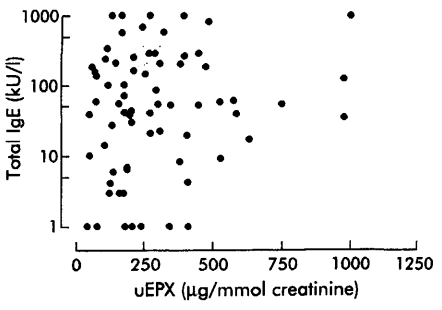

Figure 2 Scatterplot of the association between urinary eosinophil protein X (uEPX) levels and serum IgE levels during acute PVW ($r_s = 0.17$, p = NS).

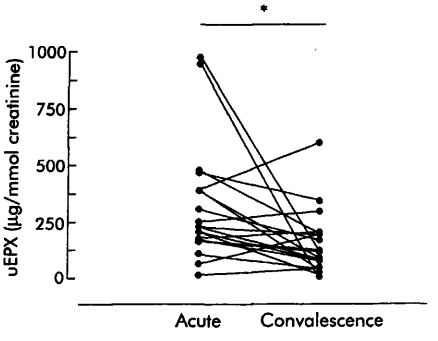

Figure 4 Change in urinary eosinophil protein X (uEPX) levels between the acute and convalescent phase of PVW. *p<0.05, 95% CI −217 to −31 µg/mmol creatinine, n = 20, Wilcoxon signed rank test.

Table 2 Two year follow up of children with preschool viral wheeze (PVW) who had reached 5 years of age in 2003

	Wheeze (n = 18)*	No wheeze (n = 7)*
Age at PVW episode (months)	37 (31 to 43)	34 (25 to 46)
Age at follow up (months)	77 (74 to 86)	83 (73 to 95)
IgE during PVW episode (kU/l)	141 (57 to 235)	31 (10 to 294)
uEPX during PVW episode (μg/mmol creatinine)	245 (166 to 387)	310 (203 to 451)

Data are presented as median (IQR). There were no differences between the two outcome groups (p = NS, Mann-Whitney U test).
*Parental reported symptoms over the previous 6 months

activation, independent of atopic mechanisms. Further studies are needed which combine markers of neutrophil activation with eosinophil markers in order to elucidate the sequence of inflammatory events in PVW.

Could a confounding factor account for the disassociation between serum IgE and uEPX levels? Corticosteroids down-regulate eosinophil activation,[15] although there is a time lag of several hours between oral steroid therapy and its systemic effects.[16] All children received a single dose of oral prednisolone on admission, which may therefore have disrupted an association between IgE and uEPX. However, uEPX levels in samples taken within the first hours of admission were no different from those obtained later (fig 3), a pattern that does not indicate therapeutic suppression. Eczema is another potential confounder, since active dermatitis is associated with increased uEPX levels[17] and a significant minority (32%) of children with PVW had mild eczema. However, eczema was not associated with increased uEPX levels, either during the acute or the convalescent phase of PVW.

The normalisation of uEPX on convalescence implies that eosinophil activation in children with PVW is an acute, but not a chronic, phenomenon. Indeed, eosinophil activation was not found in a bronchoalveolar lavage study of children with a history of episodic viral triggered wheeze.[18] The fall in uEPX levels on convalescence from PVW suggests a role for eosinophils in the pathogenesis of wheeze. However, we have not excluded systemic eosinophil activation with colds per se, although this is unlikely since uEPX levels are not increased in young children with trivial upper respiratory tract illnesses.[19]

In conclusion, we found evidence of eosinophil activation in children with severe acute PVW which normalised with resolution of symptoms. Eosinophil activation in PVW is independent of serum IgE, clinical presentation, and outcome 2 years later. We speculate that eosinophil activation in PVW is not caused by an atopic inflammation but is a result of direct stimulation by non-atopic inflammatory cells.

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Urinary leukotriene E_4 in preschool children with acute clinical viral wheeze

A. Oommen, J. Grigg

Urinary leukotriene E_4 in preschool children with acute clinical viral wheeze. A. Oommen, J. Grigg. ©ERS Journals Ltd 2003.

ABSTRACT: Cysteinyl leukotrienes (cystLTs) are important mediators of wheeze in atopic asthma, but the role of cystLTs in the pathogenesis of preschool viral wheeze (PVW) is unclear. Therefore, evidence for increased production of cystLTs in PVW was sought.

Urinary leukotriene E_4 ($uLTE_4$) and serum total immunoglobulin (Ig)E were measured in children (1–5 yrs) with PVW during an acute attack (n=44) and in the convalescent phase (n=19), and compared with normal controls (n=15). The effect of atopic sensitisation was assessed in a separate group of atopic controls (n=6) in whom only $uLTE_4$ was measured.

The levels of $uLTE_4$ were similar in normal and atopic controls and increased in acute PVW (median (interquartile range) 165 (101–285) *versus* 125 (82–163) ng·mM creatinine^{-1}). Stratification by IgE showed that whereas $uLTE_4$ was increased in 23 children with acute PVW and IgE >95th percentile (median 211 (118–312) ng·mM creatinine^{-1}), $uLTE_4$ was not increased in the 21 children with acute PVW and IgE ≤95th percentile. In the convalescent phase, $uLTE_4$ fell in the subgroup with high IgE but not in the subgroup with low IgE.

It is concluded that increased cysteinyl leukotriene production during acute preschool viral wheeze is associated with high serum immunoglobulin E.

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In atopic asthma, increased production of cysteinyl leukotrienes (cystLTs) within the lung causes bronchoconstriction [1]. Leukotriene E_4 (LTE_4) is a potent bronchoconstricting cystLT [1] and is the end product of cystLT metabolism. LTE_4 is excreted in the urine and total urinary LTE_4 ($uLTE_4$) represents ~ 5% of pulmonary production [2]. Thus, $uLTE_4$ can reflect disease severity in atopic asthma. For example, there is an association between $uLTE_4$ and the degree of airflow obstruction in asthmatic children with chronic symptoms [3], and $uLTE_4$ is increased during acute attacks of atopic asthma then falls in the convalescent phase [4]. Viral colds are a common trigger for acute asthma attacks in school-age children and for attacks of wheeze in preschool children [5, 6]. However, epidemiological studies suggest that most children with preschool viral wheeze (PVW) do not have atopic asthma [7]. First, 60% of children with PVW will become asymptomatic by 6 yrs of age (transient wheeze), whereas wheeze will continue in only 40% (persistent wheeze) [8]. Secondly, the transient pattern of wheeze is not associated with increased serum immunoglobulin (Ig)E at birth or increased atopic sensitisation at 6 yrs of age [8]. It has been speculated that the different phenotypes of PVW are associated with different patterns of pulmonary inflammation [9], but to date this remains unclear.

An understanding of the pattern and timing of the pulmonary inflammation in PVW is important since it may identify subgroups that are responsive to specific therapies. In atopic asthma (where pulmonary cystLT production is increased) cystLT receptor blockade is a therapeutic option [10]. By contrast, there is no consistent evidence for increased pulmonary cystLT production in acute PVW. Increased levels of LTC_4 in upper respiratory tract secretions occur in infants during episodes of recurrent wheezing [11]. However, BALFOUR-LYNN *et al.* [12] found no increase in $uLTE_4$ in hospitalised infants with acute PVW. One explanation for these conflicting results is that increased upper airway cystLT production is not associated with increased production in the lower airway. Alternatively, increased pulmonary cystLT production may be limited to the subgroup of preschool children with risk factors for atopic sensitisation. Markers of increased risk for atopic sensitisation have been shown to affect the clinical severity of wheeze. For example, a high total IgE increases the risk of emergency care for rhinovirus-triggered

wheeze in children 2–16 yrs of age [5]. However, to date there are no data on whether increased levels of IgE influence cystLT production in PVW.

In this study, the authors sought to determine whether pulmonary cystLT production is increased in patients with PVW that was severe enough to warrant admission to hospital. To achieve this aim, $uLTE_4$ was used as a marker for pulmonary cystLT production and IgE was measured to determine levels in the subgroup of children at increased risk for atopic sensitisation.

Methods

Patients

Children were recruited from those referred by their general practitioner to the admissions unit of the Leicester Royal Infirmary Children's Hospital (Leicester, UK) with acute PVW. The study inclusion criteria were as follows: 1) age 1–5 yrs; 2) physician-diagnosed wheeze; 3) clear symptoms of a viral cold in the 48 h preceding the wheeze attack; and 4) no wheezing in the 5 days before the clinical cold. The exclusion criteria were as follows: 1) premature birth; 2) a clinical diagnosis of bacterial infection; 3) any other chronic respiratory pathology; and 4) oral corticosteroid therapy prior to admission. On admission, each child received a single dose of oral prednisolone and "as required" nebulised salbutamol. The presence of wheeze was confirmed by a clinician, a clinical history was obtained from the parents and clinical evidence of a viral cold (*i.e.* rhinitis) was sought. A urine sample was then collected for $uLTE_4$ analysis within 36 h of admission and a blood sample obtained for IgE. After discharge, some children were visited at home within 6 weeks. If there had been no respiratory symptoms in the preceding 7 days, a "convalescent" urine sample was obtained.

Two separate control groups were recruited. First, "normal controls" from a random selection of children attending hospital for an elective ear, nose and throat or ophthalmic surgery. In these, a urine sample for $uLTE_4$ was obtained prior to surgery and a blood sample for IgE obtained immediately after induction of anaesthesia. Secondly, "atopic controls" from children with suspected food sensitivity who attended for a food challenge. These children had a history of a suspected allergic reaction to a food and at least one positive skin-prick test to food antigens. The atopic controls did not have a blood sample for IgE for ethical reasons. Controls were excluded if there was a history of either chronic respiratory disease, a previous attack of wheezing, active eczema or symptoms of a respiratory tract infection in the preceding week. The study required written parental consent and was approved by the Leicestershire Research Ethics Committee.

Sample collection and analysis

Urine samples were obtained using a bag or sterile potty. Urinary infection was excluded using the Multistix 10SG dipstick (Bayer, Newbury, UK) screening test. Urine samples were initially stored in a refrigerator, then transferred to storage at -70°C within 12 h. The $uLTE_4$ concentration was measured in unprocessed samples by enzyme-linked immunosorbent assay kit (ACE™ LTE_4 ELISA kit; Cayman Chemicals, Michigan, USA), which has been previously validated against $uLTE_4$ concentrations measured after high-performance liquid chromatography extraction [13]. Briefly, urine was defrosted at room temperature and 500 μL was used in the ELISA without further processing. The assay detection limit was 25 pg $LTE_4 \cdot mL^{-1}$. Urinary creatinine was measured using the Dade Behring Dimension Analyser (Dade Behring, Deerfield, IL, USA) and $uLTE_4$ levels were expressed as $ng \cdot mM\ creatinine^{-1}$. Serum for IgE was obtained from a venous blood sample that had clotted for 60 min at room temperature. Serum was separated by centrifugation and stored at -20°C. IgE was measured using the UniCAP Analyser machine (Pharmacia, Stockholm, Sweden) and expressed as $kU \cdot L^{-1}$.

Statistics

Data are summarised as median and interquartile range (IQR) unless specified and comparisons were performed using the Mann-Whitney U-test and the Chi-squared test. Correlations were determined by Spearman rank correlation (r_s). Paired data were analysed using the paired t-test. p-Values of <0.05 were considered statistically significant.

Results

A total of 44 children (32 males, 12 females) with acute PVW, 15 normal controls (nine males, six females) and six atopic controls (three males, three females) were studied. Children with PVW were slightly younger than normal controls (age 3.0 yrs (1.8–3.6) *versus* 4.5 yrs (3.4–5.5), $p<0.01$) and had higher IgE (118 $kU \cdot L^{-1}$ (23–293) *versus* 15 $kU \cdot L^{-1}$ (7–26), $p<0.01$). The median duration between a viral cold and onset of wheezing was 24 h (12–48). In eight children this was their first attack of PVW. Twenty six children with acute PVW had a history of wheezing exclusively with colds (previous attacks of PVW n=4 (2–6)) and 10 had a history of additional interval respiratory symptoms, including moderate nocturnal cough and wheeze after playing (previous PVW attacks n=7 (4–13)). In 19 children with acute PVW a convalescent urine sample was obtained.

Serum IgE of normal controls were within the 95th percentile reported for nonatopic children [14] and there was no difference in $uLTE_4$ between the normal controls and the atopic controls (fig. 1). Data from the PVW group were therefore compared with the normal controls only. When considered as a single group, children with acute PVW had higher $uLTE_4$ compared to normal controls (165 (101–285) *versus* 125 (82–163) $ng \cdot mM\ creatinine^{-1}$, $p<0.05$, fig. 1). The $uLTE_4$ level of the whole group fell during the convalescent phase, to lie within the range of the normal controls

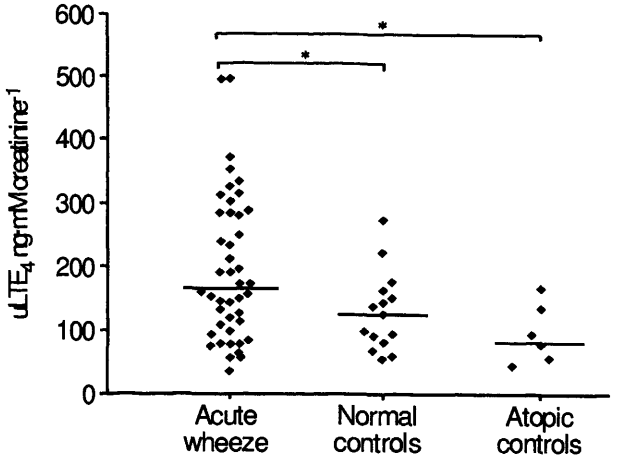

Fig. 1.–Dot plot of urinary leukotriene E_4 ($uLTE_4$) concentrations corrected for creatinine in preschool children with acute viral wheeze (n=44), normal controls (n=15) and atopic controls (n=6). Horizontal bars represent medians. LTE_4 levels are significantly higher in the acute preschool viral wheeze group. *: $p<0.05$ *versus* normal and *versus* atopic controls.

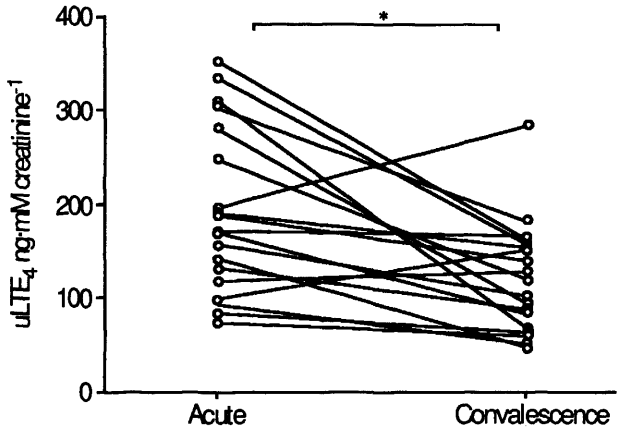

Fig. 2.–Paired change in urinary leukotriene E_4 ($uLTE_4$) between the acute and convalescent phase of preschool viral wheeze. The fall in $uLTE_4$ is significant. *: $p<0.05$, n=19, paired t-test.

(mean fall (95% confidence interval (CI)) 70 (28–112) ng·mM^{-1} creatinine, $p<0.05$, n=19 (fig. 2)).

There was a moderate correlation between $uLTE_4$ and IgE during acute PVW ($r_s=0.35$, $p<0.05$). Stratification based on the 95th percentile for IgE [14] resulted in different patterns of $uLTE_4$. The subgroup with high IgE (n=23) had elevated $uLTE_4$ during the acute phase ($p<0.05$ *versus* normal controls, table 1). The individual data plot (fig. 3) shows that the effect of IgE on acute $uLTE_4$ is apparent in children with the highest levels (>250 kU·L^{-1}). On convalescence, $uLTE_4$ fell in the high IgE subgroup ($p<0.01$ *versus* acute, n=14, table 1), to become no different from normal controls. In contrast, $uLTE_4$ in children with PVW and low IgE (n=21) was not elevated during the acute phase (*versus* normal controls) and did not change on convalescence (n=5, table 1).

Clinical parameters in children with acute PVW were similar in the high and low IgE groups (table 1). In acute PVW there was no difference in IgE or $uLTE_4$, when categorised by previous episodes of exclusive PVW (*i.e.* no interval symptoms) or history

Table 1.–Comparison between children with preschool viral wheeze (PVW) after stratification for serum total immunoglobulin (Ig)E during an acute attack

	Serum total IgE	
	≤95th percentile	>95th percentile
Subjects n	21	23
Age months	29 (17–38)	38 (31–47)
Male:female	13:8	19:4
Eczema present n	9	8
Parental history of asthma n	19	13
First attack of PVW n	4	4
Previous PVW and no interval symptoms n	12	14
Previous PVW with interval symptoms n	5	5
Number of previous attacks of PVW n	3 (2–7)	5 (2–7)
$uLTE_4$ during acute PVW ng·mM creatinine^{-1}	150 (77–217)	211 (118–312)*
Fall in $uLTE_4$ on convalescence[¶] ng·mM creatinine^{-1}	34 (-93–160)[+]	84 (36–131)[#,§]

Data are presented as median (interquartile range) unless otherwise stated. $uLTE_4$: urinary leukotriene E_4. [¶]: mean (95% confidence interval); [+]: n=5; [§]: n=14; The 95th percentile of IgE based on [14] (<3 yrs 74 kU·L^{-1}, 3–5 yrs 87 kU·L^{-1}). *: $p<0.05$ *versus* normal controls by Mann-Whitney U-test; [#]: $p<0.05$ *versus* acute levels by paired t-test.

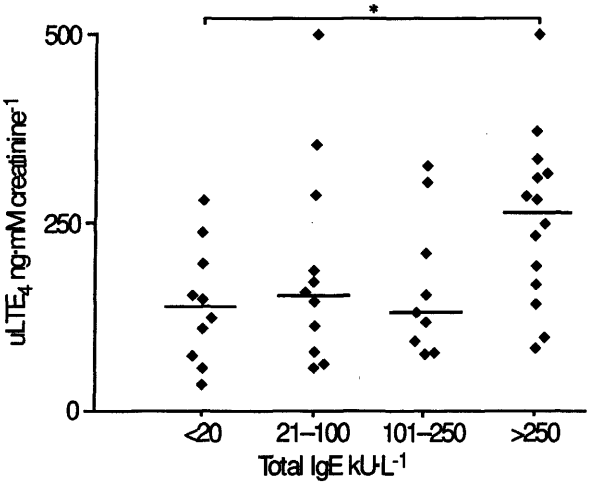

Fig. 3.–Urinary leukotriene E_4 ($uLTE_4$) of children with acute preschool viral wheeze categorised by serum total immunoglobulin (Ig)E. Horizontal bars represent medians. *: $p<0.05$, Mann-Whitney U-test.

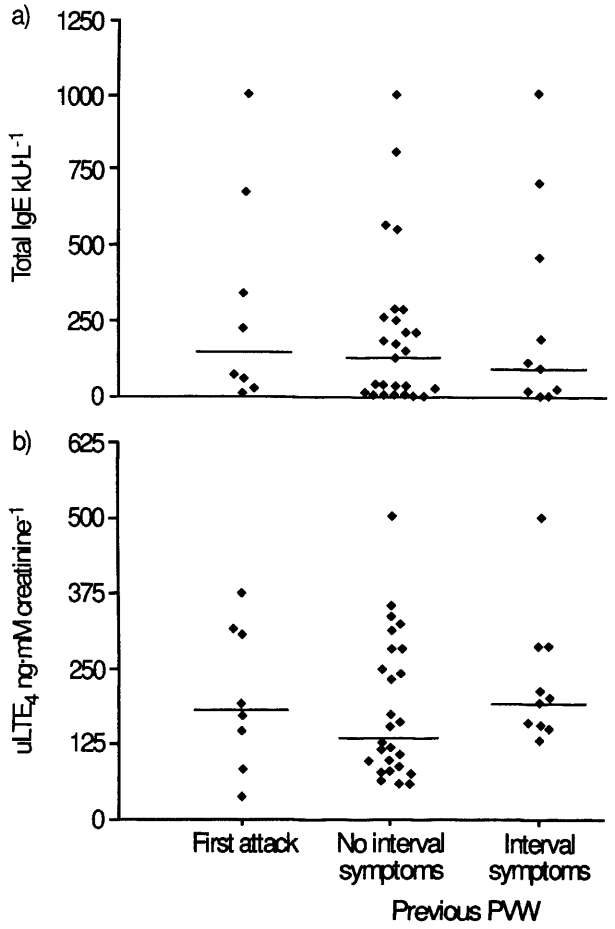

Fig. 4.–Serum a) total immunoglobulin (Ig)E and b) urinary leukotriene E_4 ($uLTE_4$) in acute preschool viral wheeze (PVW). Children were categorised as either having a first attack (no previous symptoms), no interval symptoms (previous exclusive PVW) or interval symptoms (previous PVW with interval symptoms).

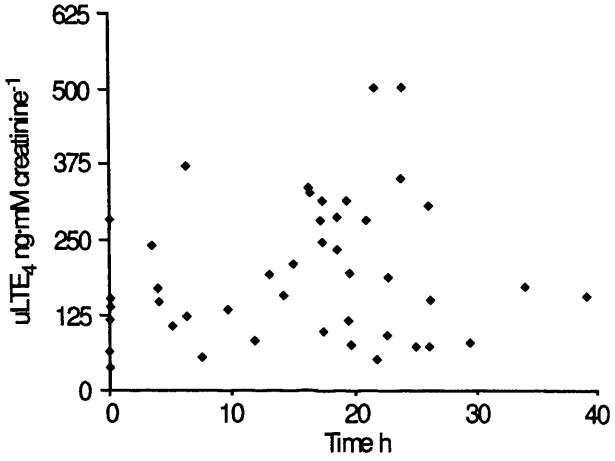

Fig. 5.–Scatter plot of the correlation between urinary leukotriene E_4 ($uLTE_4$) and the duration between steroid administration and urine sampling in acute preschool viral wheeze. The zero time point represents children sampled before oral steroid therapy. There is no correlation between the two variables by Spearman rank correlation.

of PVW and interval symptoms (fig. 4). There was no correlation between $uLTE_4$ and the time period between administration of oral steroids and urine sampling (fig. 5). $uLTE_4$ in the six children with acute PVW in whom urine was obtained before steroid therapy, was not increased compared with those (n=38) who were studied after steroid administration (fig. 5).

Discussion

The data from this study show that children admitted to hospital with acute PVW have elevated $uLTE_4$, which falls into the normal range with resolution of symptoms. However, not all symptomatic children have increased cystLT production, since $uLTE_4$ in the acute phase of PVW is not elevated in the subgroup with low serum IgE. In these children, perhaps similar to those studied by BALFOUR-LYNN *et al.* [12], no evidence was found that cystLTs contribute to symptoms. Increased $uLTE_4$ was found in the whole PVW group in the present study because children were skewed towards an increased IgE. The current authors therefore speculate that differences in the published data may be due to differences in serum IgE in the children studied [11, 12].

How could high IgE predispose to increased cystLT production during acute PVW? It is unlikely that high serum IgE or atopic sensitisation *per se* increases $uLTE_4$ since: 1) there was no association between IgE and $uLTE_4$ in the normal controls; 2) increased $uLTE_4$ did not persist in children with high IgE when asymptomatic; and 3) $uLTE_4$ was not increased in the atopic control group. This suggests that either high IgE or a mechanism that acutely increases IgE during PVW or a variable associated with elevated IgE (such as atopic sensitisation) primes the lower respiratory tract to increase cystLT production during PVW. Since skin-prick tests were not performed in children with PVW, the atopic status of the high IgE subgroup is unknown and an interaction between atopic sensitisation (rather than total IgE) and viral colds has not been excluded.

Is the inflammatory pattern of children with acute PVW and high IgE similar to a viral-triggered attack of atopic asthma? To date, there are no data comparing inflammation between acute nonviral attacks and viral-triggered attacks of atopic asthma. However, the pattern of $uLTE_4$ in acute PVW with high IgE has similarities to that reported during acute atopic asthma in older children [4]. Like atopic asthma, $uLTE_4$ in acute PVW with high IgE is elevated in the acute phase and falls on convalescence. In contrast to atopic asthma, $uLTE_4$ in acute PVW falls into the normal range on convalescence [4]. The data presented here suggest that increased pulmonary cystLT production during acute PVW rapidly returns to normal when symptoms resolve, a finding similar to that of STEVENSON *et al.* [15], who found persistent airway inflammation in asymptomatic children with "intermittent" atopic asthma, but not in children with a history of "exclusive" viral-triggered wheeze.

No clinical parameters were found that distinguished children with PVW who had either increased IgE or increased $uLTE_4$. In particular, a history of both PVW and interval symptoms was not associated with increased $uLTE_4$. CASTRO-RODRIGUEZ *et al.* [16] have reported a clinical index that calculates risk of developing asthma in young children with recurrent wheezing. Preschool children with one "major" risk factor (parental history of asthma or eczema) or two of three "minor" risk factors (blood eosinophilia, wheezing without colds and allergic rhinitis) are 9.8 times more likely to be diagnosed as active asthmatics at 6–13 yrs [16]. It remains to be determined whether this index better defines the subgroup of PVW with elevated $uLTE_4$.

There are two potential confounding variables. First, steroids attenuate cystLT release from the asthmatic lung [17] and most children had received a single dose of oral steroids prior to urine sampling. If children with low IgE were exquisitely sensitive to steroids, it is possible that cystLT levels had been normalised prior to sampling. If this were the case, children with low IgE who were sampled before, or just after, oral steroids should have had elevated $uLTE_4$. However, no association was found between $uLTE_4$ and the duration of the sampling from oral steroids, for both the low IgE subgroup (data not shown) and for the whole group (fig. 5). Furthermore, $uLTE_4$ was not different from normal controls in four children with low IgE who were studied before oral steroid therapy (data not shown). A major confounding effect of oral steroids is therefore unlikely. The second limitation is the effect of a viral cold *per se* on $uLTE_4$. The current authors did not measure $uLTE_4$ in normal children with viral colds for two reasons. First, it was ethically unacceptable to obtain blood for IgE in conscious normal children. Secondly, serendipitous blood sampling during anaesthesia was not possible during colds, since surgery was postponed. Since no increase in $uLTE_4$ was found in acute PVW with low IgE, an effect of colds *per se* on $uLTE_4$ is unlikely in this subgroup. Increased $uLTE_4$ caused by colds *per se* in the high IgE group cannot be excluded and remains a significant study limitation. Future studies of $uLTE_4$ in normal children with high IgE are required during viral colds, to assess the clinical relevance of an increased $uLTE_4$ seen in the present study. However, a recent study has demonstrated that leukotriene receptor blocker therapy improves persistent wheeze in preschool children [18], a finding compatible with a clinically relevant role for cystLTs in this age group.

In conclusion, heterogeneity in urinary leukotriene E_4 excretion in acute preschool viral wheeze was found. Elevated urinary leukotriene E_4 occurred in children with the highest serum immunoglobulin E, but whether increased levels are due to viral colds *per se* or the consequence of inflammation causing wheeze remains unclear. The authors speculate that increased cysteinyl leukotriene production during preschool viral wheeze is clinically relevant, and immunoglobulin E or urinary leukotriene E_4, or both, should be measured in trials of cysteinyl leukotriene receptor blocker therapy to prevent a potential subgroup of responders being overlooked.

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ORIGINAL ARTICLE

Systemic neutrophil activation in acute preschool viral wheeze

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See end of article for authors' affiliations

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Background: In preschool children, attacks of wheeze are usually triggered by viral colds. The inflammatory substrate in preschool viral wheeze (PVW) is unclear, but epidemiological data suggest that most PVW is not caused by allergic inflammation. We therefore speculated that the neutrophils are an important effector cell. Systemic neutrophil activation is the first stage for the development of pulmonary neutrophilia. Markers of neutrophil activation are shedding of the adhesion molecule L-selectin from the cell surface, upregulation of Mac-1 expression, and an increase in serum soluble L-selectin.
Aims: To obtain evidence for systemic neutrophil activation during PVW.
Methods: Preschool children (1–5 years) admitted to hospital with acute PVW (n = 20) and normal controls (n = 18) were studied. Adhesion molecule expression on CD16 positive neutrophils was determined in both groups and expressed as molecules of equivalent fluorochrome (MEF). Serum soluble L-selectin was analysed by ELISA.
Results: Compared with controls, children with PVW had reduced neutrophil L-selectin expression (median MEF (IQR): 69 (11 to 96) units versus 136 (109 to 163) units, $p < 0.001$) and higher serum soluble L-selectin (2.8 (2.3 to 3.1) versus 2.4 (2.2 to 2.6) μg/ml, $p = 0.04$). There was no significant difference in neutrophil Mac-1 expression.
Conclusion: Systemic neutrophil activation is associated with acute PVW.

Recent studies have focused attention on the role of non-eosinophilic mechanisms in the pathogenesis of attacks of atopic asthma triggered by colds. For example, neutrophils predominate in the sputum of asthmatic adults with colds,[1] and increased levels of interleukin 8, a potent neutrophil chemoattractant, are present in the lower airway of children with viral triggered deterioration of atopic asthma.[2] Viral colds are also an important trigger of wheeze in preschool children (preschool viral wheeze, PVW), since an upper respiratory tract virus can be isolated in more than 80% of preschool children presenting to hospital with acute wheeze.[3] Despite the common trigger factor, epidemiological studies suggest that PVW is a phenotype that is separate from atopic asthma. First, 60% of children with PVW are asymptomatic by 6 years of age.[4] Second, these "transient" wheezers do not have the normal risk factors for atopic sensitisation.[4] We have therefore previously speculated that pulmonary inflammation in PVW is different from chronic atopic asthma.[5] Indeed there are preliminary data to support a role for non-eosinophilic inflammation in PVW. Margeut and colleagues[6] performed bronchoalveolar lavage (BAL) in a highly selected population of children with a history of severe PVW. While no increase in airway eosinophils was found, this group had an increased percentage of neutrophils in the BAL fluid.

Sampling of airway cells during acute PVW is technically and ethically problematic, but evidence for the initial "activation" step in the development of pulmonary neutrophilia can be sought less invasively by the measurement of adhesion molecules. When circulating neutrophils are activated, the L-selectin receptor, an adhesion molecule that can mediate the initial "rolling step" on endothelium,[7] is shed from the cell surface, and enters the circulation as soluble L-selectin (sL-selectin).[8] Conversely, Mac-1 (CD11b), an adhesion molecule that can mediate "firm adhesion" of neutrophils on endothelial cells, is transiently upregulated on the neutrophil surface on activation.[9] Thus markers for systemic neutrophil activation are decreased neutrophil L-selectin expression, increased Mac-1 expression, and increased serum sL-selectin; either singly or in combination.[10 11]

In this study, we sought to determine whether systemic neutrophil activation occurs in PVW by measuring L-selectin and Mac-1 on systemic neutrophils, and serum sL-selectin in children admitted to hospital with PVW. Data were compared to a group of normal controls undergoing elective surgery.

METHODS

Patient details

Acute PWV was defined clinically as: (1) an episode of physician diagnosed wheeze occurring in a child aged 1–5 years; (2) a clear symptom history from the parents of a viral cold in the 48 hours preceding the wheeze attack, with clinical rhinitis on admission; and (3) requiring admission to the Children's Hospital, University Hospitals of Leicester NHS Trust. Exclusion criteria were: (1) premature birth; (2) a clinical diagnosis of bacterial infection; (3) any other chronic respiratory pathology; and (4) admission for >24 hours. Controls were recruited from children undergoing elective ear nose and throat surgery, or ophthalmic surgery. Children were excluded as controls if they had: (1) a chronic respiratory disease; (2) wheezed in the past 12 months; (3) active allergic disease; or (4) symptoms of an acute infection in the preceding week. Blood samples from children with PVW were obtained after topical anaesthesia, and samples from controls were obtained immediately after induction of general anaesthesia. PVW was treated with a single oral dose of prednisolone and "as required" nebulised salbutamol. The study required written parental consent and was approved by the Leicestershire Health Research Ethics Committee.

Abbreviations: BAL, bronchoalveolar lavage; IQR, interquartile range; MEF, molecules of equivalent fluorochrome; PVW, preschool viral wheeze

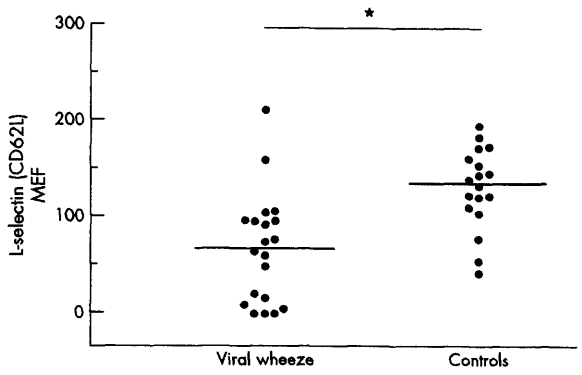

Figure 1 L-selectin (CD62L) expression on systemic neutrophils in acute preschool viral wheeze and controls. *p < 0.001, by Mann-Whitney U test. Bars represent medians.

Sample collection and analysis

One ml venous blood sample was collected into an EDTA bottle within five minutes, and transported on ice for flow cytometry. One ml of blood was allowed to clot at room temperature for 60 minutes; the serum was separated by centrifugation before storage at −70°C for analysis of sL-selectin. A full blood count was done on a 0.5 ml heparinised sample using routine hospital techniques.

Flow cytometry was conducted within 30 minutes of sample collection. The antibodies used were fluorescein isothiocyanate (FITC) conjugated monoclonal anti-CD16, phycoerythrin (PE) conjugated anti-CD 62L (L-selectin), and PE conjugated anti-CD11b (Mac-1) (Becton, Dickinson and Company, Oxford, UK). Isotype matched antibodies, PE conjugated IgG_2a and FITC conjugated IgG_1 (Becton, Dickinson and Company, Oxford, UK) were used as controls. A 50 µl aliquot each of EDTA blood was added to three tubes placed at 4°C, which already contained either control antibodies or anti-CD16 and anti-CD62L or anti-CD16 and anti-CD11b. After incubation for 10 minutes at 4°C, 1 ml of 0.8% ammonium chloride was added to lyse red cells and the samples were transferred to 37°C. Flow cytometry was performed on the samples 10 minutes later (Ortho Cytoron-Absolute, Orthodiagnostic Systems, USA). The extent of the background non-specific fluorescence, as defined by the control antibodies, was restricted to less than 1% of the total events acquired. Discrimination frames were placed around the granulocyte cluster, which was identified based on their forward and side scatter characteristics. Neutrophils within this cluster was identified by CD16 positivity.[12] The expression of CD62L and CD11b of at least 5000 events within the neutrophil population was then recorded. The mean fluorescence intensity of the gated cell population was obtained as channel numbers and was transformed to molecules of equivalent fluorochrome (MEF) using fluorescent beads (DAKO Flourospheres, Dako A/S, Denmark) according to the manufacturer's guidelines.

Serum levels of sL-selectin was analysed by ELISA according to the manufacturer's instructions (Bender Med Systems, Vienna, Austria). Briefly, serum samples were diluted 1/200, and the assay was performed in duplicate. The lower limit for sL-selectin detection was 0.3 ng/ml and the manufacturer's overall intra-assay coefficient variation was 3.7%.

Statistics

Data are summarised as median and interquartile range (IQR), and compared using the Mann-Whitney U test. Correlations were determined by Spearman rank correlation. Analysis was performed using a package for microcomputers (SPSS for Windows, version 10, SPSS Inc., Chicago, IL). Statistical significance was defined as a p value <0.05.

RESULTS

Twenty children (12 boys, 8 girls) with acute viral wheeze and 18 controls (12 boys, 6 girls) were studied. Controls underwent a variety of surgical procedures, including tonsillectomy and adenoidectomy (n = 6), insertion of grommets (n = 7), bronchoscopy (normal) (n = 2), squint (n = 1), and other (n = 2). Compared with controls, children with PVW were slightly younger (median 32 (IQR 18 to 47) months versus 54 (26 to 63) months, p = 0.04). Seventeen (85%) children with PVW had previous attacks with no interval symptoms (median number: 2.0). Three children with PVW had a history of either mild nocturnal cough or wheeze between attacks. In the subgroup where blood neutrophil numbers were measured, the majority of children with PVW and controls were within the normal range (1–8.5 × 10^9 neutrophils/l). However, the PVW group (n = 17) had a higher absolute neutrophil count (8 × 10^9/l (5 to 11) versus 4 × 10^9/l (3 to 5) p < 0.01), and increased percentage of CD16 positive neutrophils (99.5% (98.3 to 99.7) versus 97.1% (94.2 to 97.7), p < 0.001).

Neutrophil L-selectin expression in PVW (n = 20) was significantly lower than controls (n = 18) (MEF: 69 (11 to 96) units versus 136 (109 to 163) units, p < 0.001; fig 1). There was no difference in Mac-1 expression between the two groups (MEF: 934 (722 to 1287) units versus 730 (571 to 1000) units; fig 2). Neutrophil L-selectin expression was not associated with age in either viral wheeze or controls (NS by Spearman rank correlation). sL-selectin was detected in the sera of all children (PVW, n = 15; controls, n = 16), and was higher in PVW (2.8 (2.3 to 3.1) µg/ml versus 2.4 (2.2 to 2.6)

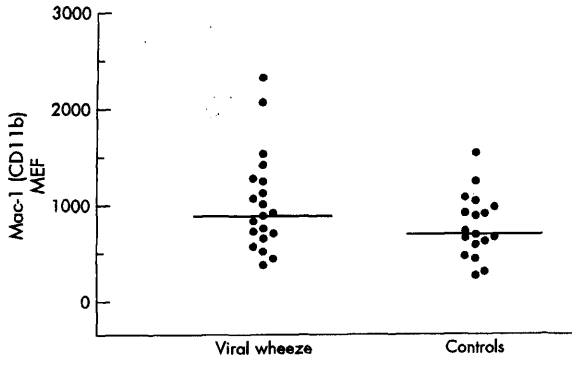

Figure 2 Mac-1 (CD11b) expression on systemic neutrophils in acute preschool viral wheeze and controls. There is no significant difference between the two groups by Mann-Whitney U test. Bars represent medians.

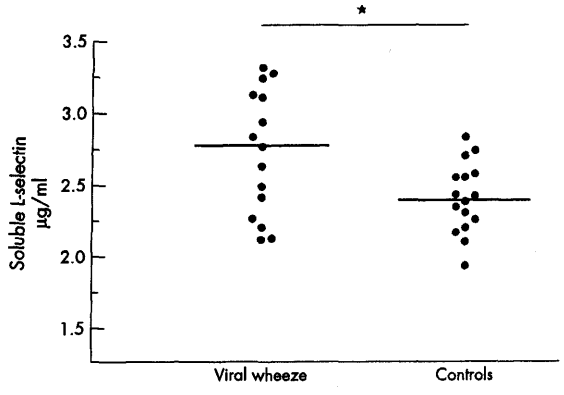

Figure 3 Serum soluble L-selectin in acute preschool viral wheeze and controls. *p = 0.04 by Mann-Whitney U test. Bars represent medians.

μg/ml, p = 0.04; fig 3). There was no association between time from oral steroid therapy and L-selectin expression in the PVW group (NS, Spearman's rank correlation).

DISCUSSION

In this study we report, for the first time, that the expression of L-selectin is reduced on systemic neutrophils, and serum sL-selectin is increased, in clinically severe PVW. These data are compatible with generalised neutrophil activation and shedding of L-selectin from the neutrophil surface into the systemic circulation.[13] We did not detect an increase in Mac-1, possibly because Mac-1 had returned to baseline levels after initial rapid upregulation, as described for neutrophils stimulated in vitro.[14]

Systemic neutrophil activation has not been studied during viral triggered wheeze in adults, and it is possible that the same changes in adhesion molecules occur in both viral triggered atopic asthma and PVW. However, previous studies have reported no association between systemic neutrophil activation and the clinical severity of atopic asthma. For example, in 't Veen and colleagues[15] found no difference in neutrophil L-selectin expression between normal (non-atopic) controls and adults with either mild or severe atopic asthma. Furthermore, Oymar and Bjerknes[16] compared serum sL-selectin in atopic asthmatic children with healthy controls, and found no increase in sL-selectin during an acute attack.

Although the present study shows the first "activation" step in the development of pulmonary neutrophilia, we cannot directly implicate the neutrophil in the pathogenesis of PVW. This is because several confounders remain to be excluded. First, trivial symptoms associated with the common cold may be associated with a similar pattern of neutrophil activation. In a very small group of children with rhinovirus colds, we have previously reported the presence of BAL fluid neutrophilia,[17] which may indicate that some neutrophil transmigration may occur during most trivial colds. We speculate that children with PVW have a propensity to recruit more neutrophils in their airways, and that neutrophil products trigger bronchoconstriction via activating other inflammatory cells, such as mast cells. Indeed, we have recently found evidence of leukotriene activation in a subgroup of children with PVW.[18] To define further the relation between neutrophil activation and the development of bronchoconstriction in PVW, more data are needed on the extent of neutrophil activation during colds in normal children. A second potential confounder is the effect of steroids. Steroids decrease the expression of L-selectin on circulating neutrophils,[19] an effect that occurs after a "lag phase" of eight hours. Decreased expression is not caused by shedding from the neutrophil surface, but the release of L-selectin "low" expressing neutrophils from bone marrow.[20] Neutrophils are not activated by corticosteroids, and serum sL-selectin is therefore not increased.[19] Since we found both increased sL-selectin in PVW, and reduced L-selectin expression on neutrophils from children with PVW sampled within three hours of oral steroids (n = 4, p = 0.01 versus controls, data not shown), it is unlikely that steroid therapy is a major confounder. A confounding effect of atopy is also unlikely since previous studies have found no affect of atopic sensitisation on neutrophil L-selectin expression or serum sL-selectin.[15 16] There are no data on the effects of anaesthetic agents on paediatric neutrophil L-selectin expression. In the normal controls, the very short period from induction of anaesthesia to sampling should have minimised any effect. Some anaesthetic agents tend to activate neutrophils. For example, ketamine further decreases L-selectin expression when adult neutrophils are stimulated by fMLP in vitro.[21] However, if anaesthesia had activated neutrophils in our control group, this would have reduced rather than increased the difference in L-selectin expression.

In conclusion, acute PVW is associated with systemic neutrophil activation. Our data are compatible with a role for neutrophils in the pathogenesis of PVW.

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Articles

Efficacy of a short course of parent-initiated oral prednisolone for viral wheeze in children aged 1–5 years: randomised controlled trial

Abraham Oommen, Paul C Lambert, Jonathan Grigg

Summary

Background Episodic wheeze triggered by viral colds is common in children aged between 1 and 5 years (preschool viral wheeze). Most affected children are asymptomatic by age 6 years. Persistence of wheeze is associated with above-average systemic eosinophil priming. Use of parental-initiated oral prednisolone is recommended at the first sign of preschool viral wheeze. However, evidence for this treatment strategy is conflicting. We therefore aimed to assess the efficacy of a short course of oral prednisolone for preschool viral wheeze, with stratification for systemic eosinophil priming.

Methods Children aged 1–5 years admitted to hospital with viral wheeze were allocated to either a high-primed or low-primed stratum according to amounts of serum eosinophil cationic protein and eosinophil protein X, and randomised to parent-initiated prednisolone (20 mg one daily for 5 days) or placebo for the next episode. The primary outcomes were the 7-day mean daytime and night-time respiratory symptom scores, which were analysed by mean differences between treatment groups.

Findings 108 children were randomised to placebo and 109 to prednisolone. Outcome data were available for 120 (78%) of 153 children who had a further episode of viral wheeze, of whom 51 received prednisolone and 69 placebo. Mean daytime (difference in means −0·01 [−0·22 to 0·20]) and night-time (0·10 [−0·12 to 0·32]) respiratory symptom scores and need for hospital admission did not differ between treatment groups. Within the high-primed (n=59) and low-primed (n=61) strata there was no difference in primary outcome between treatment groups.

Interpretation There is no clear benefit of a short course of parent-initiated oral prednisolone for viral wheeze in children aged 1–5 years even in those with above-average eosinophil priming.

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Introduction

The clinical diagnosis of asthma encompasses different phenotypes of wheeze associated with different risk factors, long term outcomes, underlying inflammation, and responses to treatment.[1–3] The predominant asthma phenotype in school-age children (6–16 years) is the classic atopic variant; a disorder characterised by widespread airflow obstruction, increased airway responsiveness to a range of stimuli, pulmonary eosinophilia, and, in vitro, a propensity of systemic eosinophils to release eosinophil cationic protein (ECP) and eosinophil protein X (EPX).[4–7] By contrast, asthma in children aged 1–5 years is characterised by recurrent, transient episodes of wheeze triggered by viral colds;[8,9] a phenotype previously labelled as wheezy bronchitis,[8] and now as preschool viral wheeze.[10] Although airway cells have not been examined in acute cases of this disorder, there is indirect evidence that its inflammatory substrate differs from atopic asthma. First, most children with preschool viral wheeze do not have risk factors for atopic sensitisation.[11] Second, most become asymptomatic by 6 years of age.[12] However, characteristic risk factors for atopic asthma, including increased systemic eosinophil priming, are associated with the few children in whom wheezing does not resolve.[11] For example, Villa and colleagues,[13] reported that in preschool children with episodic wheeze, increased serum ECP was associated with a subsequent diagnosis of current asthma at a 2-year follow-up.

Preschool viral wheeze is a transient condition, and is treated by inhaled bronchodilators as required. An additional strategy is to start a short course of systemic corticosteroids at the first sign of viral wheeze, with the aim of attenuating lung inflammation, and preventing progression to severe wheeze.[14] Indeed, extrapolation from trials in adults and older atopic asthmatic children presenting to hospital clinicians, suggests that early use of corticosteroids should reduce attack severity.[15] The consensus in the UK and the USA thus lends support to the practice of issuing parents with a course of oral steroids for treatment of viral wheeze in their young children.[5,16] However, evidence that corticosteroids given during the early stages of preschool viral wheeze improve clinical outcome is conflicting. Researchers of two placebo-controlled studies of parent-initiated treatment, that probably included young children with viral wheeze, noted that a 5-day course of oral prednisolone did not reduce respiratory symptoms.[17,18] By contrast, results of an open-label trial showed that oral prednisolone initiated by parents at the first sign of a cold resulted in a 90% reduction in admissions to hospital in preschool children with a history of recurrent severe attacks.[19]

In this investigation, we aimed to assess the efficacy of a parent-initiated short course of oral prednisolone in preschool viral wheeze. Stratification for systemic eosinophil priming was included to ensure that we could identify children at increased risk for atopic asthma. The

primary outcomes were the 7-day mean daytime and night-time lower respiratory symptom score, obtained from a parent-completed symptom diary.

Methods

Patients

Children eligible for inclusion in the study were aged between 1 and 5 years of age, and were admitted with viral wheeze to the University Hospitals of Leicester NHS Trust Children's Hospital between June 1, 1999, and June 30, 2002. We defined preschool viral wheeze as an acute episode of wheeze that arose within 2 days of the onset of coryzal upper respiratory tract symptoms. Exclusion criteria were: a history of chronic lung disease, upper respiratory tract structural abnormality, substantial non-respiratory tract disease needing continuous systemic pharmacological treatment, clinical suspicion of active systemic bacterial infection, a history of prematurity or neonatal respiratory distress, a history of chronic rhinitis with no clear pattern of acute episodes, and oral prednisolone having been given more than 1 and less than 14 days before admission. The trial was approved by the Leicestershire Health research ethics committee, and signed informed parental consent was obtained before randomisation. Within 24 h of their child's admission, parents were given a written information sheet explaining the trial, once rhinitis and wheeze had been confirmed by a paediatrician. The supervising paediatrician confirmed that parents understood the trial, and countersigned the consent form.

Procedures

We used a double-blind, randomised design, with stratification for systemic eosinophil priming. Study numbers were assigned sequentially and randomisation within strata was achieved by generating numerical codes in random permuted blocks of 10. Randomisation and packaging of placebo and prednisolone was done by Nova Laboratories, Leicester, UK. Placebo and predinisolone was packaged as identical capsules containing white powder, and was in identical containers labelled with participant number only. The research nurses and clinicians were masked to treatment allocation. The envelope with randomisation details was opened only after all data had been entered electronically.

A 2 mL blood sample was obtained from every child. 1 mL of blood was allowed to clot for 60 mins at 22°C. Serum was separated by centrifugation and stored at −70°C. Parents were provided with a preschool respiratory symptom diary card,[10,20] and taught how to fill it in. On a scale of 0 to 3, the severity of night-time symptoms, daytime symptoms, and disruption of daytime activity was recorded once daily for 7 days (table 1). Parents chose the score that best described symptom severity, and recorded frequency of use of inhaled medication.

	Night-time symptoms (cough, wheeze, or breathing difficulty)	Daytime or activity ratings (cough, wheeze, breathing difficulty, or play limitation)
Score*		
0	None	None
1	Slight; sleep not disturbed	Slight; no treatment given
2	Sleep disturbed once; no help required	Needed treatment but no outside help
3	Sleep disturbed more than once or child needed help	Severe; needed help from family practioner

*Need for hospital admission recorded separately.

Table 1: **Respiratory score used by parents to complete the symptom diary**

After discharge, and before randomisation, children were stratified into a high-primed stratum (serum ECP ≥20 μg/L, or EPX ≥40 μg/L, or a combination of both) or a low-primed stratum (serum ECP <20 μg/L and EPX <40 μg/L). An ECP cutoff value of 20 μg/L was chosen since it most accurately predicts wheeze persistence.[13,21] An EPX cutoff value of 40 μg/L was chosen since it is equivalent to 20 μg/L ECP by linear regression.[22] Serum ECP and EPX were assayed by double-antibody radioimmunoassay (Pharmacia AB Diagnostics, Uppsala, Sweden) in duplicate.

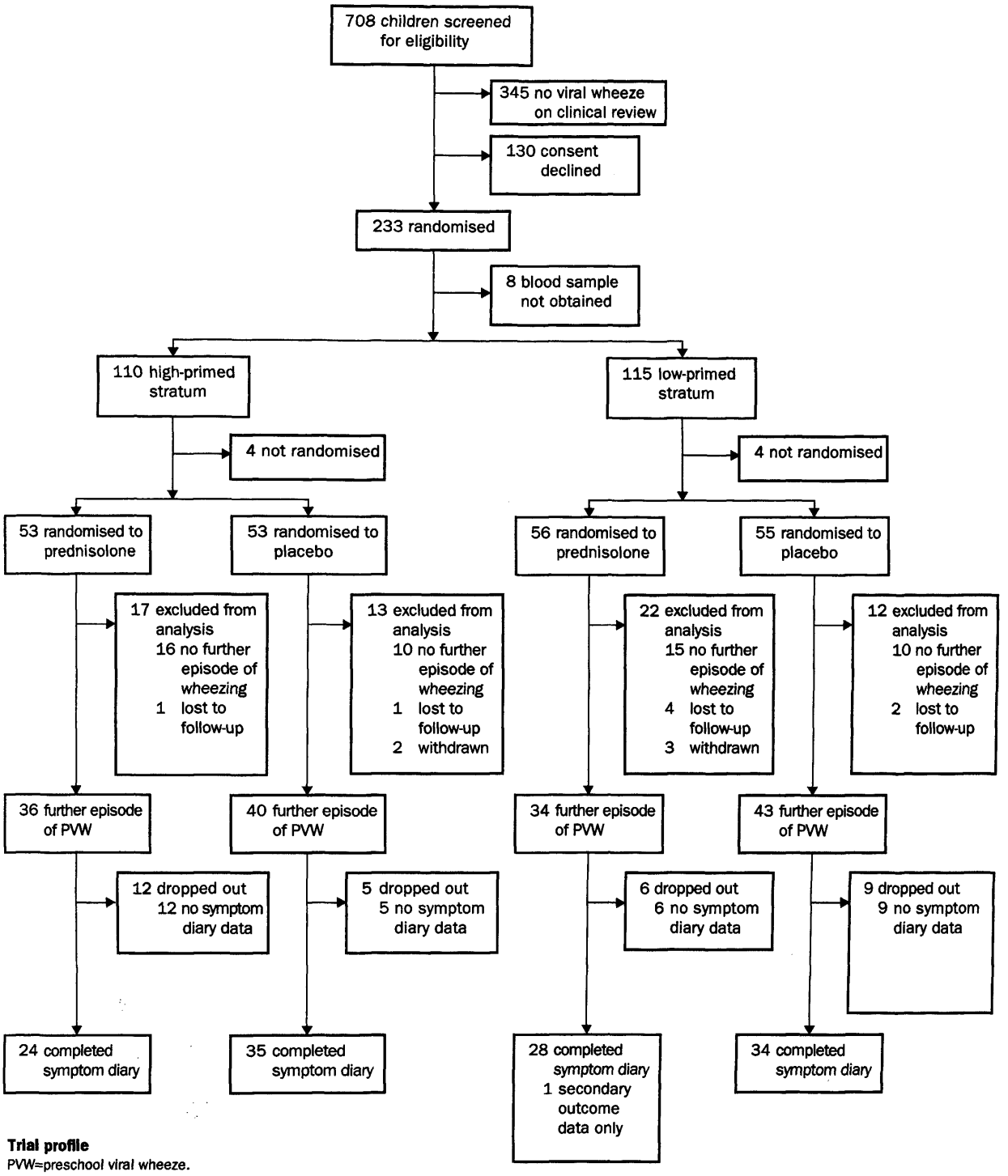
Within 6 weeks of discharge from hospital, parents were visited by the research nurse and were issued with placebo or 20 mg prednisolone capsules, one to be taken orally once a day for 5 days at the start of the next episode of viral wheeze. Preschool viral wheeze was defined as wheeze occurring within 2 days of the onset of coryzal upper respiratory tract symptoms. Parents were also asked to give their children inhaled salbutamol as needed (to a maximum dose of 400 μg, 4-hourly, by metered dose inhaler and Volumatic spacer [Allen and Hanburys, Uxbridge, Middlesex, UK]), and to record respiratory symptoms for 7 days. The trial medication could be substituted with oral prednisolone by a paediatrician or a family practitioner, if deemed clinically necessary. If a child were admitted, parents were instructed to continue to record symptoms and bronchodilator usage. Children who did not have an episode of viral wheeze within 12 months of randomisation were withdrawn from the trial. The primary outcomes were the 7-day mean daytime and night-time respiratory tract symptom scores. The secondary outcome measures were mean number of daily salbutamol actuations, hospital admission, and need for substitution of the trial medication with oral prednisolone.

Statistical analysis

The initial power calculation was done without knowledge of symptom scores in the study population (*Lancet* protocol 99PRT/19). We recalculated power from the SDs of mean daytime and night-time symptom scores over 7 days from a local population of preschool children with viral wheeze.[10] With 50 children in each treatment group the detectable difference is 0·34 assuming the SDs of mean 7-day daytime and night-time symptom scores are 0·6, at 5% significance level and 80% power. We also wanted to detect any difference in treatment effect between high-primed and low-primed strata. Assuming a 5% significance level, power of 80%, an SD of 0·6 of mean daytime or night-time symptom score over 7 days and an equal distribution of participants in high-primed and low-primed strata, there is a detectable difference of 0·69.

Differences between treatment groups for continuous outcomes were assessed by obtaining mean differences and 95% CI. The mean number of daily salbutamol actuations was log transformed before analysis since the data were positively skewed. The treatment effect thus refers to the ratio of geometric means for this outcome. If there were fewer than five valid observations for the daytime or night-time symptom score, or for secondary outcome data, values were coded as missing.

Differences between treatment groups for categorical data were reported as difference in proportions, with 95% CIs, or with Fisher's exact test for small cell frequencies. To establish whether there was a treatment difference between the high-primed and low-primed strata, we fitted a regression model to the data, and assessed the interaction between treatment groups (prednisolone and placebo) and strata (high-primed and low-primed). For



Trial profile
PVW=preschool viral wheeze.

continuous outcomes, assessment was done by fitting a linear model with normal errors. For categorical outcomes, we did the assessment by fitting a logistic regression model. The primary and secondary endpoints between treatment groups were compared first, followed by within-strata analysis.

The protocol for this study was peer-reviewed and accepted by *The Lancet*; a summary of the protocol was published on the journal's website (http://thelancet. com), and the journal then made a commitment to peer-review the primary clinical manuscript.

Role of the funding source
The sponsor of the study had no role in the collection, analysis and interpretation of the data, had no input into the writing of the report, or in the decision to submit for publication.

Results

708 children admitted with acute lower respiratory tract symptoms underwent review by a research nurse. 345 were excluded, and 130 parents refused consent. Consent was obtained from the remaining 233 parents. A blood sample

	Prednisolone			Placebo		
	All (n=109)	Analysed* (n=52)	Not analysed (n=57)	All (n=108)	Analysed (n=69)	Not analysed (n=39)
Age (months)	25 (17–37)	25 (17–25)	24 (18–38)	27 (19–38)	25 (19–38)	30 (18–37)
Boys	70 (64%)	31 (60%)	39 (68%)	77 (71%)	47 (68%)	30 (77%)
Doctor-diagnosed asthma	35 (32%)	16 (31%)	19 (33%)	35 (32%)	25 (36%)	10 (26%)
Doctor-diagnosed eczema	41 (38%)	17 (33%)	24 (42%)	36 (33%)	27 (39%)	9 (23%)†
Family history of asthma	84 (77%)	42 (81%)	42 (73%)	80 (74%)	51 (74%)	29 (74%)
Inhaled steroids	30 (28%)	18 (35%)	12 (21%)	31 (29%)	23 (33%)	8 (21%)
Previous wheeze or cough without colds	27 (25%)	10 (19%)	17 (30%)	33 (31%)	22 (32%)	11 (28%)
Age at first wheeze (months)	12 (6–21)	12 (6–22)	12 (7–20)	12 (8–23)	12 (8–22)	14 (8–24)
Previous PVW episodes	3 (1–6)	2 (1–4)	3 (1–8)	3 (1–5)	2 (1–5)	3 (2–6)
Serum ECP (μg/L)	16 (10–25)	15 (9–25)	16 (11–25)	16 (10–26)	16 (11–26)	16 (9–25)
Serum EPX (μg/L)	29 (19–42)	26 (19–34)	32 (17–51)	31 (21–42)	32 (21–45)	30 (21–40)

Data are number (%) median (IQR). PVW=preschool viral-wheeze. ECP=eosinophil cationic protein. EPX=eosinophil protein X. *One child analysed for secondary outcome only. †p=0·05 by χ^2 test *vs* analysed. p=not significant for all other comparisons for analysed *vs* not analysed.

Table 2 : **Characteristics of children randomised**

could not be taken from eight children (figure), thus 225 children with acute viral wheeze were entered into the trial: 110 in the high-primed eosinophil stratum, and 115 in the low-primed stratum (figure). Eight children were withdrawn before randomisation, and 108 were randomised to receive placebo and 109 to receive prednisolone (table 2). Baseline characteristics did not differ between strata (data not shown), treatment group (table 2), or children included or not included in the primary outcome analysis (table 2).

32 parents of 153 (68%) children who had a further episode of viral wheeze either did not give the trial medication, or did not fill in the diary (figure). Secondary outcome data only were available for one child. The mean 7-day daytime respiratory symptom score was calculated for 120 children, 51 of whom received oral prednisolone and 69 placebo. Mean daytime symptom score over 7 days did not differ between controls and prednisolone-treated groups (difference in means −0·01 [95% CI −0·22 to 0·2] table 3). The night-time score was completed for 117 children, and no difference was detected between treatment groups (0·1 [−0·12 to 0·32] table 3).

For secondary outcomes, the geometric mean number of salbutamol actuations per day was similar for the two treatment groups, as was the need for steroid substitution (table 4). There was a trend for more frequent admissions to hospital in the prednisolone group than in controls (12% *vs* 3%, p=0·06, table 4), but no clear parental preference was seen for placebo or prednisolone (table 4).

Of those children completing the trial, 59 were in the high-primed eosinophil stratum, and 62 in the low-primed stratum (figure). Secondary outcome data only were available for one child in the low-primed strata. For primary and all secondary outcomes there were similar treatment effects for both eosinophil strata, and all comparisons of the two treatment groups were not significant (tables 3, 4).

	n	Mean (SD)	Difference (95% CI)
Day-time score			
All			
Prednisolone	51	0·95 (0·56)	−0·01 (−0·22 to 0·20)
Placebo	69	0·96 (0·59)	
High-primed†			
Prednisolone	24	0·92 (0·51)	−0·01 (−0·30 to 0·28)
Placebo	35	0·92 (0·57)	
Low-primed			
Prednisolone	27	0·97 (0·60)	−0·02 (−0·33 to 0·29)
Placebo	34	0·99 (0·61)	
Mean night-time score			
All			
Prednisolone	50	0·92 (0·58)	0·10 (−0·12 to 0·32)
Placebo	67	0·82 (0·61)	
High-primed‡			
Prednisolone	23	0·96 (0·62)	0·16 (−0·17 to 0·48)
Placebo	34	0·80 (0·59)	
Low-primed			
Prednisolone	27	0·89 (0·54)	0·05 (−0·26 to 0·36)
Placebo	33	0·84 (0·63)	
Mean salbutamol actuations per day*			
All			
Prednisolone	50	1·59 (0·87)	0·93 (0·65 to 1·32)
Placebo	67	1·66 (0·99)	
High-primed§			
Prednisolone	23	1·51 (0·75)	0·92 (0·58 to 1·48)
Placebo	34	1·59 (0·96)	
Low-primed			
Prednisolone	27	1·66 (0·54)	0·92 (0·53 to 1·58)
Placebo	33	1·75 (1·04)	

Night-time score not fully completed for 3 children. *Data were log transformed before analysis (difference=ratio of geometric means). Interaction between high-primed and low-primed strata; †p=0·96. ‡p=0·63. §=0·99.

Table 3: **7-day day-time and night-time symptom score and daily uses of salbutamol**

	Number of patients (%)	Difference (95% CI)	p*
Parents considered treatment was effective†			
All			
Prednisolone	17/23 (74%)		
Placebo	21/33 (64%)	10·3% (−14·0 to 34·6)	0·42
High-primed‡			
Prednisolone	9/13 (69%)		
Placebo	11/17 (65%)	4·5% (−29·3 to 38·4)	0·79
Low-primed			
Prednisolone	8/10 (80%)		
Placebo	10/16 (62%)	17·5% (−16·8 to 51·8)	0·35
Substitution of trial medication with oral prednisolone			
All			
Prednisolone	9/52§ (17%)		
Placebo	8/69 (12%)	5·7% (−7·0 to 18·4)	0·37
High-primed¶			
Prednisolone	3/24 (12%)		
Placebo	4/35 (11%)	1·1% (−15·8 to 18·0)	0·90
Low-primed			
Prednisolone	6/28 (21%)		
Placebo	4/34 (12%)	9·7% (−9·0 to 28·3)	0·30
Need for hospitalisation			
All			
Prednisolone	6/52 (12%)		
Placebo	2/69 (3%)	8·6% (−0·9 to 18·2)	0·058
High-primed			
Prednisolone	3/24 (12%)		
Placebo	0/35 (0%)	Not done	0·062‖
Low-primed			
Prednisolone	3/28 (11%)		
Placebo	2/34 (6%)	Not done	0·65‖

*With χ^2 test. †Of those who expressed an opinion. ‡Interaction between strata, p=0·56. §Includes one child in whom only secondary outcome recorded. ¶Interaction between strata, p=0·57. ‖With fisher's exact test.

Table 4: **Comparison of binary outcomes**

Discussion

Our results show that, in children with a previous history of a clinically significant episode of viral wheeze, a 5-day course of oral prednisolone initiated by the parents at home at the start of wheezing did not have an effect on the daytime and night-time lower respiratory tract symptom score, need for inhaled salbutamol, or need for hospital admission. Furthermore, we did not record evidence for a beneficial effect of prednisolone in children with increased systemic eosinophil priming.

Our results differ from those of Brunette and colleagues[19] who treated a group of children with preschool viral wheeze with a history of severe episodes of wheeze. Intermittent oral prednisolone was given by parents during the treatment year. Compared with the previous year, there was a 65% decrease in the number of wheezing days, and a 90% fall in need for admission to hospital during the steroid-treated period.[19] No improvement was seen in an untreated group during the second year. In Brunette and colleagues' study, prednisolone was started at the first evidence of a cold, whereas parents in our trial started prednisone at the first sign of wheeze. This difference in timing might, in part, explain the different conclusions, although our trial is representative of current practice within the UK, where steroids would not be started for upper respiratory tract symptoms alone. Furthermore, Brunette and colleagues's trial[19] was not randomised or placebo-controlled, and used historical controls for a condition that improves with time.[23] By contrast, our data are compatible with the results of Webb and others[17] who prescribed placebo or prednisolone (2 mg/kg bodyweight per day for 5 days) in an outpatient setting to preschool children and infants with wheezy bronchitis—the previous term for preschool viral wheeze. In this crossover investigation, prednisolone did not reduce daily symptom scores of cough, wheeze, and breathlessness, and secondary outcome analysis found no evidence of a beneficial effect in children aged between 12 and 18 months—ie, the comparable age group with the present study.

We included stratification for eosinophil priming to ensure that responses in the subgroup of preschool viral wheeze at increased risk of persistence of wheeze could be detected,[13,21] but noted no evidence that children with increased eosinophil priming were responsive to prednisolone. Indeed, there was no interaction between treatment group and stratification for any outcome measure. However, the ability to detect a difference in symptom score within the two strata was less sensitive than that for detection of the overall effect of treatment, and a clinically relevant effect of prednisolone in the high-primed strata might have been present, but not detected. We estimate that researchers in future trials of a similar design to the present study should recruit about 800 children admitted to hospital to detect a 0·34 change in the symptom score between strata. An alternative explanation is that systematic eosinophil priming might not accurately represent eosinophilic (steroid responsive) pulmonary inflammation. However, in atopic asthmatic children (median 6·7 years), a serum ECP value higher than 20 μg/L accurately predicts substantial airway eosinophilia,[24] and systemic priming status therefore remains the best method for identification of potentially steroid-responsive children.

There are limitations to consider in interpretation of our results. First, there was a high rate of non-compliance with treatment. Parents had witnessed their child having a previous severe episode of viral wheeze, and the degree of compliance probably indicates the best that can be achieved in the UK. Second, 23% of children who were randomised did not develop a further episode of preschool viral wheeze. Delaying randomisation until the children's next episode of viral wheeze within the trial would have equalised treatment allocation, but we judged that the extra demand placed on parents to contact the research centre would have increased the failure rate. Third, if the trial medication had been substituted by oral steroids at an early stage for many children, the efficacy of prednisolone would not have been assessed. All children in this trial received at least one dose of trial medication before steroid substitution, and most received a full course of trial therapy. The negative result therefore probably indicates both failure of the treatment strategy, and lack of efficacy of prednisolone. Finally, since wheeze and cough were assessed together in the respiratory symptom score, steroid-induced reduction in wheeze could have been balanced by an increase in cough. However, wheeze, but not cough, had to be present before the trial medication could be started, and the scoring addressed the issue of most concern to parents and clinicians—ie, the degree of distress caused by lower respiratory tract symptoms.

An unexpected finding was the trend for increased admission to hospital in the prednisolone-treated group. A similar counterintuitive result was seen by Grant and colleagues[25] who reported that a single oral dose of prednisolone increased physician attendance in asthmatic children aged 2–14 years. Behavioural changes have been described in children during oral prednisolone therapy,[26] and this might affect the clinical decision to admit to hospital.[27] Our trial was not designed to detect an effect of steroids on behaviour, and future studies of oral steroids should include some form of behavioural assessment.

We did not address the efficacy of oral steroids in children with severe preschool viral wheeze admitted to hospital. A recent Cochrane review on the use of systemic corticosteroids for children admitted with acute asthma concluded that, although treatment may result in slightly shorter hospital stays, there is no effect on pulmonary function or oxygen saturation.[28] Since the benefits of steroids in children with severe wheeze and in the age range at increased risk of atopic asthma are subtle, it is perhaps not surprising that we found no benefit of a community-based strategy for preschool viral wheeze. Current British guidelines for the management of preschool asthma, recommend that parents may be provided with a course of oral steroids as part of a management plan for paediatric asthma.[5] Our findings suggest that this strategy may need re-evaluation for preschool children with viral wheeze, since there are no clear benefits to balance potential risks.

Contributors

A Oommen undertook study recuitment and assessments, supervised research nurses, collated data, contributed to interpretation of data and to drafting the report. P Lambert did statistical analyses and contributed to drafting the report. J Grigg conceived and designed the study, obtained funding, supervised data collection, and wrote the report.

Conflict of interest statement

JG has received funding to attend meetings and honoraria for educational talks from Merck Sharpe and Dohme (UK), 3M, Glaxo-Wellcome, and Astra. He has received research grant funding from Merck Sharpe and Dohme (UK) and Glaxo-Wellcome. AO has received funding to attend meetings from AstraZeneca, 3M, and Merck Sharpe and Dohme (UK), and research grant funding from Merck Sharpe and Dohme (UK).

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Appendix 5. Presentations

Oral presentations

1. **Oommen A,** Peck K, McNally T, Lambert P, Grigg J. Efficacy of a short course of parent initiated oral prednisolone for acute preschool viral wheeze. ERS International Conference Vienna 2003
2. **Oommen A,** Patel R, Peck K, McNally T, Browning M, Grigg J. Shedding of L-selectin and upregulation of Mac-1 on neutrophils in pre-school viral wheeze. BTS Winter Meeting 2000.
3. **Oommen A,** Peck K, McNally T, Grigg J. The relation between Cysteinyl leukotrienes and Eosinophil activation in pre-school children with viral wheeze. RCPCH York meeting 2000.

Poster presentations

1. **Oommen A,** Peck K, McNally T, Grigg J. Eosinophil activation in nonatopic pre-school children with episodic viral wheeze. ERS International Conference Berlin 2001
2. **Oommen A,** Peck K, McNally T, Grigg J. Cysteinyl leukotriene production and Eosinophil activation in pre-school children with viral wheeze. ERS International Conference Florence 2000

Appendix 6. Symptom diary START DATE: ☞

Day of episode		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Today my child has	a cold or a runny nose														
	a fever														
	earache or sore throat														
2. My child's wheeze or cough last night															
	I did not hear my child wheezing or coughing last night														
	I heard my child wheezing or coughing but he/she did not wake up														
	My child woke 2 to 3 times because of wheezing or coughing														
	My child was awake a lot because of wheezing or coughing														
3. My child's wheeze today															
	My child had no wheeze														
	My child was wheezing but not bothered at all														
	My child was wheezing but bothered only a little														
	My child was wheezing and bothered quite a lot														
4. Today my child visited the GP or GP was called															
5. Today my child was taken to hospital because of wheezing															
6. Name of inhaler	**Dose**	**Write in the boxes the number of puffs you gave your child each time**													
a)	Daytime														
	Night-time														
b)	Daytime														
	Night-time														
c)	Daytime														
	Night-time														
d)	Daytime														
	Night-time														
7. Urine sample (please tick the box when collected)															

Appendix 7

Leukotriene E_4 (LTE_4) assay

This assay is based on the competition between free LTE_4 and LTE_4 tracer (linked to an acetylcholinesterase molecule) for a limited number of LTE_4 specific rabbit antiserum binding sites. The amount of LTE_4 tracer able to bind to the rabbit antiserum is inversely proportional to the concentration of free LTE_4 in the well.

The rabbit antiserum-LTE_4 complex binds to the mouse monoclonal antibody previously attached to the well. The plate is then washed to remove unbound antigens and Ellman's reagent is added. The intensity of colour, determined spectrophotometrically (at 412nm) is proportional to the amount of LTE_4 tracer in the well, which is inversely proportional to the free LTE_4 in the well.

Pre-assay preparation: The LTE_4 standard is prepared and then serially diluted 8 fold. The plate is first rinsed with the wash buffer and then set up. First 8 wells are blank, maximum binding, non-specific binding, and total activity; Standards (8) are done in duplicate; samples to be studied are done in duplicate in 2 dilutions (cases - X2 and X5 and controls - X2 and X4).

The plate in incubated for 18 hours, the wells are rinsed and tracer added. The plate is then sealed, incubated and shaken for 60-90 minutes. The plate is read at 412nm.

The reading are analysed, standard curve drawn and compared to the 'quality control data sheet'. The acceptable variation was 10% and the mean values were calculated.

Many cases required repeat testing as we later realised that the urine in wheezy children were very concentrated (high creatinine) compared to controls.

Appendix 8

Human soluble L-selectin assay (sL-selectin) - Bender MedSystems

This analysis is an ELISA assay. The reagents are prepared, and samples are diluted (1:100) with the sample diluent. The Microwell strips (coated with monoclonal antibody (murine) to human sLselectin are taken out and loaded into the strip holder.
The strips are washed twice and sample diluent added to all standard wells. The sLselectin standard is added in duplicate and diluted five times.
The samples are added in duplicate and the conjugate is added and incubated for 2 hours. The strips are then washed and the substrate solution is added to all wells, incubated for 15 minutes. The enzyme reaction is stopped by adding the stop solution and the absorbance of each well on a spectrophotometer.
The average absorbance for each set of duplicate standards and samples is to be within 20% of the mean. The standard curve is drawn and concentration of sLselectin then calculated.

Appendix 9 **Raw data of respiratory score in clinical trial**

Study ID	EOS grp	TM 1pred 2plac	to include	TM opinion	d0	d1	d2	d3	d4	d5	d6	days data	n1	n2	n3	n4	n5	n6	n7	Night data	r0	r1	r2	r3	r4	r5	r6	rel: data
1100	2	2	1	3	2	2	0	0	0	0	0	7	1	1	0	0	0	0	0	7	8	8	0	0	4	0	0	7
1103	1	2	1	2	2	2	1	1	0	0	0	7	2	2	0	0	0	0	0	7	50	20	10	4	4	4	4	7
1104	1	2	1	1	1	2	1	1	0	0	0	7	2	2	0	0	0	0	0	7	4	5	4	4	1	0	0	7
1105	1	2	1	1	2	2	0	0	0	0	0	7	2	0	0	0	0	0	0	7	20	16	10	8	6	4	0	7
1107	2	2	1	1	1	1	1	0	0	0	0	7	1	1	1	0	0	0	0	7	0	8	4	0	0	0	0	7
1108	1	1	1	1	3	3	1	0	0	0	0	7	2	2	0	0	0	0	0	7	14	14	14	6	6	0	0	7
1112	1	1	1	1	2	3	0	0	0	0	0	7	3	2	2	1	0	0	0	7	4	8	6	6	6	0	0	7
1113	1	2	1	1	1	1	1	1	0	0	0	7	2	2	2	0	0	0	0	7	4	8	8	8	0	0	0	7
1114	1	2	1	3	1	2	2	2	2	0	0	7	0	2	1	2	2	2	2	7	6	12	12	3	3	0	0	7
1116	2	1	1	3	3	3	3	0	0	0	0	7	3	3	1	0	0	0	0	7	8	8	8	0	0	0	0	7
1117	1	2	1	3	1	1	0	0	0	0	0	7	1	1	0	1	0	0	0	7	13	2	0	0	0	0	0	7
1118	2	2	1	3	2	2	0	0	0	0	0	7	3	3	0	0	0	0	0	7	6	6	0	0	0	0	0	7
1119	1	1	1	3	2	2	1	0	0	0	0	7	2	2	1	1	0	0	0	7	7	6	6	1	0	0	0	7
1120	2	2	1	3	2	3	0	1	1	1	1	7	3	1	0	0	0	0	0	7	24	10	34	24	24	16	24	7
1121	1	1	1	1	1	1	1	0	0	0	0	7	0	2	1	0	0	1	1	7	4	8	8	8	8	8	6	7
1122	2	1	1	3	1	1	2	1	0	0	0	7	2	1	2	1	0	0	0	7	6	6	8	6	4	4	4	7
1125	2	2	1	3	2	1	0	0	0	0	0	7	1	0	0	0	0	0	0	7	2	4	2	0	0	0	0	7
1126	1	2	1	3	2	0	0	0	0	0	0	7	2	0	0	0	0	0	0	7	40	0	0	0	0	0	0	7
1127	1	1	1	3	2	2	1	1	1	1	1	7	0	2	1	1	1	1	1	7	30	4	4	0	0	0	0	7
1128	1	2	1	2	3	2	2	2	0	0	0	7	2	2	2	0	0	0	0	7	36	24	24	24	0	0	0	7
1129	2	1	1	3	2	2	2	1	1	0	0	7	1	1	1	1	0	0	0	7	8	8	8	8	8	4	4	7
1131	2	2	1	3	2	2	1	0	0	0	0	7	2	2	1	1	1	1	1	7	16	16	12	12	0	0	0	7
1133	1	1	1	1	2	2	3	3	2	2	1	7	2	2	2	2	1	1	1	7	8	8	12	12	12	12	12	7
1135	1	1	1	3	2	1	1	1	1	0	0	7	1	1	0	1	0	0	0	7	3	3	0	0	0	0	0	7
1136	1	2	1	2	1	1	1	1	2	2	3	7	1	1	0	2	0	1	2	7	10	8	12	15	12	14	8	7
1140	2	1	1	3	1	1	1	1	1	1	1	7	0	2	2	1	1	0	0	7	10	10	10	10	10	4	4	7
1142	2	1	1	3	3	3	3	3	1	1	1	7	3	3	3	3	1	1	1	7	8	8	8	8	8	8	8	7
1143	2	1	1	2	3	3	2	2	1	0	0	7	3	1	1	1	0	0	0	7	22	90	40	24	24	18	12	7

Eosinophil group-1 high primed, 2 low primed; TM (trial medication)-1 prednisolone, 2 placebo; days data-number of days data available; TM opinion-1 effective; 2 not effective; 3 no opinion expressed: r-reliever

Appendix 9 **Raw data of respiratory score in clinical trial**

Study ID	EOS grp	TM 1pred 2plac	to incl ude	TM opin ion	d0	d1	d2	d3	d4	d5	d6	days data	n1	n2	n3	n4	n5	n6	n7	Night data	r0	r1	r2	r3	r4	r5	r6	rel: data
1144	1	1	1	1	3	2	1	1	1	1	1	7	3	3	2	2	1	1	1	7	36	24	24	18	12	12	12	7
1145	2	2	1	2	3	3	2	2	1	0	0	7	2	3	2	0	0	0	0	7	16	20	14	6	6	2		6
1146	1	2	1	3	1	0	0	0	0	0	0	7	1	0	0	0	0	0	0	7	20	16	8	0	0	0	0	7
1147	2	2	1	1	3	3	1	0	0	0	0	7	1	2	1	1	3	1	1	7	8	4	8	2	2	2	2	7
1148	1	1	1	2	3	0	0	0	0	0	0	7	3	3	3	3	3	3	3	7	8	0	0	0	0	0	0	7
1149	2	1	1	3	0	0	0	0	0	0	0	7	2	1	1	1		1	0	6	8	9	7	4	2	2	2	7
1150	1	2	1	1	1	0	0	0	0	0	0	7	0	0	0	0	0	0	0	7	7	0	0	0	0	0	0	7
1152	1	1	1	3		0	0	0	1	1	0	6	1	1	1	1	1	1	1	7	2	6	2	2	2	0	2	7
1154	2	2	1	2	1	3	1	1	1	0	0	7	1	0	0	0	0	0	0	7	7	8	6	4	4	0	0	7
1155	1	2	1	3	1	2	1	1	1	0	0	7	3	3	2	0	0	0	0	7	10	0	0	18	18	4	0	7
1156	1	1	1	1	2	2	1	1	1	0	0	7	1	2	1	1	1	0	0	7	4	4	2	0	0	0	0	7
1157	2	2	1	3	2	3	0	0	0	0	0	7	3	0	0	0	0	0	0	7	12							1
1159	2	1	1	1	3	1	1	1	1	0	1	7	3	2	1	0	3	1	0	7	16	16	12	18	16	8	3	7
1162	2	1	1	3	3	1	1	0	0	0	0	7	2	3	0	0	0	0	0	7	18	7	4	4	0	0	0	7
1164	2	1	1	3	3	2	1	1	0	0	0	7	1	0	0	0	0	0	0	7	15	15	6	6	3	3	0	7
1165	1	2	1	3	2	1	2	1	0	0	0	7	0	0	0	0	0	0	0	7	6	8	4	6	2	0	0	7
1167	2	1	1	1	3	3	1	0	2	0	0	7	3	1	2	2	0	0	0	7	8	8	8	8	8	0	0	7
1169	1	2	1	3	2	2	2	2	2	1	2	7	2	2	2	2	2	2	2	7	8	8	8	8	8	8	8	7
1170	2	2	1	2	2	1	1	1	0	0	0	7	2	0	0	0	0	0	3	7	22	12	8	8	4	2	0	7
1171	2	1	1	1	2	2	1	0	0	0	0	7	1	1	0	0			1	5	12	12	12	12	12	0	0	7
1173	1	2	1	3	1	0	0	0	0	0	0	7	2	0	0	0	0	0	0	7	9	0	0	0	0	0	0	7
1175	1	2	1	1	1	2	1	1	1	0	0	7	1	1	1	0	0	0	0	7	8	8	8	8	8	4	4	7
1178	2	2	1	3	1	2	2	1	0	1	0	7	1	3	2	0	0	0	0	7	36	36	40	20	20	14	4	7
1180	1	2	1	3	2	2	2	1	1	1	1	7	1	2	1	1	1	1	0	7	8	10	10	8	8	8	6	7
1181	2	1	1	3	2	0	0	1	1	1	1	7	2	1	1	2	1	1	1	7	20	10	4	6	6	6	0	7
1188	2	2	1	1	1	1	1	1	1	0	0	7	0	1	0	1	0	0	0	7		16	4	16	20	4	0	6
1199	2	1	1	3	2	3	2	2	2	2	1	7	3	3	2	1	1	1	1	7	48	48	48	24	24	12	10	7
1200	2	2	1	1	2	2	1	0	2	3	2	7	3	3	3	3	3	3	2	7	12	12	4	6	4	8	12	7

Eosinophil group-1 high primed, 2 low primed; TM (trial medication)-1 prednisolone, 2 placebo; days data-number of days data available; TM opinion-1 effective; 2 not effective; 3 no opinion expressed: r-reliever

Appendix 9 **Raw data of respiratory score in clinical trial**

Study ID	EOS grp	TM 1pred 2plac	to incl ude	TM opin ion	d0	d1	d2	d3	d4	d5	d6	days data	n1	n2	n3	n4	n5	n6	n7	Night data	r0	r1	r2	r3	r4	r5	r6	rel: data
1201	1	2	1	3	3	2	2	2	1	1	0	7	1	3	3	2	1	0	0	7	19	6	6	6	6	0	0	7
1202	2	2	1	3	2	1	1	1	0	0	0	7	2	2	1	0	0	0	1	7	10	10	8	8	6	4	4	7
1204	2	1	1	3	2	0	0	0	0	0	0	7	1	1	0	2	0	0	0	7	4	4	4	4	4	0	0	7
1210	1	1	1	1	2	2	1	1	1	1	0	7	2	1	0	0	0	0	0	7	18	12	12	0	0	0	0	7
1211	1	2	1	1	2	3	3	2	2	1	1	7	2	2	3	3	1	1	0	7	48	48	48	48	48	48	12	7
1212	2	2	1	3	1	1	1	1	1	1	0	7	1	0	0	0	0	0	0	7	3	2	3	2	0	0	0	7
1213	2	2	1	1	2	2	0	1	0	0	0	7	1	1	0	0	0	0	0	7	30	20	20	20	0	0	0	7
1217	1	2	1	1	3	3	2	2	1	0	0	7	1	2	1	1	0	0	0	7	26	26	20	14	0	0	0	7
1005	2	2	1	3	1	1	1	0	0	0	0	7	1	0	0	0	0	0	0	7	8	12	4	0	0	0	0	7
1007	2	1	1	1	1	0	0	0	0	0	0	7	0	0	0	0	0	0	0	7	8	0	0	0	0	0	0	7
1008	1	1	1	2	0	1	1	0	0			5	1	0	0	0				4	6	6	6	4	4	0	0	7
1010	1	1	1	3	3	1	0	0	0	0	0	7	1	0	0	0	0	0	0	7	16	8	0	0	0	0	0	7
1013	1	2	1	3	2	3	2	2	2	1	2	7	3	2	1	1	1	2	2	7	6	6	6	6	4	4	4	7
1014	2	2	1	1	3	3	3	3	3	2	2	7	3	3		2	2	0	0	6	10	8	10	8	8	8	8	7
1018	1	1	1	3	2	3	2	1	0	0	0	7	2	2	1	0	0	0	0	7	6	12	12	8	4	6	2	7
1019	1	2	1	1	0	0	1	0	0	0	0	7	0	0	0	0	0			5	10	10	10	10	10	10	10	7
1020	2	2	1	2	3	3	3	3	3	2	2	7	3	2	2	3	2	2		6	36	36	36	36	36	36	36	7
1022	1	2	1	2	1	2	1	1	1			5	3	2	2	1				4	32	22	22	22	22			5
1023	2	1	1	1	2	0	0	0	0	0	0	7	0	1	1	1	1	1	1	7	7	0	0	0	0	0	0	7
1025	1	2	1	2	1	1	1	1	0	0	0	7	2	2	2	0	0	0	0	7	4	4	4	2	0	0	0	7
1026	1	2	1	3	0	0	1	1	1	0	0	7	0	1	1	0	0	0	0	7	0	0	0	8	4	0	0	7
1031	1	2	1	3	1	2	1	0	0	0	0	7	2	0	0	0	0	0	2	7	2	3	2					3
1032	1	2	1	3	2	2	2	2	0	0	0	7	2	2	2	1	0	0	0	7	6	16	14	8	1	2	0	7
1034	2	2	1	3	2	1	1	1	0	0	0	7	2	2	2	0	0	0	0	7	16	20	16	12	4	0	0	7
1035	2	2	1	1	2	2	1	2	1	0	0	7	2	3	1	0	1	0	0	7	2	2	1	0	0	0	0	7
1038	2	2	1	3	3	2	2	1	1	1	0	7	3	2	1	1	1	1	0	7	12	14	10	6	6	4	0	7
1040	2	2	1	1	2	2	2	1	1			5	0	0	0	0				4	20	16	4					3
1041	2	1	1	1	2		0	0	0	0	0	6	0	0	0	0	0	0	0	7	2							1

Eosinophil group-1 high primed, 2 low primed; TM (trial medication)-1 prednisolone, 2 placebo; days data-number of days data available; TM opinion-1 effective; 2 not effective; 3 no opinion expressed: r-reliever

Appendix 9 **Raw data of respiratory score in clinical trial**

Study ID	EOS grp	TM 1pred 2plac	to incl ude	TM opin ion	d0	d1	d2	d3	d4	d5	d6	days data	n1	n2	n3	n4	n5	n6	n7	Night data	r0	r1	r2	r3	r4	r5	r6	rel: data
1042	2	1	1	3	3	0						2	1							1	6							1
1044	2	1	1	2	3	2	1	1	1	2	1	7	3	0	0	2	2	0	0	7	4	6	2	0	0	0	0	7
1046	2	2	1	3	3	3	2	0	0	0	0	7	3	3	1	0	0	0	0	7	17	4	4	4	4	0	0	7
1049	2	1	1	3	2	2	1	1	1	1	1	7	2	2	1	0	0	0	0	7	8	12	12	12	12	12	12	7
1050	1	1	1	1	2	1	0	0	0	0	0	7	1	1	0	0	0	0	0	7	16	16	8	8	0	0	0	7
1052	1	1	1	3	1	2	2	2	1	1	0	7	2	2	2	2	2	2	0	7	2	8	6	6	6	6	0	7
1053	2	1	1	3	2	3	2	2	2	1	1	7	2	3	3	2	2	2	2	7	12	12	30	30	30	30	0	7
1054	2	2	1	2	2	2	2	2	2	2	2	7	1	2	2	2	1	1	1	7	30	40	40	45	45	40	35	7
1055	2	1	1	1	3	2	1	0	0	0	0	7	3	2	1	0	0	0	0	7	16	16	16	0	0	0	0	7
1056	1	2	1	1	1	0	0	0	0	0	0	7	1	0	1	0	0	0	0	7	4	6	8	8	6	0	0	7
1058	2	1	1	3	2	2	3	2	1	1	1	7	0	3	3	1	0	0	0	7		6	6	6	6	4	2	6
1059	2	1	1	3	1	1	0	0	0	0	0	7	3	1	1	0	0	0	0	7	2	1	1	0	0	0	0	7
1060	2	1	1	3	1	2	2	1	1	0	0	7	1	1	1	0	0	0	0	7	16	12	8	4	4	0	0	7
1062	1	2	1	3	1	1	0	0	0	0	0	7	0	0	0	0	0	0	0	7	6	6	6	6	6	6	0	7
1065	2	2	1	3	1	0	0	0	0	0	0	7	2	1	0	0	0	0	0	7	18	12	8	8	4	0	0	7
1066	2	2	1	3	2	2	2	1	0	0	0	7	2	2	2	1	1	0	0	7	20	24	24	20	20	0	4	7
1067	1	2	1	3	2	2	1	2	1	1	0	7	2	0	1	0	0	0	0	7	10	12	6	6	4	0	0	7
1071	1	2	1	2	2	2	2	0	0	0	0	7	2	2	2	1	3	3	0	7	5	4	4	0	0	0	0	7
1072	1	1	1	3	1	1	0	0	0	0	0	7	2	1	1	0	0	0	0	7	4	4	4	0	0	0		6
1073	1	2	1	3	1	2	2	2	2	0	0	7	2	2	2	2	2	0	0	7	4	4	2	2	2	0	0	7
1074	2	2	1	3	2	2	0	1	1	0	0	7	1	2	0	0	0	0	0	7	6	8	2	2	2	0	0	7
1075	1	1	1	3	2	1	1	0	0	0	0	7	1	1	1	0	0	0	0	7	14	2	6	6	4	4	4	7
1076	2	2	1	1	1	1	1	1	1	0	0	7	1	1	1	1	1	1	0	7	12	12	12	12	12	8	8	7
1079	1	2	1	1	2	3	3	2	2	0	0	7	1	3	1	3	3	0	0	7	12	4	4	4	2	0	0	7
1080	2	1	1	3	2	2	2	1	0	0	0	7	2	2	1	1	0	0	0	7	8	8	6	4	2	0	0	7
1081	1	1	1	2	2	2	3	2	1	2	1	7	1	1	3	1	0	0	0	7	9	12			12	12	12	7
1082	1	1	1	2	3	3	1	1	1	1	1	7	0	3	3	1	0	0	0	7	16	6	10	8	8	6	6	7
1084	1	2	1	1	1	2	1	1	1	1	1	7	2	2	2	1	1	0	0	7	2	0	0	0	0	0	0	7

Eosinophil group-1 high primed, 2 low primed; TM (trial medication)-1 prednisolone, 2 placebo; days data-number of days data available; TM opinion-1 effective; 2 not effective; 3 no opinion expressed: r-reliever

Appendix 9 **Raw data of respiratory score in clinical trial**

Study ID	EOS grp	TM 1pred 2plac	to include	TM opinion	d0	d1	d2	d3	d4	d5	d6	days data	n1	n2	n3	n4	n5	n6	n7	Night data	r0	r1	r2	r3	r4	r5	r6	rel: data
1085	2	2	1	1	1	1	1	1	0	0	0	7	1	2	1	0	0	0	0	7	4	2	2	2	0	0	0	7
1089	1	1	1	3	2	1	1	2	2	0	0	7	1	1	1	1	0	0	0	7	11	9	4	2	0	0	0	7
1090	1	2	1	3	1	1	1	1	1	0	0	7	1	1	1	1	1	0	0	7	6	6	2	6	2			5
1091	1	1	1	1	3	2	1	1	0	0	0	7	3	3	1	1	0	0	0	7	14	12	10	4	4	4	4	7
1093	2	2	1	3	0	1	2	2	0	0	0	7	0	2	2	1	0	0	0	7								0
1094	2	1	1	1	0	0	0	0	0	0	0	7	1	2	0	0	0	0	0	7	7	4	0	0	0	0	0	7
1096	2	2	1	3	1	0	0	0	0	0	0	7	1	2	1	1	2	1	1	7	8	2	2	0	2	0	0	7
1099	1	1	1	3	2	0	0	0	0	0	0	7	2	2	0	1	1	1	1	7	16	9	0	0	0	0	0	7
1225	2	2	1	2	1	1	1	1	1	1	0	7	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	7

Eosinophil group-1 high primed, 2 low primed; TM (trial medication)-1 prednisolone, 2 placebo; days data-number of days data available; TM opinion-1 effective; 2 not effective; 3 no opinion expressed: r-reliever

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