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On the trail of immunopathogenesis of asthma

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To my family

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ABSTRACT

Children with rhinovirus (RV)-induced severe early wheezing have higher risks of subsequent recurrences and asthma than those with other viral aetiologies. The immunopathogenesis of this novel association remains unclear. While all major guidelines recommend against the use of bronchodilators and corticosteroids as a treatment regimen for bronchiolitis and early wheezing, they fail to consider the emerging evidence of bronchiolitis heterogeneity. Though early RV-induced wheezing resembles recurrent wheezing and asthma, its immunopathogenesis and the efficacy of bronchodilators and corticosteroids remain poorly studied.

In this thesis we evaluated the clinical short- and long-term effectiveness of both inhaled β2-agonist with and without oral corticosteroid treatment in early wheezing, RV-affected children. Moreover, we studied the immune responses from anti-CD3/anti-CD28-stimulated peripheral blood mononuclear cells (PBMCs), and their association with short- and long-term prognoses in first-time wheezing children under different scenarios, including sole RV vs. sole respiratory syncytial virus (RSV), and sole RV vs RV while coinfecting with human bocavirus 1 (HBoV1). Lastly, the association between the initial disease severity during sole RV-associated wheezing and cytokine response from stimulated PBMCs was evaluated.

The results of this thesis support the assumption of bronchiolitis heterogeneity. Concomitant use of β2-agonist and systemic corticosteroids appears to be beneficial in children with early wheezing induced by RV. Moreover, early wheezing induced by RV and RSV result in different cytokine responses and short- and long-term prognoses, thus suggesting different immunopathology between the two primary inducers of bronchiolitis. Furthermore, HBoV1 coinfecting with RV leads to immunomodulation by suppression, indicating that coinfections during bronchiolitis may impact the overall cytokine responses. Finally, an improper balance between pro- and anti-inflammatory cytokine profiles is associated with poorer initial disease severity.

These results highlight the heterogeneity of bronchiolitis and its effect on long-term prognosis and emphasize the need for more personalised treatment strategies for children with early wheezing.

KEYWORDS: Asthma, atopy, cytokine, β2- agonist, bronchiolitis, human bocavirus 1, immunity, oral corticosteroid, peripheral blood mononuclear cells, recurrent wheezing, respiratory syncytial virus, rhinovirus, virus

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TIIVISTELMÄ

Lasten rinoviruksen (RV) aiheuttama varhainen uloshengitysvaikeus on yhteydessä suurempaan riskiin sairastua taudin myöhempiin uusiutumisiin ja astmaan kuin muilla virusetiologioilla. Tämä havainto on suhteellisen uusi ja sen taustalla oleva immunopatogeneesi on edelleen epäselvä. Vaikka kaikki keskeiset hoitosuositukset suosittelevat välttämään bronkodilataattoreita ja kortikosteroideja varhaisen uloshengitysvaikeuden hoidossa, ne eivät huomioi lisääntyvää näyttöä sairauden heterogeenisyydestä. RV-infektion aiheuttama varhainen uloshengitysvaikeus muistuttaa toistuvaa uloshengitysvaikeutta ja astmaa, mutta bronkodilataattoreiden ja kortikosteroidien tehokkuutta sen yhteydessä on tutkittu vain vähän.

Tässä väitöskirjassa arvioimme hengitettävän β2-agonistin ja suun kautta annettavan kortikosteroidin sekä niiden yhdistelmän lyhyt- ja pitkäaikaista kliinistä tehoa varhaisessa uloshengitysvaikeudessa RV-infektion aikana. Lisäksi tutkimme immuunivasteita anti-CD3/anti-CD28-stimuloiduista perifeerisen veren mononukleaarisista valkosoluista (PBMCs) ja näiden yhteyttä ensimmäistä kertaa uloshengitysvaikeudesta kärsivien lasten lyhyen ja pitkän aikavälin ennusteisiin verraten eri virusetiologioita (RV vs. respiratory syncytial virus (RSV), sekä RV vs. RV- human bocavirus 1 (HBoV1) koinfektio). Arvioimme myös akuutin sairauden vakavuuden ja sytokiinivasteiden välistä yhteyttä RV-infektioon liittyvässä uloshengitysvaikeudessa.

Tämän väitöskirjan tulokset osoittavat, että RV:n aiheuttamassa varhaisessa uloshengitysvaikeudessa sekä β2-agonistin että systeemisten kortikosteroidien yhtäaikainen käyttö vaikuttaa hyödylliseltä. Lisäksi RV:n ja RSV:n aiheuttamat varhaiset sytokiinivasteet sekä lyhyt- ja pitkäaikaisennusteet poikkeavat toisistaan, mikä viittaa erilaiseen immunopatologiaan. RV-HBoV1-koinfektio johtaa vaimentavaan immunomodulaatioon viitaten siihen, että koinfektiot voivat vaikuttaa sytokiinivasteisiin bronkioliitin aikana. Epätasapaino pro- ja anti-inflammatoristen sytokiiniprofiilien välillä on yhteydessä vaikeampaan taudinkuvaan.

Tulokset korostavat uloshengitysvaikeuden heterogeenisyyttä ja sen vaikutusta pitkän aikavälin ennusteeseen, ja korostavat tarvetta kehittää yksilöllisempiä hoitostrategioita varhaisesta uloshengitysvaikeudesta kärsiville lapsille.

AVAINSANAT: Astma, atopia, β2-agonisti, bronkioliitti, human bocavirus 1, immuniteetti, oraalinen kortikosteroidi, perifeerisen veren mononukleaariset solut, toistuva uloshengitysvaikeus, respiratory syncytial virus, rinovirus, sytokiini, virus

Table of Contents

Abbreviations

AAP	American Academy of Pediatrics
APC	Antigen-presenting cell
BALF	Bronchoalveolar lavage fluid
B-Eos	Blood eosinophil count
CCR4	C-C chemokine receptor 4
CD	Cluster of differentiation
CDHR3	Cadherin-related family member 3
CI	Confidence interval
CPS	Canadian Paediatric Society
CRP	C-reactive protein
GR	Glucocorticoid receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EGF	Epidermal growth factor
EMA	European Medicines Agency
ENA-78	Epithelial-derived neutrophil-activating peptide 78
EV	Enterovirus
F protein	Fusion protein
FAS	FS7-associated cell surface antigen
FASL	FAS ligand
G protein	Glycoprotein
GAN	Global Asthma Network
GBD	Global Burden of Disease
G-CSF	Granulocyte colony-stimulating factor
GINA	Global Initiative for Asthma
HBoV	Human bocavirus
hMPV	Human metapneumovirus
HR	Hazard ratio
ICAM-1	Intercellular adhesion molecule-1

ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
IQR	Interquartile range
IRF	Interferon regulatory factor
ISAAC	International Study of Asthma and Allergies in Children
LDLR	Low-density lipoprotein receptor
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MDA5	Melanoma differentiation-associated protein 5
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP16	Matrix metalloproteinase 16
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa B
NICE	National Institute for Health and Care Excellence
NK cell	Natural killer cell
NLR	Nucleotide-binding oligomerisation domain-like receptors
NOD	Nucleotide-binding oligomerisation domain
NOS2A	Nitric oxide synthase 2A
NPA	Nasopharyngeal aspirate
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PHA	Phytohaemagglutinin
PCR	Polymerase chain reaction
PREDICT	Paediatric Research in Emergency Departments International Collaborative
Pre-F	Pre-fusion
PRR	Pattern-recognition receptor
PWM	Pokeweed mitogen
RANTES	Regulated upon activation, normal T cell expressed and secreted
RIG-I	Retinoic acid-inducible gene I
RLR	Retinoic acid–inducible gene I like receptors
RNA	Ribonucleic acid
RR	Risk ratio
RSV	Respiratory syncytial virus

RT-PCR	Reverse transcription polymerase chain reaction
RV	Rhinovirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SIAF	Swiss Institute of Allergy and Asthma Research
SIGN	Scottish Intercollegiate Guidelines Network
SNHS	Spanish National Health System
SNP	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signalling
SP-C	Surfactant protein C
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TARC	Thymus- and activation-regulated chemokine
Tc cell	Cytotoxic T cells
TGF	Transforming growth factor
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VP1	Viral protein 1

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Hurme Pekka, Homil Kiara, Lehtinen Pasi, Turunen Riitta, Vahlberg Tero, Vuorinen Tytti, Camargo Carlos A Jr., Gern James E, Jartti Tuomas. Efficacy of inhaled salbutamol with and without prednisolone for first acute rhinovirus-induced wheezing episode. *Clinical & Experimental Allergy*, 2021; 51(9): 1121–1132.

II Hurme Pekka, Komulainen Miisa, Tulkki Marleena, Leino Annamari, Rückert Beate, Turunen Riitta, Vuorinen Tytti, Akdis Mübeccel, Akdis Cezmi A, Jartti Tuomas. Cytokine expression in rhinovirus- vs. respiratory syncytial virus-induced first wheezing episode and its relation to clinical course. *Frontiers in Immunology*, 2022; 13: 1044621.

III Hurme Pekka, Sahla Reetta, Rückert Beate, Vahlberg Tero, Turunen Riitta, Vuorinen Tytti, Akdis Mübeccel, Söderlund-Venermo Maria, Akdis Cezmi A, Jartti Tuomas. Human bocavirus 1 coinfection is associated with decreased cytokine expression in the rhinovirus-induced first wheezing episode in children. *Clinical and Translational Allergy*, 2023; 13(11): e12311.

IV Hurme Pekka, Kähkönen Miisa, Rückert Beate, Vahlberg Tero, Turunen Riitta, Vuorinen Tytti, Akdis Mübeccel, Akdis Cezmi A, Jartti Tuomas. Disease severity and cytokine expression in the rhinovirus-induced first wheezing episode. *Viruses*, 2024; 16(6): 924.

1 Introduction

Bronchiolitis is the most common illness requiring hospitalisation in young children. Up to one-third of children younger than two years experience bronchiolitis, of whom 2% to 5% need hospitalisation (Jartti et al., 2019; Mahant et al., 2022). Historically, bronchiolitis has been viewed as a single condition, leading to a scarcity of studies exploring potential endotypes. Most of the prior research has focused on comparing respiratory syncytial virus (RSV) and non-RSV agents in bronchiolitis and early wheezing, with limited data on other viral causes. However, recent evidence suggests that bronchiolitis is a spectrum of endotypes that may benefit from personalised treatment (Jartti et al., 2019; Makrinioti et al., 2022; Meissner, 2016).

The primary viral agents linked to bronchiolitis are RSV and rhinovirus (RV) (Florin et al., 2017; Meissner, 2016). RSV-associated illness is common in children younger than 12 months, whereas RV infections are the most common in older children (Jartti et al., 2019). While both viruses increase the risk of recurrent wheezing and asthma, the risk is more pronounced with RV infection. Additionally, atopic traits and other host-related factors also contribute to the risk of developing recurrent wheezing and asthma (Dumas et al., 2016; Fujiogi et al., 2022; Jartti et al., 2019; Makrinioti et al., 2022). However, the immunopathogenesis of these associations remains unelucidated.

While corticosteroids and bronchodilators are crucial for asthma management, most global guidelines advise against their use in bronchiolitis and early wheezing (Tapiainen et al., 2016). Oral corticosteroids may offer benefits with RV-associated early wheezing, particularly in case of high viral load, but the results are inconsistent (Jartti et al., 2015; Lehtinen et al., 2007). Given the significant health and economic impact, a better understanding of personalised treatment for bronchiolitis is needed.

The aims of this thesis were to assess the efficacy and short-term outcomes of inhaled salbutamol with and without oral prednisolone, and to examine the early immunological factors contributing to the acute bronchiolitis and its prognosis. Additionally, to better understand the underlying immunopathogenesis, we aimed to investigate the effect of different viral aetiologies and their independent dynamics on immune responses.

2 Review of the Literature

2.1 Definitions of bronchiolitis, wheezing, recurrent wheezing, and asthma

2.1.1 Bronchiolitis

Bronchiolitis is generally defined as inflammation of the small bronchi and the surrounding tissue induced by an acute viral infection. Importantly, in up to 100% of bronchiolitis cases, a causative viral agent can be identified (Turunen et al., 2014). The viral infection triggers a rapid immune response characterised by extensive inflammation and oedema in the bronchioles and adjacent tissue. Airway obstruction and air retention within the bronchioles are further exacerbated by excessive mucus production, epithelial cell apoptosis, necrosis, and sloughing of epithelial cells (Florin et al., 2017; Jartti et al., 2019; Meissner, 2016).

Bronchiolitis is diagnosed clinically according to the typical signs and symptoms. Generally, after an incubation period of 4 to 6 days, upper respiratory tract infection symptoms such as nasal congestion, rhinitis, and fever appear (Meissner, 2016), followed by lower respiratory tract illness characterised by cough, increased respiratory effort, feeding challenges, and decreased ventilation indicated by insufficient oxygenation (Jartti et al., 2019; Meissner, 2016). While physical examination during auscultation reveals crepitations with diffuse crackles with or without expiratory wheezing as distinctive clinical findings for bronchiolitis, the manifestations of bronchiolitis can exhibit a diverse array of symptoms and varying degrees of severity (Florin et al., 2017; Jartti et al., 2009a).

Globally, the definition of bronchiolitis is not standardised. In European countries, bronchiolitis is referred to as the acute first lower respiratory tract infection induced by a virus, and characterised by the presence of crackles, with or without wheezing during expiration, in children younger than 12 months of age (Korppi, 2015; Nenna et al., 2020; Ralston et al., 2014; Tapiainen et al., 2016). In contrast, in some countries such as the United States of America, bronchiolitis is referred to as the acute first lower respiratory infection induced by a virus in children younger than 24 months of age (Ralston et al., 2014). These differences in definitions have led to inconsistencies in terminology. For example, in European countries,

children older than 12 months experiencing the same clinical condition are described as having a virus-induced wheezing illness or asthma, while in the USA, the condition is still referred to as bronchiolitis. These variations in terminology have led to discrepancies in study protocols involving children with bronchiolitis worldwide.

2.1.2 Wheezing

Wheezing is defined as a high-pitched whistling sound accompanied by expiratory breathing difficulty (Jartti et al., 2019). Wheezing occurs as a result of the narrowing of airways within the thoracic cavity, leading to variable limitations in expiratory airflow, also referred to as obstruction. Airway obstruction arises from adverse epithelial reactions such as cell death, necrosis, epithelial sloughing, and excessive secretion of mucus with or without smooth muscle contraction within the wall of the airways (De Benedictis et al., 2017). This leads to an increased workload of breathing, clinically characterised by nasal flaring, increased breathing rate, prolonged expiration, and the use of accessory respiratory muscles (Meissner, 2016).

Wheezing episodes in young children are associated with respiratory virus infections (Jartti et al., 2019; Meissner, 2016). Due to the small calibre of the airways during infancy, many young children are prone to obstruction of bronchioles and its consequent wheezing during viral infections (El-Gamal et al., 2011). Fortunately, as these infants mature, the calibre of the airways increases, reducing the likelihood of this tendency. Nevertheless, some children continue to have wheezing episodes, known as recurrent wheezing.

Early wheezing illness and viral bronchiolitis are recognised as one of the most significant risk factors for recurrent wheezing and subsequent asthma development (both defined below) (Dalziel et al., 2022; Jartti et al., 2019; Meissner, 2016). While other viral agents, such as RSV, also increase the risk for poor short- and long-term prognosis, in RV-associated disease, the risk is notably pronounced. Surprisingly, this finding is relatively novel; previously, the focus has mainly been on overall bronchiolitis regardless of the viral agent, or RSV-associated disease. This emphasis is not surprising, considering that the latter is linked to the majority of morbidity and mortality associated with bronchiolitis, particularly in infants and neonates with additional risk factors for severe disease (Dalziel et al., 2022; Florin et al., 2017; Jartti et al., 2019; Meissner, 2016).

2.1.3 Recurrent wheezing

Recurrent wheezing is commonly defined as the occurrence of wheezing more than once. Moreover, recurrent wheezing has previously been classified clinically into

two main phenotypes: *time trend-based* and *symptom-based* phenotypes. The *time-trend based* phenotype has been further divided into *transient wheezing* (symptoms emerge and resolve before 3 years of age), *persistent wheezing* (symptoms emerge before 3 years of age and persist beyond 6 years of age), and *late-onset wheezing* (symptoms emerge after 3 years of age) (Martinez et al., 1995; Stokes et al., 2020). On the other hand, *symptom-based* phenotype has been further divided into *episodic wheezing* and *multiple-trigger wheezing* depending on whether wheezing is present between the upper respiratory tract virus infection episodes or not (Owora et al., 2018). However, assigning individual children to these phenotypes has proven to be challenging in clinical scenarios, since the manifestation of wheezing and the outcome of treatment intervention can be variable (Schultz et al., 2011; van Wonderen et al., 2016). Therefore, the classification of wheezing remains under active investigation and debate, and there is a demand for enhanced classification (GINA, 2024).

Contrary to bronchiolitis (defined above), the management of recurrent wheezing and asthma exhibits notable similarities, with bronchodilators and corticosteroids being commonly used (Stokes et al., 2020). The shared fundamental properties in both conditions pose challenges in distinguishing between the two illnesses, and therefore, it is imperative to assess the possibility of asthma in all children experiencing recurrent wheezing.

As stated before, bronchiolitis is a prevalent condition, affecting up to a third of infants (Jartti et al., 2019). However, while around 30% to 50% of children experience acute wheezing at least once before school age, less than half of these children continue to endure recurrent wheezing (Taussig et al., 2003). Of recurrent wheezing children, approximately half present aeroallergen sensitisation by school age (Guilbert et al., 2004). With increasingly advanced viral diagnostic techniques, a viral agent can be identified in up to 100% of cases involving bronchiolitis and early wheezing (Petat et al., 2021; Turunen et al., 2014). However, the viral detection rate declines in an age-dependent manner, ranging from 80% to 95% in older children (Jartti et al., 2009a).

2.1.4 Asthma

Asthma is a chronic, but heterogeneous disorder characterised by inflammation of the airways, with both variable and recurring symptoms such as wheezing, persistent cough, decreased physical endurance, and dyspnoea (Cloutier et al., 2020; GINA, 2024; Papadopoulos et al., 2012). These symptoms result from airway obstruction, air trapping, and bronchial hyperreactivity, ultimately leading to limitations in the flow of air during expiration. Typically, asthma symptoms and airflow limitations exhibit variability in both time and intensity, and are often induced by exercise,

exposure to allergens or irritants, weather changes, or viral respiratory infections (Cloutier et al., 2020; GINA, 2024).

The underlying mechanism for the limitation of airflow derives from inflammatory changes in the airways. However, the exact immunopathogenesis of asthma is unelucidated (Cloutier et al., 2020; GINA, 2024; Papadopoulos et al., 2012). Nonetheless, studies have shown that children suffering from active airway symptoms during infancy are at increased risk of having reduced lung function during childhood, suggesting that changes in the lungs leading to asthma development are progressive (Malmström et al., 2011). In contrast, infants who experience recurrent wheezing and have atopic eczema (defined below), both of which are significant risk factors for asthma, exhibit notably diminished lung function even from birth compared to those who do not have these conditions (Håland et al., 2007). These findings have prompted the need for better understanding of the immunopathogenesis of asthma.

According to clinical cluster analyses, several asthma phenotypes, emphasising the heterogeneity of asthma, have been suggested, including *allergic asthma, non-allergic asthma, adult-onset asthma, asthma with persistent airflow limitations, and asthma with obesity* (Bel, 2004; Moore et al., 2010; Wenzel, 2012). The *allergic asthma phenotype* is the most readily identifiable phenotype, often linked to a prior or family history of allergic conditions, as well as high efficacy of inhaled corticosteroids and bronchodilators (GINA, 2024).

From a pathological standpoint, asthma, particularly allergic asthma, is often described as eosinophilic bronchitis accompanied by a strong type 2 immune response, characterised by the presence of immune cells secreting interleukin (IL) 4, IL-5, and IL-13 in hosts with atopic diseases (defined below). Nonetheless, in adults, asthma can manifest without eosinophilic infiltration and the presence of cytokines associated with non-type 2 immunity (Borish, 2016). However, in the paediatric population, the majority of patients have asthma that is associated with type 2 inflammation characterised by heightened levels of type 2 immunity biomarkers (Papadopoulos et al., 2024).

Asthma is the most common chronic disease in children. Globally, the prevalence of asthma across all age groups has been documented to range from 2.4% to 4.3% (Shin et al., 2023; To et al., 2012). However, while the Global Asthma Network (GAN) reported a prevalence of 10.4% in adolescents (13-14 years old) and 9.9% (6–7 years old) in children (Asher et al., 2021), according to the International Study of Asthma and Allergies in Children (ISAAC), the overall prevalence of asthma worldwide is 13.7% in adolescents and 11.6% in children (Asher et al., 2020). The variation in asthma prevalence reported in different studies could be attributed to differences in the countries and age groups included. This is particularly significant given the notable diversity in asthma prevalence among countries and

across various stages of life, as evidenced by data from the Global Burden of Disease (GBD) and other international studies. Moreover, while the number of asthma patients has risen, the global age-standardised prevalence between 1990 and 2019 has decreased by almost a quarter, indicating that, at least partly, the global increase in asthmatic patients can be attributed to global population expansion. Nevertheless, certain regions, such as the high-income North America region, have experienced an increase in asthma prevalence (Shin et al., 2023). In addition, boys are disproportionately affected by asthma compared to girls globally (Lai et al., 2009), as well as in Finland (Kankaanranta et al., 2017). In Finland, the prevalence of paediatric asthma is reported to range from 4% to 9% (Hugg et al., 2008; Lai et al., 2009; Pekkanen et al., 1997). In a recent study, the prevalence of asthma in children aged 6-17 years in Nordic countries was found to be 4.1%, 3.5%, and 4.4% in Norway, Sweden, and Finland, respectively. Additionally, among these cases, 0.4%, 1.0%, and 0.3%, respectively were classified as severe asthma (requiring administration of high-dose inhaled corticosteroids along with long-acting β2-agonists, long-acting muscarinic antagonists, leukotriene receptor antagonists, or receiving biological therapies such as anti-immunoglobulin (Ig) E or anti-IL-5/IL5R). Interestingly, the study also indicated that asthma and severe asthma were more prevalent among females than males in the population aged over 18 years. However, in paediatric patients, both asthma and severe asthma are more prevalent in boys compared to girls, consistent with the global trend (Hansen et al., 2023).

2.1.5 Atopy and atopic eczema

Atopy is generally defined as a genetic predisposition to an excessive production of allergen-specific IgE. Furthermore, atopy is associated with an exaggerated type 2 immune response to prevalent allergens, particularly those that are inhaled or found in foods, resulting in cluster of differentiation (CD) 4 positive T helper (Th) 2 cell differentiation. Typical clinical manifestations associated with atopy include atopic eczema, food allergies, and allergic asthma (Diaz-Cabrera et al., 2021).

Atopic eczema, also referred to as atopic dermatitis, is a chronic inflammatory skin condition influenced by both genetic predisposition and environmental factors, manifesting with symptoms such as itching, redness, swelling, and cracks in common areas of the skin. The disruption of the epidermal barrier, dysregulation of immunity, and microbial dysbiosis are believed to be key factors in the development of skin inflammation. The pathogenesis of atopic eczema is characterised by excessive type 2 inflammation (defined below) (Ständer, 2021).

2.2 Immunity

Immunity refers to the host's ability to resist and protect the body against pathogenic microbes, including bacteria, viruses, fungi, and parasites, as well as harmful substances such as venoms. This protection is achieved through both anatomical barriers and the responses of primary and secondary immune cells. A well-functioning immune system primarily defends the body through the collaboration of diverse immune and immune-associated cells, recognising and neutralising foreign invaders while distinguishing them from the body's own cells and tissues (Delves et al., 2000; Zach et al., 2023).

2.2.1 Innate immunity

In the respiratory system, innate immunity, consisting of anatomical barriers (physical barriers of mucosa, as well as chemical barriers), and the activity of innate immune cells, serves as the initial wave of defence against pathogenic microbes (Johnston et al., 2021; LeMessurier et al., 2020; Riera Romo et al., 2016). If the former are breached, the latter are activated (Matsui et al., 2015; Riera Romo et al., 2016). Innate immunity relies on innate immune cells, which lack antigen-specific receptors on their surfaces, and as a result, innate immunity functions as a nonspecific defence and surveillance system. The responses of innate immunity can be divided into cellular and humoral components, and the activation of innate immunity leads to an immediate maximal response, and it does not possess immunological memory (Riera Romo et al., 2016). However, innate immunity participates in the initiation of adaptive immune responses and contributes to tissue repair (Iwasaki et al., 2015).

Cells principally participating in innate immune responses within the respiratory tract include monocytes, basophils, eosinophils, neutrophils, natural killer (NK) cells, innate lymphoid cells (ILCs), and dendritic cells (DCs) (Lamichhane et al., 2019; Marshall et al., 2018). In addition to primary innate immune cells, specific cells with alternative primary roles, such as epithelial cells, play a crucial indirect role in innate immune responses, including alarmin production such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) and subsequent chemotaxis and infiltration, as well as activation of primary innate immune cells (Holgate et al., 2000; Johnston et al., 2021; LeMessurier et al., 2020; Tan et al., 2020).

Cellular component

The cellular component of innate immunity relies on pattern-recognition receptors (PRRs) situated within various cellular compartments such as the plasma membrane, endosomes, and cytoplasm, to identify specific canonical microbial molecular structures, known as pathogen-associated molecular patterns (PAMPs). Furthermore, molecules released in tissue injury, known as damage-associated molecular patterns

(DAMPs) are also recognised by PRRs (Amarante-Mendes et al., 2018; Li et al., 2021a). PRRs belong to diverse molecular families, including toll-like receptors (TLRs) (Duan et al., 2022), C-type lectins (Hoving et al., 2014), nucleotide-binding oligomerisation domain (NOD)-like (Almeida-da-Silva et al., 2023), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLR) (Rehwinkel et al., 2020), stimulator of interferon genes (STING) (Ishikawa et al., 2009), and scavenger receptors (Taban et al., 2022). PRRs are extensively present in primary immune cells, such as macrophages and DCs but also in non-immune cells, including epithelial cells. The activation of PRRs ultimately leads to nuclear factor kappa B (NF-κB)- and/or interferon regulatory factor (IRF) family -mediated upregulation of cytokines, chemokines, adhesion molecules, and antimicrobial effectors or phagocytosis of the microbes (Amarante-Mendes et al., 2018; Honda et al., 2006; Li et al., 2021a).

Epithelial cells of the respiratory tract infected with respiratory viruses, such as RV or RSV, recognise the viral presence via PRRs including TLRs, C-type lectins, NOD-like receptors, RIG-I receptors, or melanoma differentiation-associated protein 5 (MDA5) (He et al., 2016; Heyl et al., 2014; Mayer et al., 2007; Slater et al., 2010; Slevogt et al., 2007). This ultimately leads to the expression of antimicrobial factors such as defensins and interferons (IFNs) (Denney et al., 2018), as well as cytokines and chemokines associated with the pro-inflammatory response, including IFN-γ, IL-1β, IL-6, and IL-8, resulting in chemotaxis and activation of innate immune cells (Yeo et al., 2010). Notably, environmental challenges may be associated with a reduction in the expression of PRRs and impairment in the cell's ability to recognise viruses. Interestingly, exposure to environmental air pollutants leads to downregulation of PRR messenger ribonucleic acid (mRNA) resulting in an increased RV genome load, indicating heightened susceptibility to RV infection (Müller et al., 2021). Furthermore, the challenges in the responses of the cellular component of innate immunity have been associated with poorer prognosis, as seen in a murine model, in which impaired expression of β-defensin during allergen exposure was associated with asthma (Borchers et al., 2021). On the other hand, dysregulation of PRR downstream signalling is associated with autoinflammatory diseases (Masumoto et al., 2021).

Humoral component

The humoral component of the innate immune system consists of various classes of molecules, including pentraxins (Kim et al., 2016), collectins (Holmskov et al., 2003), and ficolins (Cedzynski et al., 2009; Garred et al., 2016). These molecules serve as precursors to antibodies by commencing the activation of the complement system, opsonisation of injured cells and microbes, agglutination or neutralisation of microbes, and regulation of inflammation. Furthermore, a deficiency of L-ficolin has been associated with respiratory tract infections in atopic children (Cedzynski et al., 2009).

2.2.2 Adaptive immunity

Adaptive immunity, the second layer of protection, is recruited if the innate immunity fails to eliminate the pathogenic microbe. In contrast to innate immunity, adaptive immunity is characterised by the high pathogen- and antigen-specificity via T cell and B cell receptors (Shah et al., 2021; Yam-Puc et al., 2018). Adaptive immunity is composed of the function and various interplay of three key cell types: B cells, CD4+ T cells, and CD8+ T cells (Marshall et al., 2018). For T cells to undergo expansion and conduct the effector functions, antigen presentation by antigen-presenting cells (APCs) to naïve T cell is necessary (Smith-Garvin et al., 2009). Additionally, activated CD4+ T cells offer co-stimulation for the activation of both B cells and CD8+ T cells (Akkaya et al., 2020; Duttagupta et al., 2009; Welten et al., 2013). Activated CD8+ T cells play a critical role in eliminating infected cells directly, and the main function of CD8+ T cells is to defend the host against intracellular pathogens (Harty et al., 2000). Furthermore, the activated and differentiated B cells produce affinity-matured and isotype-switched immunoglobulins (antibodies), which function to neutralise pathogens located in the extracellular space (Doria-Rose et al., 2015). Moreover, both T and B cells may undergo differentiation into memory cells, offering a more prompt and robust response to previously encountered pathogens, thus also forming the foundation for efficient vaccination (Akkaya et al., 2020; Zhang et al., 2022a).

Upon primary infection, due to initial clonal proliferation and differentiation of the naïve lymphocytes (T and B cells) into effector lymphocytes, a noticeable delay between activation and the attainment of sufficient effector function is observed (Yunis et al., 2023). Following expansion, if the microenvironment becomes unsuitable for survival (Zhan et al., 2017), a contraction phase commences, resulting in the apoptosis of approximately 95% of effector T cells, and subsequently the surviving T cells can undergo differentiation into memory T cells (Gasper et al., 2014; Valbon et al., 2016).

Adaptive immunity can be divided into cell-mediated and humoral arms. The former is known for its direct T cell-mediated responses, and the latter is known for the production of antigen-specific antibodies by effector B cells (Annunziato et al., 2015; Sebina et al., 2018).

As the main purpose of adaptive immunity is to eliminate invading pathogenic microbes and any harmful substances they generate, it is vital that these responses are triggered exclusively by molecules of foreign origin and not by the host's own molecules. The ability to discern between foreign and host molecules is a fundamental characteristic of the adaptive immune system. Nevertheless, rarely, the adaptive immune system may falter in distinguishing between foreign and host molecules, resulting in harmful responses directed against the host, potentially leading to induction of autoimmune diseases (Marshall et al., 2018). Moreover, not

all foreign substances are pathogenic or toxic, but are considered harmless. However, in some cases, adaptive immune system may misidentify harmless molecules as threats, leading to the initiation of adaptive immune responses, resulting in allergic conditions such as allergic rhinitis, food allergy, and asthma (Wang et al., 2023). Moreover, the adverse reactions from both autoimmune and allergic diseases can vary from mild to potentially life-threatening (Turner et al., 2017; Walsh et al., 2000).

2.2.2.1 Cell-mediated adaptive immunity

In brief, cell-mediated adaptive immunity is commonly defined as the function of activated T cells that respond directly to an antigen of foreign origin displayed on the surface of the cellular membrane of the host cell. However, several additional cell types play a pivotal role directly and indirectly in the activation and regulation of T cells (Annunziato et al., 2015).

Upon a viral infection, the infected cell is primed to begin producing viral proteins. Parts of these viral proteins are degraded in proteasomes and presented on major histocompatibility complex (MHC) class I or II molecules that are further transported to the cell surface. On the cell surface, lymphocytes such as T cells can recognise and bind to these MHC molecules leading to the activation of the corresponding T cell (Wieczorek et al., 2017).

MHC molecules are divided into two main groups, referred to as MHC class I and II, according to antigen presentation. MHC class I molecules are expressed on all nucleated cells, while MHC class II molecules are primarily expressed on APCs, including DCs, macrophages and B cells (Wieczorek et al., 2017; Wu et al., 2021; Xie et al., 2003). The binding of CD8+ T cells to MHC class I molecules primes and activates the naïve cytotoxic T (Tc) cells, whereas the binding of CD4+ T cells to MHC class II molecules primes and activates naïve Th cells (Wieczorek et al., 2017).

Importantly, the immune response is influenced and characterised by the corresponding effector T cell. The activation of CD8+ T cells leads to the induction of apoptosis in the targeted cell by means of effector molecules such as perforin, granzymes, or FAS (FS7-associated cell surface antigen)/FASL (FAS ligand) pathway (Al Moussawy et al., 2022). In contrast, activation of naïve CD4+ T cells leads to differentiation and expression of cytokines and chemokines which enhances the activity of other cells, including macrophages, natural killer cells (NK cells), B cells, Tc cells (CD8+ T cells), and Th cells (CD4+ T cells) (Annunziato et al., 2015; Zhu et al., 2010).

While the naïve CD4+ T cells are activated by APC, the subsequent differentiation is driven by the cytokines expressed in the microenvironment. Notably, activated CD4+ T cell may differentiate to various subsets that all exhibit

different immunological characteristics and engage in different types of immune responses (Annunziato et al., 2015; Zhu et al., 2010). The main subsets of activated and differentiated effector CD4+ T cells are Th1, Th2, Th17, and regulatory T (Treg) cells (Annunziato et al., 2015; Luo et al., 2022; Zhu et al., 2010). Th1 cells are typically associated with proinflammatory responses, Th2 cells with allergic and parasitic diseases, Th17 cells with autoimmune diseases, and Treg cells with the suppression and regulation of immunological activity (Künzli et al., 2023; Martinez-Sanchez et al., 2018).

In a broader context, cell-mediated adaptive immune responses can be divided into three main categories according to the specific cytokines secreted by the effector CD4+ T cell: type 1, type2, and type 3 immunities. Naïve CD4+ T cells undergo differentiation into effector subsets with distinct characteristics contributing to the responses of either cell-mediated or humoral immunity (Figure 1) (Annunziato et al., 2015; Martinez-Sanchez et al., 2018).

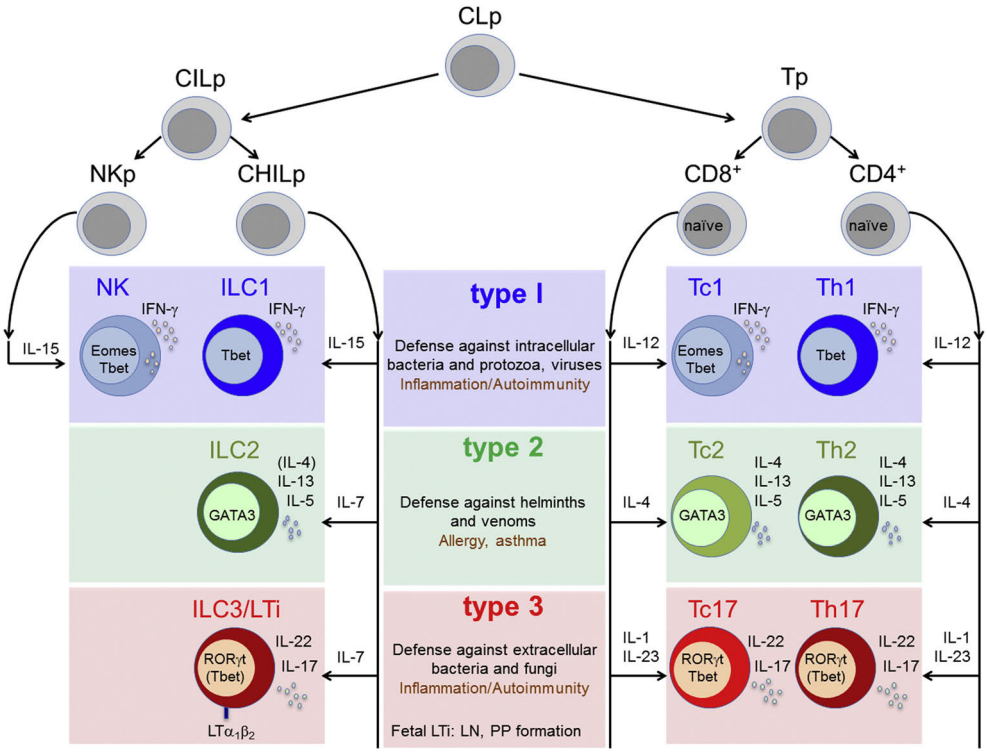

Figure 1. The three main types of cell-mediated effector immunity (innate and adaptive). CILp, Common innate lymphoid precursor; CLp, common lymphoid precursor; Tp, T cell progenitor. From the article Annunziato et al., 2015.

Type 1 immune responses are the result of intracellular pathogenic events leading to the upregulation of the transcription factor T-Bet in CD4+ T cells. Subsequently, T-Bet CD4+ T cells undergo differentiation to Th1 cells mediated by IL-12/ IFN-γ-induced signalling of signal transducer and activator of transcription (STAT) 1 and STAT4 (Annunziato et al., 2015; Farrar et al., 2002; Romagnani, 1994). Th1 cells are characterised by the ability of synthesisation and secretion of IFN-γ, and ability to enhance the activity of CD8+ T cells and macrophages (Romagnani, 1994). Furthermore, T-Bet is expressed on other types of lymphocytes such as CD8+ Tc1 (Fuchs et al., 2013; Mosmann et al., 1995), and ILC1s (Fuchs et al., 2013), and thereby, they are considered to be involved in type 1 immunity.

Type 2 immune responses are the result of responses towards extracellular pathogens, and allergens, promoting the upregulation of transcription factor GATA3 in CD4+ T cells through IL-4-mediated STAT6 signalling (Annunziato et al., 2015; Farrar et al., 2002; Romagnani, 1994). GATA3-expressing CD4+ T cells are described as Th2 cells and are characterised by the capability to secrete IL-4, IL-5, and IL-13 (Farrar et al., 2002; Romagnani, 1994). Moreover, GATA3 can be expressed in other lymphocytes such as CD8+ Tc2 cells (Jia et al., 2013; Mosmann et al., 1995), group 2 ILCs (Walker et al., 2013), which both are also involved in type 2 immunity via IL-4, IL-5, and IL-13 secretion. Additionally, IL-4, a cytokine associated with type 2 immunity, facilitates isotype switching in B cells, leading to the production of IgE (Lebman et al., 1988).

Type 3 immune responses are the result of responses towards fungal and extracellular bacterial infections, leading to the upregulation of transcription factor RORγt, promoting the CD4+ T cell to differentiate to Th17 cell (Ivanov et al., 2006). Th17 cells are signified by the production of IL-17 (Harrington et al., 2005), which is induced via STAT3 signalling (Yang et al., 2007), triggered by IL-6, IL-23, IL-1β or transforming growth factor (TGF) β (Acosta-Rodriguez et al., 2007; Cosmi et al., 2008). Additionally, RORγt can be expressed on other types of lymphocytes, such as CD8+ Tc17 cells (Intlekofer et al., 2008), and group 3 ILC (Eberl et al., 2004).

While type 1 responses induce the activity and attraction of CD8+ T cells and macrophages, and type 2 responses induce the activity and chemotaxis of eosinophils, mast cells, and basophils, type 3 responses predominantly lead to the recruitment of neutrophils to the site of inflammation (Annunziato et al., 2015). However, these reactions must be controlled. The primary function of Treg cells, which are comprised of Foxp3+ expressing CD4+ T cells (Fontenot et al., 2003), is to maintain self-homeostasis and downregulate the effects of type 1-3 immune responses carried out by conventional T cells and other cell types (Figure 2) (Josefowicz et al., 2009; Sakaguchi et al., 2008). While Treg cells secrete various immunosuppressive cytokines such as IL-10 and TGF-β (Li et al., 2007; Rubtsov et al., 2008), they can directly induce apoptosis of affected immune cells, including B

cells, T cells and APC, for example, via the granzyme B-mediated cell death (Gondek et al., 2005; Grossman et al., 2004; Zhao et al., 2006). Moreover, Treg cells robustly express IL-2R, which can deprive the micromilieu of IL-2, leading to suppression of the proliferation capabilities of other effector T cells and subsequent apoptosis via cytokine-deprivation (Pandiyan et al., 2007). In addition, constant expression of Foxp3+ is required for the Treg cell development and for sustaining the suppressive functions (Williams et al., 2007). However, conversely, in a murine model of RSV-induced pulmonary infection, Treg deprivation led to higher viral clearance at the expense of more severe immune-mediated tissue damage, ultimately contributing to poorer outcome (Loebbermann et al., 2012).

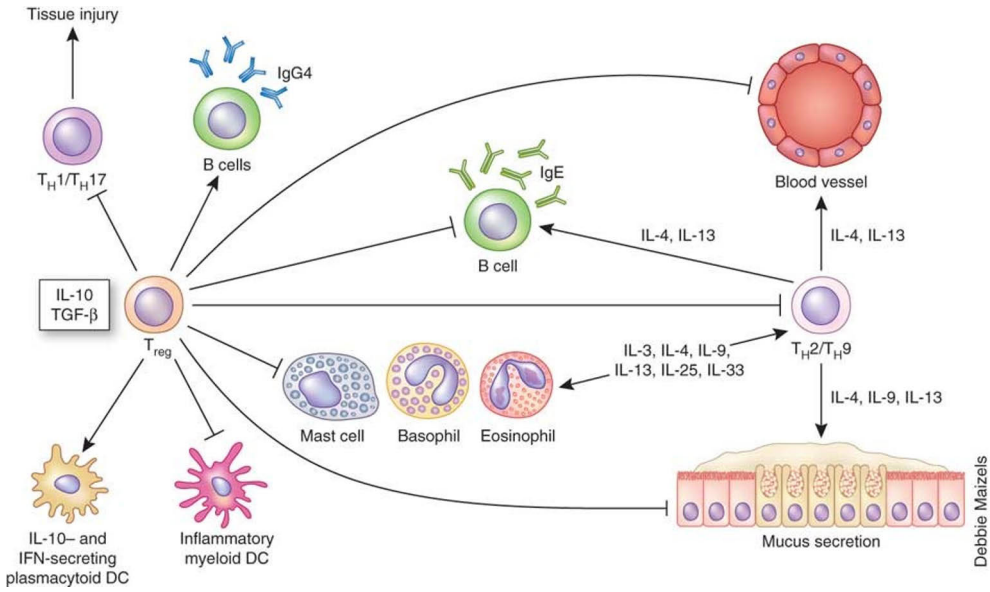

Figure 2. The role of Treg cells in relation to type 2 immunity, with regular arrows indicating stimulation and blunt arrows representing inhibition. From the article of (Holgate, 2012).

2.2.2.2 Humoral adaptive immunity

Humoral adaptive immunity refers to antigen-specific, antibody-mediated immunity characterised by the effector B cells. After encountering stimulation by an antigen via B cell receptor, B cells have the capability to process and display the antigen via MHC class II molecules. The subsequent interaction with CD4+ T cell ultimately leads to the proliferation and differentiation of B cells to high-affinity antibody (Ig) secreting plasma cells or memory cells (Batista et al., 2009; Bonilla et al., 2010; Rajewsky, 1996). While the activation of B cells predominantly relies on CD4+ T cells, B cells can be activated independently of T cells by other activators

such as foreign polysaccharides and unmethylated CpG sites of deoxyribonucleic acid (DNA) (Krieg et al., 1995; Rastogi et al., 2022).

In humans, there are five distinct isotypes of immunoglobulins based on the type of the heavy chain, including IgM, IgG, IgA, IgD, and IgE. Briefly, the isotypes vary in their biological characteristics, location of function, and capacity to respond to different antigens (Stavnezer et al., 2008; Stavnezer et al., 2014).

However, naïve B cells are known to exclusively co-express IgM and IgD (Rolink et al., 2004). Due to the specific functions tailored to various antibody isotypes, a process termed isotype or class switching becomes necessary post-activation. This process, which is dependent on the type of cytokine signal B cells receive from Th cells during antigen presentation, is essential for acquiring non-IgM and IgD effector capabilities, enabling efficient targeting and elimination of the antigen. The subsequent isotype after class switching is also dependent on the type of cytokine signal B cells receive from Th cells during antigen presentation (Bossie et al., 1991; Kühn et al., 1991; Punnonen et al., 1997; Purkerson et al., 1992; Robinson et al., 2017).

Increased levels of total serum IgE and allergen-specific IgE serve as important risk factors associated with atopic diseases such as allergic rhinitis, allergies, asthma and atopic eczema (Skaaby et al., 2017; Wong et al., 2020). IL-4, IL-5, and IL-13 which are strongly associated with type 2 immunity, can promote class switching in B cells to IgE (Punnonen et al., 1997; Purkerson et al., 1992). Furthermore, for class switching, IL-4 appears to be required, since in an IL-4-deficient murine model, class switching to IgE is impaired (Kühn et al., 1991; Robinson et al., 2017). In contrast, IFN-γ, a type 1 immunity-associated cytokine, can promote class switching to IgG2 (Bossie et al., 1991).

Antibodies can induce neutralisation, activation of the complement, and both antibody-dependent cell-mediated cytotoxicity and phagocytosis, as well as degranulation of mast cells, basophils, and eosinophils (Forthal, 2014; Joulia et al., 2015; Pantaleo et al., 2022), all of which are important defence mechanisms against harmful pathogens. Nevertheless, in certain settings, the effector functions of antibodies have the potential to intensify inflammation and cause additional harm, as demonstrated in the case of antibody-dependent enhancement observed in dengue disease (Teo et al., 2023).

2.2.3 Peripheral blood mononuclear cells (PBMCs), research tool, stimulation models

Human PBMCs consist of blood cells with a rounded nucleus, including lymphocytes, monocytes, NK cells, and DCs. A whole blood sample is segregated into two portions by means of density gradient centrifugation, and PBMCs, having

lower density, remain above the density gradient medium, while other cells (mainly red blood cells and granulocytes), having higher density, remain under the density gradient (Kleiveland et al., 2015; Stabel et al., 2023).

The majority of PBMCs are naïve or resting cells. In the absence of an ongoing immune response, T cells, which constitute the largest fraction of the isolated PBMCs, are predominantly in the form of naïve or memory T cells (Chen et al., 2020; Kleiveland et al., 2015).

In the peripheral blood, the existence of lymphocytes with single antigen specificity is limited. Thus, to stimulate these cells *in vitro*, polyclonal activators are employed since they can activate a substantial proportion of lymphocytes regardless of their antigen specificity. Depending on the stimulant, different PBMC populations may be stimulated, and the secreted and measured cytokine responses correspond to the stimulant used (Kleiveland et al., 2015). To target naïve T cell activation, antibodies that selectively bind to CD3, either alone or in conjunction with CD28, such as anti-CD3/anti-CD28, can be used (Kleiveland et al., 2015; Trickett et al., 2003). Other commonly used mitogenic stimulants include phytohaemagglutinin (PHA) and concanavalin A, primarily inducing T cell proliferation (Kleiveland et al., 2015; Lawlor et al., 2021); pokeweed mitogen (PWM), inducing both T and B cell proliferation (Bekeredjian-Ding et al., 2012; Kleiveland et al., 2015); and lipopolysaccharide (LPS), which stimulates proliferation of B cells and activation of monocytes (Kleiveland et al., 2015; Lawlor et al., 2021). However, while anti-CD3/anti-CD28 stimulates T cells directly, it has recently been identified to stimulate B cells indirectly (Lawlor et al., 2021).

2.3 Pathogenesis of bronchiolitis

2.3.1 Rhinovirus (RV)

Definition

RV is a small non-enveloped, positive-sense, single-stranded RNA (ssRNA) virus belonging to the Enterovirus genus, and therefore, is part of the Picornaviridae family (Bizot et al., 2021). RVs exhibit substantial genetic and antigenic heterogeneity. Three different species of RV, specifically RV A, B, and C, have been identified, comprising more than 160 different serotypes distinguished by variations in antigens or genetics (Bochkov et al., 2014; Bochkov et al., 2016; McIntyre et al., 2013; Simmonds et al., 2010).

RV was first isolated from a culture sample in 1953 from common cold patients (Andrewes et al., 1953). Following advancements in diagnostic testing, in 1988, the

first polymerase chain reaction (PCR)-based assay for RV detection from respiratory samples was described (Gama et al., 1988). Previously, the detection of RV infection was accomplished via viral cultures, and therefore initially, only RV A and B were identified (Horsnell et al., 1995; Savolainen et al., 2002a; Savolainen et al., 2002b). Later, however, it was revealed that not all RV species grow sufficiently in traditional laboratory-based cell cultures (Bochkov et al., 2011), and in 2006, through sequencing and phylogenetic analysis of genome sequences, RV C species were discovered (Lamson et al., 2006).) Furthermore, due to advancements in molecular diagnostics, a substantial number of new RV genotypes have been identified (Bochkov et al., 2016; McIntyre et al., 2013).

Currently, due to the delay and difficulties associated with identification via viral culture, acute RV infections are identified using reverse transcription (RT)-PCR (Gilbert et al., 1996; Rotbart et al., 2000; Wright et al., 2007). Additionally, for identification of RV, the use of serological tests is restricted to epidemiological studies, and rapid antigen tests are unavailable (Jartti et al., 2017; Rotbart et al., 2000).

Though, several genotypes of RV co-circulate throughout all seasons (Horvat et al., 2024; Jartti et al., 2008a; van der Zalm et al., 2011), a greater frequency of acute RV infections has been reported during autumn and spring (Jartti et al., 2004; Yuan et al., 2020). Notably, RV A and C are more commonly identified in circulation compared to RV B (Calvo et al., 2010; Erkkola et al., 2020; Marcone et al., 2014; Martin et al., 2018; Turunen et al., 2017), and RV C is associated with more severe illness (Calvo et al., 2010; Erkkola et al., 2020).

The transmission of RV commonly occurs through infectious aerosols and droplets, but also, through fomites and direct interpersonal contact (Andrup et al., 2023). Notably, RV can remain viable on various surfaces for up to several days and on intact skin for several hours (Andrup et al., 2023; L'Huillier et al., 2015; Winther et al., 2011).

The signs and symptoms of RV infection typically appear after an incubation period of approximately 2 days, generally lasting for 1 week (Arruda et al., 1997; Lessler et al., 2009). Nevertheless, in up to 25% of cases, some symptoms may persist for up to 2 weeks (Arruda et al., 1997). Though clinically RV infections are generally mild and can be treated without the need for medical intervention (Rotbart et al., 2000), the clinical manifestations of RV infections vary widely, ranging from asymptomatic infections to lower respiratory tract infections such as pneumonia, bronchiolitis, recurrent wheezing, and asthma exacerbations, which may require hospitalisation (Cox et al., 2018; Granados et al., 2015; Jacobs et al., 2013; Toivonen et al., 2016).

Pathogenesis of RV infection

RV primarily enters the body through the upper respiratory tract and infects the epithelial cells of the airway. RV can attach itself to both ciliated and non-ciliated cells (Jakiela et al., 2008; Tan et al., 2018), but usually, particularly RV C, seems to target ciliated cells only (Griggs et al., 2017). Interestingly, RV can also infect monocytes, but while the infection can activate monocytes, the replication is not considered sufficient (Korpi-Steiner et al., 2006; Laza-Stanca et al., 2006).

To enter the respiratory epithelial cells, the majority of RV A and B serotypes utilise the intercellular adhesion molecule 1 (ICAM-1) as a receptor for binding. However, a minority of serotype subgroups within RV A, bind to the low-density lipoprotein receptor (LDLR) instead (Uncapher et al., 1991). Notably, unlike RV A and RV B, RV C employs cadherin-related family member 3 (CDHR3) for binding and entry into the targeted epithelial cell (Bochkov et al., 2015).

Following attachment to its corresponding receptor, the RV enters the cell via receptor-mediated endocytosis, leading to internalisation into clathrin-coated endosomes. However, other types of receptors may also be used. Within the acidic endosome, pH level is decreased, leading to expansion of the virus and formation of pores in endosome via viral protein 1 (VP1) (Shingler et al., 2013).

While the endosomal pores facilitate the passage of uncoated viral RNA through to the other side of the endosome, viral proteins remaining inside the endosome are subsequently degraded and fragmented viral proteins are transported via MHC class molecules to the cell surface for presentation to immune cells. Afterward, uncoated RV RNA initiates the replication process, including the translation of viral proteins and the synthesis of the viral RNA genome, ultimately resulting in the assembly of new viral particles (Ganjian et al., 2020; Louten, 2016).

In comparison to the direct cytopathology of RSV infection, the damage caused to the airway epithelium by RV infection tends to be milder. However, RV infection can alter epithelial tight junctions, which leads to an increase in permeability of the epithelium (Looi et al., 2016; Unger et al., 2014). Moreover, RV replication thrives in damaged epithelium compared to intact tissue, as it is found in the deeper cell layers of scratched or damaged cell cultures, indicating a preference for disrupted environments (Jakiela et al., 2008).

Immune responses to RV infection

During an RV viral infection, the mucosal epithelial cells present several mechanisms of defence. Endosomal TLR3 and TLR7/8 recognise uncoated viral RNA, double-stranded RNA (dsRNA), and ssRNA, respectively, and the recognition leads to innate immune responses via NF-κB or IRF signalling (Hewson et al., 2005; Tan et al., 2018; Triantafilou et al., 2011). Further, cytoplasmic receptors, such as

MDA-5 and RIG-1 can detect replicative intermediates of the RV genome synthesis, leading to enhanced antiviral activity characterised by increased expression of type I and III IFNs and pro-inflammatory cytokines via IRF and NF-κB, respectively (Hewson et al., 2005; Sajjan et al., 2006; Slater et al., 2010; Triantafilou et al., 2011).

Following the detection of RV, the expression of early pro-inflammatory cytokines and growth factors by the airway epithelial cells occurs rapidly, and induces the activity and chemotaxis of various leukocytes, such as neutrophils, lymphocytes, and eosinophils (Jartti et al., 2019). Interestingly, RV appears to trigger more pronounced neutrophilic inflammation compared to other viral agents, such as RSV, as evidenced by a higher IL-8 response from highly differentiated human airway epithelial cells in response to a viral challenge, with the effect of RV infection further enhanced by an allergenic challenge (Chun et al., 2013). The ensuing inflammatory response causes epithelial oedema and an excessive increase in mucus production, ultimately resulting in airway obstruction and wheezing (De Benedictis et al., 2017; Jartti et al., 2019; Meissner, 2016). Interestingly, in asthmatic individuals, RV infection and IL-33 may interact, as indicated by type 2-skewed immune responses (Jurak et al., 2018).

RV infections also elicit a humoral adaptive immune response, characterised by the appearance of serotype-specific antibodies (IgG and IgA) approximately 2 weeks post-incubation (Bochkov et al., 2023; Jacobs et al., 2013). However, although the antibody levels may remain elevated for more than 1 year after the infection, due to the abundant number of serotypes and limited cross-neutralisation, the efficacy of humoral adaptive immunity in reducing the overall risk of RV infections is limited (Bochkov et al., 2023).

2.3.2 Respiratory syncytial virus (RSV)

Definition

RSV, classified within the Orthopneumovirus genus, is an enveloped, negative-sense, ssRNA virus, and a member of the Pneumoviridae family (Griffiths et al., 2017). RSV consists of two distinct antigenic groups, RSV A and B, both comprising several different genotypes. RSV epidemics are expressed seasonally, and typically, in the northern parts of the globe, epidemics manifest in the midst of winter, with nearly every child acquiring an RSV infection before 2 years of age (Kutsaya et al., 2016; Yuan et al., 2020).

RSV, which was first discovered in 1957 (Chanock et al., 1957), causes infections that constitute a major health and economic burden. Annually, approximately 30 million episodes, 3.6 million hospitalisations, and over 100,000 mortalities are estimated to be attributed to RSV infections globally (Li et al., 2022).

Importantly, risk for more severe disease is associated with young age, which is further increased by prematurity, chronic respiratory or congenital heart disease, neurological disorders, or a compromised immune system (Wildenbeest et al., 2023).

Globally, in clinical practice, rapid RSV antigen tests are commonly used to identify RSV infections. Additionally, RT-PCR may also be employed, and the sensitivity of both methods appears to be equivalent (Griffiths et al., 2017). Clinically, symptoms of RSV typically start after an incubation period of 4 days, reaching their peak around day 5 of the clinical illness and often showing signs of improvement by days 7 to 10 (Eiland, 2009; Lessler et al., 2009).

Pathogenesis of RSV infection

After the transmission of RSV, which occurs via direct contact or aerosol particles, the virus first attaches to and replicates inside epithelial cells in the upper respiratory tract subsequently advancing to the lower respiratory tract (Eiland, 2009). RSV primarily infects ciliated epithelial cells, but also pneumocytes (type I) (Moore et al., 2008; Zhang et al., 2002). Upon infection, RSV attaches itself using fusion (F protein) and glycoprotein (G protein) surface proteins to CX3CR1 on the surface of the target cell leading to receptor-mediated caveolar endocytosis that results in the internalisation of the virus (Werling et al., 1999). Subsequently, the viral replication process is initiated.

Furthermore, aside from facilitating the initial fusion between the viral and plasma membranes, the F protein also induces fusion between infected and adjoining plasma membranes, leading to the creation of syncytia, a multinucleate cell, which though uncommon, serves as a characteristic cytopathic effect of RSV infection (Pastey et al., 1999).

Immune responses to RSV infection

During RSV infection, the host cells employ various mechanisms of detection, activating antiviral defences and cytokine production via plasma membrane- and endosome-bound TLRs (such as TLR2, TLR3, TLR4, TLR6, and TLR7/8), cytosolic RLRs (including RIG-I and MDA5), and cytosolic nucleotide-binding oligomerisation domain-like receptors (NLRs) (including NOD2 and NLRP3). The signals from PRRs lead to activation of transcription factors such as NF-κB and IRF, ultimately leading to upregulation of type I IFNs, activation of DCs, and expression of proinflammatory cytokines and chemokines (Ouyang et al., 2022).

Apart from PRRs within the cell, TLR4, the primary extracellular receptor on airway epithelial cells, detects RSV via interaction with the viral F protein, which leads to the activation of NF-κB-mediated expression of proinflammatory cytokines

such as IL-1β, IL-6, IL-8, and IL-12 in different cell types (Haeberle et al., 2002; Haynes et al., 2001; Kurt-Jones et al., 2000).

Following RSV infection, the expression of plasma membrane-bound TLR4 is increased (Monick et al., 2003). However, RSV can employ various immune evasive mechanisms, such as expression of the soluble G protein, which blocks type I IFN production mediated by TLR3/4, or induction of the expression of suppressor of cytokine signalling (SOCS) proteins, such as SOCS1 and SOCS3, that inhibit pathways associated with production of IFNs and proinflammatory cytokines (Ouyang et al., 2022; Shingai et al., 2008; Yoshimura et al., 2007).

In contrast to RV infection, RSV infection leads to direct epithelial damage and necrosis, as well as ciliary disruption and destruction (Mata et al., 2012; Smith et al., 2014). As a result, the epithelial cells are sloughed, and production of proinflammatory cytokines is increased. Subsequently, the robust inflammatory response attracts innate immune cells such as ILCs, DCs, and granulocytes (Jartti et al., 2019; Jartti et al., 2017; Smith et al., 2014).

2.3.3 Human bocavirus 1 (HBoV1)

HBoV1 is a small non-enveloped, negative-sense, single-stranded DNA (ssDNA) virus belonging to the Parvoviridae family (Qiu et al., 2017). It was first discovered in 2005 while investigating nasopharyngeal aspirates (NPAs) of children experiencing respiratory infections (Allander et al., 2005). Afterwards, three additional types of bocaviruses, designated as HBoV2 to HBoV4, have been identified (Kapoor et al., 2009, 2010). Notably, whereas HBoV1 is primarily associated with respiratory tract infections, HBoV2-4 have been predominantly identified in faecal samples both in the presence and absence of gastroenteritis (Kantola et al., 2015; Paloniemi et al., 2014).

Pathogenesis and immune responses of HBoV1

The exact pathogenesis of HBoV1 infection is still not fully understood, and due to the absence of optimal *in vivo* and animal models, the knowledge of HBoV1 infections has relied predominantly on either *in vitro*, epidemiological or clinical studies based on varying diagnostic criteria such as serology, qualitative PCR of sole HBoV1 infection or identification of the presence of HBoV1 mRNA (Christensen et al., 2019; Mohammadi, 2023). However, the detection of HBoV1 DNA in asymptomatic children is also common, and HBoV1 DNA may persist for a long time in healthy subjects. Therefore, the determination of the relationship between active infection and the identification of HBoV1 DNA by qualitative PCR alone is

not advised (Byington et al., 2015; Christensen et al., 2019; Martin et al., 2015; Mohammadi, 2023).

In vitro, HBoV1 is capable of infecting differentiated laboratory-based cultured airway cells referred to as polarised epithelial cells (Shao et al., 2021). In response to HBoV1 infection these cells secrete IL-1 and IL-18 (Deng et al., 2017). Moreover, in HBoV1-associated bronchiolitis, enhancement of both type 1 and 2 immune responses has been observed, as indicated by increased levels of IFN-γ, IL-2, and IL-4 (Chung et al., 2008). Interestingly, while coinfecting with other viruses, particularly with RV, HBoV1 has been observed to suppress the overall cytokine response, but the long-term consequences of this effect are still not known (Lukkarinen et al., 2014). During acute HBoV1 infection, T cell responses appear to be predominantly mediated by CD4+ T cells, and activation through HBoV1 virus-like particles leads to elevated levels of various cytokines, including IL-10, IFN-γ, and IL-13 (Kumar et al., 2011).

Globally, HBoV1 is among the most frequently detected viruses in young children experiencing upper and lower respiratory tract infections (Jartti et al., 2019). HBoV1 can be detected in up to 25% of children experiencing symptoms of respiratory tract infection (Malta et al., 2020). Interestingly, due to the persistence of viral DNA, a similar prevalence is found in asymptomatic children. Viral coinfection with HBoV1 is frequent, with up to 75% of positive qualitative PCR HBoV1 DNA samples showing the presence of another concurrent viral agent (Calvo et al., 2016; Christensen et al., 2019). Due to prolonged shedding, this might correspond to an inactive viral infection, but importantly, in respiratory samples where HBoV1 mRNA is detected, other viruses have been identified in up to half of the cases (Christensen et al., 2013).

2.3.4 Other viruses and viral coinfections

While RV and RSV are the most prevalent viral agents identified, it is crucial to note that other viral agents, such as metapneumovirus (hMPV) (Jartti et al., 2004), parainfluenza virus (types 1-4) (Jackson et al., 2008; Kotaniemi-Syrjänen et al., 2003), influenza viruses (types A and B) (Kusel et al., 2007; Miller et al., 2013), adenoviruses (Jartti et al., 2004), human coronaviruses (specifically 229E, OC43, NL63, and HKU1) (Bisgaard et al., 2010; Jartti et al., 2008b; Kusel et al., 2007), and enteroviruses (EVs) (Jartti et al., 2009a), are also associated with viral bronchiolitis and early wheezing (Figure 3). Interestingly, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is not associated with an increased likelihood of developing bronchiolitis or early wheezing during childhood (Curatola et al., 2021).

The prevalence of these viral agents ranges from 3% to 21% among wheezing children, and the associations between the short- and long-term prognoses of these single viral infections are still not fully established (Jartti et al., 2017). However, it is noteworthy that coinfections are common (Jartti et al., 2019), and that coinfections such as RV and RSV with other viruses may contribute to an unknown interplay between the viral aetiologies and early immunity. This is indicated by a recent study observing the formation of hybrid virus particles in human lung cells when coinfected with both RSV and influenza virus (Haney et al., 2022). While bronchiolitis caused by a sole RV infection tends to require a shorter hospitalisation period compared to a sole RSV infection alone (Jartti et al., 2014), coinfections may exacerbate the severity of illness. A previous study examining viral aetiologies and hospital stay length highlighted that RV-RSV coinfection was significantly linked to longer hospitalisations, exceeding the duration seen in children infected with either RV or RSV alone (Mansbach et al., 2012). However, the data are not consistent, as suggested by a prior meta-analysis comparing the role of viral coinfections in the clinical severity among RSV infected young children. Significantly, only RSV-hMPV coinfection, characterised by an extended hospital stay and increased risk of intensive care unit (ICU) admission, was associated with poorer prognosis compared to sole RSV infection (Li et al., 2020). Yet, the severity of bronchiolitis and the broader influence of coinfection are subjects of ongoing debate, with the potential effects varying depending on the specific viral agents involved in the coinfection. In addition, since the prevalence of different viral agents is age-dependent, the variations in the upper age limit used to define bronchiolitis could impact the outcomes observed (Jartti et al., 2019).

Several of the most prevalent causes of hospital admission related to bronchiolitis include hypoxia, requirement for supplementary oxygen, feeding difficulties, and respiratory distress (Habib et al., 2022; Halstead et al., 2012; Zorc et al., 2010).

While detection of viral coinfection in children with bronchiolitis is common, and viral coinfections have been associated with more severe disease, including increased duration of hospitalisation and disease as well as increased risk for hypoxemia (Ferro et al., 2020; Harada et al., 2013; Richard et al., 2008; Tan et al., 2021), the data are not universal (Brand et al., 2012; de Souza et al., 2016; Marguet et al., 2009; Petrarca et al., 2018), and notably, bacterial infections may further complicate the data, as observed in children with severe bronchiolitis necessitating intensive care (Wiegers et al., 2019).

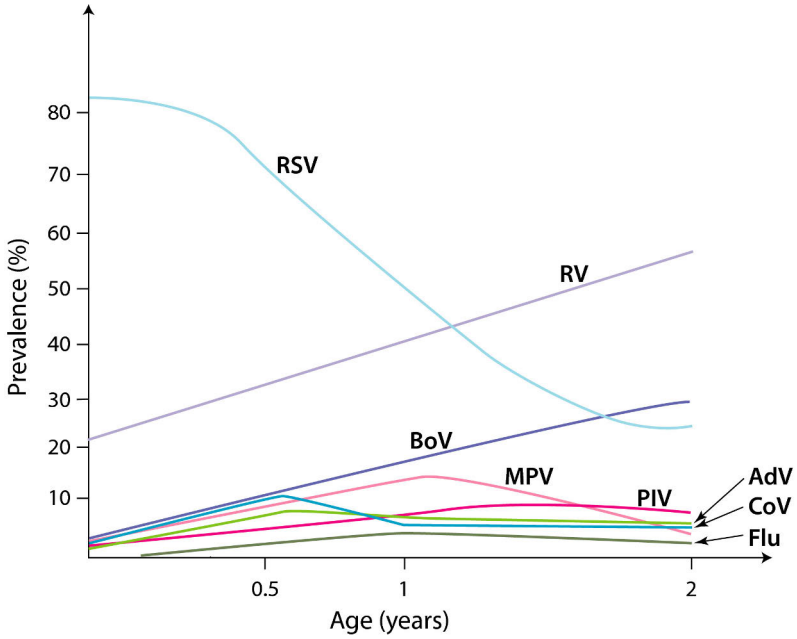

Figure 3. The frequency of viral etiologic agents according to the age of the hospitalised patients with the first episode of bronchiolitis or wheezing. RSV, respiratory syncytial virus; RV, rhinovirus; BoV, human bocavirus 1; MPV, metapneumovirus; PIV, parainfluenza virus; AdV, adenovirus; CoV, coronavirus; Flu, influenza viruses. From the article of Jartti et al 2019.

2.3.5 Viral aetiology of the early wheezing and the risk of subsequent recurrent wheezing and asthma

As described before, viral agents can be identified in up to 100% of cases of early wheezing episodes occurring within the first 2 years of life. Among these, RV and RSV are the most common and significant causes of wheezing illness. Furthermore, in recent years, HBoV1 has emerged as a notable viral agent associated with wheezing, and presently, the relationship between viral infection and wheezing episodes in childhood is well-established (Jartti et al., 2019; Petat et al., 2021; Turunen et al., 2014).

Bronchiolitis is a common illness, and depending on the definition, between 2% and 5% of infants experience severe bronchiolitis, necessitating hospitalisation, within their first 12 months of life (Díez-Domingo et al., 2014), and roughly 2% of children during the first two years of life (Mahant et al., 2022). Notably, severe bronchiolitis correlates with heightened strain on healthcare resources and diminishes the quality of life for affected families (Díez-Domingo et al., 2014; Tan et al., 2021). Significantly, the impact of severe bronchiolitis extends beyond the acute illness, as over 30% of children necessitating hospitalisation in the acute phase,

develop recurrent wheezing (Díez-Domingo et al., 2014; Gern, 2010; Zhang et al., 2020a). Moreover, in Swedish and Finnish children, the likelihood of recurrent wheezing following bronchiolitis has been observed to exceed 50% at 2–3 years of age (Piippo-Savolainen et al., 2008). While the risk of asthma following severe bronchiolitis during the first 12 months of life decreases over time, between 15% and 30% of children suffer from asthma during the early school age (Piippo-Savolainen et al., 2008), and the risk of asthma persists higher than in control patients even up to early adulthood (Sigurs et al., 2010).

Although bronchiolitis, as defined currently, is a virus-associated lower respiratory tract infection, emerging evidence suggests a connection between specific viral agents identified during bronchiolitis and the development of preschool wheezing and asthma (Kenmoe et al., 2020; Makrinioti et al., 2022).

In the past, numerous studies have shown that RSV bronchiolitis is associated with recurrent wheezing and subsequent development of asthma. However, the results are highly variable due to differences in study protocols and sample size. Compared to non-hospitalised healthy children, RSV bronchiolitis has been shown to be associated with recurrent wheezing before school-age (Bertrand et al., 2015; Bont et al., 2000; Chung et al., 2002; Kristjánsson et al., 2006; Schauer et al., 2002; Tian et al., 2009). In contrast, in prior studies comparing RSV bronchiolitis and RSV-negative-bronchiolitis, data concerning the association with recurrent wheezing has been reported as inconclusive, indicated by two smaller studies in favour of and one larger population-based study against RSV bronchiolitis as a risk factor (Cifuentes et al., 2003; Marlow et al., 2019; Valkonen et al., 2009). However, when the association between recurrent wheezing and viral aetiologies of RV and RSV bronchiolitis have been compared, the data suggest a more marked risk of recurrent wheezing after RV bronchiolitis compared to RSV bronchiolitis (odds ratio (OR) 1.5-8.8) (Bergroth et al., 2016; Da Silva Sena et al., 2021; Hasegawa et al., 2019a; Lemanske et al., 2005; Midulla et al., 2012).

When the association between asthma and post-RSV bronchiolitis has been analysed, compared to healthy asymptomatic children, RSV bronchiolitis has shown increased risk of subsequent asthma during the period before school age (OR 5.6-17.3) (Cassimos et al., 2008; Sigurs et al., 2000; Yamada et al., 2010; Zomer-Kooijker et al., 2014). Yet, when comparing RSV bronchiolitis to non-RSV bronchiolitis, the analysis of post-bronchiolitis asthma shows inconsistency (del Rosal et al., 2016; Fjærli et al., 2005; García-García et al., 2007; Henderson et al., 2005; Koponen et al., 2012; Stein et al., 1999). However, importantly, when RSV bronchiolitis and RV bronchiolitis are compared with respect to their effect on risk for subsequent asthma during childhood, RV bronchiolitis is shown to be a more marked risk factor compared to RSV bronchiolitis (OR 1.1-8.6), with Kusel et. al indicated decreased risk with RV bronchiolitis compared to RSV aetiology (OR 0.7)

(Bergroth et al., 2020; Hasegawa et al., 2019b; Jackson et al., 2008; Kotaniemi-Syrjänen et al., 2002; Kusel et al., 2007; Leino et al., 2019; Lukkarinen et al., 2013, 2017; Teeratakulpisarn et al., 2014).

Notably, RV serotype, RV C in particular, seems to be associated with the highest risk for subsequent recurrent wheezing and asthma (Bergroth et al., 2020; Hasegawa et al., 2019a). Furthermore, atopic sensitisation, during the first severe wheezing episode is associated with recurrent wheezing (hazard ratio (HR) 1.9-3.47) (Jackson et al., 2012; Lukkarinen et al., 2013), and bronchial reactivity before school-age (OR 8.8) (Leino et al., 2019), especially with the identification of concomitant RV infection.

Moreover, RV bronchiolitis is more strongly associated with atopic asthma than RSV bronchiolitis, and conversely, the association of non-atopic asthma and RSV bronchiolitis is more marked when compared to RV bronchiolitis (Lukkarinen et al., 2017).

In the past, while RSV-associated bronchiolitis has been recognised as an important risk marker for subsequent wheezing and development of asthma (Papadopoulos et al., 2004; Sigurs et al., 2000), it is noteworthy, that the increased risk has been observed in studies comparing asymptomatic healthy children to children with RSV-associated bronchiolitis (Valkonen et al., 2009). Conversely, a mounting number of studies are establishing a connection between RV-associated wheezing episode during early years of life and the development of subsequent recurrent wheezing as well as asthma in preschool-aged children (Hasegawa et al., 2018a; Makrinioti et al., 2022; Midulla et al., 2014). Furthermore, in infants with atopic predisposition and showing signs of allergic sensitisation, the association appears to be more robust (Hasegawa et al., 2019a; Lemanske et al., 2005).

While a strong epidemiological association exists between severe RSV infection during infancy and the later development of asthma (Szabo et al., 2013), a recent meta-analysis, examining whether prevention of early life RSV-associated lower respiratory tract infection was associated with reduced incidence of recurrent chronic wheezing illness, failed to show a causal association between the two (Brunwasser et al., 2020). Moreover, another recent meta-analysis comparing the impact of RSV- and RV-associated bronchiolitis on recurrent wheezing and asthma found a stronger association between RV and recurrent wheezing compared to RSV (OR 4.1). Furthermore, bronchiolitis associated with RV infection exhibited a higher likelihood of developing subsequent asthma compared to RSV (OR 2.72) (Makrinioti et al., 2022). Nevertheless, RSV bronchiolitis is associated with recurrent wheezing and asthma when compared to children without prior history of bronchiolitis.

Bronchiolitis caused by HBoV1 has also been suggested to be associated with a risk for recurrent wheezing and asthma. However, the strong evidence validating the

risk is still scarce. Nevertheless, a study observing children infected with singleton infections of HBoV1 and RSV during bronchiolitis showed that HBoV1 infection was more strongly associated with asthma (OR 1.28) and wheezing (OR 2.18) than the RSV group. Interestingly, no differences were found between the viral groups in atopic characteristics of the host or the family, and surprisingly, the viral groups were not associated with a decline in pulmonary function (del Rosal et al., 2016).

2.4 Treatment regiments of first wheezing episode

2.4.1 β2-agonist

Inhaled bronchodilators (β2-agonists) play a crucial and indisputable role in managing recurrent wheezing and asthma exacerbations. Despite their overall significance in the treatment of obstructive breathing difficulties, major guidelines such as the Scottish Intercollegiate Guidelines Network (SIGN) 2006, the Spanish National Health System (SNHS) 2010, the American Academy of Pediatrics (AAP) 2014, the Finnish Current Care 2014, the National Institute for Health and Care Excellence of the UK (NICE) 2015, the Australasian Paediatric Research in Emergency Departments International Collaborative (PREDICT) committee 2018, and the Canadian Paediatric Society (CPS) 2018 advise against their routine use as part of bronchiolitis treatment (Bakel et al., 2017; Friedman et al., 2014; O'Brien et al., 2019; Ralston et al., 2014; Ricci et al., 2015; Tapiainen et al., 2016). Only in the SNHS 2010 guideline is a salbutamol trial as part of bronchiolitis treatment regarded as optional, whereas in other guidelines the option is not addressed (Bakel et al., 2017; Friedman et al., 2014; O'Brien et al., 2019; Ralston et al., 2014; Ricci et al., 2015; Tapiainen et al., 2016). This is unsurprising since, though the efficacy of bronchodilators in treatment of bronchiolitis has been studied extensively, only a few trials have demonstrated clinical benefits of β2-agonists compared to placebos in young, first-time wheezing children (Cai et al., 2020; Flores et al., 1997; Gadomski et al., 2014; Kirolos et al., 2020). Importantly, since emerging evidence suggests that bronchiolitis is a spectrum, there is a lack of conclusive evidence regarding their efficacy in specific subgroups of bronchiolitis.

β2-agonists bind to adrenergic receptors, while importantly, exhibiting enhanced selectivity for β2-adrenergic receptors. In airway smooth muscle cells, upon the activation of the receptor, a transmembrane signal cascade is initiated, resulting in an intracellular signal cascade, which has been suggested to influence either the levels or the sensitivity of intracellular Ca2+. The decreased efficacy of intracellular Ca2+ is considered to inhibit the phosphorylation of myosin light chain, which consequently prevents the contraction of smooth muscle in the airways, leading to bronchodilation (Billington et al., 2013).

Interestingly, β2-agonists are also suggested to have the capability to trigger various inflammatory pathways in the smooth muscles of the airways, which results in a decrease in levels of intercellular adhesion molecules and chemokines, as well as the stabilisation of mast cell degranulation (Barisione et al., 2010). Notably, RSV infection has been associated with dictating the characteristics of β2-adrenergic receptors by decreasing the receptor quantity and quality (function and position). This dysregulation may explain, at least partly, the lack of observed efficacy of β2-agonists in the treatment of RSV-associated bronchiolitis (Harford et al., 2021).

Curiously, regular stimulation with β2-agonists may lead to tolerance (Haney et al., 2005), and increased airway hyperreactivity to both direct and indirect stimuli (Murphy et al., 2021). However, concomitant corticosteroid treatment may counteract these adverse reactions by increasing the upregulation of β2 adrenergic receptors, thus potentiating the effects of inhaled β2-agonists (Aksoy et al., 2002).

2.4.2 Corticosteroid treatment

Corticosteroids are essential in the management of recurrent wheezing and asthma exacerbations, and a crucial part of asthma control medication (Cloutier et al., 2020; GINA, 2024). The robust anti-inflammatory and immunosuppressive effects are mediated via intracellular glucocorticoid receptor (GR) signalling, and the activation of the GR leads to regulation (induction or inhibition) of various gene transcriptions, for instance, inhibition of NF-κB. While GR is expressed in almost every cell, it is important to note that the cellular response to glucocorticoids displays significant diversity in specificity and sensitivity, even within the same tissue depending on the targeted cell (Oakley et al., 2013; Zielińska et al., 2016).

In the airways, glucocorticoids inhibit the expression of chemoattractant and cell adhesion molecules as well as hinder the inflammatory cell survivability, leading to a decrease in the quantity of immune cells, such as eosinophils, T cells, mast cells and DCs at the site of inflammation. For instance, in response to glucocorticoid treatment, the mitigation of mucosal inflammation occurs promptly, leading to a notable decrease in immune cells, such as eosinophils within hours, which is accompanied by a reduction in airway hyperresponsiveness (Barnes, 2011; Oakley et al., 2013; Zielińska et al., 2016).

Furthermore, apart from the impacts of corticosteroids on IL-10 expression in laboratory settings, administering corticosteroids to individuals with asthma increases IL-10 production while simultaneously decreasing the levels of Th1 and Th2 effector cytokines (John et al., 1998; Richards et al., 2000). In addition, in T cells isolated from PBMCs, the use of inhaled corticosteroids enhances the expansion of Treg cells and increases the expression of the Foxp3 gene in CD4+ T cells (Karagiannidis et al., 2004). Moreover, the lack of an increase in IL-10 levels

following steroid exposure is associated with steroid-resistant illness in adults (Xystrakis et al., 2006). These findings may impact the poor response to corticosteroid treatment in bronchiolitis, especially in young atopic children with delayed Treg cell maturation (Tulic et al., 2012).

Currently, according to major guidelines, the administration of corticosteroids is not advised for the treatment of bronchiolitis despite their crucial role in recurrent wheezing and asthma exacerbations (Bakel et al., 2017; Friedman et al., 2014; O'Brien et al., 2019; Ralston et al., 2014; Ricci et al., 2015; Tapiainen et al., 2016). The efficacy of corticosteroid treatment for bronchiolitis has been previously studied extensively, and the results have been inconclusive (Fernandes et al., 2013; Kirolos et al., 2020). However, overall, the focus has been on comparing RSV-associated bronchiolitis to non-RSV-associated bronchiolitis, possibly contributing to the discouraging results. Nevertheless, recent studies have emphasised the differences in short- and long-term prognosis between RSV and RV bronchiolitis, and highlighted the need for more targeted management, as well as the potential for more personalised treatments (Makrinioti et al., 2022).

2.4.3 Other treatments

According to the majority of current guidelines, the treatment of bronchiolitis and the first wheezing illness consists mainly of supportive care, including the use of supplementary oxygen and the prevention of dehydration via nasogastric tube or parenteral fluid therapy. Additionally, the use of antibiotics, inhaled saline and bronchodilators such as adrenaline, as well as various inhaled and oral corticosteroids, has been studied without strong supporting evidence (Ralston et al., 2014; Tapiainen et al., 2016). However, as previously mentioned, these guidelines fail to consider the heterogeneity of bronchiolitis.

2.4.4 Vaccination

2.4.4.1 RV vaccination

Since RV exhibits limited cross-reactivity and a large antigenic variety across its strains, it has been difficult to create an effective vaccine against the large number of RV strains. However, anti-RV targeting, including strategies using extracellular trapping, capsid binders, binder blockers, receptor mimetics, endocytosis inhibitors, acidification inhibitors, and RNA replication inhibitors remain under active research (Real-Hohn et al., 2021).

2.4.4.2 RSV vaccination

Due to the significant impact on health and the economy, efforts have been ongoing for decades to develop an effective vaccine with long-lasting efficacy against RSV. Palivizumab, the first monoclonal antibody against RSV, was approved in 1998, and has been used to successfully decrease RSV infection severity (Romero, 2003). However, the high cost and limited availability have restricted its usage only to high-risk children.

A significant breakthrough occurred approximately 10 years ago when the structure of the virus was successfully stabilised for vaccine development, and the importance of the pre-fusion form of the virus's F protein (pre-F) as a key antigen was identified (McLellan et al., 2013). This opened a new era in the development of RSV prophylactics, and in the past year, vaccines as well as a long-acting monoclonal antibody for infants have entered the market.

In 2022, the European Medicines Agency (EMA) approved nirsevimab, a new generation antibody with an extended half-life and increased potency. It maintains sufficient serum concentration for up to 150 days, making it suitable for RSV prophylaxis in children younger than 12 months of age (Griffin et al., 2020; Hammitt et al., 2022; Rodriguez-Fernandez et al., 2021; Simões et al., 2023). Furthermore, the efficacy of nirsevimab was demonstrated in a large unblinded, European multicentre study, involving over 2000 children, in which hospitalisations associated with RSV infections were decreased by 77%, and the severe RSV infections were reduced by 85% (Simões et al., 2023).

Importantly, another strategy for protecting infants against RSV infection is based on maternal immunisation during pregnancy. By administering the vaccine to a pregnant individual, the foetus can be protected during the first few months of life through the transfer of maternal antibodies via the placenta (Saso et al., 2020). During autumn 2023, EMA approved the administration of a maternal RSV pre-F vaccine during pregnancy to protect the newborn during the first RSV epidemic. In the study supporting the approval, the maternal pre-F vaccine, administered between weeks 24-36 of gestation, demonstrated a protective efficacy of 57% against hospitalisations, and 70% against severe RSV infections up to the age of six months. However, the protective efficacy was greater during the first three months (Kampmann et al., 2023). This is unsurprising, since the maternal antibodies can decline in a time-dependent manner (Hoang et al., 2016; Munoz et al., 2014).

2.5 Immune responses and clinical outcomes of first wheezing episode

Previously, the classical Th1/Th2 balance model for the development of asthma has been extensively studied. According to this model asthma is a result of Th2-skewed

immune responses. However, the Th1/Th2 model has faced criticism for its oversimplified nature, as *in vivo*, immune responses exhibit a greater degree of heterogeneity and nuance, indicated by discoveries, including ILCs (Moro et al., 2010), macrophage polarisation (Gordon, 2003) and the recognition of additional CD4+ T cell subsets beyond Th1 and Th2 (Luo et al., 2022). Nevertheless, in early childhood, the Th1- and Th2-mediated immune responses and their differences, are suggested to influence the risk of subsequent asthma. In addition, data from infants suggest an age-related Th2-skewed immunity in all children during early life, and which is importantly emphasised by delayed Treg cell maturation in atopic children compared to non-atopic children (Tulic et al., 2012). Notably, this bias is evident in inclination toward producing Th2-associated cytokines when a naïve T cell from a newborn is stimulated *in vitro* (Roux et al., 2011). Furthermore, the occurrence of impaired Th1 responses (Tulic et al., 2012), predisposes already susceptible children to lower respiratory tract infections, including RV- and RSV-associated bronchiolitis, with subsequent type 2 immune responses (Jackson et al., 2008; Tregoning et al., 2008; Tulic et al., 2012; Wolters et al., 2024). The immune responses measured from nasal lavage fluids in children with bronchiolitis receiving outpatient care seem to be age-dependent (Cortegano et al., 2022). Furthermore, while the prior proinflammatory cytokine expression and subsequent expression of the nuclear factor-κB gene decrease during convalescence after bronchiolitis, earlier studies reported that a less significant reduction in the levels of leukotrienes is associated with recurrent wheezing in children post-bronchiolitis (Dalt et al., 2007; Sastre et al., 2020). This is interesting, since leukotrienes enhance capillary permeability, increase mucus secretion, induce bronchoconstriction and leukocyte attraction, and increase the responsiveness of the airways. Furthermore, leukotriene antagonists have been associated with attenuation of airway remodelling (Hur et al., 2018). Finally, the cell-mediated immune responses are thought to influence the outcomes depending on the viral infection. In RSV infections, a shift from a type 1 to a type 2 immune response is linked to greater disease severity, while in RV infections, the type 2 immune response contributes to the development of subsequent wheezing.

2.5.1 RV

Although the lungs may still be developing during the typical timeframe of a first severe wheezing episode caused by RV infection, they are generally more mature compared to their development during the time of an RSV infection.

In contrast to the common risk factors for RSV bronchiolitis (Murray et al., 2014), susceptibility to RV-associated illness appears to be influenced by predisposition. This is evidenced by the observation that among infants experiencing

recurrent moderate-to-severe respiratory illnesses, the prevalence of previous RV-associated bronchiolitis ranges from 50% to 80% within the first 12 months of life (Jartti et al., 2008b).

Biomarkers (cytokines, chemokines etc.)

RV infection is suggested to induce type 2 immunity-associated cytokines, including IL-4, IL-5, IL-13 and TLSP (Custovic et al., 2018; Rossi et al., 2015; Vandini et al., 2017). Moreover, a multicentre study investigating the interaction of singleton RV-associated bronchiolitis and type 2 associated cytokine responses found that elevated levels of IL-4 IL-5, IL-13 and TLSP during the acute phase were associated with asthma at 4 years, while in the RV-RSV coinfection group these were not, indicating an interplay between the RV and type 2 immune responses affecting asthma risk (Hasegawa et al., 2019b).

Polymorphisms in genes associated with IL-33 and its receptor (IL-1 receptor-like 1) are associated with increased risk of wheezing and asthma (Savenije et al., 2014). Interestingly, in a murine model, exposure to an aeroallergen during a concomitant pneumovirus-induced asthmatic state leads to the expression of IL-33 which consequently impairs the production of type I IFNs resulting in higher viral genome loads, airway smooth muscle growth and type 2 inflammation (Lynch et al., 2016).

IFNs play a pivotal role in the antiviral activity, especially in the acute phase of an initial infection to a specific pathogen, and they are divided into three main types, type I, II, and III, distinguished by their primary molecular compositions and the receptors they bind to (Hile et al., 2020). Type II IFN, also referred to as IFN-γ, induces the activation of transcription factor T-bet in type 1 immunity-associated cells, and thereby linked to the activity of Th1 cells, and subsequently suppresses the activity of transcription factor GATA3, inhibiting the activity of Th2 cells. Conversely, IL-4 exhibits the opposite influence (Chopp et al., 2023; Zissler et al., 2016). In addition, type I IFNs can also impede the differentiation of naïve CD4+ T cells into Th2 cells and destabilise the secretion of cytokines from Th2 cells by inhibiting GATA3 (Huber et al., 2010). Furthermore, whereas ILC2s participate in type 2 immune responses (Bartemes et al., 2014), type I IFNs have been reported to inhibit proliferation and induce apoptosis in ILC2s in the airways and alleviate airway hyperreactivity (Maazi et al., 2018).

Higher nasal IL-8, a potent chemoattractant for neutrophils, is associated with the severity of illness in the RV bronchiolitis (Turner et al., 1998). Moreover, both IL-8 polymorphism and cytokine expression are linked to an increased risk of subsequent atopic asthma (Charrad et al., 2017).

Innate immune responses from PBMCs towards RV infection differ between asthmatic and healthy controls (Hosseini et al., 2021). Crucially, in atopic patients, epithelial cells within distal airways exhibit a predisposition towards type 2 immune responses indicated by the release of epithelial alarmins such as TSLP, IL-31, Eotaxin-3 (CCL26), IL-25, and IL-33, triggering a localised type 2 immunity-associated response characterised by cytokines such as IL-4, IL-5, IL-9, IL-13, IL-25, IL-33, along with an elevation in the number of eosinophils upon activation (Frey et al., 2020; Hosseini et al., 2021). Furthermore, a recent meta-analysis has shown that IL-25 and IL-33 contribute to the enhancement of type 2 immunity and the expression of type I, and III IFNs were significantly impaired in individuals with asthma following RV infection (Hosseini et al., 2021; Liew et al., 2022). Moreover, when compared with RSV, RV infection has the potential to modify the microRNA profile by boosting the NFκB-signalling pathway, leading to increased expression of IL-10 and IL-13 (Hasegawa et al., 2018b). Interestingly, while IL-10 levels increase during RV-induced asthma exacerbations and decrease during convalescence, the role of IL-10 as a biomarker in early RV-induced wheezing has not been fully explored (Busse et al., 2005).

Although RV RNA is commonly detected in both symptomatic and asymptomatic children, only symptomatic RV infection triggers a strong and consistent host response, demonstrated by the elevated expression of transcription profiles linked to innate immunity and reduced expression of those associated with adaptive immunity (Heinonen et al., 2016).

Interestingly, following allergen exposure, fractalkine expression is increased in the lungs of patients with allergic asthma, potentially aiding in the recruitment of circulating Th cells (Rimaniol et al., 2003). Furthermore, RV infections have been shown to amplify house dust mite-induced fractalkine expression, which may play a role in the synergistic interaction between viral infection and allergen exposure, contributing to asthma exacerbations (Loxham et al., 2018).

Elevated levels of thymus- and activation-regulated chemokine (TARC) have been observed in NPAs of asthmatic patients infected with RV compared to non-asthmatic controls (Hansel et al., 2017). Furthermore, RV infections have been shown to promote increased expression of macrophage-derived chemokine (MDC) in NPA samples, as well as an enhanced response *in vitro* in epithelial cells from asthmatic donors and *in vivo* mouse models Interestingly, although no difference in MDC levels was observed between asthmatic and healthy controls, RV infections have been associated with a significant increase in MDC levels specifically in asthmatic individuals (Nikonova et al., 2020; Williams et al., 2021).

RV infection has been linked to increased expression of vascular endothelial growth factor (VEGF) in both *in vivo* and *in vitro* studies (Kuo et al., 2011; Leigh et al., 2008). Additionally, asthmatic children exhibit higher sputum VEGF levels

compared to healthy controls, with VEGF expression rising significantly during acute asthma exacerbations. Remarkably, in asthmatic children, VEGF levels seem to remain elevated even during periods of complete symptom remission (Hossny et al., 2009). The levels of regulated upon activation, normal T cell expressed and secreted (RANTES) have been noted to be more pronounced in RV infections compared to other viral agents, including RSV infections (Chun et al., 2013). Furthermore, heightened levels of RANTES are more commonly detected in respiratory secretions of asthmatic patients compared to non-asthmatic controls (Conti et al., 2001).

The levels of tumour necrosis factor (TNF) α increase during RV infections and are particularly elevated in wheezing infants (Balfour-Lynn et al., 1994; Gern et al., 1996). Moreover, TNF-α and RV infection synergistically enhance chemokine responses in epithelial cells, such as IL-8 and epithelial-derived neutrophil-activating peptide 78 (ENA-78), at least *in vitro* (Newcomb et al., 2007). Additionally, RV-infected asthmatics show higher levels of ENA-78 in nasal secretions compared to healthy controls (Donninger et al., 2003). Furthermore, in a murine model, inhibition of ENA-78 in RV-induced asthma exacerbation has been linked to reduced hyperactivity of airways, mucus secretion and collagen disposition (Sokulsky et al., 2020).

In children with acute respiratory symptoms, monocyte chemoattractant protein (MCP) 3 is elevated in NPAs relative to asymptomatic peers (Santiago et al., 2008). Additionally, macrophage inflammatory protein (MIP) 1β, which is linked to the recruitment of eosinophils in the respiratory tract, shows a positive correlation with symptoms of lower respiratory tract infections (Kobayashi et al., 2019; Lewis et al., 2012). Moreover, children with recurrent wheezing have been found to exhibit higher levels of both MIP-1α and MIP-1β compared to those without recurrences (Sugai et al., 2016).

Genetics

CDHR3 is responsible for encoding a transmembrane protein belonging to the cadherin family, which is expressed in epithelial cells within the airways, and crucially, the entry of RV C into the epithelial cell is facilitated by CDHR3 (Bochkov et al., 2015). Importantly, in RV infections, variations in the CDHR3 gene are associated with childhood asthma (Bønnelykke et al., 2014). Moreover, variations in the 17q21 locus, particularly in RV infections, are associated with the severity of the wheezing illness and an increased risk of asthma (Çalışkan et al., 2013; Jartti et al., 2019). Significantly, the association between asthma and these genes is predominantly observed in children with a history of RV-induced wheezing illness, further highlighting a distinct subgroup of bronchiolitis.

2.5.2 RSV

During the typical time frame of RSV infection, the respiratory tract is still under development (DenDekker et al., 2018; Hislop et al., 1986). Hence, the respiratory tract of an infant may be susceptible to long-term pathological changes, as suggested by a study observing a murine model, in which alveolar and bronchial development was impaired as a result of viral infection, leading to impaired pulmonary function (Castleman et al., 1988). Furthermore, a prospective study on children with RSV bronchiolitis, showed an impairment in pulmonary function at 6 years of age (Zomer-Kooijker et al., 2014).

Although risk of hospitalisation is elevated in recognised high-risk groups for RSV bronchiolitis, in contrast to RV bronchiolitis, the majority of infants (up to 85%) hospitalised with RSV bronchiolitis are not born prematurely and lack known predisposing risk factors for severe RSV infection (Hall et al., 2009; Murray et al., 2014). However, the reduced rates of hospitalisation due to bronchiolitis among certain high-risk groups might be attributed to the utilisation of monoclonal RSV antibodies, such as palivizumab (Blanken et al., 2013; Simoes et al., 2007; Yoshihara et al., 2013). A recent study indicated that the incidence of hospital admissions and the number of hospitalisation days among children with RV bronchiolitis are approximately 68% and 51%, respectively, compared to those with RSV bronchiolitis, and unlike the seasonal burden of RSV infection, the impact of RV infection is distributed throughout the year (Horvat et al., 2024).

Crucially, not every child infected with RSV develops bronchiolitis, and similarly, not all children with RSV bronchiolitis develop subsequent recurrent wheezing or asthma. These findings suggest that other factors, such as genetic and environmental factors, contribute to the pathophysiology. In support of this, intriguingly, Treg cells are able to display phenotypic plasticity, potentially losing their suppressive capabilities in inflammatory settings by adopting alternative phenotypes such as resembling a type 1 immunity. Notably, this alteration can be reversed by using blocking antibodies against the inflammatory cytokines (Dominguez-Villar et al., 2011). These findings are supported by a prior study performed on a murine model showing that repeated early RSV infections in ovalbumin-tolerised mice resulted in GATA3 expression and type 2 immunity-associated cytokine secretion in FOXP3 Treg cells, compromising the suppressive activity of pulmonary Treg cells and skewing towards type 2 associated immunity (Krishnamoorthy et al., 2012).

Biomarkers (cytokine, chemokines etc.)

The association between RSV bronchiolitis and poorer long-term prognosis has been observed to depend on the severity of the acute illness, with a stronger link to

subsequent asthma being evident in cases of more severe disease (Carroll et al., 2009). Moreover, the likelihood of experiencing more severe RSV infections has been associated with the skewed balance between type 1 and type 2 immune responses. Infants with more severe RSV infection have shown a reduced IFN-γ response from PBMCs (Aberle et al., 1999). In addition, reduced IFN-γ responses at the time of acute bronchiolitis have been shown to be associated with subsequent impairment of pulmonary function (Renzi et al., 1999). Furthermore, elevated IL-4 levels and reduced IFN-γ levels (IL-4/IFN-γ ratio) have been shown to exacerbate the severity of RSV bronchiolitis in infants, suggesting that in infants with more severe disease, the immune response is more skewed towards type 2 immunity (Caballero et al., 2015).

A study comparing children with RSV bronchiolitis, and age-matched controls found that in the NPAs, increased levels of RANTES were associated with RSV infection compared to controls. Furthermore, recurrent wheezing was linked to higher RANTES expression among RSV bronchiolitis patients, but notably, the RANTES levels played no part in the severity of illness (Chung et al., 2002). Moreover, another study comparing children with RSV bronchiolitis and age-matched controls observed increased levels of IL-3, IL-4, IL-10, and IL-13, but also elevated levels of IL-1β, IL-6, TNF-β, MCP-1, MIP-1α and IL-8, in bronchoalveolar lavage fluid (BALF) compared to asymptomatic controls. Furthermore, along RSV infected children, elevated expression of IL-3 and IL-12p40 in BALF were associated with recurrent wheezing. Additionally, elevated gene expression of IL-33 was observed in infants with family history of atopy, suggesting a predisposition to atopy (Bertrand et al., 2015). Interestingly, in children affected by RSV, higher viral genome loads, increased expression of genes linked with IFN and plasma cells, along with reduced expression of genes linked with inflammation and neutrophils, have been linked to less severe illness (Heinonen et al., 2020).

RSV infection has previously been shown to associate with an increase of expression of multiple chemokines such as of I-309 and TARC, RANTES, MCP-1, MDC, and MIP-1α and MIP-1β from basal epithelial cells (Zhang et al., 2001). Moreover, upregulation of I-309 has also detected in BALF from asthmatics compared to non-asthmatic patients (Mutalithas et al., 2010). Additionally, RSV infection has been linked to both acute and sustained long-term increases in VEGF expression, as observed *in vivo* and *in vitro* studies (Moreno-Solís et al., 2015; Oldford et al., 2018; Pino et al., 2009).

Furthermore, the increased severity of RSV bronchiolitis has been associated with increased viral load (Luchsinger et al., 2014; Uusitupa et al., 2020), and variations in levels of IL-33 (NPA) (Saravia et al., 2015), IL-8 (Plasma, NPA) (Brand et al., 2013; Brown et al., 2015; Choi et al., 2010; Díaz et al., 2015; Tabarani et al., 2013), TSLP (NPA) (García-García et al., 2017), periostin (NPA) (García-García et

al., 2017), IL-6 (blood, plasma, serum, NPA) (Brown et al., 2015; Díaz et al., 2015; McNamara et al., 2004; Tabarani et al., 2013), and IFN-α (blood) (Do et al., 2017; Tabarani et al., 2013; Zhang et al., 2016).

Genetics

Several studies have been concentrating on the identification of RSV bronchiolitis-associated gene variations that influence susceptibility to both the acute disease and subsequent recurrent wheezing and asthma. Interestingly, pre-term infants may be genetically predisposed to RSV infection, as demonstrated by the higher risk for RSV bronchiolitis in premature infants with the ADAM33 polymorphism. Moreover, in pre-term infants affected by RSV, single nucleotide polymorphisms (SNP) in genes associated with IL-10, nitric oxide synthase 2A (NOS2A), surfactant protein C (SP-C), matrix metalloproteinase 16 (MMP16), and vitamin D receptor (VDR) genes have been linked to heightened chronic respiratory morbidity. Furthermore, SNPs in genes associated with MMP16, NOS2A, SP-C, and VDR have been linked to reduced pulmonary function at 12 months of age (Drysdale et al., 2014). In addition, polymorphisms of IL-10, IL-13, TLR4, VDR, CCR5, and ADAM33 genes have been associated with RSV bronchiolitis and increased risk for development of asthma (Larkin et al., 2015).

One study demonstrated significant associations with SNPs in IL19, IL20, MUC5AC, TNFRSF1B, C3, CTLA4, CXCL9, IL4R and IL7 genes, and wheezing at 15 months after RSV-induced lower respiratory tract infection (Ermers et al., 2011), of which genes C3, CTLA4 and IL4R overlap with variants identified with asthma (Inoue et al., 2008). Additionally, in another study, after 6 years of follow-up, a functional SNP in IL13 gene was identified to be associated with wheezing after RSV-infection (Ermers et al., 2007).

An increase in cilia-related gene expression has been linked to a prolonged duration of hospitalisation in children with severe RSV bronchiolitis requiring intensive care treatment (Koch et al., 2022). This finding is not unexpected, considering that RSV predominantly infects and replicates within ciliated epithelial cells (Zhang et al., 2002). Following RSV bronchiolitis, both polymorphism and excessive expression of RANTES have been associated with recurrent wheezing (Tian et al., 2009).

2.5.3 HBoV1

Though HBoV1 is commonly detected in coinfections, and the exact relationship between the acute illness and the identification of coinfecting HBoV1 using conventional PCR is uncertain, the signs and symptoms of sole HBoV1 infection

vary significantly (Allander et al., 2007). Importantly, HBoV1 infection can lead to severe illness (Liao et al., 2022; Moesker et al., 2015). Moreover, a high genome load of HBoV1 is associated with more severe illness characterised by a longer duration of respiratory symptoms and hospitalisation (Deng et al., 2012). A study comparing the incidence of recurrent wheezing in hospitalised children showed marked, but not significant, differences between RV and HBoV1 aetiologies (40% vs 60%, respectively) (Lukkarinen et al., 2014). While in chronic tonsillar disease, HBoV1 is commonly identified, HBoV1 infection has been associated with inhibition of transcription factors crucial for T cell differentiation, RORγt (type 1 immunity) and FOXP3 (Treg-associated immunity), and an increased genome load leading to poorer expression of type 3 IFN (IL-28 and IL-29) and IL-13 (Ivaska et al., 2021).

3 Aims

The primary aims of this study were:

1. To assess the short- and long-term efficacy of inhaled β2-agonist with and without the use of systemic corticosteroid treatment on severe first-time wheezing in children with RV (Study I)

2. To study the differences in cytokine responses and their respective effects on short- and long-term prognosis between RV and RSV infected children suffering from severe first-time wheezing (Study II).

3. To examine the effects of HBoV1 coinfection on cytokine responses in first-time wheezing in children with RV (Study III)

4. To determine whether cytokine responses are associated with the need for hospital admission for first-time wheezing children infected with RV (Study IV).

4 Materials and Methods

The details of materials and methods are presented in the original publications.

4.1 Patients and study populations

The study population for Study I was derived from two randomised controlled trials: Vinku (n=293, NCT00494624) and Vinku2 (n=125, NCT00731575, EudraCT 2006-007100-42). In contrast, Studies II, III, and IV used participants from Vinku2 only. Both Vinku and Vinku2 were double-blinded, placebo-controlled, randomised controlled trials that compared the efficacy of oral prednisolone (2 mg/kg/d for three days) to a placebo in the first wheezing episode. In the Vinku study, the participants were randomised regardless of the viral agent, resulting in a post hoc design, while in the Vinku2 study, the participants were randomised only after a positive RV PCR test, making it prospective in design. Both studies used a uniform weight-based dosing for salbutamol and study drugs, and identical sampling and follow-up procedures, including daily symptom diaries for 2 months and follow-up visits (defined below). Guardians were also instructed to bring their child to the physician if breathing difficulties occurred.

For the Vinku study, patients were recruited between September 2000 and May 2002, and for the Vinku2 study, from June 2007 to March 2010. In Studies II-IV (Vinku2), 20% of participants were recruited from an outpatient clinic and 80% from the paediatric infectious ward at Turku University Hospital, while Study I included inpatients only.

In all studies, the identical inclusion criteria were patient aged 3-23 months, born after 36 weeks of gestation, experiencing first wheezing episode (validated through parental report and confirmed by medical records), and the detection of RV in an NPA sample by PCR. Further, identical exclusion criteria for all studies comprised the use of systemic or inhaled corticosteroids prior to study entry, the presence of chronic non-atopic diseases, and a requirement for intensive care.

In addition, in Study I, the inclusion criteria included hospitalisation and RV detected in NPA by PCR. In Study II, the inclusion criteria also required having a sole, steroid-naïve RSV or RV infection detected in an NPA sample via PCR, with exclusion criteria including the detection of a non-RSV or non-RV viral agent in the

NPA sample. In Study III, the inclusion criteria included having either a sole RV infection or a coinfection with RV and HBoV1, as indicated by the detection of viral RNA/DNA in an NPA sample via PCR and confirmed by serology for HBoV1, with exclusion criteria including the detection of a non-RV or non-HBoV1 viral agent in the NPA or serology sample. In Study IV, the inclusion criteria also encompassed having a sole RV infection detected by PCR, with exclusion criteria including the detection of a non-RV viral agent in the NPA sample.

In all studies, written informed consent by a parent or guardian was provided beforehand.

4.2 Protocols

In both original studies, at study entry, after the clinical evaluation and interviewing of the parents or guardians according to the standardised surveys on host and environmental risk factors of recurrent wheezing and asthma, peripheral blood and NPA samples were obtained. Next, the participants were randomised to prednisolone and placebo groups (in Vinku at entry, and in Vinku2 after a positive RV PCR test). Both the recruitment to the studies, and the subsequent follow-up visits at 2 weeks, 2 months, 12 months, 4 years in Vinku2 only, and 7 years were conducted by study physicians. The patients were prospectively followed up to 7 years.

4.3 Definitions

Atopy (i.e. sensitisation) was referred to as positive IgE levels (exceeding 0.35 kU/L) against any of the measured common allergens (listed below). Aeroallergen sensitisation was referred to as positive allergen-specific IgE levels against cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum*, and *Dermatophagoides pteronyssinus*. Furthermore, the presence of positive IgE antibodies to dog, cat, or *Dermatophagoides pteronyssinus* was considered to as perennial aeroallergen sensitisation. Food allergy was referred to as positive allergen-specific IgE levels against cow's milk, egg, peanut, soybean, wheat, and codfish (Jartti et al., 2015). Blood eosinophilia (i.e. elevated levels of peripheral blood eosinophils, B-Eos) was defined as eosinophil count exceeding 0.4×10^9 cells/L. Atopic eczema was defined by typical signs and symptoms such as pruritus, chronicity of illness, and typical morphological findings as well as evidence of sensitisation (Jartti et al., 2010).

4.4 Sample processing and analyses

4.4.1 Viral diagnostics

In Studies I-IV, NPA samples were collected using a standardised procedure (Allander et al., 2007; Jartti et al., 2004), stored at +4°C and analysed within 3 days of collection. Nucleic acids were extracted using either the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) or the NucliSens EasyMag automated extractor (bioMérieux, Boxtel, The Netherlands). If not analysed promptly, nucleic acids were preserved at -70°C.

The detection of RV was conducted via in-house PCR assay. The primers were developed from the highly conserved 5' noncoding region of the picornavirus genome, ensuring detection capability across nearly all RV and enterovirus (EV) genotypes (Halonen et al., 1995; Lönnrot et al., 1999). The forward primers (positive strand) used were 5' –CGGCCCCTGAATGCGGCTAA-3', and the reverse primers (negative strand) were 5'-CGCCCCCTGAATGCGGCTAA-3'.

The Vinku study used an RT-PCR hybridisation method (Lönnrot et al., 1999). For PCR cycling, a DNA Thermal Cycler (Perkin-Elmer, Cetus Corp., Norwalk, CT) was used. The amplification process involved an initial incubation at +94°C for 3 minutes, followed by 40 cycles of denaturation +94°C for 30 seconds, annealing +53°C for 45 seconds, and extensions +72°C for 60 seconds. Discrimination between RV and EV amplicons was achieved through liquid-phase hybridisation using specific probes marked with lanthanide chelates. These probes were designed with maximum homology within EVs- and RVs, as well as intra-genus homology, with maximum differentiation between EVs and RVs. The specific probes used were RV 5'-TAGTTGGTCCCITCCCG-3', EV 5'-TAITCGGTTCCGCTGC-3', and EV-RV 5'- AAAGTAGTIGGTICC-3' (Lehtinen et al., 2007).

The PCR test employed in the Vinku2 study underwent an upgrade to a real-time format, utilising SYBR Green as a double-strand DNA dye. The PCR cycling was facilitated by using a Rotogene 3000 instrument (Corbett Research, Qiagen). The amplification process involved an initial incubation at 95°C for 15 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 65-55°C for 30 seconds, and extension at 72°C for 40 seconds. A melting curve analysis was used to discriminate between EVs and RVs (Jartti et al., 2015; Österback et al., 2013).

In Vinku study, for the detection of presence of adenovirus, hMPV, influenza viruses (A and B), parainfluenza virus (types 1–4), polyomaviruses (WU and KI), RSV, and RV, PCR, time-resolved fluoroimmunoassay antigen detection methods, and virus culture were used (Jartti et al., 2004, 2015; Lehtinen et al., 2007; Turunen et al., 2014). Furthermore, in the Vinku2 study, the analysis of coronaviruses, including 229E, NL63, OC43, and HKU1, by PCR was added (Jartti et al., 2015;

Turunen et al., 2014). Acute HBoV1 infections were assessed using PCR and serology (IgM and IgG in paired sera (Kantola et al., 2011; Söderlund-Venermo et al., 2009). To ascertain the genotype specificity of HBoV1 IgG, serum samples were treated with HBoV2 and HBoV3 antigens (Kantola et al., 2011).

B-Eos and serum levels of allergen-specific IgE were analysed using routine diagnostics at the Central Laboratory of Turku University Hospital. Serum 25-hydroxyvitamin D measurements were performed by liquid chromatography-tandem mass spectrometry at Massachusetts General Hospital (Boston, MA, USA).

4.4.2 PBMC isolation, processing and analysis

In Studies II-IV, samples of peripheral whole blood for PBMCs were collected during the acute illness and convalescence (after 2 weeks). During both time points, the blood sample was stored on a rocking shaker at room temperature, and PBMC isolation from the samples were performed on the same day using density gradient centrifugation (Ficoll-Paque™ PLUS, GE Healthcare, Amersham, UK) following the manufacturer's instructions. Subsequently, PBMCs (with over 95% viability) were stimulated with anti-CD3/anti-CD28 for 24 hours. Following stimulation, the supernatants were gathered, centrifuged, and stored in a -80°C freezer until analysis. Afterwards, the supernatants were transported in dry ice containers to the Swiss Institute of Allergy and Asthma Research (SIAF) in Davos, Switzerland. Upon arrival, the samples remained frozen and were stored at -80°C until analysis.

Immediately prior to analysis, the samples were thawed and analysed using HCYTOMAG-60K-36 and HCYP2MAG-62K-20 assays (Merck KGaA, Darmstadt, Germany) on the Bio-Plex 200 System, operated with Bio-Plex Manager 6.0 Software (Bio-Rad, Cressier, Switzerland) for quantitatively assess 56 different cytokines. The internal quality controls indicated satisfactory performance across all analytes.

However, due to the limitations of quantitative multiplex ELISA profiling, in a minor subset of samples, the fluorescence was not observed within the quantitative limit of detection. Consequently, cytokines detected within the limit of quantification in over half of the samples (29 out of 56, 52%) were included in subsequent analyses. For those cytokines, samples below the detection limit were assigned a value equivalent to half of the assay's lower threshold, and samples that exceeded the limit were assigned the assay's upper threshold.

4.5 Outcomes

4.5.1 Efficacy of inhaled salbutamol with and without oral prednisolone (I)

The primary outcomes of Study I were to compare:

- The duration of time necessitating hospitalisation (i.e. time until deemed ready for discharge based on clinical scoring).
- Occurrence of a new physician-confirmed wheezing episode within 2 months post-discharge.
- Time to a new physician-confirmed wheezing episode within 2 months post-discharge.

Secondary outcomes of Study I were to compare:

- Duration of wheezing and coughing post-discharge.
- Incidence of and time to a new physician-confirmed wheezing requiring hospitalisation within a 2-month follow-up period.
- The number of bronchodilator doses within the 2 weeks post-discharge.

4.5.2 Association of cytokine responses and the short- and long-term prognoses between RV- and RSV-induced wheezing (II)

The primary outcomes of Study II were to compare:

- The cytokine responses from PBMCs stimulated with anti-CD3/anti-CD28 between RV- and RSV-associated first wheezing episodes in children during both the acute phase and the convalescent phase (after 2 weeks).
- Cytokine responses related to different RV species (A, B and C).
- Whether cytokine responses are associated with the RV genome load.
- Whether cytokine responses are associated with new physician-confirmed wheezing episodes (at 2- and 12-month follow-up) and the development of asthma by 4 years of age.

4.5.3 Immune suppression by HBoV1 on RV-induced first wheeze in young children (III)

The primary outcomes of Study III were to compare:

- The overall cytokine responses of anti-CD3/anti-CD28-stimulated PBMCs during severe first wheezing in children, specifically comparing those infected with both RV and HBoV1 to those with RV alone.
- Whether cytokine responses are associated with the disease severity (i.e., duration of hospitalisation).
- Whether cytokine responses are associated with the occurrence of recurrences (within the subsequent 2-months follow-up) and the development of asthma by 4 years.

4.5.4 Association between cytokine responses and the initial disease severity in the RV-associated wheezing (IV)

The primary outcomes of Study IV were to compare:

- Overall cytokine responses from anti-CD3/anti-CD28-stimulated PBMC of children affected by severe first-time wheezing and treated as outpatients with those treated as inpatients.
- Whether cytokine responses are linked to the occurrence of recurrences (at 2- and 12-month follow-up) and asthma by 4 years.

4.6 Statistical methods

In Studies I, II, III, and IV the differences in baseline characteristics between the study groups were analysed by using the two-sample t-test for normally distributed and the Mann-Whitney U-test for non-normally distributed data. When appropriate, the normality of distribution was assessed by the Kolmogorov-Smirnov or Shapiro–Wilk test. Due to the skewness of the data, cytokine levels were log10 or x^2 transformed when appropriate. Categorical variables were analysed using the $\chi 2$ test or Fisher's exact test.

Additionally, in Study I, logistic regression, negative binomial regression, and Cox regression were used when appropriate. The difference in duration of hospitalisation was analysed in two phases with negative binomial regression. Since in the Vinku2 study the original study drug (oral prednisolone or placebo) was initiated only after a positive RV PCR test and in the Vinku study at entry, there was a time delay in the administration of the study drug between the on-

demand/prednisolone and high-dose/prednisolone groups. Hence, the analyses were conducted in two phases. First, in the stringent analysis, between placebo study groups only (high-dose/placebo and on-demand/placebo). Second, in the loose analysis, between high-dose/prednisolone and placebo groups while excluding the on-demand/prednisolone group. The group×treatment interaction effect was included in models and if a statistically significant interaction was found, group effect (salbutamol high-dose vs on-demand) was estimated separately in the prednisolone and placebo groups. If the interaction was not statistically significant, the effects on the group and treatment were estimated from the main effects model.

In Study II, III, and IV. For other statistics, when appropriate, the two-sample t-test, Mann-Whitney U test, $\chi 2$ test, Fisher's exact test (when cell counts < 5), and multivariable linear model analysis (using the backward stepwise method to adjust for the baseline differences, only statistically significant variables ($P < .05$) were included in the final model), Kruskal–Wallis H test, and negative binomial regression were used.

A two-sided P value < .05 was considered statistically significant. Data analyses were performed using JMP software (version 13.1.0, SAS Institute, Cary, NC, USA) and SAS System for Windows (version 9.4, SAS Institute, Cary, NC, USA).

4.7 Ethics

All studies followed the ethical guidelines outlined in the Declaration of Helsinki and Good Clinical Practice. Approval for the studies was granted by the Ethics Committee of Turku University Hospital, and initiation occurred solely after securing informed written consent from the parents or the guardians.

5 Results

5.1 Study population and patient characteristics

5.1.1 Study I

The study population of Study I was derived from the Vinku and Vinku2 studies, initially enrolling 293 and 125 children, respectively. Of these, 323 children did not meet inclusion criteria for analysis, primarily due to age (>24 months, n = 118), non-RV-associated illness (n = 108), or prior history of wheezing (n = 63). Thus, 95 children were eligible for further analysis: 35 (37%) children from Vinku receiving high-dose salbutamol regularly, and 60 (63%) children from Vinku2 using salbutamol on-demand. A total of 88 (93%) children completed the 2-month follow-up (Figure 4).

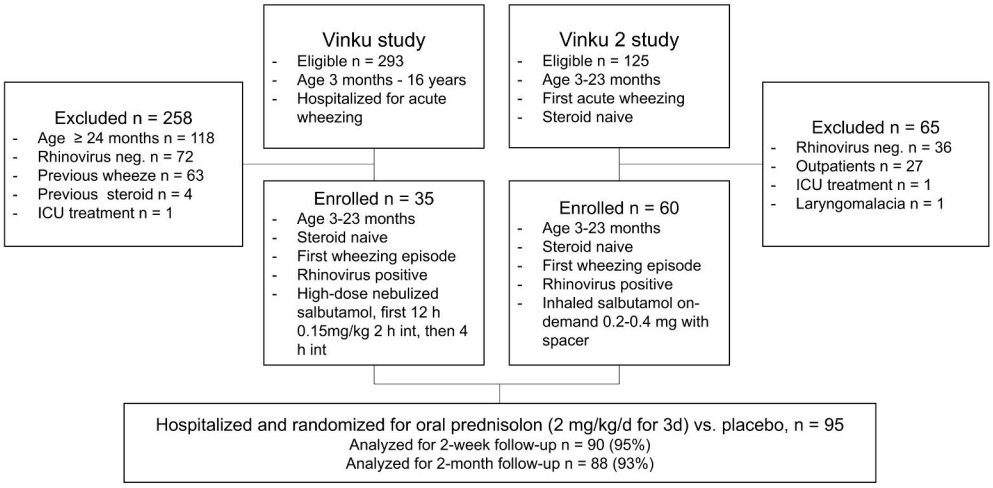

Figure 4. Study flow chart of Study I. ICU, intensive care unit; int, interval; d, day. Modified from Study I.

The study subjects, with median age of 13 months (interquartile range (IQR) 8-17 months), included 73% males, with 32% showing sensitisation and 43% having

atopic eczema. Viral coinfection was present in 43% of children overall. Treatment groups differed in sex, prior antibiotic use, and both non-RV-viral and bocavirus coinfection, aeroallergen and perennial sensitisation, and serum levels of 25-hydroxyvitamin D and D3. Subsequent analyses revealed that only viral coinfection was associated with predefined outcomes. Notably, upon analysing the prednisolone and placebo groups, all patient characteristics were evenly distributed.

5.1.2 Study II, III, and IV

Studies II-IV utilised data exclusively from the Vinku2 study, which initially enrolled 125 eligible children. After 12 children declined to continue, a total of 113 children participated in the clinical follow-up.

In Study II, of the originally enrolled 113 children, 50 were excluded due to sole non-RV or sole non-RSV aetiology along with those with viral coinfections. Additionally, PBMC samples were absent for 7 children during the acute phase, resulting in available cytokine data for 56 children (RV n = 47, RSV n = 9). In the convalescent phase, 26 children were excluded (22 due to prednisolone treatment and 4 due to sample absence), leaving 30 children for cytokine analyses. Clinical data were available for 30 children at subsequent follow-up points (2 and 12 months later) (Figure 5).

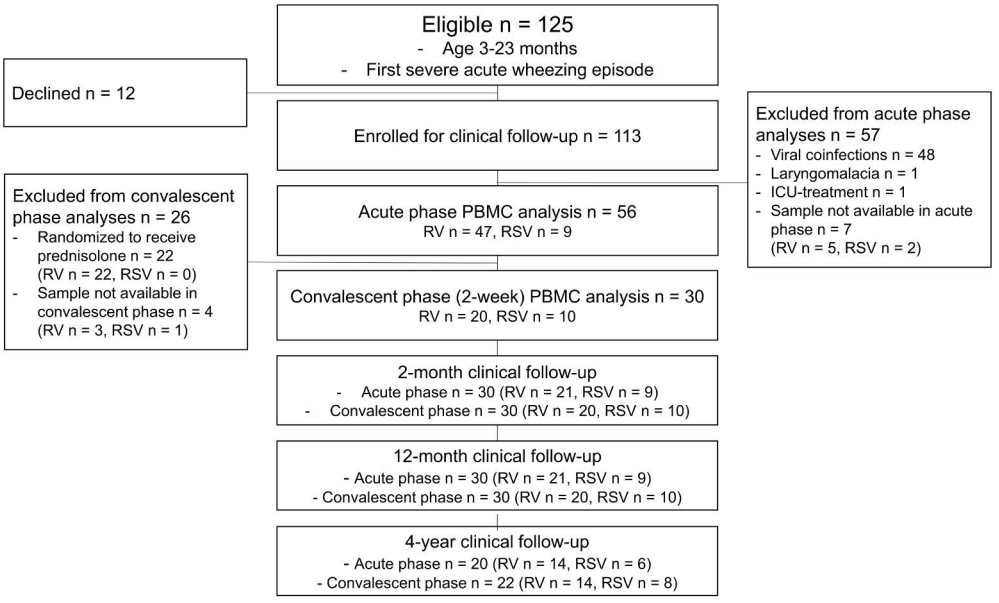

Figure 5. Study flow chart of Study II. ICU, intensive care unit; PBMC, peripheral blood mononuclear cell; RSV, respiratory syncytial virus; RV, rhinovirus. From Study II.

The children had a median age of 12.5 months (IQR 7.4-15.9). Among them, 69% were boys, 80% required hospitalisation, 29% were sensitised, and 20% had atopic eczema. Upon entry into the study, compared to those in the RSV group, children in the RV group were older, had higher body weight and B-Eos, and showed a reduced occurrence and duration of preceding symptoms like wheezing, cough, rhinitis, and fever.

In Study III, out of the originally enrolled 113 children, 52 were excluded due to non-RV and non-RV-HBoV1 aetiology, as well as those with viral coinfections. Additionally, 5 cytokine samples from the RV group were absent, leaving data from 56 children (RV n = 47, RV-HBoV1 n = 9) for cytokine analysis during the acute phase. During randomisation, 33 children received prednisolone and were excluded from the convalescent phase analyses. Ultimately, cytokine samples from 24 children were analysed during the convalescent phase. Clinical data were available for 25 children at the 2-month follow-up from the acute phase and 24 from the convalescent phase (Figure 6).

The patients had a mean age of 14.3 months (standard deviation (SD) 5.6), with 73% of the study subjects being males. Furthermore, 75% required hospitalisation, 30% were sensitised, and 24% had atopic eczema. Notably, children infected with RV were of younger age and presented with fewer preceding signs and symptoms, including wheezing, cough, and fever.

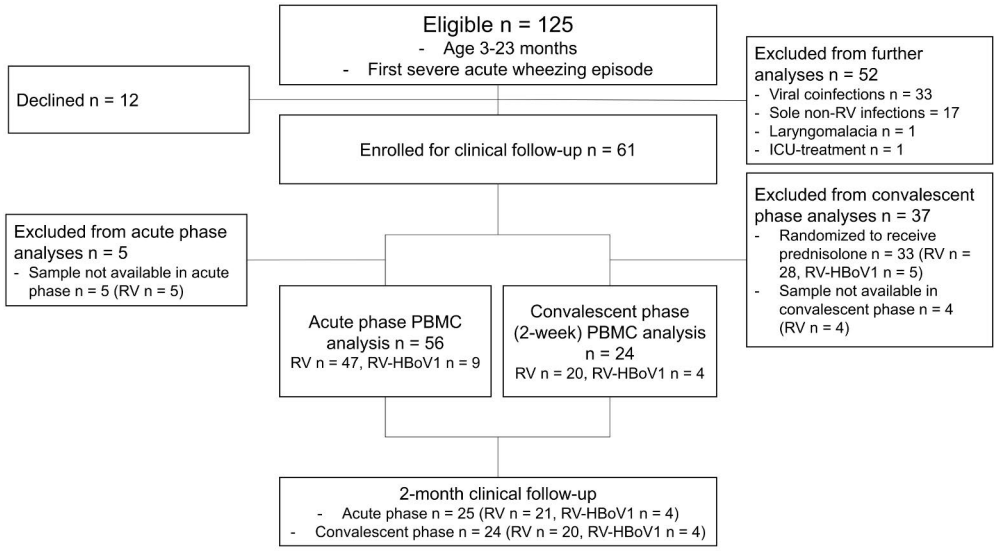

Figure 6. Study flow chart of Study III. HBoV1, human bocavirus-1; ICU, intensive care unit; PBMC, peripheral blood mononuclear cell; RV, rhinovirus. From Study III.

In Study IV, of the originally enrolled 113 children, children with viral coinfection (n = 42), and non-sole RV infected children (n = 17) were excluded, leading to 61 study subjects continuing. Furthermore, 5 children were excluded from the further analyses, due to the absence of cytokine samples, resulting in available cytokine data from 37 inpatients and 10 outpatients for further analyses (Figure 7).

The patients had a mean age of 17 months (SD 6), with 74% of the study subjects being male. Additionally, 79% required hospitalisation, 33% were sensitised, and 22% had atopic eczema. Upon study entry, the hospitalised children exhibited lower oxygen saturation and elevated C-reactive protein (CRP) levels. Moreover, in hospitalised children, the occurrence of atopy and allergic sensitisation to food allergens as well as the prevalence of parental allergies was significantly more common to non-hospitalised children (all $P < .05$).

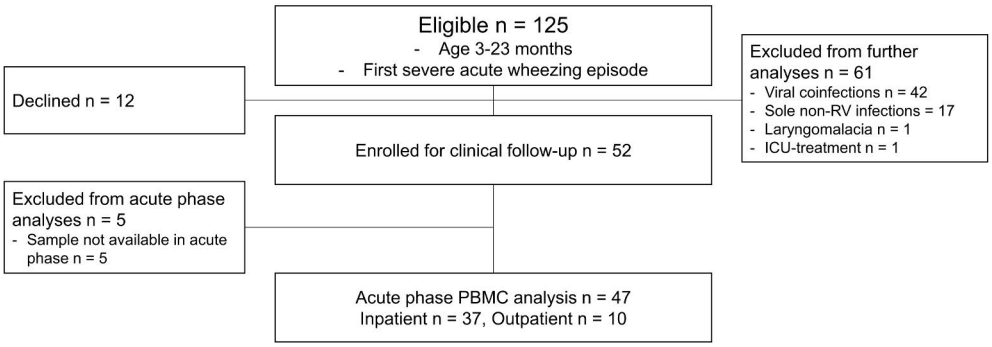

Figure 7. Study flow chart of Study IV. ICU, intensive care unit; PBMC, peripheral blood mononuclear cell; RV, rhinovirus. From Study IV.

5.2 Efficacy of inhaled salbutamol with and without oral prednisolone (I)

In Study I, the study population comprised data from Vinku and Vinku2 studies. In Vinku study, randomisation to prednisolone or placebo groups occurred at study entry, whereas in Vinku2 study, randomisation occurred after a positive RV test. This difference in study protocols led to approximately a 45-hour delay in administering the study drugs (prednisolone or placebo) in Vinku2. Consequently, the duration of hospitalisation was analysed in two phases: first, as a stringent, and second, as a loose analysis. In the stringent analysis, study groups with administration of prednisolone were excluded, while in the loose analysis, only the on-demand/prednisolone group was excluded. The study effect (group effect) statistically accounted for the delay in other outcomes. Hence, on other primary and secondary outcomes, all study groups were included.

5.2.1 Duration of hospitalisation

As stated before, the duration of hospitalisation was analysed in two phases. In the stringent analysis (prednisolone treatment arm excluded), there was no statistically significant difference in the duration of hospitalisation between the salbutamol high-dose/placebo and the salbutamol on-demand/placebo groups (risk ratio (RR) 0.71, 95% confidence interval (CI) 0.46-1.09, P =.12). However, in the loose analysis (salbutamol on-demand/prednisolone group excluded only), the salbutamol high-dose/ prednisolone group was showed a shorter hospitalisation time when compared to the salbutamol on-demand/placebo group (RR 0.58, 95% CI 0.38-0.87, P = .008). Unfortunately, due to the exclusion of study groups in both analysis methods, interaction between the treatment groups could not be estimated (Figure 8).

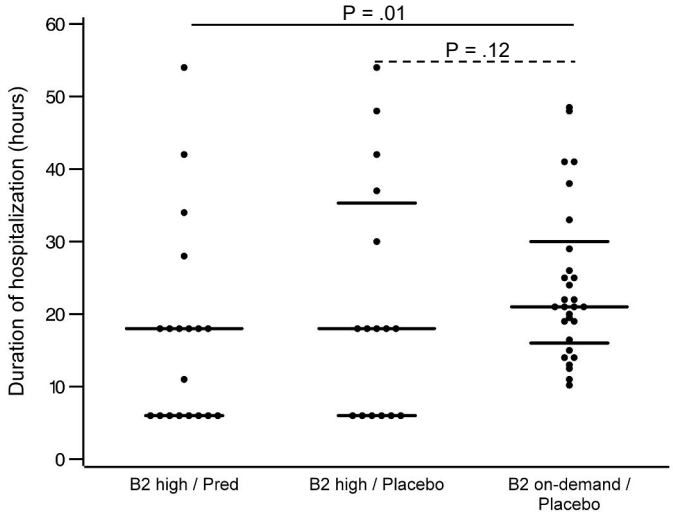

Figure 8. Duration of hospitalisation. In the loose analysis, while excluding salbutamol on-demand/prednisolone group excluded only, the salbutamol high-dose/prednisolone was characterised by a shorter hospitalisation time compared to the salbutamol on-demand/placebo group (B2 High / Pred vs. B2 on-demand / Placebo, P = .01). However, no statistically significant differences were found in the stringent analysis (B2 High / Placebo vs. B2 on-demand / Placebo P = .12). Pred, prednisolone; B2 high, high-dose salbutamol; On-demand, on-demand salbutamol. Modified from Study I.

5.2.2 The occurrence of and the appearance of to a new physician-confirmed wheezing episode

The different effect of salbutamol in prednisolone and placebo groups was indicated by significant interactions between the group effect (salbutamol high-dose vs on-demand) and the treatment effect (prednisolone vs placebo) that observed on both the

occurrence of and time to a new physician-confirmed wheezing episode (both group×treatment P = .02). The salbutamol high-dose group was characterised by fewer new wheezing episodes than the on-demand group in the prednisolone arm (OR 0.15, 95% CI 0.03-0.87, P = .03), but no difference was seen in the placebo treatment arm (OR 1.97, 95% CI 0.56-6.94, P = .29) (Figure 9). Moreover, when analysing the time to recurrences, the salbutamol on-demand group had shorter time to new physician-confirmed wheezing episode than the high-dose group (HR 0.22, 95% CI 0.05-0.98, P = .047), but no difference was seen in the placebo treatment arm (HR 1.72, 95% CI 0.68-4.35, P = .26). Importantly, the interactions were not affected by viral coinfection, since the interactions remained significant after adjustments (P = .02, and P = .04), but no statistically significant differences were detected in the prednisolone or placebo arm (all P > .05) (Figure 10). Of note, the majority (32/35, 91%) of the post-discharge relapses were confirmed at the study clinic.

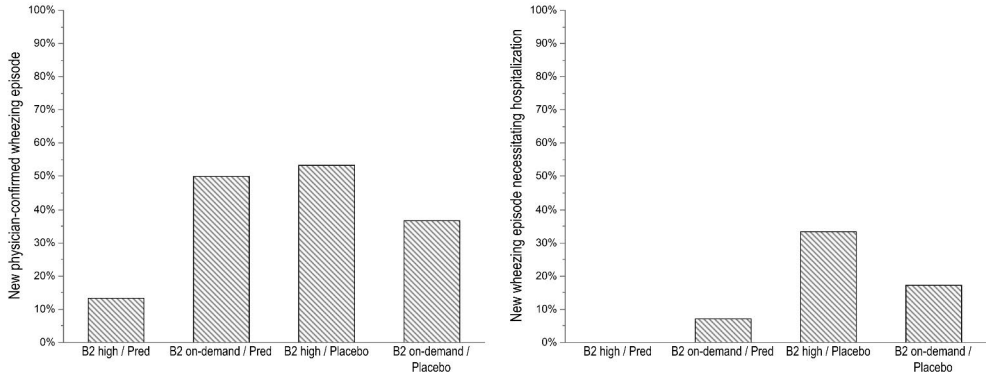

Figure 9. A new physician-confirmed wheezing episode. The interaction between salbutamol group and prednisolone treatment on new physician-confirmed wheezing episode was significant (group × treatment p = .02) (left). Salbutamol high-dose group had fewer wheezing episodes than on-demand group in prednisolone arm (p = .03), but no difference was seen in placebo treatment arm (p = .29). The group × treatment interaction effect on new physician-confirmed wheezing as inpatient was not significant (p = .30) (grey). Prednisolone treatment arm had less new physician-confirmed wheezing as inpatient than placebo arm (salbutamol group adjusted main effect of treatment p = .03). Pred, prednisolone; B2 high, high-dose salbutamol; On-demand, on-demand salbutamol.

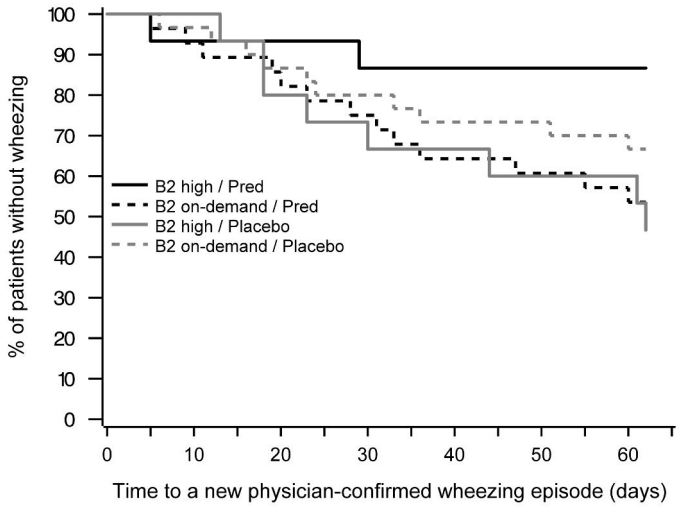

Figure 10. Time to a new physician-confirmed wheezing. The interaction between salbutamol group and treatment on a time to new physician-confirmed wheezing episode during the 2-month follow-up was significant (group × treatment p = .02). Salbutamol on-demand group had shorter time to new physician-confirmed wheezing episode than high-dose group (p = .047), but no difference was seen in placebo treatment arm (p = 0.26). Pred, prednisolone; B2 high, high-dose salbutamol; On-demand, on-demand salbutamol. From Study I.

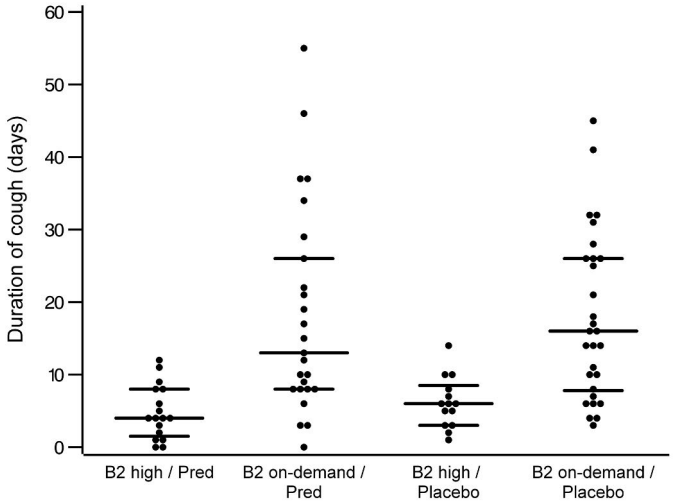

Figure 11. Duration of cough. Data are presented as medians (interquartile ranges). The group × treatment interaction effect on duration of cough was not significant (p = .46). High-dose group had shorter duration of cough than on-demand group (treatment adjusted main effect of salbutamol group p < .001). Pred, prednisolone; B2 high, high-dose salbutamol; On-demand, on-demand salbutamol. From Study I.

5.2.3 Duration of cough, and other secondary outcomes

The salbutamol on-demand group had a longer duration of cough than the salbutamol high-dose group (treatment-adjusted main effect of salbutamol group $P < .001$) (Figure 11), and the prednisolone treatment arm showed fewer new recurrences as inpatients than the placebo arm (salbutamol group-adjusted main effect of treatment $P = .03$) (Figure 10). Other secondary outcomes showed no statistically significant interactions or differences.

5.3 Association of cytokine responses and the short- and long-term prognoses between RV- and RSV-induced wheezing (II)

5.3.1 Cytokine response differences between the RV and RSV groups

In the acute phase, cytokine responses differed significantly between the RV and the RSV groups. After adjusting for baseline differences, the RV group showed lower levels of IL-1RA (97 vs. 240 pg/mL), IL-1β (3.5 vs. 30 pg/mL), and MCP-1 (6900 vs. 7500 pg/mL), but higher levels of Eotaxin-2 (740 vs. 350 pg/mL), TARC (3.9 vs. 1.8 pg/mL), and ENA-78 (900 vs. 210 pg/mL) compared to RSV (all $P < .05$) (Figure 12). Differences in IL-6, I-309, and Eotaxin-3 were notable but not statistically significant (all $P > .05$).

During convalescence, the RV group had higher levels of fractalkine than the RSV group (median 8.3 vs. 15 pg/mL, $P = .02$), but no other significant differences were found in the convalescent phase (Figure 13).

When analysing the change in the cytokine responses during the 2 weeks (between the acute and the convalescent phases), the RV group showed heightened levels of fractalkine (1.1 vs. -4.6 pg/mL) and IL-1β (5.0 vs. -6.8 pg/mL), while in the RSV group the levels were reduced (all $P < .03$). Additionally, the RV group showed decreased levels of I-309 (-8.4 vs. 10 pg/mL) and TARC (-0.96 vs. 1.4 pg/mL), whereas in the RSV group the levels were increased (all $P < .05$) (Figure 13).

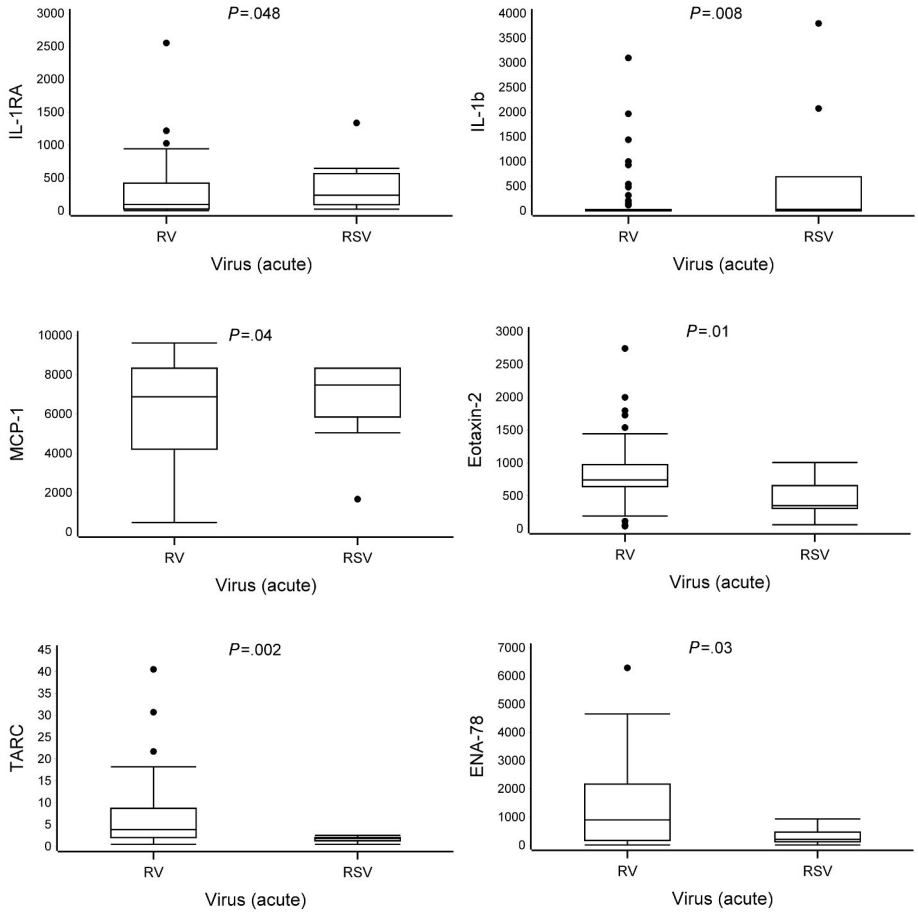

Figure 12. Differences in cytokine expression levels at study entry. Data are presented as medians (the lower (Q1) and upper (Q3) quartiles, and data falling outside the Q1–Q3 range are plotted as outliers). Cytokine concentrations are presented as pg/mL. Modified from Study II.

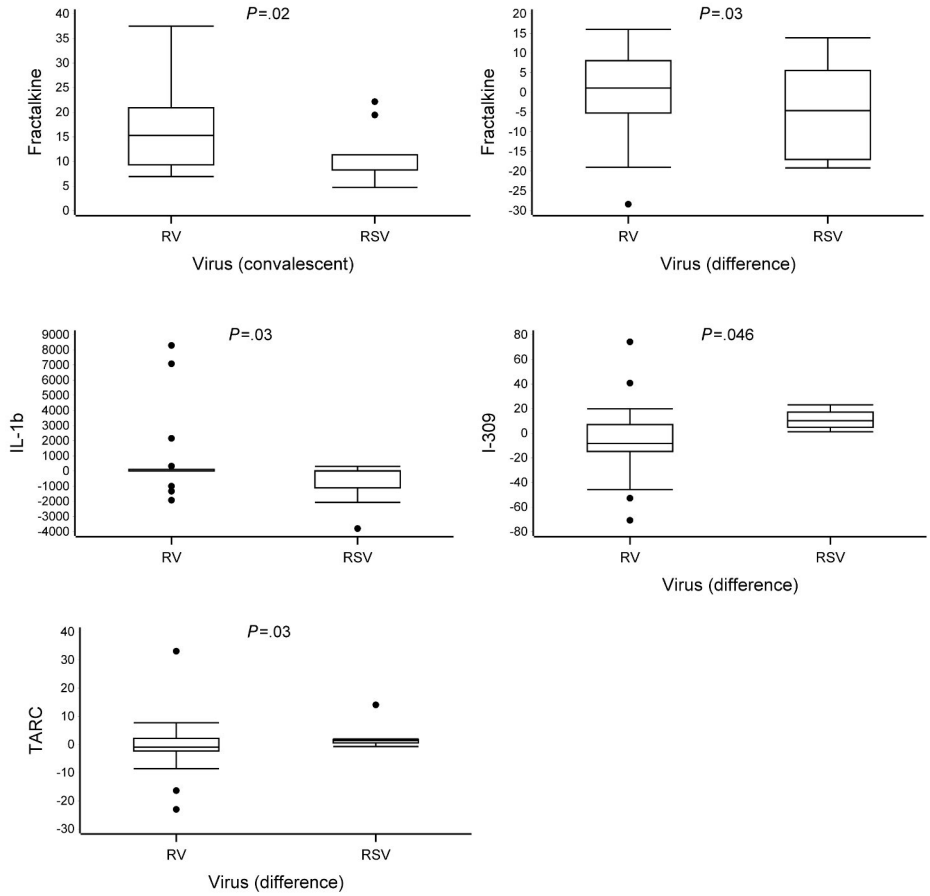

Figure 13. Differences in cytokine expression levels at convalescent phase and the difference over the study points. Data are presented as medians (the lower (Q1) and upper (Q3) quartiles, and data falling outside the Q1–Q3 range are plotted as outliers). Cytokine concentrations are presented as pg/mL. Modified from Study II.

5.3.2 Association of viral aetiology and cytokine responses and their effect on the severity of the illness

Analysis of the duration of hospitalisation revealed significant interactions between the viral group and the cytokine responses (all $P < .04$), indicating that the impact of cytokine responses from PBMCs on hospitalisation duration varied between the RV and the RSV groups. In the RSV group, higher levels of IFN-γ, MDC, IL-1RA, and VEGF were linked to shorter hospitalisation duration (all $P < .02$), whereas these associations were not significant in the RV group (all $P > .05$). Although a significant interaction between the viral group and IL-6 expression was noted, IL-6 levels were not associated with hospitalisation duration in either of the groups (all $P > .05$).

5.3.3 The occurrence of a new physician-confirmed wheezing episode during the 12-month follow-up

Though, the occurrence of a new physician-confirmed wheezing episode during the following 2 and 12 months differed between the RV and the RSV groups (52% vs. 11%, P = .02, and 81% vs. 22%, P = .002, respectively), the precise cytokine response driving these differences remained obscure due to the limited number of relapses in the RSV group.

Nonetheless, in the RV group, lower levels of I-309 (CCL1) and TARC during the acute illness were associated with the occurrence of recurrences within 2 months (median, relapse vs. no relapse, 21 vs. 48, P =.049, and 3.0 vs. 7.0, P =.03, respectively). Furthermore, during the acute illness, higher levels of IL-13 (6.0 vs. 1.5) and lower levels of I-309 (CCL1, 24 vs. 65) were associated with the occurrence of a new physician-confirmed wheezing episode during the following 12 months (all P <.05) (Figure 14). Overall, due to the scarcity of children in both the RV and RSV groups, the relationship between cytokine expression and asthma could not be evaluated at 4-year follow-up.

No statistically significant differences were observed in cytokine expression between different RV species or based on the RV genome loads.

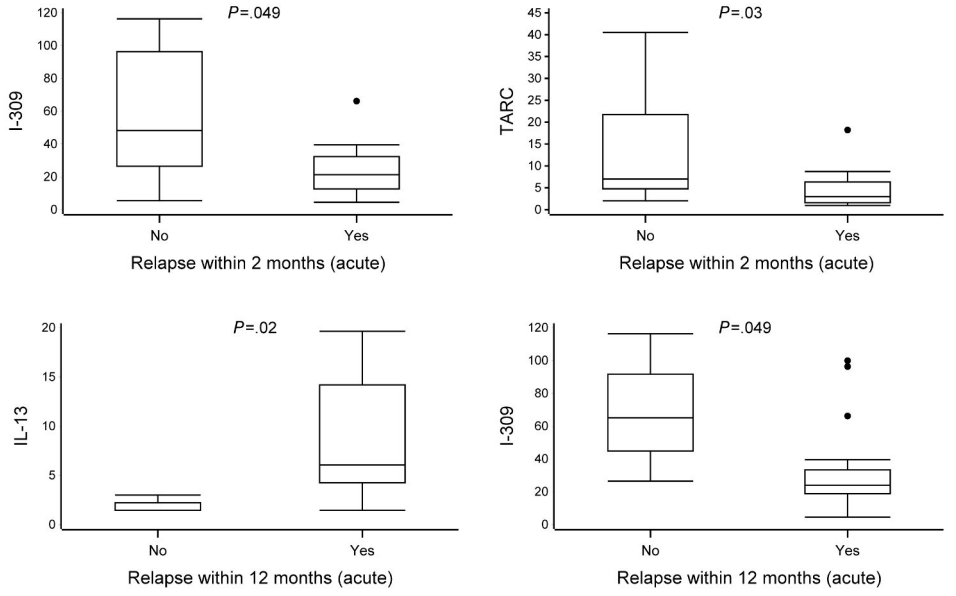

Figure 14. Association between cytokine expression and severity of acute illness (duration of hospitalisation). Data are presented as medians (the lower (Q1) and upper (Q3) quartiles, and data falling outside the Q1–Q3 range are plotted as outliers). Cytokine concentrations are presented as pg/mL. From Study II.

5.4 Immune suppression by HBoV1 on the RV-induced first wheeze in young children (III)

Significant differences in cytokine responses were noted between RV and RV-HBoV1 patients during both study points. During the acute illness, the RV-HBoV1 group was characterised by decreased levels of IL-1β (1.6 vs. 3.5 pg/mL), MIP-1β (92 vs. 210 pg/mL), RANTES (110 vs. 300 pg/mL), TNF-α (33 vs. 65 pg/mL), TARC (1.9 vs. 4.4 pg/mL), and ENA-78 (150 vs. 900 pg/mL) compared to the RV group (all $P < .05$) (Figure 15).

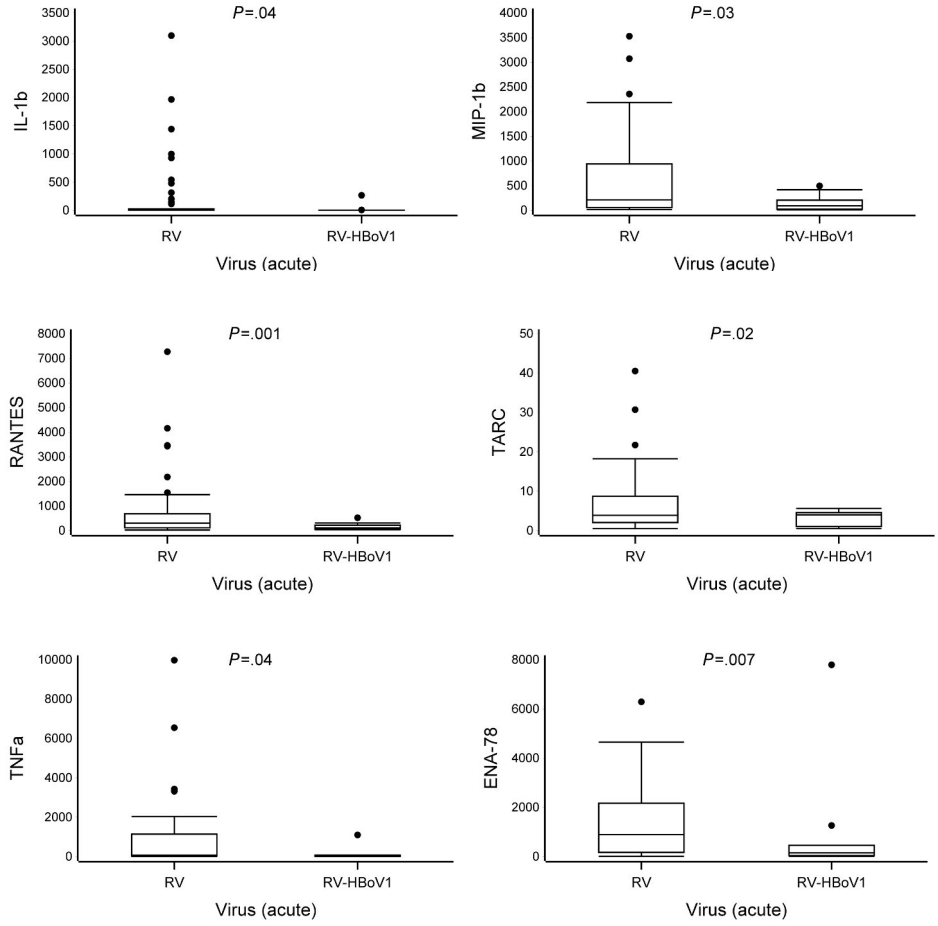

Figure 15. Association between cytokine expression and severity of acute illness (duration of hospitalisation). Data are presented as medians (the lower (Q1) and upper (Q3) quartiles, and data falling outside the Q1–Q3 range are plotted as outliers). Cytokine concentrations are presented as pg/mL. From Study II.

During convalescence, the RV-HBoV1 group was characterised by decreased levels of fractalkine (9.9 vs. 15 pg/mL), MCP-3 (10 vs. 300 pg/mL), and IL-8 (240 vs. 840 pg/mL) compared to the RV group (all $P < .05$). Differences of IL-6 and MIP-1β between the study groups were notable but not statistically significant (all $P < .05$). No significant change in cytokine expression during the time interval between acute and convalescent phases was observed between the groups (all $P > .05$) (Figure 16).

The severity of acute illness (i.e., duration of hospitalisation) was linked to viral aetiology and cytokine responses. This association was demonstrated by two significant interactions between viral groups and cytokine responses when analysing the duration of hospitalisation (all $P < .04$), indicating that the effect of cytokine response on the duration of hospitalisation differed between the two viral groups. Interestingly, higher levels of epidermal growth factor (EGF) and MIP-1β were associated with the shorter hospitalisation in the RV-HBoV1 group (all $P < .03$), but not in the RV group (all $P < .11$).

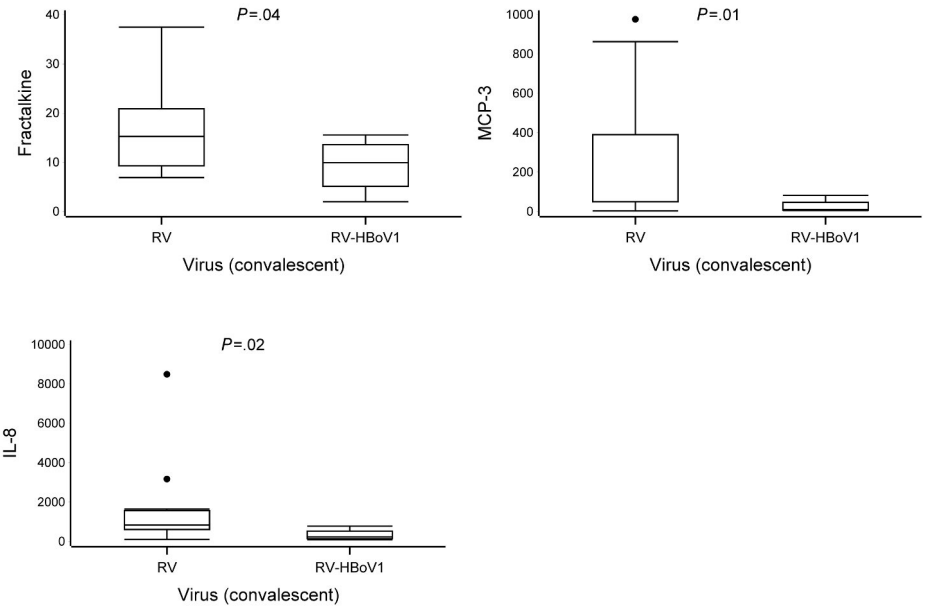

Figure 16. Differences in cytokine expression levels at convalescence phase. Data are presented as medians (the lower (Q1) and upper (Q3) quartiles, and data falling outside the Q1–Q3 range are plotted as outliers). Cytokine concentrations are presented as pg/mL. From Study III.

5.4.1 The occurrence of a new physician-confirmed wheezing episode during the 12-month follow-up.

The occurrence of relapses within 2 and 12 months after the infection seemed similar and did not reach significance between the RV and RV-HBoV1 groups (52% [11/21] vs. 0% [0/4], P > .10, and 81% [17/21] vs. 25% [1/4], P =.053, respectively). Regrettably, the scarcity of children in the RV-HBoV1 group prevented us from assessing the association between cytokine expression and the occurrence of recurrent wheezing and the development of asthma at later study points.

5.5 Association between cytokine responses and the initial disease severity in RV-associated wheezing (IV)

Following stimulation with anti-CD3/CD28, several significant differences in cytokine responses from PBMCs were observed between the hospitalised and non-hospitalised children. The non-hospitalised children exhibited higher levels of IFN-γ (median 24 vs. 1.6 pg/mL), IL-10 (110 vs. 13 pg/mL), MIP-1α (440 vs. 42 pg/mL), RANTES (1300 vs. 290 pg/mL), and TNF-α (810 vs. 52 pg/mL), and lower expression of ENA-78 (120 vs. 1400 pg/mL) compared to the hospitalised children (all P <.05) (Figure 17). The expression levels of IL-1β and IL-6 were notable but did not reach statistical significance (all P > .05).

Due to a scarcity of children in the non-hospitalised group, the assessment of differences in cytokine expression during convalescence and both the association between cytokine expression and the occurrence of recurrent wheezing at 2 and 12 months later, as well as the development of asthma during further follow-up points, could not be conducted.

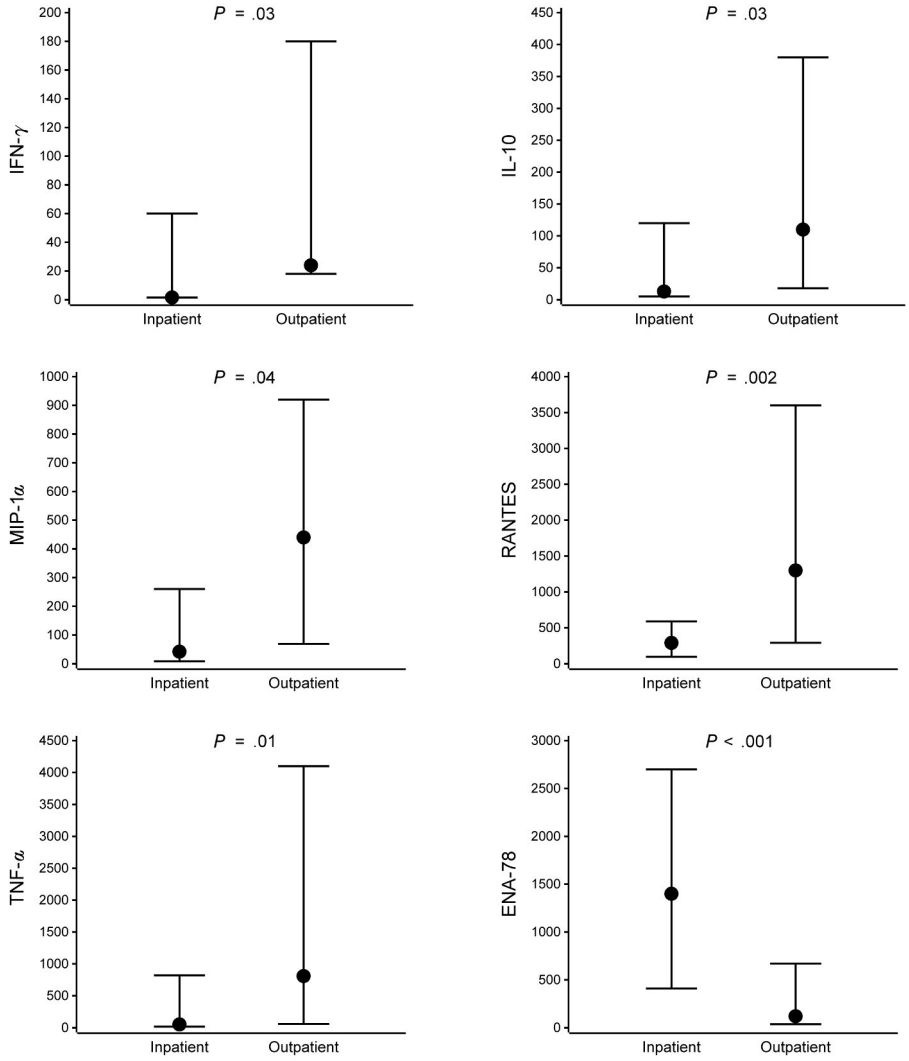

Figure 17. Differences in cytokine expression levels at the study entry (Inpatient vs. Outpatient, all P < .05). Data are presented as median, the lower (Q1) and upper (Q3) quartiles, and data falling outside the Q1–Q3 range are plotted as outliers. Cytokine concentrations are presented as pg/mL. In IFN-γ, for better visualisation, one sample from both study groups were excluded from the figure but included in the analyses [inpatient (5200pg/ml) and outpatient (2900 pg/ml)]. Modified from Study IV.

6 Discussion

6.1 The efficacy of inhaled salbutamol and oral corticosteroids in the first-time wheezing children infected with RV (I)

The first aim of this thesis was to assess the efficacy of salbutamol and prednisolone in treatment of RV-associated bronchiolitis, focusing on both short-and long-term prognoses. The previous studies, which most have found no efficacy, have not evaluated RV-associated disease alone, a distinct novel subgroup within the common definition of bronchiolitis (Anil et al., 2010; Chavasse et al., 2000; Dobson et al., 1998; Fox et al., 1996; Gadomski et al., 1994; Ipek et al., 2011; Karadag et al., 2008; Orlowski et al., 1991; Patel et al., 2002; Ralston et al., 2005; Schweich et al., 1992; Wang et al., 1992). Unlike most previous studies, we observed several beneficial differences in both short- and long-term outcomes when both interventions were used, especially with the regular administration of high-dose salbutamol with oral prednisolone compared to on-demand salbutamol with placebo. This may be attributed to different bronchiolitis endotypes, indicated by increasing evidence, suggesting that bronchiolitis is beginning to be viewed as a spectrum, with viral aetiology playing the most crucial role (Jartti et al., 2019; Makrinioti et al., 2022). These bronchiolitis endotypes may potentially exhibit various clinical and immunological characteristics. Hence, if possible, a clear need for better management of bronchiolitis remains evident.

Despite advances in viral diagnostics and more modern supportive care, the overall morbidity and mortality, as well as the short- and long-term prognoses of bronchiolitis, have remained relatively stable (Holman et al., 2003; Meissner, 2016). However, in our data, children receiving both high-dose salbutamol and oral prednisolone showed the lowest occurrence of randomisation within 2-month follow-up compared to other study groups. Further, significant interaction between the both interventions (high-dose salbutamol and prednisolone) was observed, indicating a beneficial interplay between the two treatment arms in young RV-induced bronchiolitis cases. Moreover, when assessing the hospitalisation time, and when the loose analysis was applied, the children receiving salbutamol on-demand combined with placebo were characterised by poorer short-term prognosis, indicated

by longer duration of hospitalisation when compared to the children receiving high-dose salbutamol and prednisolone. Nevertheless, the loose analysis was also influenced by the protocol differences, in which the children in the salbutamol on-demand/prednisolone group were excluded due to the delay in administration of the original study drugs (prednisolone and placebo). Furthermore, the regular administration of a β2-agonist and a systemic corticosteroid showed additional beneficial effects, evidenced by a shorter duration of post-discharge cough and fewer recurrences requiring readmission, respectively. These beneficial findings could be explained by the shared clinical and underlying pathophysiological changes between RV-associated bronchiolitis and asthma exacerbation (Jartti et al., 2019). In line with our findings, although most previous studies have not identified benefits from administering either β2-agonists or corticosteroids, a few studies have found positive consequences. In these studies, the group that experienced the most benefit has been characterised by a history of atopy (Ipek et al., 2011), which was also prevalent among our study groups. Moreover, at least two recent cluster analyses on the heterogeneity of bronchiolitis have revealed that history of atopy is strongly associated with RV aetiology of bronchiolitis (Dumas et al., 2016, 2019).

The two earlier studies, on which the current study (Study I) population is based, demonstrated the efficacy of oral prednisolone in reducing the duration of hospitalisation and respiratory symptoms, particularly in severe episodes with high RV genomic load (Jartti et al., 2006, 2015; Lehtinen et al., 2007). Moreover, oral prednisolone was observed to lower the occurrence of regular asthma control medication initiation by 30% over the subsequent 4 to 7 years (Lukkarinen et al., 2013). The current trial partially reaffirms these findings on the effectiveness of prednisolone, but notably indicates that the most promising response occurs when a high dose of nebulised β2-agonist is combined with prednisolone. Moreover, while numerous prior trials indicate that oral corticosteroids lack efficacy in treating bronchiolitis, in contrast to our study, they neglect to differentiate between various bronchiolitis endotypes. Consistent with this, additional RCTs have demonstrated that the optimal treatment response might be attained through the combination of systemic corticosteroids and adrenergic agonists in children with bronchiolitis (Alansari et al., 2013; Plint et al., 2009).

Furthermore, our data suggest that although the short-term primary outcome, time to discharge, points to a possible clinical benefit of high-dose β2-agonist treatment, long-term primary outcomes, such as randomisation and time to randomisation within 2 months, highlight the advantages of both high-dose salbutamol and oral corticosteroids. Additionally, the intermediate-term secondary outcome, duration of cough during following 2 weeks, supports the beneficence of both treatments. Interestingly, a disparity was discovered when comparing types of relapses (inpatient vs. outpatient). However, this disparity was only observed in the

salbutamol on-demand/placebo group, suggesting that relying solely on high-dose β2-agonist without prednisolone may lead to an increase in randomisation. While the exact mechanism remains unclear, this discrepancy could be attributed to increased bronchial hyperreactivity or β2-agonist tolerance (Da Silva Sena et al., 2021; Haney et al., 2005; Larj et al., 2002). Corticosteroids have been noted to mitigate this adverse effect, suggesting that combining corticosteroid and β2-agonist treatments could yield the highest efficacy (Aksoy et al., 2002). Our findings could be due to early asthma-like inflammation, which may explain the observed effectiveness. In addition, our findings are encouraging for a short course of systemic corticosteroids with possible concomitant trial of high-dose short-acting β2-agonists for bronchiolitis cases induced by RV.

6.2 Association of cytokine responses and the short- and long-term prognoses between different viral aetiologies (II and III)

6.2.1 Differences between the RV- and RSV-induced first wheezing (II)

The second aim was to evaluate whether immune responses from first-time wheezing children infected by RV and RSV differed, and whether the cytokine responses were associated with short- and long-term prognoses. As stated before, at least two recent cluster analyses on the heterogeneity of bronchiolitis have revealed that history of atopy is strongly associated with RV aetiology of bronchiolitis (Dumas et al., 2016, 2019). Moreover, RV- and RSV-associated bronchiolitis appear to influence long-term outcomes in different ways (Jartti et al., 2019; Makrinioti et al., 2022). Importantly, we were able to show significant differences in cytokine expression between the RV and the RSV groups, indicating that RV and RSV-associated bronchiolitis differ, not only clinically, but also in molecular level. Interestingly, the difference was broader at the study entry and more balanced in the convalescent phase at 2 weeks later. While overall cytokine responses were similar in both viral groups, the cytokine response in the RV group seemed to exhibit more characteristics of a type 2 immune response, whereas the RSV group showed similarities with a type 1 immune response. These differences in cytokine responses could play a significant role in the variations in long-term outcomes between the study groups.

At entry, the RV group exhibited higher expression levels of Eotaxin-2 and TARC; the former is associated with facilitating the chemotaxis of eosinophils to the respiratory tract (Provost et al., 2013), and the latter is associated with activation of type 2 immune responses via binding selectively to C-C chemokine receptor type 4 (CCR4) on the cell surface of Th2 cells, ILC2, and eosinophils (Catherine et al.,

2021), and both associated with type 2 immunity. Unexpectedly, the expression of ENA-78, which primarily facilitates the chemotaxis of neutrophils, was also higher in the RV group (Guo et al., 2021). Moreover, in contrast to the RV group, the children in the RSV group had higher expression of the type 1 and the type 3 immunity-associated cytokines, including IL-1β and its antagonist IL-1RA (Van Den Eeckhout et al., 2021). Moreover, the levels of MCP-1 were higher in the RSV group. MCP-1 is characterised as a pleomorphic cytokine, capable of inducing various immune responses in a variety of different cells. In the respiratory tract, damage to alveolar cells triggers the secretion of MCP-1, which in turn promotes the chemotaxis of both profibrotic macrophages and fibrocytes, as well as neutrophils. Additionally, MCP-1 plays a role in the polarisation of T cells (Gschwandtner et al., 2019; Yadav et al., 2010; Yang et al., 2020). However, the effects of MCP-1 are reported to depend on multiple factors such as the microenvironment, respective tissue, type of inductive pathogen and time of induction, and furthermore, the effects of MCP-1 can be associated with both type 1 and type 2 immune responses (Gschwandtner et al., 2019; Singh et al., 2021). Notably, the MCP-1-C–C chemokine receptor type 2 axis plays a pivotal role in controlling macrophage polarisation, and disruption of MCP-1 may induce the upregulation of genes linked to M1 polarisation (Gschwandtner et al., 2019; Sierra-Filardi et al., 2014). As allergic asthma is primarily associated with type 2 immune responses, the type 2-skewed cytokine expression observed in RV-affected first-time wheezing children may explain the increased risk of subsequent recurrences and the development of asthma later in childhood compared to those affected by RSV.

During convalescence (after 2 weeks), most of former disparities between study groups seemed to diminish. However, in the RV group an increased expression of fractalkine, which induces lymphocyte chemotaxis and has antiviral properties (Upton et al., 2017), was observed compared to the RSV group. Overall, these findings align with those of earlier studies conducted on NPAs and serum samples (Díaz et al., 2015; Jartti et al., 2009b; Sastre et al., 2020). Moreover, in terms of alterations in cytokine response between the study points, compared to the RSV group, the RV group exhibited a decreasing tendency of expression in type 2 immunity-associated profile (I-309 and TARC) and an increasing tendency of expression in type 1- and type 3-immunity-associated cytokines (fractalkine and IL-1β, respectively). While previous studies (utilising nasal swabs) have demonstrated variations in IFN-γ and IL-10 expression between children infected with RV and RSV (Aberle et al., 2004), a recent study indicated that this discrepancy diminishes when RV and RSV bronchiolitis coincide with wheezing (Roh et al., 2017). This discovery aligns with our findings.

Interestingly, in the RV group, cytokine expression did not associate with the severity of illness when compared to the RSV group. However, in the RSV group,

increased levels of IFN-γ, MDC, IL-1RA, and VEGF were linked to a shorter hospitalisation time. Notably, lower IFN-γ expression has previously been linked to a more serious clinical outcome (Li et al., 2020). Intriguingly, these cytokines belong to different subtypes of immunity, suggesting a complex and nuanced interplay between them. For example, IFN-γ and MDC can be categorised under type 1 and type 2 immunities, respectively. Moreover, IL-1RA is associated with type 3 immunity, and VEGF is linked to Treg responses. Of note, certain cytokines, such as MDC, exhibit properties that overlap across type 1 and type 2 immunities as well as Treg responses (Richter et al., 2014).

While data on the association between cytokine expression and long-term prognosis in young wheezing children are limited, a study indicated that an increase in MIP-1α expression was linked to subsequent recurrences (Sugai et al., 2016). However, in this study, the viral aetiologies were not differentiated. Further, a study, focusing on severe wheezing children infected with RSV, found that reduced expression of TNF-α was associated with recurrences (Kitcharoensakkul et al., 2020). Nevertheless, the study subjects were significantly younger (mean age 4.2 months). Thus, the direct comparison between earlier studies with different study protocols with ours is difficult. Nevertheless, in our dataset, heightened I-309 (CCL1) and TARC expression in the RV group associated with less randomisation within the following 2 months. Moreover, diminished IL-13 expression and increased I-309 (CCL1) expression were associated with less randomisation during the subsequent 12-month period. Notably, alterations in cytokine responses between the study points were also associated with relapses within 2 months, specifically IFNa2, and 12 months, including granulocyte colony-stimulating factor (G-CSF), fractalkine, IL-1RA, IL-1β, IL-6, and MCP-1, indicating that inappropriate timing of cytokine response could impede the successful resolution of acute viral infection. Regrettably, in the latter (12-month follow-up), the sample size in the non-recurrence group was rather limited, and consequently, the corresponding result should be regarded as hypothesis-generating in nature. Furthermore, while previous studies have noted variations in cytokine responses between RV serotypes or RV genome loads (Nakagome et al., 2014), in this study, the discrepancy remained obscured. This may be due to differences in factors such as stimulation methods, procedural protocols, or the age-dependent relationship between virus load and illness severity (Brenes-Chacon et al., 2021).

The link between severe RSV illness and worse long-term prognosis is suggested to be related to the direct disruption and destruction of pulmonary tissue and the subsequent remodelling process. In this sense, RSV may be considered more of an active perpetrator compared to RV (Fujiogi et al., 2022; Jartti et al., 2019). On the other hand, RV aetiology is more strongly associated with poorer long-term prognosis, and it is also more firmly connected with allergic sensitisation, atopic

eczema and other asthma-like characteristics (Dumas et al., 2016; Jartti et al., 2019; Lukkarinen et al., 2017; Makrinioti et al., 2022; Rubner et al., 2017). Our results may, at least partly, explain these differences between RV- and RSV-affected children.

6.2.2 The impact of HBoV1 coinfection during the RV-induced first wheezing (III)

The third aim was to determine whether coinfections influence the overall cytokine expression, and more specifically, examine the levels of cytokines and chemokines in stimulated PBMCs from young children during their first episode of wheezing, both during and after infection with either RV alone or a combination of RV and HBoV1.

Interestingly, although both study groups showed similar overall cytokine responses during the acute phase of illness, children with concurrent RV and HBoV1 infections exhibited a more significant decline across all subtypes of cell-mediated immunity (types 1, 2, and 3), as indicated by decreased expression of IL-1β, MIP-1β, RANTES, TNF-α, TARC, and ENA-78 compared to singleton RV infection. IL-1β and TNF-α, both commonly associated with type 3 immunity, are essential components of the host defence against injuries and infecting pathogens and are involved in the induction of proinflammatory proteins and the promotion of the differentiation of naïve CD4+ T cells into Th17 effector cells (Acosta-Rodriguez et al., 2007; Altieri et al., 2022). Moreover, in murine models, TNF-α has been associated with the regulation of type 1 immune responses (Zganiacz et al., 2004). MIP-1β and RANTES are typically identified as chemokines associated with type 2 immunity, recognised for their role in the chemotaxis of eosinophils, particularly in the respiratory tract (Kobayashi et al., 2019, 2022). However, recent studies have proposed broader functional capabilities for the latter (Li et al., 2021b). Furthermore, in individuals affected by RV infection, RANTES has been linked to chemotaxis of bronchial smooth muscle cells, potentially contributing to airway remodelling (Shariff et al., 2017). TARC is characterised by the ability to selectively bind to CCR4, which induces type 2 immune responses through the activation of ILC2s, Th2 cells, and pulmonary eosinophils (Catherine et al., 2021). ENA-78 predominantly serves as chemoattractant for neutrophils during respiratory tract infections, and the increased levels of ENA-78 were observed in the sole RV group when compared to children infected with RV-HBoV1 coinfection (Guo et al., 2021).

During convalescence, the earlier disparities observed at the study's entry were no longer evident. However, during the convalescent phase, children with RV-HBoV1 coinfection exhibited reduced expression of fractalkine, MCP-3, and IL-8 compared to children infected with RV only. Fractalkine serves as a crucial

chemokine and adhesion factor that is linked to both type 1 and type 2 immune responses (Bazan et al., 1997; D'Haese et al., 2010). It effectively triggers the chemotaxis of CX3CR1+ monocytes, NK cells, and CD4+ T cells (Fujimoto et al., 2001). The expression of fractalkine from RV stimulated PBMCs has been reported to be higher in the asthmatic patients compared to non-asthmatic, but notably, the exact role may be different between asthmatic and non-asthmatic patients (Upton et al., 2017). The binding of MCP-3 to CCR2 and CCR3 induces the chemotaxis of neutrophils and eosinophils, respectively, but it is also a potent chemoattractant for monocytes and macrophages in the respiratory tract (Santiago et al., 2008). MCP-3, produced by airway epithelium and macrophages, is typically induced by viral infection or cell injury, and MCP-3 promotes profibrotic state (Choi et al., 2004). In a murine model of asthma, blocking MCP-3 decreases the migration of neutrophils and monocytes to the respiratory system, although it does not seem to affect airway hyperreactivity (Girkin et al., 2015). In the respiratory tract, IL-8 promotes robust chemotaxis of neutrophils and activates various inflammatory cells via the recruitment of both innate and adaptive immune cells (Cambier et al., 2023). Moreover, IL-8 is suggested to participate in airway remodelling (Beigelman et al., 2015; Charrad et al., 2017; Wang et al., 2017). In addition, in paediatric acute respiratory distress syndrome, higher levels of IL-8 are associated with more severe disease and increased mortality, but this may be differentiated by the causative pathogen (Flori et al., 2019).

Though the cytokine response showed no association with the disease severity in the children infected with RV, the heightened levels of EGF and MIP-1β were linked to reduced length of hospital stay in the children with concurrent RV and HBoV1 infections. The former cytokine is crucial for the upkeep of the mucosal homeostasis (Tang et al., 2016), and decreased levels of circulating (serum and plasma) EGF have been suggested to be associated with greater risk of exaggerated IgE-mediated immune responses (Reinert-Hartwall et al., 2022). Consequently, children who contract concurrent RV and HBoV1 infection may be able to resolve the infection more effectively than those solely infected with RV. MIP-1β is linked to type 1 immune responses, and in adults with asthma, elevated levels of MIP-1β have been associated with diminished responses to anti-IL-5 therapy (Suzukawa et al., 2020). Furthermore, a prior investigation involving adult asthmatic individuals demonstrated reduced intracellular response of MIP-1 β from CD4+ and CD8+ T cells following in vitro stimulation compared to non-asthmatic controls, which suggest a shift in immunity polarisation away from type 1 immunity (Grob et al., 2003). These findings suggest that HBoV1 coinfection may shift the immune response more toward a type 1-dominated immunity.

The frequent co-detection of HBoV1 with other respiratory viruses arises from the prolonged persistence of HBoV1 DNA. Consequently, identifying an acute

HBoV1 infection should involve serology, quantitative PCRs, detection of viral mRNA, or identification of high DNA copy number in respiratory samples or serum (Christensen et al., 2019; Xu et al., 2017). In relation to HBoV1 infection, previous data on nasal swabs and samples from tonsil tissue have indicated reduced immune responses mediated by T cells (Ivaska et al., 2021). Moreover, B and T cells along with monocytes in tonsillar germinal centres have been found to harbour HBoV1 DNA, the consequences of which remain unknown (Xu et al., 2021). Furthermore, a prior study using serum samples observed that HBoV1 influences the immune response associated with RV infection in wheezing children (Lukkarinen et al., 2014).

While coinfections in respiratory tract diseases like bronchiolitis are frequent, there is relatively limited data on the potential interactive effect of multiple viruses in RV-induced bronchiolitis. This scarcity arises primarily from past study protocols that integrated coinfections into their analyses (Jartti et al., 2019). Therefore, the influence of viral interaction in specific coinfections has remained ambiguous in this context. Remarkably, a recent study illustrated an interaction between RSV and influenza A virus, resulting in the creation of hybrid virus particles, signifying reciprocal influence between the viral agents (Haney et al., 2022). One can speculate that these hybrid particles may function as immunomodulators.

Our study supports the growing hypothesis that HBoV1 coinfection may reduce cytokine expression during severe first wheezing episodes in children induced by RV, as indicated by changes in the overall cytokine and chemokine profiles. The data also suggest that viral proteins are likely to serve as potentially effective immunomodulators.

6.3 Association of cytokine responses and the severity of illness in first-time wheezing children infected with RV (IV)

The fourth aim was to assess the relationship between the cytokine responses and the risk for hospital admission in children experiencing a severe first-time wheezing episode infected with solitary RV infection. Remarkably, when the children treated as outpatients were compared to those treated as inpatients, significant differences in cytokine responses were found. Unexpectedly, children treated as outpatients, were found to elicit a more robust cytokine response (type 1 and type 2), but importantly, also showed a more pronounced concurrent anti-inflammatory cytokine response (Treg) compared to those treated as inpatients.

Although the cytokine responses in both study groups showed general similarity, hospitalised children displayed a more pronounced trend of decline in both pro- and anti-inflammatory cytokines. This difference was reflected in the reduced levels of

IFN-γ, IL-10, MIP-1α, RANTES, and TNF-α, coupled with increased expression of ENA-78 in the inpatient group compared to those treated as outpatients. Conventionally, IFN-γ and TNF-α are both recognised as cytokines associated with pro-inflammatory responses. IFN-γ is recognised as a pivotal immune system effector, renowned for its participation in both host defence and immune surveillance. It is known to possess immune-regulatory abilities, countering viral infections by inhibiting viral replication (Castro et al., 2018). In addition, IFN-γ serves as a significant stimulator for proliferation of CD8+ T cells (Curtsinger et al., 2012). TNF-α, on the other hand, plays a critical role in host defence against infections and injuries, inducing the production of proinflammatory proteins and promoting the differentiation of Th17 cells (Altieri et al., 2022).

MIP-1α, a chemokine predominantly originating from macrophages, is recognised for its pro-inflammatory properties, including the attraction of eosinophils, macrophages, and lymphocytes through chemotaxis (Maurer et al., 2004). Notably, both MIP-1α and RANTES are additionally linked to the proliferation of smooth muscle within the airways, contributing to remodelling of the airways (Halwani et al., 2011; Shariff et al., 2017). Further, heightened levels of MIP-1α during acute bronchiolitis have been linked to illness severity, prolonged duration of oxygen therapy, and randomisation (García et al., 2012; Garofalo et al., 2001; Sugai et al., 2016). Nevertheless, the latter discovery was specifically observed in hospitalised children without comparison to outpatient treatment.

RANTES, recognised for its role in attracting eosinophils, basophils, monocytes, and lymphocytes, is commonly identified as a chemokine associated with type 2 immune responses (Castan et al., 2017; Zhang et al., 2020b). Yet, recent studies have suggested that it may possess a more pleiotropic role in cell-mediated immunity. For instance, a recent study observed a reduced expression of RANTES from PBMCs after RV infection in asthmatic children compared to non-asthmatic children (Li et al., 2021b). ENA-78 is primarily recognised for its role in neutrophil chemotaxis, especially during the initial phases of infection (Liang et al., 2020). Additionally, the levels of ENA-78 are indirectly implicated in B cell chemotaxis through CXCL13 (Guo et al., 2021).

Notably, the children receiving outpatient care were characterised a more dominant anti-inflammatory response indicated by higher levels of IL-10 compared to those requiring hospital admission. IL-10 is crucial for countering the proinflammatory activity of type 1, 2 and 3 immune responses by downregulating the inflammatory effects. Nevertheless, data regarding the properties of IL-10 in bronchiolitis have remained inconsistent. For instance, increased levels of IL-10 have been linked to recurrent wheezing (Schuurhof et al., 2011), but conversely, decreased levels of IL-10 have been associated with more profound disease severity (Leahy et al., 2016). Furthermore, conversely, IL-10 has also been reported to play

no role in the disease severity (Mella et al., 2013). Intriguingly, IL-10 might have a dualistic effect on the severity of illness, particularly in RSV infection, indicated by attenuation in the acute phase but suggesting a potentially additive effect in the later phase, implying that the levels and impact of IL-10 are time-dependent (Sun et al., 2013). Additionally, a previous study examining cytokine responses from ILC2s demonstrated lower levels of IFN-γ and IL-10 in children with recurrent wheezing compared to those with viral bronchiolitis (Sastre et al., 2021).

Surprisingly, the children treated as outpatients exhibited a more pronounced pro-inflammatory response compared to hospitalised children, as indicated by higher levels of IFN-γ, TNF-α, and MIP-1α. This may suggest that a well-regulated balance of both pro- and anti-inflammatory responses is necessary to effectively resolve the initial infection, resulting in a less severe illness. Significantly, the dysregulation of Treg cells has been linked to more pronounced type 2-skewed immune responses, which may contribute to the development of asthma (Zhang et al., 2022b).

Our study supports the growing evidence that bronchiolitis represents a spectrum of varying endotypes. Our findings also suggest that strong and well-regulated cytokine and chemokine responses are necessary to prevent the need for hospitalisation. Our study also identifies potential new biomarkers for early asthma events in high-risk groups, specifically children experiencing their first episode of wheezing caused by RV infection. However, as demonstrated by Studies II and III, various viral agents are associated with distinct immune profiles in bronchiolitis, affecting their presentation during acute episodes.

6.4 Strengths and limitations

The strengths of the current thesis included thorough viral diagnostics, precise characterisation of the subjects, a detailed prospective follow-up in the original trials (in Study I both Vinku and Vinku2, and in Studies II-IV Vinku2 only), and in Studies II-IV, extensive analyses of cytokine profiles.

In Study I, the study design, which combined data from two previous prednisolone intervention trials, offered increased statistical power. However, crucially, the β2-agonist treatment regimens varied between the two studies. There were also a few limitations. A statistical power analysis for the intervention with salbutamol was not conducted, and the relatively small sample size limited the ability to perform optimal analyses in a multivariable model. However, in the adjusted analyses, the significant interactions persisted, and the alignment of many outcomes suggested that they are unlikely to be false positives. Furthermore, the findings of Study I may not be applicable to non-hospitalised patients, as all subjects were hospitalised. Additionally, due to variations in study protocols (a prospective RCT design in Vinku2, and a post hoc design in Vinku) the duration of hospitalisation was

not fully harmonious in relation to prednisolone treatment. In the Vinku2 study, prednisolone was given only after a positive RV PCR test, causing a delay in administration of prednisolone or placebo relative to other study groups. For other outcomes, the 45-hour delay in prednisolone administration was statistically adjusted for by the study effect (group effect).

In Studies II-IV, the initial hypothesis aimed to distinguish the study groups from each other, which resulted in the absence of a "control" group. Moreover, statistical power analyses were not performed, and the rather small sample size did not permit optimal analyses in the multivariable model. However, in all studies (II-IV), the study groups were composed of carefully characterised novel bronchiolitis subgroups. However, Studies II-IV do have some limitations. 1) Although a power calculation was performed in advance to evaluate our primary hypothesis, no power calculation was done for these specific analytic designs, and the fairly limited sample size restricted the use of optimal multivariable model analyses. No corrections for multiple comparisons were applied, and therefore, there is a possibility of an increased risk of Type I error due to testing multiple hypotheses. However, the probability of a Type II error increases with adjusted p-values, and since the sample size was already fairly small, we aimed to minimise that risk. Despite this, all study groups were drawn from novel bronchiolitis subgroups. 2) The limited volume of culture medium created difficulties in performing the dilution series, causing the fluorescence of several cytokines to exceed the upper limit of quantification, which in turn complicated the analysis of these cytokines. However, the number of affected cytokines was minimal. 3) While different RV serotypes can display unique behaviours and activate the immune system through varying mechanisms, the cases in our study were predominantly RVA- or RVC-positive, without RVB infections detected. Nonetheless, since both RVA and RVC are linked to more severe illness, this may account for the absence of variations between the serotypes. 4) Flow cytometry was not conducted, leaving open the possibility that variations in cytokine responses could, at least in part, be due to differences in the proportion of PBMCs that are T cells between the study groups. However, counter to this concern, the differences were not consistent across all cytokines, and in 80% of the analyses, no significant differences were observed between the groups, suggesting that cytokine expression was largely comparable. Additionally, cytokine responses from PBMCs stimulated with anti-CD3/CD28 might differ from those observed in other body regions, such as within the airways. 5) Finally, although early wheezing induced by RV in children has a stronger association with the risks of future recurrences and asthma compared to other viral agents, the small sample size in Studies III and IV limited our ability to evaluate the long-term prognoses.

7 Conclusions and Future Prospects

First, in our data, high-dose regularly administered β2-agonist appears to be more beneficial compared to on-demand administration in treating the novel subpopulation of bronchiolitis, specified as severe first-time wheezing children infected with RV, and the effect is further enhanced with concomitant use of oral corticosteroids, indicating a potential underlying synergistic effect between the two treatments. In these selected cases of bronchiolitis, the findings further elucidate the heterogeneity of bronchiolitis and support the use of a short course of systemic corticosteroids with at least a therapeutic trial of high-dose short-acting β2-agonist.

Second, the immune responses, as well as their association with short- and long-term prognoses, differ between the viral agents in bronchiolitis, specifically RV- and RSV-associated bronchiolitis.

Third, our findings support that coinfections may alter immune responses during the severe first-time wheezing episode. This is demonstrated by the effect of HBoV1 infection, which, when coinfecting with RV, has immunosuppressive capabilities, as evidenced by alterations in cytokine response and short-term prognosis. Our data also indicate new biomarkers for early events of asthma, particularly in these selected cases, and suggest that viral proteins can exhibit immunomodulatory potential. These findings further support the emerging assumption that bronchiolitis is a spectrum.

Fourth, the cytokine responses differ between non-hospitalised and hospitalised severe first-time wheezing children infected with solitary RV. Our data support, that both robust and highly controlled cytokine and chemokine responses are required to evade hospitalisation, and that impaired immune responses may drive the requirement for hospitalisation. Our data also indicate potential new biomarkers for the requirement of hospitalisation, specifically in first-time wheezing children infected with RV.

In conclusion, bronchiolitis is a spectrum encompassing different endotypes that may benefit from more personalised treatment according to the associated viral agent. The underlying immunopathology differs between the viral illnesses, and thus should be considered in future research as individual entities.

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