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Viral induced exacerbations of childhood asthma – clinical findings, virology and cytokine responses

by

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ABSTRACT

FACULTY OF MEDICINE

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VIRAL INDUCED EXACERBATIONS OF CHILDHOOD ASTHMA – CLINICAL FINDINGS, VIROLOGY AND CYTOKINE RESPONSES

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Viral induced exacerbations are very common in childhood asthma and a largely unmet medical need. The primary aim was to conduct an observational and feasibility study of the various components of a protocol developed in the build-up to a potential interventional study.

At baseline, children were examined, underwent skin prick testing and spirometry, and nasal washing, saliva and blood sampling for assessment of virus presence and levels of relevant cytokines and matrix metalloproteinases (MMPs). Peak expiratory flow rate (PEFR) was measured daily for one week. Parents forwarded this information together with cold/asthma symptom scores via text message (SMS). Digitally stored PEFR values were compared with transmitted values to assess compliance and data validity. A daily SMS prompted parents to report early signs of a cold.

Two home visits were conducted following report of a cold. Clinical, lung physiological and laboratory assessments were repeated. PEFR and cold/asthma symptoms were monitored for 14 days. This enabled evaluation of changes in cold and asthma symptom scores, virus status, and levels of nasal lavage cytokines and MMPs in comparison with baseline.

The final data set comprised 25 distinct cold episodes (of 32 participants). 42% of baseline and 88% of cold nasal lavages were virus positive. Detecting viruses in saliva yielded additional information. Levels of the antiviral cytokine IP-10 correlated with virus status. This study provides novel observations about virus-induced cytokine (e.g. MCP-1) and MMP (e.g. MMP-7) responses and correlation with cold and asthma severity. Compliance with PEFR measurement was high (>80%), transmission of correct values lower (65%), and the use of the SMS-system was well received by parents. Challenges encountered included low number of severe exacerbations, the difficulty to predict course of early cold symptoms, and rapid progression from upper to lower respiratory tract symptoms in children. These findings, together with the fact that the participants had insufficient burden/events, suggest that the protocol in its current form would need several adjustments for successful application for an interventional trial.

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DECLARATION OF AUTHORSHIP

I, Dr Florian Gahleitner

declare that the thesis entitled

VIRAL INDUCED EXACERBATIONS OF CHILDHOOD ASTHMA – CLINICAL FINDINGS, VIROLOGY AND CYTOKINE RESPONSES

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research.

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Definitions and Abbreviations

ACQ	Asthma control questionnaire
AHR	Airway hyper-responsiveness
AI	Asthma Index
ANOVA	Analysis of variance
ASM	Airway smooth muscle
ATP	Adenosine Triphosphate
ATS	American Thoracic Society
AUC	Area Under the Curve
BAL	Bronchoalveolar lavage
BDP	Beclomethasone dipropionate
BEC	Bronchial epithelial cell (see pBEC)
BHR	Bronchial hyper-responsiveness
bl	Baseline
BSA	Bovine Serum Albumin
BSMC	Bronchial smooth muscle cells
BTS	British Thoracic Society
cDNA	complementary Deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease or Children's outpatient department
cDNA	Complementary DNA
CF	Cystic fibrosis
CCL	Chemokine (C-C motif) ligand
CM	Conditioned medium
Cq	Quantification cycle
CRF	Case Report Form
%CV	Coefficient of variation
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DPT	Dermatophagoides pteronyssinus
DTT	Dithiothreitol
ECM	Extracellular matrix
ED	Emergency Department

EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENA-78	Epithelial-derived neutrophil-activating peptide 78 (CXCL5)
ENT	Ear Nose Throat
ERK1/2	Extracellular signal-regulated kinases-1/2
ERS	European Respiratory Society
FasL	Fas Ligand
FENO	Fractional exhaled nitric oxide (see eNO)
FEV1	Forced expiratory volume in 1 second
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FTD	fast-track Diagnostics
FVC	Forced vital capacity
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	General practitioner
hBoV	Human bocavirus
hCoV	Human coronavirus
hMPV	Human metapneumovirus
HRP	Horse radish peroxidase
HRV	Human rhinovirus
IC	Internal control
ICAM	Intercellular adhesion molecule
ICS	Inhaled corticosteroids
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IP10	IFN-γ-inducible protein 10 (CXCL10)
IRF	Interferon-regulatory factors
JAK/STAT	Janus kinase/ signal transducer and activator of transcription
JCS	Jackson Cold Score
LOQ	Limit of Quantification
LPS	Lipopolysaccharide
LRT	Lower respiratory tract
MAP	Multianalyte Profiling

MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1 (CCL2)
MDA-5	Melanoma differentiation-associated gene-5
µg	Microgram
MIP-1	Macrophage Inflammatory Proteins-1
mL	Millilitres
µL	Microlitres
mM	Millimolar
µm	Micrometre
µM	Micromolar
MMLV	Moloney Murine Leukaemia Virus
MMP	Matrix metalloproteinase
MP	Mycoplasma pneumoniae
mRNA	Messenger ribonucleic acid
MSC	Microbiological safety cabinet
NC	Negative control
Neg	Negative
NF-κB	Nuclear factor-κB
ng	Nanogram
NL	Nasal lavage
nm	Nanometre
NP	Nasopharyngeal
NTC	No Template Control
PAU	Paediatric assessment unit
pBEC	primary bronchial epithelial cell (see BEC)
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Positive control
PCR	Polymerase chain reaction
PCRN	Primary care research network
PD	PrimerDesign
PEFR	Peak expiratory flow rate
PI3K	Phosphatidylinositol 3-kinase
PKR	[Double-stranded RNA (dsRNA)-activated] protein kinase R
PMT	photomultiplier tube
Poly(IC)	Polyinosinic:polycytidylic acid

Pos	Positive
qPCR	Quantitative polymerase chain reaction
RANTES	Regulated and normal T cell expressed and secreted (CCL5)
rcf	rotational centrifugal force
RCT	Randomized Controlled Trial
RIG-1	Retinoic acid inducible gene protein-1
RNA	Ribonucleic acid
Rnase	Ribonculease
RPM	Revolutions per minute
RSV	Respiratory syncytial virus
RT	Room temperature or reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
RV	Rhinovirus (see also HRV – Human rhinovirus)
SD	Standard deviation
SMS	Short message service
SOP	Standard operating procedure
SPT	Skin prick testing
Streptavidin-PE	Streptavidin-phycoerythrin
TGFβ	Transforming growth factor β
TH	T helper cell (CD4+ T lymphocyte)
TIMP	Tissue inhibitor of matrix metalloproteinases
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TS	Throat swab
UK	United Kingdom
URTI	Upper respiratory tract infection
V1	cold visit within 48 hours of cold symptoms developing
V2	cold visit between day 4 and 6 of cold
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Asthma

1.1.1 Definition

Asthma, the most common chronic respiratory disease worldwide, is a heterogenous disease of the conducting airways and lacks a clear and uniform definition. It can best be described by its clinical, physiological and pathological characteristics. In clinical terms asthma can be described by a history of intermittent shortness of breath, especially during night time, and cough, with wheezing being the predominant feature during clinical examination and auscultation of the chest.

The main physiological feature, and main point of focus of (longterm) therapy until two decades ago, is an episodic airway obstruction with limitation of the expiratory airflow. The pathophysiological equivalent in pulmonary function tests is a reduced forced expiratory volume in 1 second (FEV_1) and peak expiratory flow (PEF). Beta2 agonist therapy produces bronchodilation for immediate relief of asthma symptoms (short-acting beta2 agonists) or in the case of long-acting beta2 agonists can be used in addition to an inhaled corticosteroid in children requiring prophylactic treatment.

Airway inflammation, characterised by influx of a variety of inflammatory cells and production of inflammatory cytokines, is the main pathological feature of asthma and at times is associated with more permanent structural airway changes (thickening of lamina reticularis, smooth muscle hypertrophy, disruption of epithelium, goblet cell hyperplasia) (Singh and Busse, 2006). The emerging awareness of the importance of airway inflammation in asthma has led to inhaled corticosteroids becoming the mainstay of preventive treatment over the last few years. The chronic inflammation is associated with airway hyperresponsiveness, another characteristic feature of asthma.

1.1.1.1 Respiratory physiological tests and asthma

Tests to determine airflow obstruction can provide support for a diagnosis of asthma and provide means to monitor disease progress. Normal results, however, do not exclude a diagnosis of asthma. Peak flow measurement, spirometry, and reversibility with bronchodilators can usually be performed in children above the age of 5 years.

The measurement of peak expiratory flow (PEF) was pioneered by Dr Basil Martin Wright and the original design of the instrument was introduced in the late 1950s.

According to definitions of a consensus paper of the ERS (Quanjer et al., 1997), PEF constitutes the highest flow achieved from a maximum forced expiratory manoeuvre from a position of maximal lung inflation. Spirometry is a respiratory physiological test used to aid the diagnosis and the monitoring of various lung conditions and is also commonly used as primary or secondary endpoints in many clinical research studies in asthma. The most common measurements, depicting how an individual inhales and exhales volumes of air as a function of time, are:

- FEV_1 – Forced expiratory volume in one second. This measurement equates to the maximal volume of air (expressed in litres) that can be forced out within one second from a position of full inspiration.
- FVC – Forced vital capacity. This measurement gives the total volume of air (in litres) forced out in one breath from full inspiration to maximal expiration.
- FEV_1 / FVC – the proportion of air in the lung, which can be blown out in one second.
- PEF – peak expiratory flow.

Widely used in the diagnosis, monitoring and assessment of response to treatment in adult lung diseases the use of spirometry poses specific challenges in younger children, especially in pre-school age, both in terms of performing as well as interpreting the spirometric manoeuvres. Spirometry requires cooperation between the child and the examiner and results depend on technical and personal factors. Guidelines exist both for the standardisation of spirometry (Miller et al., 2005, Beydon et al., 2007) as well as for interpretation of lung function tests (Pellegrino et al., 2005, Loeb et al., 2008).

The use of spirometry can be extended to include reversibility testing, i.e. to evaluate the response of the airways to bronchodilators (in most cases a beta-sympathicomimetic such as salbutamol). Reversibility testing aids in the diagnosis of respiratory diseases, more specifically it informs about the reversibility of a possible existing airflow limitation (obstruction) of the airways.

1.1.2 Epidemiology and Burden

More than 300 million people of all ages worldwide are thought to be affected by asthma. The prevalence ranges from 1% to 18% in the population of different countries. The percentage of children reported to have asthma has significantly increased. This goes along with a greater awareness of the disease leading to a decrease in the differences of asthma prevalence in different countries worldwide. Despite

improvement in the management of asthma and asthma exacerbations the mortality rate has been estimated at 250,000 per year worldwide (From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2010. Available from: http://www.ginasthma.org/).

1.1.3 Causes for asthma

Asthma is a heterogenous inflammatory disorder of the lung characterised pathophysiologically by reversible airway obstruction, airway remodelling and widespread airflow limitation. Its pathogenesis is highly complex not least because of a number of different phenotypes that have been described based on clinical and laboratory findings. 'Asthma' is the end result of a number of complex interactions of genetic, lifestyle, and environmental factors influenced also by epigenetic determinants. The exact ways how 'asthma genes' and the environment interact are still poorly understood.

Over the decades and more recently so with the rise of the so called 'asthma epidemic' a significant effort was put into exploring causative and susceptibility factors underlying asthma development as well as determining protective factors and developing preventative strategies. If one wants to give a succinct summary of evidence based, confirmed and undisputed risk factors for the development of childhood asthma, only passive smoking as an environmental factor remains on the list (Strachan and Cook, 1998, Vork et al., 2007, Burke et al., 2012). Exposure to a variety of microbes from the environment in early life (when the immune system and lung tissue matures) has repeatedly been confirmed as a protective factor (Braun-Fahrlander and Lauener, 2003, Ege et al., 2011).

Nevertheless a number of other potential risk and protective factors have been investigated and discussed over the last few years. While there is not enough evidence for any of these, progress has been hampered by a lack of large-scale studies, and therefore the current absence of firm evidence does not necessarily mean that they do not play a role. Hereditary factors are important players in the development of asthma and various genes have been listed as being linked to various biological mechanisms that play a role in defined clinical phenotypes and endotypes (March et al., 2013). Considering the time-frame it takes for genetic changes to occur it is unlikely that genetic determinants by themselves in isolation can explain the surge of asthma at the end of the last and beginning of this century. Rather the complex interaction between genes (gene-gene interactions), between genes and environment (gene-environment interaction) and not last epigenetic phenomena play an important role. It has also been

repeatedly postulated that the timing of these interactions is crucial to explain either the protective or risk-enhancing nature of lifestyle or environmental influences. This may also be one reason for the frequently contradictory result of studies looking at the role of these determinants on the development of childhood asthma.

1.1.3.1 Environmental risk and protective factors

1.1.3.1.1 Tobacco smoke (active/passive smoking)

Maternal smoking during pregnancy with exposure of her unborn child in utero (Gilliland et al., 2001, Jaakkola and Gissler, 2004, Goksor et al., 2007), parental smoking with passive exposure of a child (Strachan and Cook, 1998, Vork et al., 2007, Burke et al., 2012), and active smoking of adolescents (Strachan et al., 1996, Larsson, 1995, Withers et al., 1998, Gilliland et al., 2006) have consistently been linked with and causally related to childhood asthma and new onset asthma in teenage years respectively. A large population-based cohort study (including almost 60,000 children) showed a dose-dependent increase in risk of developing asthma among children at the age of 7 years with maternal smoking during pregnancy (Jaakkola and Gissler, 2004). Grandmaternal smoking during a mother's pregnancy was also associated with increased risk of childhood asthma in her grandchildren (Li et al., 2005). A recent meta-analysis including 79 prospective studies linked exposure to pre- or postnatal passive smoke exposure to a 21% to 85% increase in incident asthma (Burke et al., 2012). Gilliland et al. (2006) demonstrated in a prospective cohort study among 2,609 children with no lifetime history of asthma who reported new-onset smoking of 300 or more cigarettes per year a relative risk (RR) of 3.9 (95% confidence interval [95% CI], 1.7-8.5) for new-onset asthma compared with nonsmokers. The significance of passive smoking on the incidence of asthma and the effectiveness of public health measures (i.e. introduction of smoking ban in Scotland) on the reduction of hospital admissions with asthma have been demonstrated (Mackay et al., 2010) – it remains to be seen whether the long-term effect on asthma incidence can soon also be demonstrated.

1.1.3.1.2 Air pollution

Sufficient evidence supports the notion that air pollutants such as ozone and particulate matters can exacerbate pre-existing asthma in children (Tatum and Shapiro, 2005, Zhao et al., 2008). The evidence that air pollution contributes to the development of asthma remains inconsistent not least because of a number of different approaches to estimate exposure (e.g traffic proximity and density, dispersion models etc.). Increases in asthma incidence for children exposed to higher levels of air pollution have been shown in some studies (Brauer et al., 2007, Nordling et al., 2008). Asthma incidence

was positively associated with zinc in PM10 at the birth address in the PIAMA birth cohort study (Gehring et al., 2015). A large study of more than 10,000 school children in Japan using a simulation model that accounted for exposure level both at home and at school found a positive association between exposure to elemental carbon and incidence of asthma. (OR 1.07 [1.01-1.14] for each 0.1 µg/m(3) elemental carbon) (Yamazaki et al., 2014). Other studies showed no associations between asthma incidence and traffic-related air pollution (Gehring et al., 2002, Lindgren et al., 2013).

1.1.3.1.3 Allergens

The majority of childhood asthma exists on an IgE-mediated background with sensitisations to aero-allergens. Common indoor allergens include house dust mite, cat and dog dander, cockroach and certain moulds. While sensitisation and risk for developing IgE antibodies have been linked to the level of exposure of aero-allergens such as house dust mite and cat dander, no evidence has been found of an association of early indoor allergen exposure and development of childhood asthma (Lau et al., 2000). Allergen avoidance as a primary prevention of asthma (Simpson and Custovic, 2004) has not proven successful and no association between furry and feathered pet keeping early in life and asthma in school age has been found (Lodrup Carlsen et al., 2012). However, children who are already sensitized and who have significant aero-allergen exposure are at higher risk of persistent asthma and bronchial hyperresponsiveness in later childhood (Illi et al., 2006).

1.1.3.1.4 Viral Infections

The causal link between viral infections and development of asthma remains a matter of debate (von Mutius, 2001) but evidence grows stronger. Lower respiratory tract infections in infancy caused by RSV (Kusel et al., 2007, Hall et al., 2009, Sigurs et al., 2010, Wu et al., 2008) and especially human rhinovirus (Jackson and Johnston, 2010, Kotaniemi-Syrjanen et al., 2003, Lemanske et al., 2005, Jackson et al., 2008) have been linked with subsequent recurrent episodes of wheezing and the risk of developing asthma. Other studies have suggested a link between RSV infection of the LRT and subsequent wheezing but not with asthma beyond 11 years of age (Stein et al., 1999) or claimed that severe RSV infection indicated a genetic predisposition to asthma rather than being the cause of asthma (Thomsen et al., 2009).

1.1.3.1.5 Microbial substances

Over the last two decades evidence has amounted to support the theory that early life exposure to environments rich in microbial substances (e.g. such as life on a traditional dairy farm) significantly reduces risk of atopy and asthma (Braun-Fahrlander and

Lauener, 2003, Ege et al., 2011, Illi et al., 2012, Behbod et al., 2015). The timing of exposure appears to be important as in early life the immune responses and lung tissues mature. In the context of this, the use of antibiotics (e.g. interference with gut microflora) and antipyretics (Allmers, 2005) in childhood also emerges as a topic of discussion with regards to a causal relationship to asthma development. It appears to be more likely, however, that asthmatics use antibiotics and antipyretics because of their asthma (von Mutius, 2001) rather than the treatment causing asthma. There is also no evidence of vaccinations increasing the risk of asthma with a recent study claiming even a reduction in the odds of asthma with MMR vaccination through to the age of 13 years (Timmermann et al., 2015). Probiotic supplementation during pregnancy or infancy has not been shown to have a protective effect against asthma or childhood wheeze (Azad et al., 2013).

1.1.3.2 Lifestyle risk and protective factors

1.1.3.2.1 Obesity

Both asthma and obesity in childhood have increased in the last decades and the parallelism of these developments has led to studies looking into possible associations or causal links (Story, 2007). Increasing evidence suggests that obesity increases risk of subsequent asthma whereas the opposite causation is not supported by studies (Eder et al., 2006, Chinn, 2006). The significance of gender in this debate has revealed the risk of new-onset asthma to be higher in overweight boys but not in girl in one study (Gilliland et al., 2003), whereas a birth cohort study showed the opposite (Castro-Rodriguez et al., 2001). Weight loss on the other hand is associated with improvement in asthma symptoms and lung function (Schaub and von Mutius, 2005).

1.1.3.2.2 Diet

In line with the rise of obesity and asthma, the change of diet associated with westernization has been observed. Studies have looked into potential links between decreased intake of antioxidants and increase in asthma prevalence. While individual studies have claimed associations, a meta-analysis concluded that the risk of asthma was not significantly influenced by the dietary intake of antioxidants such as vitamins C, E and β-carotene (Gao et al., 2008). Similarily, inconsistent results exist with regards to the role of polyunsaturated fatty acids and asthma (Black and Sharpe, 1997, Kim et al., 2009, Dunstan et al., 2003, Almqvist et al., 2007). Support exists for increased food diversity in the first year of life for the prevention of asthma (Roduit et al., 2014).

1.1.3.2.3 Breastfeeding

A number of nutritional, sociological and health benefits have been attributed to breastfeeding. Again, the association between breastfeeding and the development of asthma remains matter of intense debate: Breastfeeding does not appear to protect infants from asthma and some studies have even claimed a positive association between breastfeeding and the development of asthma (Friedman and Zeiger, 2005, Sears et al., 2002). The third NHANES study showed significantly reduced odds for ever breastfed children (compared with never breastfed children) to be diagnosed with asthma (Chulada et al., 2003). A recent meta-analysis of 117 cohort, cross-sectional, and case control studies published between 1983 and 2012 claims a risk-reduction for the development of asthma in breastfed babies which is most pronounced up to 2 years of age but still significant at school age (Dogaru et al., 2014).

1.2 Asthma exacerbations

Asthma is a leading cause of hospital admission in children in the United Kingdom. The morbidity of childhood asthma has a significant impact on the quality of life of a child (e.g. exclusion from physical activity, missing of school days) and their family (e.g. frequent hospital visits) (Halterman et al., 2004) and bears large economic costs.

One of the significant unmet clinical needs is the sudden and often unexpected deterioration of asthma that constitutes an exacerbation, characterised by an accentuation of the clinical features of cough, wheeze, and shortness of breath and at the more severe end of the spectrum requiring intervention from a medical professional and possibly hospital admission. Exacerbations are an exaggerated response of the lower airways to an environmental trigger and can range from mild to severe and life-threatening and both symptom and lung function evaluation help in their assessment. It is important to differentiate between the current clinical control and exacerbations as the latter can occur despite adequate day-to-day asthma control and the patient not being limited in their daily activities. Despite of this awareness a clear distinction between mild asthma exacerbations and poorly controlled asthma is not always easy (Reddel et al., 1999). Acute exacerbations are responsible for much of the associated morbidity with (and also mortality of) childhood asthma (Wijesinghe et al., 2009, Sears, 2008).

1.2.1 Etiology of asthma exacerbations

A range of factors can contribute to an increase in asthma symptoms, such as respiratory infections, allergens, temperature change, air pollution and tobacco smoking (Ko et al., 2007, Silverman et al., 2003), occupational exposures in adults

(Quirce and Bernstein, 2011, Mapp et al., 2005) but also psychological stress (Sandberg et al., 2004). Atopy plays an important role in childhood and adult asthma, with more than 80% of children being sensitised to environmental allergens. The majority of asthma exacerbations though are not triggered by direct allergen exposure (Singh and Busse, 2006) but by upper respiratory tract infections (Johnston et al., 1995) and are associated with predominantly neutrophilic rather than eosinophilic inflammation. There is though increasing convincing evidence that there is a synergistic relationship between allergic sensitisation and allergen exposure and viral upper respiratory tract infection in provoking acute exacerbations of asthma (Murray et al., 2006, Contoli et al., 2005, Green et al., 2002, Quirce and Bernstein, 2011). In addition also the combination of airway pollutants and viral infections increases the likelihood of an asthma exacerbation (Chauhan et al., 2003). Each of these triggers can initiate a destructive process resulting in damage of the respiratory epithelium and augment the immune response to the other irritant (Singh and Busse, 2006).

1.2.2 Pathology and immunpathogenesis of asthma exacerbations

The final common pathway to various triggers of asthma exacerbations includes the infiltration of airways by inflammatory and immunological cells (neutrophils, eosinophils, lymphocytes) and their activation. The provoking factor determines the predominant cell type of the infiltrate (i.e. eosinophilia in allergen driven exacerbation and neutrophilia in infection) and therefore the pattern of airway inflammation though a combination of more cell types is common (Norzila et al., 2000). Allergens leading to eosinophilic infiltration support a Th2 response by lymphocytes that secrete increased levels of IL-4, IL-5 and IL-13. It remains subject to debate whether this shift towards a Th2 response explains reduced Th1 activity and therefore higher susceptibility for viral infections with subsequent exacerbation (Singh and Busse, 2006). The number of eosinophils drops quickly with treatment with systemic corticosteroids and a high number of neutrophils is then seen in bronchoalveolar lavage (BAL) fluid of patients ventilated for status asthmaticus (Lamblin et al., 1998). The release of pro-inflammatory cytokines both from this cell infiltrate as well as from the damaged epithelium enhances the airway inflammation. Oxidative stress due to increased oxygen free radical production by granulocytes in addition leads to lipid and protein oxidation (Wark and Gibson, 2006). Mucus cell hyperplasia and mucus secretion leading to mucus plugging and air trapping typically seen in acute severe asthma exacerbations are mediated by various factors such as IL-13, epidermal growth factor receptor activation (Zimmermann et al., 2003, Rogers, 2004), neutrophil elastase, mast cell chymase, eosinophil cationic protein, and leukotrienes (Hays and Fahy, 2003).

1.2.3 Viruses as key trigger

Respiratory viruses, in the context of childhood asthma, have drawn considerable interest for two main respects: firstly in relation to early life influences and pathogenesis of asthma (see 1.1.3.1.4) and secondly as infective agents considered the main culprits in triggering asthma exacerbations.

1.2.3.1 Epidemiological and clinical evidence

Data from clinical and epidemiologic studies demonstrate a significant increase in visits to the emergency department (ED) and in numbers of hospitalisation for asthma exacerbations for school age children in September about 2 weeks after returning to school after the long summer holidays (Sears and Johnston, 2007). A similar but less striking peak of asthma exacerbations with some delay compared to school-age children was noted in adults.

This substantial increase in clinically significant exacerbations at a time of the year when children gather in larger groups and spend many hours a day within classrooms, thereby spreading infections more easily, suggests the contribution of infectious agents to the etiology of asthma exacerbations. This is supported also by the frequent clinical observation of cold symptoms preceding the acute lower respiratory tract exacerbation. The development of highly sensitive laboratory detection techniques ([reverse transcriptase-] Polymerase chain reaction [RT-PCR]) for respiratory viruses has made it possible to provide evidence that up to 85% of asthma exacerbations in children (Johnston et al., 1995, Tan, 2005, Papadopoulos and Kalobatsou, 2007) and 50-60% in adults (Nicholson et al., 1993) are due to virus infections with the most severe requiring hospitalisation (Johnston et al., 1996). These viral infections can trigger severe asthma exacerbations even when there is good asthma control by compliant patients taking optimal doses of inhaled corticosteroids (ICS) (Doull et al., 1997). Such exacerbations may or may not be prevented by escalating maintenance therapy even though the regular use of inhaled corticosteroids provides some benefit and reduces the risk of ED visits and admission to hospital with acute exacerbations (Johnston et al., 2005, Murray et al., 2006).

The link between RV infection and acute exacerbations of asthma has been further explored in an experimental in vivo human challenge model of RV-16-induced asthma exacerbation (Cheung et al., 1995, Bardin et al., 1996). In this model volunteers receive an intranasal delivered inoculum of RV-16. A mild generally sub-clinical exacerbation occurs in the majority of asthmatic volunteers. There is now convincing evidence that experimental RV-16 infection of the upper airways leads to virus spread

to the lower airways and to lower respiratory tract (LRT) symptoms experienced during an asthma or chronic obstructive pulmonary disease (COPD) exacerbation. The challenge model exacerbations are associated with evidence of viral replication, release of inflammatory cytokines and with inflammatory cell recruitment and airways narrowing (Johnston et al., 1995). The challenge model has also been used to demonstrate that COPD exacerbations can be induced by rhinovirus (Mallia et al., 2006).

1.2.3.2 Laboratory evidence

The improvement in viral detection techniques enabled the discovery of a wide range of respiratory viruses present in nasal aspirates and/or nasal and throat swabs. Human picornaviruses and more specifically human rhinovirus (RV) infections account for a high proportion of the viral infections in children (60-80% of all acute asthma exacerbations) (Johnston et al., 1995, Friedlander and Busse, 2005, Khetsuriani et al., 2007). Less frequently other viruses such as Respiratory syncyticial virus (RSV), Influenza virus, coronaviruses, parainfluenza viruses, adenoviruses, bocaviruses and Human metapneumovirus can also induce asthma exacerbations. Asthma exacerbations during winter time are more likely associated with Influenza and RSV infections (Gavala et al., 2011). The only virus type significantly associated with asthma exacerbations in children between 2 and 17 years of age seem to be rhinoviruses as documented in a recent epidemiological study (Miller et al., 2009). Therefore RV-induced asthma exacerbations represent a potential target for therapeutic intervention.

RV is a small single stranded RNA virus of the Picornaviridae family with more than 100 serotypes and is classified into groups A, B and C. The more recently discovered Human Rhinovirus C (HRV-C) accounts for the majority of asthma attacks in children presenting to hospital and leads to more severe attacks than previously known groups or other viruses (Bizzintino et al., 2011).

RV infections were long thought to be confined to the upper airway only but it has been shown that RV can infect the lower airways (Friedlander and Busse, 2005, Papadopoulos et al., 2000, Gern et al., 1997). Virus induced exacerbations of asthma are a result of dissemination of the cold viruses from the infected upper airway to the lower airway even though remote mechanism may also play a role (Gern and Busse, 1999). It was shown that rhinovirus viraemia occurs in 25% of children in the early stages of an acute asthma exacerbation suggesting that rhinoviraemia may play a role in the pathogenesis of an exacerbation (Xatzipsalti et al., 2005). The spreading process

from upper to lower airway appears to be more efficient and effective (from the virus's point of view) in the case of children and adults with asthma compared to healthy individuals and often results in the clinical symptoms of an acute exacerbation. Children and adults with asthma are not at higher risk of developing cold symptoms than individuals without asthma but are more likely to progress to develop (prolonged) lower respiratory tract symptoms (Corne et al., 2002).

The findings of Duff *et al.* (1993) (Duff et al., 1993) indicate that respiratory allergies and respiratory virus infections in asthma may have synergistic inflammatory effects on the lower-airway that greatly increase the risk of wheezing. This also leads to an increased risk of hospital admission in children (Murray et al., 2006). This importance of potential co-factors is supported by the finding that RV inoculation of asthma sufferers does not necessarily always lead to acute asthma symptoms (DeMore et al., 2009, Fleming et al., 1999).

1.2.3.3 Types of viruses

Most studies report that over 80% (range between 40-90%) of children with acute wheezing episodes have a respiratory virus infection of which human rhinoviruses are the most commonly detected viruses (Johnston et al., 1995).

Picornaviridae (human rhinovirus, enteroviruses)

Human rhinoviruses are single stranded, positive-sense, non-enveloped RNA viruses with remarkable genetic and antigenic heterogeneity; there are over 100 serotypes. Apart from causing common colds (Makela et al., 1998) and asthma exacerbations (Thumerelle et al., 2003) they are also the principal aetiology of the September asthma epidemic (Johnston et al., 2005). Rhinovirus appears to be the only virus type that is significantly associated with asthma exacerbations in the 2-17 years age-group (Khetsuriani et al., 2007). Several other respiratory viruses have been discussed in the literature and are detected at varying frequencies during childhood asthma exacerbations. The much lower detection rates mean that it is unclear whether these viruses (and atypical bacteria) definitely cause asthma exacerbations, are just "innocent bystanders" or cause chronic rather than acute asthma symptoms.

The prevalence of enteroviruses in cohort studies of children with asthma exacerbation has been reported as 9.8 – 18% (Freymuth et al., 1999, Thumerelle et al., 2003, Kotaniemi-Syrjanen et al., 2003), with one study that collected samples during summer months only reaching a prevalence of 29% (Rawlinson et al., 2003).

Coronaviridae

Four different groups of human coronaviruses (single-stranded, positive-sense RNA viruses) have been discovered (hCoV-229E, hCoV-OC43, hCoV-NL63, hCoV-HKU1) and are considered important common cold viruses. Their role in acute exacerbations of asthma is questionable, mainly due to the very low detection rates reported in many studies (Kotaniemi-Syrjanen et al., 2003, Johnston et al., 2005, Freymuth et al., 1999, Bosis et al., 2008). They are more frequently found as co-infections with other viruses (Bosis et al., 2008). Co-infections between different viruses are well known but there is little information about the interaction of these viruses and their individual and possibly synergistic roles in triggering asthma exacerbations (Korppi et al., 2004, Papadopoulos et al., 2011).

Paramyxoviridae (respiratory syncytial virus, human metapneumovirus, parainfluenza virus)

Respiratory syncytial virus (RSV), a single-stranded, enveloped, negative-sense RNA virus occurring in two serotypes (A and B), has a peak infection time during the winter months. It is a well-known major pathogen responsible for bronchiolitis in infancy, wheezing in pre-school children (Foulongne et al., 2006, Manoha et al., 2007) and to a lesser extent associated with acute asthma exacerbations (4-12%) (Johnston et al., 1995, Johnston et al., 2005). Human metapneumovirus (hMPV) is a more recently discovered virus that is also a paramyxovirus and is closely related to RSV. It is more prevalent in winter and early spring, has a proclivity for children less than 5 years of age and causes lower respiratory tract infections and wheezing illnesses (Foulongne et al., 2006, Sloots et al., 2006). A recent study identified no significant association between hMPV infection and asthma exacerbation in older children (Papadopoulos et al., 2011).

The role of parainfluenza viruses in triggering asthma exacerbations has been questioned. Parainfluenza viruses are present at low frequency in acute asthma exacerbations (Freymuth et al., 1999, Johnston et al., 1995, Khetsuriani et al., 2007) although rates of detection are similar in those with a non-wheezy lower respiratory tract infection (Kusel et al., 2007), healthy controls (Heymann et al., 2004) and stable asthmatics (Johnston et al., 2005).

Adenoviridae (adenovirus)

Adenoviruses are non-enveloped, double-stranded DNA viruses. Adenovirus frequencies are not increased in children admitted with wheezing LRTI compared to controls (Heymann et al., 2004).

Orthomyxoviridae (influenza virus)

Influenza viruses belong to the orthomyxoviruses and lead to yearly epidemics during winter. While their prevalence in childhood asthma exacerbations is generally low (up to 7%) (Khetsuriani et al., 2007, Johnston et al., 1995) children are more likely to be hospitalised with influenza exacerbation (Glezen, 2006, Miller et al., 2008), especially if they are atopic (Kwon et al., 2014).

Parvoviridae (human bocavirus)

More recently a parvovirus called human bocavirus (hBoV), a non-enveloped, single-stranded DNA virus, was detected in children with lower respiratory tract infection (Allander et al., 2005). Reported detection rates in healthy individuals is near-zero, in asthma exacerbations detection rates lie between 1.5% and 13% (Vallet et al., 2009, Papadopoulos et al., 2011) and co-infection with other viruses is common (Kusel et al., 2006).

Atypical bacteria (Mycoplasmataceae and Chlamydiaceae)

The association of atypical bacteria such as *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* (MP) with acute asthma exacerbations in childhood is also still a matter of investigation. While they are clearly an important cause of human respiratory tract disease, one study showed equally frequent detection rates in asthma patients during asymptomatic periods (Cunningham et al., 1998). Some data point towards an effect of MP in initiation and recurrence of asthma (Biscardi et al., 2004, Zaitsu, 2007).

While it seems intuitive to assume that the isolation of respiratory viruses (especially rhinovirus) from the airways during an asthma exacerbation have a causative link to the exacerbation itself, a number of studies have detected these viruses both in asymptomatic healthy children as well as asymptomatic children with asthma. Johnston *et al*. demonstrated a frequency of 12% (8/65) of samples positive for picornavirus from asymptomatic children(Johnston et al., 1995). Leung *et al*. showed human rhinovirus positivity in 13% of subjects with stable asthma who did not experience any symptoms or signs of disease exacerbation (Leung et al., 2010). In a

longitudinal study of healthy children the percentage of asymptomatic RV infections was even higher (20.6%) (Winther et al., 2006) and it is becoming clearer that one needs to be cautious to assume causality between HRV detection and a specific pattern of respiratory tract illness, and especially so between HRV and asthma symptoms (Olenec et al., 2010).

1.2.3.4 Upper airway sampling for pathogen detection

The nasal passages not only often present the first point of entry for respiratory pathogens but the importance of the nose in context of asthma has more recently also been emphasised in the “united airway disease hypothesis”. Previously regarded and treated as separate disorders, rhinitis and asthma are considered now as manifestations of a single inflammatory process and this concept extends to upper and lower airway diseases more generally (Togias, 2003).

Forming an interface between blood and air the upper and lower airways are significantly involved in regulatory functions shaped and challenged by the interactions between human body and its environment. Adaptive and innate immune systems have pivotal roles in these functions, which are directed by the respiratory epithelium.

Rhinitis is an inflammatory condition of the nasal mucosa and clinically characterised by itching, sneezing, rhinorrhoea and nasal congestion (Maurer and Zuberbier, 2007). Rhinitis in children is usually due to either allergy or (mainly viral) infection and it is often the combination of these two major forms that triggers asthma exacerbations and leads to poor asthma control (Murray et al., 2006).

It is essential to obtain a good sample of nasal secretions to determine the presence of respiratory pathogens in children with colds and asthma exacerbations. It is crucial, especially in the paediatric population, also to look at the patient discomfort and acceptability of sampling techniques. A recent study investigated the efficacy and patient acceptability of four techniques: swab, aspirate, brush and wash in adult patients with common cold symptoms. Nasal washes yielded the highest detection rate of respiratory viruses with minimal patient discomfort (Spyridaki et al., 2009). In another study nasopharyngeal sampling was more sensitive than oropharyngeal sampling, and nasopharyngeal washes lead to higher detection rates compared with nasopharyngeal swabs (Lieberman et al., 2009). An Australian study looked at the sensitivity of combined nose-throat specimens versus nasopharyngeal aspirates. The sensitivity of nose-throat swab specimens was regarded as adequate for use in the clinic/outpatient setting and for large-scale community studies through parent collection (Lambert et al., 2008) and associated with less patient discomfort.

Another study looked at detection rates of bocavirus in nasal samples and saliva samples and found the sensitivity of detection to be higher in saliva samples (Martin et al., 2009). One study looked at the detection rates of a number of respiratory viruses in throat swabs or saliva specimens with nasopharyngeal specimens as the gold standard. Testing of saliva specimens yielded a 74% detection rate compared with nasopharyngeal specimens (Robinson et al., 2008).

1.2.4 Immunpathogenesis of viral induced asthma exacerbations

The understanding of the immunpathogenesis of (rhino-) virus induced asthma exacerbations is the key to the successful prevention and management of asthma exacerbations as current therapeutic strategies are aimed at minimising the day-to-day variations in asthma control but are of limited value during exacerbations (Harrison et al., 2004, Doull et al., 1997, Wilson and Silverman, 1990).

In general the main feature of an antiviral response is a Th1 phenotypic response with increased levels of IFN-γ and CD8 cell recruitment. In patients with asthma a shift towards Th2 phenotypic response with increase of IL-4, IL-5 and IL-13 appears to be reducing the ability to effectively clear the virus (Gern et al., 2000). While the exact mechanisms are still unknown viral infection appears to lead to airway obstruction in individuals with asthma through inflammatory cell influx, oedema, and increased mucus secretion. In addition direct neuronal stimulation by the virus has also been claimed as a potential mechanism to induce bronchospasm (Singh and Busse, 2006).

1.2.4.1 The role of the bronchial epithelium

The bronchial epithelium is a physical barrier, immunologically active and plays an important role in both innate and adaptive immune processes but also in inflammation. The bronchial epithelial cell is also the first point of contact between respiratory virus and host (Bals and Hiemstra, 2004). The intrusion of respiratory viruses leads to replication within the epithelial cells and subsequent secondary effects such as destruction of normal airway epithelium and tissue with damage to cilia and impairment of mucociliary clearance. These destructive processes are enhanced by a cascade of pro-inflammatory immune responses that are initiated within the epithelial cell on contact with the virus. The subsequent release of chemokines recruits cells such as neutrophils, mononuclear cells, and eosinophils to the airways. A complex interaction of cytokines aimed to eliminate the viral pathogen often leads to further airway tissue destruction.

Most research has focussed on rhinovirus infection, its direct and indirect effects on the bronchial epithelial cells and the cells of the innate and adaptive immune system and

the following paragraphs will reflect this. While other viruses have been implicated and linked to asthma exacerbations, a strong association has only been shown with human rhinovirus.

1.2.4.2 Human rhinovirus – attachment, replication and intracellular signalling cascades

Human rhinoviruses attach to epithelial cells either via intercellular adhesion molecule 1 (ICAM-1) (major serotypes) or via low-density lipoprotein receptor (minor serotypes) and lead to up-regulation of these receptors, which enhances the infective process (Grunberg et al., 2000, Papi et al., 2000). Human rhinoviruses have also been shown to be able to infect airway smooth muscle cells and fibroblasts *in vitro* (Hakonarson et al., 1999, Ghildyal et al., 2005).

Following the ligation of the virus to surface receptors viral RNA is released into the cytoplasm and replication takes place. Endosomal receptors, such as toll-like receptor 3 (TLR3) and 7 (TLR7), can detect this viral RNA, initiate downstream signalling and induction of gene expression of interferons (IFNs) that bind to interferon receptors (autocrine/paracrine signalling) and initiate a Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling cascade(Gavala et al., 2011).

Further intracellular molecules have been described that can also detect HRV, including dsRNA/protein kinase receptor (PKR), and the cytoplasmic RNA helicases retinoic acid inducible gene protein-1 (RIG-1) and melanoma differentiation-associated gene (MDA-5)(Gavala et al., 2011). The activation of PKR has been described as crucial for HRV-mediated IFN release from primary epithelial cells (Chen et al., 2006) (see Figure 1.1).

Type I IFNs (IFN-α and IFN-β) and type III IFNs (IFN-λ) are part of the innate immune system and important to fight viral infections. Reduced expression of IFN-β in cultures of primary epithelial cells of asthmatic patients (Wark et al., 2005) as well as deficient IFN-λ expression in human experimental infection models of adults with mild-to-moderate asthma (Contoli et al., 2006) has been noted. These findings are considered essential for explaining increased virus replication in the asthmatic airway that may lead to asthma exacerbations. Infection with rhinoviruses and the associated airway hyper-responsiveness (AHR) were also found to be longer lasting (up to 6 weeks after hospitalisation for asthma exacerbation) in children with asthma (Kling et al., 2005).

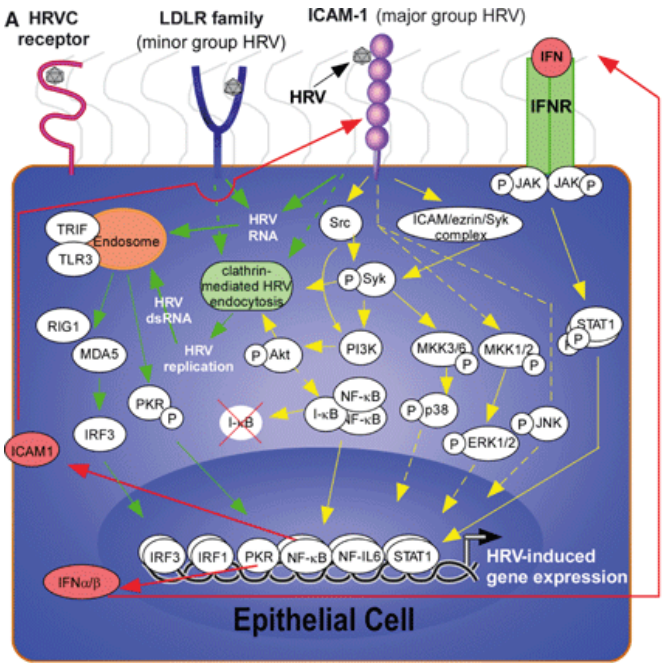


Figure 1.1 Human rhinovirus, receptors and intracellular signalling cascades leading to induction of gene expression of cytokines

Figure reproduced from reference (Gavala et al., 2011) with permission from John Wiley & Sons, Inc .

In contrast to the described viral replication dependent pathways HRV infection can also induce replication-independent signalling via activation of the tyrosine kinases Src and Syk and the downstream activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling cascade(Lau et al., 2008, Bentley et al., 2007). This cascade can lead to nuclear factor-κB (NF-κB) activation via translocation of this transcription factor into the nucleus. The induction of expression of several factors, such as IL-6, CXCL8, CXCL10, MMP-9, VEGF have been linked to NF-κB (Leigh et al., 2008, Tacon et al., 2010, Funkhouser et al., 2004, Zhu et al., 1997, Edwards et al., 2007, Volonaki et al., 2006) and higher levels of NF-κB have been found in bronchial epithelial cells from children with asthma compared to non-asthmatic children (Zhao et al., 2001). NF-κB, on the other hand, has been described as an essential player in the host response to viral infections via interferon induction (Thanos and Maniatis, 1995) (see Figure 1.1).

Activation of Syk also leads to replication-independent p38 mitogen-activated protein kinase (MAPK) activation that induces expression of a number of cytokines, such as IL-6, G-CSF, ENA-78, VEGF and IL-8 (Leigh et al., 2008, Griego et al., 2000, Wang et al., 2006). Another replication-independent pathway (extracellular signal-regulated kinases-1/2 [ERK1/2] MAPK pathway) leads to induction of IL-8 and the angiogenic

growth factor VEGF(Leigh et al., 2008), while its inhibition increases HRV-dependent expression of interferon-regulatory factors (IRF) and IP-10 (Zaheer et al., 2009, Zaheer and Proud, 2010).

Next to transcription factors such as NF-κB and STAT interferon-regulatory factors play an important role in HRV-mediated signalling cascades in the bronchial epithelial cell and have been linked to the induction of the expression of IFN-β, IFN-γ, RIG-1, MDA-5 and IP-10 (Zaheer and Proud, 2010).

1.2.4.3 Cytokines, cell recruitment and inflammation

There is an ongoing debate about the extent of direct cell and tissue damage caused by human rhinovirus but it is accepted that release of pro-inflammatory cytokines and chemokines from the initially affected epithelial cells, recruitment of inflammatory cells, and release of further factors by these cells often leads to further damage (see Figure 1.2).

The cascade of immune responses, primarily aimed at elimination of the viral pathogen, also induces neutrophilic (via CXCL8/IL-8, a potent chemoattractant for neutrophils), lymphocytic (via CXCL10/IP-10, interferon-inducible protein 10), and eosinophilic (via CCL5/RANTES) inflammation processes in the airways (Jackson and Johnston, 2010). Growth factors (such as GM-CSF and G-CSF) that are released from epithelial cells in response to HRV infection prolong the life of these infiltrating inflammatory cells and in addition contribute to airway remodelling processes (Gavala et al., 2011).

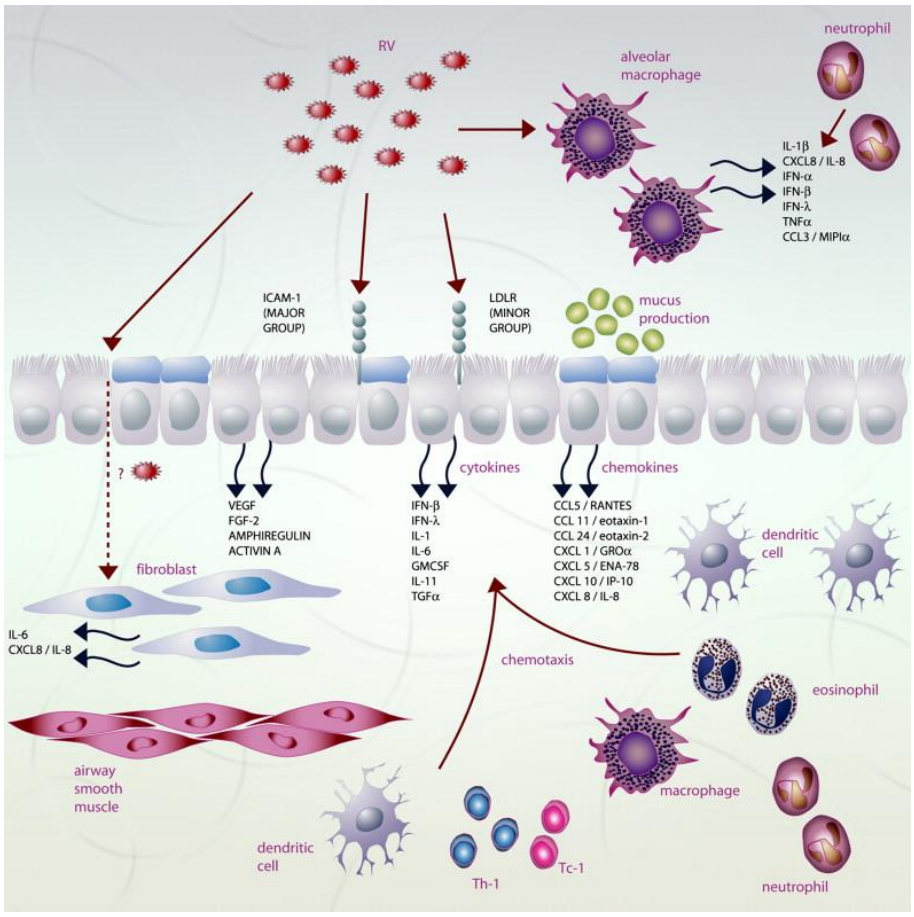


Figure 1.2 Human rhinovirus infection, cytokine production, cell recruitment and inflammation

Figure reproduced from reference (Jackson and Johnston, 2010) with permission from Elsevier.

1.2.4.3.1 Cell response of the innate immune system (Macrophages, Neutrophils, Eosinophils)

Macrophages are the most common cell type in the airway lumen and interaction with rhinovirus leads to secretion of pro-inflammatory cytokines including IL-8, TNF-α, IFN-γ, and macrophage inflammatory protein (MIP)-1α (Gern et al., 1996, Wolpe and Cerami, 1989, Parry et al., 2000, Johnston et al., 1997, Laza-Stanca et al., 2006). Macrophages are a source of type I IFNs that may contribute to combating the viral infection (Subauste et al., 1995, Laza-Stanca et al., 2006) but are also able to modulate immune responses by inhibition of antigen presentation by dendritic cells, B-cell immunoglobulin production, and T-cell activation (Holt, 1986, Holt et al., 1993). HRV induced signalling mechanisms in monocytic cells are less well understood but, in view of absent replication of rhinovirus in these cells, appear to be reliant on replication-independent

pathways. CCL2 (MCP-1) secretion from primary monocytic cells is dependent on NF-κB activation and phosphorylation of the transcription ATF-2 (Hall et al., 2005).

Neutrophilic influx is a dominant feature of viral induced asthma exacerbation and IL-8 secreted by bronchial epithelial cells and macrophages a powerful chemo-attractant for these cells. Increased levels of IL-8, G-CSF and neutrophil counts in nasal lavage fluid, sputum, and bronchoalveolar lavage (BAL) fluid of allergic or asthmatic subjects undergoing experimental rhinovirus infection have been shown (Fleming et al., 1999, Grunberg et al., 2000, Gern et al., 2000). Neutrophil degranulation products such as neutrophil elastase are found to be elevated in the sputum of asthmatic patients with virus-induced asthma exacerbations (Wark et al., 2002). These products lead to up-regulation of goblet cell mucus secretion causing airway obstruction as a feature of asthma exacerbation.

Rhinovirus infected bronchial epithelial cells also produce CCL5/RANTES, a potent chemotactic cytokine and activator of eosinophils, leading to influx of eosinophils into the airways. This eosinophilic infiltrate is present in both healthy individuals and asthmatic patients after experimental rhinovirus infection but is still present after 6-8 weeks only in the latter group (Fraenkel et al., 1995). Higher levels of RANTES have been shown in children with wheezing illness compared to those with cold symptoms alone (Pacifico et al., 2000) and rhinovirus-induced eosinophil activation correlates with changes in AHR and shown by higher levels of eosinophilic cationic protein in sputum supernatants (Grunberg et al., 2000).

1.2.4.3.2 Cell response of the adaptive immune system (T lymphocytes)

Bronchial epithelial cell infected with rhinovirus secrete CCL5 and CXCL10/IP-10, potent chemokines for T-cells. Levels of IP-10 are elevated in virus-induced asthma exacerbations and can be used to differentiate virus-induced from non-virus induced exacerbations (Wark et al., 2007).

Viral infection is usually characterized by type 1 T-cell responses and stimulation of IL-2 and IFN-γ production in order to clear the virus. On the other hand, the asthmatic airway is characterized by a type 2 T-cell and cytokine (IL-4, IL-5, and IL-13) microenvironment that could hamper an effective clearance of the viral infection.

This deficient induction of Th1 cytokines in asthmatic patients was shown in an experimental infection study where Th1 cytokines and IL-10 were protective against exacerbations, while Th2 cytokines corresponded with disease severity (Message et al., 2008). Reduced Th1 responses alongside elevated Th2 responses may also be

responsible for increased susceptibility to HRV infection and more frequent viraemia in children with acute asthma exacerbations (Xatzipsalti et al., 2005).

1.2.4.4 Matrix Metalloproteinases in viral exacerbations of asthma – airway inflammation and remodelling

Matrix Metalloproteinases (MMPs) are a group of extracellular proteolytic enzymes able to cleave components of the extracellular matrix. Depending on their preferential substrate, MMPs are subdivided into groups, such as collagenases, gelatinases, stromelysins, membrane-type MMPs and others. MMPs are secreted as Pro-MMPs into the extracellular space and activated by different mechanisms.

MMPs are important regulators of development (e.g. branching morphogenesis) and various physiological processes (e.g. angiogenesis, wound healing) by degradation of extracellular matrix (ECM), support of cell migration, alteration of the ECM microenvironment and by influencing and modulating the activity of cytokines and growth factors (Vu and Werb, 2000, Van Lint and Libert, 2007). In the healthy airways and lungs, a number of structural cells produce MMPs and their physiological inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs), and a balance exists within this proteolytic-antiproteolytic network (Gueders et al., 2006).

MMPs and TIMPs are also produced by various inflammatory cells on stimulation and have been implicated as important factors involved in inflammation and remodelling processes in pathological conditions (Mott and Werb, 2004). ECM does not only provide mechanical support for cells but also hosts a number of cytokines and growth factors. Degradation of ECM by MMPs therefore allows for cell migration but also releases bioactive molecules that lead to inflammatory cell recruitment and activation (Mott and Werb, 2004, Shapiro, 1998) (see Figure 1.3). Imbalances in the MMPs-TIMPs network can result in either exaggerated ECM turnover with remodelling processes that involve impaired repair or scar formation or to accumulation of ECM (Gueders et al., 2006) leading to fibrosis.

1.2.4.4.1 MMPs, asthma and infection

Chronic airway inflammation with influx of inflammatory cells and development of tissue remodelling with structural airway changes are important features of disease processes in asthma. MMPs are considered important players in both of these processes by orchestrating and modulating the cell recruitment, activation and ECM degradation as described above (see Figure 1.3).

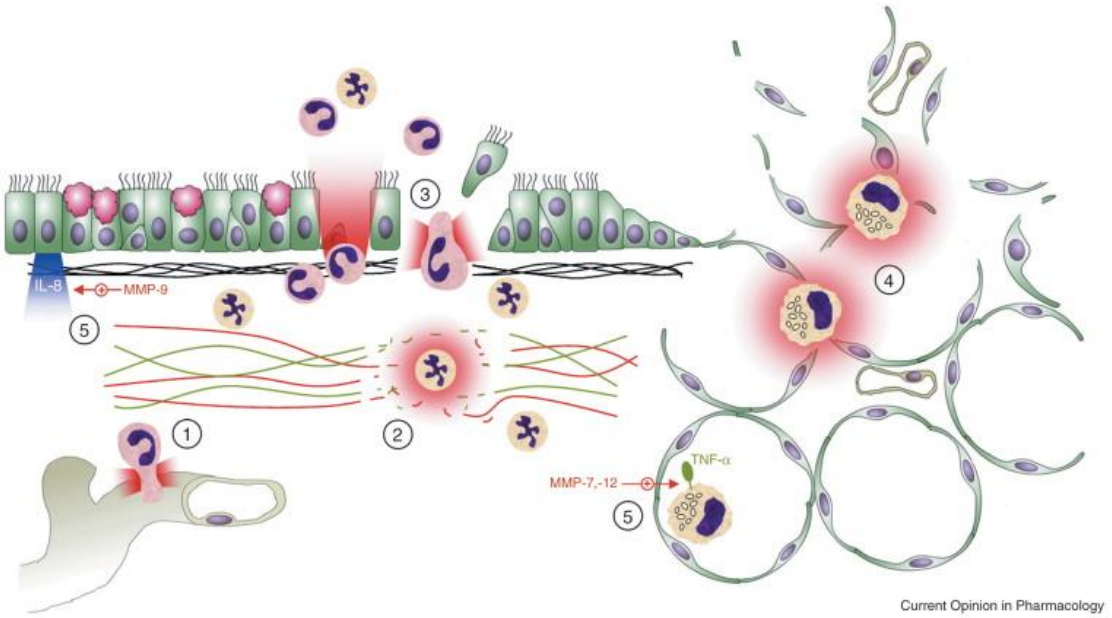


Figure 1.3 Possible pathogenic actions of MMPs in asthma

1. Involvement in extravasation of inflammatory cells from blood to interstitium; 2. Degradation of ECM proteins by MMPs allowing further migration of inflammatory cells; 3. MMPs facilitate movement of inflammatory cells through the epithelium by acting on cell-cell and cell-matrix adhesions; 4. Large amounts of MMPs may lead to direct tissue destruction; 5. Effect of MMPs on chemokines and pro-inflammatory cytokines. Figure reproduced from reference (Demedts et al., 2005) with permission from Elsevier.

MMP-9

Several MMPs have been studied for their involvement in asthma pathology; many studies have focused on MMP-9 and its role in inflammatory cell migration in asthma. Increased levels of MMP-9 have been found in serum (Bosse et al., 1999), induced sputum (Cataldo et al., 2000) and bronchoalveolar lavage fluid (Mautino et al., 1997) of patients with asthma and increased numbers of neutrophils (Cataldo et al., 2001) and eosinophils (Ohno et al., 1997) in the airways seem to be responsible for this. Levels and activity of MMP-9 were also found to be increased during acute asthma exacerbations both in serum (Oshita et al., 2003, Mak et al., 2013) and in sputum (Lee et al., 2001) where they significantly correlated with inflammatory cell count and decreased with therapy including systemic steroids. Severity of asthma also has a significant influence on MMP-9 levels in both serum (Belleguic et al., 2002) and sputum (Mattos et al., 2002) with higher levels in patients with severe asthma compared to mild asthma. 10- to 160-fold increases of MMP-9 concentrations were found in epithelial

lining fluid of patients ventilated because of acute severe asthma exacerbations (Lemjabbar et al., 1999). Elevated levels of MMP-9 in BAL correlating with disease severity have also been described in pneumonia (Schaaf et al., 2008) in adults. Nasopharyngeal levels of MMP-9 (and MMP-8) were increased during acute viral respiratory infections in children younger than 5 years but no association with disease severity was found (Brand et al., 2012). In the latter study there was also no association between serum MMP-9 levels and disease severity. Rhinovirus was found to upregulate the expression of both MMP-9 and MMP-2 in fibroblasts of nasal polyps (Wang et al., 2009).

MMP-2

Sputum levels of MMP-2 are elevated in patients with asthma(Cataldo et al., 2000) and MMP-2 promotes inflammatory cell migration into the airway lumen. In healthy lungs, fibroblasts, bronchial epithelial cells and smooth muscle cells are producers of MMP-2. MMP-2 is an important mitogen and controller of proliferation of airway smooth muscle cells(Johnson and Knox, 1999) and therefore relevant in airway remodelling processes. IL-1β, a potent pro-inflammatory cytokine, is degraded by MMP-2, MMP-3 and MMP-9 (Ito et al., 1996).

MMP-8

MMP-8 was found to be stored in secondary granules of neutrophils and released on stimulation. Increased expression and activation of MMP-8 was detected in bronchial biopsies and in BAL from asthmatic patients(Prikk et al., 2002). Studies on mice have postulated a role for MMP-8 in regulating processes leading to neutrophil clearance while lack of MMP-8 has led to severe granulocytic inflammation(Gueders et al., 2005). On the basis of this a protective role of MMP-8 in asthma was proposed (Vandenbroucke et al., 2011). During viral respiratory infections in children younger than 5 years increased expressions of MMP-8 in peripheral blood monocytes and granulocytes and higher MMP-8 plasma levels were found and these correlated with disease severity. Elevated nasopharyngeal levels did not correlate with disease severity (Brand et al., 2012). One study showed a significant inverse correlation between FEV_1 (as marker of airway obstruction) in patients with asthma and MMP-8 levels and activation in bronchoalveolar lavage (Prikk et al., 2002).

MMP-1 and MMP-13

MMP-1 is produced by a number of structural and inflammatory cells in the lung, among them fibroblasts, smooth muscle cells and alveolar macrophages (Gueders et

al., 2006). MMP-1 can lead to airway smooth muscle cell hyperplasia by activation of the insulin-like growth factor (IGF) axis (Rajah et al., 1999) and release of fibroblast growth factor (FGF) (Whitelock et al., 1996). A recent study showed MMP-1 expression in airway smooth muscle cells and reticular basement membrane of patients with asthma but not in normal airways and it was postulated that it may play an important part in increased airway contraction (Rogers et al., 2014). MMP-13, another collagenase, has been shown to be increased in allergic nasal mucosa with a strong correlation with the number of eosinophils(Mori et al., 2012).

MMP-12

MMP-12 amplifies the inflammatory cascade by direct activation of inflammatory cells (Lagente and Boichot, 2010) and activating the latent form of tumour necrosis factor-α on the surface of macrophages (Parks et al., 2004). Selective inhibition of MMP-12 in a mouse model of allergic airway inflammation restricts the increase in eosinophils in the BAL fluid after antigen challenge (Yu et al., 2010). A very recent study described an antiviral property of MMP-12 based on the function of MMP-12 as transcription factor for IκBα, leading to interferon-α secretion. Following the amplification of the inflammation, MMP-12 then leads to degradation of extracellular IFN-α, thereby limiting and resolving inflammation (Marchant et al., 2014).

MMP-3

Bronchial lavages from patients with acute asthma exacerbations were found to contain elevated levels of MMP-3 (Lemjabbar et al., 1999) and MMP-3 is associated with enhanced production of procollagen I in bronchial fibroblasts of patients with mild-to-moderate asthma. This in turn leads to reduced lung function and airway hyperresponsiveness (Todorova et al., 2010). MMP-3, like MMP-1, can release bound fibroblast growth factor (FGF) as well as TGF-β and process the pro-inflammatory cytokine IL-1β precursor to its active form (Schonbeck et al., 1998) but is also involved in degradation of IL-1β to limit its effect (Ito et al., 1996).

MMP-7

Active forms of MMP-7 have been demonstrated in ciliated epithelial cells of normal human airways. The expression of MMP-7 protein in a poorly differentiated asthmatic airway epithelium in comparison was found to be abnormal as MMP-7 is strongly expressed throughout the epithelium and also in basal cells (Wadsworth et al., 2010). Cleavage of Fas ligand (FasL) by MMP-7 releases soluble FasL, which furthers airway inflammation and epithelial cell damage. The interaction of Fas (CD95), an apoptosis-

inducing receptor that is ubiquitously expressed, and FasL (expressed on immune cells) triggers the apoptosis of the Fas-presenting cell (Hamann et al., 1998). This process has been described in the airway remodelling process of patients with cystic fibrosis (Durieu et al., 1999). FasL is also a potent neutrophil chemoattractant, which adds to the inflammatory cascade (Ottonello et al., 1999). The role of MMP-7 in creating a chemokine gradient and thereby controlling the transepithelial efflux of neutrophils has been explored and confirmed in a murine model of colonic injury (Swee et al., 2008) and in the context of acute lung injury (Li et al., 2002).While dysregulated and increased MMP-7 appears to contribute to airway inflammation and pathological tissue remodelling in asthma (Wadsworth et al., 2010), the constitutive expression of MMP-7 by ciliated epithelial cells in normal airways may indicate a role in the innate mucosal immunity of the airways (Lopez-Boado et al., 2001). Alongside MMP-12, MMP-7 is able to activate the pro-inflammatory tumour necrosis factor-α on the surface of macrophages (Parks et al., 2004) (see Figure 1.3).

1.2.4.5 Individual cytokines

Cytokines are a diverse group of proteins that act as mediators between cells. Cytokines, initially identified as secretory products of immune cells, are now also known to be produced by other cell types and of having effects on these cells as well. They bind to specific receptors on target cells in an autocrine, paracrine or endocrine fashion.

1.2.4.5.1 Cytokines directly influenced by interferon-β

Interferon-β has repeatedly been shown to be difficult to measure as the protein may be unstable and determining interferon-β levels potentially risks showing falsely low results (Edwards et al., 2013). Surrogate markers for the effect of interferon-β however are to be found downstream of the interferon-β pathway with IP-10 (CXCL10) the best known inflammatory chemokine with IFN-β related antiviral activity. IFN-β is known to potently upregulate IP-10 (Wark et al., 2007, Buttmann et al., 2007), especially also on exogenous administration of IFN-β in *vitro (*Cakebread et al., 2011), in mice and in *vivo* (Petry et al., 2006, Djukanovic et al., 2014). IFN-β also directly leads to an increase in human endothelial MCP-1 (CCL2) production (Buttmann et al., 2007). Direct effects of IFN-β on cytokines (inhibitory or suppressing) have been described for the pro-inflammatory cytokines MIP-1β (CCL4), IL-8 (CXCL-8) (Laver et al., 2008), and IL-1β (Guarda et al., 2011), and the growth factor VEGF (Fukuda et al., 2004, Roh et al., 2013).

IP-10 (CXCL10; IFN-γ induced protein)

IP-10 is a chemokine of the CXC subfamily and ligand for the receptor CXCR3 expressed at high levels on Th0 and Th1 lymphocytes but also on eosinophils. Binding of this protein to its receptor leads to pleiotropic effects, including stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression. IP-10 is produced by various cell types in response to IFN-γ (Weng et al., 1998). The release of IP-10 by monocytic cells is virus replication-independent but requires type I interferon receptor ligation and activation of STAT1 (Korpi-Steiner et al., 2010)

Increased levels of IP-10 can lead to mast cell migration and activation of mast cells residing in the ASM (Brightling et al., 2005). Infection of the bronchial epithelial cells with HRV trigger release of IP-10 with subsequent worsening of pre-existing airway inflammation and enhancing bronchoconstriction due to stimulation of mast cells (Hansbro et al., 2008). Increased serum levels of IP-10 were specifically associated with infection and correlated with the degree of airflow obstruction in adults with acute asthma exacerbation triggered by RV (Wark et al., 2007b). Increased levels of IP-10 have also been found in nasal secretions following HRV infection (Spurrell et al., 2005) and in nasal aspirates of asthmatic children during natural colds with various respiratory viruses (Lewis et al., 2012). In the latter no correlation was found with self-reported respiratory tract symptoms.

A study looking at the interaction of IP-10 and eosinophils showed enhanced eosinophil adhesion to ICAM-1, and induction of various cytokines and chemokines by eosinophils thereby up-regulating the effector functions of eosinophils (Takaku et al., 2011).

MIP-1β (CCL4, macrophage inflammatory protein-1β)

This cytokine is a mitogen-inducible monokine (i.e. produced by mononuclear phagocytic cells) and has chemotactic and pro-inflammatory functions but can also promote homeostasis. MIP-1 expression and production can be induced by a number of pro-inflammatory agents, such as LPS, viral infection, TNF-α, IFN-γ, IL-1α/β. Dexamethosone, IL-4, IL-10, and other anti-inflammatory signals lead to down-regulation of MIP-1 expression. MIP-1β acts via G-protein-coupled cell surface receptors (CCR1,3,5) and leads to chemotaxis, degranulation, phagocytosis, and mediator synthesis as part of a wider acute and chronic inflammatory response (Maurer and von Stebut, 2004) . MIP-1 proteins can also have protective effects by inducing

inflammatory responses against viruses such as influenza (Menten et al., 2002). Members of the MIP-1 family have been linked to a number of respiratory diseases (Holgate et al., 1997, Ziegenhagen et al., 1998, Capelli et al., 1999). MIP-1β levels determined in nasal aspirate samples from children with asthma rose significantly during naturally occurring colds and also correlated significantly with respiratory symptom scores including lower respiratory tract symptoms (Lewis et al., 2012).

IL-8 (CXCL8)

IL-8 is a chemokine produced by macrophages and other cells (e.g. epithelial cells, airway smooth muscle cells, etc.) and a major contributor to the inflammatory response. It functions as a chemoattractant for neutrophils and other granulocytes, induces phagocytosis and is also a potent angiogenic factor. Infection with major group HRV leads to IL-8 release from epithelial cells via activation of the phosphatidylinositol 3-kinase (PI3K/Akt) signalling cascade and NF-κB activation (Newcomb et al., 2005) or via the tyrosine kinase Syk and the p38 MAPK pathway (Griego et al., 2000).

In an experimental infection study with rhinovirus, participants with symptomatic cold had increased levels of IL-8 in their nasal secretions compared to non-symptomatic or sham-inoculated volunteers (Turner et al., 1998) . IL-8 has also been linked with reduced lung function in experimental RV infection (Grunberg et al., 1997) and IL-8 production is considered a crucial component of virally induced in comparison to allergen-driven asthma exacerbations (Wark et al., 2002, Kallal and Lukacs, 2008). IL-8 levels in nasal aspirate samples from children with asthma increased significantly during natural colds and correlated significantly with respiratory symptom scores (Lewis et al., 2012).

MCP-1 (CCL2; chemokine ligand 2)

This cytokine is a member of the CC chemokine subfamily and shows chemotactic activity for monocytes and basophils. MCP-1 is induced in the lung during asthma exacerbation (Daldegan et al., 2005) and raised levels were found in primary airway smooth muscle (ASM) cell supernatants from asthmatic patients compared with healthy volunteers (Singh et al., 2014). Increased levels were also demonstrated in blood (Singh et al., 2014) of asthmatic patients, BALF (Alam et al., 1996), and in sputum (Singh et al., 2014) of patients with bronchial wall thickening. MCP-1 has been shown to be linked with extracellular matrix changes as seen in airway remodelling by increasing MMP-1 expression in cells from bronchial secretions of asthmatic patients (Cataldo et al., 2004).

In a study on mice sensitized and challenged with Ovalbumin (OVA) and exposed to rhinovirus significant increases in MCP-1 levels were found in comparison to RV negative mice. In the same study bronchoalveolar macrophages from children with asthma produced more MCP-1 when exposed to HRV *ex vivo* than cells from non-asthmatic controls and a link between MCP-1 and airway hyperresponsiveness was postulated (Schneider et al., 2013).

Human enteroviruses also show the ability to stimulate secretion of MCP-1 (among other cytokines) from infected human primary lower respiratory tract cells(Renois et al., 2010). MCP-1 levels in nasal aspirate samples from asthmatic children increased significantly during naturally occurring colds and also correlated significantly with respiratory symptoms (Lewis et al., 2012).

IL-1β

IL-1β is a member of the interleukin 1 cytokine family and is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.

Increased levels of IL-1β are found in the asthmatic airway where it activates various inflammatory genes that are expressed in asthma and leads to airway neutrophilia (Barnes, 2008a). Administration of the receptor antagonist IL-1ra leads to reduction in allergic AHR in mice but was not found to be effective in the treatment of asthma in humans (Rosenwasser, 1998). A recent study investigating how infections could exacerbate respiratory diseases such as asthma or COPD showed the link of raised airway ATP levels with the release of IL-1β from extracellular vesicles *in vitro* and in bodily fluids and subsequent neutrophilic inflammation (Eltom et al., 2014). Raised levels of IL-1β were also found in nasal secretions of non-asthmatic participants in a study of experimentally induced colds and levels corresponded with cold severity (Proud et al., 1994). In another study this increase in IL-1β in non-asthmatic volunteers was not found, but demonstrated in the group of asthmatic participants (de Kluijver et al., 2003).

VEGF (vascular endothelial growth factor)

VEGF is a mitogen that specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis. HRV

infection of bronchial epithelial cell leads to production of VEGF via activation of p38 MAPK pathways (Leigh et al., 2008, Griego et al., 2000, Psarras et al., 2006), and is also regulated by ERK1/2 MAPK pathways (Leigh et al., 2008). VEGF is a crucial growth factor in the asthmatic airway, regulating angiogenesis and vascular leakage (Siddiqui et al., 2007, Feltis et al., 2006).

HRV infection leads to significant increases in VEGF in primary airway fibroblasts (Ghildyal et al., 2005) but was only found raised in nasal secretion of asthmatics with verified rhinovirus and respiratory syncytial virus (RSV) co-infection but not following rhinovirus infection alone (De Silva et al., 2006). Elevated VEGF levels were found in nasopharyngeal aspirates of children experiencing an HRV-related asthma exacerbation (Psarras et al., 2006) and sputum supernatant of children during an acute asthma attack contained significantly higher levels of VEGF compared to healthy controls and levels did decrease but not return to normal levels after 6 weeks of treatment (Abdel-Rahman et al., 2006).

1.2.4.5.2 Other cytokines and growth factors

G-CSF (granulocyte colony-stimulating factor)

G-CSF is a cytokine that controls the production, differentiation, and function of granulocytes.

HRV infection of bronchial epithelial cells leads to p38/MAPK mediated production of G-CSF (Leigh et al., 2008, Griego et al., 2000). G-CSF and IL-8 regulate neutrophil recruitment and activation and are considered important players in the pathogenesis of virus induced respiratory symptoms and airway hyperresponsiveness (Grunberg et al., 1997, Fleming et al., 1999). An experimental infection study with rhinovirus looking at the relationship of upper and lower airway cytokines showed rapid increases of nasal G-CSF following inoculation. A corresponding blood neutrophilia suggested a systemic effect of locally produced G-CSF. Lower airway G-CSF levels determined in sputum also corresponded to neutrophil count but were unrelated to changes in lung function (Gern et al., 2000). Serum levels of G-CSF were found to be significantly elevated in children with acute versus stable asthma and remained elevated in the acute phase despite treatment including systemic corticosteroids (Kato et al., 2010).

ENA-78 (CXCL5, epithelial-neutrophil activating peptide-78)

ENA-78, a CXC chemokine, is proposed to bind the G-protein coupled receptor chemokine (C-X-C motif) receptor 2 to recruit neutrophils (Bisset and Schmid-Grendelmeier, 2005), to promote angiogenesis and to remodel connective tissues.

Eosinophils are a source of ENA-78 and therefore are able to recruit and activate neutrophils via CXCR2 at sites of inflammation and contribute to immunoregulatory functions and tissue remodelling (Persson et al., 2003). In a recent study Bronchial Smooth Muscle cells (BSMC) from asthmatic and non-asthmatic patients were cultured and conditioned medium (CM) collected to test for angiogenic capacity. The observed increase in angiogenic capacity derived from CM from asthmatic BSMC corresponded with increases in levels of ENA-78 and other angiogenic factors (Keglowich et al., 2013). Sputum ENA-78 was found to be increased during acute asthma exacerbations and correlated positively both with the severity of exacerbation and with eosinophil activation and negatively with the peak expiratory flow rate. Nasal ENA-78 in asthmatic participants with rhinovirus infection was more elevated compared to nasal ENA-78 without RV infection in another study (Donninger et al., 2003).

1.2.4.6 The specific role of IFN-β

Bronchial epithelial cells (BECs) represent the primary site of rhinovirus infection in the lower airways (Gern et al., 1997). A defect in the ability of primary bronchial epithelial cells (pBECs) derived from adult asthmatic subjects has been detected which limits their ability to prevent replication of rhinovirus (Wark et al., 2005). It was observed that in comparison to pBECs from healthy volunteers, asthmatic pBECs following RV infection failed to up-regulate the antiviral and immunoregulatory cytokine IFN-β which is part of an integral pathway in the antiviral defence (by inducing anti-viral proteins such as PKR and RNase L in infected cells and by apoptosis preventing virus replication) (Malmgaard, 2004, Haller et al., 2006). *In vitro* administration of IFN-β at doses designed to restore levels to those produced by healthy pBECs attenuated viral replication preventing cytopathic cell death and inflammatory mediator release (Wark et al., 2005, Cakebread et al., 2011).

In addition, subsequent in vitro studies of the antiviral effect of exogenously added IFN-β1a (1) following rhinovirus infection of bronchial epithelial cells from long term smokers and patients with COPD (Haywood et al., 2010), (2) following 2009 H1N1 ('swine flu') infection of human alveolar epithelial cells (Boxall et al., 2010) and (3) following H3N2 ('seasonal flu') infection of human alveolar epithelial cells (Tear *et al.,*

2010; ERS Conference:poster 2920) also demonstrated suppression of viral replication and infection likely due to reinstated antiviral defense.

Furthermore data suggest that there is a similar deficiency in IFN-β production in primary bronchial epithelial cells from atopic and non-atopic asthmatic children (Baraldo et al., 2012) and in severe therapy resistant atopic asthmatic children (Edwards et al., 2013). In asthmatic children with decreased baseline lung function interferon-signalling pathways were also shown to be reduced (Bosco et al., 2010). IFN-β via the inhalational route, where systemic absorption constitutes only a small fraction (about 10%) of the total dose administered, is now being evaluated as a potential prevention strategy against viral induced exacerbations of asthma in adults by Synairgen (a University of Southampton spin out company). A randomised, double-blind, placebo-controlled proof of concept Phase II study in adults was comparing the efficacy and safety of inhaled IFN-β1a to placebo. Following the onset of a viral infection of the upper respiratory tract in adults with asthma, the study medication (or placebo) was administered with the aim to prevent or attenuate symptoms of asthma exacerbation (Djukanovic et al., 2014).

As part of the development of a corresponding Phase II interventional study of nebulised IFN-β use in children aged 5 - 11 years with asthma, an observational/feasibility study was performed. In addition IFN-β related antiviral cytokines such as IP-10, cytokines that are suppressed by IFN-β (such as MCP-1 and VEGF), and other potentially important cytokine and MMP networks were explored. Participating children were assessed during asymptomatic periods and followed up until they developed colds with potential associated asthma exacerbations. At the onset of upper respiratory tract infection symptoms nasal washings for virus detection and saliva and blood samples for assessment of antiviral response on mRNA and cytokine level were collected.

1.2.5 Clinical tools to assess asthma exacerbations

While validated clinical tools exist, both for adults and children, to assess the quality of life (e.g. Pediatric Asthma Quality of Life Questionnaire (Juniper et al., 1996),) and also asthma control (Childhood Asthma Control Test (Liu et al., 2007, Liu et al., 2010)), criteria for asthma exacerbation currently lack standardization even though the following classification for use in clinical trials was suggested by Reddel *et al* (2009) (Reddel et al., 2009): (i) severe exacerbation (defined as at least one of the following): (1) hospitalization due to asthma exacerbation, requiring systemic corticosteroids, (2) use of systemic corticosteroids for at least 3 days); (ii) moderate exacerbation (defined

as presence of one or more of the following): deterioration in symptoms, deterioration in lung function, increased use of rescue bronchodilator use or increased use of inhaled corticosteroids for at least 2 consecutive days, ED visit for asthma but not requiring systemic corticosteroids. It is evident that these criteria, while they may provide orientation points to stratify the severity of exacerbations, are intrinsically vague and may be largely influenced by the assessment and judgement by the (more or less experienced) clinician. In addition no definition of mild exacerbation is provided, which may be partly explained by the difficulty of distinguishing between a more gradual transient loss of asthma control and an actual sudden exacerbation of asthma. Pauwels *et al* (1997) (Pauwels et al., 1997) used similar exacerbation criteria for asthma exacerbations in adults which included defining mild exacerbation as 2 or more consecutive days of a peak expiratory flow rate (PEFR) of 80% of baseline mean morning PEFR or less, nocturnal awakenings caused by asthma symptoms, or an increase of 3 or more puffs per day over baseline albuterol use. Severe exacerbation was defined as 2 or more consecutive days of a PEFR of 70% of baseline morning PEFR or less or the need for system corticosteroid treatment.

The absence of a system or instrument that presents both subjective and objective asthma exacerbation criteria in a quantitative manner has led Sorkness *et al* (2008) (Sorkness et al., 2008) to develop a continuous variable to define asthma exacerbations and also to enable a longitudinal assessment of changes associated with exacerbation triggers.

This instrument was developed studying 47 adults between 18 and 52 years with physician-diagnosed asthma and 22 non-atopic adults without asthma who recorded once daily the numbers of puffs of beta-agonist used and nocturnal awakening due to asthma symptoms and twice daily PEFRs and symptom scores. Symptom scores were based on a 0-3 grading scale of the patient-perceived severity of wheezing, chest tightness, shortness of breath, and cough respectively. A twice daily subjective sum was calculated adding up symptoms scores and the number of puffs of beta-agonist and night time awakenings. The subjective score comprised 4 consecutive subjective sums. Each recorded PEFR was put into relation to a reference PEFR (= average evening PEFR during an asymptomatic baseline week with normal asthma control) and expressed as a percentage decrease. The PEFR score was computed for any rolling 48 hour period by averaging 4 consecutive PEFR% decrease scores.

Sorkness *et al* (2008) (Sorkness et al., 2008) gave as explanation for using the mean for the PEFR score and the total sum for the subjective score the aim to assign similar

weights to subjective and objective criteria. The sum of the PEFR score and the subjective score led to the asthma score, the subtraction of the mean of the asthma scores during the baseline week from each individual asthma score resulted in the Asthma Index (AI). In summary the AI was described as '*an indicator of changes in both PEFR and subjective variables relative to the individual's stable baseline and smoothed over a 48-hour period*' (Sorkness et al., 2008).

1.3 Collecting data and monitoring a child in a clinical study

One very important aspects of a clinical trial is the instrument and document that is used to acquire the data. There is an undeniable relationship between the quality of the data collected and the quality of that instrument. One can be as committed as possible and spend as much time and effort on conducting a clinical trial, a meaningful analysis is only possible if the correct data points were collected.

1.3.1 Conventional/Traditional data collection in research

The researcher can use personal interviews (face-to-face or via telephone), observation, questionnaires (paper, web-based) or paper diaries completed by the participants for collection of data. Each of these collection methods comes with specific advantages and disadvantages. Questionnaires are familiar to many people and often enable the respondent to reflect on the response, to answer more intimate or embarrassing questions, especially if they can choose to remain anonymous. On the other hand written questionnaires assume the participants have sufficient literacy skills to understand and respond, and web-based questionnaires in addition rely on participants' access to a computer. Interviews allow the personal contact between researcher and participant and the response to questions is immediate. This may be an advantage but could also be seen as a disadvantage as respondents may choose to be short in their answer and may choose to please rather than be entirely honest and accurate. Paper diaries allow the collection of carefully selected data from an individual over a longer period of time but clear instructions are necessary about what to record. Paper diaries are notorious for the low compliance and low reliability (Stone et al., 2002) as patients are known to falsify and backfill diaries.

1.3.2 SMS – a novel way of data collection

Short Message Service (SMS) is a text messaging service component of phone, web, or mobile communication systems. It uses standardized communications protocols that enable the exchange of text messages between fixed line or mobile phone devices. Used more and more frequently in everyday life as a way of communication especially

among younger generations, SMS has also been discovered as a novel and alternative way of data collection in medical research (Johansen and Wedderkopp, 2010, Bexelius et al., 2009, Kew, 2010) . According to the Ofcom UK Communications Market Review (2010) the numbers of text messages sent by mobile users continued to climb, growing by nearly one-third to 104.4 billion messages in 2009 (representing an average of over four a day for every person in the UK), a faster rate of growth than in 2008 (24.8%) but on a similar level to annual growth between 2005 and 2007. The advantages of SMS, both for everyday use but also for research settings, are manifold: Relatively cheap and user-friendly (free text messages often included in price plans) the messages, often in simplified and short "SMS language", are delivered instantaneously to individuals at any time or place, they can be stored and retrieved and be answered at the recipient's convenience and at a time that suits the individual. In addition the same message can be sent to multiple recipients simultaneously. In combination with the appropriate software SMS, in the research setting, can be transferred automatically and directly to an electronic data file that can be accessed by the researcher. This more or less real-time capture of data also avoids the pitfalls of backfilled and falsified paper diaries as responses are tagged with time information in the server storing the data (Johansen and Wedderkopp, 2010).

1.3.3 Mobile phones/SMS in the health care context

Neville et al (2008) (Neville et al., 2008) speak of the '*discordance between formality of traditional health service interactions face-to-face or by telephone and the anarchic appeal and convenience of SMS unconstrained by spelling, grammar or formality*'.

SMS is widely accepted as a way of communication among various population groups in general but more specifically among adolescents and (less educated) young adults, socioeconomically disadvantaged populations and those who rent or frequently change addresses (Franklin et al., 2003, Faulkner and Culwin, 2005, Koivusilta et al., 2007, Fjeldsoe et al., 2009). Considering that a high level of mobile telephone use is also associated with lower levels of self-rated health and higher likelihood of engaging in health-compromising behaviours (Koivusilta et al., 2007, Fjeldsoe et al., 2009) the mobile telephone in general and more specifically SMS technology could be regarded as an ideal medium of delivering health-related (educational) messages, health behaviour interventions, and a way of engaging the individual in the self-management of their respective (chronic) disease.

SMS has been used in the promotion of healthy behaviour (i.e. promoting sunscreen application (Armstrong et al., 2009), supporting weight-loss program (Patrick et al.,

2009), promoting HIV prevention (Coomes et al., 2011)), in preventive medicine (i.e reminder for Hepatitis vaccination (Vilella et al., 2004), paediatric vaccination reminders (Kharbanda et al., 2009), mammogram reminders (Lakkis et al., 2011), diabetes education and prevention (Somannavar et al., 2008). It has also been used for disease monitoring, self-management, improving medication adherence and facilitation of more frequent communication by providing education, monitoring and feedback outside and between scheduled clinic visits (i.e. SMS support to improve anti-retroviral treatment adherence and rates of viral suppression (Lester et al., 2010, Pop-Eleches et al., 2011), daily SMS to increase adherence to asthma treatment (Strandbygaard et al., 2010), diabetes self-management (review of 18 studies in adults and children (Krishna and Boren, 2008)). Furthermore SMS messages have successfully been used as a tool for health behaviour intervention in smoking cessation programmes (Rodgers et al., 2005, Obermayer et al., 2004).

1.3.4 SMS in medical research

In the field of medical research SMS has been used for data collection in several studies. In the context of primary care patients were asked about their satisfaction with the GP consultation. The response rate was 80.2% using the text messaging system and 85.6% using a card response method (Haller et al., 2009). A randomised controlled trial (RCT) comparing SMS, paper and online diaries in collecting weekly sexual behaviour found that SMS as a data collection tool was regarded as convenient and timely and the fact that it can be sent from anywhere was seen as an advantage. The online diary was though preferable and more complete (Lim et al., 2010). To compare data obtained via retrospective telephone interview with weekly SMS data Johansen *et al*. (2010) (Johansen and Wedderkopp, 2010) sent two SMS to 260 patients with low back pain over 53 weeks to collect number of days with back pain and number of days off work due to back pain. While the agreement between telephone interview and SMS diary was good for 1-week and 1-month recall there was a huge discrepancy for 1-year recall therefore favouring the SMS data collection (Johansen and Wedderkopp, 2010). 62 patients with bipolar disorder provided mood data in response to weekly SMS or emails with a response rate of 75%. The reply to the SMS prompt was with a code of numbers between 0 and 3. All replies were time-stamped with 83% of the response messages obtained within 12 hours of the prompting message (Bopp et al., 2010).

The feasibility of a Short Message Service (SMS) for symptom and behaviour monitoring and the provision of tailored feedback as maintenance treatment was demonstrated in a study with 40 overweight children following cognitive behavioural therapy (Bauer et al., 2010). A mobile phone text message service consisting of daily

reminders to use an inhaler, health education tips, and safety messages was used with 32 people with asthma between 10 and 46 years (Neville et al., 2002) in a feasibility and acceptability study. The system was well received as it integrated these health related reminders with lifestyle related text messages about sport, celebrity gossip, and horoscopes written in contemporary text jargon and they were sent by a *'virtual friend with asthma'* named Max. Ninety-seven adolescents with mild-to-moderate asthma were asked to enter their forced expiratory volume in 1 second (FEV_1) and peak expiratory flow (PEF) values daily into a designated web-application or to send them via SMS over a four-week period in a study trying to uncover barriers to and benefits of internet-based self-management. They received an immediate reply message with the FEV_1 and PEF expressed as a percentage of the expected or personal best value but this reply was not accompanied by any specific interpretation, general or treatment advice (van der Meer et al., 2007). Fifteen children between 9 and 15 years were asked to report pain six times daily for a week using their own cell phones to record pain data. This study used numerical responses to state intensity, duration and pain-related disability (Alfven, 2010).

1.3.5 The use of SMS in this study

This study used SMS technology alongside paper-based questionnaires to collect data from children with asthma throughout their participation in the study at varied intensity. Due to the age profile of participating children (5 to 11 years) it was considered more appropriate for these data to be transmitted via their parents. The responses were date- and time-tagged and delivered a more or less real-time picture of the symptoms experienced by the study participants. Participants were asked to provide written feedback about the use of the text message system based data collection for this study (see appendices D and E). A specifically designed short questionnaire was provided at the beginning of the study to collect information about their expectations and at the end of the study about their opinion about the feasibility and user friendliness of this technology for data collection in research.

The parents received the Jackson Cold Symptom questionnaire twice a day for one week after their baseline visit and for two weeks with a cold. The Asthma Index (AI) questionnaire (Sorkness et al., 2008) was used to assess asthma control. The AI questionnaire started on the day after the subject's Screening Visit and continued for 7 days to establish a baseline. Once subjects had reported respiratory virus symptoms, the AI questionnaire commenced immediately and continued for two weeks.

1.4 Aims, Hypotheses and Study Outline

1.4.1 Aims

This study was an observational study and a feasibility study of the various components of a protocol developed in the build-up to a potential interventional trial (with the hypothesis that a treatment with a course of nebulised IFN-β at the start of upper respiratory tract infection symptoms would prevent it triggering an exacerbation of asthma).

Aim 1

The initial aim was to characterize viral induced asthma exacerbations in young school children by exploring general and asthma specific features of participating children and documenting the frequency of viral induced common cold symptoms and frequency and severity of asthma exacerbations. The Jackson Cold Score (Jackson et al., 1958) and Asthma Index (Sorkness et al., 2008) were used and validated as scoring tools to provide quantitative data and to be able to identify potential cut-off values for the definition of colds or asthma severity grades.

The hypotheses were that:

i) The observed frequency of viral induced common cold symptoms during the study period would be comparable with published figures in the literature and in line with the parental report at the time of enrolment.

ii) The frequency and severity of asthma exacerbations during the study period would be comparable with published figures in the literature and in line with the parental report at the time of enrolment.

iii) The Jackson Cold Score provides a useful cut-off value to identify the presence of a cold in a young school age child.

iv) The Asthma Index is a valid tool to define the severity of an asthma exacerbation in a young school age child.

Aim 2

The second aim was to pilot a text-based monitoring system applied in this study to collect data to be used for clinical scores (Jackson Cold Score, Asthma Index). The combined use of a digital peak flow meter recording the PEF manoeuvres and of a time-tagged SMS system allowed validation of data points. Feedback questionnaires enabled assessment of the acceptability of this tool for data collection.

The hypotheses were that:

i) The compliance of study participants (and their parents) with responding to the SMS transmitted questions ('SMS-diary-compliance') would be high and allow the calculation of the relevant scores.

ii) The compliance of study participants with regards to performing PEFR measurements ('PEFR-meter-compliance') would be high and allow the calculation of the asthma index.

iii) The comparison between SMS-transmitted PEFR data points with PEFR-meter stored values would proof the validity of SMS-transmitted PEFR data points and allow their inclusion in the calculation of the asthma index.

iv) The SMS-data collection would be an acceptable tool for parents of participating children.

Aim 3

The aim was to describe the detection of respiratory pathogens in a cohort of school-age children with asthma with regards to frequency of occurrence, type, and seasonality, and to establish the incidence of multiple infections with two or more respiratory pathogens. Nasal lavage detection rates of pathogens were compared with saliva detection rates. Potential associations of virus status with different clinical syndromes (asymptomatic, cold only, cold + asthma), with AUC and peak of Asthma Index (Sorkness et al., 2008) and Jackson Cold Score (Jackson et al., 1958), and with symptoms/signs as reported by parents were explored.

The hypotheses were that:

i) The detection rates of respiratory pathogens in this cohort of children would be high at the time of a cold/asthma exacerbation and in line with previously published data.

ii) The detection rate of viruses in saliva and nasal lavage during cold episodes would be comparable.

iii) Virus status (positive/negative) during cold episodes would correlate with Jackson Cold Score and Asthma Index.

Aim 4

The aim was to assess the practicality of assaying a known directly IFN-β dependent biomarker (C-X-C motif chemokine 10 (CXCL10 = IP-10)) and potential novel markers

of airway inflammation (cytokines and matrix metalloproteinases (MMPs)) in nasal fluid. In addition the aim was to characterize fluctuations in nasal lavage cytokines and MMPs between baseline (asymptomatic) periods and during naturally occurring respiratory viral infections. Potential correlation between levels of nasal lavage cytokines and MMPs and severity of viral induced asthma exacerbations, virus status (positive/negative), and cold and asthma scores were explored.

The hypotheses were that:

i) IP-10 and other cytokines and MMPs can be determined in nasal lavage fluid of children with asthma during asymptomatic and cold episodes.

ii) Cytokine and MMP levels in nasal lavage will differ between asymptomatic and cold episodes and will be increased during viral colds.

iii) Cytokine and MMP levels will be increased during virus positive compared to virus negative baseline and cold episodes.

iv) Upper airway inflammatory biomarkers (cytokines and MMPs) will correlate with cold and asthma scores (Jackson Cold Score, Asthma Index).

1.4.2 Specific objectives

1. Exploration of the general and asthma specific features of participating children (chapter 3)
2. Documentation of the frequency of viral induced asthma symptoms and severity of asthma exacerbations (chapter 3)
3. Identification of the Jackson Cold Score which best identifies a cold (chapter 3)
4. Validation of the Asthma Index and comparison of original and modified versions (chapter 3)

   Assessment of the compliance of study participants (and their parents) in terms of responding to the SMS transmitted questions (‘SMS-diary-compliance’) and with regards to performing PEFR measurements (‘PEFR-meter-compliance’) (chapter 4)

2 Materials and Methods

2.1 Study design

As part of the development of a Phase II study interventional study in children, an observational/feasibility study was conducted. Children with a history of viral induced exacerbations of asthma were assessed when asymptomatic to establish a baseline and with home visits conducted by the study team when they had developed an upper respiratory tract infection (up to a maximum of 2 common colds).

2.1.1 Participants

Children aged 5 to 11 years with doctor diagnosed asthma, with a history of interval symptoms and exacerbations with upper respiratory tract infections (at least two such exacerbations in the previous year), were recruited from the paediatric outpatient clinics at Southampton General Hospital, the paediatric assessment unit, the emergency department and via community asthma nurses. Advertising with posters and leaflets was also performed in a number of local GP practices (via PCRN – primary care research network) and in a nearby district general hospital (Winchester). See Figure 3.1.

Specific inclusion criteria were:

1 Aged 5-11 years

2 Clinical diagnosis of asthma on basis of typical symptoms, at least a 15% increase in FEV_1 with bronchodilator or at least 15% diurnal variability in peak expiratory flow rates

3 Two respiratory viral associated exacerbations of asthma in the previous year.

4 Treatment with inhaled corticosteroids.

Specific exclusion criteria were:

1 Inability to perform lung function measurement

2 Poor compliance with medication

3 Treatment with more than 1000µg inhaled beclomethasone equivalent a day

4 History of other lung disease.

Participating children were initially consented to be enrolled in the study for a maximum of 6 months and covering one cold episode (± exacerbation) only. 10 months into the study, following ethical approval and re-consenting of parents and children, the monitoring period of children with asthma was extended until two distinct colds had developed or to a maximum of 10 months of daily monitoring (and monthly follow ups following two cold episodes for a total of 12 months from the time point of enrolment into the study). See details for rationale of change of protocol and extension of monitoring period in 3.5.2. Only one cold per participant was included in the final statistical analysis as it was deemed important that all data points were independent of each other.

2.1.2 Baseline assessment

All successfully enrolled participants attended a baseline visit during which a concise asthma and general medical history was taken and a quality of life questionnaire was completed. Children were also examined, underwent skin prick testing to determine atopy status; spirometry for assessing their current asthma status; and nasal washing, saliva and blood sampling for assessment of virus presence, gene expression and cytokine levels relevant to asthma.

At their baseline visit participating children received a digital peak flow meter Mini-Wright^{TM} Digital (*Clement Clarke International, UK*) for monitoring PEFR (and FEV_1) at home. They were required to measure their PEFR twice a day (morning and evening) at home during a baseline week when their asthma control was good (usually during the 7 days following the baseline visit) and for 14 days during a cold episode. The measurement and digital storing of their PEFR and the documentation and SMS-transmission of subjective cold and asthma symptom scores, both during the baseline week and during a cold episode (see 2.1.4), formed the basis of the calculation of the asthma index as outlined below.

2.1.3 Cold episode

Within 48 hours of the start of cold symptoms and 4-6 days into the cold history taking, clinical examination, lung function testing, nasal washing and saliva and blood sampling were repeated during home visits.

More detail about the clinical methods can be found in section 2.2.

2.1.4 Text message system

The researchers sent SMS messages to parents of participating children and received their responses via a third party SMS provider called ClinicalSMS Ltd (*Bournemouth,*

UK). This is a two-way SMS messaging server that sent, received and stored time-tagged messages. No identifiers other than mobile phone numbers were stored. The schedule of SMS messages used in this study is shown in table 2.1.

2.1.4.1 SMS during baseline and cold episode

All parents of children participating in the study received as a standard six text messages a day (4 in the morning, 2 in the evening) for seven days during the baseline week (total number: 42 SMS) and five text messages a day (3 in the morning, 2 in the evening) for fourteen days during a cold episode (total number: 70 SMS). The measurement and digital storing of their PEFR and the documentation and SMS-transmission of subjective cold and asthma symptom scores both during the baseline week and during a cold episode formed the basis of the calculation of the asthma index (Sorkness et al., 2008).

2.1.4.2 Daily monitoring for colds

All participants were assessed daily (daily monitoring) using a text based system until they developed their first and second cold (or up to 10 months, whichever was earlier). Parents were asked to respond to the following questions via SMS text:

Does your child have a sore throat, more nasal symptoms than normal or a cold or flu?

If they respond with a yes, they received twice daily texts assessing cold and asthma symptoms for the following 2 weeks.

Parents chose a time in the morning (7:00 – 10:00) and in the evening (17:00 – 20:00) when they wanted to receive the text messages. They received a reminder after one and after two hours if they had not responded to the messages.

2.1.4.3 Monthly monitoring for colds

Participants were followed up once monthly following their cold episode(s). Assessments were by text to assess the frequency of viral respiratory tract infections and associated asthma symptoms in this period.

Text message	Name	Morning and/or evening	Text message sent	Example of response	Baseline 7 days	Daily monitoring	Cold episode 14 days
SMS 1	Daily Cold question	am	*Does your child have a sore throat, more nasal symptoms than usual (not hayfever/allergy related), or a cold or flu? (Y/N)*	'Y' *e.g in parent's view child has cold symptoms*	√	√	
SMS 2	Cold symptoms	am & pm	*Sore throat? Runny nose? Sneeze? Nasal cong? Tired? Fever? Headache? Hoarseness? Earache? Cough? (Score each 0-3)*	*'1012200112'* e.g. mild sore throat no runny nose etc.	√		√
SMS 3	Asthma symptoms I	am & pm	*Chest tightness? (0-3) Wheeze? (0-3) Cough? (0-3) Short of breath? (0-3) PEFR value? (030-999)*	*'1220420'* e.g. mild chest tightness, moderate wheeze and cough, no SOB, PEF 420	√		√
SMS 4	Asthma symptoms II	am	*No of times woken during the night to use inhaler? (0-9) No of extra puffs used in 24hrs for acute symptoms? (0-99)*	*'212'* *e.g. used inhaler twice over night and used 12 extra puffs in previous 24hrs*	√		√

Table 2.1 Data collected via SMS system

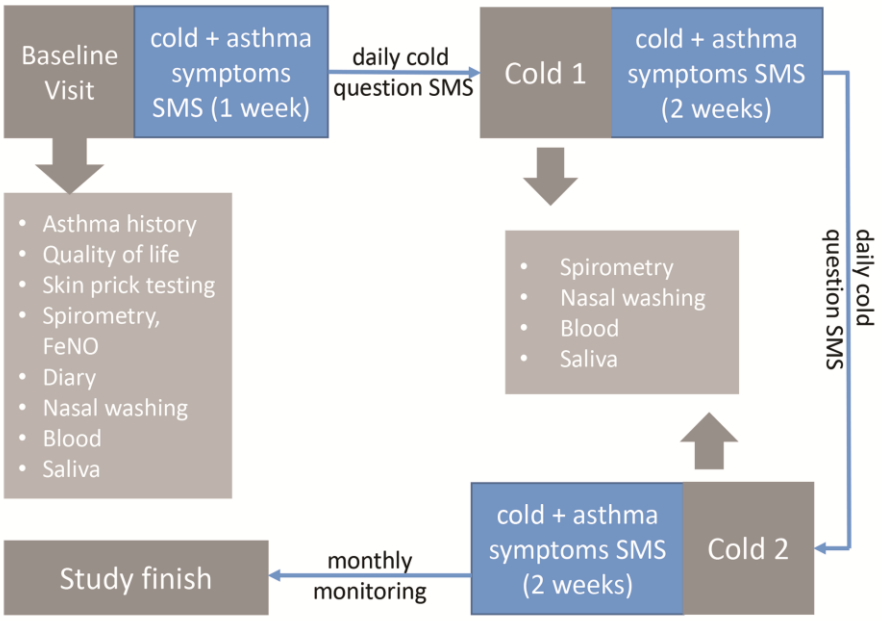


Figure 2.1 Study Design

2.1.5 Study schedule

Study months	0	1	2	3	4	5	6	7	8	9	10	11-12	Cold
Informed consent	X												
Medical history	X												
Examination	X												
Height, weight	X												
Skin allergy test*	X												
Spirometry	X												
Text based assessment**	X	X	X	X	X	X	X	X	X	X	X	X	X
Home spirometry	X												X X
Nasal lavage	X												X X
Saliva sample	X												X X
Blood	X												X
Feedback SMS system	X												X

Table 2.2 Study Schedule

*Skin allergy testing was undertaken unless assessed in previous 2 years.

**Assessments via text message. Twice daily assessment for 1 week post screening visit; then daily screening for cold; once develop a cold, two weeks of twice daily assessment; then again daily screening for cold until second cold, then two weeks of twice daily assessment. Then only one text message monthly for two months or until end of 12 months (whichever is earlier).

2.2 Scoring systems for colds and asthma exacerbations

2.2.1 Calculation of Jackson Cold Score

The Jackson cold symptom score was received twice a day for a week after baseline screening and for 2 weeks with a cold. Parents were asked (via SMS text) to what extent the following symptoms had affected their child in the past 12 hours:

A. Sore throat

B. Runny nose

C. Sneeze

D. Nasal congestion (blocked or stuffy nose)

E. Malaise (tiredness)

F. Fever (feverish/chills)

G. Headache

H. Hoarseness

I. Earaches

J. Cough

They were asked to score each symptom as follows:

0 No Symptom

1 Mild Symptom

2 Moderate Symptom

3 Severe Symptom

The profile of the Jackson Cold Score (JCS), both during the baseline week and the cold episode, was plotted against time and the peak and the AUC of the JCS were

determined. The sum of the JCS over any 48 hour period was calculated (i.e. sum of two morning JCS or sum of two evening JCS) and the peak of these summated JCS was examined. The temporal relationship between cold symptoms (JCS) and asthma symptoms were documented by plotting both in one graph against time (objective iii.).

2.2.2 Calculation of original and modified asthma index

One of the perceived disadvantages in the approach to calculate the (original) asthma index by Sorkness et al. (2008) (Sorkness et al., 2008) specifically in the application in the paediatric population, was the lack of quantifiable data during the first 48 hours of cold symptoms, as the earliest point an asthma index is calculated is after 48 hours of collection of objective and subjective exacerbation data. Parallel to the originally presented calculation of the asthma index by Sorkness *et al*., a slightly modified approach was pursued so that the value of the new Subjective Score reflected the mean time point between the measurements. In addition values for the new PEFR score were calculated as the mean of 2, 3 or 4 consecutive PEFR%Decreases, which allowed to obtain calculated PEFR scores for the first 48 hour period. Instead of originally 11 asthma scores during the stable baseline week 14 asthma scores were therefore achieved.

This modified approach to the calculation of the asthma index was expected to be advantageous in this study population since the progression from initial cold symptoms to an exacerbation is much more rapid than in adults and at times the asthma symptoms may even precede the cold symptoms. In the following a short summary of the approach to the computation of the original asthma index and the alternative or new approach is given. A more detailed summary can be found in the Appendix Section F.

2.2.2.1 Computation of Asthma Index (including AUC and peak)

The Asthma Index (AI) incorporates Peak Expiratory Flow Rate (PEFR), use of short-acting bronchodilators and subjective variables relative to an individual's baseline. The score is a rolling average over a 48-hour period.

The following information needs to be recorded (and sent by SMS or stored on PEFR meter respectively):

Morning:

a. Number of puffs of salbutamol (or equivalent) used in the past 24 hours

b. Number of times the subject woke up at night and required salbutamol

c. Peak Expiratory Flow Rate (highest of 3 efforts)

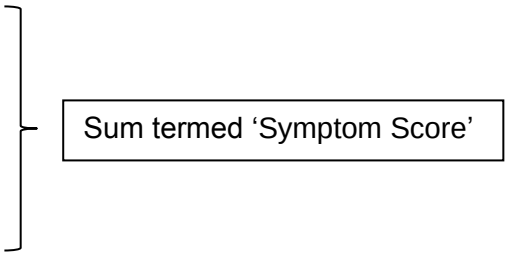
d. Each of the following scored 0 - (not present); 1 - mild (noticeable, but not bothersome); 2 - moderate (bothersome, but you can perform normal activities); 3 – severe (interferes with your normal life activities, and/or requires therapy)

i. Chest tightness

ii. Wheeze

iii. Cough

iv. Shortness of Breath



Evening:

a. Peak Expiratory Flow Rate (highest/best of 3 efforts)

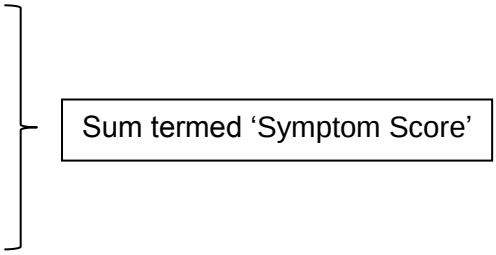
b. Each of the following scored 0 – 3:

i. Chest tightness

ii. Wheeze

iii. Cough

iv. Shortness of Breath



Computation of Baseline Reference PEFR and Reference Asthma Score (‘Reference Combined Score’)

1. Morning and Evening data as outlined above need to be recorded for 7 days.

2. The Baseline Reference PEFR is computed by taking the average of the 7 evening PEFR readings

3**.** The Reference Asthma Score (Reference Combined Score) is derived from the morning and evening data as follows**:**

The morning Subjective Sum is computed by adding together the salbutamol use in 24 hours, the asthma-related wakening episodes overnight and the Symptom Score as detailed above. The evening Subjective Sum is equal to the evening Symptom Score described above.

The *original* Subjective Score is then derived by adding together the 4 consecutive Subjective Sums. The *new* Subjective Score is computed by multiplying the average of 4 consecutive subjective sums by 4, shifting the score though so the value reflects the mean time point between the measurements. This new approach will enable to obtain Subjective Scores for the first 48 hours.

Subsequently the PEFR score (% change in PEFR from the Reference PEFR) is calculated using PEFR%Decrease values:

The *original* PEFR Score is calculated for any rolling 48 hour period by averaging 4 consecutive PEFR% Decrease scores. The *new* PEFR Score is computed for any rolling 48 hour period by averaging between 2 and 4 consecutive PEFR% Decrease scores. As this approach allows the averaging of 2 and 3 PEFR%Decrease values will also be obtained for the first 48hr period.

To compute the Asthma Score (Combined Score), rolling (48hr) Subjective Scores and PEFR Scores need to be added up. Over a 7 day period, there should be 11 *original* Asthma Scores and 14 *new* Asthma Scores as the new approach yields Subjective Scores and PEFR Scores also for the first 48hr period.

The Reference Asthma Score (Reference Combined Score) is then computed by averaging the 11 or 14 Combined Scores respectively**.**

Computation of the Asthma Index during the cold episode (and during baseline)

The Asthma Index is computed by deducting the Reference Asthma Score (Reference Combined Score) from each half-daily Asthma Score:

Original Asthma Index = half-daily Asthma Score minus the *original* Reference asthma score (in original publication this is done during cold episode only but in this study this was also calculated for the baseline period)

New Asthma Index = half-daily Asthma Score minus the *new* Reference asthma score during baseline and cold episode.

Computation of the Area under the Curve (AUC) and the Peak Asthma Index

Original:

The AUC of the Asthma Index is calculated by adding up 2 consecutive *original* Asthma Indices, dividing by 2 and multiplying by 0.5 (to reflect the half-day time periods). The values generated this way are then added up to yield the *original* Area under the Curve.

This calculation is based on the trapezoidal rule for calculating AUC :

$$\Delta AUC_{1-2} = \frac{(Cp_1 + Cp_2)}{2} \times (t_2 - t_1)$$

The trapezoidal rule is a numerical method that approximates the value of a definite integral (Whittaker, 1967).

The adjusted AUC of the asthma index (to yield positive AUC values for all subjects) is calculated according to a new formula: ((asthma index day 0.5 + 40 + asthma index day 1 +40)/2)*0.5. The 10 values generated this way are then added up to yield the adjusted *original* Area under the Curve.

The *original* Peak Asthma Index and the adjusted *original* Peak Asthma Index are also determined**.**

New:

The AUC of the asthma index is calculated by adding up 2 consecutive *new* Asthma Indices, dividing by 2 and multiplying by 0.5 (to reflect the half-day time periods). The 13 values generated this way are then added up to yield the *new* Area under the Curve.

The adjusted AUC of the asthma index is then calculated according to the new formula as outlined above.

The *new* Peak Asthma Index and the adjusted *new* Peak Asthma Index are also determined.

2.2.3 Severity classification

The severity classification for asthma exacerbations for use in clinical trials as suggested by Reddel *et al* (2009) (Reddel et al., 2009) was used to divide the participants into 3 severity groups:

(i) severe exacerbation (defined as at least one of the following): (1) hospitalization due to asthma exacerbation, requiring systemic corticosteroids, (2) use of systemic corticosteroids for at least 3 days;

(ii) moderate exacerbation (defined as presence of one or more of the following): deterioration in symptoms, deterioration in lung function, increased use of rescue

bronchodilator use or increased use of inhaled corticosteroids for at least 2 consecutive days, ED visit for asthma but not requiring systemic corticosteroids;

(iii) cold only: The last classification of 'cold only' was added and is not part of the original classification by Reddel *et al*. who do not include a specific definition for mild or no exacerbation.

In order to distinguish a "cold only" episode from a "moderate exacerbation" the following 3 criteria were quantified: Beta-agonist use, the asthma symptom score, and the lung function measurements. Criterion 4 (oral corticosteroid use) determined a severe exacerbation as described above.

Beta-agonist use (criterion 1), the asthma symptom score (criterion 2, transmitted by SMS), and the lung function measurements (criterion 3) of each cold episode of each participant were analysed and a final classification determined for each episode by taking the highest score of the following criteria:

Criterion 1 – beta-agonist use: The cold episode analysed received a score of 2 (moderate exacerbation) if there was an increased use of beta-agonist over at least 2 consecutive days and the increase per day was at least double the average baseline use. Otherwise a score of 1 (cold only) was given.

Criterion 2 – asthma symptom score: Participants submitted scores (0-3) for wheeze, chest tightness, cough, and shortness of breath twice a day both during baseline and during a cold episode. Criterion 2a considered the absolute value of the symptom score and the percentage increase from baseline. Based on this criterion a cold episode received a score of 2 (moderate exacerbation) if the asthma symptom score was ≥ 4 and if there was at least a 25% increase from baseline. Criterion 2b looked at the number of distinct symptoms during any day of the cold episode. If there were at least 2 more symptoms (e.g. wheeze AND cough) compared to any day of the baseline week (e.g. chest tightness only) the cold received a score of 2 (moderate exacerbation). If these definitions were not achieved a score of 1 (cold only) was given.

Criterion 3 – lung function: If either the best PEFR during first week of cold was ≥ 20% less than best PEFR during the baseline week OR the best FEV_1 during first cold visit ≥ 12% less than the best FEV_1 at baseline visit the cold episode received a score of 2 (moderate exacerbation). Otherwise a score of 1 (cold only) was given.

Criterion 4 – corticosteroid use: If a participant received oral corticosteroids as part of their asthma treatment during a cold episode the criterion for a severe exacerbation was fulfilled and a score of 3 was given.

2.2.4 Decision process on what cold episode (in case of two episodes to include in analysis)

The decision to amend the protocol and re-consent participants for a longer monitoring period in order to catch two colds was based on the observation that a majority of the initial participants developed colds that did not lead to any asthma exacerbations which are the main focus of this research. Nine participants were both monitored and received home visits with nasal lavage, saliva and blood samples being taken during two episodes of cold symptoms +/- asthma exacerbation. For the purpose of statistical analysis and after discussion with a statistician only one cold episode per participant was included. An important objective of this research study was the assessment of viruses and markers of airway inflammation and the variation of the latter with the severity of respiratory viral infection and exacerbation of asthma. Nasal lavage, saliva and blood samples were therefore collected both during baseline and during the cold episode. The sampling was not always possible because of various reasons (e.g. child not happy on the day, equipment not working). In order to maximise the chances to yield useful information cold episodes were prioritised during which the sampling of biological samples was (near) complete. This process did not lead to any change in the inclusion process of cold episodes as far as the severity grading (see 2.2.3 above) was concerned but helped to decide between two cold episodes of one participant with the same severity grading.

2.3 Specific clinical methods

2.3.1 History: general and asthma specific information

A detailed history was taken at baseline which included general information such as birth circumstances, breastfeeding, respiratory illness in infancy, atopy, family history of atopy, social and environmental factors (living conditions, smoke and pet exposure etc.). In addition a more detailed asthma history included asthma triggers, seasonal predominance of asthma symptoms, use of medications and number of asthma exacerbations. This information was explored and compared between participants with no cold episodes and participants with one or two cold episodes during their involvement in the study.

2.3.2 Physical examination

All children were examined by one paediatric registrar (clinical fellow) at the baseline visit, and during the home visits while having a cold. While it involved a respiratory, cardiovascular, gastrointestinal, ear-nose-throat (ENT), dermatological (and a gross neurological and musculoskeletal) examination during the baseline visit, it was restricted to a respiratory and ENT examination during the other visits. Findings during the physical examination were documented in the case report form.

2.3.3 Spirometry (including reversibility)

Equipment: see Appendix N

Information required:

Height

Weight

Date of birth

Name

Study number

Respiratory history and medication details

Calibration:

Calibration was always carried out on the day of the challenge immediately before the procedure and a record of the calibration was logged.

Procedure:

The FVC manoeuvre can be divided into three distinct phases; (1) full inspiration, (2) exhalation with maximal force, and (3) continued complete exhalation to the end of the test. (Miller et al., 2005)

The child was instructed to stand up straight throughout the testing with good view of the laptop screen displaying an incentive game. A new single-use mouth filter was placed on the Koko spirometer. If deemed necessary the technique was demonstrated to the child using a different Koko filter. The child was actively encouraged as they performed the test, receiving clear instructions about "*taking in a deep breath*", "*blasting it out as hard and fast as you can*", and to "*keep blowing, blowing, blowing*

until your lungs are empty". Particular attention was paid to a good seal of the lips around the mouthpiece.

The child was instructed to perform up to a maximum of 8 efforts, depending on the technical quality and repeatability of their blows.

Following the baseline blows 600µg (i.e. six puffs) of Salbutamol were administered using a volumatic spacer and the lung function test was repeated after 15 minutes as described above.

2.3.4 Nasal Lavage

Nasal lavages were performed at three different time points, i.e. at baseline (while children are stable and asymptomatic), within 48 hours of cold symptoms being recognised and reported by parents, and at one time point between four to six days into the cold. The purpose of performing nasal lavage was to obtain nasal fluid for subsequent analysis for presence of airway pathogens (viruses, see chapter 5) and to determine markers (cytokines, see chapter 6) corresponding with cold symptoms and/or worsening of asthma symptoms.

Equipment: see Appendix N

Procedure:

After explaining the procedure to the child and parents the operator washed their hands and put on protective clothing. The child could wear an apron for protection if desired. The child was instructed to hold the universal specimen container with the collecting funnel standing up inside it. The funnel collected any fluid escaping from the nostril. The appropriate sized olive fits firmly in the child's nostril to make a good seal and prevent premature leakage of the lavage fluid. The olive was inserted into one nostril, normally about half way to obtain a good seal. A 5mL syringe was filled with 2.5mL of normal saline and attached to the end of the olive. The child sat in an upright position with their head forward at an angle of about 30-45 degrees. This position should prevent fluid from reaching and irritating the nasopharynx. The fluid was repeatedly (5 times) pushed gently from the syringe into the nasal cavity and withdrawn again. Full flushes were attempted but this was not always possible in a small child or if the nose was obstructed. Frothing of fluid was avoided. The fluid finally withdrawn from the nasal cavity was placed in the universal specimen container and the child remained in the same position for a few seconds to collect drops leaving the nose. The procedure was then repeated on the other side. The fluid collected was immediately placed on ice and the total volume of lavage was recorded.

2.3.5 Saliva Sampling

Saliva sampling was performed to easily obtain diagnostic samples for virus detection and cytokine analysis. The Salivette® system from Sarstedt (*Nürnbrecht, Germany*) was used to collect saliva samples at baseline and during colds of participants.

Participating children were instructed not to eat for one hour prior to sampling and they rinsed their mouth with clear water 10 minutes before sampling. If either was not possible this was clearly documented in the CRF.

The child put the cotton roll of the Salivette® tube into the mouth, chewed it for 60 s, and kept it in the mouth for an additional minute.

The cotton roll was placed into the flat bottom upper tube of the Salivette®, sealed with the stopper and put immediately on ice before processing.

2.3.6 Blood Sampling

Blood sampling was performed by specifically trained and experienced paediatric staff at baseline and within 48 hours of a cold developing. Topical anaesthetic or ethyl chloride spray was used according to the child's preference. No more than two attempts were made to obtain blood at each occasion.

Equipment: see Appendix N

Blood sampling schedule:

	Baseline	**Cold 1 (first 48 hours)**	**Cold 2 (first 48 hours)**
Type of sample (max volume)	EDTA (6 mL)	X	X
Type of sample (max volume)	Serum (6 mL)	Serum (6 mL)	Serum (6 mL)
Type of sample (max volume)	PAXgene (2.5mL)	PAXgene (2.5mL)	PAXgene (2.5mL)

Table 2.3 Blood sampling schedule

Obtaining blood for RNA processing using PAXgene blood RNA system

Blood was sampled using a needle (butterfly needle with extension tubing) and syringe technique. This constituted a deviation from the existing SOP aimed at adult participant

as the proposed vacuum system (BD vacutainer safety-lok system) would cause collapse of usually small veins in children preventing the successful collection of blood.

The tourniquet was released to allow 2.5mL of free flowing blood to be sampled according to the manufacturer's instructions. This was then added to the PAXgene tube and the PAXgene tube immediately inverted 10 times and put on ice prior to further processing.

2.3.7 Skin prick testing

Children underwent skin prick testing (SPT) to a panel of aero- and food-allergens in order to determine their atopy status. SPT was performed by specifically trained staff on the day of the child's baseline visit.

Equipment: see Appendix N

Panel of allergens:

Allergen	**Allergen**
Negative Control	Aspergillus
DPT	Alternaria
Farinae	Cladosporium
Mixed Grass	Cow's Milk
Cat	Egg
Dog	Peanut
Mixed Trees	*Positive Control*

Table 2.4 Panel of Allergens for SPT

Procedure:

Bottles containing allergens were kept refrigerated at all times between uses to maintain potency of solutions. SPTs were performed according to standardised techniques. Briefly, following explanations given to the child and parent and ensuring their understanding, droplets of test allergens were placed on the skin of the inner aspect of one forearm in the order they were printed on the respective CRF sheet. The

tip of a lancet was then inserted into the skin at right angles to the surface of the skin (90°), with a different lancet being used for each allergen to prevent cross-contamination. Once all allergens had been introduced superficially under the skin the surplus of the allergen containing droplets was blotted away. After 15 minutes the wheal size was recorded if present.

Interpretation:

A test was regarded as positive if the wheal diameter was greater than 3mm. The validity of the test was determined by a negative control and a positive histamine control.

2.3.8 Peak Flow and FEV_1 measurements

Obtained from flow-volume curve data, PEF is expressed in L x s^{-1}, when obtained from portable monitoring instruments it is expressed in L x min^{-1}.

PEF measurements vary according to a child's height, sex and race and depend on the technique. The technique of using the digital peak flow meter Mini-Wright™ Digital (*Clement Clarke International, UK*) provided in this study was demonstrated to the child and parent during the baseline visit and the child was observed performing up to three successful and repeatable PEF manoeuvres. The digital peak flow meter also allowed for the parallel measurement of FEV_1 (forced expiratory volume in one second). The device stored up to 240 FEV_1 and 240 PEF.

PEF and FEV_1 were measured at home by the child twice daily during the baseline week (for a total of 8 days) and twice daily during a cold (for a total of 14 days). The children were asked to blow into the peak flow meter 3 times each time, recording the highest PEF values achieved. Parents were asked to send this value via text message to the study team. An instruction leaflet by the manufacturer was provided with the device. Children and parents were made aware that the PEF meter was storing the results of the blows and that they would be downloaded at the end of the study.

2.3.9 Questionnaires and explanation sheets

Baseline asthma questionnaire

See appendix A.

Asthma Quality of Life Questionnaire

See appendix B.

Text message system – explanation sheet

See appendix C.

Text message questionnaires (baseline + follow up)

See appendices D. and E.

2.4 Specific Laboratory Methods

2.4.1 Pre-Processing

2.4.1.1 Pre-Processing of Nasal lavage samples

General:

The "VIPA Study nasal lavage worksheet" was filled in throughout the processing procedure. Any deviations which could affect sample quality were clearly recorded on the worksheet. Nasal lavages were processed in a class II microbiological safety cabinet (MSC). The MSC was situated in a laboratory that is designated for working on primary samples that have a greater risk of infection. A beaker of 2% virkon solution (or appropriate alternative) and/or autoclave bin was available for discarding/disposing contaminated plastic-ware.

Equipment: see Appendix N

Procedure:

- The nasal lavage was performed by a member of the clinical research team as outlined in section 2.2.4. The nasal lavage samples were kept on ice until they could be processed.
- The centrifuge was pre-cooled to 4°C
- The nasal lavage sample was filtered through a 100µm nylon cell strainer into a 50mL polypropylene conical tube to remove any mucus and debris. If the filter became blocked with mucus, multiple filters were used.

- The nasal lavage sample was transferred into a 15mL polypropylene conical tube. The approximate volume of the nasal lavage sample was determined to establish how many tubes needed to be labelled. Approximately 2.5-3.5mL (i.e. five to seven 0.5mL aliquots) of nasal lavage sample from 5mL saline were obtained each time.
- Filtered nasal lavage sample were centrifuged for 10 minutes at 4°C at 400xg
- The appropriate number of 0.5mL amber sample tubes was labelled with the information below. The aliquotted nasal lavage sample was stored in these tubes.
    - Subject number + Visit number (e.g. V055 VISIT 2)
    - Sample type (Nasal lavage or Nasal lav cells)
    - Volume of aliquot (i.e. 500µL)
    - Date (in DD MM YYYY format)
- The supernatant was transferred into a new 15mL conical polypropylene tube using a fine tipped pastette and care was taken in order not to disturb the cell pellet (which often was very small only or not visible).
- The tube was inverted to mix nasal lavage supernatant.
- 500µl aliquots were pipetted into labelled 0.5mL amber sample tubes using a pipette (with a barrier tip) and tubes were capped with yellow lid.
- The cell pellet was re-suspended with 200µl of RLTplus Buffer and transfer into 1.5mL microfuge tubes.
- Aliquots and re-suspended cell pellet were immediately stored at ≤ 70°C or below.
- The worksheet was completed including details about the storage location.

2.4.1.2 Pre-Processing of Saliva samples

Equipment: see Appendix N

Procedure:

- Salivettes were centrifuged for 4 minutes at 1000 rcf (rotational centrifugal force = g) at 4°C. The centrifugation procedure forced any mucous material or solid substances into the conical base of the tube.
- The appropriate number of 0.5mL amber sample tubes was labelled with the information below. The aliquotted saliva sample were stored in these tubes.

o Subject number + Visit number (e.g. V055 VISIT 2)

o Sample type (SALIVA)

o Volume of aliquot (500ul)

o Date (in DD MM YYYY format)

- The flat bottom tube from the Salivette was removed and the clear saliva pipetted from the bottom of the V-tube, aliquotted (0.5mL) and stored at -80°C.
- The stopper, cotton wool swab and suspended insert were disposed of as one unit.

2.4.2 Downstream analysis

2.4.2.1 Pathogen detection in nasal lavage (and saliva)

(adapted from Protocol *L173 SG005 Pathogen detection in nasal lavage v3* from Synairgen Research Ltd, Brooke Laboratories, Southampton, UK – Author: Rona Beegan)

2.4.2.1.1 Principles and Objectives

Viral nucleic acids were extracted from the nasal lavage supernatant and the saliva using column technology. A one-step RT-PCR reaction was performed in multiplex reactions against a panel of 21 respiratory pathogens. An internal control was included to ensure that the extraction and PCR are acceptable.

2.4.2.1.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

2.4.2.1.3 Nucleic acid extraction

Nasal lavage was processed as outlined above (section xxx) and stored at ≤-70°C until analysis. A negative control (NC) supplied by FTD was included in each batch of samples being extracted. Internal control (supplied by FTD) was added to each sample during the lysis step of the extraction. The extraction method was based on a user-developed protocol provided by Qiagen (Purification of viral RNA and DNA from 1000µL of plasma, serum and cell-free body fluids using the QIAamp® MinElute® Virus Vacuum Kit (QA32 Jul-10)).

Preparing Kit Components

The following was carried out in a class II MSC to avoid contamination of reagents

- 310µg lyophilized carrier RNA was reconstituted with 310µL Buffer AVE to obtain a 1µg/µL solution. This was stored at –20°C in 85µL aliquots.
- QIAGEN Protease from the kit was reconstituted in 4.4mL Buffer AVE and stored at 4°C (for up to 1 year or until the kit expiration date if that was sooner). QIAGEN Protease that was ordered separately was reconstituted in 7mL DNase/RNase free water and stored at 4°C (for up to 2 months).
- 25mL ethanol was added to AW1 and stored at room temperature (for up to 1 year or until the kit expiration date if that was sooner).
- 30mL ethanol was added to AW2 and stored at room temperature (for up to 1 year or until the kit expiration date if that was sooner).

Immediate Preparation

- 2 x 500µL aliquots of each sample to be tested plus one negative control (NC) and one internal control (IC) from the FTD kit were defrosted.
- Oven was heated to 56°C.
- The bench surrounding the vacuum manifold was cleaned.

Setting Up the Vacuum Manifold

- It was ensured that the waste bottle was not full but contained enough non corrosive biocidal/virucidal detergent (e.g. biocleanse) to disinfect the waste.
- The manifold was placed on a flat, planar surface, ensuring that the lower outlet on the manifold was on the left side (as per Figure 2.2).
- The tubing from the main vacuum valve was connected to the quick coupling on the QIAvac manifold (which should be attached to the lower threaded hole on the manifold on the left).
- It was ensured that the screw cap valve was attached to the upper threaded hole of the manifold (on the right) and that it was in the closed position (i.e. the valve was pushed down flat towards the manifold).
- The main vacuum valve was opened (see Figure 2.2).
- Any unused luer slots on the manifoldereclosed with luer plugs.

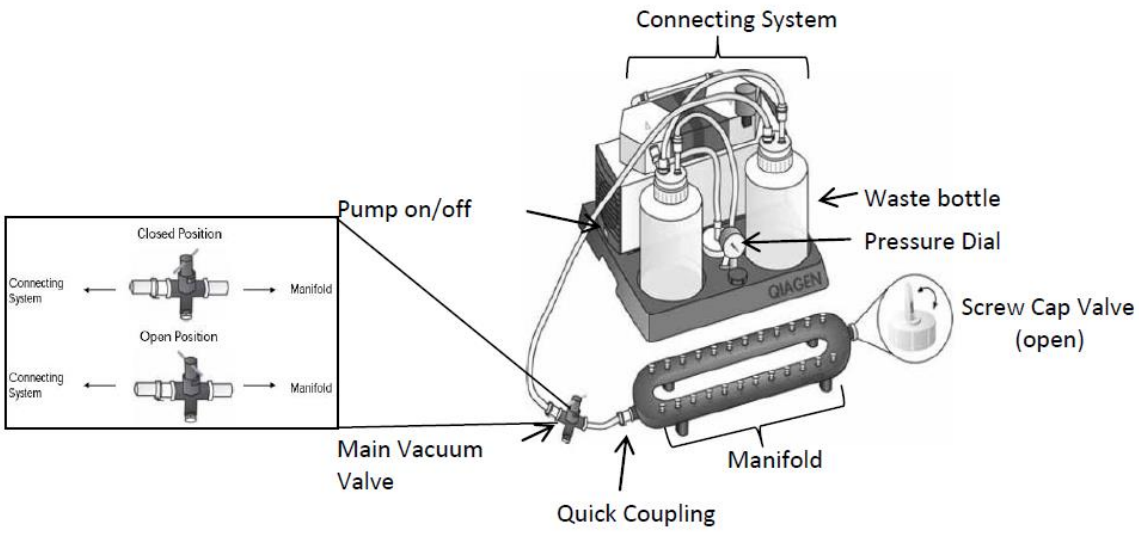


Figure 2.2 Vacuum Manifold

Extraction method

The lysis was completed in a class II MSC. The MSC was situated in a laboratory that is designated for working on samples that have a greater risk of infection

- Appropriate numbers (1 per sample, plus NC, plus Mastermix 1) of 15mL polypropylene tubes was labelled.
- The Mastermix 1 (Buffer AL, carrier-RNA in Buffer AVE and FTD kit internal control (IC)) for the required number of samples was produced according to the table below.
- The Mastermix was inverted 10 times to gently mix

	Volume for 1 sample	Volume for 13 samples
Buffer AL	0.55 mL	7.15 mL
Carrier-RNA (in Buffer AVE)	6.2 µL	80.6 µL
FTD kit internal control (IC)	2 µL	26 µL

Table 2.5 Mastermix preparation for nucleic acid extraction

- 75µL QIAGEN protease was added to each labelled 15mL polypropylene tube designated for a sample or negative control.
- 500µL nasal lavage supernatant/NC was added to each 15mL tube.
    - Nasal lavage was stored in 500µL aliquots, so 450µL from each tube was added. Any nasal lavage remaining in the tubes was discarded in 2% Virkon solution for disinfection.
    - FTD kit NC only contains 200µL, so was made up to 500µl by adding 300µL sodium chloride 0.9% W/V. A new vial of sodium chloride was opened each day to ensure no contamination occurred.
- 500µL of Mastermix 1 was added to each 15mL tube.
    - The cap was closed and the tube content was mixed by pulse-vortexing for 15 seconds.
    - The tube was incubated at 56°C for 15 minutes and then pulse-spun

- 600µL of ethanol (96–100%) was added to each 15mL tube.
    - The cap was closed and the tube content mixed thoroughly by pulse-vortexing for 15 seconds.
    - The tube was incubated for 10 minutes at room temperature and then pulse-spun.
- The appropriate number of QIAamp MinElute columns was labelled.
- A VacConnector was inserted into the luer slots to be used on the vacuum manifold. The QIAamp MinElute columns were inserted into the VacConnectors
    - An extension tube was inserted into the open QIAamp MinElute columns.
    - The collection tube was kept to use later.
- All of the lysate (contents of 15mL tube) was carefully added into the extension tube of the QIAamp MinElute column.
    - The vacuum pump was turned on.
    - After all lysates had been drawn through the QIAamp MinElute column, the vacuum pump was switched off and the pressure released to 0 mbar
- 600µL of Buffer AW1 was added to the QIAamp MinElute column.
    - The extension tube was removed and discarded before the vacuum was switched on.
    - After all of Buffer AW1 had been drawn through the QIAamp MinElute column, the vacuum pump was turned off and the pressure released to 0 mbar.
- 750µL of Buffer AW2 was added to the QIAamp MinElute column.
    - The lid of the column was left open, and the vacuum pump switched on.
    - After all of Buffer AW2 had been drawn through the QIAamp MinElute column, the vacuum pump was switched off and the pressure released to 0 mbar.
- 750µL of ethanol (96–100%) was added to the QIAamp MinElute column.

- The lid of the column was left open, and the vacuum pump was switched on.
- After all of ethanol had been drawn through the column, the vacuum pump was switched off and the pressure released to 0 mbar.

- The lid of the QIAamp MinElute column was closed, the column removed from the vacuum manifold, and the VacConnector was discarded.
  - The QIAamp MinElute column was placed in a clean 2mL collection tube (saved from earlier) and centrifuged at 12,000 rpm for 3 min.
  - The collection tube with the filtrate was discarded.
- The QIAamp MinElute Column was placed into a new 2mL collection tube
  - The lid was opened and the column was incubated at 56°C for 3 min to dry the membrane completely.
- The QIAamp MinElute column was placed in a 1.5mL microcentrifuge tube
  - 40µL of Buffer AVE was applied to the membrane.
  - The lid was closed and the tube incubated at room temperature for 5 min.
  - The tube was then centrifuged at 12,000 rpm for 1 min.
- Another 40µL of Buffer AVE was added to the column membrane.
  - The tube was incubated for a further 5 min at room temperature, then centrifuged at 12,000 rpm for 1 min.
- The column was discarded and the lid of the 1.5mL microfuge tube was closed.
- Tubes were labelled (subject code, visit number, extraction number and date) and stored at -20°C (or ⩽-70°C if > 1 month until qPCR analysis).

2.4.2.1.4 qPCR Plate Map Setup

A plate map was produced for each batch using the CFX manager software prior to setting up any PCR reactions.

To create a new plate map Bio-Rad CFX Manager was opened, File and ‘New’ → ‘Plate’ were selected. ‘Settings’ (top left hand side of the screen) and ‘Plate Type’ →

'BR White' were selected. To select 'Scan Mode' the dropdown arrow next to the icon (on top panel) was clicked on and 'All Channels' was selected.

All required wells were highlighted and the 'Fluorophore' button (right hand panel of the screen) was selected. 'SYBR' was deselected by clicking in the adjacent box to remove the tick and 'FAM, VIC, ROX and Cy5' were selected. The wells required for the samples only were highlighted, the 'Sample Type' the drop down arrow (on right hand panel of the screen) was clicked and 'Unknown' selected. The wells required for the negative controls only were highlighted, the 'Sample Type' drop down arrow (on right hand panel of the screen) was clicked and 'Negative Control' selected. The wells required for the positive controls only were highlighted, the 'Sample Type' drop down arrow (on right hand panel of the screen) was clicked and 'Positive Control' selected.

Each well then had a sample type and abbreviated name:

o Unknown – Unk in blue

o Negative – Neg in pink

o Positive – Pos in grey

The blue 'Unk' wells were highlighted and each of the four boxes on right hand panel of the screen under the 'Load' heading FAM, VIC, ROX and Cy5 was clicked. This was done for each sample type (i.e. Neg and Pos). Each 'Unk' well was highlighted individually and by clicking in the 'Sample Name' box (on right hand panel of the screen) the subject code and visit number could be written in.

The 'Load' box next to the Sample Name was selected and this was done for each unknown and for each negative control. The completed plate was saved by clicking the disk icon (top left hand corner of the screen).

2.4.2.1.5 RT-PCR

Guidelines followed for performing PCR

Only RNase/DNase free consumables and designated PCR pipettes and filter/barrier tips were used. The working area was cleaned prior to starting work with RNase away according to manufacturer's instructions. Then DNA Remover was used as per manufacturer's instructions. The area was then rinsed with DNase/RNase-free water.

Designated PCR areas (away from air conditioning units) were used and disposable gloves and lab coat were changed prior to entering different designated PCR areas.

The PCR plate was placed in a PCR cooler when plating out the reaction. Nucleic acid samples were defrosted, vortexed and pulse spun prior to addition to PCR plate. Samples were stored on ice during plating and placed back in freezer after use.

PCR kits

The FTD respiratory pathogens 21 kit contains primer/probe (p/p) mixes for 5 different 'batches' of virus (see Table 2.3). A separate mastermix needed to be made for each 'batch' of viruses.

Batches of FTD primers and probes	FTD terminology for each pathogen	Pathogen	Reporter dye
Flu/Rhino	Flu A	Influenza A	FAM
	Rhino	Rhinovirus	VIC
	Flu A H1N1 swl	Influenza A (H1N1) swine	Cy5
	Flu B	Influenza B	ROX
COR	Cor 229	Coronavirus 229E	FAM
	Cor 63	Coronavirus NL63	VIC
	Cor 43	Coronavirus OC43	Cy5
	Cor HKU	Coronavirus HKU1	ROX
Para 2/3/4	Para 3	Parainfluenza 3	FAM
	Para 2	Parainfluenza 2	VIC
	IC (BMV)	Internal control	Cy5
	Para 4	Parainfluenza 4	ROX
Bo/Mp/Pf1	Para 1	Parainfluenza 1	FAM
	HMPV A/B	Human metapneumovirus A&B	VIC
	Mpneu	Mycoplasma Pneumoniae	Cy5
	HBoV	Bocavirus	ROX
RS/EPA	RSV A/B	Respiratory syncytial virus A&B	FAM
	PV	Parechovirus	VIC
	AV	Adenovirus	Cy5
	EV	Enterovirus	ROX

Table 2.6 FTD respiratory pathogens 21 kit

In addition to the 'Rhino' primers and probes supplied in the FTD kit, rhinovirus was also measured using primers and probes designed by PrimerDesign (HRV all species). This is because validation work by Synairgen laboratory scientists showed that the two p/p sets appear to have different sensitivities for different rhinovirus serotypes.

Plate maps and Mastermix calculations

Calculations:

Reagents	1 reaction	96 reactions
P/P mix	1.5 µL	144 µL
2x RT-PCR buffer	12.5 µL	1200 µL
25 x RT-PCR enzyme	1 µL	96 µL
Total	15 µL	1440 µL

Table 2.7 FTD mastermixes for each batch of p/p's

Reagents	1 reaction	96 reactions
P/P mix	1 µL	96 µL
2x RT-PCR buffer	12.5 µL	1200 µL
25 x RT-PCR enzyme	1 µL	96 µL
Total	14.5 µL	1392 µL

Table 2.8 Primer Design RV mastermix

PCR Methodology

1. 15µL per well of FTD mastermix was added into appropriate wells of white 96 well PCR plate.
2. 14.5µL per well of PD mastermix was added into appropriate wells of white 96 well PCR plate.
3. 10µL nucleic acid sample or NC was added to appropriate wells.
4. Plate seal was cut to ensure it covered the wells containing samples and NC and plate seal was attached.
5. The next step was performed in an area not designated for PCR to minimise contamination:
    - 10µL positive controls (PC) was added to appropriate wells and the plate seal was attached over PC wells.
6. The next steps were again performed in designated PCR area:
    - The sealed PCR plate was spun and the plate run immediately

Loading the plate

The CFX96/C1000 was switched on and the Bio-Rad CFX Manager software was opened on the attached laptop. The ‘Create a new Run’ button was checked and ‘OK’ clicked. A ‘Run Setup’ window opened with the ‘Protocol’ tab open. The ‘Select Existing’ button and the FTD protocol were clicked.

Programme details:

50°C for 15min hold

95°C for 10min hold

40 cycles of: 95°C for 8 sec

60°C for 35 sec

‘next’ was then clicked to go to the ‘Plate’ tab and the ‘Select Existing’ button was clicked.

A preview of the plate map was opened and it was ensured that the ‘Plate Type’ was ‘BR White’ and the ‘Scan Mode’ was ‘All Channels’. For FTD plates all four fluorophores (FAM, VIC, ROX and Cy5) were selected. For the Primer Design RV plate, VIC, ROX and Cy5 were deselected.

‘next’ was clicked to go to the ‘Start Run’ tab. The machine lid was opened by clicking the ‘Open Lid’ button at the bottom of the window. The plate was positioned so that it was aligned correctly in the plate holder. The machine lid was closed by clicking the ‘Close Lid’ button at the bottom of the window. The ‘Start Run’ button on the bottom right of the window was clicked . Once each run was complete, ‘View’ → ‘Run Log’ was clicked and data generated briefly scanned for anomalies.

Data analysis

For each individual plate the virus type specific for each fluorophore was added. The positive control traces were changed to grey for easy identification on the Amplification chart.

The run data were set up in the following way:

The baseline set by the CFX manager software was left un-altered (auto-calculated). Traces were set as baseline subtracted. Fluorescence drift correction was applied.

Internal controls

Internal controls were assessed using ‘single threshold Cq determination mode’. The threshold line on the Amplification chart was kept above the ‘baseline noise’ and on the linear section of the traces when viewed on the ‘log’ scale.

Positive controls and samples

Positive controls and samples were analysed using the regression Cq determination mode. All sample traces were checked by eye on the regression mode. Samples that were assigned a Cq value in the regression mode were considered positive, unless the trace was clearly not positive ‘by eye’ using the ‘log’ mode. Sample traces were also checked ‘by eye’ on single threshold Cq determination mode to ensure there were no ambiguous samples which might have been excluded by regression mode.

Data interpretation

Controls

Internal control (IC) traces were accepted if Cq < 31. Any traces with a Cq value ≥ 31 were carefully assessed and discussed with the Synairgen laboratory science team.

Both samples and negative controls needed to yield acceptable IC traces while positive controls should not amplify for IC.

A positive control (PC) was considered positive if it showed an exponential trace and was assigned a Cq value < 33 by the regression Cq determination mode. Any traces with a Cq value ⩾ 33 were carefully assessed and discussed with the Synairgen laboratory science team.

Samples

A sample was considered positive if it showed an exponential trace and was assigned a Cq value by the regression Cq determination mode.

Any sample with no trace was considered as negative as long as the controls were acceptable.

PP mix	Pathogen	Reporter	positive Fluorescent trace in the green chanel	positive Fluorescent trace in the yellow chanel	positive Fluorescent trace in the red chanel	positive Fluorescent trace in the orange chanel
FLU/ Rhino	FluA	FAM	POS			
	Rhino	VIC		POS		
	FluA (H1N1) swl	Cy5			POS	
	FluB	ROX				POS
	If Flu A **AND** FluA(H1N1)swl are positive, the patient is FluA(H1N1)positive.If just FluA is positive the patient is FluA positive. If Rhino **AND** EV (RS EPA PP) are postive the patient is EV positive. If just Rhino is positive the patient is Rhino positive.					
Para 2/3/4	Para 3	FAM	POS			
	Para 2	VIC		POS		
	IC (EAV)	Cy5			POS	
	Para 4	ROX				POS
	The IC has to be positive for each extracted material (patients and NC)					
COR	Cor 229	FAM	POS			
	Cor 63	VIC		POS		
	Cor 43	Cy5			POS	
	Cor HKU	ROX				POS
RS EPA	RSVA/B	FAM	POS			
	PV	VIC		POS		
	AV	Cy5			POS	
	EV	ROX				POS
	If just EV (no Rhino) is postive the patient is EV positive.					
BoMpPf1	Para1	FAM	POS			
	HMPV A/B	VIC		POS		
	Mpneu	Cy5			POS	
	HBoV	ROX				POS

The table shows the possible results with the FTD Respiratory 21 test. Pos= positive; empty = negative

Table 2.9 Possible results with the FTD Respiratory 21 test

2.4.2.2 Whole blood poly(IC) stimulation assay

(adapted from Protocol *L147 Whole blood poly(IC) stimulation assay for SG007* from Synairgen Research Ltd, Brooke Laboratories, Southampton, UK – Author: Rona Haynes)

2.4.2.2.1 Principles and Objectives:

At the baseline visit blood was collected into tubes containing anticoagulant and stimulated with poly(IC) (or sterile water for control sample) for 6hrs at 37°C. The blood was then added to PAXgene RNA blood tubes in order to lyse the cells and stabilise the mRNA profile. The blood was stored frozen in the PAXgene tubes prior to

RNA extraction. The effect of poly(IC) on interferon (and related genes) induced mRNA expression was analysed later by qPCR.

2.4.2.2.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

2.4.2.2.3 Methods

Prior to blood collection

A bottle of distilled water and 1.5mL microfuge tubes were autoclaved and a solution of 1mg/mL poly(IC) was prepared (10mL of the endotoxin free water provided was used to re-suspend the vial containing 10mg poly(IC). Complete solubilisation was achieved by vortexing, heating to 50°C and then re-vortexing. Aliquot were stored frozen at -20°C in autoclaved microfuge tubes.

On day of blood collection

A blood processing worksheet was filled in to record incubation times etc throughout procedure.

The MSC was set up with tube rack, absorbent tissue and 1% virkon solution (or alternative disinfectant) and 2 new 3mL EDTA blood collection tubes were labelled appropriately in the MSC (6hr control, 6hr poly(IC)).

6mL blood was collected in an EDTA blood collection tube. The blood collection tube was inverted 10 times immediately after the blood had been taken. The blood was processed within 30 min of being taken. In the lab the tube was inverted a couple of times.

Using a stripette 3mL blood was added into each of the new EDTA blood collection tubes

- 300µL sterile water was added to the control tube
- 300µL 1 mg/mL poly(IC) was added to the 100µg/mL poly(IC) tubes,

If less blood had been taken (instead of 6mL), the volumes were reduced accordingly. The tubes were capped and inverted 10 times. The tubes were placed in a biohazard labelled Tupperware box and placed in the incubator at 37°C.

After 6hrs, the control and poly(IC) treated tubes were removed from the incubator and inverted a couple of times. Working in an MSC, 2.5mL blood were removed from each EDTA blood collection tube with a stripette and added to a labelled PAXgene tube. The PAXgene tubes were capped and inverted 10 times and stored upright in racks at ⩽ -70°C.

2.4.2.3 Whole Blood RNA extraction using PAX gene

(adapted from Protocol *L121 Whole blood RNA Extraction using PAXgene* from Synairgen Research Ltd, Brooke Laboratories, Southampton, UK – Author: Rona Haynes)

2.4.2.3.1 Principles and Objectives

The PAXgene Blood RNA System consists of a blood collection tube (PAXgene Blood RNA tube) and nucleic acid purification kit (PAXgene blood RNA kit). The blood RNA tube allows collection, storage, transport and stabilisation of the RNA profile all in one tube. The samples can be stored frozen until they are processed.

This method describes the isolation of intracellular RNA from 2.5mL whole blood using the PAXgene Blood RNA kit. The samples were then DNase treated and reverse transcribed. Changes in mRNA expression could subsequently be measured by qPCR.

2.4.2.3.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

2.4.2.3.3 Methods

Blood collection and storage

For details on blood collection, please refer to 2.2.5.

Blood tubes were stored vertically at ≤-70°C for freezing. On the day of extraction, they were defrosted at room temperature (20-25°C) for 1hr, and then equilibrated to room temperature for a further 2hrs before starting the extraction procedure. Tubes were inverted twice during these 3 hours.

Kit reconstitution (according to PAXgene Blood RNA Kit Handbook (version 2 April 2008)

Buffer BR4 was diluted with 44mL 100% Ethanol to obtain a working solution prior to initial use.

Alteration of protocol

The on-column DNase provided with the kit had not been found to remove all genomic DNA in previous experiments by Synairgen laboratory scientists. The on-column DNase step as described in the handbook was therefore omitted and an extra DNase incubation using Ambion DNA free kit was included after the RNA preparation (see 3.2.4).

Method

1. The heat block was warmed to 55°C before start of the procedure.

2. The PAXgene tubes were centrifuged for 10 minutes at 3,000-5,000xg in swing-bucket centrifuge. The original tube stopper was discarded and the supernatant tipped out into virkon solution (2%). Any remaining supernatant was removed with a fine tipped pastette without disturbing the cell pellet.

3. 4mL RNase-free water (RNFW) was added to the cell pellet, the tube was closed with a new Hemogard closure, and the tube was vortexed at maximal speed to re-suspend the cell pellet (approximately 2min per sample).

4. The PAXgene tubes were centrifuged for 10 min at 3,000-5,000xg in swing-bucket centrifuge. The supernatant was discarded into virkon solution and any remaining supernatant removed with a fine-tipped pastette.

5. 350µL Buffer BR1 was added and the tube closed with the same Hemogard closure. The tube was vortexed at maximal speed to thoroughly re-suspend the cell pellet (approximately 2 min per sample). The lysate was transferred to a labelled 1.5mL microcentrifuge tube.

6. 300µL Buffer BR2 and then 40µL Proteinase K was added to each tube. This was mixed by vortexing for 5 seconds and then the tubes were incubated at 55°C for 10 min in the pre-warmed heat block. Samples were briefly vortexed after 5 min and upon completion of the incubation.

7. A purple Qiagen Qiashredder column (in 2mL processing tube) was labelled for each sample (with a permanent marker). The entire lysate was transferred to the Qiashredder column and spun at maximum speed (13,000xg) for 3 min.

8. The Qiashredder colum was discarded and the flow-through transferred into a labelled 1.5 mL microcentrifuge tube. Care was taken not to transfer the pellet that may have formed in the bottom of the collection tube.

9. 350µL 100% ethanol was added to the flow-through (lysate) and briefly mixed by vortexing. The tube was pulse spun for 1-2 seconds to pool the liquid at the bottom.

10. 600µL of the lysate was loaded onto a labelled pink PAXgene RNA spin column and spun at 13,00xg for 1 min. The flow-through was discarded and the processing tube retained.

11. The remaining lysate (if any) was loaded onto the PAXgene spin column and spun at 13,000xg for 1 min. The flow-through was discarded and the processing tube retained.

12. 350µL Buffer BR3 was added to the PAXgene spin column and spun at 13,000xg for 1 min. The flow-through was discarded and the processing tube retained.

13. A further 350µL Buffer BR3 was added to the PAXgene spin column and spun at 13,000xg for 1 min. The flow-through was discarded and the processing tube retained.

14. 500µL Buffer BR4 (working solution) was added to the PAXgene spin column and spun at 13,000xg for 1 min. The flow-through was discarded and the processing tube retained.

15. A further 500µL Buffer BR4 (working solution) was added to the PAXgene spin column and spun at 13,000xg for 3 min. The PAXgene spin column was transferred to a new processing tube. The old processing tube was discarded.

16. The empty PAXgene spin column was centrifuged at 13,000xg for 1 min to ensure that the membrane was dry and that there was no residual Buffer BR4 left behind. The PAXgene spin column was transferred to a new labelled 1.5mL microcentrifuge tube. The old processing tube was discarded.

17. 40µL Buffer BR5 was added directly to the PAXgene spin column membrane ensuring the entire membrane was wet. The tubes were centrifuged at 13,000xg for 1 min.

18. The 40µL Buffer BR5 was removed from the microcentrifuge tube and added back to the PAXgene spin column membrane. The tubes were centrifuged at 13,000xg for 1

min. The PAXgene spin column was removed and discarded and the microfuge tube lids were closed.

19. The RNA was denatured by incubating at 65°C for 5 min in the heat block. Following this incubation step the tubes were immediately chilled on ice.

20. All tubes were labelled appropriately and the RNA stored frozen at ≤-70ºC before DNase treatment, quantification, reverse transcription and qPCR.

2.4.2.4 DNAse treatment of purified RNA

(adapted from Protocol *L143 DNAse treatment of purified RNA using Ambion DNA-free kit v2* from Synairgen Research Ltd, Brooke Laboratories, Southampton, UK – Author: James Roberts)

2.4.2.4.1 Principles and Objectives

RNA prepared from whole cells is never 100% free from contaminating genomic DNA. DNA-free kit and TURBO DNA-free kit from Ambion are both designed to remove up to 50µg DNA per mL RNA to a level which is not detectable by RT-PCR. DNA-free kit and TURBO DNA-free kit can be used in RNA preparations at a concentration up to 500ng/µL RNA. The DNase is inactivated using an inactivation reagent which also removes divalent cations which can catalyse RNA degradation when the RNA is heated.

TURBO DNase is an engineered version of wildtype DNase I with 350% greater catalytic efficiency and a higher affinity for DNA than conventional DNase I, thus it is more effective in removing trace quantities of DNA contamination. Therefore the TURBO DNA-free kit should be used in preference to the DNA-free kit.

Although DNase steps are included with some Qiaspin columns, the DNase works more efficiently in solution. The DNase step is therefore omitted from the column extraction and performed on the RNA solution.

2.4.2.4.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

2.4.2.4.3 Methods

Ambion TURBO DNA-free kit reagents were defrosted on ice.

A blank sample was prepared using the RNA elution reagent and volume used in RNA preparation (40µl buffer BR5 for Paxgene kits). This is for use as a blank on the Nanodrop.

A mastermix of 10x TURBO DNAse I buffer (4µL) and TURBO DNase I (2µL) was prepared and added to the samples (40µL).

All samples were mixed gently and incubated at 37°C in a heat block for 20-30 min.

The inactivation reagent was thoroughly vortexed prior to adding to the samples to resuspend the slurry. 0.2 volumes of inactivation reagent (8µL) were added to each sample.

The samples were incubated at room temperature for 2 min, and the samples mixed 2-3 times during the incubation to keep the inactivation reagent suspended.

If samples were used immediately, they were centrifuged at maximum velocity for 1-2 min to pellet the inactivation slurry. If time did not allow, RNA samples were stored at ⩽-70°C until the RNA concentration could be measured.

The RNA concentration of each sample was determined prior to performing the RT reaction (see 3.2.5 Nucleic Acid concentration and quality determination using NanoDrop), using the blank sample taken through the DNAse procedure as a blank.

2.4.2.5 Nucleic Acid Concentration and Quality Determination Using Nanodrop

(adapted from Protocol *L106 Nucleic Acid Concentration and Quality Determination Using Nanodrop v2* from Synairgen Research Ltd, Brooke Laboratories, Southampton, UK – Author: Rachel Pestridge)

2.4.2.5.1 Principles and Objectives

NanoDrop is a full spectrum (220-750nm) spectrophotometer that measures 1-2µL sample with high accuracy and reproducibility using retention technology to hold the sample in place. It has the capacity to measure highly concentrated samples without the need for dilution eliminating the need for cuvettes or other devices that could lead to possible contamination.

Values for A260, A280, 260/280, 260/230 and concentration (ng/L) appear on the screen after each sample measurement. The 260/280 is the ratio of the sample absorbance at 260 and 280nm. It is used to assess the purity of the nucleic acid. A pure ratio for DNA is 1.8 and for RNA is 2.

The 260/230 is the ratio of the sample absorbance at 260 and 230nm. This is a secondary measure of purity. A pure sample is 1.8-2.2.

2.4.2.5.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

2.4.2.5.3 Method

The bench was cleaned with RNase away and a 2% solution of Trigene was made up in case of spills. An aliquot of RNase free water was poured into a universal container.

The NanoDrop software (ND-1000) was opened and 'Nucleic Acids' was selected from the start screen to measure RNA. A water sample needed to be loaded by lifting the sampling arm and pipetting 1-2µL of distilled water onto the lower measurement pedestal ensuring that a liquid column forms between the upper and lower pedestals when the sampling arm is down. The sampling arm was lowered and OK was clicked to initialize the instrument. After initialization the sampling arm was opened and the water wiped off from the upper and lower pedestals with a soft tissue. RNA-40 was selected for 'Sample Type'.

Before making a sample measurement, a blank was measured. A blank sample (buffer BR5) was loaded onto the lower measurement pedestal and the 'Blank' button was clicked. After the measurement the blank sample was wiped off firmly with a tissue. An aliquot of the blank was analysed as though it were a sample by reloading 1-2µL, typing 'blank' in 'Sample ID' and then 'Measure'. The result should be a flat spectrum.

Each of the samples was loaded and labelled by filling in the 'Sample ID'. 'Measure' was clicked to get a spectrum. Values for A260, A280, 260/280, 260/230 and concentration (ng/L) appeared on the screen after each sample measurement.

A blank sample was measured ('Measure') after every 6 samples to ensure the pedestal had been cleaned effectively and there was no carryover of sample. These were labelled as 'blank' in the 'Sample ID'. If RNA was present in the 'blank' sample,

the pedestal was re- cleaned and another 'blank' was measured. If decontamination was necessary 0.5% sodium hypochlorite (e.g. a freshly prepared 1:10 dilution of common commercial bleach solution) was used to clean the pedestal to ensure that no biologically active material was present.

After the last measurement, the pedestals was cleaned with 1-2µL distilled water and wiped thoroughly.

2.4.2.5.4 Report formatting

Data were automatically saved in a default user folder after each measurement as 'Nucleic Acid' and the date (as an NDJ file). However the data were easiest to analyse in 'report' form and they were therefore saved in the following way before exiting the programme:

'Show Report', 'Reports' then 'Save Report' was clicked and the 'Full Report' was saved. This was a NDV file which could be loaded back into the software on any computer. The report was opened on a laptop by clicking 'Reports' and then 'Load Report' and browsing for the specific NDV report file.

The report tab listed the samples and included the sample ID, time, date, ng/L, A260, A280, 260/280, 260/230 and measurement type. Choosing 'Configure Report' (found in 'Reports' button on the toolbar) allowed deleting or modifying the columns shown.

The plot tab showed the overlayed spectrum of the samples. Positioning the cursor over 'Legend' brought up a visual display matching the sample name to plot colour. Clicking on a specific line displayed the specific sample information on the right. Up to 20 plots could be seen on one graph.

Transferring data to Excel

To transfer the report into Excel to modify columns or exclude samples, 'Reports' and 'Save Report' was clicked and 'Export Report Table Only' was selected. This saved a text file which could be opened in Excel.

2.4.2.6 Reverse Transcription

(adapted from Protocol *L169 Precision nanoScript Reverse Transcription v2* from Synairgen Research Ltd, Brooke Laboratories, Southampton, UK – Author: Kerry Lunn)

2.4.2.6.1 Principles and Objectives

nanoScript (*Primer Design, Southampton, UK*) is a novel, mutated (multiple point mutations) form of the Moloney Murine Leukaemia Virus (MMLV) enzyme. NanoScript

retains greater activity over a wider range of temperatures than other modifiend MMLV enzymes and has greater thermo-stability. Reactions performed at higher temperatures are faster and increase the total complementary DNA (cDNA) yield. Higher temperature reactions also produce longer transcripts and are more reproducible due to lower levels of secondary structure in the template. The enzyme has an enhanced affinity for primer template complexes enabling efficient transcription of very low concentrations of RNA.

The following describes the preparation of cDNA from total RNA preparations using the nanoScript Reverse Transcription kit (PrimerDesign). The nanoScript reverse transcriptase enzyme is recommended for use with between 2ng and 2µg RNA.

2.4.2.6.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

2.4.2.6.3 Methods

General remarks

Strict precautions (disposable gloves; good microbiological technique; use of sterile DNase/RNase-free disposable plasticware; use of barrier tips; disinfection of working surfaces with RNase away and DNA remover) were taken to prevent RNase contamination.

Reverse transcription

RNA samples were defrosted on ice and centrifuged to ensure pelleting of DNase inactivation agent (1 min, ≥ 9,000rpm, 4°C). Labelled 8 well PCR tube strips were prepared for all samples plus controls. One ‘No RT’ tube was prepared for each subject to control for the presence of any remaining/contaminating genomic DNA and one ‘No RNA’ tube for each RT run.

Mastermix 1 was prepared as below for the number of samples and control tubes plus two extra for pipetting inaccuracies:

Random nonamers (red lid) 1µL/sample

Oligo dT (yellow lid) 1µL/sample

2µL of the mastermix was added to each tube.

1µg of RNA from each sample was added into a tube (up to 12µL of RNA was added; if less than 12µL was added, RNase/DNase-free water was added to each tube to make the volume up to 12µL).

The 'No RNA' control was made by adding 12µL RNase/DNase-free water to the appropriate tube instead of RNA. The 'No RT' controls were made by adding 1µg of an RNA sample that is likely to have expression of the gene(s) of interest. The nanoScript enzyme was then omitted from the 'No RT' tubes at a later stage.

Following the addition of RNA the 8 well strips were capped, pulse spun and heated to 65°C for 5 minutes using the thermocycler. Then, immediately after, they were placed on ice to cool rapidly (> 3 mins).

Mastermix 2 was prepared for the number of samples and 'No RNA' control tubes plus 2 extra.

qScript 10x Buffer	2µL/sample
dNTP	1µL/sample
DTT	2µL/sample
qScript enzyme	1µL/sample

Mastermix 3 was prepeared for the "No-RT contols" samples plus 2 extra.

qScript 10x Buffer	2µL/sample
dNTP	1µL/sample
DTT	2µL/sample
RNAse/DNAse free W	1µL/sample

6µL of the appropriate mastermix was added to each tube. The tubes were capped, vortexed and pulse-spun and incubated at room temperature for 5min. They were then incubated at 55°C for 20min, followed by 75°C for 15min (Thermocycler). Samples were pulse-spun, 180µL of distilled water was added to each sample and samples were mixed. Samples were stored at -20°C for ongoing qPCR work.

2.4.2.7 Preparation of qPCR plates and Real Time PCR Analysis

(adapted from Protocols *L104 Preparing qPCR plates and using the Bio-Rad CFX96* (Author: Lauren Cracknell) and *L156 Real Time PCR Analysis using Bio-Rad CFX Manager* (Author: Chrissy Boxall) from Synairgen Research Ltd, Brooke Laboratories, Southampton, UK)

2.4.2.7.1 Principles and Objectives

qPCR is a highly sensitive method with potential to measure very small amounts of nucleic acid. mRNA expression may be detected for specific genes either relative to designated reference genes or relative to a standard curve.

The following describes how the PCR plates were prepared and the use of the Bio-Rad CFX96.

2.4.2.7.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

2.4.2.7.3 Method

General considerations

The working area was cleaned with RNase away, DNA remover and DNase/RNase-free water. RNase/DNase free consumables and designated PCR pipettes and filter/barrier tips were used. Only water from certified DNase/RNase free sources were used and aliquotted prior to use.

Primer and probes were delivered in the lyophilised form and were reconstituted according to manufacturer's instructions with the required volume of DNase/RNase-free water.

Samples were compared to reference genes ATB5B and CYC1.

Plating qPCR reaction

A plate layout was prepared that included the samples , a negative control (water instead of cDNA template), 'No RT' controls for each subject assayed, and a 'No RNA' control (no template control) for each RT reaction.

The volume of mastermix (MM) required was calculated according to the manufacturer's instructions:

	1 rxn
Primer/probe	1µL
2 X Mastermix	10µL
Water	4µL

In order to keep reagents in the 96-well plate cool but to prevent freezing, the PCR cooler was removed from the -20°C freezer and allowed to warm for about 15min prior to use. All other samples and reagents were kept on ice when not in use.

Once the PCR template (cDNA) had thawed, it was vortexed to mix and then pulse-spun. 15µL of Mastermix was added to each of the wells where samples or controls were to be added using an electronic pipette. 5µL of sample cDNA or controls was then added to the wells as per the plate template. An adhesive seal was then placed on the completed 96 well plate, ensuring that the seal adhered properly without forming bubbles. The sealed 96 well plate was placed in the centrifuge and pulse-spun to ensure that all the reaction mix was at the bottom of each well. The plate was then run immediately on the Bio-Rad CFX96.

Running the plate

The CFX96/C1000 was switched on and the Bio-Rad CFX Manager software was opened on the attached laptop. The 'Create a new Run' button was checked and 'OK' clicked. A 'Run Setup' window opened with the 'Protocol' tab open. The 'Select Existing' button was clicked and the appropriate protocol clicked.

Programme details:

50°C for 15min hold

95°C for 10min hold

40 cycles of: 95°C for 8 sec

60°C for 35 sec

'next' was then clicked to go to the 'Plate' tab and the 'Select Existing' button was clicked.

A preview of the plate map was opened and it was ensured that the 'Plate Type' was 'BR White' and the 'Scan Mode' was 'All Channels'.

Analysing qPCR plates

qPCR plates were analysed by comparing samples to an experimental control once samples had been normalized to two reference genes (relative quantification). CFX Manager Software v2.0 was used.

Analysis of CFX files

The saved data file (Bio-Rad optical file) was opened.

1. Setting the baseline

The baseline is the noise level in early cycles where there is no detectable increase in fluorescence due to amplification products. The baseline was set according to Qiagen guidelines that suggest that the baseline should be set so that the growth of the amplification plot begins at a cycle number greater than the highest baseline cycle number.

As the default setting for the CFX software is 'Baseline Subtracted Curve Fit', this was changed to 'Baseline subtracted'. In many cases the CFX software set the baseline at an acceptable number of cycles but occasionally this had to be adjusted if a sample appeared to 'drift'.

2. Setting the Threshold Value

According to Qiagen guidelines the threshold was adjusted to a value above the background and significantly below the plateau of the amplification plot. It was placed within the linear region of the amplification curve, as close as possible to the base of the exponential phase.

The CFX manager often placed the threshold relatively high and it was therefore lowered manually while viewing the data in the logarithmic amplification plot view to enable easy identification of the log-linear phase of the PCR. The threshold was always kept above the noise of the baseline.

3. Plate Map Setup

The plate map was annotated with sample type (i.e. UNK (unkown), POS (positive control), Neg (negative control), NTC (no template control), NRT (no RT control). A target name (name of gene) was allocated to each fluorophore.

One gene was run per plate.

4. Negative controls

All negative controls were checked before the plate was fully analysed. A PCR plate was deemed acceptable if there was > 5 Cq separation between the samples and the No Template Controls and the samples and all of the negative controls. Negative control had to give a Cq value of > 35 and no sample with a higher Cq then a negative control was included in the analysis.

5. Exclusion criteria

The following standard exclusion criteria for duplicates were applied:

- Samples were excluded if there was >1Cq separation between replicates
- Samples were excluded if one of the replicates produced a poor amplification trace and the other replicate produced a nicely amplified trace with a Cq above the LOQ
- Samples were excluded if a known accidental error was made whilst preparing the PCR plate

If samples were excluded based on any of the above criteria, the PCR for these samples was repeated once.

2.4.2.8 Nasal Lavage Biomarker Measurement using Fluorokine® Multianalyte Profiling (MAP) and Luminex Technology

2.4.2.8.1 Principles and Objectives

Fluorokine MAP bead-based kits for the Luminex platform are highly efficient multiplex assays that have been validated for the quantification of factors in complex matrices. They are able to simultaneously detect multiple analytes and only require small sample volume (< 50 µL).

Analyte-specific antibodies are pre-coated onto color-coded microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. A final wash removes unbound Streptavidin-PE and the microparticles are resuspended in buffer and read using a Luminex analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound (*adapted from Package insert of: Luminex® Performance Assay, R&D Systems Inc*.).

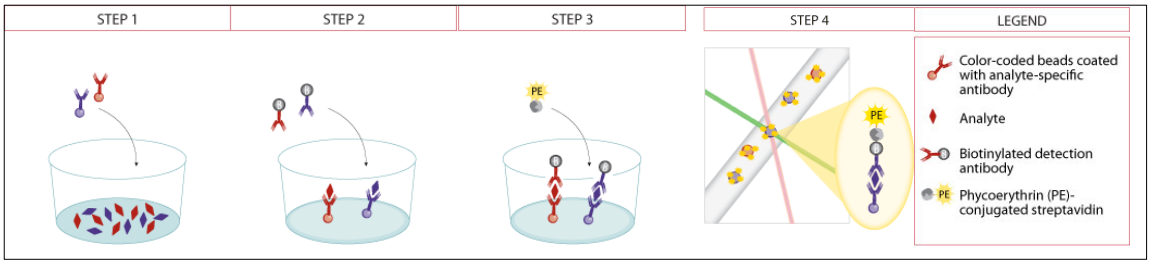


Figure 2.3 Luminex® Bead-based Multiplex Assay Principle.
Reproduced from website:
http://www.rndsystems.com/product_detail_objectname_MultiplexAssayPrinciple.aspx

2.4.2.8.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

Luminex Performance Assay analyte-specific kit:

(1) Human Cytokine Base Kit A – R&D Systems #LUH000

Analyte	Sensitivity (pg/mL)	Analyte	Sensitivity (pg/mL)
CCL2/MCP-1	0.47	**IL–1ra/IL–1F3**	10.91
CCL3/MIP-1α	1.45	**IL–1β/IL–1F2**	0.57
CCL4/MIP-1β	0.72	**IL–2**	2.23
CCL5/RANTES	1.91	**IL–4**	4.46
CXCL8/IL–8	1.97	**IL–5**	0.71
CXCL5/ENA-78	4.14	**IL–6**	1.11
FGF basic	4.91	**IL–10**	0.30
G-CSF	1.48	**IL–17**	1.10
GM-CSF	1.98	**TNF-α**	1.50
IFN-γ	1.27	**Tpo***	9.94
IL–1α/IL–1F1	0.36	**VEGF**	1.84

Table 2.10 Cytokines included in Human Cytokine Base Kit A (R&D Systems)

* included in kit but not analysed in this study

(2) Human MMP Base Kit – R&D Systems

Analyte	alternative enzyme name	Sensitivity (pg/mL)	Analyte	alternative enzyme name	Sensitivity (pg/mL)
EMMPRIN*		5.6	**MMP-8**	Collagenase 2	16.6
MMP-1	Collagenase 1	1.1	**MMP-9**	Gelatinase B	13.7
MMP-2	Gelatinase A	12.6	**MMP-10***		3.2
MMP-3	Stromelysin 1	7.3	**MMP-12**	Macrophage Metalloelastase	0.7
MMP-7	Matrilysin	6.6	**MMP-13**	Collagenase 3	63.5

Table 2.11 Human matrix metalloproteinases (MMPs) included in Human MMP Base Kit (R&D Systems)

* included in kit but not analysed in this study

2.4.2.8.3 Methods

General principles of Multianalyte assay:

All reagents and samples were brought to room temperature before use and all samples and standards were assayed in duplicates. All reagents, working standards, and samples were prepared as per manual for Luminex® Performance Assay, R&D Systems Inc.

Detailed method of Multianalyte assay:

The filter-bottomed microplate was pre-wet with 100µL of Wash Buffer and the liquid was removed through the filter at the bottom of the plate using a vacuum manifold.

The diluted microparticle mixture was resuspended by vortexing and 50µL of the microparticle mixture added to each well of the microplate.

50µL of Standard or sample (nasal lavage) was added per well. The microplate was then covered with a foil plate sealer and incubated for 3 hours at room temperature on a horizontal orbital shaker at 500rpm.

Using a vacuum manifold, the microplate was washed by removing the liquid, filling each well with 100µL of Wash Buffer and removing the liquid again ensuring that all liquid was removed to avoid any loss of microparticles. The wash procedure was performed three times.

50µL of diluted Biotin Antibody Cocktail was added to each well. The microplate was then covered with a foil plate sealer and incubated for 1 hour at room temperature on a horizontal orbital shaker at 500rpm.

Using a vacuum manifold, the microplate was washed by removing the liquid, filling each well with 100µL of Wash Buffer and removing the liquid again ensuring that all liquid was removed to avoid anly loss of microparticles. The wash procedure was performed three times.

50µL of diluted Streptavidin-PE was added to each well. The microplate was then covered with a foil plate sealer and incubated for 30 minutes at room temperature on a horizontal orbital shaker set at 500rpm.

Using a vacuum manifold, the microplate was washed by removing the liquid, filling each well with 100µL of Wash Buffer and removing the liquid again ensuring that all

liquid was removed to avoid anly loss of microparticles. The wash procedure was performed three times.

The microparticles were resuspended by adding 100µL of Was Buffer to each well. The microplate was then incubated for 2 minutes on the shaker set at 500rpm.

The plate was then read within 90 minutes using a Luminex 200 analyzer.

2.4.2.9 IP-10 ELISA using using R&D Systems Quantikine® ELISA Human IP-10

2.4.2.9.1 Principles

"This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IP-10 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IP-10 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IP-10 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IP-10 bound in the initial step. The color development is stopped and the intensity of the color is measured." (from: R&D Systems Quantikine® ELISA Human CXCL10/IP-10 Manual)

2.4.2.9.2 Materials

Equipment: see Appendix N

Consumables: see Appendix N

Reagents: see Appendix N

Solution preparation

Wash buffer - 0.05% Tween 20 in PBS

Reagent Diluent - 1% BSA in PBS, 0.2 um filtered

Substrate Solution - 1:1 mixture of color reagent A and B (R&D Systems # DY999)

Stop Solution - R&D Systems # DY994

2.4.2.9.3 Methods

Procedure (theR&D Systems Quantikine® ELISA Human CXCL10/IP-10 Manual was followed)

1. All reagents, working standards, and samples were prepared as directed in the previous sections of the manual.

2. Excess microplate strips were removed from the plate frame and returned to the foil pouch containing the desiccant pack

3. 150 µL of Assay Diluent RD1-56 was added to each well.

4. 100 µL of Standard, control, or sample was added per well and covered with the adhesive strip provided and incubated for 2 hours at room temperature.

5. Each well was aspirated and washed, and the process was repeated three times for a total of four washes.

Washing was performed by filling each well with Wash Buffer (400 µL) using a manifold dispenser. Complete removal of liquid at each step was ensured. After the last wash, any remaining Wash Buffer was removed by decanting The plate was inverted and blotted against clean paper towelling.

6. 200 µL of IP-10 Conjugate was added to each well, covered with a new adhesive strip and incubated for 2 hours at room temperature.

7. The aspiration/wash was repeated as in step 5.

8. 200 µL of Substrate Solution was added to each well and incubated for 30 minutes at room temperature while ensuring protection from light (aluminium foil).

9. 50 µL of Stop Solution was added to each well.

10. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm with wavelength correction set to 570 nm.

Fifty-seven assays were evaluated by the manufacturer and the minimum detectable dose (MDD) of IP-10 ranged from 0.41-4.46 pg/mL. The mean MDD was 1.67 pg/mL.

2.5 Statistical analysis

2.5.1 Characterization of viral induced asthma exacerbations

2.5.1.1 Description of general and asthma specific information

Data were presented as mean (standard deviation (SD)), median (interquartile range (IQR)) and frequency (percentage), unless stated otherwise. Normality of the quantitative (numeric) data distributions was tested by the Shapiro-Wilk test.

2.5.1.2 Frequency of viral induced common cold symptoms and frequency and severity of asthma exacerbations

Participants were monitored and cold episodes documented for each participant comparing report of increased cold symptoms with clinically confirmed colds. Frequency of cold symptoms was calculated in relation to number of children monitored for colds each month.

2.5.1.3 Identification of Jackson Cold Score that best identifies a cold

The Area under the Curve and the peak Jackson Cold Score as well as the peak of the 48 hour sum of the JCS were calculated for the baseline and the cold episode of each participant. For validation purposes virus positivity was taken as the factor that determined a “true cold”. Sensitivity, specificity, positive and negative likelihood ratios, and positive and negative predictive values were calculated and data presented as percentages (95% confidence interval (CI)) or ratios (95%CI). In addition a Receiver Operating Characteristics (ROC) curve analysis was performed to evaluate the test with the aim to determine a cut-off that best distinguishes between a cold and no cold.

2.5.1.4 Validation of original and modified asthma index

In a 3-step approach the original asthma index was validated, the modified with the original asthma index compared, and subsequently the modified asthma index validated: The original asthma index was validated using clinical data from this study and the classification of severity of asthma exacerbations according to Reddel *et. al.* and the data presented as column scatter plots. Analysis of variance (ANOVA) was performed. If p-value suggested a significant difference between the three groups post hoc 2 sample t-test analysis with Bonferroni correction for multiple testing was performed and p-values were presented. The modified asthma index was compared with the published asthma index using Bland Altman Plots showing mean difference and 95% limits of agreement. In a final step the modified asthma index was validated using clinical data from this study and the classification of severity of asthma

exacerbations according to Reddel *et al*. ANOVA and post-hoc 2 sample t-test analysis with Bonferroni correction was performed as above.

2.5.2 Text based monitoring

2.5.2.1 Assessment of compliance

Compliance with the SMS data collection system was assessed using the proportion of sent text messages (SMS 3; see Table 4.1) to the number expected ('SMS-diary-compliance'). The proportion of digitally stored PEFRs to the number expected was calculated to determine the compliance to the PEFR-monitoring ('PEFR-meter-compliance'). Results are reported as percentage (standard deviation).

2.5.2.2 Assessment of validity

Participating children and their parents were informed that the digital peak flow meters stored the measurements of peak flows and these would be downloaded at a later stage. Parents were asked to score asthma related symptoms and to send these together with the PEFR (measured by the digital peak flow meter) by text message system (SMS 3; see Table 4.1) twice daily during the baseline period and cold episode (one only where more than one was monitored). The PEFR data sent by SMS were compared with the PEFR data stored on the digital peak flow meter (both types of data were time-tagged). Data points were split into the following 5 categories: (1) PEFR sent/PEFR measured/corresponding ('correct'); (2) PEFR sent/PEFR measured/not corresponding ('incorrect'); (3) PEFR sent/PEFR not measured ('self-invented'); (4) PEFR not sent/PEFR measured ('not sent'); (5) PEFR not sent/PEFR not measured ('missing'). Median percentages (25^{th} and 75^{th} percentile) are presented. Additionally, the median difference between paired SMS and digital peak flow meter PEFR measurements were calculated for individual participants and representative Bland-Altman plots generated.

2.5.2.3 Assessment of acceptability

A total of 24 parents received a baseline questionnaire (see appendix D.) focused on their everyday use of mobile phones, SMS messaging, and their feeling about using text messages in the context of a research study. All parents received a text message information sheet (see appendix C.) explaining the use of SMS in this research study. This also included a trouble-shooting guide and contact details for the research team. A follow up questionnaire (see appendix E.) at the end of the study more specifically asked about the parents' experience with the text message system, the reason why they might not have replied, and whether they would be happy if this was used in a

future study where they (or their child) might take part. Both questionnaires allowed for free comments in addition to either yes-no or Likert scale questions.

2.5.3 Viruses and asthma

To describe the infecting viruses in a cohort of children with asthma with regards to frequency of occurrence, type, and seasonality (objective i), the frequency and type of infecting viruses in this cohort and how these vary with season was tabulated. For each of the baseline and symptomatic periods the frequencies and types of viral infections in the warmer (April-September) and cooler (October to March) months were compared using Chi square test.

To establish the incidence of multiple infections with two or more respiratory pathogens (objective ii), the number of isolated viruses was compared during baseline and follow up as for objective i.

To describe the detection rates of viruses in saliva in comparison to nasal lavage (gold standard) during cold episodes (objective iii), cross tabulation with kappa analysis was performed to assess the degree of concordance.

To explore the potential association of virus status with different clinical syndromes (asymptomatic, cold only, cold + asthma), with AUC and peak of Asthma Index and Jackson cold score, and with symptoms/signs as reported by parents (objective iv), analysis of variance (ANOVA) was performed.

2.5.4 Cytokines and Asthma Exacerbations

Cytokine levels were not normally distributed (even after log transformation), therefore non-parametric tests were used for statistical analysis.

To characterize fluctuations in nasal lavage cytokines and matrix metalloproteinases between baseline (asymptomatic) periods and two visits (V1 – within 48 hours of cold symptoms developing; V2 – on day 4-6 of cold) during a naturally occurring respiratory viral infections in children with asthma (objective i) Friedman test was performed. Pairwise comparison (Wilcoxon Signed Rank test) was performed if Friedman test showed overall statistically significant difference with adjustment of significance levels in consideration of multiple comparisons.

Pairwise comparison was performed in two ways: (1) inclusion of only complete sample groups (i.e. valid results available for baseline visit, V1 and V2) and (2) inclusion of all paired samples (i.e. baseline visit compared with V1 even if V2 sample (result) not available).

To explore the potential correlation between levels of nasal lavage cytokines / MMPs and severity of viral induced asthma exacerbation (objective ii) Kruskal Wallis test was performed.

To explore the potential correlation between levels of nasal lavage cytokines / MMPs and virus status (positive/negative) during baseline and cold episodes (objective iii) Mann-Whitney U test was performed.

To determine the relationship between upper airway inflammatory biomarkers (cytokines) and cold and asthma scores (objective iv) Spearman's Rank Correlation was performed.

2.6 Ethics

2.6.1 Ethical approval

The study was approved by the Southampton & South West Hampshire Research Ethics Committee B (Ref. 10/H0504/52). See approval document Appendix G.

2.6.2 Substantial amendments

The following substantial amendments were submitted between December 2010 and October 2011:

2.6.2.1 Amendment 1

Despite being part of the original protocol and mentioned in the consent form, we neglected to mention in both the child and parent PISs that a nasal washing will also be collected at the first visit. In addition we neglected to include details of the saliva sample collection in both the PIS and consent form. The necessary amendments have been made to the child and parent PISs and also the consent and assent forms. In addition we have made the following clarifications: "A teaspoon amount (5 ml) of salty water will be gently squirted up your child's nose with a small syringe and will be collected as it runs back out. This feels a bit like having a runny nose".

Rationale: This change was necessary in order to perform both nasal washing and saliva sampling with participants and parents being fully informed and having consented for all procedures.

2.6.2.2 Amendment 2

We have realised that a written information sheet to participants (and their parents) about the text messaging system we will use would be useful. This sheet will give

information and advice about what texts they will receive and how to respond as well as a trouble-shooting guide.

Rationale: The written information sheet proved very useful as it contained all the necessary information and contact details if problems occurred with the text messaging system.

2.6.2.3 Amendment 3

Our protocol states that we were aiming to recruit twenty children. Since we started enrolling children into our study we have seen considerable differences in cold and asthma symptoms between children. There is a very real possibility that a small group of only 20 children will not provide sufficient numbers in each of these subgroups to allow the data to be interpreted. This would significantly compromise the value of the data. Therefore we are submitting an amended protocol to increase the size of the group to 40 children.

Rationale: Following the change in protocol the number of patients recruited into the observational study could be increased.

2.6.2.4 Amendment 4

The specific exclusion criteria in our protocol (Version 1) state:

- Inability to perform lung function or FeNO measurement

The measurement of FeNO does not provide any information from an efficacy and safety point of view and we therefore have removed “the inability to do FeNO measurements” as an exclusion criterion and changed this sentence in the corresponding paragraph of the protocol to:

- Inability to perform lung function measurements

Rationale: Measurement of FeNO proved to be challenging especially for younger participants who struggled with the technique. As it would have been an exclusion criterion without the result contributing majorly to the study it was beneficial to take FeNO out of the list of exclusion criteria.

2.6.2.5 Amendment 5

(1) In our current protocol children are being monitored until they have recovered from their first cold and then are only followed up with monthly text messages until the end of the study (maximum 6 months).

Proposed change: We would like to monitor children with asthma until they have developed two distinct colds, up to a maximum of 10 months of monitoring for colds.

Rationale: In our experience so far, most colds have not been associated with a signficant exacerbation of asthma. Our research interest is to look at the relationship between colds and asthma exacerbations and so there is a potential that we may not have sufficient exacerbation events in our final data set. By following up participants through two colds, we increase our chance to monitor them during at least one cold episode that leads to increased asthma symptoms. This will provide better data to ensure that a future interventional trial doses participants when they are most at risk of developing an asthma exacerbation.

In our experience to date, participants and their families have been very happy to be followed up by the daily text messages during colds. Our informal conversations with them would suggest that they will be happy for follow up to be extended to include two colds.

(2) In our current protocol children are only seen once during their cold (home visit within first 48 hours) with lung function being performed and nasal lavages, saliva and blood samples being collected.

Proposed change: We want to conduct 2 home visits with each cold (one on day 1 and one between day 3 and 6) and repeat lung function measurements as well as sampling of nasal lavage, saliva and blood (blood sample only with the day 1 visit so we would only be increasing the total number of blood samples in the study from 2 to 3 and participants would continue to have the option of not providing a sample).

Rationale: As few participants are having a cold induced exacerbation of their asthma, the home visit with the second cold, will increase the chance that we are able to collect samples from participants during the start of a viral induced exacerbation of asthma. If few colds are leading to an exacerbation of asthma, we need to find a system for predicting who will exacerbate. This may involve waiting until 3-6 days into the cold when patients typically start to develop some lower respiratory tract symptoms. To document this, we propose a second home visit at day 3-6 of the cold with repeated investigations (but not a further blood test).

In our experience to date with the home visits, participants and their families have been very happy to be assessed at home in these circumstances. Additionally the nasal washing, saliva and blood sampling have been extremely well tolerated. We do have two participants (of currently 17) who have declined the blood tests but all have been

happy with the nasal washings and saliva sampling. We would continue with our policy of giving participants the choice as to whether or not they provide blood samples.

3 Characterization of viral induced asthma exacerbations in children

3.1 Introduction

Asthma is a very common respiratory condition characterised clinically by chest tightness, shortness of breath, wheeze and cough, physiologically by airway hyper-responsiveness and decreased lung function. Acute exacerbations of asthma constitute one of the leading causes of hospital admission in children in the United Kingdom. The chronicity of the disease and the unpredictability of acute deteriorations particularly when triggered by a respiratory viral infection, often put a big burden on the child and the family and impact their quality of life. The aims of the clinician, in collaboration with the patient and other allied health professionals, are to achieve best-possible asthma control and to prevent acute exacerbations, goals that have increasingly been recognized and targeted in asthma management but also as primary endpoints in clinical trials involving patients with asthma.

3.2 Specific objectives

The specific objectives of characterizing viral induced asthma exacerbations in young school children were to:

i. explore the general (birth, atopy and social factors) and asthma specific (triggers, medication, exacerbations) features of participating children

ii. document frequency of viral induced common cold symptoms and frequency and severity of asthma exacerbations

iii. identify the Jackson Cold Score (Jackson et al., 1958) which best identifies a cold

iv. validate the Asthma Index (Sorkness et al., 2008) and compare original and modified versions

3.3 Results

3.3.1 Screening and recruitment

Between January 2011 and February 2012 32 children between 5 and 11 years of age were recruited to participate in this study. Figure 3.1 shows the clinical areas where children and their parents were approached to participate and the subsequent recruitment figures. Children were recruited from the paediatric outpatient clinics at Southampton General Hospital, the paediatric assessment unit, the emergency department and via community asthma nurses. Advertising with posters and leaflets was also performed in a number of local GP practices (via PCRN – primary care research network) and in a nearby district general hospital (Winchester). Figure 3.2 is a flow diagram showing the number of children screened and enrolled in the study and the result of their participation (i.e. developed 1 or 2 colds, withdrawn from study, or no reported colds).

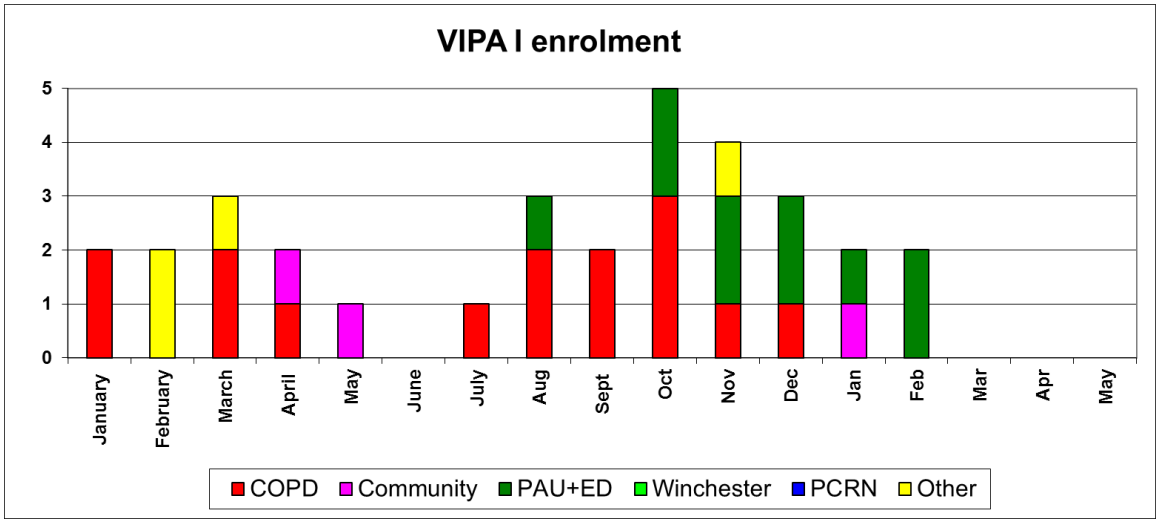


Figure 3.1 VIPA – viral induced paediatric asthma study – enrolment figures (Jan 2011 - May 2012)

COPD – children's outpatient department; PAU – paediatric assessment unit; ED – emergency department; PCRN – primary care research network.

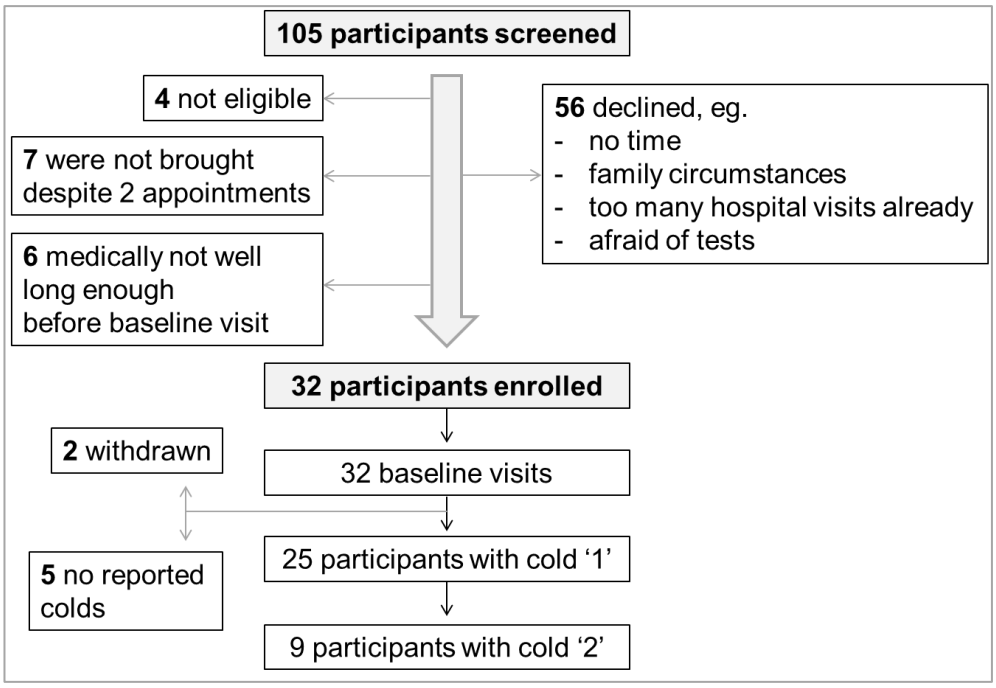


Figure 3.2 Flow sheet of all participants screened for participation in study

	All (N=32)	no colds (N=7)	1 cold (N=16)	2 colds (N=9)
Age, years	7.7 (1.78)	7.7 (1.89)	7.8 (1.74)	7.5 (1.96)
Sex, male(%)/female(%)	18(56)/14(44)	5(71)/2(29)	9(56)/7(44)	4(44)/5(56)
Treatment, n (%)				
- **inhaled corticosteroid**	32 (100)	7 (100)	16 (100)	9 (100)
- **LABA**	11 (34)	1 (14)	8 (50)	2 (22)
- **Montelukast**	15 (47)	4 (57)	9 (56)	2 (22)
steroid dose (µg)*	250 (200, 500)	400 (287, 700)	400 (200, 500)	200 (100, 200)
%FEV1 - pre	94.76 (15.87)	95.25 (13.39)	87.84 (15.51)	106.68 (11.58)
%FEV1 - post	104.42 (11.80)	106.94 (11.77)	97.96 (9.95)	112.50 (9.39)
%reversibility*	7.26 (2.19, 18.01)	13.61 (6.93, 18.40)	9.81 (5.41, 23.59)	2.23 (1.6, 5.6)

Table 3.1 Description of participants.
Numbers are means (SD) or total numbers (percentages) except those marked with asterix (*) which are medians (25th, 75th percentile). LABA – Long acting beta-agonist.

Table 3.1 summarises age, sex, treatment, %FEV1 and bronchodilator response of the participating children. Tables 3.2 and 3.3 expand on key asthma features of study participants and general demographic and health data of the participants respectively (objective i.). The tables are split into four result columns, column 1 showing all participants, column 2 (group 1) participants who either did not develop a cold (n=5) or withdrew from the study after a period of 3-6 months without having developed a cold (n=2), column 3 (group 2) participants who were monitored during one cold episode and column 4 (group 3) participants who had 2 colds during their observation period. Despite the division into participants with no, one cold or two colds in this tables it is important to note that this division is not necessarily based on an inherent increased frequency of colds in the last group but rather reflects the fact that some participants joined the study at a later time point when approval for the monitoring of 2 cold episodes was in place. Some participants who joined the study at the beginning (before approval for monitoring of two cold episodes was in place) developed a cold within the first 3 months and monitoring for further colds was then only performed by monthly retrospective SMS. Furthermore some participants were enrolled in the last 3-4 months of the study and may have also developed only one cold before the study was ended. A true qualitative and quantitative difference between the two groups would only have been possible if all participants had been enrolled at the same time point to rule out the bias introduced by the duration of monitoring and the month/season the participant was enrolled. Therefore differences between the groups (e.g. numbers of children on montelukast) are unlikely to be due to biological differences.

Keeping this in mind there is a slightly higher number of female participants in group 3 with 56% versus 44% in group 2 and 29% in the group with no monitored colds. Whereas weather conditions were identified as a trigger for asthma exacerbations in all participants of groups 1 and 2, only 78% of group 3 regarded it as a relevant trigger. Pollen and cigarette smoke on the other hand were noted as triggers in all (smoke) or all but one participant (pollen) who were monitored during 2 cold episodes (group 3) while only 44% of those in group 2 indicated these 2 factors as triggers for exacerbations. Only 29% of the group that did not develop a cold episode regarded cigarette smoke as relevant but 100% mentioned pollen as a potential trigger. Interestingly, 43% of participants in group 1 and 63% of participants in group 2 had at least one family member who smoked, while none of group 3 was exposed to smoking in their home environment.

Children with a background history of hayfever were more common in groups 1 (100%) and 3 (78%) compared to group 2 (44%) and also paternal atopy was more prevalent.

Participants in group 3 were more likely to be on lower doses (100-200 µg) of preventer inhalers (78%) than participants in group 1 (15%) and group 2 (31%). Groups 1 and 2 were also two-and-a-half times as likely on montelukast as an additional medication compared to group 3. About half of the children in group 2 had bronchiolitis and chest infections in infancy, while this was the case in only less than a quarter of children in group 3.

The group of children that did not report a cold during their monitoring period interestingly reported a higher number of exacerbations due to cold/flu in the previous year (median 4 versus 2 in the other 2 groups) at their baseline visit.

	All participants (N=32)	Participants with no cold (Group1, N=7)	Participants with 1 cold (Group 2, N=16)	Participants with 2 colds (Group 3, N=9)
Age (range) at time of enrolment (mean, years)	7.7 (5.3 – 11.7)	7.7 (5.5 – 10.3)	7.8 (5.3 – 11.5)	7.5 (5.6 – 11.7)
Gender (male/female)	18/14	5/2	9/7	4/5
Age at asthma diagnosis (years)*	2 (1-5)	2 (1-4)	1 (1-4)	2 (1-5)
Age when reliever inhaler first prescribed (years)*	2 (1-5)	2 (1-4)	2 (1-4)	2 (1-5)
Age when preventer inhaler first prescribed (years)*	3 (1-9)	3 (1-9)	3 (1-5)	3 (2-8)
Time when asthma worse, %(n/N)				
- *Dec – Feb*	40.6 (13/32)	28.6 (2/7)	62.5 (10/16)	11.1 (1/9)
- *Mar – May*	18.8 (6/32)	28.6 (2/7)	12.5 (2/16)	22.2 (2/9)
- *Jun – Aug*	15.6 (5/32)	28.6 (2/7)	6.3 (1/16)	22.2 (2/9)
- *Sep – Nov*	56.3 (18/32)	28.6 (2/7)	75 (12/16)	44.4 (4/9)
- *No specific month*	34.4 (11/32)	71.4 (5/7)	18.8 (3/16)	33.3 (3/9)
Asthma –triggers, % (n/N)				
- *Weather*	93.8 (30/32)	100 (7/7)	100 (16/16)	77.8 (7/9)
- *Pollen*	68.8 (22/32)	**100 (7/7)**	**43.8 (7/16)**	**88.9 (8/9)**
- *Emotions*	40.6 (13/32)	57.1 (4/7)	37.5 (6/16)	33.3 (3/9)
- *Fumes*	31.3 (10/32)	28.6 (2/7)	37.5 (6/16)	22.2 (2/9)
- *Dust*	68.8 (22/32)	71.4 (5/7)	62.5 (10/16)	77.8 (7/9)
- *Pets*	43.8 (14/32)	71.4 (5/7)	31.3 (5/16)	44.4 (4/9)
- *Cold/flu*	100 (32/32)	100 (7/7)	100 (16/16)	100 (9/9)
- *Cigarette smoke*	56.3 (18/32)	**28.6 (2/7)**	**43.8 (7/16)**	**100 (9/9)**
- *Foods/drinks*	21.9 (7/32)	14.3 (1/7)	18.8 (3/16)	33.3 (3/9)
- *Soaps/sprays*	21.9 (7/32)	14.3 (1/7)	25.0 (4/16)	22.2 (2/9)
- *Exercise*	75.0 (24/32)	71.4 (5/7)	81.3 (13/16)	66.7 (6/9)
Preventer medication, % (n/N)				
- Beclomethasone	59.4 (19/32)	71.4 (5/7)	43.8 (7/16)	77.8 (7/9)
- Fluticasone	31.3 (10/32)	28.6 (2/7)	37.5 (6/16)	22.2 (2/9)
- Budesonide	9.4 (3/32)	0	18.8 (3/16)	0
Preventer daily dose, % (n/N)				
- *100 – 200µg*	40.6 (13/32)	**14.7 (1/7)**	**31.3 (5/16)**	**77.8 (7/9)**
- *250 – 400µg*	28.2 (9/32)	42.8 (3/7)	31.3 (5/16)	11.1 (1/9)
- *500 – 800µg*	21.9 (7/32)	14.3 (1/7)	31.3 (5/16)	11.1 (1/9)
- *1000µg*	6.3 (2/32)	14.3 (1/7)	6.3 (1/16)	0
- *Unknown*	3.1 (1/32)	14.3 (1/7)	0	0
Missing preventer doses, % (n/N)				
- *Never*	21.9 (7/32)	14.3 (1/7)	25 (4/16)	22.2 (2/9)
- *Occasionally*	56.3 (18/32)	71.4 (5/7)	56.3 (9/16)	44.4 (4/9)
- *Once a week*	18.8 (6/32)	14.3 (1/7)	12.5 (2/16)	33.3 (3/9)
- *Half the time*	3.1 (1/32)	0	6.3 (1/16)	0
- *Most of the time*	0	0	0	0
Other medication, % (n/N)				
- *Seretide*	21.9 (7/32)	14.3 (1/7)	25 (4/16)	22.2 (2/9)
- *Symbicort*	3.1 (1/32)	0	6.3 (1/16)	0
- *Salmeterol*	9.4 (3/32)	0	18.8 (3/16)	0
- *Montelukast*	46.9 (15/32)	**57.1 (4/7)**	**56.3 (9/16)**	**22.2 (2/9)**
Exacerbations /year*	5 (1-15)	5 (1-10)	4 (2-10)	6 (2-15)
Exacerbations due to cold/flu*	2.5 (0-9)	4 (0-9)	2 (2-8)	2 (2-6)
Courses of steroids / year*	1.5 (0-10)	1 (0-10)	2 (0-6)	1 (0-6)
Missed school days / year*	5 (0-30)	5 (0-30)	5 (1-28)	5 (0-30)
Hospital admissions total*	2.5 (0-30)	2 (0-30)	4.5 (0-20)	1 (0-8)
Hospital admission last year*	0 (0-6)	0.5 (0-6)	0 (0-3)	0 (0-2)

Table 3.2 Key asthma features of study participants Numbers are percentages (n/N) or means (SD) except those marked with asterix (*) which are median (range)

	All participants (N=32)	Participants with no cold (N=7)	Participants with 1 cold (N=16)	Participants with 2 colds (N=9)
Birth weight (kg)*	3.69 (1.47-4.34)	3.40 (3.09 –4.34)	3.28 (1.47-4.30)	3.77 (3.40–4.17)
Gestation at birth*	40 (33-42)	40 (37-42)	40 (33-42)	40 (37 – 42)
Mode of delivery, % (n/N)				
- Normal vaginal	59.4 (19/32)	28.6 (2/7)	62.5 (10/16)	77.8 (7/9)
- Instrumental	9.4 (3/32)	14.3 (1/7)	12.5 (2/16)	0
- Caesarean	31.3 (10/32)	57.1 (4/7)	25.0 (4/16)	22.2 (2/9)
Any period of breastfeeding, % (n/N)	65.6 (21/32)	57.1 (4/7)	62.5 (10/16)	77.8 (7/9)
Bronchiolitis in infancy, % (n/N)	43.8 (14/32)	**42.9 (3/7)**	**56.3 (9/16)**	**22.2 (2/9)**
Chest infections in infancy, % (n/N)	40.6 (13/32)	28.6 (2/7)	50.0 (8/16)	33.3 (3/9)
Eczema, % (n/N)	75.0 (24/32)	100 (7/7)	68.6 (11/16)	66.7 (6/9)
Hayfever, % (n/N)	65.6 (21/32)	**100 (7/7)**	**43.8 (7/16)**	**77.8 (7/9)**
Food allergies, % (n/N)	50.0 (16/32)	28.6 (2/7)	50.0 (8/16)	66.7 (6/9)
Allergic to animals	53.1 (17/32)	85.7 (6/7)	37.5 (6/16)	55.6 (5/9)
- *Cat*	*43.8 (14/32)*	*57.1 (4/7)*	*31.3 (5/16)*	*55.6 (5/9)*
- *Dog*	*28.1 (9/32)*	*28.6 (2/7)*	*31.3 (5/16)*	*22.2 (2/9)*
- *Other*	*25.0 (8/25)*	*57.1 (4/7)*	*6.3 (1/16)*	*33.3 (3/9)*
Maternal atopy				
- Asthma	34.4 (11/32)	71.4 (5/7)	25.0 (4/16)	22.2 (2/9)
- Eczema	15.6 (5/32)	28.6 (2/7)	12.5 (2/16)	11.1 (1/9)
- Hayfever	53.1 (17/32)	85.7 (6/7)	37.5 (6/16)	55.6 (5/9)
- Food allergy	3.2 (1/31)	0	0	11.1 (1/9)
Paternal atopy				
- Asthma	37.5 (12/32)	42.9 (3/7)	25.0 (4/16)	55.6 (5/9)
- Eczema	19.4 (6/31)	28.6 (2/7)	13.3 (2/15)	22.2 (2/9)
- Hayfever	41.9 (13/31)	28.6 (2/7)	33.3 (5/15)	66.7 (6/9)
- Food allergy	3.2 (1/31)	0	6.7 (1/15)	0
Sibling with atopy				
- Asthma	29.6 (8/27)	20.0 (1/5)	30.8 (4/13)	33.3 (3/9)
- Eczema	29.6 (8/27)	60.0 (3/5)	15.4 (2/13)	33.3 (3/9)
- Hayfever	25.9 (7/27)	20.0 (1/5)	30.8 (4/13)	22.2 (2/9)
- Food allergy	3.7 (1/27)	0	0	11.1 (1/9)
Smoking				
- Smoker in family	40.6 (13/32)	**42.9 (3/7)**	**62.5 (10/16)**	**0**
- Smoking in house	15.6 (5/32)	28.6 (2/7)	18.8 (3/16)	0
Housing				
- Dampness on walls	28.1 (9/32)	57.1 (4/7)	25.0 (4/16)	11.1 (1/9)
- Mould on walls	28.1 (9/32)	42.9 (3/7)	25.0 (4/16)	22.2 (2/9)
- Dust impermeable mattresses	50.0 (16/32)	71.4 (5/7)	43.8 (7/16)	44.4 (4/9)
Animal contact – cats				
- None	40.6 (13/32)	28.6 (2/7)	50.0 (8/16)	33.3 (3/9)
- Not in house	31.3 (10/32)	42.9 (3/7)	18.8 (3/16)	44.4 (4/9)
- Including in house	21.9 (7/32)	28.6 (2/7)	25.0 (4/16)	11.1 (1/9)
- Including bedroom	6.3 (2/32)	0	6.3 (1/16)	11.1 (1/9)
Animal contact – dogs				
- None	18.9 (6/32)	0	18.8 (3/16)	33.3 (3/9)
- Not in house	43.8 (14/32)	57.1 (4/7)	43.8 (7/16)	33.3 (3/9)
- Including in house	31.3 (10/32)	42.9 (3/7)	25.0 (4/16)	33.3 (3/9)
- Including bedroom	6.3 (2/32)	0	12.5 (2/16)	0

Table 3.3 General information about study participants Numbers are percentages (n/N) or means (SD) except those marked with asterix (*) which are median (range).

3.3.2 Days of monitoring, days to cold episodes

Participating children were initially consented to be enrolled in the study for a maximum of 6 months and covering one cold episode (± exacerbation) only. Several months into the study, following ethical approval and re-consenting of parents and children, the monitoring period of children with asthma was extended until two distinct colds had developed or to a maximum of 10 months of daily monitoring (and monthly follow ups following two cold episodes for a total of 12 months from the time point of enrolment into the study). The rationale behind this change was that the majority of reported colds to that point had not been associated with an exacerbation of asthma. As the principle aim of the study was to examine the relationship between colds and asthma exacerbations, it was felt that an extended period of monitoring was necessary in order to maximise the possibility of studying sufficient exacerbation events. By following up participants through two colds, the aim was to increase the chance to monitor them during at least one cold episode that would lead to increased asthma symptoms. At the time of this protocol change 9 participating children had already either completed the study having been monitored during one cold episode (n=6), had completed the study without having developed a cold during 6 months of monitoring (n=2), or had withdrawn from the study (n=1).

Table 3.4 gives an overview of the number of days of daily monitoring, number of days of total enrolment in study and number of days of monitoring till first cold. The maximum time of daily monitoring/monitoring including monthly follow ups exceeds the stated time periods of monitoring as one participant was not actively monitored for a time period of 52 days until ethical approval for extension was in place and participant re-consented. The range reflects the total number of days from initial start of enrolment till last monthly follow up text message.

	Mean (Std. Deviation)	Median (Range)
Number of days to first cold (n=25)	70 (± 64)	52 (6 – 286)
Number of days between cold 1 and cold 2 (n=9)	61 (± 26)	61 (26 – 84)
Number of days of daily monitoring (n=32)	139 (± 81)	136 (26 – 352)
Number of days of monitoring including monthly follow ups	177 (± 71)	177 (42 – 411)

Table 3.4 Duration of monitoring of children for colds

3.3.3 Frequency of colds and exacerbations

3.3.3.1 Frequency of colds (objective ii.)

Children were recruited to the study between January 2011 and February 2012 and the study was completed in May 2012. A total of 32 children were recruited into the study and the average duration of daily monitoring with text messages was 139 (± 81) days, including the monthly retrospective monitoring this resulted in an average of 177 (± 71) days of monitoring (see Table 3.4). A total number of 75 'colds' or 'increased symptoms' were reported during the monitoring period. 12 of these colds were reported during the retrospective monthly monitoring. These 'monthly' colds were neither clinically or virologically confirmed nor followed up by telephone conversations but regarded as true colds on the basis that parents should be able to make a retrospective judgement whether their child had a cold or not in the previous month. The other 63 reports of increased symptoms via SMS were followed up by telephone conversations with parents and in 46 cases (73%) a clinical judgement, based on a combination of parental report of symptoms and parental opinion, confirmed the presence of (the initial stages of) a cold. In 17 cases of reported symptoms a cold was not confirmed (i.e. symptoms hayfever-related, symptoms disappeared, incorrect reporting by parent). A total number of 34 episodes of colds of 25 participants (9 of which developed two colds) were followed up with home visits, daily monitoring of cold and asthma symptoms, and in most cases with sampling of saliva, nasal lavage +/- blood. In 12 cases of confirmed colds a home visit could not be arranged due to unavailability of either study participants or study team.

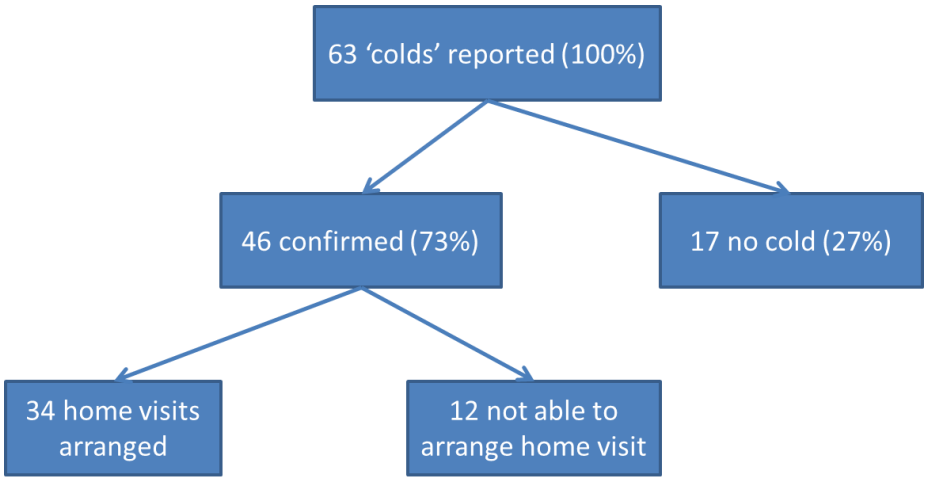


Figure 3.3 Flow diagram of reported colds

	children with daily (d) monitoring[1]	children with monthly (m) monitoring[2]	total number of children monitored (d+m)[3]	increased reported symptoms (d)[4]	clinically confirmed colds (d)[5]	colds with home visits + samples[6]	virus positive colds[7]	increased reported symptoms (d+m)[8]	clinically confirmed colds (d+m)[9]	cold reports on monthly monitoring[10]
Jan-11	2	-	2	2	2	1	1	2	2	-
Feb-11	4	-	4	0	0	0	0	0	0	-
Mar-11	6	1	7	3	2	1	1	3	2	0
Apr-11	8	1	9	3	1	0	0	3	1	0
May-11	8	2	10	1	0	0	0	2	1	1
Jun-11	7	2	9	5	3	2	2	5	4	0
Jul-11	7	3	10	0	0	0	0	1	1	1
Aug-11	9	2	11	1	0	0	0	1	0	0
Sep-11	10	1	11	3	2	2	2	3	2	0
Oct-11	13	1	14	3	0	0	0	5	2	2
Nov-11	14	2	16	8	6	5	5	8	7	0
Dec-11	18	2	20	10	9	5	4	10	9	0
Jan-12	19	3	22	8	7	6	6	9	8	1
Feb-12	19	5	24	8	7	7	6	12	11	4
Mar-12	15	7	22	4	4	2	1	6	6	2
Apr-12	12	5	17	4	3	3	1	5	4	1
May-12	11	3	14	0	0	0	0	0	0	0

Table 3.5 Quantification of colds A

Quantification of reported increase in symptoms[4,8] and confirmed[5,9] colds in relation to the number of participants being followed up during each month by either daily[1], retrospective monthly[2] or daily *and* monthly[3] monitoring. Colds during daily monitoring were regarded as (clinically) confirmed[5] if, following telephone contact with parents, they definitely confirmed that their child had a cold (based on symptom reports). If a home visit could be arranged within 48 hours of first cold symptoms, samples were taken and PCR was performed to determine the virus status[6,7]. Cold reports on retrospective monthly monitoring were regarded as true (confirmed) colds.

	Daily monitoring			Daily + monthly monitoring		
	number of children monitored	reported increase in symptoms[1] [n (%)]	confirmed colds[1] [n (%)]	number of children monitored	reported increase in symptoms[2] [n (%)]	confirmed colds[2] [n (%)]
Jan-11	2	2 (100)	2 (100)	2	2 (100)	2 (100)
Feb-11	4	0 (0)	0 (0)	4	0 (0)	0 (0)
Mar-11	6	3 (50)	2 (33)	7	3 (43)	2 (29)
Apr-11	8	3 (38)	1 (13)	9	3 (33)	1 (11)
May-11	8	1 (13)	0 (0)	10	2 (20)	1 (10)
Jun-11	7	5 (71)	3 (43)	9	5 (56)	4 (44)
Jul-11	7	0 (0)	0 (0)	10	1 (10)	1 (10)
Aug-11	9	1 (11)	0 (0)	11	1 (9)	0 (0)
Sep-11	10	3 (30)	2 (20)	11	3 (27)	2 (18)
Oct-11	13	3 (23)	0 (0)	14	5 (36)	2 (14)
Nov-11	14	8 (57)	6 (43)	16	8 (50)	7 (44)
Dec-11	18	10 (56)	9 (50)	19	10 (53)	9 (47)
Jan-12	19	8 (42)	7 (37)	21	9 (43)	8 (38)
Feb-12	19	8 (42)	7 (37)	24	12 (50)	11 (46)
Mar-12	15	4 (27)	4 (27)	18	6 (33)	6 (33)
Apr-12	12	4 (33)	3 (25)	14	5 (36)	4 (29)
May-12	11	0 (0)	0 (0)	12	0 (0)	0 (0)

Table 3.6 Quantification of colds B

Overview of number of children with daily monitoring only and daily + monthly monitoring each month. Reported increases in symptoms and confirmed colds are shown as total numbers and in brackets as [1] percentages of the number of children with daily monitoring or [2] percentages of number of children with daily + monthly monitoring each month.

Tables 3.5 and 3.6 give an overview of reported increases in cold/asthma symptoms in relation to the number of participants being followed up during each month by daily, retrospective monthly or daily *and* monthly monitoring. During the monitoring period on average 25% of participants (that were monitored daily) developed a clinically confirmed cold each month. During a number of months there were no clinically confirmed colds (February, May, July, August, October 2011 + May 2012) while 50% (n=9) of monitored children had clinically confirmed colds in Dec 2011 and 100% (n=2) in January 2011. In September 2011 only 20% (n=2) developed an increase in symptoms.

3.3.3.2 Frequency of exacerbations (objective ii.)

Based on the classification by Reddel *et al* (2009) (Reddel et al., 2009) and as outlined in paragraph 3.4.5.1 the severity of viral induced asthma exacerbation of the participants was determined. In total there were 34 cold episodes - 5 that did not result

in any asthma exacerbation, 23 with a moderate exacerbation and 6 with a severe exacerbation. As 9 participants developed 2 cold episodes, and in order to avoid duplication, the final dataset included 25 separate cold episodes. The decision process on which cold episode to include is described in 2.4. and was based on the most complete data set available. This process did not bias the results in terms of severity grading of exacerbations. The majority of cold episodes happened between November and February, which may be partly explained by the higher number of children enrolled in the study at that time and not only by the obvious association of viral infections and winter months.

Figure 3.4 shows the ratio of severe and moderate exacerbations and 'cold only' for all colds, for cold 1 and cold 2 separately, and for those colds that were included in the final data analysis.

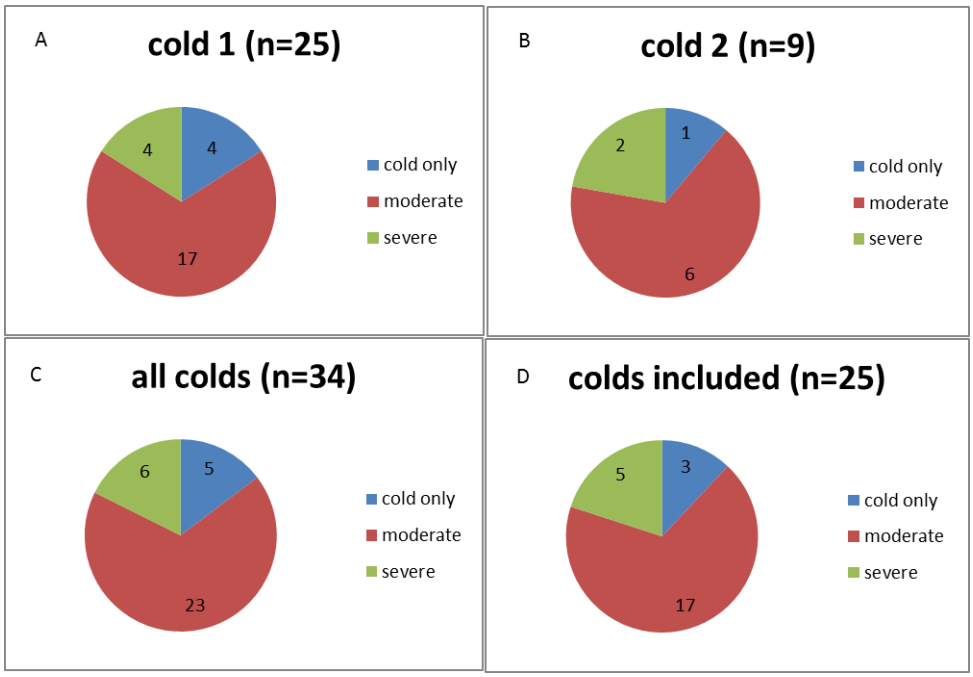


Figure 3.4 Severity grading (asthma exacerbations) of colds

A. first cold episodes; B. second cold episodes; C. all colds combined; and D. final analysis group.

3.3.4 Jackson Cold Score (JCS)

The Area under the Curve and the peak Jackson Cold Score as well as the peak of the 48 hour sum of the JCS were calculated for the baseline and the cold episode of each participant. An adult study that looked at the prevention of viral induced asthma exacerbation defined a clinical virus infections as achieving a Jackson Cold score >14 over any two day period in the first 7 days (Djukanovic et al., 2014). This approach was extrapolated to the participating children. In consideration of the bigger challenge of

applying the symptom scoring to children and no clear definition of a cut-off three groups of cold episodes were determined based on the 48 hour sum of the JCS:

(1) Scoring 9 or less

(2) Scoring 10 – 13

(3) Scoring 14 and above

Six cold episodes scored 9 or less, eight cold episodes between 10 and 13, and twenty fell into group 3 with a score of 14 and above. The mean 48 hour score was 15, with a maximum score of 37 in one participant (Figure 3.5 and Table 3.7).

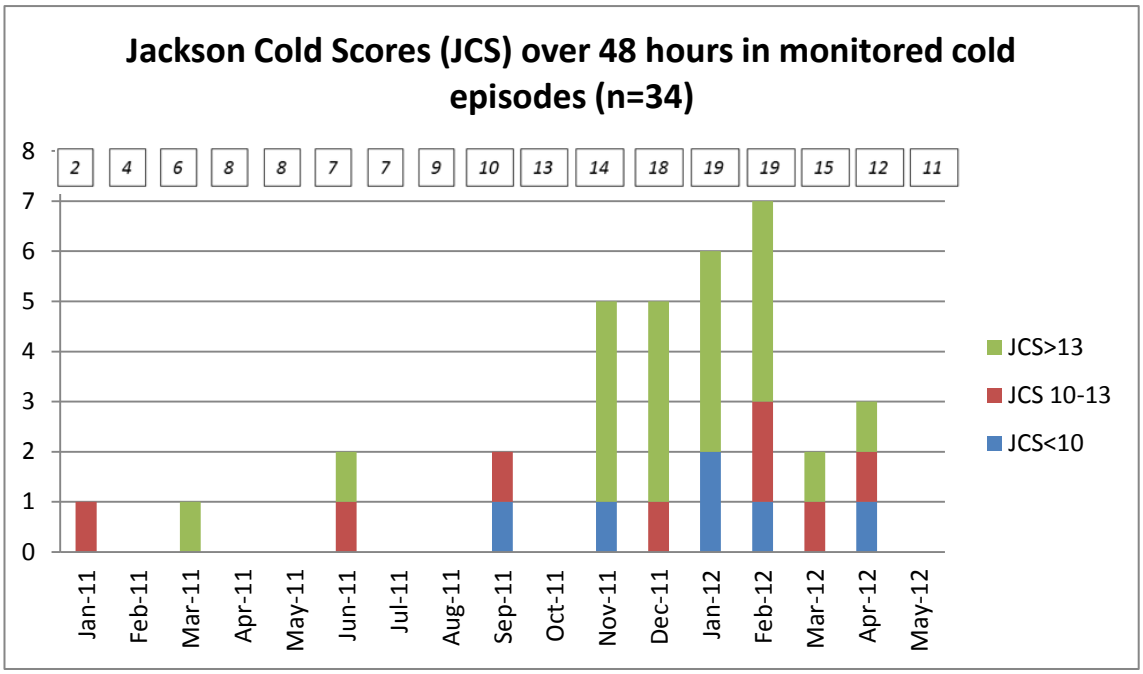


Figure 3.5 Jackson Cold Score over 48 hours in monitored cold episodes

The figure shows the number of SMS-monitored cold episodes each month during the study period, and splits the cold episodes into colds that achieved a JCS less than 10, 10 to 13, or more than 13 as a sum over 48hr. The numbers show the total number of participants enrolled and monitored in the study each month.

	numbers daily monitored	colds with home visits + samples	JCS48hpeak≤9	JCS48hpeak=10	JCS48hpeak=11	JCS48hpeak=12	JCS48hpeak=13	JCS48hpeak>13
Jan-11	2	1	0	0	0	1	0	0
Feb-11	4	0	0	0	0	0	0	0
Mar-11	6	1	0	0	0	0	0	1
Apr-11	8	0	0	0	0	0	0	0
May-11	8	0	0	0	0	0	0	0
Jun-11	7	2	0	0	0	1	0	1
Jul-11	7	0	0	0	0	0	0	0
Aug-11	9	0	0	0	0	0	0	0
Sep-11	10	2	1	1	0	0	0	0
Oct-11	13	0	0	0	0	0	0	0
Nov-11	14	5	1	0	0	0	0	4
Dec-11	18	5	0	0	1	0	0	4
Jan-12	19	6	2	0	0	0	0	4
Feb-12	19	7	1	1	0	0	1	4
Mar-12	15	2	0	0	1	0	0	1
Apr-12	12	3	1	1	0	0	0	1
May-12	11	0	0	0	0	0	0	0
All colds		**34**	6	3	2	2	1	20

Table 3.7 Colds and Jackson Cold Score

The number of monitored colds with a sum of the Jackson cold score (JCS) over any 48 hour period equal to or less than 9, 10, 11, 12, 13 and 14 and above respectively (at any time during the cold monitoring period) are shown.

3.3.4.1 Jackson Cold Score that best identifies a cold (objective iii)

There is not a pre-defined cut-off for the JCS for children to determine the presence of a cold. The clinical judgement based on parental reporting and perception formed the basis of defining presence of a cold in this study. Therefore all 34 cold episodes were definite colds with 29 cold episodes virus-positive and 5 virus-negative. This approach was taken to perform further statistical analysis and to determine the rest of the objectives of the study. On that basis, in the absence of true negative or false positive episodes, a validation of the Jackson cold score is however impossible. For validation purposes virus positivity was taken as the factor that determined a "true cold" and the results are shown in Table 3.8.

	JCS cut off = 8	**JCS cut off = 9**	**JCS cut off = 10**	**JCS cut off = 11**	**JCS cut off = 12**	**JCS cut off = 13**	**JCS cut off = 14**
true positive	28	28	24	22	21	19	18
false negative	1*	1	5	7	8	10	11
true negative	0	1	1	2	3	3	3
false positive	5	4	4	3	2	2	2**
Sensitivity	96.55% (82.24 to 99.91)	96.55% (82.24 to 99.91)	82.76% (64.23 to 94.15)	75.86% (56.46 to 89.70)	72.41% (52.76 to 87.27)	65.52% (45.67 to 82.06)	62.07% (42.26 to 79.31)
Specificity	0.00% (0.00 to 52.18)	20.00% (0.51 to 71.64)	20.00% (0.51 to 71.64)	40.00% (5.27 to 85.34)	60.00% (14.66 to 94.73)	60.00% (14.66 to 94.73)	60.00% (14.66 to 94.73)
Positive Likelihood Ratio	0.97 (0.90 to 1.03)	1.21 (0.77 to 1.88)	1.03 (0.65 to 1.65)	1.26 (0.60 to 2.66)	1.81 (0.60 to 5.42)	1.64 (0.54 to 4.95)	1.55 (0.51 to 4.71)
Negative Likelihood Ratio	---	0.17 (0.01 to 2.33)	0.86 (0.13 to 5.91)	0.60 (0.17 to 2.11)	0.46 (0.18 to 1.16)	0.57 (0.24 to 1.38)	0.63 (0.27 to 1.48)
Disease Prevalence	85.29% (68.94 to 95.05)	85.29% (68.94 to 95.05)	85.29% (68.94 to 95.05)	85.29% (68.94 to 95.05)	85.29% (68.94 to 95.05)	85.29% (68.94 to 95.05)	85.29% (68.94 to 95.05)
Positive Predictive Value	84.85% (68.10 to 94.89)	87.50% (71.01 to 96.49)	85.71% (67.33 to 95.97)	88.00% (68.78 to 97.45)	91.30% (71.96 to 98.93)	90.48% (69.62 to 98.83)	90.00% (68.30 to 98.77)
Negative Predictive Value	0.00% (0.00 to 97.50)	50.00% (1.26 to 98.74)	16.67% (0.42 to 64.12)	22.22% (2.81 to 60.01)	27.27% (6.02 to 60.97)	23.08% (5.04 to 53.81)	21.43% (4.66 to 50.80)

Table 3.8 Validation of Jackson Cold Score (JCS over 48 hours):

29 virus-positive episodes and 5 virus-negative episodes. Numbers are percentages (95%CI) or ratios (95%CI). * one virus-positive episode had a JCS of 4; ** two virus-negative episodes had a JCS of 19 and 21 respectively.

In addition a Receiver Operating Characteristics (ROC) curve analysis was performed to evaluate the test and to determine a cut-off that best distinguishes between a cold and no cold. The AUC with a value of 0.617 (95%CI: 0.349 to 0.886), however, shows that the Jackson Cold Score is a poor test to determine a cold on the basis of virus positivity. A Jackson Cold Score of around 11.5 achieved the combination of best sensitivity (around 0.7) and best specificity (around 0.6). However, in order to increase sensitivity (to around 0.95) a cut-off of the JCS of 9 was determined.

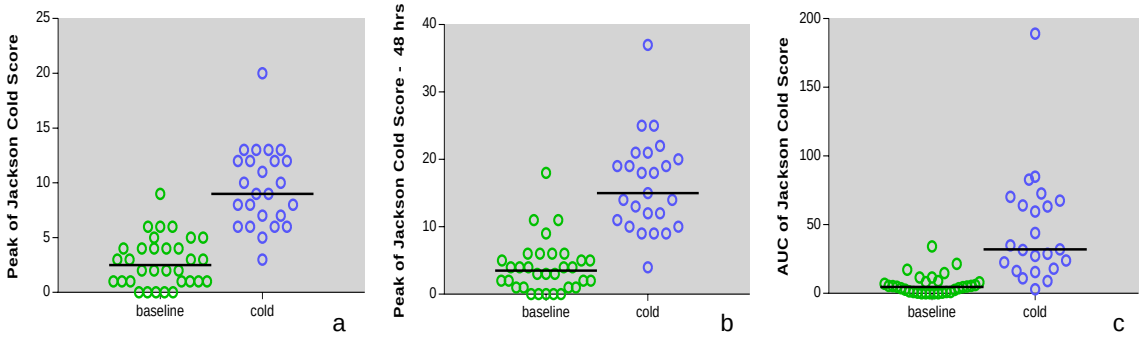


Figure 3.6 Jackson Cold Score - comparison between baseline (n=32) and cold (n=25)

Comparison of a. peak of Jackson Cold Score (JCS), b. peak of JCS over 48 hours, and c. AUC of JCS between baseline and cold. Figures are column scatter plots with bar representing group median.

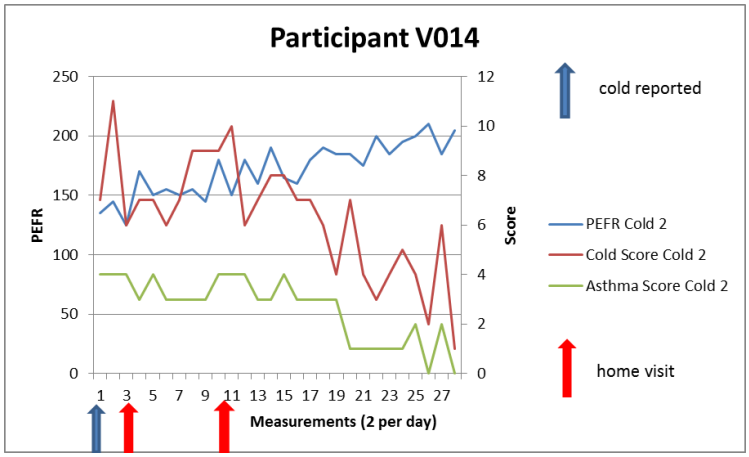


Figure 3.7 Temporal relationship - cold and asthma symptoms and PEFR

Example (participant V014) of temporal relationship between cold symptoms, asthma symptoms and peak expiratory flow rate (PEFR) (objective iii.) and timing of home visits. This figure shows a reduced PEFR and a raised asthma score at the on-set (and reporting) of cold symptoms.

3.3.5 Asthma index

The original and new (modified) asthma index were calculated for each participating child as outlined in the method section and the asthma index (red), the percentage decrease of the PEFR (blue) from a reference PEFR (computed from values obtained during baseline week) and the subjective sums (sum of symptom scores, green) plotted as graphs (objective iv.). See Figures 3.8 to 3.12 below as examples.

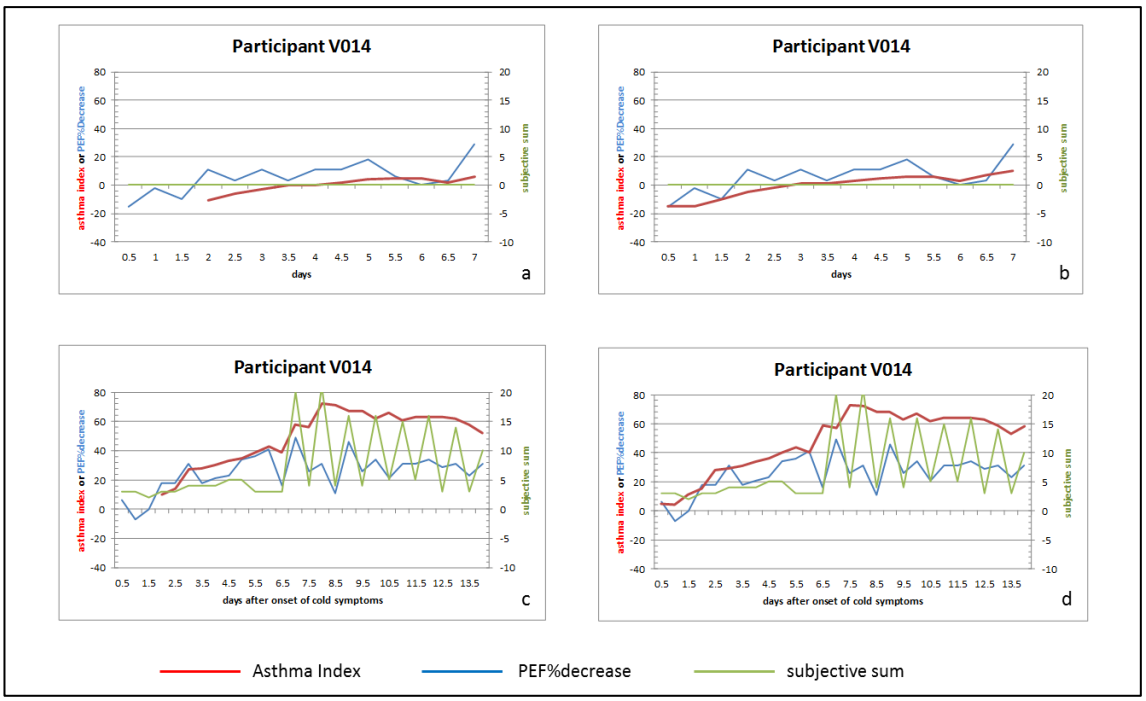


Figure 3.8 Original and new asthma index - example 1 (Participant 14)

a. original AI during baseline, b. new AI during baseline, c. original AI during cold, d. new AI during cold. This figure shows that both, subjective (symptom related) and objective (PEFR), elements of the asthma index change during the cold episode and the lines represent an increase in symptoms (green), a higher decrease in PEFR (blue) and an asthma index peaking (70) on day eight into the cold episode. The calculation of the Area under the Curve of the asthma index results in a positive number.

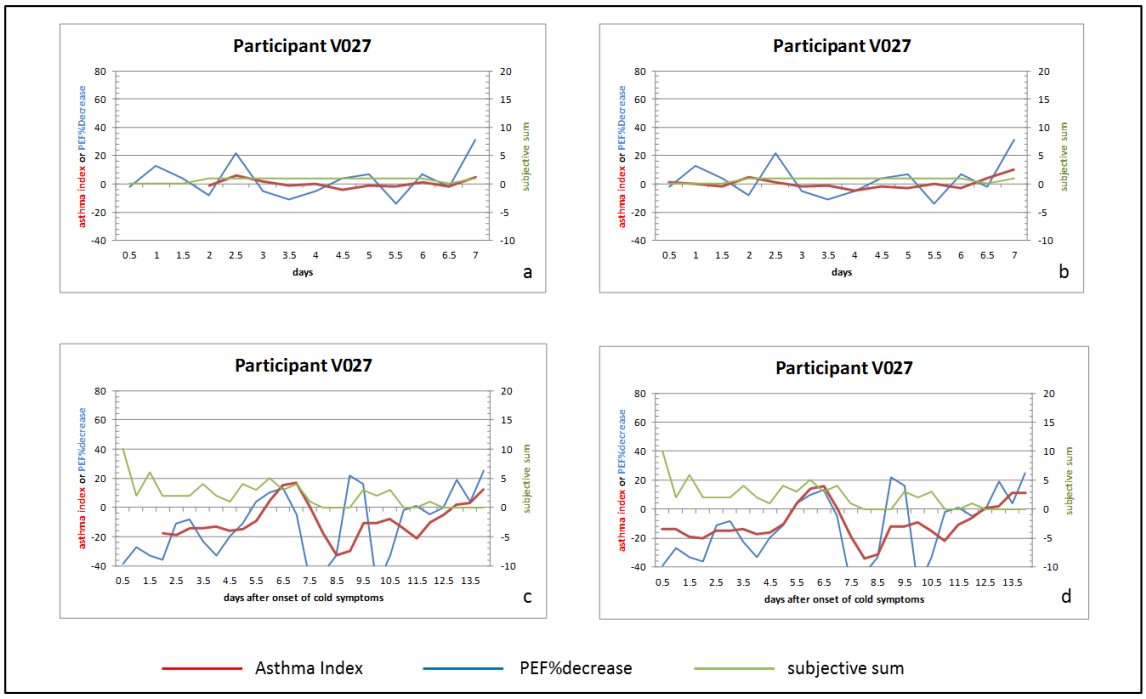


Figure 3.9 Original and new asthma index - example 2 (Participant 27)

a. original AI during baseline, b. new AI during baseline, c. original AI during cold, d. new AI during cold. This figure shows a predominantly negative asthma index during the cold episode that only becomes briefly positive on day 7 and 13 of the cold. Despite a positive symptom score, the influence of the improved PEFR (in relation to the reference PEFR) pushes the asthma index into negative figures and also leads to a negative Area under the Curve.

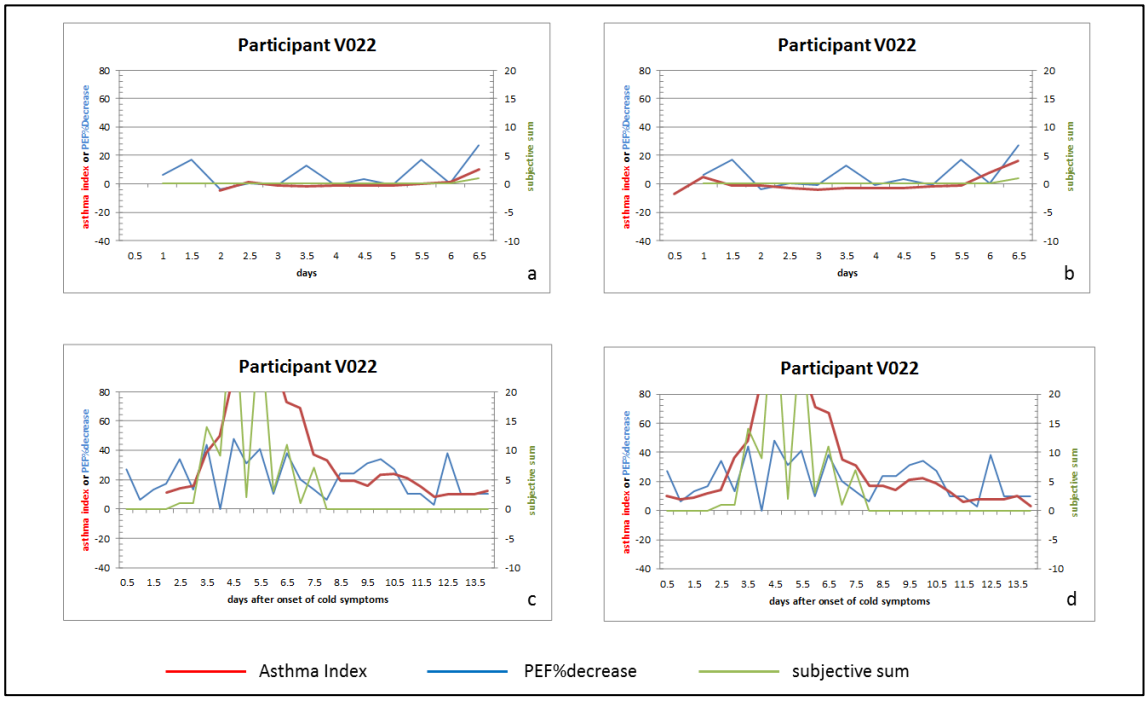


Figure 3.10 Original and new asthma index - example 3 (Participant 22)

a. original AI during baseline, b. new AI during baseline, c. original AI during cold, d. new AI during cold. This figure shows an asthma index that, using the proposed scale for asthma index and subjective sum by Sorkness *et al*. (2008) (Sorkness et al., 2008), peaks between day 4 and 6 of the cold episode off the scale. Both elements of the asthma index change more or less synchronously to yield a positive asthma index and AUC.

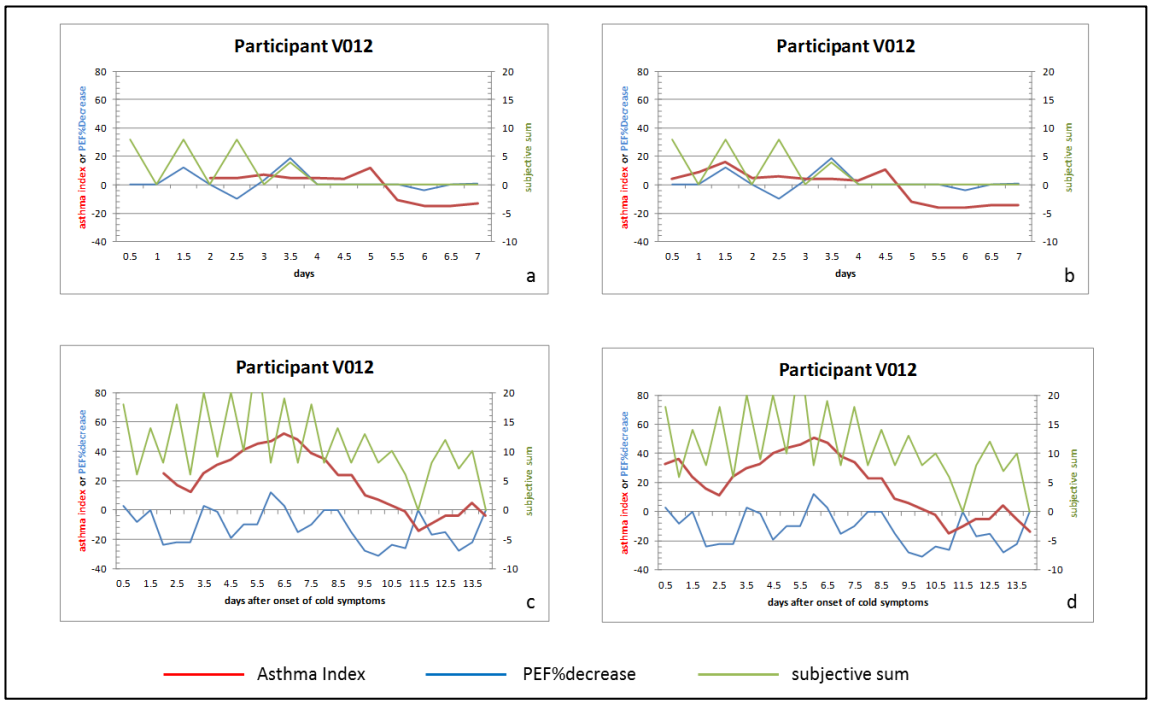


Figure 3.11 Original and new asthma index - example 4 (Participant 12)

a. original AI during baseline, b. new AI during baseline, c. original AI during cold, d. new AI during cold. This figure shows a predominantly positive asthma index in view of high symptom scores despite PEFR improvements compared to baseline. Participant 12 received a 3 day course of oral steroids by their GP.

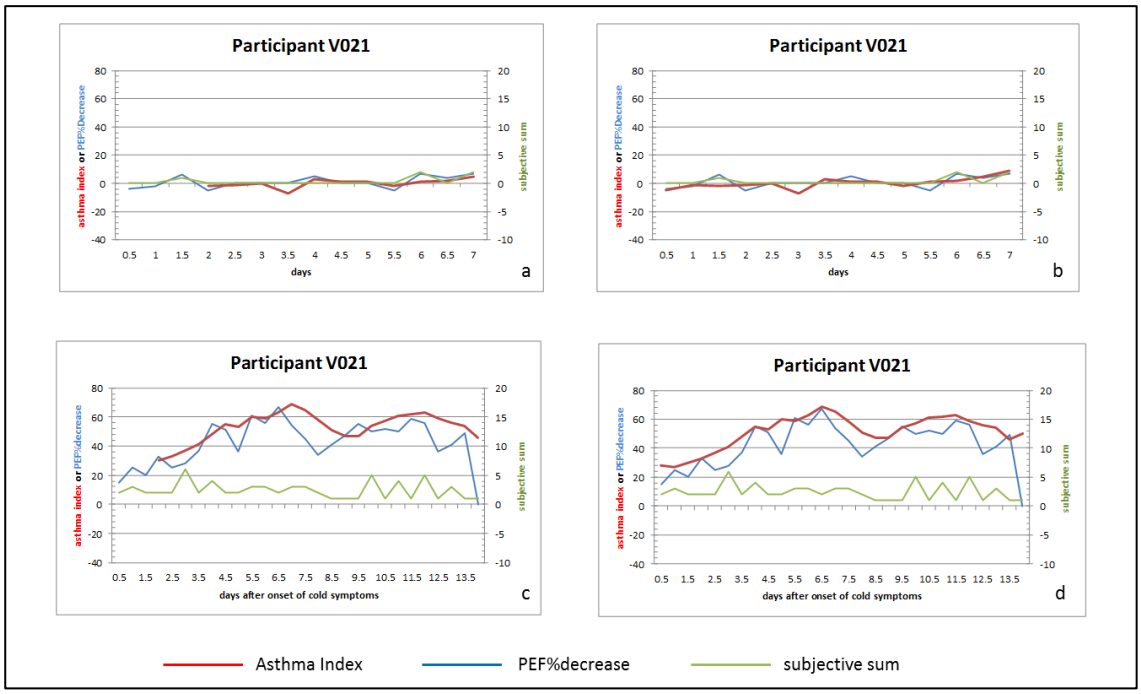


Figure 3.12 Original and new asthma index - example 5 (Participant 21)

a. original AI during baseline, b. new AI during baseline, c. original AI during cold, d. new AI during cold. In contrast to Figure 3.11 this shows a clearly positive asthma index with the main contribution by a much reduced PEFR while the patient reports relatively few symptoms.

A first step to improve the graphic display of the asthma index and its components was to adjust the scale of the primary and secondary y-axis (Figure 3.13). This was necessary as in the case of several participants the asthma index (and/or components) went beyond the scale suggested by Sorkness *et al*. (for example Figures 3.9 and 3.10 above).

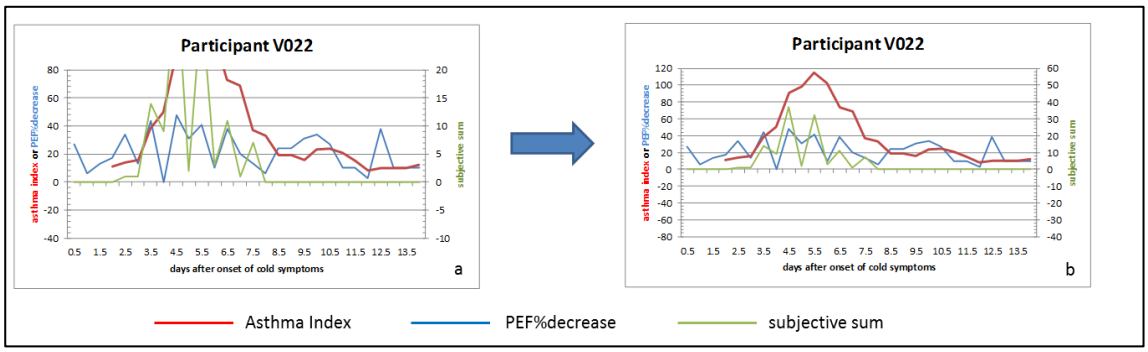


Figure 3.13 Asthma index - adjustment of the scale of the primary and secondary y-axis

In a second step, necessitated by negative AUCs of the asthma index of several participants, the asthma index was shifted by a value of ‘40’ to yield positive AUCs (Figure 3.13).

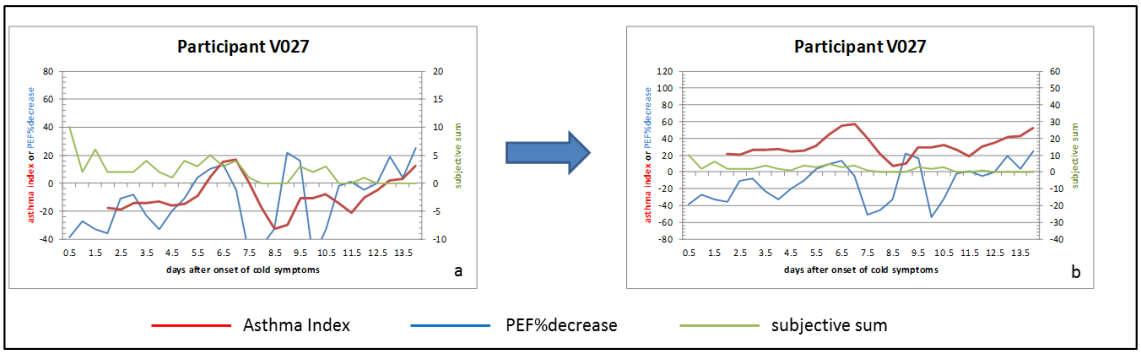


Figure 3.14 Asthma index - shift by a value of '40'

3.3.5.1 Asthma Index and severity of exacerbation – validation of the asthma index (objective iv.)

The asthma index (Sorkness *et al*., 2008) was developed as a continuous variable reflecting both subjective and objective components of asthma exacerbations. It has been applied in adult populations but so far has not been validated in a paediatric population.

Step 1: Validating the original asthma index using clinical data from this study and the classification of severity of asthma exacerbations according to Reddel *et al*.

The severity classification for use in clinical trials as suggested by Reddel *et al.* (Reddel et al., 2009) was used to divide participants into 3 severity groups: (i) severe exacerbation - defined as at least one of the following): (1) hospitalization due to asthma exacerbation, requiring systemic corticosteroids, (2) use of systemic corticosteroids for at least 3 days); (ii) moderate exacerbation - defined as presence of one or more of the following: deterioration in symptoms, deterioration in lung function, increased use of rescue bronchodilator use or increased use of inhaled corticosteroids for at least 2 consecutive days, ED visit for asthma but not requiring systemic corticosteroids; (iii) cold only. The last classification of 'cold only' was added and is not part of the original classification by Reddel *et al*. who do not include a specific definition for mild or no exacerbation.

The Area Under the Curve (AUC) and the Peak of the asthma index was then plotted against this severity grading (Figures 3.15 and 3.16).

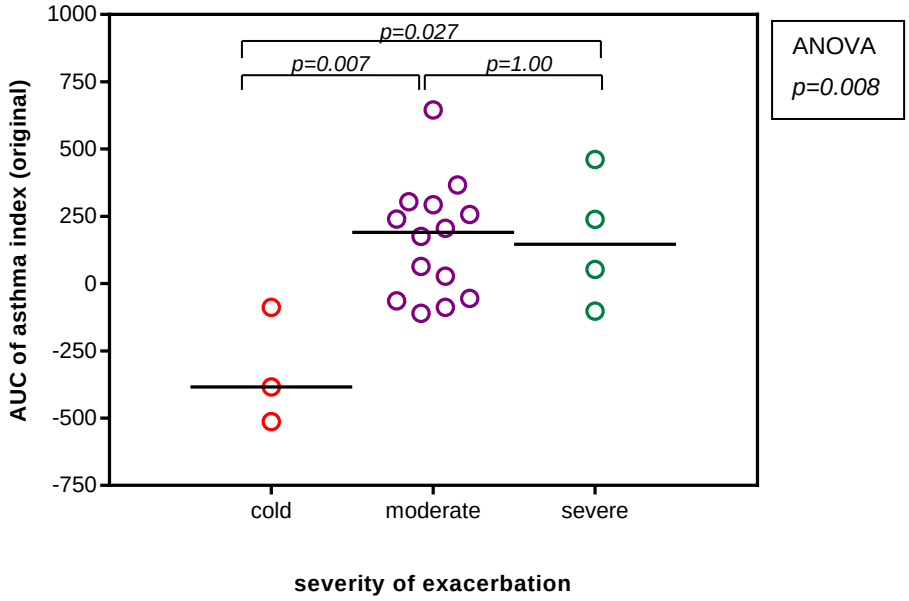


Figure 3.15 Area Under the Curve of Asthma Index (original) and severity of exacerbation.

Data showing scatter plot of the Area Under the Curve of the asthma index (original). Solid bars are group medians. In case of two cold episodes only one was included per participant. Asthma exacerbation severity defined according to ATS/ERS Recommendations for classifying asthma exacerbations in clinical trials (Reddel, Taylor et al. 2009). ANOVA p-value suggests a significant difference between the three groups. Other p-values refer to post hoc 2 sample t-test analysis with Bonferroni correction for multiple testing.

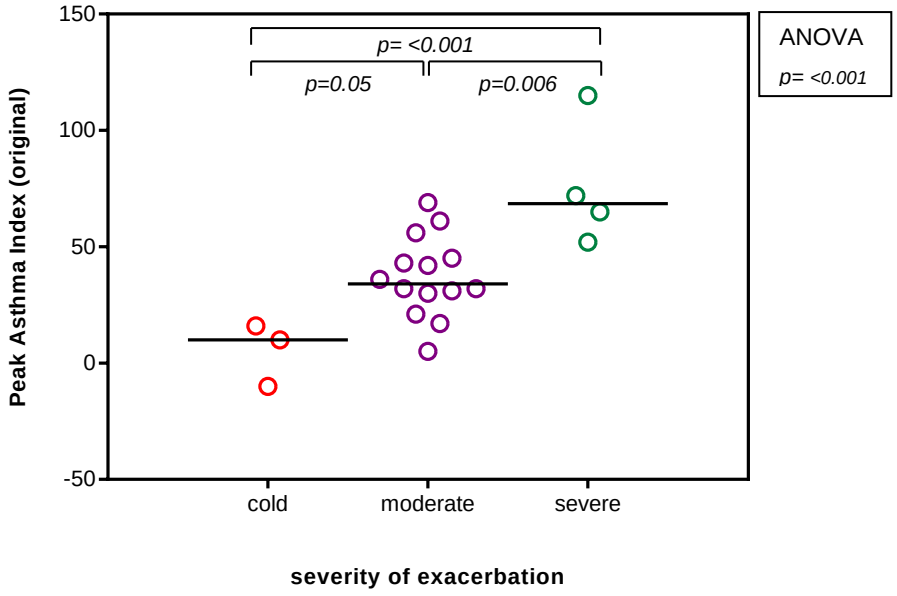


Figure 3.16 Peak Asthma Index (original) and severity of exacerbation

Data showing scatter plot of the Peak asthma index (original). Solid bars are group medians. In case of two cold episodes only one was included per participant. Asthma exacerbation severity defined according to ATS/ERS Recommendations for classifying asthma exacerbations in clinical trials (Reddel, Taylor et al. 2009). ANOVA p-value suggests a significant difference between the three groups. Other p-values refer to post hoc 2 sample t-test analysis with Bonferroni correction for multiple testing.

This shows that the AUC of the original asthma index distinguishes between any and no exacerbation but is unable to discriminate between moderate and severe exacerbation. The peak of the original asthma index, however, shows a good discrimination between all groups that is statistically significant between 'cold only' and 'severe exacerbation' as well as between 'moderate exacerbation' and 'severe exacerbation' but just misses statistical significance between 'cold only' and 'moderate exacerbation'. In view of the lack of numbers especially in the 'cold only' and 'severe exacerbation' groups, however, the results must be interpreted with caution.

Step 2: The modified asthma index was compared with the published asthma index using Bland Altman Plots (Figures 3.17 and 3.18) showing mean difference and 95% limits of agreement.

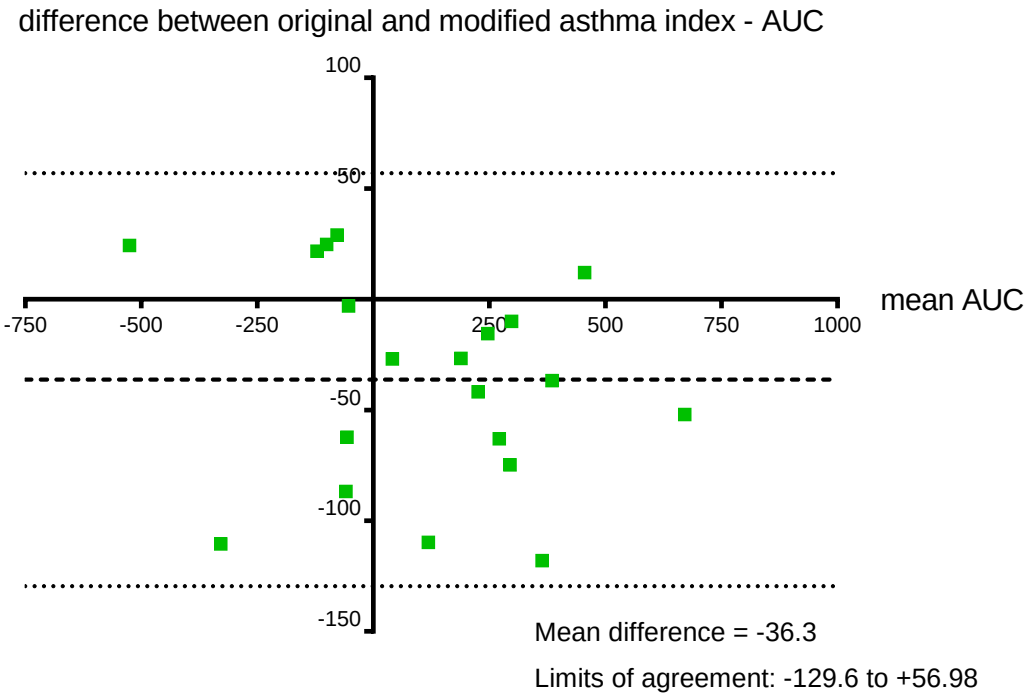


Figure 3.17 Bland Altman Plot of the AUC of the original and modified asthma index. The broken horizontal lines correspond with the mean difference (dark) and limits of agreement (lighter).

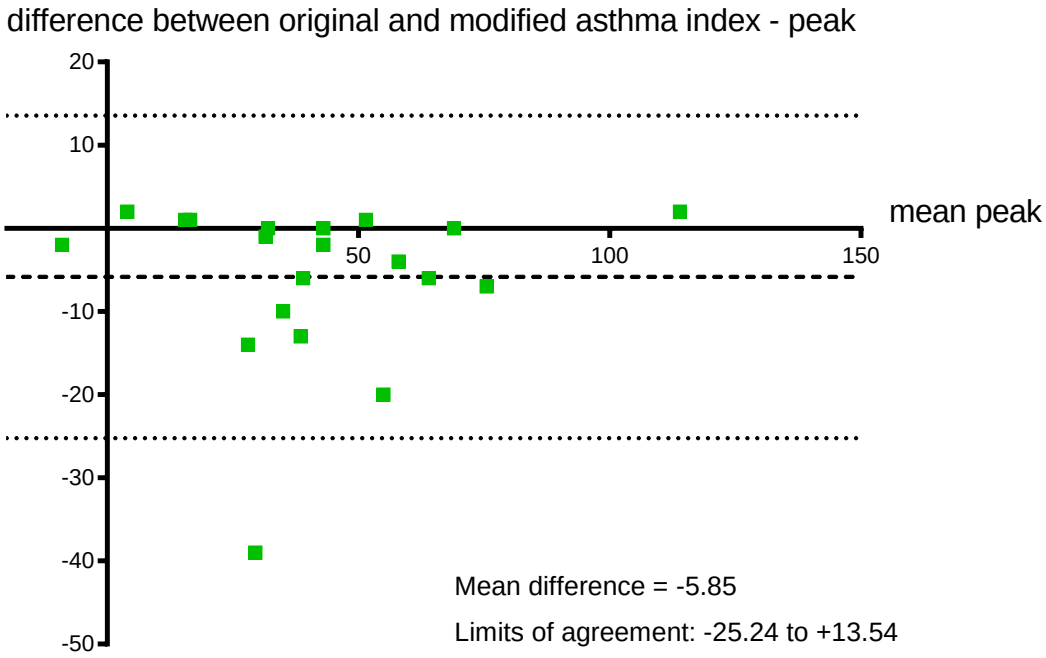


Figure 3.18 Bland Altman Plot of the peak of the original and modified asthma index. The broken horizontal lines correspond with the mean difference (dark) and limits of agreement (lighter).

Step 3: Validating the modified asthma index using clinical data from this study and the classification of severity of asthma exacerbations according to Reddel *et al*. (Figures 3.19 and 3.20).

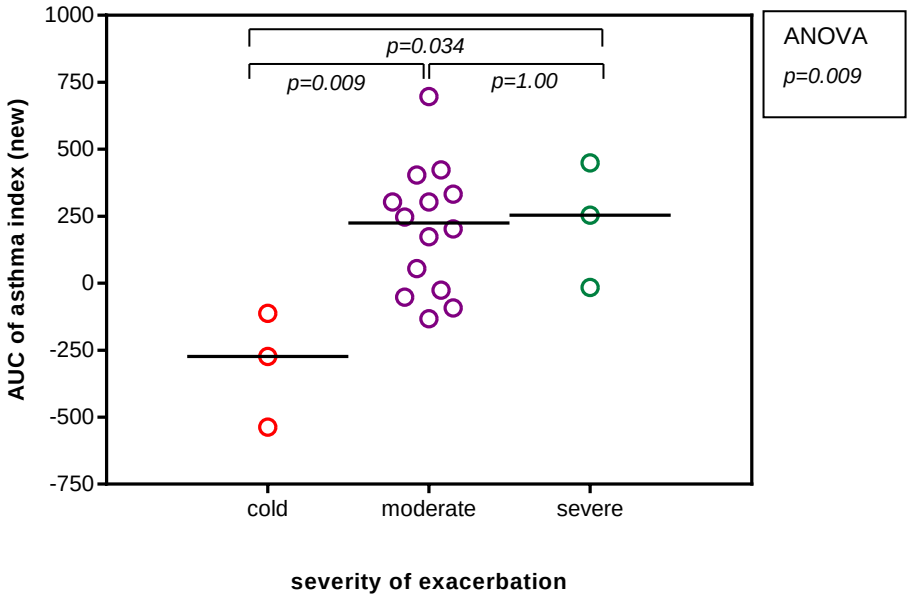


Figure 3.19 Area Under the Curve of Asthma Index (new) and severity of exacerbation

Data showing scatter plot of the Area Under the Curve of the asthma index (new). Solid bars are group medians. In case of two cold episodes only one was included per participant. Asthma exacerbation severity defined according to ATS/ERS Recommendations for classifying asthma exacerbations in clinical trials (Reddel, Taylor et al. 2009). ANOVA p-value suggests a significant difference between the three groups. Other p-values refer to post hoc 2 sample t-test analysis with Bonferroni correction for multiple testing.

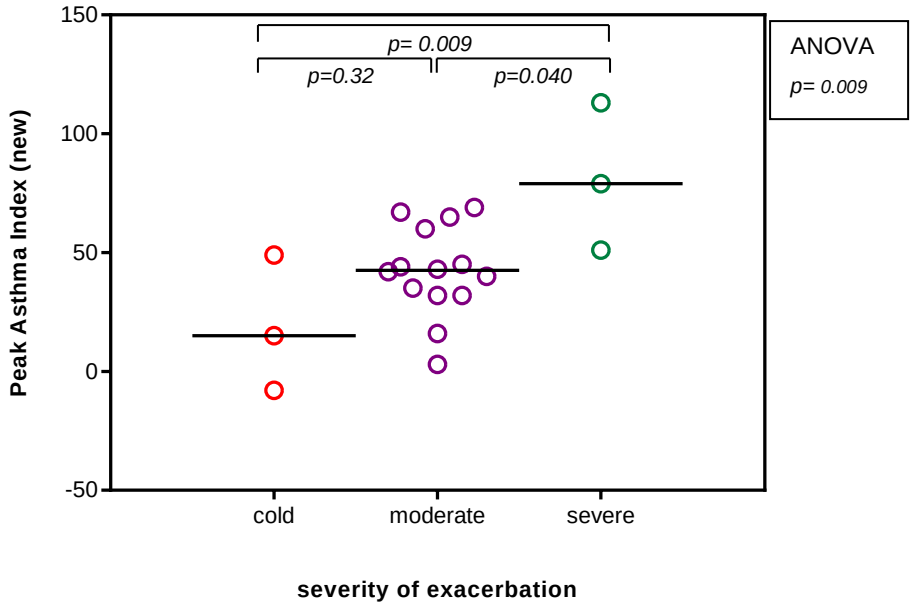


Figure 3.20 Peak Asthma Index (new) and severity of exacerbation

Data showing scatter plot of the Peak asthma index (new). Solid bars are group medians. In case of two cold episodes only one was included per participant. Asthma exacerbation severity defined according to ATS/ERS Recommendations for classifying asthma exacerbations in clinical trials (Reddel, Taylor et al. 2009). ANOVA p-value suggests a significant difference between the three groups. Other p-values refer to post hoc 2 sample t-test analysis with Bonferroni correction for multiple testing.

This shows that the AUC of the new asthma index (in line with the original asthma index) distinguishes between any and no exacerbation but is unable to discriminate between moderate and severe exacerbation. The peak of the new asthma index (in line with the original peak asthma index), however, shows a good discrimination between all groups that is statistically significant between ‘cold only’ and ‘severe exacerbation’ as well as between ‘moderate exacerbation’ and ‘severe exacerbation’ but just misses statistical significance between ‘cold only’ and ‘moderate exacerbation’. The peak asthma index (see Figures 3.16 and 3.20) appears to be a more suitable indicator of exacerbation severity compared to the AUC of the asthma index (see Figures 3.15 and 3.16). In view of the lack of numbers especially in the ‘cold only’ and ‘severe exacerbation’ groups, however, the results must be interpreted with caution.

The modification of the asthma index has not provided any advantage in terms of distinguishing exacerbations of different severity and therefore was not pursued for this study and cannot be recommended for this purpose for a future study.

3.4 Discussion

This study recruited 32 children aged 5-11 years with a diagnosis of asthma and treatment with inhaled corticosteroids and a history of at least two respiratory viral associated exacerbations of asthma in the preceding 12 months. It was an observational/feasibility study of the components of a protocol for a planned interventional study with the aim of preventing viral induced asthma exacerbations in children by treatment with nebulised Interferon-β following the completion of a similar study by *Synairgen Research Ltd*. (University of Southampton spin-out Company) conducted in adults (Djukanovic et al., 2014) in parallel to this observational study.

As a first objective (objective i.) the general and asthma specific features of participants were explored. Most participating children and their parents reported at their baseline visit worse asthma symptoms between the months of September and February compared to the rest of the year. The start and peak of the “exacerbation season” with the month of September is well described in the literature (Johnston et al., 2005). While the debate is ongoing regarding the significance of early life respiratory virus infections and the development of asthma in later life (Sly et al., 2010, Holt and Sly, 2012) (A. the asthmatic phenotype predisposes to infant bronchiolitis or chest infections or B. the viral lower respiratory tract infection marks the beginning of the later development of childhood asthma by causing lung damage) there is little doubt that viral upper respiratory tract infections can lead to exacerbations in both children (Johnston et al., 1995) and adults (Nicholson et al., 1993) with established asthma.

The main triggers identified by families were common cold and flu (100%), weather changes (94%), exercise (75%), pollen and dust (both 69%). Cigarette smoke was a relevant trigger in 56%, with an interesting division between non-smoking households all of which regarded cigarette smoke as a potential culprit and smoking households that did not assign the same significance to smoke exposure. A recent study (Mackay et al., 2010) has shown a clear link between passive smoke exposure and asthma morbidity in children: After the introduction of a smoking ban in public in Scotland, a reduction of 18.2% per year in the rate of asthma-related hospitalizations in children followed. Prior to implementation, admissions for asthma were increasing at a mean rate of 5.2% per year. On average children in this study had 5 exacerbations in the preceding year, half of which were associated with viral upper respiratory tract infections (URTI). Considering that the average child undergoes a minimum of 4 to 8 URTIs per year (Padberg and Bauer, 2006, Stickler et al., 1985, Lorber, 1996) and there is no evidence for more colds in people with asthma (Corne et al., 2002) this

confirms that, while certainly contributing to asthma morbidity, there is no absolute correlation between viral URTIs and asthma associated illness (Olenec et al., 2010). This poses a clear challenge when aiming to intervene at the correct time point to prevent a viral induced asthma exacerbation. In addition, many viral infections are detected in asymptomatic individuals, as shown both in this study and reported in the literature (Jansen et al., 2011, DeMore et al., 2009). Participants were given between 0 and 10 courses of steroids (median 1.5) during the 12 month prior to study enrolment. A significant proportion of children showed other aspects of atopy such as eczema (75%) or food allergies (50%) and sensitization on the basis of skin prick test was confirmed in 78% (25/32) of children.

As a second objective (objective ii.), the frequency of viral induced common cold symptoms and frequency and severity of asthma exacerbations in the study group were explored. Children were monitored daily for 139 (± 81) days and 25/32 developed at least one cold episode (9 participants were monitored during 2 cold episodes), 5/32 children did not develop (or report) viral URTI symptoms, and 2/32 children were withdrawn from the study not having developed a cold up to that time point. The phenomenon of reduced frequency of (reported) URTI symptoms during the study compared to the reported frequency at the time-point of recruitment to the study might be due to a number of factors: parental exaggeration at the time point of enrolment, parental underreporting (i.e. inconvenient time-point for family to have study visits) while in the study or a real decrease in URTI frequency (as the child gets older and the immune system matures). A limitation of the study in this respect was also the enrolment of children at different times/seasons of the year and initially for a pre-specified time period of 6 months only leading to drop out of participants who for example had not developed any cold between the “warmer months” of March and September. The underreporting of both upper and lower respiratory tract symptoms (as well as decreases in PEFR) was shown in a community based study that monitored 108 children aged 9-11 years with asthma for 13 months (Johnston et al., 1995). This study found the mean (SD) number of episodes per child to be 5.33 (4.99) for lower respiratory tract symptoms, and 7.24 (4.47) for upper respiratory tract symptoms (Johnston et al., 1995). Johnston *et al.* (1995) enrolled a cohort of children for 13 months while this study was approved to closely monitor children with lung function, nasal lavage, saliva and blood sampling for initially one cold and, following protocol amendment, for two colds. Despite the aim to capture further cold and asthma exacerbation episodes for epidemiological purposes by monthly SMS-based

retrospective monitoring, the low response rate did not allow firm conclusions in this respect.

The 25 cold episodes (in order to avoid duplication only one cold per participant) included in this study were divided into 12% (3/25) 'cold only' episodes with no evidence of lower respiratory tract involvement, 68% (17/25) moderate exacerbations, and 20% (5/25) severe exacerbations according to the severity classification by Reddel *et al* (2009) (Reddel et al., 2009). This severity classification, that includes need for increased treatment and medication, differs from the one applied by Johnston *et al*. (1995) where severity was expressed on the basis of the maximum total symptom score during the episode for upper and lower respiratory tract episodes and as the largest drop from the median expiratory flow rate for peak expiratory flow episodes. Most episodes on that basis were described as 'severe'. In the study that is described in this thesis children measured their peak flow during an asymptomatic baseline and a symptomatic cold episode. No peak flow measurements were obtained in the immediate period leading up to a cold and also not more than two weeks after the inception of a cold. This is important to note as some other studies use PEFR changes as criteria for as assessment as increased diurnal variations in peak flow have been described as being related to symptoms and airway hyperresponsiveness (Brand et al., 1997) and one clinical trial reported that a 90-day average seasonal 5% reduction in peak flow was associated with a 22% increase in the risk of an asthma exacerbation (Covar et al., 2008). Peak flow measurements were not considered in isolation for the study that is described in this thesis but only as part of the asthma index where they constituted the objective parameter of evaluating the severity of an asthma exacerbation. PEFR changes as criteria for triggering an assessment however have also been reported in other studies. Lee *et al*. performed a prospective cohort study of 114 children aged 6-14 years who were monitored for respiratory symptoms over a full year. In this study participants would attend if they met a predefined symptom score, if parents subjectively felt them developing a cold, or if the PEFR dropped below 80% of their baselines. In this study, however, the viral detection rate during unscheduled clinic visits was low at 36.7%, which may be partly explained by the inclusion of PEFR drops without necessary presence of any cold symptoms (Lee et al., 2011).

The current BTS/SIGN Asthma guidelines (Revision 2014) define the PEFR value to qualify as moderate asthma exacerbations as ≥ 50%, a severe asthma exacerbation as 33-50% of the individual best PEFR. In this study the mean drop (standard deviation) of PEFR was 31% (17) – calculated as the percentage drop of the lowest PEFR from the average PEFR of the asymptomatic baseline (= personal best). This corresponds with

the findings of Olenec *et al*. who monitored children between 6 and 8 years of age with asthma for infections and respiratory illness during the common cold season with aim to evaluate rhinovirus infections and to analyze the relation to illness severity. Their maximum decrease in PEFR was 29% (15) for virus positive versus 25% (14) for virus negative episodes (Olenec et al., 2010).

An adult randomized controlled trial for the prevention of viral induced asthma exacerbations defined a significant cold as achieving a Jackson Cold Score >14 over any two day period in the first 7 days (Djukanovic et al., 2014). The Jackson Cold Score has been used but has not been validated in children and the cut-off for defining a viral URTI may be different. As a further objective (objective iii.), a cut-off of the JCS was defined that would best identify a cold. Only 20 cold episodes (of all 34 cold episodes) would have reached the cut-off of 14 while six scored 9 or less and eight between 10 and 13. The vast majority of parent-reported and clinically confirmed colds in this study were also virus-positive so the sensitivity in detecting virus-positive colds was low with the original cut-off of 14. For the purpose of this study and for all subsequent analysis the parental report and clinical confirmation of colds was used for the definition of a 'cold'. However, adjusting the definition of a 'cold' to a virus-positive episode allowed validation of the JCS. ROC curve analysis was limited by an overall low AUC which depicted the JCS as a poor test to distinguish a cold on the basis of virus positivity. The highest combined sensitivity/specificity was reached with a cut-off of around 11.5. However, to maximise sensitivity a JCS of 9 was chosen for pragmatic reasons. Parental impression alone was regarded a moderately accurate predictor of viral URTI in children in one study that showed virus presence in only about 57% of children with 'colds' (Taylor et al., 2010) but in this study all but 3 participants for which home visits were arranged post parental reporting of cold symptoms had confirmed virus presence. An attempt to distinguish viral positive from viral negative asthma exacerbation in a group of patients > 7 years with asthma on the basis of the Common Cold Questionnaire (CCQ) (Wiselka et al., 1991) was not successful (Powell et al., 2008). In addition another study including 917 (non-asthmatic) adults showed that neither patients nor doctors could reliably predict the severity or duration of a common cold and assigned an only moderate predictive value to the Wisconsin Upper Respiratory Symptom Survey (WURSS) (Longmier et al., 2013). All children participating in this study were school-aged children but a severity rating of at least some symptoms may still provide a challenge for younger participants. In addition they were required to tell their parents who then documented and forwarded the information to the study team. The challenges of symptom scoring especially in younger children

have previously been discussed in the literature and have led to development of other scales and scoring systems (Taylor et al., 2010, Barrett et al., 2006). Interestingly, neither the Jackson Cold Score nor the 44-item WURSS was able to provide useful rules to reliably predict infection (Barrett et al., 2006). One study used daily symptom records for 10 days in school-aged healthy children and found that children more frequently report nasal discharge, nasal obstruction, and cough than adults in the first days of a common cold (Pappas et al., 2008) while some elements of the Jackson cold score, such as headache and sore or scratchy throat, around 10% of parents found difficult to determine in their children (Taylor et al., 2010).

The asthma index, developed as a tool and continuous variable to quantitatively incorporate both subjective (symptom scores) and objective (PEFR) elements of asthma exacerbations, was used in this study. It was originally developed with a group of 47 adult asthma patients and 22 non-asthmatic non-atopic subjects who were studied during naturally occurring or experimentally induced colds. The asthma index reflects changes in subjective and objective domains relative to the individuals asymptomatic or stable baseline (Sorkness et al., 2008). No information regarding its use in children has been found in the literature. To validate the asthma index using established asthma exacerbation criteria (objective iv.), the exacerbation criteria of Reddel *et al.* (Reddel et al., 2009) were used. Keeping the limitation of low subject numbers in the 'cold only' and 'severe exacerbation' groups in mind, the peak asthma index correlated well with the severity groups as defined by Reddel *et al*. In contrast to the adult data (who correlated with exacerbation criteria of Pauwels *et al*. (Pauwels et al., 1997)), the asthma index also discriminated well between moderate and severe exacerbations. It may be tempting to postulate, based on these data, that the cut-off between 'cold only' and exacerbation was lower (17 or greater) than in the adult group (who suggested that a score of 30 or greater was an appropriate threshold for the definition of an exacerbation). The low overall number of subjects and the higher intersubject variability, however, clearly shows the need for adequately powered studies exploring this matter further.

To tackle one of the perceived disadvantages in the approach to calculate the (original) asthma index by Sorkness *et al*. (Sorkness et al., 2008), the lack of quantifiable data during the first 48 hours of cold symptoms, a slightly modified approach (new, in parallel to the original) was taken. The aim was that the value of the new Subjective Score reflected the mean time point between the measurements. In addition values were calculated for the new PEFR score as the mean of 2, 3 *or* 4 consecutive PEFR%Decreases, which allowed obtaining calculated PEFR scores also for the first

48 hour period. This modified approach to the calculation of the asthma index was deemed advantageous when looking at individual children in this study population since the progression from initial cold symptoms to an exacerbation is much more rapid than in adults. The overall effect on group comparisons was not significantly affected, there was no added value to the original asthma index and therefore this modification cannot be recommended for further studies. The first 48 hours did not appear to alter the peak of the asthma index. Despite features of an exacerbation of asthma being present the peak appeared to happen at a later stage. As a further modification not only the peak of the asthma index but also the AUC was calculated in order to determine whether this would give a better reflection of an asthma exacerbation. Again, the AUC of the 'cold only' and exacerbation groups was significantly different, while this approach did not discriminate between the moderate and severe exacerbation classification by Reddel *et al* (2009) (Reddel et al., 2009). One of the reasons may be that 'severe exacerbation' is defined by systemic corticosteroid use, which is more likely to be prompted by a peak on the one hand, but also may lead to a quicker resolution of symptoms/improvement of PEFR on the other hand. Therefore the AUC between moderate (without oral corticosteroid use) and severe exacerbation group over 14 days are similar despite a higher peak in the severe group. Eight participants had negative AUC values during their cold episode and while unexpected there are a number of possible explanations: First of all none of these participants had a severe exacerbation and three were classified as 'cold only' with no asthma exacerbation. Secondly the main contribution to the negative AUCs came from improved PEFR values in conjunction with relative modest subjective symptom reports. There may be various reasons for these improved PEFR values including potential better medication adherence while in the trial, an improvement in PEFR technique over time especially in younger participants, and an increase in PEFR due to child getting older and growing. Some colds happened 6-10 months after baseline PEFR values were obtained and a significant growth spurt with associated lung growth in some children cannot be excluded. The peak of the AI appears to be the correct measure of severity of the asthma exacerbation. It was used as a continuous rather than a categorical variable in the study but future investigations including a larger number of children should aim to define potential cut-offs distinguishing severity grades in children.

4 The validity and acceptability of a text based monitoring system for paediatric asthma studies

4.1 Introduction

A crucial aspect of a clinical trial is the instrument used to acquire the data for analysis. The quality of the data collected is dependent on the quality of that instrument. A meaningful analysis is only possible if the collected data is valid. Traditionally face-to-face or telephone interviews, observation, paper questionnaires or diaries completed by participants have been used. Questionnaires allow the respondent to reflect on the response, and answer more intimate or embarrassing questions. Written questionnaires assume the participant has sufficient literacy skills to understand and respond. Interviews allow personal contact between researcher and participant but the respondent may choose to please rather than be entirely honest and accurate. Paper diaries allow the collection of carefully selected data from an individual over time but they are renowned for low compliance and low reliability (Stone et al., 2002). Web-based systems are limited by the need for participants to have access to a computer and the internet.

Short Message Service (SMS) is a text messaging service component of phone, web, or mobile communication systems. It is very commonly used in everyday life as a way of communication, especially among younger generations. SMS has recently been used as a novel way of collecting data in research (Bexelius et al., 2009, Johansen and Wedderkopp, 2010, Kew, 2010). This system is either free or relatively cheap and is user-friendly with messages delivered instantaneously to individuals at any time or place. Messages can be stored and retrieved and be answered at the recipient's convenience. The same message can be sent to multiple recipients simultaneously. In combination with the appropriate software, SMS data can be transferred automatically and directly to an electronic data file accessible by the researcher. This real-time capture of data also avoids the pitfalls of backfilled and/or falsified paper diaries as responses are time-tagged.

In this study the parents of children aged 5 to 11 years were asked to respond to regular text messages sent by the study team. These text messages related to

symptom reporting and to expiratory peak flow values recorded by digital peak flow meters. Compliance, validity of collected data and acceptability of the method were evaluated.

4.2 Objectives

The objectives of this part of the study were to:

i. Assess the compliance of study participants (and their parents) in terms of responding to the SMS transmitted questions ('SMS-diary-compliance') and with regards to performing PEFR measurements ('PEFR-meter-compliance').

ii. Evaluate the validity of SMS-transmitted PEFR data points by comparing with PEFR-meter stored values.

iii. Assess the acceptability of SMS-data collection in research with questionnaires to parents of research participants.

4.3 Results

A total of 32 children were recruited into the study and baseline visits were performed. Two children withdrew from the study and five children did not develop a cold episode during the study period. This left 25 children who were monitored by regular PEFR measurements and 2 home visits during each cold episode (see Figure 4.2). Nine of these children developed a second cold episode during the observation period.

Three participants (V007, V008, V013), who did not develop a cold and did not return their digital peak flow meter at the end of the study, were excluded from this analysis as all their peak flow meter data were missing. One participant (V004), who withdrew from the study before developing a cold, reported exposure of the peak flow meter to strong magnetic field and no data could be downloaded; they were therefore also excluded. Two participants (V009 and V010) continued to use their peak flow meter on a daily basis beyond the recommended monitoring as part of the study involvement and their data exceeded the data storage limit of the digital peak flow meter leading to the initial baseline data to be overwritten. V010 developed a cold and the SMS and peak flow meter data during the cold episode were available for analysis.

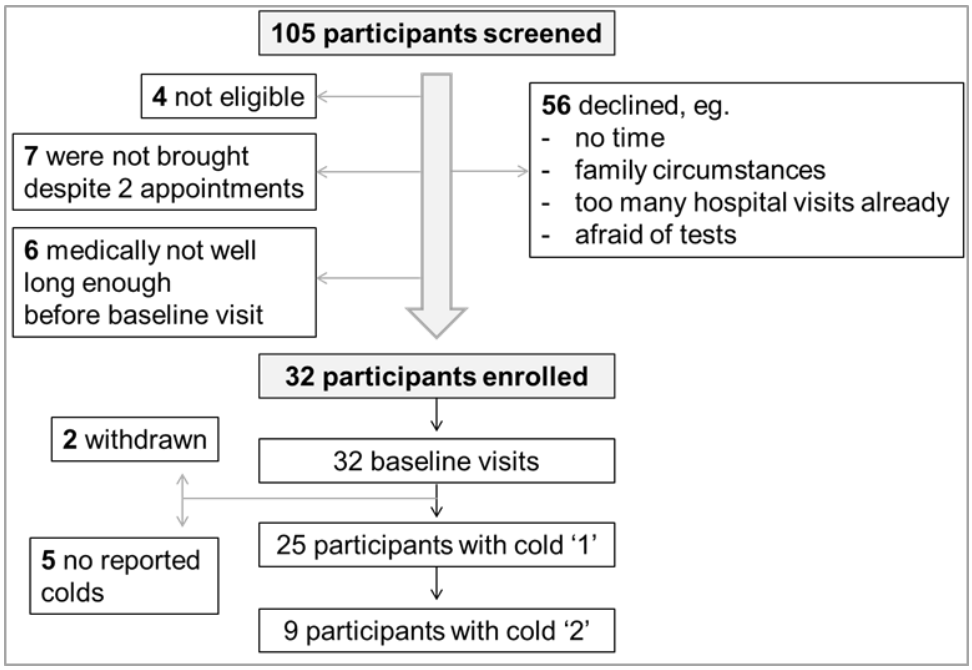


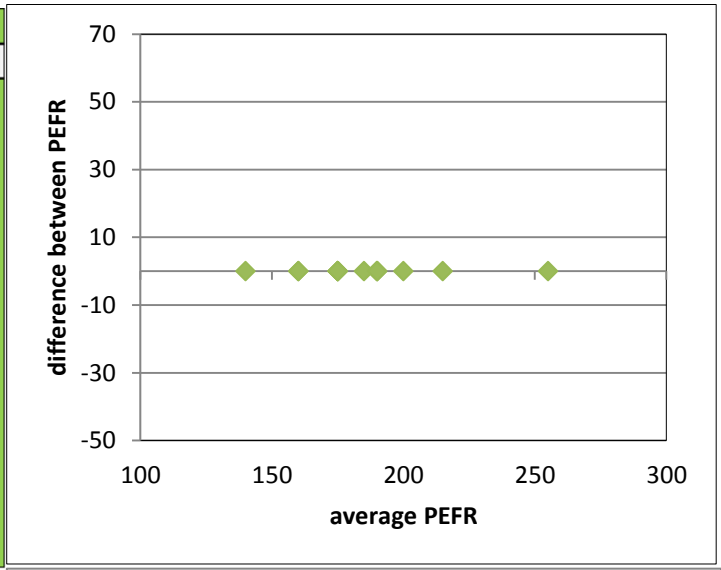
Figure 4.1 Flow sheet of all participants screened for participation in study

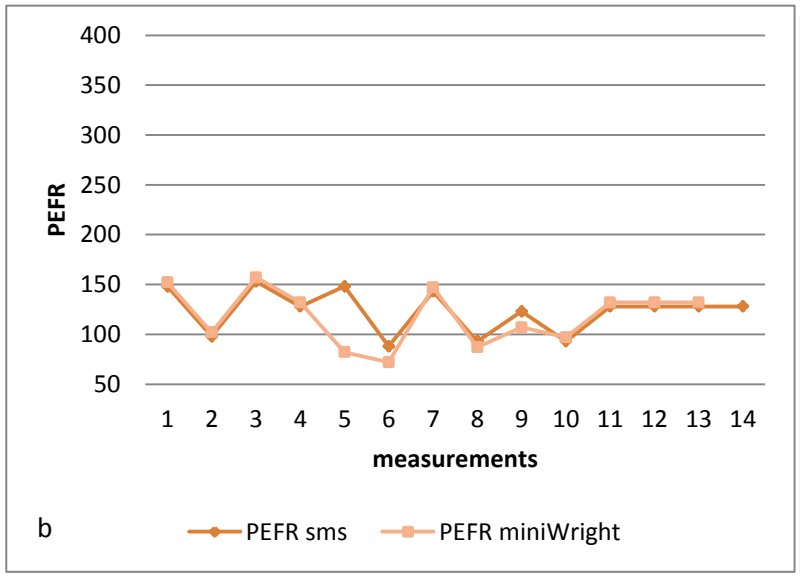
4.3.1 Compliance

The overall 'SMS-diary-compliance' during baseline (32 participants) and during a cold episode (25 participants) was 96% (±8) and 91% (±12) respectively. The overall 'PEFR-meter compliance' during baseline (26 participants) and during a cold episode (25 participants) was 84% (±21) and 82% (±20) respectively. Illustrative examples of participants with different levels of compliance are given in Figure 4.3.

a

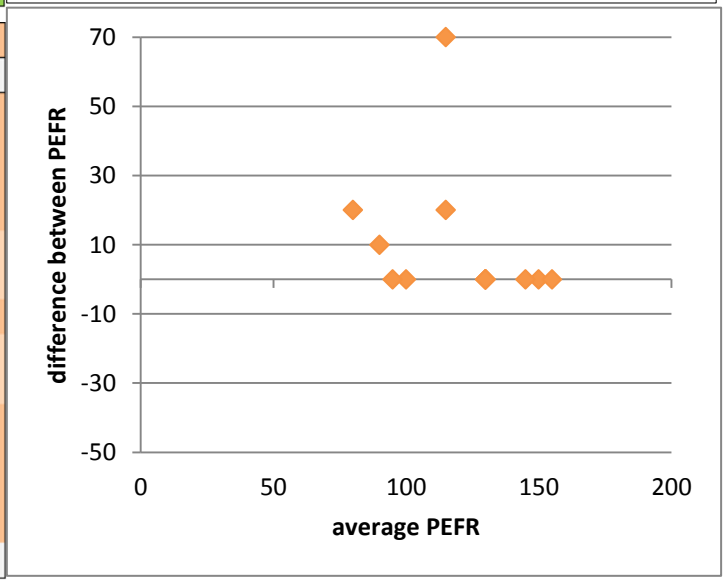
	Participant V014	
	sms	PEF meter
D1 am	200	200
D1 pm	215	215
D2 am	175	175
D2 pm	190	190
D3 am	175	175
D3 pm	190	190
D4 am	175	175
D4 pm	175	175
D5 am	160	160
D5 pm	185	185
D6 am	255	255
D6 pm	190	190
D7 am	140	140
D7 pm	160	160

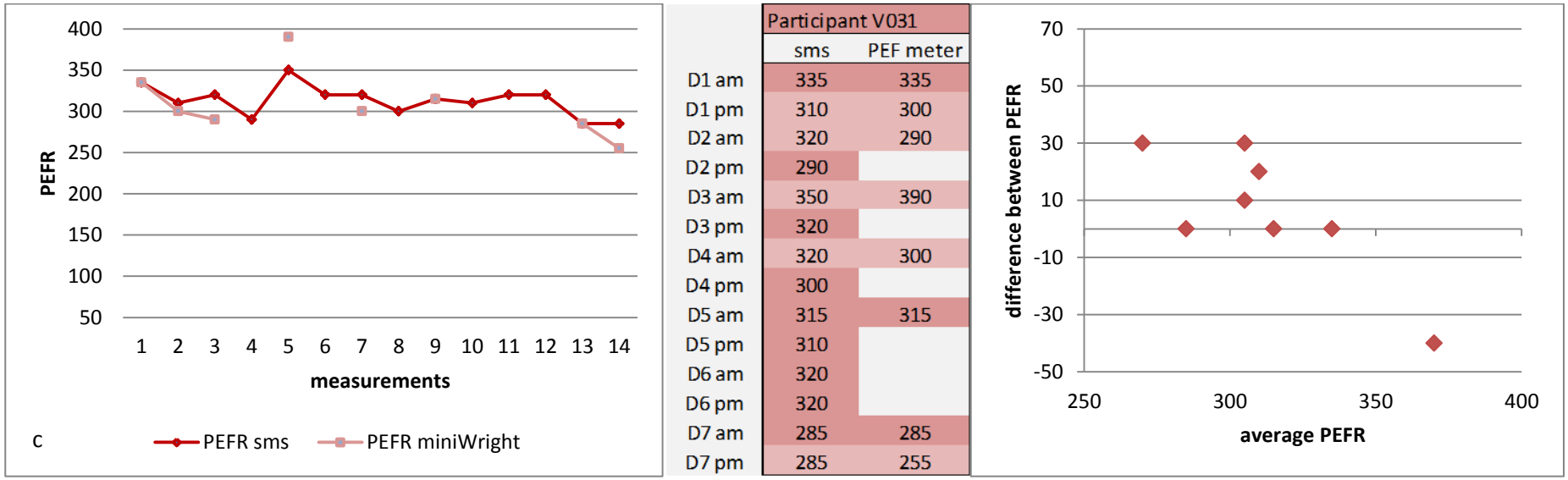




b

	Participant V018	
	sms	PEF meter
D1 am	150	150
D1 pm	100	100
D2 am	155	155
D2 pm	130	130
D3 am	150	80
D3 pm	90	70
D4 am	145	145
D4 pm	95	85
D5 am	125	105
D5 pm	95	95
D6 am	130	130
D6 pm	130	130
D7 am	130	130
D7 pm	130	





	Participant V031	
	sms	PEF meter
D1 am	335	335
D1 pm	310	300
D2 am	320	290
D2 pm	290	
D3 am	350	390
D3 pm	320	
D4 am	320	300
D4 pm	300	
D5 am	315	315
D5 pm	310	
D6 am	320	
D6 pm	320	
D7 am	285	285
D7 pm	285	255

Figure 4.2 Peak flow data during baseline - comparison of SMS and digitally stored data

Peak flow data during baseline week – comparison of data sent via SMS and digitally stored on PEFR meter as diary charts (left pane), comparison of measurements (central pane) and Bland-Altman plot (right pane). a. Participant V014 with 100% 'SMS-diary-compliance' and 'PEFR-meter-compliance' and 100% 'correct' values. b. Participant V018 with 100% 'SMS-diary-compliance', 93% 'PEFR-meter-compliance' and 64% 'correct' values. c. Participant V031 with 100% 'SMS-diary-compliance', 57% 'PEFR-meter-compliance', 21% 'correct' values and 43% 'self-invented' values.

4.3.2 Validity

The median percentage of correctly reported PEFR values for all the participants (baseline and cold weeks combined) was 65.5% (32.7, 82.1) with the lowest value being 7.1% in one participant and up to 100% in others (see Table 4.1). There was a statistically non-significant difference between reporting during the baseline week, and the first and second cold weeks (see Table 4.2). In 8.3% (2.4, 15.5) of all cases PEFR values sent were 'self-invented' and did not have a time-tagged correlate on the PEFR meter (57% in one participant) and 2.4% (0.0, 0.0) of values were missing. For the purpose of this study the actual digitally stored PEFR values were used for further analysis of data and these were available in 83% of all cases. Figure 4.2 shows three examples of participants' compliance with SMS-diary and PEFR-meter and the validity of data.

4.3.3 Acceptability

92% (22/24) and 83% (20/24) of parents completed the baseline and follow up questionnaires respectively. Parents of participating children were in general very familiar with the use of mobile phones and text messages: 86% (19/22) have had a mobile phone for more than five years, 59% (13/22) had a mobile phone contract and 73% (16/22) sent at least 50 SMS a month. A quarter of parents were familiar with the use of text messages in the context of appointment and school reminders, car parking, bills, surveys and buying concert tickets. None of them had used SMS in the context of a research study before.

All (22/22) were 'very happy' or 'happy' to use SMS for this study. 95% (19/20) found the system user-friendly and 55% (11/20) said that they would more likely participate in a study if it was using SMS for data collection. 25% (5/20) felt 'sometimes unhappy' about receiving the SMS with 'lack of time' or being 'at work' mentioned as reasons. Issues with mobile phone reception (signal) were mentioned by two participating parents. See Table 4.3 for more details.

	Baseline week	Cold - first week	Cold – second week	All weeks
PEF sent / PEF measured / corresponding	**71.4** (42.9, 78.6)	**50.0** (17.9, 82.1)	**64.3** (32.1, 82.1)	**65.5** (32.7, 82.1)
PEF sent / PEF measured / not corresponding	**14.3** (7.1, 28.6)	**14.3** (7.1, 28.6)	**14.3** (3.6, 35.7)	**15.5** (10.1, 23.2)
PEF sent / PEF not measured	**7.1** (0.0, 21.4)	**7.1** (0.0, 17.9)	**7.1** (0.0, 17.9)	**8.3** (2.4, 15.5)
PEF not sent / PEF measured	**0.0** (0.0, 0.0)	**0.0** (0.0, 0.0)	**0.0** (0.0, 0.0)	**0.0** (0.0, 0.0)
PEF not sent / PEF not measured	**0.0** (0.0, 0.0)	**7.1** (0.0, 7.1)	**0.0** (0.0, 3.6)	**2.4** (0.0, 4.8)
Number of text messages	**350**	**322**	**322**	**994**

Table 4.1 Validity of PEFR values

Validity of PEFR values during Baseline week compared with Cold Week 1 and Cold Week 2. Figures are median (25th, 75th percentile) percentages of correct, incorrect, self-invented, not sent, and missing values. ‘sent’ refers to values transmitted via SMS. ‘measured’ refers to values digitally stored on PEFR meter. Each potential scenario (i.e. correct, incorrect, self-invented, not sent, and missing values) is considered separately in the data of the table. Therefore, a median of all numbers in one scenario does not directly relate to the median of another and the sum of the medians will not equal 100 percent.

Text message system – Feedback Questionnaires					
Baseline Questionnaire					
1. How do you feel about using text messages as a way of providing information to the research team about your child's cold and asthma symptoms? **(n=22)**	Very happy **16 (73%)**	Happy **6 (27%)**	Neutral **0**	Unhappy **0**	Very unhappy **0**
Follow up Questionnaire					
1. Did you reply to the text messages every day? **(n=20)**	All of the time **10 (50%)**	Most of the time **10 (50%)**	Some of the time **0**	Hardly ever **0**	
2. At times you did not reply to the text messages, what was the reason for it (name all)? **(n=20)**	Lack of time **4 (20%)**	At work **2 (10%)**	Forgot about it **1 (5%)**	No credit on phone **2 (10%)**	Other* **4 (20%)**
3. Did you feel annoyed or unhappy at any stage about receiving text messages related to our research study? **(n=20)**	No, never **14 (70%)**	Rarely **1 (5%)**	Sometimes **5 (25%)**	Once a week **0**	Every day **0**
4. If you decided to take part in another research study, would the fact that it also uses text messaging as a way of gathering data…	Make it more likely for you to participate in the study? **11 (55%)**		Not influence your decision to take part either way? **9 (45%)**		Put you off from participating? **0**

Table 4.2 Acceptability of using SMS for collecting research data

* mostly related to poor phone reception.

4.4 Discussion

SMS has previously been used for data collection in several medical studies. Response rates were compared between a text messaging system (80.2%) and a card response (85.6%) method in a survey about satisfaction with GP consultations (Haller et al., 2009). An RCT comparing SMS, paper and online diaries to collect details of weekly sexual behaviour found SMS to be a convenient, timely data collection tool (Lim et al., 2010). A mobile phone text message service consisting of daily reminders to use an inhaler, health education tips, and safety messages was used in a feasibility and acceptability study of 32 people with asthma between 10 and 46 years of age. The system was well received (Neville et al., 2002).

The parents of children participating in this study received and had to respond to 42 text messages during a baseline week and 70 text messages over fourteen days during a cold episode. In addition, children were asked to use a digital peak flow meter twice a day to record their PEFR and then transmit this information as part of this SMS diary. This clearly asked for a considerable commitment to the study. Despite this, the average 'SMS-diary-compliance' during baseline period and cold episode was 93.5% and the majority of SMS transmitted PEFR data was validated by recorded digital peak flow meter data with only a small minority of data recorded inaccurately. In general, therefore, the SMS message system was a very acceptable approach for families to collecting research data.

Parents were aware that the digital peak flow meters stored the PEFR values which would be downloaded at the end of the study. It may therefore seem surprising that the overall SMS-transmission rate of correct PEFR values was only 65.5%; additionally in 8.3% of cases PEFR values appeared to be 'self-invented' by parents. This phenomenon is usually a feature of falsified paper-diaries that often get retrospectively completed with invented values just before the clinic visit (Stone et al., 2002). In a study trying to uncover barriers and benefits associated with internet-based self-management, 97 adolescents with asthma were asked to enter PEFR values daily into a designated web-application or to send them via SMS over a four-week period (van der Meer et al., 2006). They did not know that the spirometer stored the values and that data could be downloaded subsequently. The overall compliance was 90% with correct reporting of PEFR values on 79% of days. The compliance of the participants in this study was slightly lower, perhaps because they were not routinely followed immediately by feedback and/or therapeutic consequences as parents were advised to obtain medical advice as they would usually do. This might have negatively influenced

compliance but would be an issue that could be addressed relatively easily. PEFR values were also less likely to be correctly reported in this study than in the adolescent study (van der Meer et al., 2006). Around 15% of PEFR values recorded on the peak flow meter were either wrongly transmitted via SMS and some were not sent at all. This may partly be a reflection of the study design as, due to the age of the children, parents were asked to send the PEFR via their mobile phone and not the children themselves. This has led to a split between the performance of PEFR meter measurements by the children and the electronic transmission of the PEFR value by parents. While some children may have been closely supervised performing the PEF manoeuvre and the PEFR value immediately read and transmitted by parents, others may have performed measurements independently and only informed their parent of a value if asked for it. This would lead to an increased risk of errors that can possibly be avoided if the operator of the digital PEFR meter and the mobile phone are the same or the PEFR meter transmitted the data electronically to the researcher's data base. However, despite a surprisingly high percentage of incorrect values transmitted via SMS, a closer look at the actual PEFR values (see Tables 4.4 and 4.5) reveals that the median difference (and 25^{th} and 75^{th} percentile) between SMS transmitted PEFR values and PEFR meter stored values in most cases are zero.

All parents were happy to use SMS messaging for transmission of research data despite none having previously used SMS in a research context. More than half of them were more likely to participate in a study again if SMS were used for collecting research data, which may point to the convenience and ease of using SMS for data transfer. During the study parents received very frequent SMS and were subsequently reminded if no response had been received. Given the frequency of transmission, it was unavoidable that an SMS would, on occasion, be received at an inconvenient time. Although participants could choose convenient times at the start of the trial, a more flexible approach to adjust timing during the study may have improved this issue.

The real-time capture of data appears to be well accepted and could avoid some of the pitfalls of backfilled paper diaries although the validity of the data is not absolute. Some of the discrepancies between PEFR meter values and SMS-transmitted values may be avoided in older children or teenagers who could operate both devices (PEFR meter and mobile phones) independently. SMS messaging is a relatively low cost approach to collecting data in follow up studies involving children and young people.

Participant	No of SMS data points (max N=14)	No of MW data points (max N=14)	Mean PEFR SMS data	SD SMS data	Mean PEFR MW data	SD MW data	Median difference SMS vs MW	25th, 75th percentile
V001	14	14	162	12,65	165	19,16	0	-10, 0
V002	14	8	120	9,81	118	14,39	0	0, 3.75
V003	14	6	175	7,84	168	14,02	2.5	0, 8.75
V004	14	-	259	29,17				
V006	14	13	170	28,59	186	53,63	0	0, 0
V007	14	-	235	51,96				
V008	14	-	137	25,02				
V009	14	-	189	12,62				
V010	13	-	128	25,44				
V011	14	9	126	21,43	122	27,04	0	0, 10
V012	10	6	208	22,75	214	23,54	0	0, 0
V013	14	-	174	39,17				
V014	14	14	185	27,42	185	27,42	0	0, 0
V015	10	14	152	18,27	157	19,29	0	0, 0
V016	14	13	208	20,36	207	20,86	0	0, 0
V017	14	14	201	11,07	201	10,77	0	0, 0
V018	14	13	125	22,05	116	28,13	0	0, 0
V019	14	14	216	48,13	208	41,40	0	0, 0
V020	14	14	206	24,76	214	21,32	0	0, 0
V021	14	11	386	18,90	381	18,18	0	0, 25
V022	13	10	133	14,37	133	14,76	0	0, 0
V023	11	5	145	10,60	142	13,51	0	0, 0
V024	14	13	235	26,35	233	28,69	0	0, 0
V025	14	12	100	27,91	100	28,76	0	0, 10
V026	14	13	193	24,78	192	26,35	0	0, 0
V027	14	14	140	22,66	162	21,00	-12.5	-43.75, 0
V028	14	14	160	21,49	176	26,30	-12.5	-36.25, 6.25
V029	13	14	157	48,24	161	45,86	0	0, 0
V030	12	14	153	20,28	153	17,72	0	0, 0
V031	14	8	313	18,37	309	40,16	5	0, 22.5
V032	14	13	145	32,88	146	36,12	0	0, 0
V033	13	12	196	44,54	190	42,34	0	0, 0

Table 4.3 Validity of PEFR data - Median difference and 25th and 75th percentile of SMS versus PEFR meter data.

Validity of PEFR values showing mean and standard deviation of PEFR values sent by SMS and PEFR meter recorded values during baseline week for individual research participants and median difference and 25th and 75th percentile of SMS versus PEFR meter data. MW = miniWright® (digital PEF meter); SD = standard deviation.

Participant	No of SMS data points (max N=28)	No of MW data points (max N=28)	Mean SMS data	SD SMS data	Mean MW data	SD MW data	Median difference SMS vs MW	25th, 75th percentile
V001	27	26	170	17,32	174	22,05	-5	-10, +5
V002	27	10	119	12,33	137	12,26	-5	-17.5, 0
V003	28	19	168	25,06	174	32,01	0	-15, 0
V004	-	-						
V006	25	26	121	22,58	131	30,96	0	-5, 0
V007	-	-						
V008	-	-						
V009	-	-						
V010	27	18	146	18,73	136	28,33	0	-3.75, +5
V011	16	4	100	0,00	80	21,60	30	25, 35
V012	25	25	241	34,63	251	27,94	0	-10, 0
V013	-	-						
V014	28	27	150	29,60	148	28,53	0	0, 0
V015	23	27	168	18,39	168	18,55	0	0, 0
V016	24	24	229	27,88	229	27,88	0	0, 0
V017	23	25	189	11,70	188	13,82	0	0, 0
V018	28	26	140	14,04	141	14,51	0	0, 0
V019	15	25	174	46,29	193	46,88	0	0, 0
V020	28	26	160	33,01	156	23,68	0	0, 0
V021	28	26	348	43,78	350	45,85	0	0, 0
V022	28	27	109	23,95	113	19,03	0	0, 0
V023	28	28	143	28,56	143	28,59	0	0, 0
V024	26	24	193	31,85	189	37,63	0	0, 0
V025	25	20	125	37,73	122	42,07	0	0, 0
V026	-	-						
V027	28	27	187	37,30	188	38,11	0	0, 0
V028	28	24	153	22,23	163	25,66	-10	-15.5, -5
V029	-	-						
V030	27	27	162	23,30	162	23,30	0	0, 0
V031	26	17	296	27,10	291	36,08	0	0, 5
V032	28	22	160	25,31	160	30,12	0	0, 0
V033	24	22	273	40,19	277	34,52	0	0, 0

Table 4.4 Validity of PEFR data - Median difference and 25th and 75th percentile of SMS versus PEFR meter data.

Validity of PEFR values showing mean and standard deviation of PEFR values sent by SMS and PEFR meter recorded values during cold week for individual research participants and median difference and 25th and 75th percentile of SMS versus PEFR meter data. MW = miniWright® (digital PEF meter); SD = standard deviation.

5 Respiratory viruses and asthma

5.1 Introduction

Respiratory viruses, in the context of childhood asthma, have drawn considerable interest for two main respects: firstly in relation to early life influences and pathogenesis of asthma and secondly as infective agents considered the main culprits in triggering asthma exacerbations.

Asthma induction in early childhood appears to be driven by two main environmental factors: early postnatal sensitization to perennial aeroallergens and viral infections of the lower respiratory tract (Wu et al., 2008, Kusel et al., 2007, Jackson et al., 2008, Stein et al., 1999, Sly et al., 2008). Multiple studies have documented the influence of these two factors on asthma development. While both appear to be able to independently further the development of asthma in early childhood, the risk seems to be higher if they happen to trigger early airway inflammation in concert (Holt and Sly, 2012).

There are now a large number of studies that, supported by advances in molecular detection techniques such as RT-PCR, have shown the strong association of asthma exacerbations with viral respiratory tract infections. Most studies report that over 80% (range between 40-90%) of children with acute wheezing episodes have a respiratory virus infection of which human rhinoviruses are the most commonly detected viruses (Johnston et al., 1995). While it seems intuitive to assume that the isolation of respiratory viruses (especially rhinovirus) from the airways during an asthma exacerbation have a causative link to the exacerbation itself, a number of studies have detected these viruses both in asymptomatic healthy children as well as asymptomatic children with asthma. Johnston *et al*. demonstrated a frequency of 12% (8/65) of samples positive for picornavirus from asymptomatic children (Johnston et al., 1995). Leung *et al*. showed human rhinovirus positivity in 13% of subjects with stable asthma who did not experience any symptoms or signs of disease exacerbation (Leung et al., 2010). In a longitudinal study of healthy children the percentage of asymptomatic RV infections was even higher (20.6%) (Winther et al., 2006) and it is becoming clearer that one needs to be cautious to automatically assume causality between HRV detection and a specific pattern of respiratory tract illness, and especially so between HRV and asthma symptoms (Olenec et al., 2010).

5.2 Objectives

The objectives of this part of the study were:

i. To describe the detection of respiratory pathogens in a cohort of children with asthma with regards to frequency of occurrence, type, and seasonality

ii. To establish the incidence of multiple infection with two or more respiratory pathogens

iii. To describe the detection rates of viruses in saliva in comparison to nasal lavage (gold standard) during cold episodes

iv. To explore the potential association of virus status with different clinical syndromes (asymptomatic, cold only, cold + asthma), with AUC and peak of Asthma Index (Sorkness et al., 2008) and Jackson cold score (Jackson et al., 1958), and with symptoms/signs as reported by parents

5.3 Results

5.3.1 Participant monitoring and classification

Between January 2011 and February 2012 thirty-two children between 5 and 11 years of age participated in the study. All 32 participants underwent a baseline visit during which nasal lavage and saliva samples were collected. A total of 25 participants developed colds, 9 of these developed 2 cold episodes. If possible, visits were conducted within 48 hours of initial cold symptoms and a further visit between the 4th and 6th day of the cold. Eighty-two nasal lavage samples and 84 saliva samples were collected, pre-processed and stored for further analysis in this process.

Participants were monitored for 7 days post their initial baseline visits with a twice daily SMS-transmitted Jackson Cold Score questionnaire and an asthma symptom questionnaire. Both questionnaires were also answered for 14 days following initial cold symptoms.

Based on the classification by Reddel *et al*. (2009) (Reddel et al., 2009) and as outlined in paragraph 2.2.3 the severity of viral induced asthma exacerbation of the participants was determined. In total there were 34 cold episodes - 5 that did not result in any asthma exacerbation, 23 with a moderate exacerbation and 6 with a severe exacerbation and an overview of these results are presented in 5.3.2.1. As 9

participants developed 2 cold episodes, and in order to avoid duplication, the final dataset contained 25 separate cold episodes.

5.3.2 Samples included in analysis for respiratory pathogens

A total number of 166 biological samples (82 nasal lavages/84 saliva samples) from 32 children were collected throughout the study. The following pragmatic approach to analysis was taken:

Sample included	Rationale	Number of samples
Baseline nasal lavage samples	To demonstrate "virus-free" status of asymptomatic children	31
Nasal lavage samples within 48 hours of first cold symptoms	To demonstrate presence of virus(es) in children's nasal passages during cold symptoms	31 [cold 1 (23), cold 2 (8)]
Saliva samples within 48 hours of first cold symptoms	To demonstrate whether we can replicate the nasal lavage results (validation study)	24 [cold 1]
	Total Number of Samples	86

Table 5.1 Samples included in analysis for respiratory pathogens

5.3.2.1 Overview over ALL cold episodes

The analysis of all nasal lavage samples and saliva samples obtained during all cold episodes (34) yielded 29 virus-positive cold episodes (85%) with 35 viruses (28 viruses between October and March), and 7 multiple infections (Figure 5.1): 6 dual infections and 1 triple infection (representing 21% of the 33 episodes for which samples were available). A breakdown of the detection of multiple infection in nasal lavage and saliva individually and combined will be provided in paragraph 5.4.6. No specimen or specimen combination contained more than three pathogens. The triple infection comprised bocavirus, coronavirus OC43 and *Mycoplasma pneumoniae*. Further combinations found were rhinovirus and bocavirus (6.1% of episodes, 2/33), rhinovirus and parainfluenza virus 3, parechovirus and parainfluenza virus 3, rhinovirus and coronavirus OC43, and rhinovirus and *Mycoplasma pneumoniae* (3.0%, 1/33 each).

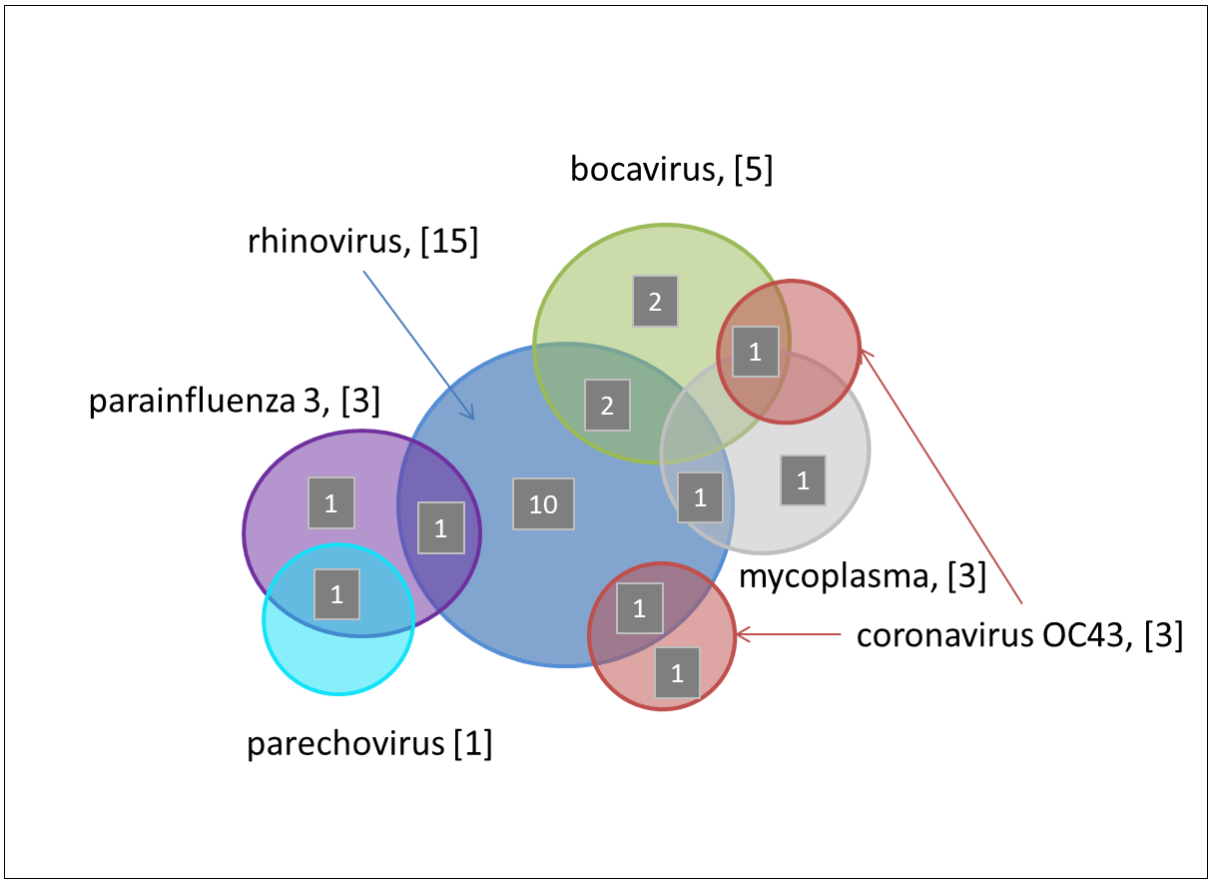


Figure 5.1 Graphical depiction of multiple respiratory pathogen infections (all cold episodes included)

Each circle represents an individual respiratory pathogen. Dual infection is represented by circle intersection. Figures in intersections represent total number of episodes where pathogen(s) was identified in either nasal lavage or saliva or both. Figures in square brackets indicate the total number each pathogen was identified during the cold episodes. There was also one dual infection during baseline (not shown).

5.3.3 Viruses detected in baseline samples

Respiratory pathogens were identified in 13/31 (41.9%) of the baseline nasal lavage samples analysed (Figure 5.2). A total number of 14 viruses were detected, as one child showed presence of 2 viruses (rhinovirus and bocavirus) in their nasal lavage. The majority of viruses at baseline were rhinoviruses (9, 64%), followed by bocavirus (3, 21%). Metapneumovirus and HKU1, a human coronavirus, were only discovered once each.

5.3.3.1 Monthly distribution (seasonality)

The study period covered January 2011 to May 2012. Baseline visits were conducted up until the end of February 2012. Figure 5.3 represents the distribution of pathogen

positive baseline nasal lavage samples on a monthly basis. Baseline visits during January 2011 and 2012 were merged as were those conducted during February 2011 and 2012. During the colder 6 months (October – March), 10 samples were positive representing 43.5% (10/23) positive samples per participants assessed during this period. This compares to 3 positive samples representing 33.3% (3/9) positive samples per participants assessed during the warmer half of the year (April – September). A chi-square test was performed and no relationship was found between month of baseline and virus status, X2 (1, N = 32) = 0.385, p =0.54. Rhinovirus was the only type of virus detected at baseline visits conducted between and including the months of March and September.

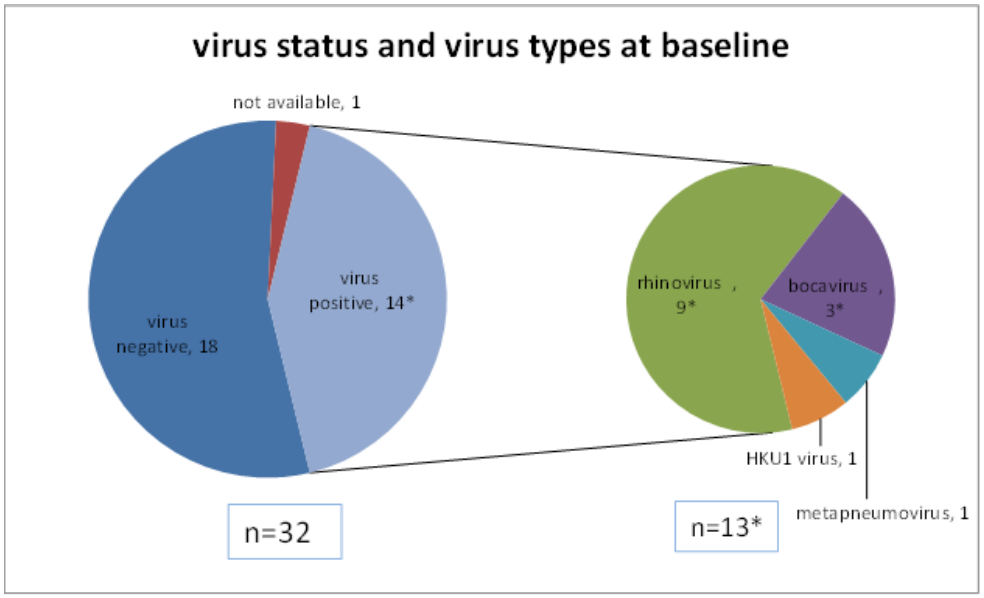


Figure 5.2 Virus status during baseline visits

Virus status as determined by RT-PCR of nasal lavage samples obtained during baseline visits. Types of viruses found in baseline samples. *One child had dual presence of rhinovirus and bocavirus, which explains the slight discrepancy when adding up the numbers.

	Baseline		Cold episode	
	episodes	samples	episodes	samples
	n=32	*n=31*	*n=25*	*n=24*
Nasal Lavage (NL)				
Virus positive	13 (40.6%)	**13 (41.9%)**	21 (84.0%)	**21 (87.5%)**
Virus positive (⩾2 viruses)	1 (3.1%)	**1 (3.2%)**	2 (8.0%)	**2 (8.3%)**
Virus negative	18 (56.3%)	**18 (58.1%)**	3 (12.0%)	**3 (12.5%)**
No sample / not tested	1	0	1	0
	episodes	samples	episodes	samples
	n=32	*n=31*	*n=25*	*n=20*
Saliva (S) samples				
Virus positive	-	-	13 (52.0%)	**13 (65.0%)**
Virus positive (⩾2 viruses)	-	-	2 (8.0%)	**2 (10.0%)**
Virus negative	-	-	7 (28.0%)	**7 (35.0%)**
No sample / not tested	32	31	5	0
	episodes	samples	episodes	paired samples
	n=32	*n=31*	*n=25*	*n=25*
Total (NL + S)				
Virus positive	13 (40.6%)	13 (41.9%)	**22 (88.0%)**	22 (88.0%)
Virus positive (⩾2 viruses)	1 (3.1%)	1 (3.2%)	**4 (16.0%)**	4 (16.0%)
Virus negative	18 (56.3%)	18 (58.1%)	**3 (12.0%)**	3 (12.0%)
No sample / not tested	1		**0**	

Table 5.2 Summary of virus status in nasal lavage and saliva during baseline and cold episode

One cold episode per participant was included only in case of 2 episodes monitored. The denominator for NL samples during cold episode is 24, as one participant refused NL. The denominator for saliva samples during cold episode is 20 as one participant did not have saliva sampled and the specific saliva for the included cold episode had not been tested in four participants. For each cold episode at least one sample type (NL or S) was available.

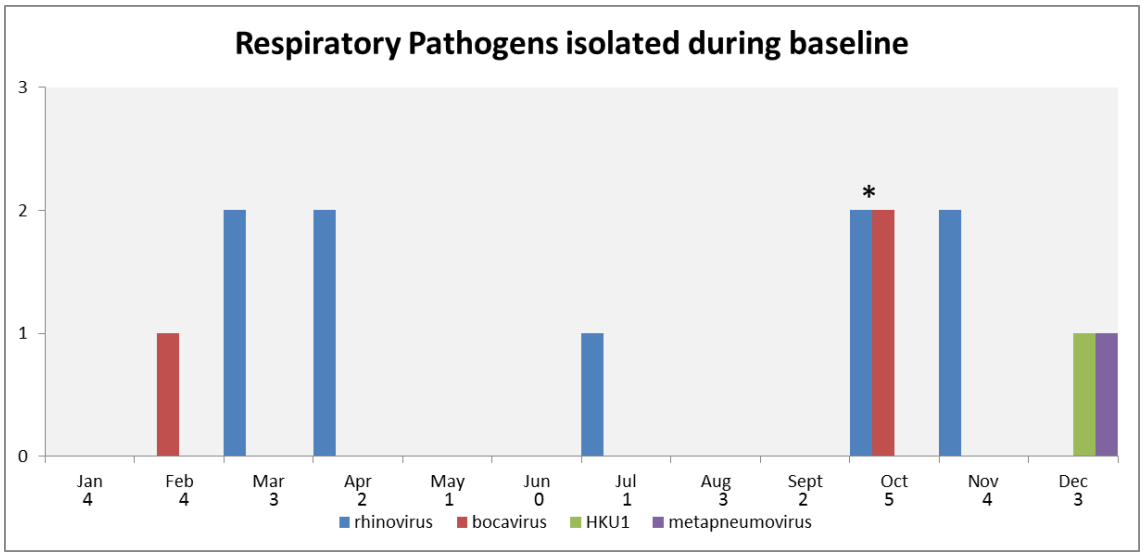


Figure 5.3 Monthly distribution of respiratory pathogens isolated during baseline
The asterix indicates one dual infection of rhinovirus and bocavirus. The numbers next to the x-axis signify numbers of baseline visits during each month.

5.3.4 Viruses detected in samples during cold episodes

Nasal lavage samples and saliva samples obtained during cold episodes tested positive for respiratory pathogens in 21/24 (87.5%) and 13/20 (65%) respectively (Table 5.2). No virus was detected in 2 (8.3%) nasal lavage and 7 (35%) saliva samples, and 2 samples of both sample types contained more than 1 viral pathogen. Rhinovirus was the most frequently detected pathogen being identified in 44% (11/25) of cold episodes followed by bocavirus (16%, 4/25), metapneumovirus, coronavirus OC43, parainfluenza virus 3 and influenza A virus (8%, 2/25).

5.3.4.1 Monthly distribution (seasonality)

The study period covered January 2011 to May 2012. Where more than one cold episode was captured, only one was included in this analysis (see paragraph 3.4.5 for algorithm which cold episode was included). Figure 5.4 represents the distribution of pathogen positive nasal lavage and saliva samples on a monthly basis. Cold visits during January 2011 and January 2012 were merged as well as the subsequent (duplicate) months till May. 19 viruses (17 cold episodes) were detected during the months of October to March with an average number of 18 (13-23) children being monitored for colds each month. 7 viruses (5 cold episodes) were detected during the months of April to September with an average number of 12 (7-20) children being monitored for colds each month (see Table 3.4 in clinical chapter). A chi-square test was performed and no relationship was found between season of cold episode and virus status, $X2$ (1, N = 25) = 0.163, p =0.69.

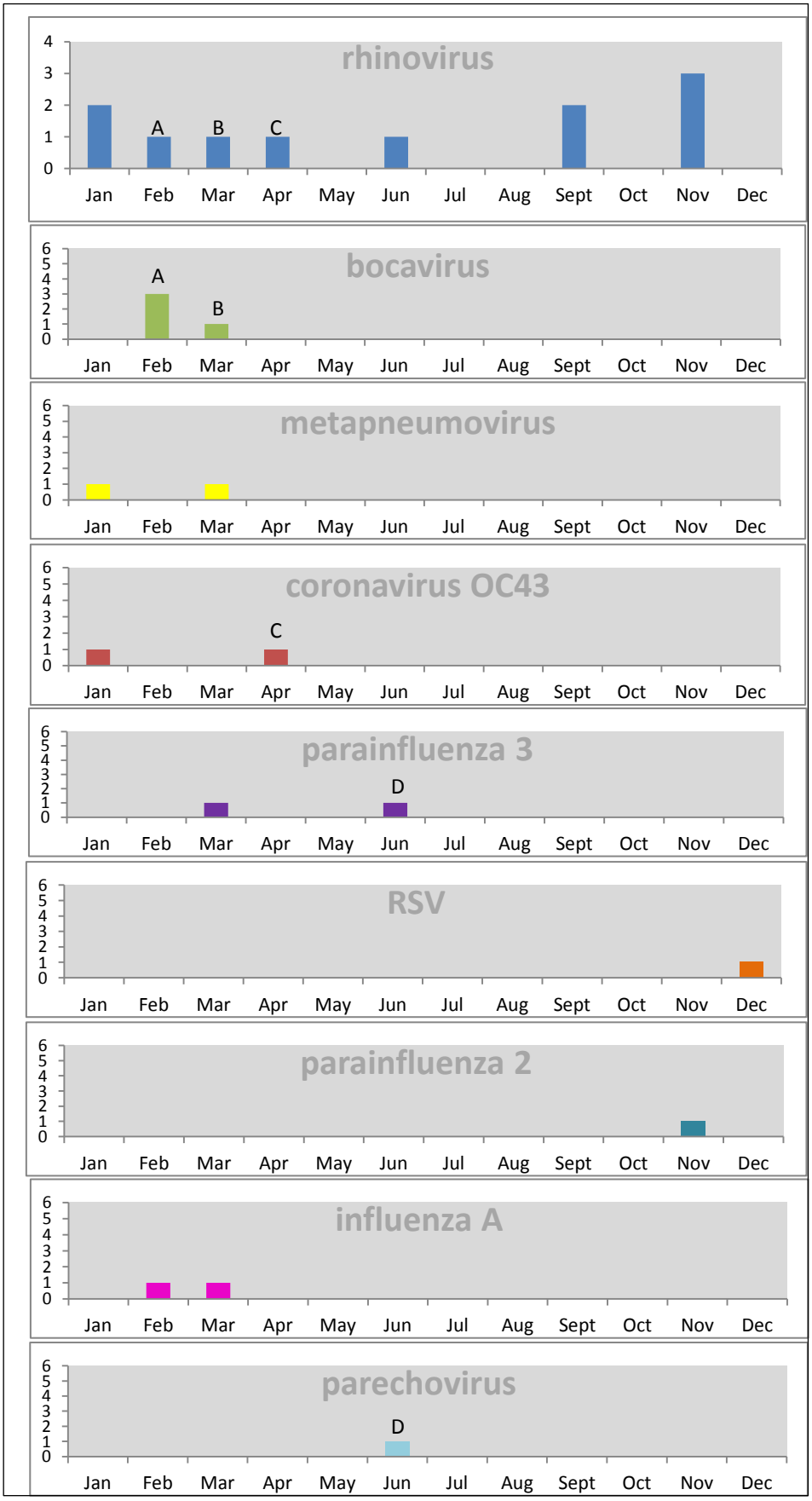


Figure 5.4 Monthly distribution of respiratory pathogens identified in nasal lavage and saliva
(one cold episode per participant). Multiple infections are depicted by the letters A-D.

5.3.5 Incidence of multiple infections

5.3.5.1 Baseline samples

Dual pathogen infection (with rhinovirus and bocavirus) was identified in 1 (3%) of the 31 baseline nasal lavage samples analysed (see Figures 5.2 and 5.3).

5.3.5.2 Cold episode samples

The analysis of the nasal lavage and saliva samples of the 25 cold episodes included in the analysis (one cold episode per participant) yielded 4 dual infections (representing 16% of cold episodes). One dual infection (parainfluenza virus 3 and parechovirus) was found in saliva only (with parainfluenza 3 also present in nasal lavage), one dual infection (rhinovirus and bocavirus) was found in nasal lavage only (with rhinovirus also present in saliva), one dual infection (rhinovirus and bocavirus) was found in both nasal lavage and saliva and one dual infection (rhinovirus and coronavirus OC43) arose from the combination of nasal lavage and saliva results (see Figure 5.5).

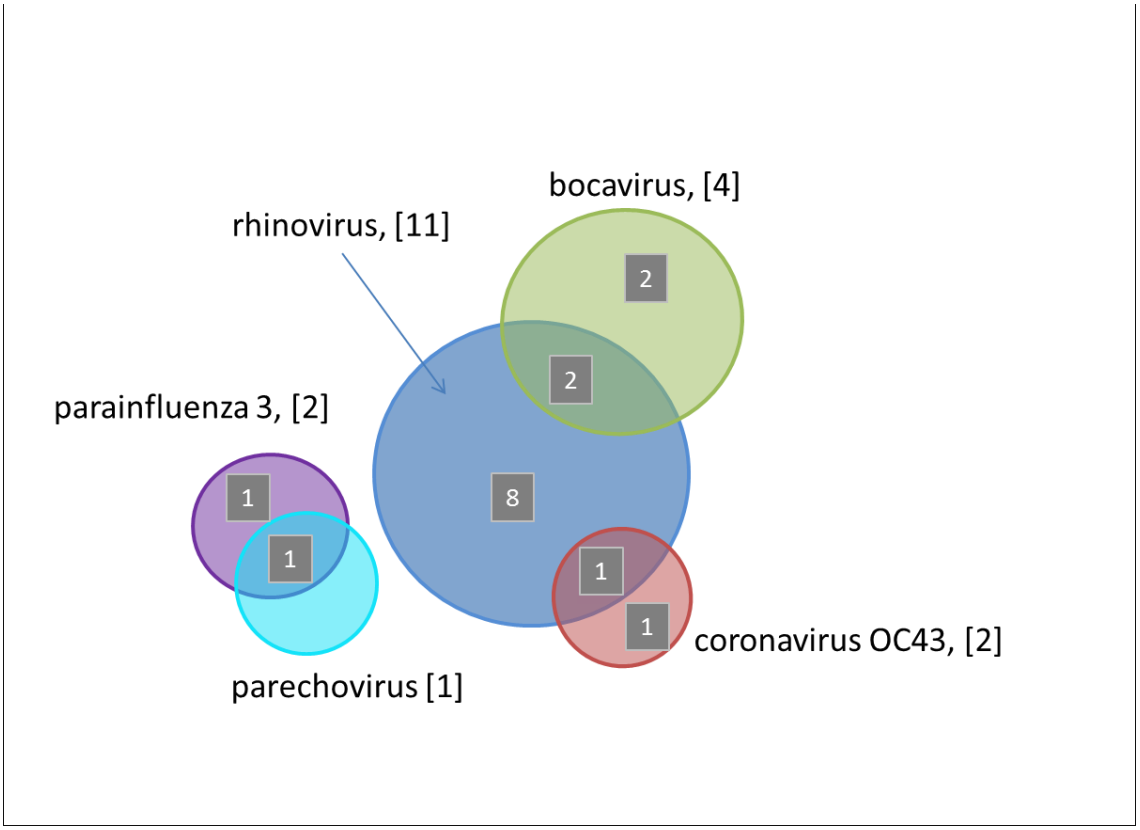


Figure 5.5 Graphical depiction of multiple respiratory pathogen infections (one cold episode per participant)

Each circle represents an individual respiratory pathogen. Dual infection is represented by circle intersection. Figures in intersections represent total number of episodes where pathogen(s) was identified in either nasal lavage or saliva or both. Figures in square

brackets indicate the total number each pathogen was identified during the cold episodes (one episode per participant).

5.3.6 Detection rates of respiratory pathogens in saliva in comparison to nasal lavage

23 (complete) pairs of nasal lavage and saliva were available for analysis for presence of respiratory pathogen DNA/RNA. These paired samples are all derived from the first cold of each participant (which in 4 cases was not the cold episode included in the rest of the analysis presented earlier). Table 5.4 gives an overview of the results by specimen type individually and looking at paired nasal lavage and saliva samples. The latter yielded a total virus detection rate of 87% (nasal lavage: 83%, saliva: 61%) and a multiple virus detection rate of 26% (nasal lavage: 17.4%, saliva: 13%). In 12 cases (8 single virus infections, 1 dual virus infection, 3 negative PCRs) nasal lavage and saliva PCR yielded the exact same result. In one participant the combination of two pathogens detected in nasal lavage and two pathogens detected in saliva yielded a triple infection (see Figures 5.7 and 5.8).

The 23 nasal lavage samples combined contained 23 respiratory pathogens, 13 of which were found in the corresponding saliva samples also. 10 nasal lavage samples and 4 saliva samples yielded positive PCR results that were not replicated in the corresponding paired specimen type (see Figure 5.6). Cohen's κ was run to determine if there was agreement between the PCR-determined virus status of the two sample types (Table 5.4). There was fair agreement between nasal lavage and saliva virus status, $\kappa = .345$, $p = .016$ with neither nasal lavage or saliva detecting all viruses (Landis and Koch, 1977).

	Nasal lavage only (n=23)	Saliva only (n=23)	Nasal lavage + saliva (n=23 pairs)
No virus detected	4 (17.4%)	9 (39.0%)	3 (13.0%)
Single virus detection	15 (65.2%)	11 (47.8%)	14 (60.9%)
Multiple virus detection	4 (17.4%)	3 (13.0%)	6 (26.1%)

Table 5.3 Overview of positive and negative viral PCR

by looking at nasal lavage only, saliva only, and looking at paired nasal lavage and saliva samples.

		Nasal lavage			Total
		none	single	multiple	
Saliva	none	3 (75%)	5 (33%)	1 (25%)	9
	single	1 (25%)	9[a] (60%)	1 (25%)	11
	multiple	0 (0%)	1 (7%)	2[b] (50%)	3
Total		4 (100%)	15 (100%)	4 (100%)	23

Table 5.4 Nasal lavage*Saliva Crosstabulation

Figures are numbers (column percentage). a. in one case two distinct pathogens were detected in the samples. b. in one case the combination of two dual infection yielded a triple infection in the paired sample.

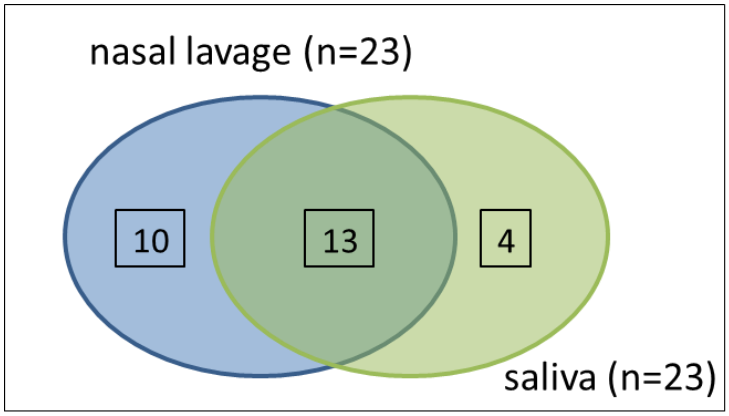


Figure 5.6 Graphical depiction of positive viral PCR results comparing saliva with nasal lavage.

Concurrent results (the same pathogen was detected in nasal lavage and saliva) are represented by circle intersection. Figures in squares represent number of distinct pathogens.

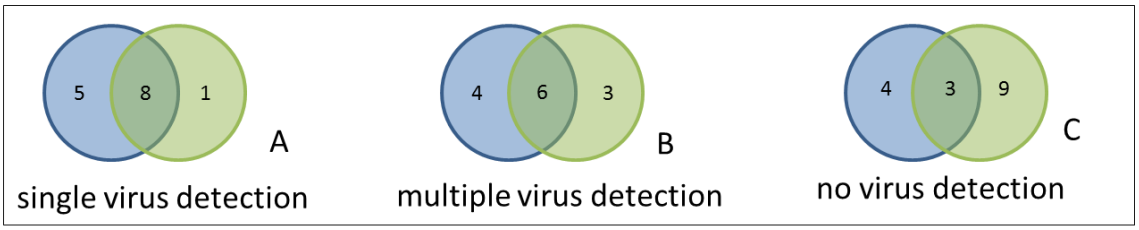


Figure 5.7 Graphical depiction of single and multiple virus detection by looking at paired nasal lavage (blue) and saliva samples (green).

A Single virus detection (n=14): in 8 cases the same virus was detected in both nasal lavage and saliva sample. B Multiple virus detection (n=6): in one case two dual infections in both saliva and nasal lavage sample yielded a triple infection. C No virus detection (n=3): in three cases no virus was detected.

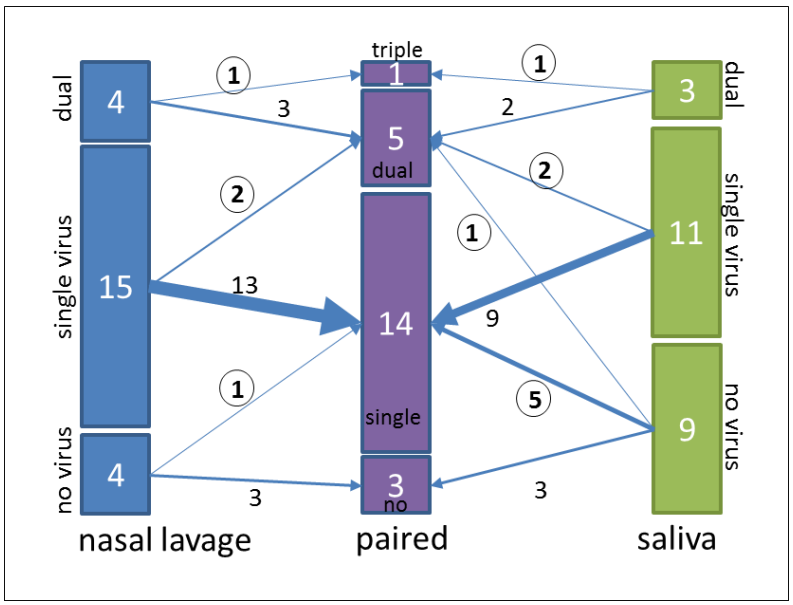


Figure 5.8 Graph showing the additional information gained by combining the results of nasal lavage and saliva PCR.

Figure 5.8 shows the additional information gained when combining the results of viral PCR from nasal lavage and saliva. In 4 cases adding saliva results to nasal lavage yielded additional pathogens. In 9 cases adding nasal lavage results to saliva yielded additional pathogens. Figure 5.9 shows the types of viruses (including multiple infections) according to specimen type. The only virus that was only found in saliva (but only once), and not in nasal lavage, was parechovirus. The combination of parainfluenza 3 and parechovirus was therefore also only found in saliva (once only). In addition the combination mycoplasma and coronavirus OC43 was also only found in

saliva (once only). Parainfluenza 2 and influenza A was only found in nasal lavage; the combinations of rhinovirus & mycoplasma and coronavirus & bocavirus were also only found in nasal lavage. The low number of patients in this study does not allow performing comparative statistical analysis with any higher degree of precision. Therefore, the overall low number of samples does not allow drawing the conclusion that this is a significant finding. As most viruses (apart from rhinovirus and bocavirus) were found only once or twice each a correlation of virus types with clinical features was not performed.

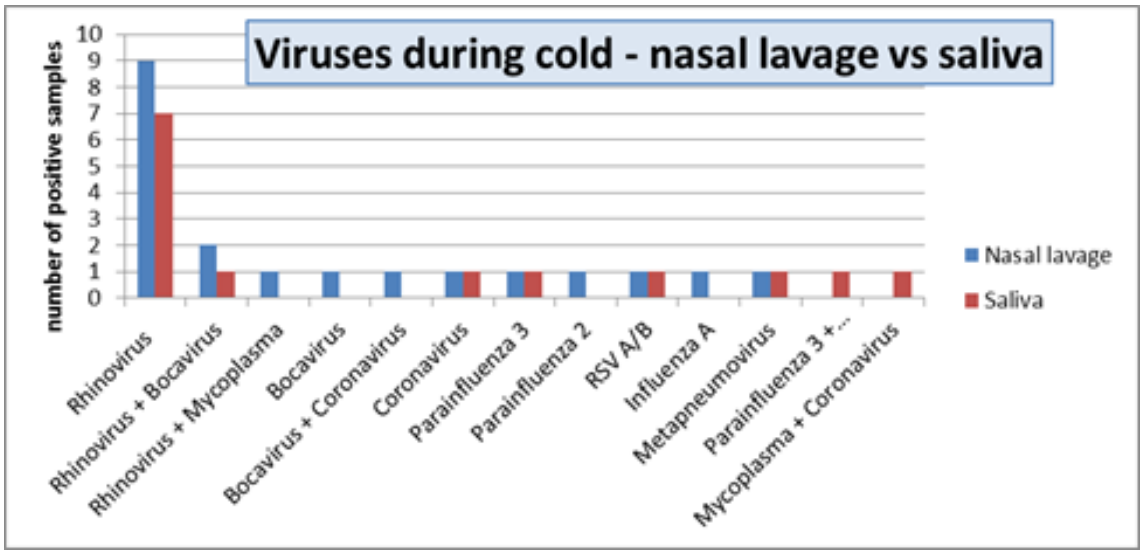


Figure 5.9 Types of viruses found in nasal lavage and saliva

5.3.7 Virus status in the context of clinical syndromes and symptom scores

5.3.7.1 Virus status and Asthma Index/Jackson Cold Score

Analysis of variance (ANOVA) was performed to explore the potential associations of the virus status during a cold episode (one cold included only) with a number of clinical syndromes and symptom scores: (1) AUC of Asthma Index, (2) Peak of Asthma Index, (3) AUC of Jackson Cold Score, (4) Peak of Jackson Cold Score (see Table 5.6). This analysis was limited by the low number of virus negative episode and the only significant associations found were with AUC and Peak of the Asthma Index. Figure 5.10 shows histograms comparing Jackson Cold Score (AUC, peak, 48 hour sum) between virus negative and positive participants. Subgrouping the virus positive episodes into single virus and multiple virus cold episodes and re-performing did not yield any statistically significant associations (see Table 5.6). In addition a chi-square

test was performed and no relationship was found between severity of exacerbation and virus status, X2 (2, N = 25) = 2.20, p =0.33 for virus status (positive/negative) and X2 (4, N = 25) = 3.72, p=0.45 for virus status (no/single/multiple virus).

5.3.7.2 Virus status and summative clinical symptom scores (cold/asthma)

A table was compiled (presenting individual values of the three virus-negative episodes, median and IQR) exploring the potential associations of the virus status during a cold episode (one cold included only) with summative symptom scores of subjective elements of the Jackson Cold Score (Sore throat, runny nose, sneezing, blocked nose, tiredness, fever, headache, hoarseness, earache, cough) and of subjective elements of the Asthma Index (cough, chest tightness, wheeze, shortness of breath). No positive associations were found in respect of any of the parameters (see Tables 5.7 and 5.8).

	Virus status (n)	Mean	Standard error	F value	Significance
AUC of Asthma Index (original, adjusted)	negative (2)	168.13	201.38	6.095	***p= .023***
	positive (19)	614.20	55.18		
Peak of Asthma Index (original)	negative (2)	3.50	13.50	4.489	***p= .048***
	positive (19)	43.84	5.95		
AUC of Jackson Cold Score	negative (3)	23.67	10.49	1.143	*p= .297*
	positive (20)	50.04	9.27		
Peak of Jackson Cold Score	negative (3)	9.67	2.03	0.003	*p= .958*
	positive (22)	9.55	0.80		
AUC of Asthma Index (original, adjusted)	no virus (2)	168.13	201.38	3.566	***p= .050***
	single virus (15)	643.37	57.50		
	two viruses (4)	504.81	154.43		
Peak of Asthma Index (original)	no virus (2)	3.50	13.50	2.311	*p= .128*
	single virus (15)	45.53	7.01		
	two viruses (4)	37.50	11.45		
AUC of Jackson Cold Score	no virus (3)	23.67	10.44	1.029	*p= .376*
	single virus (17)	53.63	10.70		
	two viruses (3)	29.67	3.72		
Peak of Jackson Cold Score	no virus (3)			0.002	*p= .998*
	single virus (18)				
	two viruses (4)				

Table 5.5 Association between virus status (negative/positive & no virus/single virus/two viruses) and clinical status:
Area under the curve (AUC) and Peak of both Asthma Index and Jackson Cold Score. Significant p-values in bold.

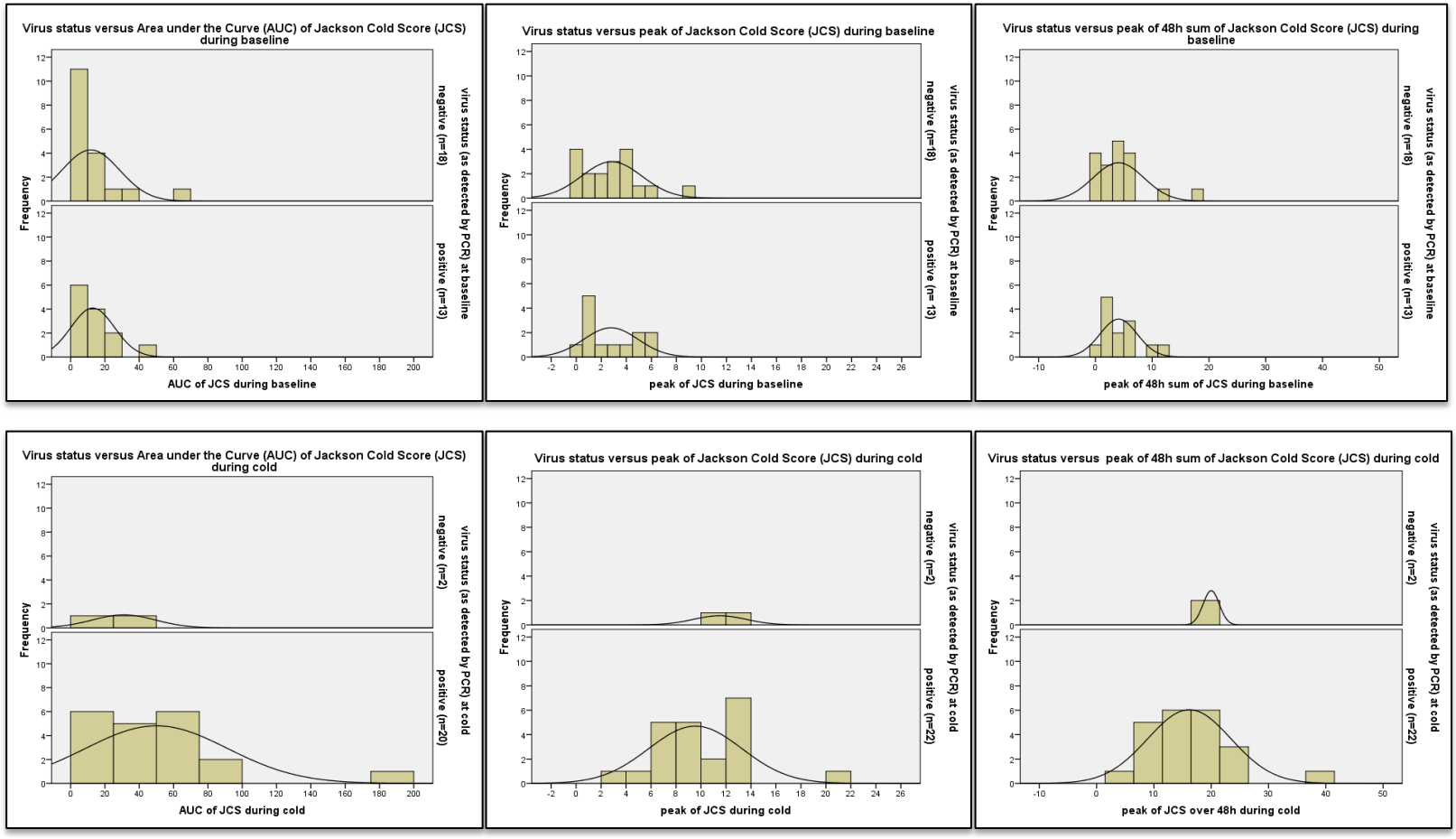


Figure 5.10 Virus status versus Jackson Cold Score:

Histograms comparing AUC (first column), peak (second column) and peak of 48 hour sum of JCS between virus positive and virus negative participants. Top row showing baseline and bottom row cold episodes.

Sum of	Virus status (n)	median	IQR	values
Sore throat score	Negative (3)	.00		0,0,16
	Positive (21)	3.00	10	
Runny nose score	Negative (3)	7.00		3,7,10
	Positive (21)	14.00	18	
Sneezing score	Negative (3)	8.00		0,8,9
	Positive (21)	8.00	15	
Blocked nose score	Negative (3)	6.00		6,6,19
	Positive (21)	16.00	25	
Tiredness score	Negative (3)	10.00		0,10,18
	Positive (21)	8.00	15	
Fever score	Negative (3)	1.00		0,1,7
	Positive (21)	.00	3	
Headache score	Negative (3)	8.00		0,8,10
	Positive (21)	1.00	7	
Hoarseness score	Negative (3)	1.00		0,1,2
	Positive (21)	1.00	9	
Earache score	Negative (3)	.00		0,0,3
	Positive (21)	.00	5	
Cough score	Negative (3)	10.00		2,10,10
	Positive (21)	22.00	24	
Total cold score	**Negative (3)**	**53.00**		**20,53,93**
	Positive (21)	**72.00**	**94**	
Chest tightness	Negative (3)	4.00		0,4,18
	Positive (21)	5.00	13	
Wheeze score	Negative (3)	3.00		1,3,5
	Positive (21)	7.00	11	
SOB score	Negative (3)	3.00		0,3,18
	Positive (21)	4.00	7	
Total asthma score	**Negative (3)**	**19.00**		**3,19,49**
	Positive (21)	**43.00**	**44**	

Table 5.6 Association between virus status (negative/positive) and summative clinical symptom scores (cold and asthma) during cold episode. Numbers in last column represent actual values of the virus negative cases (n=3).

Sum of score	Virus status (n)	Mean	SE	F value	Sig.
Sore throat	no virus (3)	5.33	5.33	.430	*p= .656*
	single virus (17)	8.47	3.26		
	two viruses (4)	2.50	1.10		
Runny nose	no virus (3)	6.67	2.03	1.594	*p= .227*
	single virus (17)	19.06	3.68		
	two viruses (4)	10.00	1.23		
Sneezing	no virus (3)	5.67	2.85	.527	*p= .598*
	single virus (17)	11.47	2.84		
	two viruses (4)	7.75	1.03		
Blocked nose	no virus (3)	10.33	4.33	.829	*p= .450*
	single virus (17)	20.24	4.32		
	two viruses (4)	11.50	3.93		
Tiredness	no virus (3)	9.33	5.21	.460	*p= .637*
	single virus (17)	13.82	3.88		
	two viruses (4)	6.75	1.65		
Fever	no virus (3)	2.67	2.19	.089	*p= .915*
	single virus (17)	3.12	1.88		
	two viruses (4)	1.50	0.87		
Headache	no virus (3)	6.00	3.06	.518	*p= .603*
	single virus (17)	4.18	1.60		
	two viruses (4)	1.50	0.65		
Hoarseness	no virus (3)	1.00	0.58	.687	*p= .514*
	single virus (17)	6.35	2.24		
	two viruses (4)	3.25	1.44		
Earache	no virus (3)	1.00	1.00	.950	*p= .403*
	single virus (17)	3.71	1.49		
	two viruses (4)	0.00	0.00		
Cough	no virus (3)	7.33	2.67	1.432	*p= .261*
	single virus (17)	23.29	3.98		
	two viruses (4)	19.25	6.30		
Total cold	**no virus (3)**	**55.33**	**21.11**	**1.048**	***p = .368***
	single virus (17)	**114.82**	**22.62**		
	two viruses (4)	**66.50**	**5.95**		
Chest tightness	no virus (3)	7.33	5.46	1.048	*p= .368*
	single virus (17)	10.94	3.17		
	two viruses (4)	3.75	1.93		
Wheeze	no virus (3)	3.00	1.16	1.015	*p= .379*
	single virus (17)	12.12	3.41		
	two viruses (4)	5.25	2.29		
SOB	no virus (3)	7.00	5.57	.298	*p= .745*
	single virus (17)	7.47	2.42		
	two viruses (4)	3.50	1.44		
Total asthma	**no virus (3)**	**23.67**	**13.48**	**.972**	***p= .433***
	single virus (17)	**53.35**	**11.47**		
	two viruses (4)	**32.75**	**7.33**		

Table 5.7 Association between virus status (no virus/single virus/two viruses) and summative clinical symptom scores

5.4 Discussion

Highly sensitive molecular techniques have led to a step change in our quest for determining an infectious agent as presumed culprits for asthma exacerbations. The frequent detection of respiratory pathogens, however, not only in children symptomatic with asthma (i.e. asthma exacerbations of varying severity) but also in asymptomatic children with asthma and healthy controls raises questions about a direct causal link. The increased sensitivity may therefore come with a price and this raises the concern that a non-quantitative approach may be misleading in determining the causes for asthma exacerbations.

In this study 32 children between 5 and 11 years were seen at varying time points, both when asymptomatic and clinically stable (baseline) and during episodes of viral cold that possibly but not necessarily led to an asthma exacerbation. Unsurprisingly, and in line with previous larger epidemiological studies (Johnston et al., 1995), the detection rate of respiratory pathogens during cold and exacerbation episodes was 88% when looking at paired nasal lavage and saliva results, and 87.5% and 65% when looking at nasal lavage and saliva individually. Rhinovirus was the most frequently detected virus (44%), which supports the common notion of a significant link between this virus and asthma exacerbations (Thumerelle et al., 2003, Khetsuriani et al., 2007, Johnston et al., 2005). Bocavirus was the second most frequent respiratory agent that was only first discovered 10 years ago and since then found to be associated with asthma exacerbations and frequently discovered in co-infections.

More unusual and sparking the debate about the actual significance of respiratory viral nucleic acid detection is the finding of 42% positivity rate in baseline nasal lavage samples and the presence of bocavirus in 3 samples next to a high presence of rhinovirus. Bocavirus has rarely been identified in control samples in previous studies (Papadopoulos et al., 2011). Baseline samples in this study are samples taken from children with stable asthma who are not displaying any cold symptoms. In general, the prevalence of respiratory viruses in asymptomatic children is known to be high, but in this study the prevalence rate of 42% is closer to that expected for asymptomatic infants (44%) rather than older children (27%) (Jansen et al., 2011). The time course of viral infections and acute asthma exacerbations has not yet been completely unravelled, it remains a distinct possibility that viruses may persist for several weeks (Kling et al., 2005) or cause frequent re-infections (Jartti et al., 2008).

The occurrence of infection with multiple viruses at the same time is well known and has been reported for some viruses (such as bocavirus (Kusel et al., 2006) and human

coronavirus (Bosis et al., 2008)) more frequently than for others. The importance of this finding and the possible interaction between different viruses remains poorly understood in the context of asthma exacerbations. In this study the detection rate of dual infections was 3% in the baseline samples and 16% in the samples collected during the cold episode. Three of these dual infections were rhinovirus/bocavirus co-infections, one of which was found in an asymptomatic child. When looking at potential associations between number of viruses and clinical status, no statistically significant associations were found. This may though be due to overall low numbers of cold episodes. Different viruses have developed various inhibitors of the virus-induced IFN-α/β response loop (Haller et al., 2006) and so a synergistic effect could lead to a more effective infection.

Obtaining invasive samples from children always poses a significant challenge that requires child-friendly explanations in simple language, a very calm approach, reassurance and, on occasion, the use of distraction techniques. Nevertheless collection of samples from the respiratory tract poses a burden on any child and efforts have to be made to look for less invasive techniques. Advances in sensitive molecular diagnostics allow for the collection of less invasive specimens in a number of areas. In adult patients, nasal washes have been studied and found to be both diagnostically highly sensitive for virus detection and associated with less patient discomfort in comparison to nasopharyngeal aspirates and nasal brushings (Spyridaki et al., 2009). Robinson *et al.* compared nasopharyngeal (NP) specimens, throat swabs (TS) and saliva specimens in a group of 137 children and found a virus in 105 NP specimens, and 87 (83%) TS and 77 (74%) of corresponding saliva specimens. TS and saliva samples were analysed by PCR only if the corresponding NP specimen had been positive. No comment was made about additional or different viruses being detected in the different sample types (Robinson et al., 2008). This study looked at 23 paired nasal lavage and saliva samples and found that neither of the two sample types individually detected all viruses but that the combination of both led to a higher yield, both in absolute viral pathogen detection and in identifying co-infections: in one case a virus was only found in saliva, in another case a single virus was found in nasal lavage and two viruses in the corresponding saliva sample, a triple infection arose out of the combination of both sample types in another child and two different viral species were detected in a further specimen pair. Not only is the collection of saliva non-invasive and well accepted by children, but it may actually yield additional diagnostic information that nasal lavages by themselves would miss. The reason for detecting different viral nucleic acid in some of these two different sample types requires further investigations.

Looking at the literature, there is a lack of studies exploring the yield of respiratory viruses from saliva specimens. One study compared the detection rate of human bocavirus from nasal and saliva specimens from children with and without respiratory illness and found hBoV in saliva samples during asymptomatic periods in 3% (5/149) and during respiratory illness in 2% (2/106) of the cases. This compared to a detection rate of hBoV in only 1/149 asymptomatic and 0/106 illness nasal samples. Potential explanations discussed included ongoing viral shedding from a previous (asymptomatic) viral infection with bocavirus and high viral load in respiratory tract with shedding into oral cavity (Martin et al., 2009). Robinson et al. detected respiratory viruses (RSV, influenza A and B viruses, parainfluenza virus, adenovirus, and human metapneumovirus) in 105 (RSV=83) of 137 NP specimens. The same virus was detectable in 77 (74%) of 104 saliva specimens and the conclusion was that saliva samples were inferior to NP specimens but acceptable in screening settings (Robinson et al., 2008).

A number of studies have compared nasopharyngeal (NP) with oropharyngeal (OP) specimens obtained by swabs: In a study with 533 children a virus was detected in 339 (64%) NP samples and 268 (50%) OP samples. The added value of the OP sample ranged from 9% (rhinovirus) to 31% (adenovirus) (Hammitt et al., 2011). OP swabs were more sensitive than NP swabs for detection of the 2005 H5N1 influenza virus (Kandun et al., 2006). A further study on a large number of patients comparing NP and OP swabs confirmed that the relative performance of specimen type varied by virus type. Looking at eight different respiratory viruses, NP swabs were more sensitive for influenza B virus, parainfluenza virus 2 and 3. OP swabs were more sensitive for influenza A virus and adenovirus (Kim et al., 2011). In the case of adenovirus the authors discuss that this difference may be due to the fact that the main place of initial replication of adenoviruses is the non-ciliated respiratory epithelium of the oropharynx. The sensitivity of the OP swab for parainfluenza virus 2 was the lowest of any virus in this study. The fact that nasal washes and aspirates lead to the highest rates of viral recovery for parainfluenza viruses was previously reported (Henrickson, 2003). The tropism of viruses to different types of cells of the oropharynx and upper and lower respiratory tract (potentially at different stages of their life cycles) may explain some of the differential findings of this study. Saliva contains a range of proteins (MUC5B, scavenger receptor cysteine rich glycoprotein 340, histatins, and human neutrophil defensins) that were shown to be able to inhibit Influenza A virus and therefore represents an important initial barrier to influenza A virus infection (White et al., 2009).

The complexity of salivary immune defence against viruses may also partially explain inconsistencies of findings between nasal and salivary virus yields.

The saliva collection technique applied in this study required a degree of cooperation on part of the paediatric study population that may be avoided by altering the saliva collection method in the infant and toddler group. Nasal washes were also found to be well tolerated by this group of 5-11 year olds, this is based on personal observation by the study team though and has not been specifically investigated.

In summary the data are broadly in keeping with previously published studies looking at prevalence of viral infections in childhood asthma exacerbations. The very high prevalence of viruses in asymptomatic children adds to the debate about the significance of finding viral nucleic acid and the causal relation to exacerbation, the persistence of viruses over time and the rate of re-infections in this group of patients. Future studies should include quantitative PCR (qPCR) approaches to determine changes of viral load at different time points rather than simply documenting presence of viral ribonucleic acid. If the viral load at asymptomatic baseline was not lower the paradigm that viruses themselves cause asthma exacerbations per se could be questioned and/or a ‘second hit’ hypothesis (i.e. virus plus allergic stimulus) could be raised. Saliva was found to be an acceptable non-invasive method of specimen collection. While less sensitive than nasal lavage additional information can be gained that may aid in clinical diagnosis and management. This would suggest that a combination of nasal/nasopharyngeal and saliva sampling might usefully increase the sensitivity of viral detection.

6 Cytokines and asthma exacerbation

6.1 Introduction

Respiratory virus infected airway tissue (with epithelial cells as the principal target) releases various pro-inflammatory cytokines and mediators (chemokines) that recruit cells to the airway. It is thought that this, in combination with other immune responses, leads to the development of airway inflammation rather than direct cell injury secondary to RV infection as rhinoviruses themselves cause little tissue destruction (Gern and Busse, 1999). On the other hand RV-induced epithelial cytotoxicity may play an important role in the induction of asthma exacerbation in an already compromised epithelium (Bossios et al., 2005).

A number of neutrophilic, lymphocytic and eosinophilic chemokines (such as IL-8, IP-10, and RANTES) released from airway epithelial cells have an important role in initiating an inflammatory cell influx into the airways at the initial stages of viral induced asthma exacerbations (Jackson and Johnston, 2010). The life-span of these cells is prolonged via growth factors (e.g. G-CSF) that are partly released from epithelial cells in response to viral infection (Gavala et al., 2011) and partly released from extracellular matrix (ECM) degraded by activated matrix metalloproteinases (MMPs) (Van Lint and Libert, 2007). Degradation of ECM is crucial for inflammatory cell migration but MMPs are also involved in activation of a number of pro-inflammatory cytokines. Exaggerated ECM turnover can lead to airway remodelling processes with impaired repair, scar formation or accumulation of ECM components (Gueders et al., 2006).

The bronchial epithelial cell has also an important role in anti-viral defence via innate immune responses leading to production of type I interferons (IFNs)-α and –β and type III IFN-λ. On viral infection the expression of IFN-α4 and IFN-β is induced first and these interferons subsequently enhance this virus-mediated expression of themselves and other IFN-αs in autocrine and paracrine fashion (Taniguchi and Takaoka, 2002). The concerted effort of these type I IFNs is directed towards induction of multiple IFN-inducible genes that contribute to antiviral defence and lead to apoptosis in infected cells (Takaoka et al., 2003, Wark et al., 2005, de Veer et al., 2001).

IFN-β has been shown to be unstable as a protein and difficult to measure (Edwards et al., 2013). Surrogate markers for the effect of interferon-β however are to be found downstream of the interferon-β pathway with IP-10 (CXCL10) the best known inflammatory chemokine with IFN-β related antiviral activity. IFN-β leads to

upregulation of IP-10 (Wark et al., 2007, Buttmann et al., 2007), especially also on exogenous administration of IFN-β in *vitro (*Cakebread et al., 2011) and in *vivo* (Petry et al., 2006, Djukanovic et al., 2014). IFN-β also directly leads to an increase in human endothelial MCP-1 (CCL2) production (Buttmann et al., 2007) and suppresses pro-inflammatory cytokines MIP-1β (CCL4), IL-8 (CXCL-8) (Laver et al., 2008), and IL-1β (Guarda et al., 2011), and the angiogenetic growth factor VEGF (Fukuda et al., 2004, Roh et al., 2013).

6.2 Objectives

The objectives of this part of the study were:

i. to characterize fluctuations in nasal lavage cytokines and MMPs between baseline (asymptomatic) periods and during naturally occurring respiratory viral infections in children with asthma

ii. to explore the potential correlation between levels of nasal lavage cytokines and MMPs and severity of viral induced asthma exacerbation

iii. to explore the potential correlation between levels of nasal lavage cytokines and MMPs and virus status (positive/negative) during baseline and cold episodes

iv. to determine the relationship between upper airway inflammatory biomarkers (cytokines and MMPs) and cold and asthma scores

6.3 Results

6.3.1 Participant monitoring and classification

Between January 2011 and February 2012 thirty-two children between 5 and 11 years of age participated in the study. All 32 participants underwent a baseline visit during which nasal lavage samples were collected. A total of 25 participants developed colds, 9 of these developed 2 cold episodes. If possible, visits were conducted within 48 hours of initial cold symptoms (in the following termed V1) and a further visit between the 4th and 6th day of the cold (termed V2). Eighty-two nasal lavage samples were collected, pre-processed and stored for further analysis in this process.

Participants were monitored for 7 days post their initial baseline visits with a twice daily SMS-transmitted Jackson Cold Score questionnaire (see 2.2.1) and an asthma

symptom questionnaire (see 2.2.2). Both questionnaires were also answered for 14 days following initial cold symptoms.

Based on the classification by Reddel *et al*. (Reddel et al., 2009) and as outlined in paragraph 2.2.3 the severity of viral induced asthma exacerbation of the participants was determined. The asthma index was calculated according to Sorkness *et al.*(Sorkness et al., 2008).

In total there were 34 cold episodes - 5 that did not result in any asthma exacerbation, 23 with a moderate exacerbation and 6 with a severe exacerbation and an overview of these results are presented in 5.3.2.1. As 9 participants developed 2 cold episodes, and in order to avoid duplication, a final dataset comprised 25 separate cold episodes.

6.3.2 Overview of cytokine/MMP assay results

6.3.2.1 Cytokine assays

Following the IP-10 ELISA and multiplex cytokine assay a total of 9 cytokines were included in the subsequent analysis. For statistical purposes, levels below the lower limit of the standard curve were assigned a value of half the lower limit of quantification (LLOQ) for each specific cytokine. Levels above the upper limit of the standard curve were assigned a value of double the upper limit of quantification (ULOQ) for each specific cytokine. Results of duplicate samples with a coefficient of variation (%CV) > 30 were excluded.

Two cytokines (FGFb – fibroblast growth factor basic; MIP-1α – macrophage inflammatory protein-1α) were excluded on the basis that the negative control yielded positive results. FGFb assay also yielded results for all samples only just below or at the lower end of the standard curve. Other cytokines (GM-CSF, IFN-γ, IL-6, IL-10, TNF-α, IL-17, MIP-1α, RANTES, IL-5) were only detected in very few samples each. IL-2 and IL-4 were not detected at all. More than 80% of samples yielded results far above the upper limit of the standard curve for IL-1ra. For details see Table 6.1.

Cytokine	Comment	included
FGFb	Standard curve: 10 -5700pg/mL; negative control positive; sample values between 8-14pg/mL	
G-SCF		√
GM-CSF	only detected in one sample	
IFN-γ	only detected in two samples	
IL-1β		√
IL-6	only detected in very few samples, many %CV>30	
IL-8		√
IL-10	only detected in 5 samples	
TNF-α	only detected in very few samples	
VEGF		√
ENA-78		√
IL-1α		√
IL-17	only detected in 3 samples	
MCP-1		√
MIP-1α	only detected in very few samples; negative control positive	
MIP-1β		√
RANTES	only detected in very few samples, many %CV>30	
IL-1ra	>80% of samples above the upper limit of standard curve	
IL-2	not detected	
IL-4	not detected	
IL-5	only detected in very few samples	
separate assay:		
IP-10		√

Table 6.1 Overview of cytokines analysed in nasal lavage samples
Comments about detection rate and reason for exclusion from statistical analysis.

6.3.2.2 MMP assays

All MMP assays of nasal lavages yielded satisfactory results of standard curves and negative controls and therefore all MMPs were included in the subsequent analysis. For statistical purposes, levels of each MMP below the lower limit of the standard curve were assigned a value of half the lower limit of quantification (LLOQ) for each specific

cytokine. Levels above the upper limit of the standard curve were assigned a value of double the upper limit of quantification (ULOQ) for each specific cytokine. Results of duplicate samples with a coefficient of variation (%CV) > 30 were excluded.

6.3.3 Nasal lavage cytokines and MMPs at asymptomatic periods and during naturally occurring respiratory viral infections in children with asthma

Comparing nasal lavage cytokines and MMPs at baseline with those obtained during two cold visits (V1 – within 48 hours of cold symptoms, V2 – between day 4 and 6 of cold) yielded statistically significantly increased levels of MMP-7, G-CSF, ENA-78, and MCP-1 at both cold visits (V1 and V2) when compared with baseline levels. MMP-3 and MMP-8 levels were statistically significantly increased at V2 compared to baseline and differences between baseline and V1 reached borderline significance (p= .019 and .018 respectively). IL-1β, IL-8, IP-10 and MIP-1β were significantly increased at V1 compared to baseline while VEGF was only increased at V2. No statistically significant difference in levels was found between V1 and V2 for any of the analysed cytokines or MMPs. For details see Table 6.2 and Figures 6.1 and 6.2.

In all but one cytokine/MMP (i.e. MMP-3) the inclusion of all pairs of sample (versus just those with baseline, V1 *and* V2 data) in the pairwise comparison maintained the statistically significant difference between baseline and V1 cytokine/MMP levels. The same was true when comparing baseline and V2 cytokine/MMP levels. In most cases the increase in sample numbers included in pairwise comparison lead to lower p-values (see Table 6.2).

	N	N (complete)	median (25^{th}, 75^{th} percentile)	Significance [a]	bl to V1		bl to V2		V1 to V2	
					N	Significance [b]	N	Significance [b]	N	Significance [b]
MMP-1										
baseline	25		82.83 (41.51, 161.64)	.918						
V1	21	10	78.28 (54.49, 127.45)							
V2	13		75.13 (54.26, 152.86)							
MMP-2										
baseline	25		2557.62 (1729.35, 3180.46)	.895						
V1	19	9	2666.92 (2014.76, 2904.62)							
V2	13		2361.74 (1535.97, 2920.68)							
MMP-3										
baseline	27		134.44 (90.33, 272.25)	.016	17	.019	12	**.003**	11	.328
V1	20	9	292.31 (120.72,533.58)		*9*	*.015*	*9*	*.011*	*9*	*.767*
V2	14		310.78 (236.99, 656.74)							
MMP-7										
baseline	29		790.87 (525.41, 1832.54)	.002	21	**.006**	13	**.001**	13	.861
V1	22	12	1854.80 (809.51, 5419.31)		*12*	*.008*	*12*	*.002*	*12*	*.695*
V2	14		3458.43 (1774.14, 4901.23)							
MMP-8										
baseline	26		20573.46 (7981.95, 65212.60)	.002	19	.018	12	**.006**	13	.173
V1	22	11	31199.79 (17386.05,133400.00)		*11*	*.075*	*11*	*.005*	*11*	*.173*
V2	14		100604.98 (42045.96, 133400.00)							
MMP-9										
baseline	27		23049.13 (7743.67, 83000.00)	.074						
V1	21	11	83000.00 (12970.30, 83000.00)							
V2	14		83000.00 (83000.00, 83000.00)							
MMP-12										
baseline	26		232.76 (157.61, 289.16)	.131						
V1	20	10	194.40 (106.06, 249.66)							
V2	13		229.99 (61.45, 269.10)							
MMP-13										
baseline	25		104.37 (39.78, 257.38)	.200						
V1	20	9	129.16 (39.78, 318.10)							

V2	13		189.25 (39.78, 276.29)							
	N	**N (complete)**	**median (25th, 75th percentile)**	Significance[a]	**bl to V1** N	Significance[b]	**bl to V2** N	Significance[b]	**V1 to V2** N	Significance[b]
G-CSF										
baseline	28		82.08 (9.71, 170.42)	.001	19	**.004**	13	**.016**	11	.477
V1	21	11	208.64 (75.68, 828.35)		*11*	*.013*	*11*	*.003*	*11*	*.477*
V2	13		299.71 (223.10, 671.57)							
IL1β*										
baseline	30		14.37 (1.65, 35.51)	.033	21	**.003**	12	.136	10	.203
V1	21	10	24.16 (13.28, 138.52)		*10*	*.066*	*10*	*.037*	*10*	*.203*
V2	12		37.86 (24.78, 97.69)							
IL-8*										
baseline	28		381.17 (124.17, 639.75)	.006	19	**.011**	9	.066	6	.249
V1	19	6	670.58 (388.16, 5900.00)		*6*	*.028*	*6*	*.028*	*6*	*.249*
V2	9		736.25 (368.32, 3421.93)							
VEGF*										
baseline	24		112.42 (87.29, 151.93)	.030	15	.061	10	**.005**	10	.646
V1	20	8	129.82 (102.65, 215.84)		*8*	*.069*	*8*	*.012*	*8*	*.889*
V2	12		175.36 (115.43, 232.13)							
ENA-78										
baseline	29		264.45 (87.43, 597.73)	.005	20	**.015**	14	**.002**	12	.272
V1	20	12	549.28 (96.38, 1659.61)		*12*	*.019*	*12*	*.005*	*12*	*.272*
V2	14		463.68 (308.72, 1321.63)							
IL-1α										
baseline	25		14.73 (4.57, 22.96)	.159						
V1	16	8	7.31 (1.79, 20.32)							
V2	11		9.67 (1.27, 14.53)							
MCP-1*										
baseline	29		6.96 (4.74, 10.88)	.003	21	**.002**	13	**.002**	12	.814
V1	22	12	16.42 (5.40, 33.71)		*12*	*.008*	*12*	*.003*	*12*	*.814*
V2	13		15.41 (9.81, 23.50)							
MIP-1β*										
baseline	28		4.25 (4.25, 22.99)	.005	19	**.002**	14	.028	13	.583
V1	21	13	52.51 (10.03, 98.63)		*13*	*.008*	*13*	*.041*	*13*	*.583*
V2	14		53.00 (18.76, 95.31)							

	N	N (complete)	median (25th, 75th percentile)	Significance[a]	bl to V1		bl to V2		V1 to V2	
					N	Significance[b]	N	Significance[b]	N	Significance[b]
IP-10*										
baseline	31		241.90 (70.24, 779.67)	.045	22	**.003**	13	.054	12	.632
V1	22	12	1785.36 (364.53, 4146.18)		*12*	*.017*	*12*	*.069*	*12*	*.632*
V2	13		758.83 (277.89, 4067.07)							

Table 6.2 Levels of matrix metalloproteinases (MMPs) and cytokines at baseline (bl) versus first (V1 – within 48 hours of cold symptoms developing) and second (V2 – on day 4-6 of cold) cold visit.

N in first column shows total number of samples available for each visit. Median (25th, 75th percentile) cytokine level shown for all samples. Friedman test was performed on complete sets of samples from all three time points [N (complete)] to show difference of MMP and cytokine levels between visits. If statistically significant difference shown (significance level[a] $p < 0.05$) pairwise comparison (Wilcoxon-signed-rank test) was performed on all available sample pairs with adjustment of significance level[b] to account for multiple comparison ($p < 0.017$). In addition pairwise comparison (Wilcoxon-signed-rank test) was performed on those samples only where complete set of samples was available and the results are shown in *italics*. Cytokines whose expression is directly influenced by IFN-β are marked with an asterisk (*). IP-10 levels post log transformation showed normal distribution and ANOVA with repeated measures and t-test for pairwise comparisons were performed.

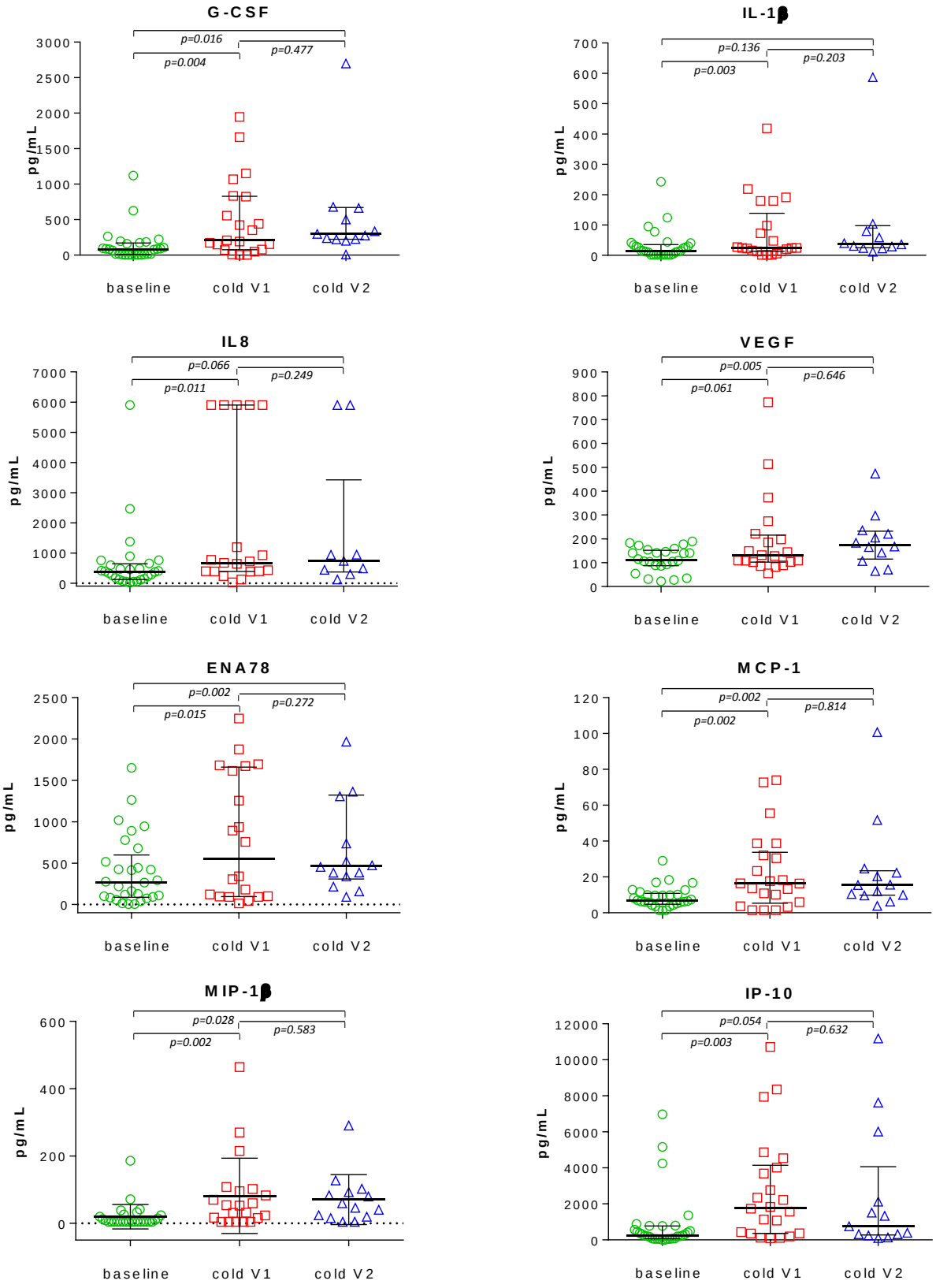


Figure 6.1 Column-scatter-graphs of cytokine levels (pg/mL) in nasal lavage shown at baseline cold visit 1 (within 48 hours of cold symptoms) and cold visit 2 (on day 4-6 of cold). Median and interquartile range shown for each cytokine. p-values given for pairwise comparison (significance level p<0.017). One outlier omitted from graphs G-CSF, IL-1β, ENA-78 (cold V2) for presentation purposes.

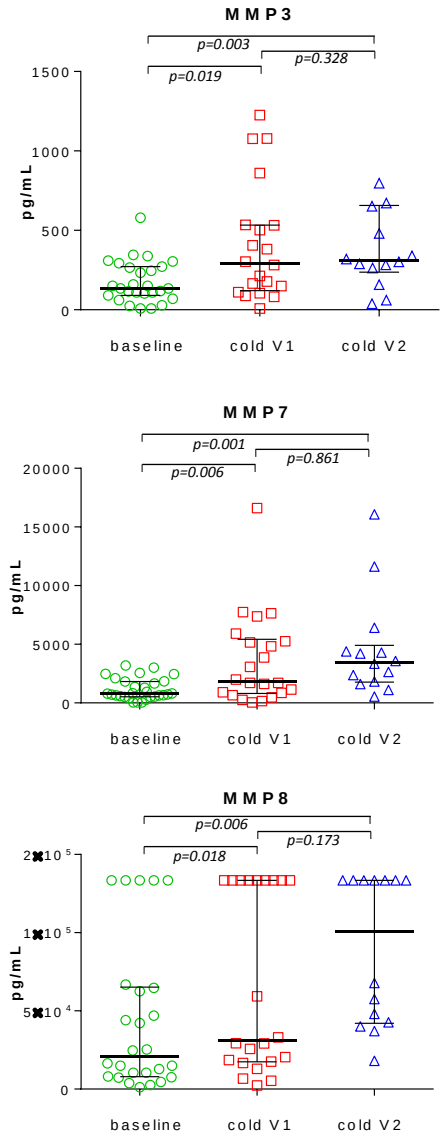


Figure 6.2 Column-scatter-graphs of matrix metalloproteinase (MMP) levels (pg/mL) in nasal lavage

at baseline cold visit 1 (within 48 hours of cold symptoms) and cold visit 2 (on day 4-6 of cold). Median and interquartile range shown for each cytokine. p-values given for pairwise comparison (significance level $p<0.017$). One outlier omitted from graph MMP-3 (cold V2) for presentation purposes.

6.3.4 Nasal lavage cytokines and MMPs and severity of viral induced asthma exacerbation (objective ii.)

No statistically significant differences in MMP or cytokine levels were detected when comparing samples of participants with cold only, moderate asthma exacerbation and severe asthma exacerbation respectively (see Table 6.3).

6.3.5 Nasal lavage cytokines and MMPs and virus status during baseline and cold episodes (objective iii.)

There was a statistically significant difference in IP-10 levels at baseline between participants with virus presence in nasal lavage compared to participants who were virus negative ($p = .037$). There was also a significant difference ($p = .036$) in nasal G-CSF levels between virus positive and negative children at the first cold visit but only two participants were contributing data to the virus negative group.

No other statistically significant difference was found in any other cytokine/MMP for any of the samples. See Table 6.4 for details.

6.3.6 Relationship between nasal lavage cytokines and MMPs and Jackson cold score / asthma index (objective iv.)

Correlations between cytokine/MMP levels and area under the curve (AUC) and peak of both asthma index and Jackson cold score (JCS) were explored. The only statistically significant correlations between cytokine/MMP levels and asthma index were found in relation to the matrix metalloproteinase MMP-7 at V1 during the cold (AUC: $p=0.025$ and peak: $p=0.018$) and in relation to the cytokine MCP-1 at V2 (AUC: $p=0.008$ and peak: $p=0.031$).

Correlation between AUC of the JCS during the cold episode and cytokine/MMP levels at V1 only was statistically significant for MMP-1, MMP-3, MMP-7, MCP-1 and MIP-1β and at V2 only for MMP-9. Correlation between AUC of the JCS and cytokine/MMP levels at both V1 and V2 was statistically significant for MMP-8 and IL-1β. The correlation with the peak of the JCS was only significant for MMP-13 at V2 (see Table 6.5).

	V1							V2						
	cold only		moderate		severe		Sig.	cold only		moderate		severe		Sig.
	N	median (25th, 75th centiles)	N	median (25th, 75th centiles)	N	median (25th, 75th centiles)		N	median (25th, 75th centiles)	N	median (25th, 75th centiles)	N	median (25th, 75th centiles)	
MMP-1	1	109.68	16	77.42 (52.77, 119.22)	4	102.63 (22.05, 452.91)	.717	0	-	9	75.13 (59.82, 229.72)	4	69.35 (18.93, 97.07)	.487
MMP-2	1	2893.42	15	2529.64 (1938.89, 2809.48)	3	4145.90 (2512.85, -)	.197	0	-	9	2473.68 (2216.25, 4477.53)	4	1569.55 (308.75, 2703.14)	.217
MMP-3	1	405.65	15	281.71 (104.27, 534.06)	4	357.54 (136.84, 933.97)	.793	0	-	10	391.36 (252.07, 702.76)	4	291.49 (110.86, 334.10)	.322
MMP-7	1	1711.42	16	2393.67 (515.36, 7001.60)	5	1998.19 (1000.23, 4517.18)	.989	0	-	10	4246.91 (1774.14, 7720.17)	4	2504.30 (993.67, 3342.27)	.157
MMP-8	1	133400	16	44239.49 (17185.77, 133400.00)	5	29365.39 (15256.59, 83217.10)	.525	0	-	10	62690.72 (42045.96, 133400.00)	4	133400.00 (46917.50, 133400.00)	.546
MMP-9	1	83000	15	83000.00 (13098.68, 83000.00)	5	14429.98 (9597.55, 61676.41)	.385	0	-	10	83000.00 (83000.00, 83000.00)	4	83000.00 (37817.23, 83000.00)	.417
MMP-12	1	220.26	15	187.56 (134.14, 253.43)	4	148.98 (29.52, 357.65)	.893	0	-	9	229.99 (152.70, 263.82)	4	135.07 (7.12, 286.38)	.756
MMP-13	1	256.97	15	125.62 (39.78, 327.82)	4	125.62 (59.47, 287.60)	.825	0	-	9	246.58 (39.78, 282.01)	4	114.52 (39.78, 240.65)	.341

	V1							V2						
	cold only		**moderate**		**severe**		**Sig.**	**cold only**		**moderate**		**severe**		**Sig.**
	N	median (25/75)	N	median (25/75)	N	median (25/75)		N	median (25/75)	N	median (25/75)	N	median (25/75)	
G-CSF	1	1150.84	15	208.64 (79.40, 833.17)	5	154.96 (37.53, 454.13)	.264	0	-	9	299.71 (231.55, 1681.65)	4	278.38 (61.12, 593.43)	.440
IL-1β*	1	24.16	15	24.16 (13.61, 179.63)	5	24.30 (3.61, 103.05)	.835	0	-	9	35.77 (26.12,333.41)	3	58.83 (11.46, -)	.926
IL-8*	1	396.87	13	670.58 (390.38, 5900.00)	5	771.53 (254.49, 1060.08)	.738	0	-	7	935.87 (486.61, 5900.00)	2	279.59 (119.58, -)	.078
VEGF*	1	197.46	15	128.15 (89.23, 273.96)	4	127.01 (108.65, 147.69)	.737	0	-	8	194.26 (88.62, 281.96)	4	166.54 (121.34, 207.37)	.610
ENA-78	1	893.01	14	243.80 (94.41, 1361.79)	5	1614.98 (490.82, 1774.65)	.507	0	-	10	497.03 (201.68, 1514.45)	4	420.23 (351.38, 665.89)	.671
MCP-1*	1	18.27	16	17.05 (4.22, 37.06)	5	13.22 (6.14, 27.57)	.761	0	-	10	15.51 (10.31, 29.72)	3	9.65 (6.23, -)	.398
MIP-1β*	1	31.72	15	53.56 (4.25, 101.89)	5	31.09 (10.03, 273.66)	.981	0	-	10	43.14 (18.76, 80.46)	4	97.67 (26.43, 121.00)	.288
IP-10*	1	1575.79	16	1739.17 (358.23, 4652.99)	5	1832.01 (969.02, 3377.42)	.972	0	-	10	1424.97 (296.88, 6414.10)	3	310.94 (100.39, -)	.091

Table 6.3 Levels of cytokines at cold visits V1 (within 48hrs of cold symptoms) and V2 (day 4-6 of cold) depending on severity of exacerbation. Figures represent medians (25th, 75th centiles). Significance represents p-values based on Kruskal Wallis test looking at whether levels are similar at each time point. Cytokines whose expression is directly influenced by IFN-β are marked with an asterisk (*)

	baseline					V1					V2				
	virus positive		virus negative		Sig	virus positive		virus negative		Sig	virus positive		virus negative		Sig.
	N	median (25^{th}, 75^{th} centiles)	N	median (25^{th}, 75^{th} centiles)		N	median (25^{th}, 75^{th} centiles)	N	median (25^{th}, 75^{th} centiles)		N	median (25^{th}, 75^{th} centiles)	N	median (25^{th}, 75^{th} centiles)	
MMP-1	11	75.13 (53.55, 102.13)	14	101.75 (31.49, 266.66)	.529	19	78.28 (57.94, 122.81)	2	95.84 (5.01, -)	.857	11	75.13 (60.68, 103.41)	2	714.48 (5.01, -)	.921
MMP-2	11	2344.95 (1790.91, 2834.16)	14	2812.30 (1518.83, 3842.52)	.352	18	2598.28 (1995.79, 2991.59)	1	-	.361	11	2361.74 (2081.92, 2887.82)	2	4663.95 (81.66, -)	.844
MMP-3	11	150.55 (90.33, 232.94)	16	116.89 (70.86, 302.08)	.902	18	342.56 (137.96, 615.57)	2	138.55 (111.23, -)	.257	12	310.78 (268.44, 609.04)	2	5069.61 (60.02, -)	.855
MMP-7	12	767.39 (541.85, 1792.16)	17	790.87 (521.47, 1969.51)	.825	20	2538.19 (962.97, 5743.94)	2	758.75 (657.24, -)	.171	12	3458.43 (1965.70, 4369.31)	2	8306.74 (533.42, -)	1.00
MMP-8	9	46945.36 (11519.66 , 133400.00)	17	16485.30 (7447.97, 53346.39)	.268	20	46174.24 (17185.77, 133400.00)	2	23386.33 (17607.97, -)	.408	12	100604.98 (44046.28, 133400.00)	2	75745.00 (18090.00, -)	.559
MMP-9	10	53024.56 (6716.60, 83000.00)	17	21562.88 (7611.49, 83000.00)	.677	19	83000.00 (13098.68, 83000.00)	2	47920.97 (12841.93, -)	.897	12	83000.00 (83000.00, 83000.00)	2	52878.15 (22756.31, -)	.099
MMP-12	11	280.78 (236.63, 348.73)	15	222.17 (153.51, 315.29)	.855	18	194.40 (124.78, 242.12)	2	154.08 (7.12, -)	.950	11	229.99 (115.77, 263.03)	2	892.64 (7.12, -)	.843
MMP-13	11	153.92 (39.78, 256.97)	14	95.52 (39.78, 298.47)	.695	18	155.68 (39.78, 330.67)	2	39.78	.094	12	217.92 (39.78, 277.95)	1	-	.271

	baseline					V1					V2				
	virus positive		**virus negative**		**Sig**	**virus positive**		**virus negative**		**Sig**	**virus positive**		**virus negative**		**Sig.**
	N	median (25/75)	N	median (25/75)		N	median (25/75)	N	median (25/75)		N	median (25/75)	N	median (25/75)	
G-CSF	12	89.10 (28.55, 253.84)	16	16.00 (4.59, 102.92)	.136	19	351.03 (147.92, 833.17)	2	6.88 (3.09, -)	**.036**	11	299.71 (225.11, 663.79)	2	4503.90 (7.81, -)	1.00
IL-1β*	13	29.87 (1.65, 61.01)	17	12.46 (1.65, 26.19)	.243	19	24.30 (13.61, 179.13)	2	10.79 (1.65, -)	.130	10	37.86 (27.46, 85.74)	2	960.74 (11.46, -)	1.00
IL-8*	12	534.75 (168.46, 1253.13)	16	271.92 (124.17, 466.45)	.078	17	720.65 (392.51, 5900.00)	2	258.54 (124.47, -)	.108	7	736.25 (439.61, 943.86)	2	3009.79 (119.58, -)	.883
VEGF*	9	109.39 (60.95, 161.55)	15	88.86 (115.44, 153.94)	.976	18	138.01 (102.82, 234.97)	2	95.66 (81.83, -)	.208	11	183.38 (142.08, 235.94)	1	-	.311
ENA-78	12	269.78 (102.27, 620.91)	17	163.58 (65.70, 704.16)	.773	18	549.28 (94.41, 1676.45)	2	518.72 (99.95, -)	.801	12	463.68 (257.57, 1164.97)	2	7069.87 (339.74, -)	.584
IL-1α	12	13.89 (3.83, 18.95)	13	14.73 (5.33, 26.74)	.513	14	7.31 (1.27, 18.34)	2	14.99 (5.30, -)	.522	10	8.69 (1.27, 14.56)	1	-	1.00
MCP-1*	13	6.39 (4.74, 10.64)	16	7.85 (4.17, 12.21)	.599	20	16.42 (7.03, 36.67)	2	16.74 (1.44, -)	.647	11	15.41 (9.97, 22.37)	2	53.50 (6.23, -)	.844
MIP-1β*	13	4.25 (4.25, 28.96)	15	4.25 (4.25, 24.17)	.646	19	52.51 (15.80, 95.37)	2	56.14 (4.25, -)	.904	12	53.00 (20.85, 90.43)	2	147.31 (4.25, -)	.927
IP-10*	13	561.80 (213.47, 836.38)	18	186.34 (56.31, 397.46)	**.037**	20	2028.15 (595.25, 4402.42)	2	160.09 (120.83, -)	.068	11	758.83 (310.94, 2122.24)	2	5640.33 (100.39, -)	1.00

Table 6.4 Levels of matrix metalloproteinases (MMPs) and cytokines at baseline and cold visits V1 (within 48 hours of cold symptoms) and V2 (day 4-6 of cold) depending on virus status. Figures represent medians (25^{th}, 75^{th} centiles). Significance represents p-values based on Mann-Whitney U test looking at whether levels are similar whether or not virus positive. *Cytokines directly influenced by IFN-β.

		Asthma Index				Jackson Cold Score			
		AUC (original, adjusted)		Peak (original)		AUC		Peak	
		Spearman's rho	*Significance*	*Spearman's rho*	*Significance*	*Spearman's rho*	*Significance*	*Spearman's rho*	*Significance*
MMP-1	V1	.096	.715	0.185	.477	0.475	**.040**	0.145	.530
	V2	-0.27	.937	-0.196	.564	0.249	.436	-0.137	.656
MMP-2	V1	-0.106	.696	-0.031	.910	0.164	.529	0.095	.700
	V2	-0.091	.790	-0.278	.408	-0.126	.697	-0.417	.156
MMP-3	V1	0.375	.152	0.438	.090	0.475	**.040**	0.164	.491
	V2	-0.503	.095	-0.378	.225	0.011	.972	-0.120	.684
MMP-7	V1	0.527	**.025**	0.551	**.018**	0.475	**.034**	0.140	.533
	V2	-0.049	.880	-0.049	.880	0.082	.789	-0.381	.179
MMP-8	V1	0.018	.943	0.096	.706	0.457	**.043**	0.090	.692
	V2	0.047	.885	0.359	.251	0.657	**.015**	0.341	.233
MMP-9	V1	-0.232	.369	-0.098	.708	0.334	.162	0.043	.853
	V2	0.306	.334	0.394	.205	0.620	**.024**	-0.080	.786
MMP-12	V1	-0.050	.854	0.315	.235	-0.003	.990	-0.094	.694
	V2	-0.032	.926	0.018	.957	-0.183	.569	-0.080	.786
MMP-13	V1	0.282	.290	0.434	.093	0.164	.516	-0.016	.946
	V2	0.298	.374	0.238	.481	-0.181	.573	-0.628	**.022**

		Asthma Index				Jackson Cold Score			
		AUC (original, adjusted)		Peak (original)		AUC		Peak	
		Spearman's rho	*Significance*	*Spearman's rho*	*Significance*	*Spearman's rho*	*Significance*	*Spearman's rho*	*Significance*
G-CSF	V1	0.440	.077	0.235	.363	0.396	.093	-0.120	.605
	V2	-0.191	.574	-0.096	.780	0.189	.557	-0.297	.325
IL-1β*	V1	0.182	.484	0.258	.318	0.535	**.018**	0.193	.401
	V2	-0.055	.881	0.024	.947	0.700	**.016**	0.430	.163
IL-8*	V1	0.184	.512	0.166	.555	0.417	.096	0.076	.758
	V2	0.180	.699	-0.378	.403	0.360	.342	-0.214	.581
VEGF*	V1	0.415	.110	0.319	.228	0.457	.056	0.129	.586
	V2	0.600	.067	0.321	.365	0.445	.170	0.088	.785
ENA-78	V1	0.203	.451	0.165	.542	0.360	.142	0.194	.413
	V2	-0.566	.055	-0.375	.230	-0.027	.929	-0.100	.735
IL-1α	V1	-0.004	.991	-0.164	.611	0.351	.219	0.130	.632
	V2	0.136	.728	-0.136	.728	0.514	.106	0.544	.084
MCP-1*	V1	0.009	.972	-0.166	.511	0.559	**.010**	0.147	.513
	V2	-0.745	**.008**	-0.647	**.031**	0.070	.829	-0.041	.893
MIP-1β*	V1	-0.022	.933	0.178	.494	0.570	**.011**	0.270	.236
	V2	-0.336	.286	0.144	.656	0.454	.119	0.326	.255
IP-10*	V1	0.344	.162	0.189	.453	0.356	.123	0.098	.665
	V2	-0.209	.537	-0.588	.057	0.028	.931	-0.433	.140

Table 6.5 Levels of cytokines at cold visits V1 (within 48hrs of cold symptoms) and V2 (day 4-6 of cold) and their correlation with peak and area under the curve (AUC) of asthma index and Jackson Cold Score

Figures represent Spearman's rho with significance representing the associated p-value. Cytokines whose expression is directly influenced by IFN-β are marked with an asterisk (*).

6.4 Discussion

This part of the study looked at the feasibility of measuring mostly pro-inflammatory cytokines and matrix metalloproteinases in nasal lavage and at fluctuations in nasal cytokine and MMP levels between asymptomatic periods and during naturally occurring colds in children aged 5-11 years with asthma (objective i.). In addition it explored the correlation between levels of these cytokines and MMPs and exacerbation severity (objective ii.), virus status (objective iii.) and the asthma index as well as the Jackson cold score (objective iv.). It confirms some of the findings of previously reported studies but also provides some novel observations about virus-induced cytokine and MMP responses in the context of viral respiratory tract infections and asthma. Many cytokine levels in nasal lavage (MIP-1β, IL-8, G-CSF, IL-1β, and VEGF) correlated well with those published in the literature both in respect of baseline levels as well as during colds/asthma exacerbations (Lewis et al., 2012, Noah et al., 1995, Manthei et al., 2014). MCP-1, IP-10, and ENA-78 levels were lower when compared with another study but incremented during colds in a comparable fashion (Lewis et al., 2012, Donninger et al., 2003). Observed differences between levels are most likely due to three factors: (1) the methods of nasal lavage or nasal mucus sampling and the fluid amount used, (2) the study population (children versus adults, healthy versus asthmatic), (3) the times of sampling (baseline and cold/asthma, cold/asthma versus resolution of cold).

Significantly increased levels of cytokines and MMPs were found during viral colds in comparison with asymptomatic periods (objective i.) either at the first cold visit (V1) which was conducted within 48 hours of cold symptoms (IL-1β, IL-8, IP-10, and MIP-1β), at the second cold visits (V2) 4-6 days into the cold (VEGF, MMP-3, MMP-8), or at both cold visits (MMP-7, G-CSF, ENA-78, and MCP-1). The initial analysis performed only included comparison of participants where valid sample results from all three time points were available. The subsequent inclusion of all pairs of valid nasal lavage samples led to a decrease of the p-value, suggesting that increasing the number of participants would have potentially shown an even more significant difference of cytokine levels between asymptomatic and cold episodes. No significant difference in levels was found for any of the analysed cytokines or MMPs between first and second cold visit pointing towards persistently raised levels over a period of a few days during a cold without major fluctuations. Sustained increases in certain cytokines for a week following viral infection have previously been demonstrated in another study (Lewis et al., 2012).

In an attempt to elucidate the significance of cytokine and MMP level fluctuations in relation to severity of cold symptoms on the one hand and asthma exacerbations on the other hand correlation with symptom scores (Jackson Cold Score [JCS] (Jackson et al., 1958), Asthma Index [AI] Score (Sorkness et al., 2008)) were performed (objective iv.). Correlation between AUC of the JCS during the cold episode and cytokine/MMP levels at V1 only was statistically significant for MMP-1, MMP-3, MMP-7, MCP-1 and MIP-1β and at V2 only for MMP-9. Correlation between AUC of the JCS and cytokine/MMP levels at both V1 and V2 was statistically significant for MMP-8 and IL-1β. The correlation with the peak of the JCS was only significant for MMP-13 at V2. These findings support the notion that sustained cold symptoms over a period of time (as reflected in the AUC) rather than the acute and short-lived peak in cold symptoms lead to an increase in certain pro-inflammatory cytokines and a number of MMPs with airway remodelling capacities.

The Asthma Index was calculated daily as described in a publication by Sorkness *et al*. (Sorkness et al., 2008) . In a modification, in addition to the peak of the Asthma Index also the AUC was calculated and correlated with cytokine and MMP levels. The only statistically significant correlations between cytokine/MMP levels and AI were found in relation to the matrix metalloproteinase MMP-7 at V1 during the cold and in relation to the cytokine MCP-1 at V2. A recent study did not show a relation of MCP-1 levels with symptom scores but an inverse relationship of baseline MCP-1 levels from nasal samples to peak viral titres (DeMore et al., 2009) and postulated a protective anti-viral effect through recruitment of monocytes to the airways. Correlations between sputum cytokines and the Asthma Index Score (peak) were conducted in a recent study looking at the relationship between lower airway inflammation and severity of an exacerbation and confirmed for IFN-α1, IFN-β1 and IFN-γ mRNA levels (Schwantes et al., 2014).

The lack of correlation between most cytokines/MMPs analysed in this study and the AI may have a number of explanations: Firstly, analysing nasal lavage samples may not necessarily reflect the situation of the lower airways in respect of cytokine levels but induced sputum was not collected in this study and bronchoalveolar lavages would not have been feasible. In two studies, nasal epithelial cells, in comparison to bronchial epithelial cells, constitutively expressed higher levels of certain cytokines (IL-6, IL-8, RANTES, MMP-9) (McDougall et al., 2008) and this was also specifically demonstrated in a group of children in relation to IL-6, IL-8 and G-CSF (Pringle et al., 2012). The same *ex vivo* studies on airway epithelial cells from both nasal and bronchial origin have however demonstrated comparable increases of a number of cytokines (IL-6, RANTES, VEGF, MCP-1, MMP-9 and TIMP-1) in cells of both origins on stimulation

with IL-1β and TNF-α (McDougall et al., 2008). Very recent transcriptomic studies (Poole et al., 2014) also appear to provide some evidence of similarity of cytokine mRNA expression throughout the airway in line with the “united-airway” hypothesis (Grossman, 1997). Secondly, the majority of children developed only minor increases in asthma symptoms while only three subjects were given oral prednisolone during an exacerbation. The subtlety of changes during viral respiratory infections in the study participants may have not produced a big enough effect on asthma symptoms. Clear correlations with cold symptom scores were demonstrated for a number of cytokines/MMPs as outlined above. The significance of these findings however are limited by repeated statistical analysis to look for associations as there is a risk of finding an association by chance. Theoretically, and in the context of current knowledge about the role of the described cytokines and MMPs, all associations are biologically plausible (as described in the introduction and above) but due to the overall low sample numbers only a statistical significance can be described. A possibility remains that associations found are potentially false positive.

A broad panel of cytokines (including Th1- and Th2-type lymphokines, pro-(and anti-) inflammatory cytokines, growth factors and chemokines) and matrix metalloproteinases were included in the analysis. All of them have been discussed in the literature to varying extent in the context of immunpathogenesis of asthma in general and (virus-induced) asthma exacerbations specifically as outlined in the introduction. While some of these factors are more obviously associated with asthma and viral respiratory tract infections (e.g. IP-10, RANTES, MMP-9), others have only more recently been considered and studied in more detail (e.g. ENA-78, MMP-12). Many of the studied cytokines are directly or indirectly influenced by IFN-β, either as IFN-β related proteins with antiviral activity (IP-10), pro-inflammatory cytokines (MCP-1, MIP-1β, IL-1β, IL-8) or angiogenic growth factors (VEGF). IFN-β itself has been reported to be unstable and difficult to be measured reliably (Edwards et al., 2013). A potential interventional trial using nebulised IFN-β therefore can rely on the measurement of these cytokines that are either up- or downregulated by IFN-β.

Interestingly, IL-4 and IL-5 (both Th2-type cytokines) were either not detected (IL-4) or only in very few samples/participants (Il-5) and, if present, at levels just below or above the bottom end of the standard curve of the assay. This is a surprising finding, as there is an increase in the number of CD4+ cells in the asthmatic airway, which are predominantly of the Th2 subtype (Barnes, 2008b) and producers of IL-4 and -5 (and -13) and a majority of the study participants were atopic. In a study of allergic and non-allergic asthmatic children with chronic rhinosinusitis it was shown that both groups had

a typical Th2 cytokine pattern and in addition there was a correlation between high levels of IL-4 and low levels of IFN-γ (Riccio et al., 2002). The most plausible explanation for the lack of detecting Th2 cytokines is two-fold: a dilutional effect by using 5mL of 0.9% saline solution for nasal lavages and (at least for IL-4; 4.46 pg/mL) a potential lack of sensitivity of the assay used. In one study measuring IL-6 and IL-4 in nasal lavage samples of 12 participants with experimental influenza A infection (sensitivity 3.0 pg/mL) IL-4 was not found in any of the baseline samples and only in one participant during infection (Gentile et al., 1998). Another study also was unable to detect IL-4 in any nasal lavage samples of atopic and non-atopic adults with common colds (Linden et al., 1995). In 15 patients with perennial rhinitis and asthma with moderate-to-severe nasal obstruction on the other hand mean IL-4 levels in nasal lavage of 11.8 ± 3.3 pg/mL (controls: 0.8 ± 0.4 pg/mL) were detected (Ciprandi et al., 2004). One exclusion criterion of that study, however, was acute or chronic upper respiratory tract infection. Bossley et al. (2012) noted the lack of Th2-cytokine expression in a group of carefully characterized children with severe therapy-resistant asthma both in the airway mucosa and the lumen (Bossley et al., 2012). The failure to detect IL-2 and IFN-γ, both Th1 cytokines, is perhaps less surprising, as the expression of IFN-γ is reduced in T cells from the airways of asthmatic patients in comparison to non-asthmatic patients (Finotto et al., 2002), although levels have been found to be elevated during acute exacerbations (Schwantes et al., 2014), viral infections (Linden et al., 1995, Lewis et al., 2012) and in patients with more severe disease (Kumar et al., 2006). Again, it could be that dilution in the nasal lavage sampling process may have led to a negative assay result. Fleming *et al.* described in their study of experimental colds in healthy non-atopic and atopic asthmatic adults that IL-5, IFN-γ, and RANTES were detected only in nasal lavages from two asthmatic subjects, who had the most severe colds (Fleming et al., 1999) and in another experimental cold study only about a third of subjects during acute colds had detectable IFN-γ responses (DeMore et al., 2009).

Viruses have been clearly associated with asthma exacerbations both in adults (Nicholson et al., 1993) and in childhood (Johnston et al., 1995) but the exact mechanisms leading up to the pathophysiological features of multicellular inflammation, mucus overproduction, enhanced bronchial responsiveness, and airflow obstruction are not fully understood. The challenges in investigating these processes lie in the complexity of gene-environment interactions between the host of the infection and the viral pathogen itself (Busse et al., 2010), the host innate and adaptive immune response and the cascades of pro- and anti-inflammatory cytokine responses and

antiviral defence systems and their interaction with cells of the airways and lungs that may lead to increased airway responses. The interaction with other possible triggers such as allergens has also sparked a considerable amount of interest in studies investigating asthma exacerbations (Green et al., 2002, Murray et al., 2006). Additional challenges arise from the fact that a large number of different respiratory viruses with an even larger number of strains can lead to respiratory tract infection but may trigger different pathways with involvement of distinct cellular and cytokine responses (Kato et al., 2011, Spann et al., 2014b, Schaller et al., 2006, Spann et al., 2014a) that may converge to a common pathway leading to asthma exacerbations in predisposed individuals. Some studies have reported similarities between cytokine production following infection with different respiratory viruses. Both the number of viruses and the number of cytokines investigated were limited (Noah et al., 1995, Fritz et al., 1999) and their analysis does not rule out potential differing pathways depending on virus type. Due to the limited number of participants in the study with a number of distinct viral pathogens this aspect may have had an impact on the analysis because all viral infections were combined in one group as looking at individual viruses and their cytokine responses would not have been meaningful.

A further limitation of this study is the lack of a healthy control group that would allow comparison of cytokine/MMP fluctuations between this group and children with asthma. Participating children were their own controls in the sense of providing samples during asymptomatic periods as well as during naturally occurring colds and 8 cytokines (G-CSF, IL-1β, IL-8, VEGF, ENA-78, MCP-1, MIP-1β, IP-10) as well as 3 MMPs (MMP-3, MMP-7, MMP-8) showed distinct responses with elevated levels during cold episodes. A surprising finding of the study was the high number of viral nucleic acids positive asymptomatic periods (baseline) but no significant difference in cytokine levels between virus-positive and virus-negative baseline samples was demonstrated apart from elevated IP-10 levels in the virus-positive group (objective iii.). This is consistent with findings of another study when viral persistence up to 6 weeks post recovery from an acute asthma exacerbation was associated with increased IP-10 and IL-10 mRNA expression (Wood et al., 2011). Participants in the study with virus presence at baseline were not known to have had an asthma exacerbation but it is possible that they had a cold up to 4 weeks prior to enrolment in the study. It can therefore be concluded that IP-10 levels can remain elevated several weeks following a common cold in these children even if they had not experienced an increase in asthma symptoms or an exacerbation. As pointed out in the study by Djukanovic et al. '*CXCL10 [=IP-10] may be a useful biomarker for clinical development of IFN-β*'

(Djukanovic et al., 2014) and therefore this is likely a significant and biologically very plausible finding and may indicate a persistent low grade activation of the interferon pathway leading to IP-10 in the presence of viruses. A comparison of cytokine and MMP level differences between virus-positive and virus-negative cold episodes (+/- exacerbations) in the study (objective iii.) was limited by the fact that in only 3 children viral nucleic acid was absent and nasal lavage samples for cytokine studies were only available for 2 of these children. Therefore no significant differences were demonstrated apart from significantly elevated levels of G-CSF in the virus-positive group.

G-CSF production is stimulated by HRV infection (Gern et al., 2000, Leigh et al., 2008) and is a regulator of neutrophil recruitment and involved in the pathogenesis of virus induced respiratory symptoms and airway hyperresponsiveness (Fleming et al., 1999). Therefore the result would be biologically plausible. Due to the lack of numbers, however, this could be regarded a probable false positive result in this specific context and does not allow to draw further conclusions. IL-8 and IL-1β are also known to be involved in neutrophil recruitment and were significantly elevated during colds. This is consistent with findings of previous studies of both naturally occurring (Lewis et al., 2012) and experimentally induced (Proud et al., 1994, Turner et al., 1998, de Kluijver et al., 2003) colds. IL-1β during acute viral infections was also to some extent predictive of virus-induced asthma exacerbations in one study (Manthei et al., 2014) and a reduction in IFN-β has been shown to lead to an increase in IL-1β secretion (Guarda et al., 2011). Due to very low numbers of participants with no exacerbation or severe exacerbation this predictive value could not be confirmed. By upregulating the expression of the receptor for rhinoviruses (ICAM-1) IL-1β contributes to the rhinoviral spread (Paolieri et al., 1997). The IL-1β receptor antagonist IL-1ra was also part of the cytokine panel investigated in the study but levels of IL-1ra were greater than the upper limit of the Luminex standard curve in more than 80% of samples and were therefore not included in further analysis (as additional aliquots not available for dilution and retesting). IL-1ra possibly modulates IL-1β mediated inflammation as an endogenous inhibitor of RV-induced airway inflammation and higher baseline levels were demonstrated in non-asthmatic compared to placebo (i.e. no steroids) – treated asthmatics (de Kluijver et al., 2003).

VEGF, a crucial growth factor mediating increased vascular leakage and inducing angiogenesis, was significantly increased in nasal lavage 4-6 days following initial cold symptoms but no significant correlation with either cold or asthma symptom scores could be demonstrated. Previous reports on VEGF measurements in induced sputum

(Abdel-Rahman et al., 2006, Hossny et al., 2009) and nasal lavage (Psarras et al., 2006) from children and nasal lavage from adults (Lee and Lee, 2001) also showed increased levels during acute asthma exacerbations. The magnitude of VEGF response during acute infection was predictive of virus-induced asthma exacerbation in a recent study that included 59 children (Manthei et al., 2014). The importance of VEGF in the context of viral infection and asthma exacerbations seems convincing considering its role for increased vascular permeability (Sedgwick et al., 2002), allowing inflammatory cell migration and increased vascular density with augmenting potential lower airway obstruction. ENA-78, also a promoter of angiogenesis and connective tissue remodelling as well as chemo-attractant for neutrophils, was significantly elevated during viral cold episodes (both at V1 and V2). Unlike one study (Donninger et al., 2003), that showed increased levels of ENA-78 in acute asthma depending on RV presence, it was not possible to demonstrate this association due to the low numbers of children without viral nucleic acid detection. The sustained elevation of ENA-78 levels at V1 and V2 indicates that ENA-78 supports ongoing neutrophil traffic from the vascular compartment to interstitial tissue spaces contributing to ongoing airway inflammation.

The cytokine MCP-1 (with chemotactic activity for monocytes and basophils) and the matrix metalloproteinase MMP-7 (with chemotactic activity for neutrophils) were both significantly raised during cold episodes when compared with baseline levels (objective i.) and both correlated significantly with cold symptom score (Jackson Cold Score) and asthma severity as expressed by the asthma index (which integrates PEFR and asthma related lower airway symptoms) (objective iv.). A recent study, investigating nasal lavage cytokines in a cohort of asthmatic children, similarly demonstrated raised MCP-1 levels correlating with subjective (upper and lower respiratory) symptom scores (Lewis et al., 2012) but did not take into account objective measures of lung function such as PEFR. The findings of this study therefore emphasize the importance of MCP-1 in the context of viral induced asthma exacerbations. MCP-1 contributes to airway inflammation via its ability to stimulate surface IgE-positive or IgG-4 positive B cells for enhanced IgE/IgG4 production (Kimata et al., 1996) and induction of degranulation of cytotoxic T cells and natural killer cells (Taub et al., 1995). While in this study elevated MCP-1 levels were demonstrated in nasal lavage, augmented release of MCP-1 was previously demonstrated also in airway epithelial cells from distal segments of the bronchial tree in the context of human enterovirus infection (Renois et al., 2010) and in bronchoalveolar macrophages of children with asthma (Schneider et al., 2013). MMP-7, a matrix metalloproteinase with broad substrate specificity and ability to degrade elastin,

proteoglycans, type IV collagen among other components found in lung matrix (Dunsmore et al., 1998), has indirect chemotactic activity for neutrophils by creating chemokine gradients and participates in matrix remodelling also by inactivating α1-antitrypsin (Sires et al., 1994). Its function and role in controlling repair of the airway epithelium has been well described. Dunsmore *et al.* describe in a series of *ex vivo* and *in vitro* studies the specific importance of MMP-7 in epithelial cell migration and re-epithelialization of airway wounds (Dunsmore et al., 1998). As MMP-7 is constitutively expressed by airway epithelial cells important functions in innate mucosal immunity of airways (Lopez-Boado et al., 2001) as well as a role in digesting glycoproteins and membrane debris and thereby facilitating mucous flow in the airway (Dunsmore et al., 1998) have been postulated. A more recent study showed that patients with chronic (untreated) asthma expressed MMP-7 in their distal airspaces (but not healthy controls) and, in response to a potent allergenic stimulus, had significantly more nasal production of MMP-7. It was claimed that airway epithelial expression of MMP-7 was a crucial component for the development of asthma-like disease (Goswami et al., 2009).

The specific importance of MMP-7 becomes clearer in the context of type-2-driven inflammation. Beale et al. have shed light in a recent study on the relation between rhinovirus infection and type-2-driven immune responses and inflammation (Beale et al., 2014). They explore the role of IL-25, as a member of the interleukin-17 family, in triggering type-2-driven inflammation. It is described how not only typical type-2-inducing stimuli (e.g. allergens) but also rhinovirus interacts with bronchial epithelium resulting in the promotion of a type-2 immunity which is central to the immunpathogenesis of asthma. They hypothesised that rhinovirus-induced IL-25 was necessary for advancing the immune cascade leading up to allergic airways inflammation during rhinovirus-induced asthma exacerbations. They showed in vitro that rhinovirus-infected cultured asthmatic BECs expressed higher levels of IL-25 (which was in correlation with their donor atopic status, i.e. increased RV-induced IL-25 expression was associated with sensitization to a greater number of common environmental allergens). In vivo human IL-25 expression in airways was higher in patients with asthma, both at baseline and during experimental rhinovirus infection. In mice, rhinovirus infection both induced IL-25 expression and also augmented allergen induced IL-25. Maximum activity of IL-25, however, can only be achieved in presence of epithelial MMP-7 as described in the study by Goswami et al (Goswami et al., 2009). Goswami et al. describe increased IL-25 production in the airway as a result of proteinase allergen stimulation and the subsequent induction of a Th2 immune response with marked lung eosinophilia, mucus hyperproduction and increase in

expression of Th2 cytokines. In a mouse study they showed that airway epithelial cells expressed MMP-7 following exposure to proteinase allergen or recombinant IL-25 and that MMP-7 was essential for cleaving IL-25, thereby augmenting its effect on Th2 differentiation. In vivo experimental exposure to ragweed extract led to significantly higher levels of MMP-7 in nasal secretions of atopic patients. In humans with chronic asthma MMP-7 and IL-25 expression in distal lung was shown (bronchial biopsy specimens). While healthy volunteers also showed IL-25 expression, MMP-7 was exclusively seen in airways of asthmatic patients. The group concludes that all their results provided evidence that MMP-7 served as a pro-inflammatory mediator that augments the effect of IL-25 on Th2 immune responses both by modulating IL-25 expression and cleaving IL-25.

In conclusion, the novel finding that MMP-7 levels in nasal lavages were not only significantly raised in the context of upper airway inflammation in children with asthma but also correlated with cold symptom scores and asthma index during acute exacerbations, makes biological sense. IL-25 was however not part of the cytokine panel explored in the study. The current results suggest that MMP-7 has a significant role in viral induced asthma exacerbations through augmentation of airway inflammation and that it would be worthwhile exploring the relation of MMP-7 and IL-25 in the context of respiratory virus induced asthma exacerbation in children. In this study MMP-7 levels were determined independently of its inhibitor (TIMP). Rather than looking at MMP-7/TIMP ratios, comparisons of MMP-7 levels were made in nasal lavage obtained at different time-points (e.g. asymptomatic and cold episodes) and a significant difference was demonstrated. Future studies should include the assessment of the inhibitor of MMP-7 to provide a more comprehensive picture of the complex MMP/TIMP network.

Two further matrix metalloproteinases, MMP-3 and MMP-8, were also shown to be significantly raised during cold exacerbations and correlated with cold symptom scores but not with asthma symptoms. Interestingly, mainly based on studies in mice, a protective role of MMP-8 in asthma has been postulated as MMP-8 contributes to neutrophil clearance (Gueders et al., 2005) as well as inactivation of the pro-inflammatory macrophage inflammatory protein-1α (Quintero et al., 2010). MMP-3 is linked to enhanced procollagen I production in bronchial fibroblasts of asthmatic patients and subsequent reduced lung function (Todorova et al., 2010). There is, however, a significant lack of studies exploring the role of MMP-3 and MMP-8 in viral respiratory tract infections as well as acute asthma and this study is the first to demonstrate significant correlations with cold symptoms in children with asthma. A

significant correlation with cold symptoms was also shown for the macrophage inflammatory protein-1β (MIP-1β) at the start of the cold (V1), which possibly reflects its importance as a pro-inflammatory cytokine involved in chemotaxis, degranulation, phagocytosis and mediator synthesis in the early stages of viral respiratory tract infections. One study showed a clear correlation of MIP-1β with lower respiratory tract symptoms (Lewis et al., 2012), a finding that was not replicated in this study.

In summary 8 cytokines in nasal lavage samples (G-CSF, IL-1β, IL-8, VEGF, ENA-78, MCP-1, MIP-1β, IP-10) as well as 3 MMPs (MMP-3, MMP-7, MMP-8) showed distinct responses with elevated levels during cold episodes that remained elevated over the course of a week (objective i.). Correlation between AUC of the Jackson Cold Score during the cold episode and cytokine/MMP levels was statistically significant for MMP-1, MMP-3, MMP-7, MCP-1 and MIP-1β at V1, MMP-9 at V2 and for MMP-8 and IL-1β at V1 and V2 (objective iv.). There was a distinct lack of correlation of most cytokines with the asthma index, a score which integrates PEFR changes and asthma related lower airway symptoms. Only the cytokine MCP-1 and the matrix metalloproteinase MMP-7 were both significantly raised during cold episodes when compared with baseline levels and both correlated significantly with cold symptom scores and asthma severity as expressed by the asthma index (objective iv.). MCP-1 and MMP-7 have chemotactic and pro-inflammatory properties and their roles in the context of viral induced asthma exacerbations should be further explored. In addition, the significant increase of further matrix metalloproteinases, MMP-3 and MMP-8, during colds and correlation with cold symptom scores in a group of asthmatic children calls for further studies examining the function of matrix metalloproteinases in this context. The property of IP-10 levels to distinguish virus positive from virus negative episodes (objective iii.), even and especially in clinically asymptomatic children, should be further explored in longitudinal studies. It may allow identification of children with asthma who could be in a chronically pro-inflammatory state driven by respiratory viral infection.

7 Discussion

The primary aim was to conduct an observational and feasibility study with a group of children aged 5 to 11 years with asthma and piloting various components of a protocol developed in the build-up to a potential interventional trial (with the hypothesis that a treatment with a course of nebulised IFN-β at the start of upper respiratory tract infection symptoms would prevent it triggering an exacerbation of asthma). In a very recent editorial (Jackson, 2014) it was described as being *"of great interest to bring this strategy to the pediatric population"*.

In order for the protocol to be successfully applied in an interventional trial a cohort of mild-to-moderate asthmatic children burdened by frequent viral induced exacerbations despite otherwise reasonably well-controlled asthma needed to be identified. Firstly, the aim was to characterise their baseline characteristics during asymptomatic periods in terms of lung physiological parameters (PEFR, FEV_1), cold and asthma symptoms (Jackson Cold Score and Asthma Index respectively), virus status (in nasal lavage and saliva), and inflammatory cytokine networks (in nasal lavage). Secondly, the aim was to compare these findings to findings during symptomatic cold episodes and determine correlations between severity of colds (and potential cold induced asthma exacerbations) and clinical, lung physiological and laboratory parameters.

The specific objectives included the exploration of demographic and asthma specific features of participants, documentation of the frequency of common colds and viral induced asthma symptoms, identification of a cut-off for the Jackson Cold Score to describe a cold, and the validation of the Asthma Index and evaluation of original and modified versions of the Asthma Index in the light of established asthma severity criteria. Further important objectives were the assessment of the compliance of study participants with a text based system to monitor cold and asthma symptoms, and of the compliance with performing PEFR measurements and the evaluation of the validity of SMS-transmitted PEFR data points by comparison with PEFR-meter stored values. To establish the acceptability of SMS-data collection parental feedback via questionnaires was evaluated. Furthermore the infecting viruses in a cohort of children with asthma were described in terms of frequency, type, and seasonality and the incidence of multiple virus infections was established. When conducting research one always has to consider the burden put on study participants. A further objective of this study therefore was to evaluate whether determining viral nucleic acid in saliva (which can be easily collected) would be equal to detection in nasal lavage (that requires a slightly more

invasive approach). Potential associations of virus status with cold symptoms and the Asthma Index were then explored.

The final objectives included the assessment of the practicality of assaying known and potential novel markers of airway inflammation (cytokines and matrix metalloproteinases [MMPs]) in nasal fluid and explore fluctuations of these markers between between asymptomatic periods and episodes with cold symptoms and of potential correlations with severity of respiratory viral infection and exacerbation of asthma. Furthermore correlations between levels of nasal lavage cytokines and MMPs and virus status (positive/negative) as well as Jackson Cold Score and Asthma Index were explored.

In this final chapter findings of the present work will be summarised and discussed in the light of the initial hypotheses and objectives. It will reflect on the novel findings, possibilities for alternative approaches and further research and includes the strengths, some of the challenges and the weaknesses/limitations of this study.

7.1 Text-based (SMS-) monitoring of research participants

Any trial that involves patients with asthma, where symptoms can frequently change (especially so during an exacerbation), relies on the possibility to capture changes in symptoms, lung function and medication use promptly, reliably (Reddel et al., 2009) and without putting too much burden on study participants. To assess these fluctuations, asthma symptoms are usually assessed twice-daily as this potentially allows capturing episodes of asthma-associated morbidity in a timely manner before the occurrence of the actual event of interest (Stull et al., 2009).

In this study an SMS-based system was used to capture cold and asthma symptoms and PEFR data in more or less real-time twice daily both during asymptomatic and symptomatic episodes and to capture the time-point when parents considered their child to show first signs of cold symptoms. This system sent software-generated text-messages to the parents of study participants, sent reminder messages in case of failure to reply, and received and stored time-tagged responses including PEFR data and a symptom diary. The average compliance with the SMS-diary was 93.5% and this high compliance provided sufficient data to reliably calculate the relevant cold and asthma scores in this study. Response rates reported in the literature vary massively (from 15% (Bexelius et al., 2009) to 100% (Kew, 2010)) and factors identified for

improved compliance included (not-surprisingly) meeting between researcher and participant prior to data collection and use of reminders (Kew, 2010). All parents in this study were happy to use SMS messaging for transmission of research data, and more than half of them said they were more likely to participate in a study again if SMS were used for research data collection. This is in line with two other asthma-related studies that received positive participant feedback for using text-based data collection (Neville et al., 2002, Anhoj and Moldrup, 2004). In this study parents were asked to transmit the PEFR generated by their child on a digital PEFR meter that stored PEFR data. This allowed comparison of SMS-transmitted and digitally stored PEFR data. Overall compliance with measuring PEFR was above 80% and there was no significant difference between the first and second week of monitoring. Other studies have found similar compliance rates but documented a rapid decrease in compliance if monitoring was conducted over a 3 to 5 week period (Redline et al., 1996, Kamps et al., 2001, Meuric et al., 2005). A surprising finding was that the overall transmission rate of correct PEFR values (i.e. corresponding with data on PEFR meter) was only 65.5%, while 15% were incorrectly transmitted and in 8.3% of cases values were 'self-invented'. Large inter-subject variations were observed both in terms of compliance with PEFR measurement and in the relationship between digitally recorded PEFR values and SMS-transmitted values. Parents needed to either read the value correctly from the digital PEFR meter or rely on the reporting of the PEFR value by their child and then include the correct value in an SMS on their phone. As the time of PEFR measurement and time of receiving the computer-generated text message may not exactly coincide an element of failing to remember an exact number (such as PEFR) may be part of the explanation for incorrect transmissions. In addition parents may have felt the wish to please the researcher and send an invented value rather than admitting that the PEFR had not been measured. 'Self-invention' of values varies in other studies from close to 0%-4.6% (van der Meer et al., 2006) to 12% (Meuric et al., 2005) and even up to 22% (Kamps et al., 2001, Verschelden et al., 1996). Most data were received in a more or less real-time manner; therefore the problem of retrospective completion of diary entries ('parking-lot problem') well known in paper-and-pencil diaries (Stone et al., 2002) was not an issue in this study. The use of time-tagged data is clearly an advantage and one of the pre-requisites of momentary assessments where time of data entry can be as important as the accuracy of the entry itself. The comparison between SMS-transmitted PEFR data points with PEFR-meter stored values however showed that these data still need to be carefully scrutinized and that the calculation of the asthma index should be based on the digitally stored data rather than the SMS-transmitted values.

In this study parents also submitted symptom scores for their children twice daily, the accuracy of which was not assessed separately and therefore this is certainly a limitation of the study. Not only do these symptom scores rely on the ability of a child (and their parent) to interpret symptoms (such as shortness of breath or headache) correctly, but also assign a severity grade (0-3) to the symptom, and inform their parent who transmits this information as a code of numbers to the researcher. An interview study of parents of children up to the age of 6 years with respiratory problems showed that even parents vary considerably in their perception and interpretation of respiratory symptoms (Young et al., 2002). The fact that the study participant performing the PEFR manoeuvre and experiencing symptoms differs from the person who records, potentially interprets, and then transmits the data is a limitation of any study involving young children and therefore also of this study. This may partly explain the higher percentage of correctly reported PEFR values (79%) in a study of 97 adolescents who both performed PEFR manoeuvres and then reported the results themselves by either entering them on a specifically designated web application or sending them via SMS (van der Meer et al., 2006). The overall compliance in this study with performing PEFR measurements was almost identical to this study (83%) (van der Meer et al., 2006) and adolescents found this way of data transmission easy and fast (van der Meer et al., 2007).

In this study parents were sent a daily SMS asking them about whether they thought their children had developed symptoms of a cold in order to enable the research team to perform a home visit to capture early changes in lung function, nasal lavage inflammatory markers and to determine the presence of respiratory viruses. While difficult to proof and not evaluated in the study, this daily SMS may have also served the purpose of reminding parents that their child needed to take their steroid inhaler and therefore improved medication adherence. This assumption is based on the discovery that most exacerbations children experienced while in the study only required increase of β-agonist inhalers (and were therefore classified as ‘moderate’) rather than use of systemic corticosteroids. Based on the Childhood Asthma Management Program, a multicenter clinical trial of 1,041 children, treatment with inhaled corticosteroids was predictive of having no severe asthma exacerbations (Wu et al., 2011).

The use of SMS in this study enabled time-tagged capturing of symptoms and PEFR data and was well-received by parents of study participants as documented in their feedback about this data collection tool. It will allow careful monitoring of study participants in an interventional trial and deliver real-time data that inform the

researcher about subtle changes instantly and provide an adequate reflection of the events during a viral induced asthma exacerbation. The bias and error in recollected reports of handwritten diaries are largely avoided. The limitations of this form of data collection include the need for correct transfer of data into a code of numbers, the problem of lack of mobile phone reception in some areas (which was also part of the feedback I received), and the split between the device measuring PEFR and the mobile phone used for data transmission leading to errors. There are other pitfalls that are more inherent to the questions and questionnaires used (i.e. symptom interpretation by children and parents) rather than to the data capturing system.

Technological innovations are rapidly changing and advancing the way of real-time data capturing for both research purposes but also for improving patient care. Nowadays there is a large number of hardware platforms used as electronic subject diaries such as special-purpose devices for collecting one measure by button push, pen-based multifunctional portable computers, or interactive voice response systems (Raymond and Ross, 2000). In the context of asthma research, devices that provide an integrated electronic diary with a peak flow meter (Ireland et al., 2012) and in combination with Bluetooth technology (Moor et al., 2005) that transfers data instantly into an e-Diary, PDA or mobile phone may be the way forward.

7.2 Viral induced asthma exacerbations

In consideration of the objectives mentioned at the beginning of the chapter, a main point of interest of this observational and feasibility study was the timely capturing and monitoring of viral induced asthma exacerbations with description of symptom and lung function (PEFR) changes, as well as documentation of aetiological viral agents and inflammatory pathways.

Children in this study had on average 5 exacerbations in the year prior to enrolment in the study, half of which were associated with viral upper respiratory tract infections (URTI). Despite this, 5 children did not report any cold episodes during their enrolment in the study. This phenomenon might be due to either parental exaggeration at the time point of enrolment, parental underreporting while in the study or a real decrease in URTI frequency. This phenomenon poses a challenge for a study like this (in terms of identifying the correct study group) but is not new (Connett and Lenney, 1993). Johnston et al. (1995) in their 13 months community based study of 108 children between 9-11 years also allude to this issue of underreporting. Their mean (SD) number of episodes per child was 5.33 (4.99) for lower respiratory tract symptoms, and 7.24 (4.47) for upper respiratory tract symptoms during this time period. A weakness of

the study presented in this thesis in this respect was also the enrolment of children at different times/seasons of the year (unlike the study by Johnston et al. (1995)) and initially for a pre-specified time period of 6 months only leading to drop out of participants who for example had not developed any cold between March and September. The low frequency of viral induced common cold symptoms and significant asthma exacerbations was surprising and did not correspond with the parental report at the time of enrolment.

In order to be able to intervene with a potential preventative measure symptoms leading up to a viral induced asthma exacerbations need to be recognized and reported. This study relied on parental perception of their child displaying symptoms of a cold and reporting a cold by answering a daily SMS. This triggered a phone call to the parent to discuss the symptoms and, if consistent with an early cold, to arrange for a home visit within 24-48 hours. The fact that all but three reported colds were associated with positive viral PCRs provided reassurance about this approach in capturing colds appropriately. In line with previous larger epidemiological studies (Johnston et al., 1995), the detection rate of respiratory pathogens during cold and exacerbation episodes was 88% when looking at paired nasal lavage and saliva results, and 87.5% and 65% when looking at nasal lavage and saliva individually. However, there was no correlation between virus status (negative/positive) and Jackson Cold Score and Asthma Index respectively. This analysis was however significantly compromised by the overall low numbers of virus negative cold episodes and therefore poses a significant weakness of the study.

The timeframe until conductance of the first home visit was on the one hand chosen for pragmatic reasons as all relevant parties (the child, the parent(s), a research nurse and the research fellow) needed to be available. On the other hand this was considered a feasible time-frame for an interventional trial that would require administration of a study drug during these 24-48 hours. One of the findings however was that children who developed moderate or severe exacerbations (and therefore most of the participants) very often had already developed lower respiratory tract symptoms such as wheeze or shortness of breath during the first 24-48 hours and required increased use of their inhaler. Prodromal symptoms preceding the overt exacerbation have been studied in a group of 134 children with asthma and included respiratory (e.g. rhinorrhoea and cough) as well as other symptom, including behavioural (e.g. irritability, anxiety) and involving other organ systems (e.g. abdominal pain, itching) (Beer et al., 1987). This study did not specifically screen for other symptoms to avoid lowering the specificity of the question but rather asked the parents to answer to this daily SMS:

'*Does your child have a sore throat, more nasal symptoms than normal or a cold or flu?'.* In the study of Beer *et al*. (1987) (Beer et al., 1987), depending on the nature of the prodromal symptoms (respiratory, non-respiratory), a mean (SD) interval of 24.5 (12.2) hours and 21.5 (8.5) hours, respectively, was found between the initial symptom and the overt asthma exacerbation. They also describe a third group of children that had a time interval less than 6 hours between initial symptoms and asthma attack. Furthermore, the younger the child, the more likely the initial symptoms were non-respiratory in nature. In concordance with the findings of this study this introduces the challenge of providing a potential study drug within 24 hours (probably though best within 12 hours of the first cold symptoms) with the aim to prevent viral induced asthma exacerbations. This may only be practically possible if parents would be supplied with the study drug/placebo at the baseline visit of the study. This strategy was used in other trials where parents initiated treatment with inhaled budesonide (Connett and Lenney, 1993) or fluticasone (Ducharme et al., 2009) in pre-school wheezers or montelukast (Robertson et al., 2007) in 2-14 year old children at the onset of cold symptoms.

The timing of an interventional strategy for prevention of a viral induced asthma exacerbation is clearly crucial but this already presumes that the actual event of interest (i.e. the early stages of a cold with the potential to lead up to a significant (severe) exacerbation) has been correctly identified. While this sounds self-explanatory it needs to be considered that asthma exacerbations are often triggered by multiple factors (Singh and Busse, 2006) (e.g. children in this study reported weather, exercise, and pollen and dust as triggers in 94%, 75% and 69% respectively). These factors also interact with each other (Murray et al., 2004) and it may be difficult to establish the relative contribution of these factors to individual exacerbations . In addition not all viral upper respiratory tract infections lead up to significant exacerbations (Lee et al., 2011, Olenec et al., 2010, Walter et al., 2008) despite the anecdotal parental claim that 'whenever my child catches a cold (s)he has an asthma attack'. In this study 12% (3/25) were 'cold only' episodes with no evidence of lower respiratory tract involvement, 68% (17/25) were moderate exacerbations requiring increase of β-agonist inhaler use only, and 20% (5/25) severe exacerbations according to the severity classification by Reddel *et al*. (2009) (Reddel et al., 2009). The inability/difficulty of predicting the severity and duration of a common cold (in non-asthmatic adults) by patients, doctors, and validated cold questionnaires has recently been shown in a study involving 917 adults (Longmier et al., 2013). A study of 413 adults with asthma investigating predictive factors of worsening asthma control post-cold found the severity of a cold measured within the

first 2 days using the Wisconsin Upper Respiratory Symptom Survey (WURSS)-21 to be a predictor of subsequent clinically significant worsening of asthma control (Walter et al., 2008). Unfortunately, the day 1 symptom score by itself was not predictive, only the day 2 or the combined day 1 and 2 score (Walter et al., 2008). Not only has this tool not been validated in children (and an equivalent predictive questionnaire was not found), but the delay of two days would also reduce its applicability in a study testing a preventative anti-viral treatment such as IFN-β as the progress from upper to lower respiratory tract symptoms in children appears to be more rapid as discussed above.

An interventional trial of a preventative treatment aimed to restore anti-viral defence would need to ensure that the proposed treatment is given to the correct (severity) group of children at the earliest possible time point during a cold episode which is likely to lead up to a significant exacerbation. This is not only crucial in order to show a possible effect, but also in order to minimise harm. An adult study of inhaled IFN-β for example demonstrated in participants with mild asthma (BTS step 2) increased symptoms during the first week when compared with placebo (Djukanovic et al., 2014). The potential pro-inflammatory effects of exogenous IFN-β in a group that possibly does not lack endogenous IFN-β (Sykes et al., 2014) have been discussed (Jackson, 2014) as potential reasons for this. Therefore it may be necessary to determine the "IFN-β – status" (and other innate immune responses) of children at the time of study enrolment and prior to exposure to study medication. As lower airway cells are difficult to source, nasal epithelial cells may have to replace them as surrogates as previously suggested (McDougall et al., 2008). A recent study of atopic and non-atopic, non-asthmatic children aged 2-10 years with a history of viral wheeze (Spann et al., 2014a) looked at *ex vivo* nasal and tracheal epithelial cell production of IFN-β and –λ in response to respiratory syncytial virus or human metapneumovirus infection. This study did not show a strongly deficient IFN response (unlike other studies looking at IFN responses to RV infection (Wark et al., 2005, Contoli et al., 2006, Baraldo et al., 2012)) in response to RSV or hMPV despite demonstrating increased viral shedding in cells from children with wheeze and/or atopy compared to healthy controls. The children with history of wheeze included in the study were not diagnosed with asthma and did not take inhaled corticosteroids. In contrast, bronchial epithelial cells from children with severe therapy-resistant asthma lacked an effective antiviral IFN response to infection with rhinovirus (Edwards et al., 2013). This leads to the conclusion that the antiviral response of airway epithelial cells is multifactorial. On the other hand some factors or pathways may predominate in specific subgroups of children and adults with asthma and therefore addressing this specific lack in the antiviral defence may lead to some

improvement by reduction of the viral load for example. This could explain some of the beneficial effects of inhaled IFN-β in more severe asthmatic patients seen in the adult study (Djukanovic et al., 2014).

Together with the findings of this study this indicates that children with more severe (difficult to treat) asthma who experience more frequent exacerbations may be the more appropriate group of patients to involve in an interventional trial of inhaled IFN-β. The Jackson Cold Score was unable to predict the course of a viral URTI and therefore one has to rely on adequate parental assessment of whether their child has early signs of a cold. The Jackson Cold Score did also not provide a reliable cut-off for distinguishing a “true cold” from an episode with “cold symptoms”. The analysis however was hampered by the overall very low number of virus negative episodes as the “gold standard” for defining a “true cold”.

The parental report of cold symptoms in combination with a discussion with a clinician has been successfully applied in this study and currently appears to be the only reliable method to be able to potentially intervene early in the course of a cold. Considering the rapid progression from upper to lower airway symptoms in children, an intervention (i.e. study drug) would have to take place within 12-24 hours of first symptoms. This can realistically only be achieved if the parents were provided with either placebo or study drug already at the baseline visit.

7.3 Airway inflammation – Interferons, cytokines and MMPs

Significantly increased levels of cytokines and MMPs during viral colds in comparison with asymptomatic periods were found either at the first cold visit (IL-1β, IL-8, IP-10, and MIP-1β), at the second cold visits (VEGF, MMP-3, MMP-8), or at both cold visits (MMP-7, G-CSF, ENA-78, and MCP-1). The cold visits were between 2-4 days apart and no significant difference in levels of cytokines or MMPs was shown pointing towards a sustained release over this period of time (first week of cold). These cytokines and MMP have a variety of roles both in viral respiratory tract infections and asthma exacerbations, such as anti-viral, cell recruiting, pro- and anti-inflammatory, and injury remodelling properties (Manthei et al., 2014, Watelet et al., 2006). A significant increase in nasal aspirate IL-8, IP-10, MCP-1, and MIP-1β has recently also been demonstrated by Lewis et al. in 16 children with asthma during viral colds (Lewis et al., 2012). The same study also showed increased IFN-γ levels while in this study IFN-γ could be detected in only 2 samples. The method of sampling of nasal aspirates however differed significantly.

The comparison of cytokine and MMP levels between virus positive compared to virus negative baseline and cold episodes was compromised by the very low number of virus negative cold episodes and no difference could be demonstrated. However, virus positive baseline episodes (42% of all baseline episodes) showed significantly higher levels of IP-10, the IFN-β related antiviral cytokine, which indicates that the IFN-β pathway may be activated in these situations despite no overt signs or symptoms of a cold. This is consistent with findings of another study when viral persistence up to 6 weeks post recovery from an acute asthma exacerbation was associated with increased IP-10 mRNA expression (Wood et al., 2011). It may be interesting to explore whether this “low-level” inflammation may be protective against further viral infection or actually provides a microenvironment conducive for early re-exacerbation. However, this was not the remit of this study.

Correlation between AUC of the Jackson Cold Score (as a measure of cold severity) during the cold episode and cytokine/MMP levels was statistically significant for MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, MCP-1, MIP-1β, and IL-1β. The correlation with the peak of the JCS was only significant for MMP-13 at V2. The only statistically significant correlations between cytokine/MMP levels and Asthma Index (as a measure of exacerbation severity) were found in relation to MMP-7 at V1 during the cold and in relation to the cytokine MCP-1 at V2.

IL-1β is a potent pro-inflammatory cytokine and raised levels are found in nasal lavages of subjects with cold symptoms (Proud et al., 1994). Its involvement in the recruitment of neutrophils (Barnes, 2008a), upregulation of ICAM-1 (Paolieri et al., 1997) (receptor for rhinoviruses), increasing vascular permeability, its association with cold severity (Proud et al., 1994) and predictive value for asthma exacerbations (Manthei et al., 2014) have been described. Increased levels may also indirectly reflect a reduction in IFN-β (Guarda et al., 2011). For these reasons IL-1β is an important cytokine in the context of viral induced exacerbations. The predictive value for exacerbations was not confirmed in this study and IFN-β was not measured to be able to support these findings. Macrophage inflammatory protein-1β (MIP-1β) is another pro-inflammatory cytokine significantly raised during colds and correlating with cold severity in the study and confirming the findings of a previous study (Lewis et al., 2012) in children that in addition demonstrated significant correlations with lower respiratory tract symptoms.

A novel finding of the study was that the cytokine MCP-1 and the matrix metalloproteinase MMP-7 were not only significantly raised during cold episodes when compared with baseline levels during asymptomatic periods but also correlated

significantly with cold symptom scores and asthma severity as expressed by the asthma index. In addition, MMP-3 and MMP-8 were also shown to be significantly raised during cold exacerbations and correlated with cold symptom scores but not with asthma symptoms. A recent study (Lewis et al., 2012), investigating nasal lavage cytokines in a cohort of asthmatic children, similarly demonstrated raised MCP-1 levels correlating with subjective (upper and lower respiratory) symptom scores but did not take into account objective measures of lung function such as PEFR. Matrix metalloproteinases have not yet been systematically investigated in the context of asthma and asthma exacerbation. Most work so far has concentrated on mainly MMP-9 and there is limited literature on MMP-3, MMP-7 and MMP-8 in asthma.

Matrix metalloproteinases are important regulators of development and various physiological processes by degradation of ECM, support of cell migration, alteration of the ECM microenvironment and by influencing and modulating the activity of cytokines and growth factors (Vu and Werb, 2000, Van Lint and Libert, 2007). In the healthy airways and lungs, a number of structural cells produce MMPs and their physiological inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs and TIMPs are also produced by various inflammatory cells on stimulation and have been implicated as important factors involved in inflammation and remodelling processes in pathological conditions. A short-coming of this study was to not determine the relevant TIMPs in addition to the MMPs as the balance and ratios of MMPs and TIMPs play a crucial role influencing the individual pathways these proteinases are involved in. The findings of the study however suggest that MMPs and the networks of cytokines they are involved in should be explored in more depth in the context of viral induced asthma exacerbations.

7.4 Conclusions

An observational/feasibility study was conducted to explore the components of a protocol designed for testing the hypothesis that treatment of children with a course of nebulised IFN-β at the start of upper respiratory tract infection symptoms would prevent it triggering an exacerbation of asthma.

First of all representative group of children aged 5 to 11 years with a diagnosis of asthma and a history of viral induced exacerbations was recruited, their demographic and baseline characteristics (general and asthma history, medication, trigger factors etc.) determined, and their lung physiology, viral status and cytokine levels in nasal lavage determined.

The results and main challenges in this respect were:

i. Patients were recruited mainly from the acute admission unit (i.e. paediatric admission unit (PAU)), general paediatric and paediatric respiratory outpatient clinics (in a large tertiary medical centre), and via community asthma nurses. Advertising was also indirectly performed via leaflets and posters in other local hospitals and some GP practices. Despite this effort, recruitment was challenging. In addition it became apparent that many paediatric asthma patients are being treated in primary care only, both in respect of their chronic illness but also during their acute exacerbations. A recruitment via the Primary Care Research Network (PCRN) was pursued but possibly too late in the course of the observational study to show effect. For any future study targeting a patient group of mild-to-moderate asthmatic children the PCRN should be included from the beginning. In addition, the recent changes in quality standards for paediatric asthma patients may increase the likelihood that these patients are followed up in secondary (or tertiary) care. This would also enable this group to be more easily accessed and be more readily involved in asthma studies in the future.

ii. One of the main challenges encountered was the relative scarcity of events of interest (i.e. significant exacerbations), as the prevention of these would be the main aim of an intervention such as the administration of nebulised IFN-β. The reasons for this are possibly manifold and cannot be entirely avoided. They are also a well-recognized issue described in other studies in the literature (and indeed in the recently published adult study of nebulised IFN-β). A future study may have to focus on children with potentially more severe asthma (BTS steps 3-5) and the inclusion criterion may have to focus on numbers of significant exacerbations requiring oral corticosteroid use ± hospital admission in the preceding year.

iii. In this study, 42% of baseline nasal lavages (i.e. nasal lavages performed during asymptomatic periods) were positive for viral nucleic acid. While much lower and also similar detection rates are described in the literature, this certainly sparks the debate about the significance of detecting viruses in nasal lavage both during asymptomatic and symptomatic periods. Interestingly, the presence of viral nucleic acid during baseline was associated with higher IP-10 levels in nasal lavage.

Following an initial baseline assessment participants were monitored by using a digital peak flow meter and a text-based monitoring system that captured cold symptom, asthma symptom and PEFR data during an asymptomatic week. Following this a daily

SMS prompted the parents to assess whether their child had early signs of a cold and to report this to the study team.

The results and main challenges in this respect were:

i. Overall compliance with measuring PEFR was above 80% while the overall transmission rate of correct PEFR values via SMS (i.e. corresponding with data on PEFR meter) was only 65.5%, while 15% were incorrectly transmitted and in 8.3% of cases values were 'self-invented'. The data analysis was performed using the digitally stored PEFR data but findings clearly emphasize the need to continuously scrutinise the data recorded by research participants. The text based system appears to be a useful tool for real-time data capture and monitoring of study participants. In addition it is well-received by parents of participating children. To facilitate data transfer and to omit the step between PEFR measurement and SMS transmission, the use of peak flow meters with direct data transfer to online systems (e.g. via Bluetooth technology) may enhance the validity of data even further.

ii. For the period of one week during (asymptomatic) baseline, children were required to rate 10 items of a cold symptom score (Jackson Cold Score). In addition, once parents assessed their child as having early symptoms of a cold, they also rated their cold symptoms. This yielded useful data for retrospective analysis but the cold symptom score on day one was not useful for predicting the course of the cold. In addition there is no validated cold symptom score that would serve as an early indicator of a more severe cold or of a cold more likely leading to an asthma exacerbation. The challenge for an interventional study would be to exactly determine these early indicators.

iii. The combination of parental opinion and clinical assessment via telephone was highly accurate in terms of determining viral positive colds (>85%) and this seems to be a feasible approach to determine colds in children early on irrespective of their severity later on.

If a child was deemed to have early signs of a cold, 2 weeks of monitoring of cold and asthma symptoms and PEFR were performed. Home visits to perform nasal lavage and saliva sampling and clinical and lung physiological assessments were conducted.

The results and main challenges in this respect were:

i. The first home visit was conducted within 24-48 hours. Many children were found to have already developed lower respiratory tract symptoms and increased β-agonist

requirements by that time. The rapid progression from upper to lower respiratory tract signs poses a significant challenge for a study that aims to prevent exactly this progression via use of an interventional drug. To overcome this, a study drug (or placebo) would possibly have to be supplied to parents at the beginning of the study so that it could be used at the earliest time point possible in the course of a viral cold.

ii. Nasal lavage sampling was well tolerated but saliva sampling is an even less invasive procedure especially for children. Determining viral status in nasal lavage was superior to saliva but the combination of both yielded a higher number of virus positive episodes and especially of multiple viral infections. Some viruses were only detected in saliva. As both sampling techniques are well tolerated, the combination of both may be considered a favourable approach for a future study.

iii. The previously described Asthma Index was validated in the study using the severity definitions of Reddel *et al*. (2009). The peak asthma index correlated well with the severity definitions of Reddel *et al.* (2009). In order to determine a defined numeric cut-off between different severity levels, further studies including larger numbers of participants will be needed.

Finally, the nasal lavage levels of a number of cytokines and matrix metalloproteinases with potential roles in the pathogenesis of viral induced asthma exacerbations was determined. Findings of other studies were confirmed but more importantly it was demonstrated for the first time that the cytokine MCP-1 and the matrix metalloproteinase MMP-7 were not only significantly raised during cold episodes when compared with baseline levels during asymptomatic periods but also correlated significantly with cold symptom scores and asthma severity as expressed by the asthma index. While one has to be aware of the pitfalls and shortcomings of a reductionist study of individual cytokines (or MMPs for that matter) these findings encourage exploring the networks that these cytokines/MMPs are involved in.

<u>Ongoing and future work</u>

In consideration of the link between IP-10 levels in nasal lavage and virus status, even and especially in clinically asymptomatic children, IP-10 serum levels as well as IP-10 mRNA expression in blood taken from the group of study participants will be evaluated.

It will be interesting to investigate whether presence of viral nucleic acid during asymptomatic periods induces a systemic response in the form of raised IP-10 levels.

In addition to IP-10 mRNA also 2'-5' oligoadenylate synthetase (OAS1) expression will be explored. The gene encodes a member of the 2-5A synthetase family, essential proteins involved in the innate immune response to viral infection. The encoded protein is induced by interferons and leads to viral RNA degradation and inhibition of viral replication via activation of latent RNase L.

Taking into account the findings of this observational/feasibility study, a study protocol for an interventional trial will be developed.

8 Implications of study results for a future interventional study

8.1 Introduction

The aim of this study was on the one hand to perform an observational study with a group of children aged 5-11 years with asthma and frequent viral induced exacerbations and on the other hand a feasibility study of various components of a potential interventional trial. Some of the questions the study aimed to answer in respect of a future interventional trial were: Is it feasible to recruit to such a trial? Has the correct study population been identified in terms of inclusion/exclusion criteria? Can one rely on parental notification of colds and reporting of cold symptoms/peak expiratory flow measurements? Is the Jackson Cold Score reliable in predicting the course of a cold and does it provide a cut-off for discriminating “real cold”? Can the asthma index, a tool that had previously only been used and validated in adults, be used to define the severity of asthma exacerbations in children? Should a text-based monitoring be used in an interventional trial? In addition, as the study progressed and yielded results, further questions evolved in respect of the design and delivery of an interventional trial that were not necessarily considered as a specific challenge in the first instance: When in the time course of a cold and/or exacerbation should an intervention be delivered? The reflection on this was influenced by the experience of rapid progression from upper to lower respiratory symptoms in children. Does a positive viral PCR always mean that the responsible culprit for a cold or asthma exacerbation has been identified? This initial assumption was challenged by an exceptionally high positivity rate of baseline nasal lavage samples.

The following paragraphs aim to answer the questions above and highlight the implications of the study results on the development of a protocol for an interventional trial with the aim to prevent viral induced asthma exacerbations in school aged children.

8.2 Results and Conclusions

Multi-centre study with consideration of various recruitment strategies and inclusion of children with greater disease and treatment burden

The difficulty experienced recruiting a higher number of children into this study, would suggest that a future interventional study would need to be a multi-centre study with adjusted recruitment criteria and augmented recruitment strategies. The majority of

children in this study reportedly had a reasonably high disease burden at the point of enrolment but the disease and treatment burden appeared less significant during the study. Consideration should be given to include children with even greater disease and treatment burden with an appropriate adjustment of inclusion criteria.

Reliance on parental reporting of cold symptoms in conjunction with clinical assessment but peak flow should be monitored using peak flow meters with digital storing of data (and/or direct data transmission)

This study suggests that overall parents report their child's symptoms reliably and in a timely manner using the text based monitoring system and therefore enable the study team to calculate cold and asthma scores. The ability to rely on parent reported symptoms depends largely on individual circumstances. It is not always possible to have objective confirmation of symptoms such as data obtained from a digital peak flow meter. It is therefore vital that parental reporting is as reliable as possible. In an interventional trial the researchers will most likely depend on parental reporting of early symptoms of a cold and on parents keeping a symptom diary. The fact that all but 3 cold episodes in this study were associated with positive virology in nasal lavage and/or sputum within 48 hours of the first reported symptoms is reassuring although the possible underreporting of colds cannot be accurately assessed. Establishing trust between the research team and the research candidate (and family) is probably the most important factor in this 'real-life scenario'. While the request for sending a PEFR result via SMS can help as a regular reminder for parents to encourage their child to perform PEFR measurements the researcher should use data stored on a digital peak flow meter or directly transmitted via blue-tooth or other technology.

Evaluation of other cold scores will be necessary

The Jackson Cold Score used in this study did not reliably inform about the progress of a cold and, in view of the high prevalence of virus positive cold episodes, did not provide a satisfactory cut-off for identifying a cold. The evaluation of the Jackson Cold Score may however have been limited by the restricted numbers of participants and cold episodes. Other cold scores such as the common cold questionnaire or the Wisconsin Upper Respiratory Symptom Survey (Walter et al., 2008) may need evaluating for their validity in monitoring the cold symptoms of children in an interventional trial.

The peak Asthma Index can be used to define severity of asthma exacerbation. The modified version and AUC of the asthma index cannot be recommended.

The peak asthma index as a continuous variable correlated well with the severity groups as defined by Reddel *et al.* and would therefore be appropriate for use in an interventional study. The low number of participants and exacerbations however did not allow to define clear cut-offs and this may have to be explored in an appropriately powered study. The modified version of the asthma index did not any additional value on a group level and therefore cannot be recommended. The AUC of the asthma index in comparison to the peak asthma index does not appear to be an adequate reflection of the event of interest (i.e. the severity of exacerbation) and should therefore also not be used.

A text-based monitoring system provides real-time data and is an acceptable tool for data-capturing.

The text-based monitoring system used in this study provided an acceptable tool for parents to transmit data on a twice daily basis about objective and subjective aspects of their child's health. This allowed the calculation of cold and asthma scores, potentially reminded parents and children to perform PEFR measurements, and avoided retrospective completion of symptom diaries. While more modern technologies (e.g. App on smart phones etc.) may provide more elegant solutions, the accessibility of SMS technology and its widespread use may be a pragmatic and less costly approach for data capturing in an interventional study.

A quantitative PCR should be performed to determine virus nucleic acid levels during asymptomatic and cold episodes.

This study found that more than 40% of asymptomatic baseline samples were virus positive. The increased sensitivity of modern molecular detection techniques may therefore come with a price and this raises the concern that a non-quantitative approach may be misleading in determining the causes for asthma exacerbations. Virus persistence from preceding cold episodes, frequent viral re-infections, and asymptomatic virus colonization may be some of the potential explanations. In future studies, and especially an interventional study, one should determine the actual viral load with the help of quantitative PCR (qPCR) rather than just the presence of virus. This would enable an assessment of viral load changes between asymptomatic and cold episodes as well as post treatment/intervention.

A combination of nasal lavage and saliva sampling yields a higher number of virus positive episodes and multiple virus infections than one technique alone

The findings of this study suggest that neither PCR of nasal lavage nor saliva samples alone detect all viruses: in 4 cases adding saliva results to nasal lavage yielded additional pathogens and in 9 cases adding nasal lavage results to saliva yielded additional pathogens. Both sampling techniques were well accepted in this study and it may be advantageous for the completeness of the results of an interventional trial to include both techniques for detecting viruses.

The study intervention needs to be delivered by the care giver at the onset of cold symptoms

This study suggests that there is a rapid progression from upper to lower airway symptoms in children. This means that an intervention (i.e. study drug) for viral induced asthma would have to take place within 12-24 hours of first symptoms to be most effective. This can realistically only be achieved within a study protocol if the parents had either placebo or study drug at home to given at the start of symptoms rather than relying on the coordination of a study visit within that time frame. A home visit by the research team or a visit by the participant to the study centre within 24-48 hours should be arranged. This alternative approach however may require a prior test dose to be given under observation during an asymptomatic period to assess tolerability and safety and ensure adequate administration by the care giver.

The effects of exogenous IFN-β may be determined by measuring directly and indirectly related cytokines in nasal lavage

This study demonstrated the feasibility of determining levels of the antiviral cytokine IP-10 (CXCL10), the pro-inflammatory cytokines MIP-1β (CCL4), MCP-1 (CCL2), IL-1β and IL-8 (CXCL8), as well as the angiogenic growth factor VEGF. It also demonstrated their responsiveness to viral infections. The expression of these cytokines is influenced by IFN-β and therefore they could be used to determine the effects of exogenous IFN-β in an interventional trial with the aim to demonstrate prevention of viral induced asthma exacerbations in children.

8.3 Summary

This study has answered many of the questions and provides valuable guidance for the development of a protocol for a future interventional trial that would have to involve more centres and patients with greater disease burden. In addition it has highlighted

some of the challenges and problems that one needs to be aware of and ways of approaching them. The subjective component of symptom reporting will not be completely avoidable but real-time data delivery and digital data storing are successful strategies to optimize data validity. This study has also raised a number of additional questions that may have to explored separately prior to embarking on an interventional trial. The current inability to reliably predict the course of early symptoms of a cold and its impact on the child with asthma and the absence of validated scoring tools is probably the biggest challenge in that respect. The further exploration of potential biomarkers such as IP-10 may provide an alternative approach to tackling this issue in the future.

9 Appendices

9.1 Appendix A. VIPA Baseline Questionnaire

VIPA Baseline Questionnaire

Subject_ID [ ] DOB (dd/mm/yy) [ ] Initials [ ]

Date of baseline visit? (dd/mm/yy) [ ]

Your asthma

What age (years) did your asthma start? [ ]

What age (years) did you start using a blue reliever inhaler (eg salbutamol, ventolin, terbutaline, bricanyl)? [ ]

What age (years) did you start using a regular preventer inhaler (eg beclomethasone, becotide, budesonide, pulmicort, etc)? [ ]

Which months is your asthma worse?

December-February?	*Yes*	*No*	September-November?	*Yes*	*No*
March-May?	*Yes*	*No*	No specific month	*Yes*	*No*
June-August?	*Yes*	*No*			

What triggers your asthma?

Weather?	*Yes*	*No*	Cigarette smoke?	*Yes*	*No*
Pollen?	*Yes*	*No*	Foods / drinks?	*Yes*	*No*
Emotions?	*Yes*	*No*	Soaps / sprays / detergent?	*Yes*	*No*
Fumes?	*Yes*	*No*	Exercise?	*Yes*	*No*
Dust?	*Yes*	*No*	Other things?	*Yes*	*No*
Pets?	*Yes*	*No*	Please specific what other things		
Cold /flu?	*Yes*	*No*	[ ]		

What type of reliever medication do you use? [ ]

What type of reliever device do you use? [ ]

How many doses of your reliever do you usually use? [ ]

What type of preventer inhaler do you use? [ ]

What type of preventer device do you use? [ ]

What dose (mcg) of preventer do you use? [ ]

How many times a day do you use your preventer? [ ]

Do you use servent/oxis/seretide? [ ]

Do you use singulair/montelukast? [ ]

Do you use other asthma medications? [ ]

How often do you miss your preventer inhalers?	*never miss*	*occasionally*	*once a week*	*half the time*	*most of the time*

Do you use any other medication?

Have you used any other complementary (alternative) medications for your asthma? *Yes No*

If yes, what do you use?

How many asthma exacerbations did you have in the last year?

How many of these exacerbations were associated with a cold/flu?

How many courses of steroid (prednisolone) did you need in the last year?

How many days of school did you miss in the last year because of your asthma?

How many admissions to hospital have you had because of your asthma?

How many of these admissions were in the last year?

Have you ever been admitted to intensive care because of your asthma? *Yes No*

About your birth, infancy and other illnesses

How heavy were you at birth? (Kg)

How many weeks gestation where you when you were born?

How were you born? *Normal delivery* *Instrumental (eg forceps)* *Caesarian section*

Where you breast fed for any time as a baby? *Yes No*

Did you have bronchiolitis as a baby? *Yes No*

Did you have chest infections as a baby? *Yes No*

Do you have eczema? *Yes No*

Do you have hayfever in the summer? *Yes No*

Have you ever had a problem with sneezing, or a runny or a blocked nose when you do not have a cold? *Yes No*

Do you have food allergies? *Yes No*

Are you allergic to any animals? *Yes No*

If yes, which animal - cat? *Yes No* - dog? *Yes No* - other? *Yes No*
If other, please specify?

About your family and home

What age (years) did your mother leave school? ☐

What is your mother's occupation? ☐

What age (years) did your father leave school? ☐

What is your father's occupation? ☐

How would you describe the ethnic origin of your family?

White/English/Scottish/Welsh	*Black Carribean*	*Bangladeshi*
White Irish	*Black other*	*Chinese*
White other	*Indian*	*Other*
Black African	*Pakistani*	

Does your mother have:	asthma?	*Yes*	*No*
	eczema?	*Yes*	*No*
	hayfever?	*Yes*	*No*
	food allergy?	*Yes*	*No*
Does your father have:	asthma?	*Yes*	*No*
	eczema?	*Yes*	*No*
	hayfever?	*Yes*	*No*
	food allergy?	*Yes*	*No*
Do any of your brothers or sisters have:	asthma?	*Yes*	*No*
	eczema?	*Yes*	*No*
	hayfever?	*Yes*	*No*
	food allergy?	*Yes*	*No*

Does anyone in the family smoke? *Yes* *No*

Does anyone smoke in the house? *Yes* *No*

Is there any damp on the walls at home? *Yes* *No*

Is there any mould on the walls at home? *Yes* *No*

Do you have dust impermeable mattresses? *Yes* *No*

Do you have any contact with cats?	*none*	*yes but not in house*	*yes including in house*	*yes including my bedroom*
Do you have any contact with dogs?	*none*	*yes but not in house*	*yes including in house*	*yes including my bedroom*
Do you have any contact with other animals?	*none*	*yes but not in house*	*yes including in house*	*yes including my bedroom*

If other animal, please specify what ☐

Thank you

9.2 Appendix B. Asthma quality of life questionnaire

You should not be guided by your parents when answering questions; there are no correct/right answers!

I want you to tell me how much you have been bothered by your asthma during the past week. Pick a description that best describes how much you were bothered by each problem.

DURING THE PAST WEEK:

1. How often did your asthma make you feel FRUSTRATED?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

2. How often did your asthma make you feel TIRED?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

3. How often did you feel WORRIED, CONCERNED or TROUBLED because of your asthma?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

4. How often did your asthma make you feel ANGRY?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

5. How often did you feel IRRITABLE / CRANKY because of your asthma?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

6. How often did you feel DIFFERENT or LEFT OUT because of your asthma?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

7. How often did you feel FRUSTRATED BECAUSE YOU COULD NOT KEEP UP WITH OTHERS because of your asthma?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

8. How often did your asthma WAKE YOU UP DURING THE NIGHT?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

9. How often did you feel UNCOMFORTABLE because of your asthma?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

10. How often did you feel OUT OF BREATH?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

11. **How often did you feel you COULD NOT KEEP UP WITH OTHERS because of your asthma?**

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

12. How often did you have trouble SLEEPING AT NIGHT because of your asthma?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

13. How often did you feel FRIGHTENED BY AN ASTHMA ATTACK?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

14. How often did you have difficulty taking a DEEP BREATH?

All of the time	most of the time	quite often	some of the time	once in a while	hardly any of the time	none of the time

List three activities during which your asthma bothers you. If necessary the following list may be used as a prompt: hockey, rounders, baseball, basketball, dancing, football, playing with pets, playing with friends, riding a bike, running, sleeping, swimming, volley ball, walking, walking up hill, laughing, studying, helping around the house, singing, gymnastics, rollerblading, skateboarding, athletics and climbing.

List the three activities chosen: 1. 2. 3.

DURING THE PAST WEEK:

15. How much have you been bothered by your asthma in activity 1?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

16. How much have you been bothered by your asthma in activity 2?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

17. How much have you been bothered by your asthma in activity 3?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

18. How much did COUGHING bother you?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

19. How much did your ASTHMA ATTACKS bother you?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

20. How much did WHEEZING bother you?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

21. How much did TIGHTNESS IN YOUR CHEST bother you?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

22. How much did SHORTNESS OF BREATH bother you?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

23. Think about all the activities you did in the last two weeks. How much were you bothered by your asthma doing these activities?

Not bothered	Hardly bothered	Bothered a bit	Quite bothered	Very bothered	Extremely bothered

(Juniper et al., 1996)

9.3 Appendix C. Text message Explanation Sheet

VIPA Study: How to Answer the Text Messages

- Save the following number to your mobile phone: **07786207497**
- When you receive a message, press "forward" to answer. (If you press "reply" you will lose the message from the screen).
- The six digit code at the beginning of the message allows us to identify you. Do not delete this.
- Do not change or delete any part of the original message.
- Answer each question using the scoring system indicated in the question.
- Add your answer to the end of each question.
- Send your reply to the number you have saved on your phone.

Trouble Shooting:

What to do if you receive one of the following messages:

"Your message was not recognised. When you receive a question message FORWARD it to this number with your answers added at the end. Thank you".

None of your answers to that question were recognised. Try answering the message again making sure you:

- Press forward, not reply.
- Do not add or delete any words, letters, numbers or brackets. Add only your answer.

"The answers to <123456> were incorrect. Please try again making sure every answer is in the range specified e.g. (0-3). Also, make sure you answer every question".

Try answering the message again, and check the following:

- Your answers are within range of possible answers.
- Your have not added or deleted any words, letters, numbers or brackets from the message. Add only your answer.
- You have added too many or too few answers.

"We have not yet received your reply to message <123456>. Please can you FORWARD the original message complete with your answers".

If you forget to reply to the text message or your phone is switched off, you will be sent this message 1 hour after the initial message was sent.

Text Message 1 – Daily Cold question

You will receive this message once a day until your child has a confirmed cold / flu:

<123456> Does your child have a sore throat, more nasal symptoms than usual (not hayfever/allergy related), or a cold or flu?(Y/N)

Every day we would like you to answer 'Yes' (Y) or 'No' (N) to this question.

A reply might look like this:

<123456> Does your child have a sore throat, more nasal symptoms than usual (not hayfever/allergy related), or a cold or flu?(Y/N)N

With this question we would like to know from you whether your child is developing a cold. If your child suffers with hayfever or allergy and you strongly suspect that symptoms such as sneezing or runny nose are hayfever or allergy related you should answer "N" ("No") to this question. If you are unsure or you think your child is developing a cold please answer "Y" ("Yes") and you will automatically be sent text messages 2 and 3 (see below) and we may additionally contact you by phone to clarify.

Text Message 2 – Cold symptoms

You will receive this message once in the morning and once in the afternoon during your child's Baseline period and from the time your child has a confirmed cold/flu:

<123456> Sore throat? Runny nose? Sneeze? Nasal cong? Tired? Fever? Headache? Hoarseness? Earache? Cough? (Score each 0-3)

This is asking you to what extent each of these symptoms have affected your child in the past 12 hours:

A. Sore throat
B. Runny nose
C. Sneeze
D. Nasal congestion (blocked or stuffy nose)
E. Tiredness
F. Fever (feverish/chills)
G. Headache
H. Hoarseness
I. Earaches
J. Cough

Please score each symptom as follows:

0	No symptoms
1	Mild symptoms
2	Moderate symptoms
3	Severe symptoms

An example reply might look like this:

<123456> Sore throat? Runny nose? Sneeze? Nasal cong? Tired? Fever? Headache? Hoarseness? Earache? Cough? (Score each 0-3) 0123012301

This would mean that your child does not have a sore throat, feel tired or have earache. Your child has a mild runny nose, fever and cough, moderate sneezes and headache, and severe nasal congestion and hoarseness.

Text Message 3 – Asthma symptoms

You will receive this message once in the morning and once in the afternoon during your child's Baseline period and from the time your child has a confirmed cold/flu:

<123456> Chest tightness? (0-3) Wheeze? (0-3) Cough? (0-3) Short of breath? (0-3) PEFR value? (030-999)

Please assess to what degree your child has experienced the following symptoms in the last 12 hours:

A. Chest tightness?
B. Wheeze?
C. Cough?
D. Shortness of breath?

Please score these as follows:

0	not present
1	mild (noticeable, but not bothersome)
2	moderate (bothersome, but your child shows normal activity)
3	severe (interferes with your child's normal life activities, and/or requires therapy)

We would also like you to tell us your child's **best** peak flow reading (PEFR) which they can do on their hand held device. This will be a 3 digit number between 030 and 999, for example 270.

An example reply might look like:

<123456> Chest tightness? (0-3) Wheeze? (0-3) Cough? (0-3) Short of breath? (0-3) PEFR value? (030-999) 0000270

This would show that your child had no asthma symptoms and a PEFR of 270.

Text Message 4 – Asthma symptoms

You will receive this message only in the morning during your child's Baseline period and from the time your child has a confirmed cold/flu:

<123456> No of times woken during the night to use inhaler? (0-9) No of extra puffs used in 24hrs for acute symptoms? (0-99)

This message is asking:

- How many times did your child wake up at night and require salbutamol?

  Your answer should be between 0 and 9

- Number of puffs of salbutamol (or equivalent) did your child use in the past 24 hours (excluding puffs taken pre-exercise)

  Your answer should be the number of times that your child used his/her inhaler.

An reply might look like this:

<123456> No of times woken during the night to use inhaler? (0-9) No of extra puffs used in 24hrs for acute symptoms? (0-99)110

This would indicate that your child woke once during the night and used his/her inhaler and in the last 24 hours he/she administered 10 puffs of salbutamol (or equivalent) to him/herself.

If you have any questions, please give us a call on 07796 991981 or 023 8079 4479 (Emma Holland) or 023 8079 4887 (Florian Gahleitner)

9.4 Appendix D. Baseline Questionnaire 1

Clinical SMS text message system

As part of this study we are using an automated text messaging system to collect data about cold and asthma symptoms from our research participants. This is a new way of interacting with participants in a research study. We would be grateful if you could fill out this basic questionnaire about your experience with using text messages (sms) in general and your expectations about using it in the context of this research study. At the end of the study we will ask you to give us feedback about the use of the text message system in this study.

1. How long have you had a mobile phone?

Less than a year ☐ *1-5 Years* ☐ *More than 5 years* ☐

2. Do you have a contract or a "pay as you go" phone?

Monthly Contract ☐ *Pay as you go* ☐

3. Roughly, how many text messages (sms) do you send a week?

0-10 ☐ *10-30* ☐ *More than 30* ☐

4. Roughly, how many text messages (sms) do you send a month?

0-50 ☐ *50-100* ☐ *More than 100* ☐

5. Do you use text messages (sms) for any other purpose than texting friends & family members (e.g. tickets for car parking, concerts etc)?

Yes ☐ *No* ☐ if yes, please specify: _____

6. Have you ever used text messages (sms) to provide information for a research study?

Yes ☐ *No* ☐ if yes, please specify: _____

7. How do you feel about using text messages as a way of providing information to the research team about your child's cold and asthma symptoms?

Very happy ☐ *Happy* ☐ *Neutral* ☐ *Unhappy* ☐ *very unhappy* ☐

8. Any comments:

_____

_____

_____

VIPA Study
Viral Induced Paediatric Asthma Study

Thank you !

9.5 Appendix E. Follow up Questionnaire 2

Clinical SMS text message system

As part of this study we were using an automated text messaging system to collect data about cold and asthma symptoms from your child. We ask you to fill out this feedback form about your experience over the last few weeks / months with using text messages (sms) in the context of this research study. This will inform us whether we should be using an automated text messaging system in future studies.

1. Did you find the text message information sheet useful?

Yes ☐ *No* ☐

If not, what could be improved?

_____

_____

2. Did you find the text message system for this study easy to use?

Yes ☐ *No* ☐

If not, what were the problems you experienced?

_____

_____

3. Did you reply to the text messages every day?

All of the time ☐ *Most of the time* ☐ *Some of the time* ☐ hardly ever ☐

4. At times you did not reply to the text messages, what was the reason for it (name all):

Lack of time ☐ *At work* ☐ *Forgot about it* ☐ *No credit on phone* ☐

Other: _____

5. Did you feel annoyed or unhappy at any stage about receiving text messages related to our research study?

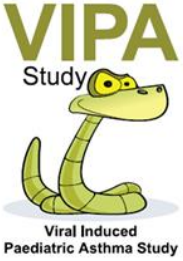
No, never ☐ *Rarely* ☐ *sometimes* ☐ *once a week* ☐ *every day* ☐

6. If you decided to take part in another research study, would the fact that it also uses text messaging as a way of gathering data

   a. Make it more likely for you to participate in the study ☐

   b. Not influence your decision to take part either way ☐

   c. Put you off from participating in this research study ☐



7. Any other comments

_____

_____

9.6 Appendix F. Calculation of original and modified asthma index

Step-by-step computation of Asthma Index (including AUC and Peak Asthma Index)

The Asthma Index (**AI**) incorporates Peak Expiratory Flow Rate **(PEFR**), use of short-acting bronchodilators and subjective variables relative to an individual's baseline. The score is a rolling average over a 48-hour period.

Section 1) Data to be recorded

The following information needs to be recorded:

1. AM (morning)

a. Number of puffs of salbutamol (or equivalent) used in the past 24 hours (excluding puffs taken pre-exercise or during study visits) (**salbutamol_24h**) sms

b. Number of times the subject woke up at night and required salbutamol (**salbutamol_night**) sms

c. Peak Expiratory Flow Rate (highest of 3 efforts) (**PEFRAM**) mw

d. Each of the following scored 0 - (not present); 1 - mild (noticeable, but not bothersome); 2 - moderate (bothersome, but you can perform normal activities); 3 – severe (interferes with your normal life activities, and/or requires therapy)

i. Chest tightness (**ChestAM**) sms

ii. Wheeze (**WheezeAM**) sms

iii. Cough (**CoughAM)** sms

iv. Shortness of Breath (**SoBAM)** sms

Sum termed Symptom Score

2. PM (evening)

a. Peak Expiratory Flow Rate (highest/best of 3 efforts) (**PEFRPM**) mw

b. Each of the following scored 0 - (not present); 1 - mild (noticeable, but not bothersome); 2 - moderate (bothersome, but you can perform normal activities); 3 – severe (interferes with your normal life activities, and/or requires therapy)

i. Chest tightness (**ChestPM**) sms

ii. Wheeze (**WheezePM**) sms

iii. Cough (**CoughPM**) sms

iv. Shortness of Breath (**SoBPM**) sms

Sum termed Symptom Score

Subject	FileID/Name	Date	recordedwhen*	Time	FEV1	PEF	
15	V015	24/08/2011	1	11:30	1.4	180	BL1am
15	V015	24/08/2011	2	18:30	1.05	145	BL1pm
15	V015	25/08/2011	1	06:25	0.95	115	BL2am
15	V015	25/08/2011	2	16:50	1.2	150	BL2pm
15	V015	26/08/2011	1	07:30	1.2	145	BL3am
15	V015	26/08/2011	2	18:30	1.2	185	BL3pm
15	V015	27/08/2011	1	05:55	1.1	135	BL4am
15	V015	27/08/2011	2	18:30	0.95	160	BL4pm
15	V015	28/08/2011	1	07:50	1.15	155	BL5am
15	V015	28/08/2011	2	18:20	1.15	160	BL5pm
15	V015	29/08/2011	1	10:40	1.1	180	BL6am
15	V015	29/08/2011	2	18:35	0.9	150	BL6pm
15	V015	30/08/2011	1	07:20	0	170	BL7am
15	V015	30/08/2011	2	18:35	1	170	BL7pm
15	V015	31/08/2011	1	08:10	1	180	BL8am
15	V015	31/08/2011	2	18:35	0.95	155	BL8pm

Example (participant 15) of PEFR measurements on digital mini Wright showing participant ID, date and time of recording (1 = AM, 2 = PM), FEV1 and PEF. BLx = BaseLine week, x= number of day.*

Subject	SentDate	sentwhen	salbutamol_night	salbutamol_24h	Symptom Score
14	29-Nov-11	1	0	0	
14	29-Nov-11	2			3
14	30-Nov-11	1	0	0	3
14	30-Nov-11	2			2
14	01-Dec-11	1	0	0	3
14	01-Dec-11	2			3
14	02-Dec-11	1	0	0	4
14	02-Dec-11	2			4
14	03-Dec-11	1	0	0	4
14	03-Dec-11	2			5
14	04-Dec-11	1	0	0	5
14	04-Dec-11	2			3
14	05-Dec-11	1	0	0	3
14	05-Dec-11	2			3
14	06-Dec-11	1	0	16	4
14	06-Dec-11	2			4
14	07-Dec-11	1	0	18	3
14	07-Dec-11	2			4
14	08-Dec-11	1	0	12	4
14	08-Dec-11	2			4
14	09-Dec-11	1	0	12	4
14	09-Dec-11	2			5
14	10-Dec-11	1	0	12	3
14	10-Dec-11	2			5
14	11-Dec-11	1	0	12	4
14	11-Dec-11	2			3
14	12-Dec-11	1	0	12	2
14	12-Dec-11	2			3
14	13-Dec-11	1	0	6	4

Example (participant 14) of sms data obtained showing subject ID, date and time of sending (1=AM, 2=PM), symptom score, night time awakening due to asthma and use of salbutamol over 24 hours.

Section 2) Computation of Baseline Reference PEFR and Reference Combined Score

1. AM and PM data needs to be recorded for 7 days.

2. The **Reference PEFR (Ref_PEF)** is computed by taking the average of the 7 **PEFRPM** readings and rounding to the nearest whole number. Note this is PM scores only, not AM.

3**.** The **Reference Asthma Score (Ref_comb_score)** is derived from the AM and PM data as follows**:**

a. Compute AM and PM Subjective Sums as follows

i. The **AM Subjective Sum (Subj_Sum)** is computed by adding together:

1. salbutamol_24h

2. salbutamol_night

3. ChestAM

4. WheezeAM

5. CoughAM

6. SoBAM

Sum termed Symptom Score

ii. The **PM Subjective Sum (Subj_Sum)** is computed by adding together

1. ChestPM

2. WheezePM

3. CoughPM

4. SoBPM

Sum termed Symptom

b. (i) Compute the *original* **Subjective Score (Subj_Score_orig)** by adding together the 4 consecutive **Subjective Sums**.

For example, the first original **Subjective Score** will be the sum of:

Day 1 **AM Subjective Sum**, Day 1 **PM Subjective Sum**, Day 2 **AM Subjective Sum**, and Day 2 **PM Subjective Sum**.

The second **Subjective Score** will comprise Day 1 **PM Subjective Sum**, Day 2 **AM Subjective Sum**, Day 2 **PM Subjective Sum** and Day 3 **AM Subjective Sum**, i.e. drop the Day 1 **AM Subjective Sum** and add in the Day 3 **AM Subjective Sum.**

b. (ii) Compute the *new* **Subjective Score (Subj_Score_new)** by multiplying the average of 4 consecutive subjective sums by 4, shifting the score though so the value reflects the mean time point between the measurements (i.e. based on the assumption of AM measurements/recordings at 8am and PM measurements/recordings at 8pm the new Subjective Scores are shifted to 2am and 2pm respectively)

This approach will enable to obtain Subjective Scores for the first 48 hours unlike the original Subjective Score approach.

c. In order to end up with the **Asthma Score**, which will later be averaged to become the baseline reference point (**Reference Asthma Score)** from which subsequent changes are measured), the **PEFR Score (PEFscore_orig or PEFscore_new)** needs to be calculated, which essentially is the % change in **PEFR** from the **Reference PEFR (Ref_PEF)** as follows:

i. Compute **PEFR% Decrease (PEF_dec)** by taking the **PEFRAM** or **PEFRPM** less the **Reference PEFR (Ref_PEF)** divided by the **Reference PEFR (Ref_PEF)** and then multiply by 100.

As an example, if the **PEFRAM** was 457 and the **Reference PEFR** was 482, the **PEFR % Decrease (PEF_dec)** would be 5, calculated as [-(457-482)/482]*100 (rounded to nearest 1 percent).

If the **PEFR** reading was 488, you would record “-1” (calculated as -(488-482)/482).

ii.i. Compute the *original* **PEFR Score (PEFscore_orig)** for any rolling 48 hour period by averaging 4 consecutive **PEFR% Decrease (PEF_dec)** scores. The resultant number should be rounded to the nearest whole number (0.5 being rounded to 1, below this being rounded to zero, etc).

ii.ii. Compute the *new* **PEFR Score (PEFscore_new)** for any rolling 48 hour period by averaging 2-4 consecutive **PEFR% Decrease (PEF_dec)** scores. The resultant number should be rounded to the nearest whole number (0.5 being rounded to 1, below this being rounded to zero, etc). Time frame will be shifted compared to original score - so values lie at 2am or 2pm respectively. As this approach allows the averaging of 2 and 3 PEFR%Decrease scores values will be obtained for the first 48hr period (unlike the original approach).

d. To compute the **Asthma Score (comb_score_orig or comb_score_new),** add together rolling (48hr) **Subjective Score (subj_score_orig or subj_score_new)** and **PEFR Score (PEFscore_orig or PEFscore_new)**.

Over a 7 day period, there should be 11 *original* **Asthma Scores (comb_score_orig)** (there are 11 scores because the first score occurs after 48 hours, thus during a 7 day period there is an opportunity to create 11 scores in total).

Over a 7 day period, there should be 14 *new* **Asthma Scores (comb_score_new)** as the new approach yields Subjective Scores and PEFR Scores also for the first 48hr period.

e. Compute the **Reference Asthma Score (Ref_comb_score_orig or Ref_comb_score_new)** by averaging the 11 or 14 **Combined Scores** and round to the nearest whole number (0.5 being rounded to 1, below this being rounded to zero, etc).

Section 3) Computation of the Asthma Index during the cold period (and during baseline)

1. The data are recorded in the same way as set out in Section 1.

2. The data does not have to start with an AM reading.

3. The **Subjective Score (Subj_Score_orig or Subj_Score_new)** should be calculated in the same way as set out in Section 2.

4. The **PEFR% Decrease (PEF_dec)** will again be computed off the **Reference PEFR (Ref_PEF)** as computed in Section 2.

5. The **PEFR Score (PEFscore_orig or PEFscore_new)** will be computed as Section 2.

6. The **Asthma Score (comb_score_orig or comb_score_new)** will be computed by adding the **Subjective Score** to the **PEFR Score**.

7. The **Asthma Index (Asthma_Index_orig or Asthma_Index_new)** will be computed by deducting the **Reference Asthma Score (Ref_comb_score_orig or Ref_comb_score_new)** from the half-daily **Asthma Score**.

Original Asthma Index (Asthma_Index_orig) = half-daily asthma score minus the original Reference asthma score **(Ref_comb_score_orig)** (in original publication during cold episode only but here also calculated for baseline period)

New Asthma Index (Asthma_Index_new) = half-daily asthma score minus the Reference asthma score **(Ref_comb_score_new)** during baseline and cold episode.

Section 4) Computation of the Area under the Curve and the Peak Asthma Index

Original:

The **AUC of the asthma index (AUC_AI_orig)** will be calculated by adding up 2 consecutive *original* **Asthma Indices (Asthma_Index_orig)**, dividing by 2 and

multiplying by 0.5 (to reflect the half-day time periods). The 10 **AUC_AI_orig** generated this way will then be added up to yield the **total Area under the Curve (AUC_AI_orig_total).**

The **adjusted AUC of the asthma index (AUC_AI_orig_adj)** will be calculated according to a new formula: ((asthma index day 0.5 + 40 + asthma index day 1 +40)/2)*0.5. The 10 **AUC_AI_orig_adj** generated this way will then be added up to yield the total Area under the Curve **(AUC_AI_orig_total_adj). This approach is necessary to yield positive AUC values for all subjects.**

The **original Peak Asthma Index (Peak_AI_orig)** and the **adjusted original Peak Asthma Index (Peak_AI_orig_adj)** will be determined**.**

New:

The **AUC of the asthma index (AUC_AI_new)** will be calculated by adding up 2 consecutive *new* **Asthma Indices (Asthma_Index_new)**, dividing by 2 and multiplying by 0.5 (to reflect the half-day time periods). The 13 **AUC_AI_new** generated this way will then be added up to yield the **total Area under the Curve (AUC_AI_new_total).**

The **adjusted AUC of the asthma index (AUC_AI_new_adj)** will be calculated according to a new formula: ((asthma index day 0.5 + 40 + asthma index day 1 +40)/2)*0.5. The 13 **AUC_AI_new_adj** generated this way will then be added up to yield the total Area under the Curve **(AUC_AI_new_total_adj). This approach is necessary to yield positive AUC values for all subjects.**

The **new Peak Asthma Index (Peak_AI_new)** and the **adjusted new Peak Asthma Index (Peak_AI_new_adj)** will be determined**.**

3.4.2 Calculation of Jackson Cold Score

The Jackson cold symptom score was received twice a day for a week after screening and for 2 weeks with a cold. Parents were asked (via SMS text) to what extent the following symptoms had affected their child in the past 12 hours:

A. Sore throat

B. Runny nose

C. Sneeze

D. Nasal congestion (blocked or stuffy nose)

E. Malaise (tiredness)

F. Fever (feverish/chills)

G. Headache

H. Hoarseness

I. Earaches

J. Cough

They were asked to score each symptom as follows:

0 No Symptom

1 Mild Symptom

2 Moderate Symptom

3 Severe Symptom

The profile of the Jackson cold symptom score (JCS), both during the baseline week and the cold episode, was plotted against time and the peak and the AUC of the JCS were determined. The sum of the JCS over any 48 hour period was calculated (i.e. sum of two morning JCS or sum of two evening JCS) and the peak of these summated JCS was examined. The temporal relationship between cold symptoms (JCS) and asthma symptoms (asthma index) were documented by plotting both in one graph against time.

9.7 Appendix G. Ethical approval document

NHS
National Research Ethics Service
Southampton & South West Hampshire REC (B)
Building L27
University of Reading
London Road
Reading
Berkshire
RG1 5AQ

Telephone: 0118 9180566
Facsimile: 0118 9180559

01 November 2010

Dr Graham Roberts
Reader
University of Southampton
University Child Health
Level F, South Academic Block (MP803)
SOUTHAMPTON
SO16 6YD

Dear Dr Roberts

Study Title: **IFN-β in childhood asthma – observational study**
REC reference number: **10/H0504/52**

Thank you for your letter of 06 October 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Alternate Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to

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The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

Other conditions specified by the REC

Please provide a CV for Dr Gahleitner to the Committee.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Investigator CV		01 May 2010
Protocol	1	12 July 2010
CV - Donna Davies		
REC application		28 July 2010
Covering Letter		22 July 2010
Covering Letter		06 October 2010
Letter from Sponsor		21 July 2010
Questionnaire: VIPA Baseline Questionnaire	1	25 July 2010
Advertisement	2	17 September 2010
Letter of invitation to participant	2	29 September 2010
GP/Consultant Information Sheets	1	01 July 2010
Participant Information Sheet: Information for parents - standard	2	17 September 2010
Participant Information Sheet: PIS - Gifting of samples	1	20 September 2010
Response to Request for Further Information		06 October 2010
Participant Information Sheet: Information for parents - enhanced	2	17 September 2010
Participant Information Sheet: Information for children - standard	2	17 September 2010
Participant Information Sheet: Information for children - enhanced	2	17 September 2010
Participant Consent Form: Consent form - standard	2	17 September 2010
Participant Consent Form: Consent Form - Gifting of samples	1	20 September 2010
Participant Consent Form: Consent form - enhanced	2	29 September 2010

Participant Consent Form: Assent form for children - standard	2	29 September 2010
Participant Consent Form: Assent form for children - enhanced	2	29 September 2010
CV - Victoria Cornelius		
CV - Julian Legg		
Referees or other scientific critique report		15 July 2010
Review - Dr Gary Connett		10 July 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H0504/52	**Please quote this number on all correspondence**

Yours sincerely

pp

Dr Giles M Y Tan
Alternate Vice-Chair

Email: scsha.SWHRECB@nhs.net

Enclosures: "After ethical review – guidance for researchers"
Copy to: *Ms Christine McGrath*

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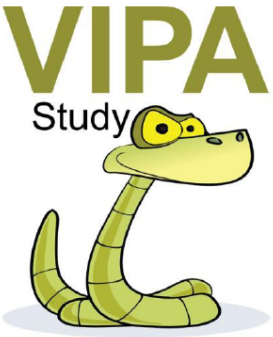
9.8 Appendix H. Parent Information Sheet

UNIVERSITY OF Southampton

Southampton NHS
University Hospitals NHS Trust

The VIPA Study

(Viral Induced Paediatric Asthma Study)



Viral Induced Paediatric Asthma Study

INFORMATION SHEET FOR PARENTS

We would like to invite your child to take part in a study which aims to provide valuable information about paediatric asthma.

- Part 1 of this information sheet tells you the purpose of this study and what will happen to your child if your child takes part.
- Part 2 gives you more detailed information about the conduct of the study.

Please take time to read the following information and discuss it with your family or GP if you wish. If anything is not clear and you require more information before you decide whether or not your child should take part in the study, please telephone the study team on 07796 991981 or 023 8079 4479.

Part 1: What is the purpose of the study?

Asthma affects one in seven children and one in ten adults in the U.K. Asthma is a condition that affects the airways – the small tubes that carry air in and out of the lungs. An asthma attack occurs when something irritates the airways (a trigger) causing the muscles around the airways to tighten and the lining of the airways to start to swell and produce sticky mucus or phlegm. As a result the airways become narrower, making it harder to breathe. This leads to asthma symptoms such as wheeze and shortness of breath.

Viral infections such as the common cold are known to be the most frequent cause for an asthma attack in children with asthma. However, it is not entirely clear how viral infections cause asthma attacks and what predisposes some children to have an asthma attack with viral infections.

This study aims to try and understand how viruses cause asthma attacks. We think that some children do not fight viral infections as well as others and that this and other factors can lead to an asthma attack. To do this we will be studying a group of children aged between 5 and 11 years old who have had asthma attacks associated with colds.

This study aims to follow this group of children up to 12 months to establish the effects of viral infections on their asthma. If successful, it may help us to develop ways of preventing children developing asthma attacks.

Why has my child been chosen?

We have asked your child to take part because he/she has had asthma attacks associated with colds in the last 12 months.

Does my child have to take part?

It is up to you to decide whether or not your child takes part in this research. It is entirely voluntary. If you decide to enrol your child in this study, you will be asked to keep this information sheet and to sign a consent form. You are free to withdraw your child at any time without giving a reason. This will not in any way affect the standard of care your child receives.

What will happen to me / my child if we take part?

Initial assessment:

- If your child is completely well they ideally should avoid using their reliever (blue) inhaler for six hours before the initial meeting.
- We will complete a short questionnaire with you and your child, this will tell us about your child's health and family history.
- We would then do a skin prick test. This is a quick and simple allergy test, where drops of liquid containing grass pollen and other allergens are placed on the skin and scratched.
- We will then perform some fun blowing games, which will allow us to assess your child's lung capacity (lung function testing) and measure the amount of inflammation (redness) in your child's lungs. Inflammation is seen in asthma, this is a standard test for patients with asthma.
- We would also like to collect 10-15 millilitre (2 to 3 teaspoons) of blood.
- A teaspoon amount (5 ml) of salty water will be gently squirted up your child's nose with a small syringe and will be collected as it runs back out. This feels a bit like having a runny nose. We will also ask your child to spit into a little pot to collect a sample of saliva.
- We will then teach your child how to blow into a meter (PIKO) that will help us look at his/her lung capacity at home. This is a very simple meter that we use routinely in many patients with asthma.

Following the initial assessment:

- During the first week we want to find out about your child's cold and asthma symptoms: we will send you four texts a day for the first week to find out whether things like fever, runny nose or earache have affected your child and also about the asthma control. All your answers will be as a code of numbers only. As this is a new way of collecting data for research we will also ask you to complete a short questionnaire at the beginning and at the end of the study to find out whether we should use this method in the future.
- Throughout the study we will send you one text to your mobile phone once a day to ask whether your child has a sore throat or symptoms of a cold or flu.
- If you reply "yes" to the text we would then visit you twice at home (day 1 and between day 3 and 6) and each time will use a small amount of salty water to collect a specimen from your child's nose so that we can try to find out which virus your child has caught and we will collect a saliva sample. We would also like to repeat the blood test (day 1 only). We would then like you to get your child to blow into the PIKO meter in the morning and in the evening every day for the next two weeks following our first home visit. During that 2 week period we will also send you text messages each day asking you how your child is doing.
- We will reimburse you for all text messages.

Following your child's first cold:

- Following your child's first cold and the 2 weeks of follow up as described above, we will restart our daily cold question once your child has recovered (earliest 2 weeks later) until a further cold episode when the home visits and test will be repeated as above (blood optional).

Following your child's second cold:

- Following your child's second cold and 2 weeks follow up with text messages we will only contact you by text once a month (for 2 months) to ask you how your child has been doing.

What are the possible disadvantages and risks of taking part?

There may be a little discomfort associated with a skin prick test and blood test.

What are the possible benefits of taking part?

Your child's treatment will not be altered by participation in this study. The information we collect during this study will hopefully help us to improve our ability to treat childhood asthma in the future.

Do I have to take part?

You do not have to take part. It is entirely up to you to decide. If you do decide to participate, you will be given this information sheet to keep and asked to sign a consent form. The first part of the consent form (Part A) asks for your consent to participate in the study. The second part (Part B) asks for permission to store any unused blood or other samples for use in future research into allergic diseases. The samples will only be used for studies approved by the Local Research Ethics Committee (an independent committee which oversees any research done within Southampton General Hospital). The samples will be fully anonymous to the researchers who use them but will contain codes that would allow the clinical study team who collected them to link them back to you. You are free to choose to just sign Part A and not sign Part B. You will receive a copy of the signed consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect your medical care.

What if you have any questions or concerns?

Please contact Dr Julian Legg (023 8079 4829),

Dr Graham Roberts (023 8079 6160),

Dr Florian Gahleitner (023 8079 4887) or

Emma Holland (07796 991981 or 023 8079 4479).

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential.

Contact for further information

Please contact Dr Julian Legg, Dr Graham Roberts, Dr Florian Gahleitner or Emma Holland (telephone numbers above).

This completes Part 1 of the Information Sheet. Part 2 will give you more detailed information about the conduct of the study.

Part 2: What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (see contacts for further information above). If you still have questions or concerns, you can contact Research and Development, Southampton University Hospitals NHS Trust. In the very unlikely event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against Southampton University Hospital NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints procedure will still be available to you.

Will my taking part in this study be kept confidential?

The personal information collected in this study will be kept confidential. The data we collect from your child will not be labelled with your child's personal details and will be stored securely. Data collected during the study may be shared with our research collaborators; however they will not know who the information belongs to as your name and address will only be kept at Southampton University Hospital NHS Trust. Only the study personnel will have access to your child's personal details. The sponsor of the study (Southampton University Hospital NHS Trust) may also wish to access the records as part of their monitoring of ongoing research. Your child will not be individually identified in any reports or publications resulting from the study. We will keep your child's data on file for use in future studies approved by the Research Ethics Committee.

Who will have access to my health records?

Senior Investigators on this project will need to look at your child's health records to ensure safe conduct of the study procedures.

Involvement of the General Practitioner

We would like your permission to notify your General Practitioner (GP) of your child's participation in this study. With your permission we will send your GP the results of your child's allergy tests as they may be useful for their future medical care. We would not send your GP any other results from the study.

What will happen to any samples I give?

The nasal and saliva (spit) specimens will be analysed for the presence of viruses. The blood sample would be used to study markers of allergy and to see whether there are genes which are specific for asthma. Some of the blood sample and nasal specimen might be stored securely at the University of Southampton for later tests to be done in connection with this study or other research studies. The samples will not be labelled with your child's name or address so that the researchers analysing them will not know that the sample belongs to you.

What will happen to the results of the research study?

We aim to publish the results of the study in medical journals so that other doctors and researchers can make use of them. This is likely to be accompanied by an article in the local press. It will not be possible to identify any individuals involved in this study from these published results.

Who is organising and funding the research?

The researchers at the University of Southampton are organising and carrying out this study. The study is being funded by the National Institute for Health Research.

Who has reviewed the study?

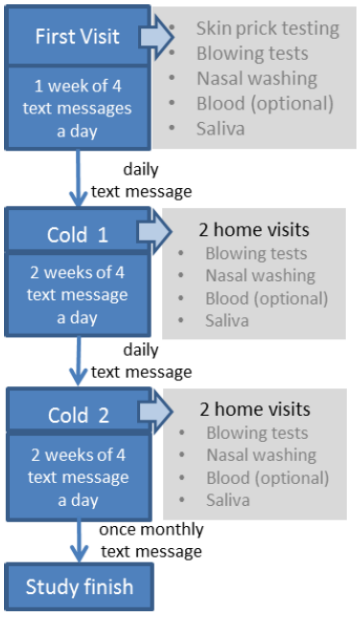
This study was given a favourable ethical opinion for conduct in the NHS by the Southampton and South West Hampshire Local Research Ethics Committee (B).

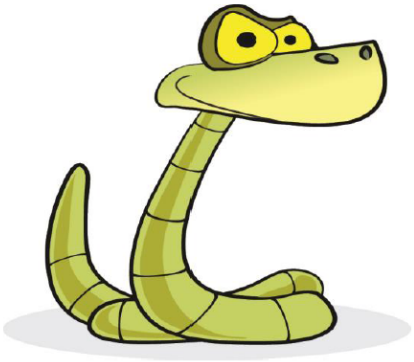
How long do I have to decide whether I should take part?

Your decision to participate in this study is entirely voluntary. You should take as much time as you need.

Thank you for taking time to read this information sheet.

Overview:





9.9 Appendix I. Child Information Sheet

Q.What happens afterwards?

We will continue to text your parents to see how you have been and will visit you again to repeat the tests (as above) if you happen to get another cold.

Q. How long does the study last?

We will continue to text your parents for further 2 months after your second cold to see how you have been getting along. After this the study will finish.

If you have any questions make sure you ask the nurses when you arrive at the hospital.

We look forward to seeing you soon!

REC: 10/H0504/52
VIPA Study Children Information Sheet - enhanced
Version 5 3 October 2011

UNIVERSITY OF Southampton

Southampton NHS
University Hospitals NHS Trust

VIPA Study

Viral Induced Paediatric Asthma Study

The VIPA Study

INFORMATION SHEET FOR CHILDREN

Dr Julian Legg, Dr Graham Roberts and Dr Florian Gahleitner
Respiratory Biomedical Research Unit
Southampton General Hospital
Telephone: 07796 991981 or 023 8079 4479

Q. What is the VIPA Study?

The VIPA study is a research project that is looking at children who have asthma to try and find out why their asthma gets worse when they catch a cold.

Some children get more wheezy and have a tight chest when they have a cold. We would like to try and work out why this happens.

Q. Why have you given me this leaflet?

Your doctor has told us that your asthma is sometimes made worse when you have a cold.

One of our research team will talk to you and your parents about whether you would like to take part in our research. If you decide not to take part that is perfectly fine and your care will not be any different at all.

Q. Why are you doing this research?

We are trying to find new ways of improving treatment for children with asthma. We would like you and your parents to visit us in the hospital and then we would like to keep in contact with you and your parents to see how you are. It is important to talk to your parents about whether or not you would like to take part in this research

Q. What will happen when you visit us?

When you arrive in hospital you will meet the nurses. They will then talk to you and your parents about how you have been.

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Version 5 3 October 2011

We will then get you to do some fun blowing games to see how hard you can blow. We will give you a special machine to take away with you that you can blow into at home.

The nurses will then mark the inside of your forearm with a waterproof pen and put some small drops of fluid on it. The nurse will then make a tiny scratch through each drop. This can make your skin become a bit itchy.

The nurses will then put some magic cream on the back of your hand. This will make your skin numb, so that we can take a small blood sample.

We will also wash the inside of your nose: we will gently squirt a small amount of salty water into your nose and collect it into a pot as it runs back out. This feels a bit like having a runny nose. At the end we will also ask you to spit into a little pot.

Q. What happens next?

We will text your parents regularly to see how you are. If you catch a cold, we will ask you to blow into the special machine we have given you for a few days.

We will then visit you at home to check that you are doing well and to again wash the inside of your nose with a small amount of salty water. We would also like to take another small blood sample.

9.10 Appendix J. Parent Information Sheet – Gifting

PARTICIPANT INFORMATION SHEET

Gifting of samples

The VIPA Study

Your child is participating in VIPA Study. As part of this study blood, saliva and nasal washing samples will be collected. We will use these samples to gain valuable information about your child´s asthma. The saliva and nasal samples will be analysed for viruses and markers of inflammation. The blood can tell us how your child is responding to any infections that they have.

The saliva, nasal and blood samples will initially be stored in Southampton's Faculty of Medicine's research facility at Southampton General Hospital. They will be analysed in the Faculty of Medicine.

What will we do with any spare specimens?

We may not use all the samples in this study. If you are happy for us to keep your child´s remaining samples at the end of the study you can donate them as a gift. They will then be used for medical research in the future. This may include genetic tests; these tests would be unlikely to have any direct implications for your child.

The spare sample would be kept in Faculty of Medicine's Human Tissue Bank in Southampton where they will be stored under the Human Tissue Act. The study investigators will be responsible for the samples that you/your child donate. The specimens would be kept anonymised, ensuring

that your child cannot be identified as the source of the sample. Your child´s unique identification number will be used for this.

How would we use any spare specimens?

In the future, if we are undertaking a study that could make use of these spare specimens, we will request permission for this from the Local Research Ethics Committee. The tests we do on the specimens may generate findings that may become the subject of commercial research and development. You will not be able to derive any personal financial benefit from this study, other than the amount paid to you for your time and inconvenience. This means that you have no claim to any revenue received by any academic institution or commercial entity as a result of their use of the samples or data. This includes revenue received as a result of any inventions or discoveries made as a result of this research.

Consent form, Part B

In order to gift your child´s samples you will need to complete the consent form (Part B) that is provided with this information sheet. Please note that you and your child are not obliged to gift these samples and the decision to not gift your child´s samples will not affect your child´s study participation in the main study, the standard of care he/she receives or his/her legal rights.

If you decide not to donate any unused specimens

If you do not want to donate your child´s samples, any unused samples will be destroyed at the end of the study in compliance with the Human Tissue Act. If you decided to withdraw your consent (either from the entire study or the gifting of the samples) at anytime, the samples that have been taken can be destroyed in compliance with the Human Tissue Act at your request.

Any questions

Thank you once again for reading this sample gifting information sheet, and for considering gifting your child´s samples for future research. Please contact Emma Holland (07796 991981 or 02380794479), Florian Gahleitner (02380794887), Graham Roberts (02380796160) or Julian Legg (02380794829) if there is anything that is not clear or if you would like more information. Take your time to decide whether or not you wish to take part.

Thank you for considering this.

9.11 Appendix K. Consent Form

Southampton NHS
University Hospitals NHS Trust

UNIVERSITY OF Southampton
School of Medicine

The VIPA study – Viral Induced Paediatric Asthma study
PARTICIPANT CONSENT FORM

PART A: Consent for the main study

Title of Project: The VIPA study – Viral Induced Paediatric Asthma study

Name of Researcher: Dr Graham Roberts

Please initial box if you agree with each section

1. I confirm that I have read and understand the information sheet dated **3rd October 2011 (version 7)** for the above study and have had the opportunity to ask questions. ☐
2. I understand that my child's participation is voluntary and that I am free to withdraw my child at any time, without giving any reason, without their medical care or legal rights being affected. ☐
3. I agree that three blood samples, five nasal washings and five saliva samples can be taken from my child and used for the purpose of this study. ☐
4. I understand that relevant sections of my child´s medical notes and data collected during the study, may be looked at by individuals from the study team, from regulatory authorities or from the NHS Trust, where it is relevant to my child taking part in this research. I give permission for these individuals to have access to my child´s records. ☐
5. I give my permission for my General Practitioner to be informed of my child's participation in this study. ☐
6. I agree for my child to take part in the above study. ☐

Name of parent / guardian	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for parent, 1 for researcher, 1 to be kept with hospital notes

9.12 Appendix L. Consent form – Gifting of samples

Southampton NHS
University Hospitals NHS Trust

UNIVERSITY OF Southampton
School of Medicine

The VIPA study – Viral Induced Paediatric Asthma study
PARTICIPANT CONSENT FORM

PART B: GIFTING SAMPLES CONSENT FORM

Title of Project: The VIPA study – Viral Induced Paediatric Asthma study
Name of Researcher: Dr Graham Roberts

Please initial box if you agree with each section

	Yes	No
1. I confirm that I have read and understand the information sheet dated 29th November 2010 (Version 2) - Gifting of samples - and have had the opportunity to ask questions.	☐	☐
2. I give permission for my child´s samples (saliva, nasal washings and blood) and any derived cells to be stored (potentially for many years) for other studies (see 3 and 4). I understand that some of these projects may be carried out by other researchers, including researchers working for commercial companies. I understand that I can alter these decisions at any stage by letting the research team know and that these studies will not directly benefit my child´s health.	☐	☐
3. I give permission for the samples to be used for research about medical conditions relating to virus infection in asthma, allergy and lung disease and potential tests and treatments thereof.	☐	☐
4. If it is deemed to be appropriate to the research, I give permission for a member of the research team to look at my child´s medical records to obtain information on his/her medical history. I understand that the information will be kept confidential and ethical approval will be obtained for such studies.	☐	☐
5. I would like to be informed if the results of any genetic tests conducted on my child´s samples may affect his/her health now or in the future. I understand that I may be informed of these findings any time in the future and that this may potentially be many years.	☐	☐

Name of Parent/Guardian	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for parent, 1 for researcher, 1 to be kept with hospital notes

REC: 10/H0504/52 VIPA Study Consent Form – Gifting of samples Version 2 29th November 2010 Page 1 of 1

9.13 Appendix M. Assent Form

Southampton NHS
University Hospitals NHS Trust

UNIVERSITY OF Southampton
School of Medicine

ASSENT FORM FOR CHILDREN

(to be completed by the child and their parent/guardian)

Project title: **The VIPA study – Viral Induced Paediatric Asthma study**

Name of Researcher: Dr Graham Roberts

Child (or if unable, parent on their behalf) /young person to circle all they agree with:

Have you read (or had read to you) about this project?	Yes / No
Has somebody else explained this project to you?	Yes / No
Do you understand what this project is about?	Yes / No
Have you asked all the questions you want?	Yes / No
Have you had your questions answered in a way you understand?	Yes / No
Are you happy for us to wash the inside of your nose with a tiny amount of salty water?	Yes / No
Are you happy to spit into a little pot to give us a sample of your saliva?	Yes / No
Are you happy for us to take a small blood sample at the beginning of the study and again when you have a cold?	Yes / No
Do you understand it's OK to stop taking part at any time?	Yes / No
Are you happy to take part?	Yes / No

If any answers are 'no' or you don't want to take part, don't sign your name!

If you do want to take part, you can write your name below

Your name _____

Date _____

The doctor who explained this project to you needs to sign too:

Print Name _____

Sign _____

Date _____

Thank you for your help.

1 for patient, 1 for researcher, 1 to be kept with hospital notes

VIPA study Assent enhanced V3 29112010.doc REC: 10/H0504/52 Page 1 of 1

9.14 Appendix N. Itemised Lists of Equipment

9.14.1 Spirometry

Koko Spirometer (nSpire, UK) connected to designated computer

Koko Filter (single person use)

3L calibration syringe

Environmental thermometer

Calculator

(Nose clip)

Rescue medication & equipment:

(Prescription chart)

Salbutamol

Volumatic spacer

(Nebuliser with O2 supply)

9.14.2 Nasal Lavage

Gloves

Apron for staff and volunteer

5 mL syringe

0.9% saline (sterile) at room temperature

Nasal olive (metal)

Plastic/metal collecting funnel

Universal specimen container

Tissues

Storage container with ice

9.14.3 Blood sampling

Blood bottles

- PaxGene
- EDTA
- Serum

Butterfly needle

5mL Luer-lock syringes

Alcohol swabs

Gauze or cotton wool

Plaster

Ametop® Gel (4%w/w) - Smith & Nephew Healthcare Ltd, Hull, UK

Cryogesic® (Ethyl chloride) Spray – Fannin, Dublin, Ireland

9.14.4 Skin Prick Testing

Allergen solutions in dropper bottles (manufactured by ALK-Abello, UK)

SPT 1mm stylus lancets

Sharps Disposal Bin

Soluprick® Pen (ALK, Denmark)

Numbered Tape and clear tape

Clear plastic ruler and Reaction gauge

Timer

Tissues

Blue paper towel

Pillow

Anaphylaxis kit

9.14.5 Pre-Processing of Nasal Lavage Samples

100 µm nylon cell strainers – Marathon Laboratories #352360

Sample storage tubes (amber) and lids (yellow, green) – Alpha Laboratories Ltd # CP5913A (0.5mL tubes), #CP5940Y (yellow lids), #CP5940G (green lids)

Disposable gloves, e.g. Nitrile gloves

Absorbent laboratory tissue

1mL Aerosol barrier/resistant tips e.g. Promega #DY1131

Fine tipped pastettes e.g. Alpha Laboratories Ltd #LW4237

RNase/DNase free 1.5mL and 0.5mL microfuge tubes e.g. Sigma #T3566

15mL conical polypropylene tubes e.g. Greiner #188261

50mL conical polypropylene tubes e.g. Greiner #210261

Printer labels e.g. Triple Red #PTL-29-427

Printer ink ribbon e.g. Triple Red #R4310

Black permanent marker pens (fine tip)

Virkon or equivalent for disinfecting tips

Phosphate buffered saline (PBS) e.g. Dulbecco A tablets (Fisher Scientific #BR0014G) in UHQ Water

RLT plus Buffer

9.14.6 Pre-processing of saliva samples

Sample storage tubes (amber) and lids (yellow, green) – Alpha Laboratories Ltd. # CP5913A (0.5mL tubes), #CP5940Y (yellow lids), #CP5940G (green lids)

Disposable gloves, e.g. Nitrile gloves

Absorbent laboratory tissue

1mL Aerosol barrier/resistant tips e.g. Promega #DY1131

Fine tipped pastettes e.g. Alpha Laboratories Ltd #LW4237

Printer labels e.g. Triple Red #PTL-29-427

Printer ink ribbon e.g. Triple Red #R4310

Black permanent marker pens (fine tip)

Virkon or equivalent for disinfecting tips

9.14.7 Pathogen detection in nasal lavage (and saliva)

Pipettes with 0.1µL-1mL capacity

LTS EDP3+ pipette 20-200µLl (E3-20, Rainin)

CFX96 Real-Time system with C1000 Thermal cycler

Centrifuge with capacity to spin 96 well plates and 15mL tubes

Ice and ice bucket

Vortex mixer

Picofuge

Vacuum manifold

Class II Microbiology Safety Cabinet (MSC)

Oven that heats up to 56ºC

PCR cooler

DNase/RNase-free 1.5mL microfuge tubes e.g. Sigma #T3566

ART Barrier Pipette Tips for use with RNA capacity

- 10µLe.g. Promega #DY1051
- 20µL e.g. Promega #DY1071
- 200µL e.g. Promega #DY1121
- 1000µL e.g. Promega #DY1131

LTS barrier tips for Rainin multidispense pipette (20-200uL) e.g. Anachem #RT-L200F

Gloves safeskin nitrile e.g. NHS supplies; small #FTG125, medium #FTG126, large #FTG127.

2mL collection tube e.g. Qiagen #19201

VacConnectors e.g. Qiagen #19407

White 96 well Hard-Shell PCR plates e.g. Bio-Rad #HSP-9655

PCR plate seal e.g. Bio-Rad #MSB-1001

Biocidal/virucidal non corrosive detergent e.g. Biocleanse

Virkon tablets e.g Fisher #HYG-205-010P

Water (DNase/RNase-free, molecular biology grade) e.g. Sigma #95284

RNAse away e.g. Fisher #D/0048/08

DNA-Remover e.g. Cambio #15-2200

QIAamp MinElute kit - Qiagen #57714

Kit contains:

- QIAamp MinElute Columns
- Extension Tubes
- Collection Tubes
- Buffer AL
- Buffer AW1
- Buffer AW2
- Buffer AVE
- Protease Resuspension Buffer (not required)
- Carrier RNA
- QIAGEN Protease

Buffer AL - Qiagen #19075

QIAGEN Protease - Qiagen #19155 for 7.5AU or #19157 for 30AU

Sodium chloride 0.9% W/V – Fannin

Ethanol (96–100%) e.g Sigma #32221

Respiratory pathogens 21 - FTD #2-96/12

Kit contains:

- Flu/Rhino P/P mix
- COR P/P mix
- Para 2/3/4 P/P mix
- Bo/Mp/Pf1 P/P mix
- RS/EPA P/P mix
- Positive control (PC)
- Negative control (NC)
- Internal control (IC)

RV pathogen detection primer/probes - Primer Design #Path-HRVsp-std

Ag-Path one-step RT-PCR - Ambion #AM1005

- 2 x RT-PCR buffer
- 25 x RT-PCR enzyme mix

9.14.8 Whole blood poly(IC) stimulation assay

Non-polystyrene racks to hold blood collection tubes (during processing and in freezer) e.g. Fisher – STK-610-050P

Pipettes

Pipetteboy

Label printer e.g. Brady thermal printer TLS2200 Fisher #LAB-900-F

Beaker/wash bottle for detergent

Autoclave bin

Incubator / oven at 37°C

Plastic tupperware for containing tubes in incubator, or suitable container

⩽ -70º C Freezer or -20° C freezer

Absorbent laboratory tissue

Permanent marker pen

12mL and 3mL (Lavender capped) EDTA BD Vacutainer blood collection tube

PAXgene blood RNA tubes – BD biosciences #762165

15mL conical polypropylene tubes e.g. Greiner #188261

1.5mL polypropylene microfuge tubes e.g. Sigma #T3566

Barrier tips for 200µl pipette e.g. Promega #DY1121

5mL stripettes e.g. Greiner #606180

Disposable gloves e.g. NHS supplies Small #FTG125 medium #FTG126 large #FTG127.

Biohazard labels

Printer labels e.g. Triple Red #PTL-29-427

Poly(IC) – Autogen Bioclear #TLRL-PIC

Detergent for disinfecting equipment e.g. Trigene or Biocleanse

Disinfectant e.g. Virkon - Fisher Scientific #330001/ 330002/ 330003

9.14.9 Whole Blood RNA extraction using PAX gene

Swing bucked centrifuge with adaptors for 12mL round bottom tubes and 3000g capacity

Refrigerated microfuge with > 10,000g capacity

Heat block or water bath

Mini-Vortexer

Tube rack for freezer storage e.g. Fisher scientific # STK-610-050P

Tube rack e.g. Alpha Laboratories # LW6220

Pipette boy

Pipettes with 0-1mL capacity

Label printer e.g. Brady thermal printer TLS2200

Biohazard receptacles for liquid and solid waste

RNase-free 1.5mL microfuge tubes e.g. Sigma #T3566

RNase-free 0.5mL microfuge tubes e.g. Sigma #T244

3mL fine tip pastettes e.g. Alpha Laboratories #LW4232

5mL stripette e.g. Fisher #FB55483

Barrier tips

- 10µL e.g. Promega #DY1051
- 20µL e.g. Promega #DY1071
- 200µL e.g. Promega #DY1121
- 1000µL e.g. Promega #DY1131

Disposable nitrile gloves

Printer labels e.g. Triple Red #PTL-29-427

Printer ink ribbon e.g. Triple Red #R4310

Permanent marker pen

PAXgene Blood RNA Collection Tubes (100) – BD Bioscience #762165

PAXgene Blood RNA Reagents Kit (50) – Qiagen #762174

Kit contains:

RNAse-free water

Buffer BR1

Buffer BR2

Buffer BR3

Buffer BR4 concentrate

Buffer BR5

DNase 1

DNA digestion buffer

DNase resuspension buffer

Proteinase K solution

2mL processing tubes

1.5 mL microcentrifuge tubes

PAXgene RNA spin columns

PAXgene shredder spin columns

Secondary Hemogard Closures

100% Ethanol e.g. Sigma #32221

RNAse away – Fisher #D/0048/08

Detergent for spills e.g. Trigene – Envirotec U.K. #TR106

Virkon e.g.Fisher #HYG-205-230B

9.14.10 DNAse treatment of purified RNA

Heraeus Fresco or equivalent 1.5 mL microcentrifuge.

Heat block or water bath set at 37°C

Pipettes with 0-1mL capacity

DNase/RNase-free 0.5 mL microfuge tubes e.g. Sigma #T2441

Barrier PipetteTips for use with RNA

- 10µl Promega #DY1051

- 20µl Promega #DY1071
- 200µl Promega #DY1121
- 1000µl Promega #DY1131

Disposable gloves e.g. NHS supplies; small #FTG125, medium #FTG126, large #FTG127.

RNAse free water e.g. Sigma #95284

RNAse away – Fisher #D/0048/08

DNase kit – Ambion DNA free # 1906

- including 10x DNase I buffer, DNase I enzyme and inactivation reagent

DNase kit– Ambion TURBO DNA-free # 1907

- including 10x TURBO DNase I buffer, TURBO DNase I enzyme and inactivation reagent

Elution buffers from appropriate RNA extraction kits (if applicable)

9.14.11 Nucleic Acid Concentration and Quality Determination Using Nanodrop

Nanodrop

Gilson pipettes

Safeskin nitrile gloves small – NHS supplies #FTG125, medium #FTG126, large #FTG127

30mL universal container – Sterilin #128B

Tissues - NHS Supplies MRT161

ART Barrier PipetteTips -10 l Promega #DY1051, 20µl - Promega #DY1071,

200µl - Promega #DY1121, 1000 l Promega #DY1131

CF-1 (aqueous potassium dichromate) - Nanodrop

RNase away - E&K scientific #EK-3352

RNase free water – Sigma #95284

9.14.12 Reverse Transcription

Heraeus Fresco or equivalent 1.5mL microcentrifuge

Heat source for 8 well PCR tube strips (Eppendorf thermal cycler) with the capability to reach 55°C, 65°C and 75°C

Mini microcentrifuge (to pulse spin 200µL tube 8 well strips)

Pipettes with 0-1mL capacity

DNase/RNase-free 0.5mL microfuge tubes, e.g. Sigma #T2441

DNase/RNase-free 1.5mL microfuge tubes, e.g. Sigma #T3566

0.2mL 8well strips, e.g. Star labs #I1402 2900

Barrier PipetteTips for use with RNA

- 10µL Promega #DY1051
- 20µL Promega #DY1071
- 200µL Promega #DY1121
- 1000µL Promega #DY1131

Safeskin nitrile gloves small – NHS supplies #FTG125, medium #FTG126, large #FTG127

RT Kit – PrimerDesign #RT-nanoScript (includes: Oligo-dT primers, random nonamer primers, dNTP mix, DTT, nanoScript enzyme, 10x reaction buffer, RNAse/DNAse free water)

RNAse away – Fisher #D/0048/08

DNA remover – Cambio #15-2025

RNAse free water, e.g. Sigma #95284

DNase kit – Ambion #1906

DNase kit – Qiagen #79254

Gene specific RT primers (if appropriate), e.g. from RNA/DNA internal control kits – Primer Design

9.14.13 Preparation of qPCR plates and Real Time PCR Analysis

Pipettes with 0.1µL-1mL capacity

Electronic multi-dispensing pipette, e.g. LTS EDP3+ pipette 20-200µL (E3-20, Rainin)

CFX96 Real-Time system with C1000 Thermal cycler, Bio-Rad

Centrifuge with capacity to spin 96-well plates

Ice and ice bucket

Vortex mixer

Mini microcentrifuge (to pulse spin 200µL tube 8-well strips)

PCR cooler

Safeskin nitrile gloves small – NHS supplies #FTG125, medium #FTG126, large #FTG127

Tissues

Blue lab roll

Barrier PipetteTips

- 10µL Promega #DY1051
- 20µL Promega #DY1071
- 200µL Promega #DY1121
- 1000µL Promega #DY1131

LTS barrier tips for Rainin multidispense pipette (20-200µL), e.g. Anachem #RT-L200F

DNase and RNase free polypropylene tubes

DNase/RNase-free 1.5mL microfuge tubes, e.g. Sigma #T3566

DNase/RNase-free 0.5mL microfuge tubes, e.g. Sigma #T2441

0.2mL 8 strip PCR tubes with flat caps, e.g. Star labs #I 1402 2800

White 96 well Hard-Shell PCR plates, e.g. Bio-Rad #HSP-9655

PCR plate seal, e.g. Bio-Rad #MSB-1001

RNAse away – Fisher #D/0048/08

DNA remover – Cambio #15-2025

2x qPCR Master Mix (MM) – PrimerDesign #mastermix-iC

Primer/Probe mix – PrimerDesign #PP-hu

Water (DNase/RNase-free, molecular biology grade), e.g. Sigma #95284

9.14.14 Nasal Lavage Biomarker Measurement using Fluorokine® Multianalyte Profiling (MAP) and Luminex Technology

Luminex 200 including MCV plate IV (calibration/cleaning plate)

Microplate vacuum manifold

Pipettes and barrier pipette tips with 0.1µL-1mL capacity

Deionized or distilled water.

Multi-channel pipette with 300µL capacity

50 mL and 500 mL graduated cylinders.

Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of

500 ± 50 rpm.

Microcentrifuge

Vortexer

DNase/RNase-free 0.5 mL Polypropylene test tubes for dilution of standards and samples

Aluminium foil

96 well V-bottomed polypropylene plates (Luminex 96-well Filter plate)

Plate Covers (Foil plate sealers)

90mm Petri dish

Disposable nitrile gloves

Absorbent laboratory tissue

Standard Cocktail 1 (+ Standard Value Card 1)

Standard Cocktail 2 (+ Standard Value Card 2)

Microparticle Diluent

Calibrator Diluent RD5K Concentrate

Wash Buffer Concentrate

Biotin Antibody Diluent 2

Streptavidin-PE

Reagents for running Luminex 200:

Deionised water

10% bleach

70% isopropanol

xMAP sheath fluid

Bio-Plex calibration beads

Bio-Plex validation kit 4.0

9.14.15 IP-10 ELISA using using R&D Systems Quantikine® ELISA Human CXCL10/IP-10

IP-10 Quantikine® ELISA Human CXCL10/IP-10

Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.

Pipettes and pipette tips.

Deionized or distilled water.

Squirt bottle, manifold dispenser, or automated microplate washer.

500 mL graduated cylinder.

Polypropylene test tubes for dilution of standards and samples.

Wash buffer - 0.05% Tween 20 in PBS

Reagent Diluent - 1% BSA in PBS, 0.2 um filtered

Substrate Solution - 1:1 mixture of color reagent A and B (R&D Systems # DY999)

Stop Solution - R&D Systems # DY994

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