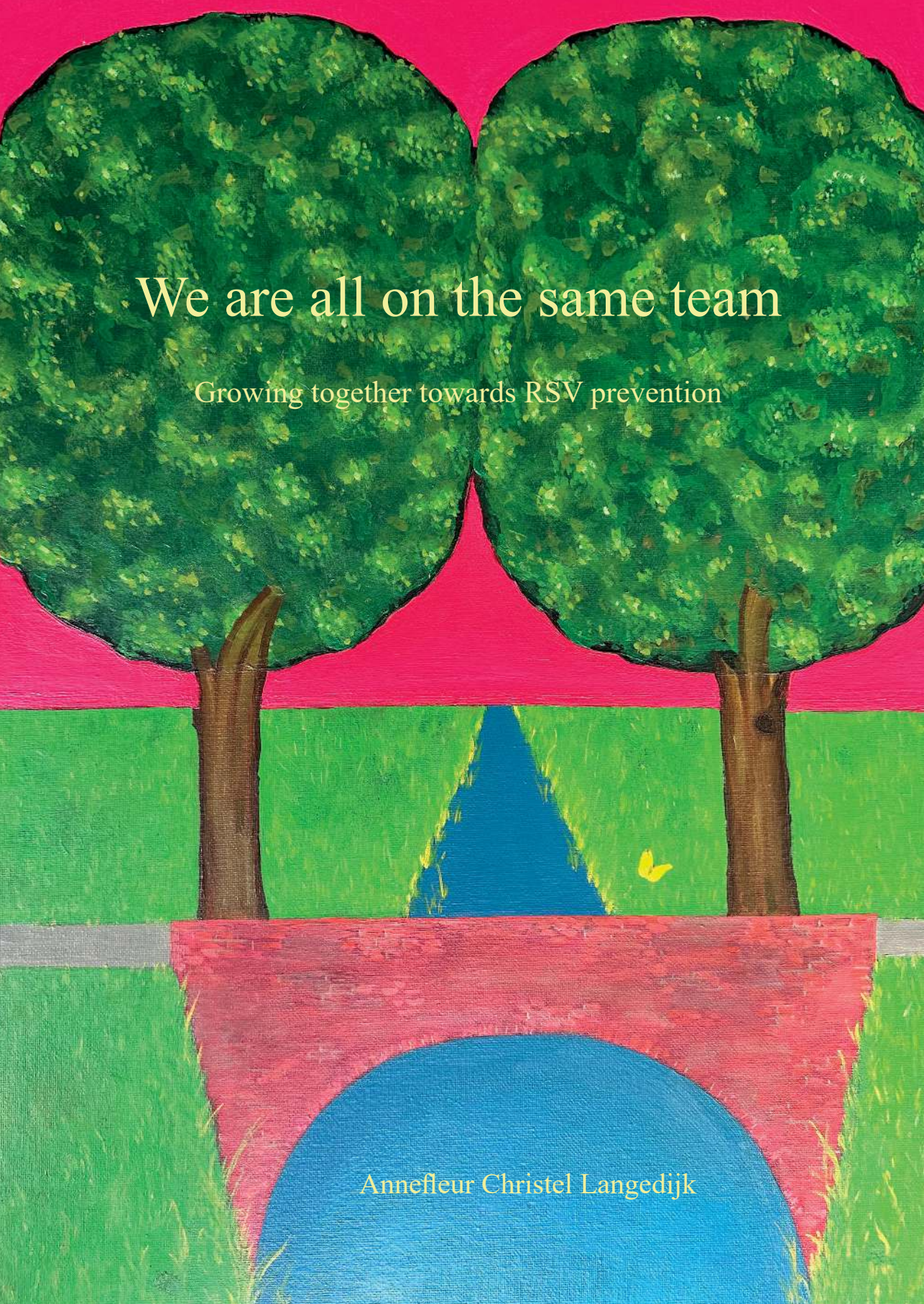
We are all on the same team

Growing together towards RSV prevention



Annefleur Christel Langedijk

WE ARE ALL ON THE SAME TEAM

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WE ARE ALL ON THE SAME TEAM
GROWING TOGETHER TOWARDS RSV PREVENTION

HET VERBINDEN VAN DE ACADEMISCHE WERELD EN FARMACEUTISCHE INDUSTRIE IN DE PREVENTIE VAN RSV
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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TABLE OF CONTENTS

Chapter 1

Introduction

"If you want to go fast, go alone.
If you want to go far, go together."
— African proverb

PUBLIC-PRIVATE PARTNERSHIPS

1.1 RSV infection and the development of novel products

Respiratory syncytial virus (RSV) is a leading cause of paediatric death[1]. More than 97% of this mortality burden is in low-and-middle income countries (LMIC) where community deaths are substantial[1,2]. In high-income countries (HIC), RSV contributes significantly to infant hospitalisations: in fact, one out of every 56 healthy full-term infants is hospitalised due to RSV infection[3]. It is important to note that RSVs burden is not limited to infants; it has also been recognised as a problem in older adults comparable to the burden of influenza[4]. The burden of RSV disease may be even greater than that of influenza in hospitalised older adults[5]. Despite the substantial impact, the development of antiviral treatments for RSV, aside from ribavirin, has progressed slowly. As it stands, the primary approach remains supportive care, encompassing interventions like oxygen supplementation and intensive care unit-based ventilation.

The current landscape in RSV research is characterized by noteworthy advancements: two vaccines for use in older adults, one for pregnant women, and a monoclonal antibody (mAb) for all infants have recently been approved[6]. In clinical trials, GSK's vaccine had 83% efficacy against RSV lower respiratory tract infection (LRTI)[7] and Pfizer's vaccine even showed 87% efficacy[8]. Additionally, Pfizer's vaccine is also indicated for use in pregnant women showing an efficacy of 82%[10]. The mAb nirsevimab developed by AstraZeneca and Sanofi was 75% effective at preventing LRTI in infants who required medical attention[9]. The widespread implementation of RSV prophylaxis emphasises the need for active surveillance. Such surveillance is instrumental not only in comprehending the global ramifications of these interventions across time but also in the prompt identification of viral escape mutants to novel products.

For a more comprehensive introduction to this topic, please refer to **Chapter 2**.

1.2 Biomarkers of infection

To effectively target safe and efficacious mAbs and vaccines, it is important to identify biomarkers related to diagnosis. The diagnostic potential of both mucosal and serum biomarkers has received increasing attention. Moreover, identifying viral biomarkers that differentiate mild from severe cases is critical for developing interventions for RSV. However, the timeline for the development of diagnostic tools is a protracted one:

1. The academic realm establishes a robust scientific foundation for achieving a level of performance that is at least on par with existing standards (spanning 3-5 years).
2. Forward-thinking enterprises embrace the concept, embarking on the development of a marketable device (within a timeframe of 3-5 years).
3. Rigorous clinical validation, undertaken collaboratively by academia and industry, culminates in regulatory endorsement (expected within 1-3 years).

4. Following successful market entry, the device transitions into the domain of major biotechnology players (a transition estimated to take 1-2 years).
5. Gradually, the technology becomes an integral part of both regulatory guidelines and clinical protocols, further solidifying its position (an anticipated process spanning 1-2 years).

1.2.1 Saliva as a source for respiratory virus biomarker research – the FRIENDS project

Accurate diagnostic tests and even more specifically equitable access to testing are essential for controlling RSV. The current gold standard for RSV diagnosis is RT-PCR from nasopharyngeal swabs. Saliva-based sampling for RSV detection has the potential to address many barriers associated with nasopharyngeal swab sampling. Saliva has demonstrated high sensitivity and specificity while being less invasive relative to nasopharyngeal swabs for Streptococcus pneumoniae and SARS-CoV-2. The collection procedure is not only non-invasive, but also easy and inexpensive. We initiated the Finding Respiratory viruses In Diagnostic Saliva (FRIENDS) project for developing a saliva-based test for RSV diagnosis using the current SARS-CoV-2 saliva assay. In addition to proving the principle of viral detection in saliva, we aim to evaluate collection devices as collection of saliva samples in infants poses additional challenges.

1.2.2 Host biomarkers of bacterial co-infection – the HERACLES project

Our research group has previously investigated a three-host protein-based assay to differentiate bacterial from viral infection in children with RTI demonstrating the diagnostic value of the biomarkers CRP, TRIAL, and IP-10 in this population[11]. During the early days of the COVID-19 pandemic, we decided to leverage our expertise in order to supplement these discoveries. Our goal was to establish baseline values for these three biomarkers in healthy individuals. This phase of my PhD journey was marked by considerable challenges, largely due to the suspension of all RSV-related studies by the medical ethics boards in response to the pandemic. However, we adeptly transformed this setback into an opportunity by launching the HERACLES project. Within this initiative, we collected blood samples from >300 healthcare professionals over a span of twee weeks at the Wilhelmina Children's Hospital.

1.2.3 Viral biomarkers of RSV infection during infancy – the role of viral load

The RESCEU (REspiratory Syncytial virus Consortium in Europe) project has created a sustainable and multidisciplinary multi-stakeholder community to provide an infrastructure for future trials for RSV vaccines and therapeutics. It is essential to identify target populations where trials of therapeutic and preventative measures could be directed This study is 1 of 4 clinical studies in the RESCEU project and explores host demographic and viral factors

associated with clinical characteristics of RSV infection. There is conflicting evidence on the association between RSV viral load and disease severity: the majority of previously conducted studies have reported a positive relationship indicating that high viral load may be associated with severe disease[12]. While the concept may be intuitive to comprehend, the imperative lies in the need to systematically collect samples from previously healthy infants across several years and numerous countries.

1.3 Exploring collaborative success

I embarked on my PhD journey with a straightforward yet impactful endeavor: the INFORM-RSV project, which aims at defining the global molecular epidemiology of RSV infection. Throughout the course of my doctoral studies, I not only initiated the INFORM-RSV project but also catalyzed and contributed significantly to a range of concurrent projects. These diverse endeavors collectively enriched my understanding of epidemiology, virology, and immunology, interweaving their findings into the fabric of my thesis. Notably, a common thread unifying these studies was their affiliation with various forms of public-private partnerships (PPPs), emphasizing the pivotal role of collaborative efforts.

As I approach the conclusion of PhD journey, a clear narrative emerges: the backbone of my thesis resides in the intricate realm of PPPs. This overarching theme has not only ignited my inspiration but has also profoundly molded my perspective as a scientist. Moreover, its guiding influence has seamlessly directed me towards the next chapter of my professional journey.

1.3.1 PPP is a form of co-creation

Navigating the transformation of a raw creative concept into a approved product involves a pertinent question: who bears the financial responsibility? It is a role that neither academic institutions nor governments typically fulfil; research is largely supported by industry. Industry heavily relies on the expertise of academic investigators for steering product development, capitalizing on their networks, and tapping into the patient demographics under the care of clinicians. Interestingly, approximately a quarter of academic investigators in biomedical research have received funding from industry sources[13]. In this context, PPPs emerge as collaborative forums, harmonizing cross-disciplinary prowess between academia and industry *to co-create value by touching upon crucial factors such as dialog and transparency.* A notable precedent was set in 2011 when the Vaccine Alliance Gavi orchestrated a PPP to negotiate reduced prices for human papillomavirus vaccines in LMICs[14], exemplifying how collaboration can impact the health landscape. More recently the COVID-19 pandemic has uniquely spotlighted the potential of PPPs, offering insights applicable to other like RSV. The popularity of PPPs has surged in the realm of RSV, with influential players such as the Bill & Melinda Gates Foundation and Gavi collaboratively striving into enhance accessibility to RSV vaccines and mAbs in LMICs[16].

1.3.2 Different forms of PPP

Clinical research can be funded in three ways: (1) the investigator's department supports the study; (2) the investigator can collaborate with a company that has interest in the product or concept; or (3) a study can be supported by an independent public organization such as the National Institute of Health or a foundation[13]. All three are "investigator-initiated research" meaning that the academic investigator controls the study. The other model is for a company to initiate, fund, and manage a study referred to as a "sponsored trial" which differs distinctly from investigator-initiated research in that the sponsoring company is involved in the development of the protocol, study execution and manuscript preparation. This makes industry-funded research critical to new product development. *Without sponsored trials, academic research would stagnate with little innovation.* Some even argue that innovation would not happen without industry funding.

The manuscripts included in this thesis are a result of various PPPs within the RSV field with all PhD research collaborations summarised in the table below.

Table: PPP in this thesis.

Study name / collaboration	**Study goal**	**Industry partner**	**Academic partner**
INFORM	To better understand the global RSV dynamics in infants and to timely detect escape mutants.	AstraZeneca	>17 universities, KU Leuven, UMC Utrecht
HERACLES	To examine the dynamics of the host response to viral respiratory infection in healthcare workers.	MeMed	UMC Utrecht
FRIENDS	To evaluate RSV detection in paired nasopharyngeal swabs and saliva samples in infants.	Merck	Yale University, UMC Utrecht
RESCEU	To identify host and viral biomarkers of severe RSV ARTI in infants.	AstraZeneca, GSK, Janssen, Novavax, Pfizer, Sanofi (IMI)	>5 universities, UMC Utrecht, RIVM (IMI)
HARTI (no data included in this thesis)	To better understand how RSV viral surface proteins are evolving.	Janssen	UMC Utrecht
BRICE (no data included in this thesis)	To estimate the burden of severe RSV ALRI in children ≤2 years.	Merck	Local hospitals the United Kingdom, France, Germany, Spain and Italy, UMC Utrecht
Inno4Vac RSV challenge model validation (no data included in this thesis)	To identify the inoculation dose needed to induce RSV infection with a new strain, making use of RSV isolates from the UMC Utrecht.	Sanofi, GSK (IMI)	Hannover University, RIVM, Imperial College London, UMC Utrecht, RIVM (IMI)

Abbreviations: GSK, GlaxoSmithKline; IMI, Innovative Medicines Initiative; RIVM, National Institute for Public Health and the Environment

1.4 Thesis outline

The overarching aim of this PhD project is to identify and address the implications and challenges associated with RSV product development, with the primary objective of advancing the development of safe and effective interventions to enhance public health outcomes.

1.4.1 Part I. Product development of RSV immunoprophylaxis

We wrote a state-of-the-art review paper describing novel RSV interventions including the development of immunoprophylaxis in **Chapter 2**.

Data on disease burden in healthy infants are necessary to determine RSV immunisation policies. In **Chapter 3**, we estimate the incidence of RSV-associated hospitalisations in the first year of life to guide the roll out of mAbs and vaccines.

With the approval of a mAb for all infants as well as the first-ever RSV vaccine, the need for global monitoring of RSV has become increasingly important in evaluating the effectiveness of those mAbs and vaccines. **In Chapter 4**, we aim to identify knowledge gaps in recent RSV literature to study global RSV evolution and transmission patterns and, at the same time, provide guidance for monitoring mAbs before and after the granting of license.

In **Chapter 5**, we build on the finding of a single spontaneous mutation that negatively impacted antibody binding of the mAb suptavumab. We state that palivizumab will probably be replaced by next-generation mAbs in the coming years and that therefore molecular evolution of RSV is complex and a critical factor to be recognized.

To bridge these gaps in knowledge, the INFORM-RSV study (International Network for Optimal Resistance Monitoring of RSV) has been initiated to better understand the dynamics of global RSV transmission and to timely detect mAb resistance mutations.INFORM-RSV is the largest clinical study worldwide monitoring currently circulating RSV strains in children under 5 years of age. We describe the study design in **Chapter 6**.

In **Chapter 7**, we analyse the samples collected during the first INFORM-RSV season (2017-2018). These sequences establishe an important molecular baseline of RSV strain distribution and sequence variability.

The geo-temporal evolution of potential escape variants in recent RSV seasons has not been thoroughly investigated. Therefore, we assess the nirsevimab binding site conservation based on the latest prospective surveillance studies including INFORM-RSV in **Chapter 8**.

Surveillance and prevention of RSV at a global level relies heavily on the understanding of RSV spread. By applying phylodynamic approaches, we uncover how selection and neutral epidemiological processes shape RSV diversity, and we explore the dynamics of global RSV circulation and its driver in **Chapter 9**.

In **Chapter 10**, we investigate whether fatal RSV infections in Zambia could stem from variations in viral strains or potentially be indicative of non-virological factors such as limited access to supportive medical care.

1.4.2 Part II. Product development of biomarkers

In **Chapter 11**, we investigate whether saliva may be an alternative diagnostic option compared to nasopharyngeal swabs. Ultimately, detection in saliva will make large-scale and frequent clinical and community sampling more feasible.

In **Chapter 12**, we assess a novel blood test capable of distinguishing between bacterial and viral infections by measuring the concentrations of three host biomarkers. Our findings establish the baseline values in healthy individuals.

In **Chapter 13**, we evaluate host demographic and viral factors associated with RSV disease severity in infants under 1 year of age from 3 European countries. These results deepen the understanding of risk factors and identify target populations for therapeutic and preventive measures.

Chapter 14 delves into a reflective analysis within an ethical framework of the insights garnered from the endeavors outlined in this thesis. Within this chapter, we engage in a comprehensive examination of the lessons derived from personal experiences within PPPs, addressing not only the valuable takeaways but also acknowledging any limitations and hurdles encountered along the way.

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PART I

Product Development of RSV Immunoprophylaxis

Chapter 2

Respiratory Syncytial Virus Infection and Novel Interventions

Nature Reviews Microbiology, 2023

"If people are not laughing at your goals,
your goals are too small."
— Azim Premji, Indian businessman and philanthropist

Respiratory Syncytial Virus Infection and Novel Interventions

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ABSTRACT

The large global burden of respiratory syncytial virus (RSV) respiratory tract infections in young children and older adults has gained increased recognition in recent years. Recent discoveries regarding the neutralization-specific viral epitopes of the pre-fusion RSV glycoprotein have led to a shift from empirical to structure-based design of RSV therapeutics, and controlled human infection model studies have provided early-stage proof of concept for novel RSV monoclonal antibodies, vaccines and antiviral drugs. The world's first vaccines and first monoclonal antibody to prevent RSV among older adults and all infants, respectively, have recently been approved. Large-scale introduction of RSV prophylactics emphasizes the need for active surveillance to understand the global impact of these interventions over time and to timely identify viral mutants that are able to escape novel prophylactics. In this Review, we provide an overview of RSV interventions in clinical development, highlighting global disease burden, seasonality, pathogenesis, and host and viral factors related to RSV immunity.

INTRODUCTION

Respiratory syncytial virus (RSV) presents a serious health threat to children, particularly infants who are 6 months of age or younger or those with comorbidities, and to older adults[1,2]. RSV is so named because of its propensity in pathological samples to cause respiratory epithelial cells to fuse into multi-nucleated giant cells (syncytia). There are two major subtypes (RSV-A and RSV-B) determined by duplications in RSV G protein sequences and antigenic drift, but also supported by genomewide sequence divergence including within RSV F protein. In the absence of specific treatment, supportive care is the mainstay of therapy. Until the recent registration of the world's first RSV vaccine, efforts mainly focused on preventing RSV respiratory tract infection (RTI) through passive immunization. The experience with an earlier formalin-inactivated RSV vaccine that increased the severity of RSV infection after natural exposure has had a negative impact on vaccine development and exposed our lack of understanding of the immune response against RSV[3]. Subsequent investigations suggested that the vaccine had amplified T cell-mediated responses and non-neutralizing antibodies, enhancing disease severity. That observation has focused efforts on protection solely through antibodies, either via maternal vaccination (as only IgG antibodies cross the placenta from the vaccinated mother) or via monoclonal antibodies (mAbs)[4]. Before the approval of the mAb nirsevimab in 2022, only one mAb (palivizumab) — a mAb targeting the surface fusion (F) glycoprotein — had been licensed, and that occurred over 20 years ago[5]. Ribavirin is the only antiviral therapy recognized for RSV treatment, but its safety profile and poor efficacy limit its use in practice[6].

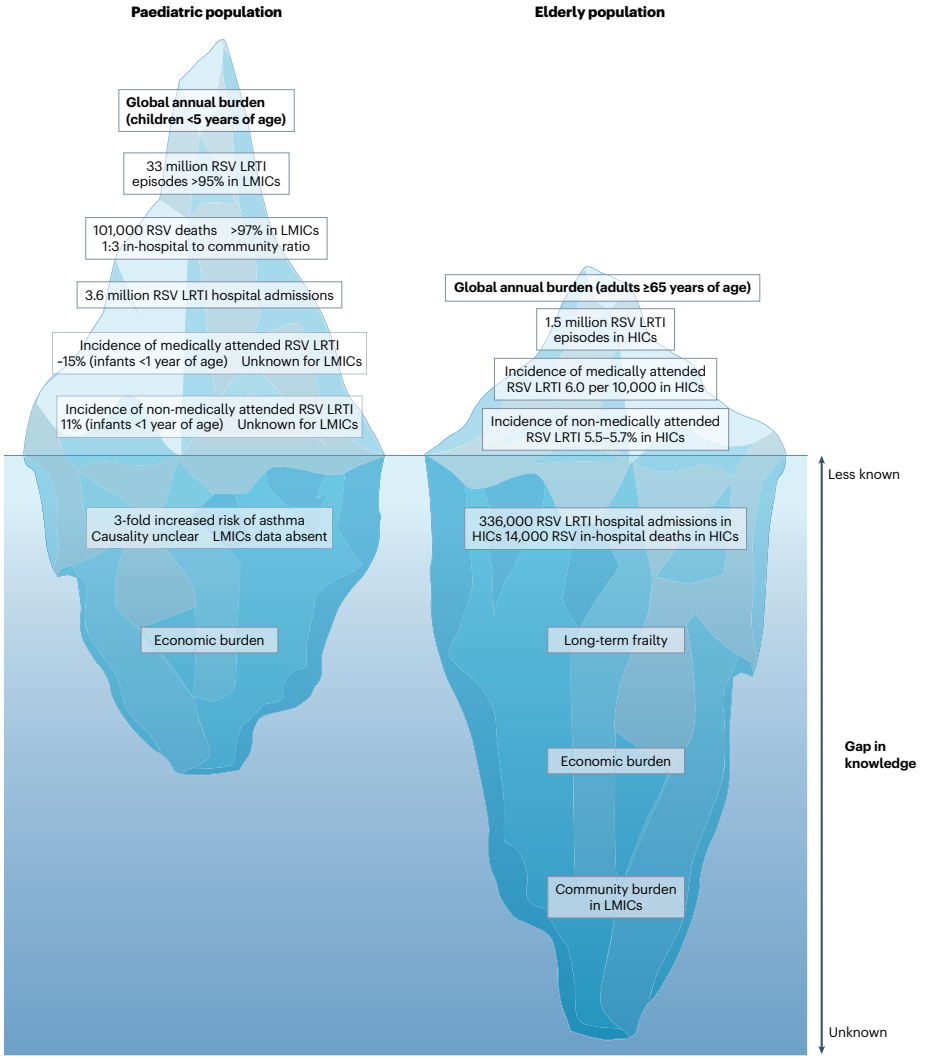
Much has happened since a previous review in 2019 explored RSV entry mechanisms and how those insights can guide the development of vaccines and therapeutics[7]. Today, we are in an exciting phase as the US Food and Drug Administration (FDA) just approved the world's first RSV vaccine[8] on 3 May, 2023 (company press release[9]) and the second vaccine was approved on 31 May, 2023 (company press release[10]), and the mAb nirsevimab received European Medicines Agency (EMA) approval on November 4, 2022 (company press release[11,12]). These may be followed shortly by the approval of a maternal vaccine[13], another vaccine for older adults (company press release[14]) and a fusion inhibitor for the treatment of infants[15], all showing positive phase III trial results. In this Review we discuss our current understanding of the burden of disease, of virus biology and pathogenesis, and of host and viral factors related to immunity as a segue towards the development of novel RSV interventions.

BURDEN OF DISEASE AND EPIDEMIOLOGY

RSV has been recognized as a leading cause of lower RTIs (LRTIs) in young children and substantially contributes to the disease burden, especially during the first 6 months after birth[16]. Our understanding of the disease burden in low-income and middle-income countries (LMICs) is limited (Fig. 1). More than 97% of RSV-related deaths across all age groups are in LMICs[1]. Fatal RSV LRTI is concentrated in community settings among younger children. Assessing the burden of RSV mortality is challenging because of limited surveillance in general, and particularly for capturing the burden of community deaths in LMICs[17,18]. Sixty-seven per cent of paediatric RSV deaths occur in the community setting before any healthcare is sought[18]. Therefore, currently reported RSV incidence rates, which are largely based on extrapolations from cases identified in hospitalized children, probably underestimate the true global burden[1]. RSV is highly contagious and nearly all children will become infected with RSV in the first 2 years after birth[19]. Most RSV-related hospitalizations occur in the first 2–3 months after birth, whereas the peak incidence of RSV infection is at 6–12 months of age[20]. The incidence of RSV-associated hospitalization is 1.7% in the general population under 5 months of age in the United States[21] and 1.8% in the first year after birth of healthy term infants in Europe[22]. Pre-existing medical conditions such as prematurity, congenital heart disease, lung disease and Down syndrome all strongly increase the risk of RSV hospitalization[23]. RSV mortality rates are highest among children with known risk factors. However, these children represent a minority of severely affected infants. Most children hospitalized for RSV infection are previously healthy term infants[24,25]. In addition to the direct effects of RSV disease, children who have recovered from RSV bronchiolitis often experience subsequent chronic wheezing, and possibly also asthma. However, the causal relationship between RSV and subsequent wheezing remains inconclusive[26]. In response to RSV LRTI, bronchial epithelial cells can release alarmins, which are mediators that are effective in stimulating type 2 innate lymphoid cells to produce TH2 cytokines, ultimately causing airway inflammation in mice[27]. Research in humans is required to determine how human type 2 innate lymphoid cells mediate pathology in wheezing and asthma. A recent observational study showed a lower risk of asthma at 5 years of age in children who did not have evidence of RSV infection during their first RSV season. However, these results should be interpreted with caution until large RSV prevention trials with long-term follow-up can confirm a causal link between RSV infection and asthma development[28,29].

Most older children and adults often present with nonspecific cold symptoms. However, the elderly population is at increased risk of severe RSV LRTI. Despite its importance, we know little about the RSV burden in older adults (Fig. 1). In a recent meta-analysis, the incidence of RSV LRTI in people over 65 years of age was estimated to be 6.7 cases per 1000 people per year[30]. In the case of RSV infection of frail older adults, the risk of hospitalization is relatively low[31]. Although the individual risk of developing severe RSV LRTI is lower than

Fig. 1 | Global disease burden of respiratory syncytial virus.



The global disease burdens of respiratory syncytial virus (RSV) infections in the paediatric population (left) and the elderly population (right) are shown. Our understanding of the global disease burden can be viewed as 'the tip of the iceberg'[21,21,22,26,31,188–195]. The area under water shows the less-known or unknown aspects of the global burden of RSV, such as the causal relationship between RSV and asthma and economic burden. For older adults, fewer aspects are known including frequencies of hospital admissions and in-hospital deaths, longterm frailty and community burden in LMICs. The blue gradient demonstrates the knowledge gap. HICs, high-income countries; LMICs, low-income and middle-income countries; LRTI, lower respiratory tract infection.

in infants, the absolute number of elderly individuals at risk in high-income countries (HICs)[32] is higher and is increasing because of the ageing population. RSV also poses a serious threat to adults with chronic medical conditions such as congestive heart failure, chronic obstructive pulmonary disease, diabetes mellitus, coronary artery disease, cerebrovascular accidents and end-stage renal disease[2,33,34]. Thus, young children are not the only RSV-vulnerable popula-

tion: older adults, in particular those with medical comorbidities, are also at increased risk of severe RSV infection.

Before the COVID-19 pandemic, RSV epidemiology was characterized by a seasonal pattern in most places around the globe. Countries around the equator may have more perennial transmission[35,36]. Interestingly, COVID-19 has exerted substantial effects on RSV epidemiology. In fact, RSV disease was largely absent in many countries globally during the first pandemic winter. A delayed summer epidemic was observed in many places around the world[37-39]. Out-of-season RSV activity may be explained by decreased population immunity following a prolonged period of reduced RSV exposure ('immunity debt'), in this case due to COVID-19 nonpharmaceutical interventions[40]. Paradoxically, these prolonged periods of low exposure have proven to be a threat to the health-care system as the capacity of supportive care systems was overwhelmed during the rebound RSV season in summer[41,42]. Virus–virus interactions affect the infection dynamics of RSV. Recent research showed that RSV blocks SARSCoV- 2 replication by triggering an antiviral response[43]. Conversely, RSV replication is reduced during coinfection with SARS-CoV-2 (ref. 44).

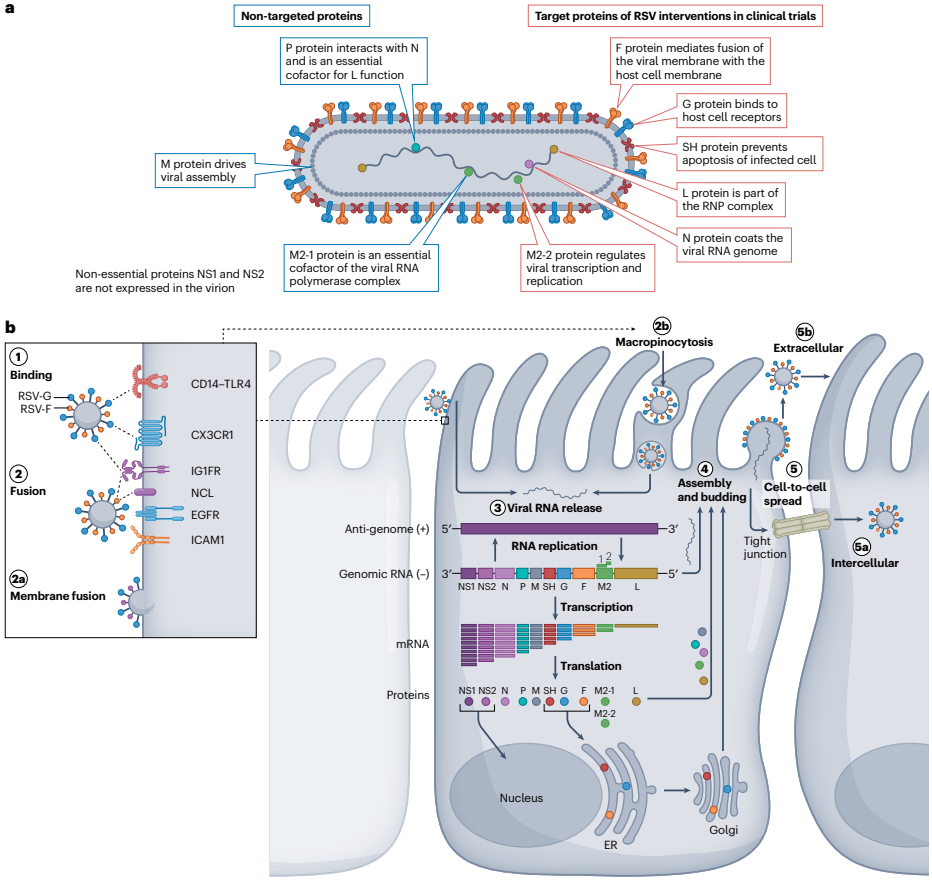
RSV BIOLOGY AND PATHOGENESIS

Virion and genome

RSV is a negative-sense, single-stranded RNA virus that belongs to the *Pneumoviridae* family. The RSV genome contains 10 genes that encode 11 proteins (Fig. 2a): non-structural protein 1 (NS1) and NS2, nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), small hydrophobic protein (SH), attachment protein (G), fusion protein (F), the M2-1 and M2-2 cofactor proteins, and the polymerase protein (L) (listed in order of transcription). The transmembrane glycoproteins F and G have an important role in virus entry through attachment (G) of RSV to the epithelium and fusion (F) of the viral and host cell membranes[7]. The role of the third surface glycoprotein, the SH protein, is less clear. Anti-SH antibodies do not have neutralization capacity, although the SH protein prevents apoptosis in RSV-infected cells and its absence leads to viral attenuation[45,46]. Some RSV genes encode virulence factors that interfere with host antiviral responses. For example, the genes for the non-structural proteins NS1 and NS2 are the first to be transcribed, and both proteins interfere with host innate antiviral immunity. NS1 is structurally similar to the M protein and modulates host interferon responses by hindering the recruitment of transcription regulators[47,48]. NS2 induces autophagy by modulating Beclin-1 protein stabilization. Autophagy prevents the production of inflammatory cytokines and thereby impedes activation of apoptosis, a key immunological defence to viral infection[49].

The structural details of the RSV virion have recently been untangled. Virions and glycoproteins covering the viral envelope have an extensive helical order. These envelope

Fig. 2 | The respiratory syncytial virus virion and replication cycle.



a, The respiratory syncytial virus (RSV) virion consists of nine structural proteins of which attachment protein (G)[196], fusion protein (F)[197], small hydrophobic protein (SH)[198], nucleocapsid protein (N)[199], cofactor protein M2-2 (ref. 200) and polymerase protein (L)[82] are targeted by RSV interventions in clinical trials. By contrast, phosphoprotein (P)[201], matrix protein (M)[202] and cofactor protein M2-1 (ref. 203) are not targeted. The RSV genome also encodes two multifunctional non-structural (NS) proteins, NS1 and NS2, that are not part of the viral particle and are therefore not shown. **b,** Binding and entry of RSV into the host epithelial cell. RSV particles enter the cell through cell surface entry or macropinocytosis by interacting with host cell receptors that bind to RSV G (such as CX3CR1, HSPs and CD14–TLR4) and to RSV F (such as IGF1R, NCL, EFGR and ICAM1). Following entry, the release and uncoating of the incoming genomic RNA subject it to RNA replication, transcription and translation. The proteins are translated and processed into the non-structural proteins (NS1, NS2), which move via the nucleus and structural proteins. The glycoproteins F, G and SH are assembled at the plasma membrane, whereas the other proteins transfer via free ribosomes to the cell surface. At the cell surface, RSV assembles into viral filaments after which membrane fusion occurs to cleave the assembled viral particles from the cell membrane. ER, endoplasmic reticulum; RNP, ribonucleoprotein.

glycoproteins cluster in pairs on filamentous virions, which may influence the conformation of the F protein epitope that is the main target for vaccine and mAb development[50]. Another recent discovery includes the possible role of defective viral genomes (DVGs) in influencing disease outcomes. DVGs are truncated viral genomes that are produced during the replication of RSV when the viral polymerase detaches from the template and resumes elongation at a

later point by copying the 5′ end of the newly replicated DNA strand. This replication process results in a shorter viral genome with a possible hairpin-loop structure. DVGs generated from the 5′ end of the virus cannot be transcribed to produce the proteins necessary for replication and therefore require complementation with a helper virus to complete a viral replication cycle. DVGs have a higher propensity to generate double-stranded RNA genomes, which are strong stimulators of innate immunity. The kinetics of DVG accumulation and duration could predict clinical outcome of RSV-A infection in humans and could be used a prognostic tool to identify patients at risk for developing severe disease[51].

Attachment and fusion glycoproteins

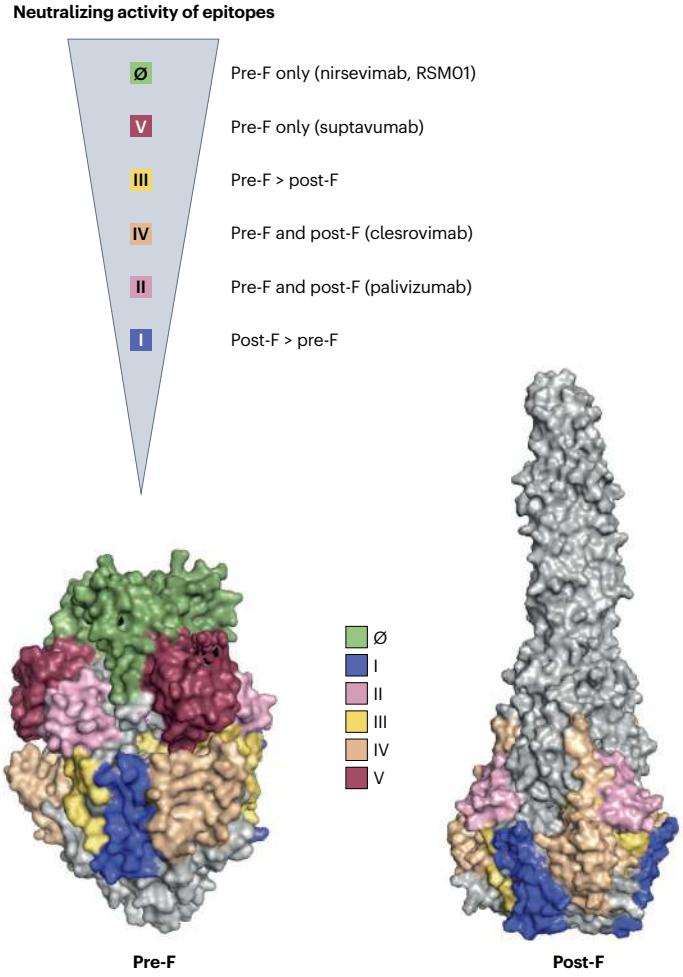
The F protein has a critical role in host cell infection. The inactive precursor F assembles into a trimer that is folding-competent. Protein folding and maturing in the endoplasmic reticulum are subject to quality control after which proteolytic maturation occurs in the Golgi apparatus. The F protein continues to refold irreversibly into the stable post-fusion (post-F) conformation. Productive refolding requires the presence of a target membrane and is triggered by receptor binding[7,52]. The prefusion (pre-F) and post-F conformations offer different challenges for vaccine and mAb design because they have distinct epitopes available to neutralizing antibodies. Whereas the target epitopes of some mAbs are only present in the pre-F state (nirsevimab), others are present in both pre-F and post-F conformation (palivizumab, clesrovimab)[53] (Fig. 3). Earlier vaccines provided insufficient protection as they used the post-F conformation as the vaccine antigen. Stabilization of the pre-F conformation has made it possible to develop effective subunit vaccines[54]. Vaccines and mAbs will be further discussed in the section ‘Clinical interventions’. Crystal structures of neutralizing antibodies bound to the G protein have been published previously[55]. However, the crystal structure of the complete G protein is unknown.

Pre-F can be stabilized by structure-based engineering, such as the introduction of a disulfide bond and cavity-filling mutations[54]. A limited number of unique mutations have been identified that stabilize the pre-F conformation of RSV F and substantially increase expression levels[54]. Structure-based design showed stabilized versions of RSV F that maintained antigenic site Ø when exposed to extremes of pH, osmolality and temperature. On an atomic level, crystal structures of site Ø-stabilized variants of RSV F (including DS, Cav1, DS-Cav1 and DS-Cav1-TriC) have been identified, showing that cysteine residues and filled hydrophobic cavities improve stability[56].

RSV subtypes and genotypes

Historically, RSV has been classified into two antigenically different subtypes, RSV-A and RSV-B, based on variations in the G protein. There are no major differences in disease outcomes, so the importance of RSV-A and B has largely been in terms of epidemiological surveillance. Although antigenic sites of the F protein are generally thought to be well-

Fig. 3 | Crystal structures of the respiratory syncytial virus fusion protein in pre-fusion and post-fusion state and its neutralizing epitopes.



Crystal structures of the respiratory syncytial virus fusion (F) protein in the pre-fusion (pre-F; lower left) and post-fusion state (post-F; lower right) are shown. The neutralizing epitopes are indicated. The crystal structures have been created using PyMOL version 2.5.0. The antigenic sites are depicted at the top of the figure, showing the neutralizing activities of the various epitopes. Two sites (Ø and V) are only present in pre-F, whereas the other sites are present in both conformations. Four sites (Ø, II, IV and V) are currently targeted by monoclonal antibodies (mAbs), as indicated. No mAbs bind to sites I and III. Neutralizing activity is higher for pre-F than for post-F for site III, but higher for post-F than for pre-F for site I. For the other two sites that are present in two conformations (II and IV), the neutralizing activity is comparable for pre-F and post-F. Adapted with permission from ref. 204, Elsevier.

conserved, there are differences between RSV-A and RSV-B. RSV Long was the first strain to be isolated, obtained from a child with bronchiolitis back in 1956. Although its F protein differs by 6 amino acids from the chimeric A2 strain, both belong to the same GA1 clade of RSV-A[57]. The RSV-A subgroup has fewer amino acid changes than RSV-B when compared to the historical RSV-A Long strain[58]. Vaccines and mAb candidates have generally been

developed using the RSV-A Long strain[58]. More variability has been found in the pre-F sites of RSV-B than in the historical RSV-A genotype. Amino acid changes in the antigenic sites occurred at a frequency of >90% (ref. 58). The relevance of RSV subtypes A and B to vaccine development depends on the degree to which the antigenic targets for mAbs or vaccines vary between subtypes. The RSV DS-Cav1-based vaccine seemed to have 2–3-fold higher neutralizing titres against RSV-A than against RSV-B, although the clinical relevance of this finding is not yet clear[59]. Neutralizing antibody responses elicited by a subtype RSV-A-based F protein vaccine had excellent activity against both subtypes, which is probably due to the high degree of conservation of the F protein irrespective of subtype[60]. To ensure that vaccines have equal efficacy against RSV-A and RSV-B, a bivalent vaccine containing pre-F glycoproteins from both RSV subgroups has been developed[61]. Results of phase III trials will ultimately determine the differences in efficacy of individual vaccines against RSV-A and RSV-B.

The two RSV subtypes can be further subdivided into multiple genotypes[62]. To date, RSV-A has been classified into 9 genotypes, whereas 32 genotypes have been described for RSV-B, although as yet there is no clear consensus defining the criteria for genotyping[63,64]. In addition to the existing genotypes, new genotypes appear periodically and sometimes become predominant circulating strains globally[64]. Recent efforts have been taken to harmonize RSV strain nomenclature and classification around the diverse G protein to avoid confusion and thereby better understand the dynamics of RSV transmission worldwide[65-67]. Genetic diversity of the viral genome, and thereby the variability of the F and G surface glycoproteins, has a key role in RSV pathogenesis by mediating immune escape.

RSV entry process and life cycle

Cell entry of RSV is facilitated by the attachment of the G protein to host cell receptors followed by fusion through the F protein (Fig. 2b). Binding of the host cell to both the F protein and the G protein is essential for entry[68-70]. In this process, CX3CR1 (ref. 71), heparan sulfate proteoglycans[72] (HSPGs) and CD14–Toll-like receptor 4 (TLR4)[73] are host cell receptors that bind to the RSV G protein. However, HSPGs are not expressed on the apical surface of ciliated epithelial cells (where RSV initiates infection of human bronchial epithelial cells) or in vivo. Instead, it seems that CX3CR1 is a receptor for the RSV G protein in human bronchial epithelial cultures[70]. In addition, the RSV F protein interacts with insulin-like growth factor-1 receptor (IGF1R)[71], nucleolin (NCL)[71], epidermal growth factor receptor (EGFR)[71,74] and intercellular adhesion molecule 1 (ICAM-1)[75]. These RSV receptors may be targets for mAbs and antivirals. Recently, it was reported that the RSV F protein interacts with the entry receptor IGF1R to stimulate recruitment of the co-receptor NCL. Modest binding of RSV G to IGF1R also occurs[71,76] (Fig. 2b). Similar to the response to other viruses, pattern recognition receptors, including the CD14–TLR4–MD2 complex, are involved at the start of the innate immune response against RSV. A paediatric patient with a homozygous CD14 frameshift

mutation demonstrated that RSV F-mediated activation of the innate immune response is CD14-dependent[73,77].

Two distinct entry pathways exist for RSV: cell surface entry and endosomal entry (macropinocytosis) (Fig. 2a). During macropinocytosis, the RSV F protein undergoes cleavage to become fusioncompetent[78,79]. After fusion, the viral RNA is released into the host cell cytoplasm[80] (Fig. 2b). Transcription and replication take place in the cytoplasm in inclusion bodies[81]. In virus particles and infected cells, the viral RNA is encapsidated by the N protein. Viral synthesis uses an RNA synthesis ribonucleoprotein (RNP) complex consisting of the N, L, P and M2-1 proteins[82]. It is known that transcription in RSV follows a gradient: the extent to which a gene is transcribed falls with its distance from the 3′ promoter. For example, there is quite a difference in the quantity of NS1 mRNA transcribed compared to L mRNA, which is reflective of the transcription gradient[83]. During transcription, an increasing number of L proteins dissociate from the viral genome as they progress in a 3′ to 5′ direction from one transcription unit to another along the genome[84]. The non-structural proteins NS1 and NS2 are produced in high amounts during the early hours of infection and move into the nucleus to regulate transcription through Mediator subunits[48,80]. The glycoproteins F, G and SH move via the endoplasmic reticulum to the Golgi apparatus to be assembled at the plasma membrane. The other proteins transfer via free ribosomes to the cell surface[81]. At the cell surface, RSV assembles into viral filaments (Fig. 2b). F, M, N and P proteins are required for this filament formation with the M protein as the driving force for the assembly of RSV filaments. As a final step, membrane scission cleaves the assembled viral particle from the host cell membrane[85] (Fig. 2b). The RSV F protein initiates fusion in the absence of any other viral component; its presence on the surface of cells is sufficient to initiate fusion, which leads to the formation of syncytia. Clinical signs are mostly a consequence of immunopathogenesis[86,87], as is discussed in the next section. Another way of infecting neighbouring epithelial cells may be through tight junctions[88,89].

HOST AND VIRAL FACTORS RELATED TO RSV IMMUNITY

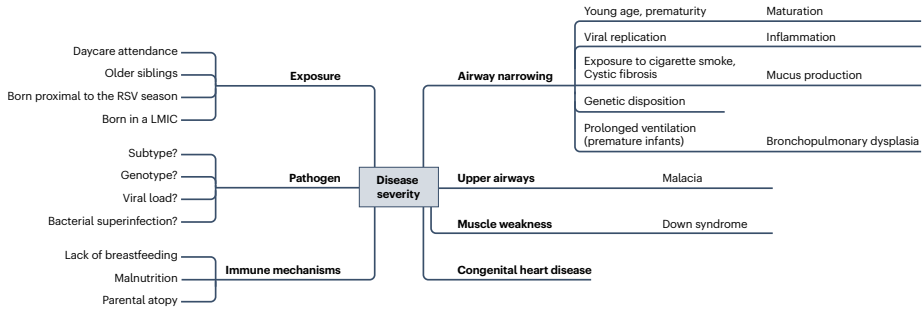
Immune response

The clinical syndrome of RSV bronchiolitis is substantially caused by the host inflammatory response to RSV, and not simply by virusinduced cytopathology. It is likely that this at least partially explains the lack of a clear association between RSV viral loads and clinical severity[90,91]. A combination of clinical, virological and environmental factors may contribute to severe disease in children (Fig. 4). RSV immune responses are short lived: protective antibodies and T cells decline within weeks or months, explaining why RSV re-infections are common[92,93]. Acquisition of maternal antibodies protects against most severe RSV infection

during the first weeks after birth. Passive immunity via transplacental antibodies is critically dependent on gestational age at delivery with little transfer in children born before 28–32 weeks of gestation[94].

The first line of innate immune defence to RSV is essential in reducing disease burden. A necessary first step for the induction of a robust innate immunity response is binding of RSV to the nasal epithelial cells[77,95]. The early inflammatory response is further activated by TLR-2, TLR-3, TLR-4 and TLR-7 (ref. 96) followed by the production of cytokines such as interleukin-6 (IL-6), IL-8 and type I and III interferon (IFN) by alveolar macrophages and epithelial cells. This leads to further recruitment of innate immune cells — especially neutrophils, monocytes and dendritic cells — to the lung[97]. Soluble G protein, NS1 and NS2 inhibit the host type I IFN response, indicating that any of these three proteins may be targeted therapeutically[98].

Fig. 4 | Factors associated with respiratory syncytial virus disease severity in infants.



Key factors include exposure to external factors, pathogen characteristics, immune mechanisms, airway narrowing, conditions of the upper airways, muscle weakness and congenital heart disease. Question marks are factors that have not conclusively been shown to be contributing factors. LMIC, low-income and middle-income country; RSV, respiratory syncytial virus.

Viral evasion of immune responses

Severe RSV bronchiolitis is caused by a delayed, deranged or prolonged innate immune response[97]. Neutrophils have a prominent role in RSV infections and are the most abundant leukocyte cell type to infiltrate the lungs post-infection[99]. They can limit viral replication and spread, as well as stimulate an effective viral immune response[100]. However, the roles of neutrophils in lung injury are complex, with protective and harmful effects at the same time. Neutrophil functions in RSV pathogenesis include the release of proteolytic enzymes into the environment through degranulation, the production of reactive oxygen species, the formation of neutrophil extracellular traps (NETs) by the cell death programme known as NETosis[100] and the stimulation of mucus production. Human challenge studies showed that susceptibility to RSV infection is associated with pre-infection airway neutrophil activation[101]. RSV human challenge studies are further discussed in Box 1.

Box 1

Lessons learned from experimental infection

Controlled human infection model (CHIM) studies have the potential to rapidly advance the development of novel drugs and vaccines. CHIM studies can offer early-stage proof of concept and are therefore contributing to a better understanding of transmission, dose escalation, pathogenesis of immune response and infection-derived immunity. Moreover, CHIM studies enable the investigation of events before symptom onset, enabling comparison of immune activation in the airway at baseline to the early presymptomatic period during the course of infection[101]. CHIM studies have recently become more frequently used in research and development to test the efficacy of antiviral drugs and during the past year to test vaccine candidates[205]. Although CHIM studies have taught us about mucosal inflammation during respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI)[101] and correlates of protection in older adults[206], CHIM studies also enable the generation of efficacy data during early development. This can avoid expensive large-scale trials that could cost as much as US$500 million[207,208]. However, so far, antiviral candidates have not shown much progress after being successful in CHIM studies. Antivirals that were discontinued despite success in CHIM studies include the fusion inhibitor presatovir (GS-5806) in a phase II trial[209], the nucleoside analogue ALS-008176 (ref. 210) and the fusion inhibitor ALX-0171 in a phase IIb trial[123]. Only EDP-938 is still in clinical development after demonstrated efficacy in CHIM[129]. Currently, six RSV vaccine and monoclonal antibody candidates have been tested in CHIM: RSVPreF vaccine[211], Ad26.RSV.preF/RSV preF vaccine[212], MV012-968 vaccine[213], MVA-BN-RSV vaccine[214], clesrovimab[215] and palivizumab biosimilar[216]. However, CHIM studies are limited by their study population as they cannot be conducted in the target population for ethical reasons. Another drawback mentioned by the World Health Organization (WHO) is that drug efficacy is tested against a single challenge strain[217]. Only RSV-A strains are used in CHIM studies, leaving the question unanswered whether drugs and vaccines will be effective against RSV-B strains[59,60]. Moreover, CHIM studies can only cause infections that lead to upper respiratory illness, thus making it impossible to examine how lower respiratory disease develops and how it may be prevented[218]. A final disadvantage of CHIM trials is that antiviral administration is often initiated as soon as respiratory samples are positive for RSV (detected via PCR with reverse transcription (RT-PCR)), which is often hours following infection and well before any signs of clinical symptoms. Therefore, the experimental set-up does not always accurately reflect the clinical situation. Although RSV CHIM studies have clearly contributed to our knowledge of RSV immunity, the added value of CHIM studies for the successful development of RSV interventions remains to be confirmed.

Innate immune responses to RSV are involved both in the onset of severe disease and in convalescence. Most patients recover before a full adaptive immune response has been mounted[93]. Lack of type 1 immunity during acute infection has been associated with RSV LRTI severity[102,103]. Naturally induced T cell immunity against RSV protects against re-infections, which are mostly mild[97]. Nevertheless, even in the absence of antigenic variation, RSV can induce recurrent symptomatic LRTI. The role of antibodies in the development of RSV re-infection is insufficiently understood. It is possible that non-neutralizing antibodies may sterically hinder neutralizing antibodies from binding to RSV[104]. Human challenge studies showed that low mucosal IgA titres are predictive of susceptibility to RSV LRTI[101,105].

MICROBIAL INTERACTIONS

The respiratory tract microbiota has been linked to the pathogenesis of RTIs. During RSV bronchiolitis, the local microbiome seems to have an immunomodulatory role. Specific microbiota profiles seem to be associated with RTI susceptibility. For example, airway microbiome maturation in the first 2 months after birth is associated with increased expression of IgA, polymeric immunoglobulin receptor for secretory IgA (PIGR) and HLA-II, which

could all be related to protection against RSV infection during infancy[106,107]. The use of next-generation sequencing enables a better understanding of the possible functions of the microbiome in the susceptibility to respiratory infection[106,108]. The development of the respiratory microbiome depends on several host and environmental factors, including mode of birth, feeding type, antibiotic treatment, presence of siblings and day-care attendance. In turn, specific microbiota profiles in the upper respiratory tract have been associated with severity of RSV disease[106]. The upper respiratory tract is a complex ecosystem that comprises bacterial, viral and fungal pathogens[108,109]. Early dysbiosis may have a role in the causal pathway leading to RSV LRTI[108,109].

Several respiratory viruses can co-circulate at the same time and can concurrently or sequentially infect the respiratory tract, which leads to so-called viral interference. A negative association between RSV and co-detection of other respiratory viruses has been observed, which suggests that suppressing RSV infection by RSV mAbs might increase the risk of other viral infections[110-112]. Positive virus–virus interaction might also result in increased disease severity, as shown for co-infection by RSV and human metapneumovirus[113].

A new paradigm in respiratory infection is the possible correlation between RSV and *Streptococcus pneumoniae*, a commensal organism in the RTI but considered invasive when found in the blood or spinal fluid. Clinical observations of the respiratory microbiological ecosystem indicate that RSV may alter the infant nasopharyngeal microbial profile[106]. An epidemiological correlation between RSV and *S. pneumoniae* is supported by common seasonality in the population[114] as well as the parallel disappearance of RSV and pneumococcal disease during the COVID-19 pandemic[115,116]. RSV affects the host susceptibility to pneumococcal disease by enhancing pneumococcal adherence[117]. The causal relationship between RSV and pneumococcal infection will remain uncertain until probe studies have been performed, such as the pneumococcal vaccination trial in South Africa[118]. Mouse studies demonstrated that infection with pneumovirus, the mouse RSV analogue, results in increased pneumococcal density in the nasopharynx of infected animals and increased transmission to contact animals[119]. Unravelling the complex interaction between RSV and *S. pneumoniae* is needed to understand the full potential impact of vaccine strategies against both pathogens as well as potential synergy between these strategies.

CLINICAL INTERVENTIONS

Therapeutics

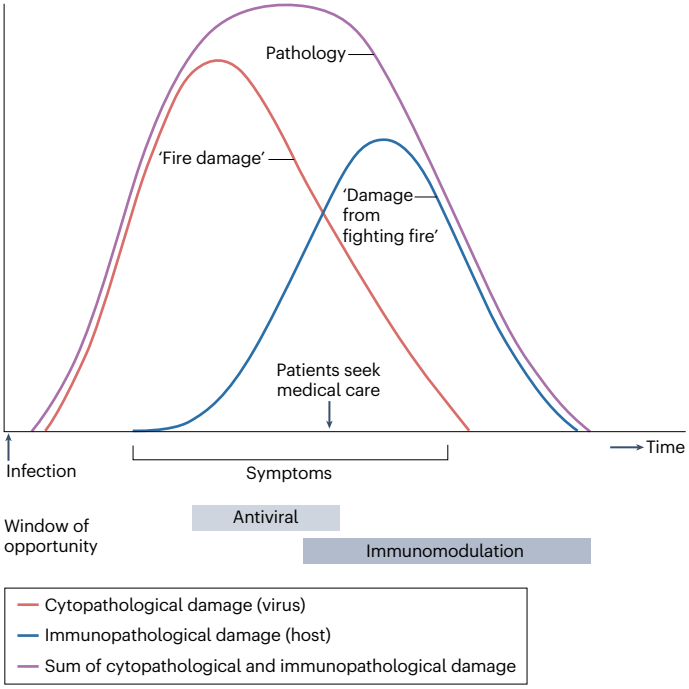
Ribavirin is a nucleoside analogue and is the only licensed antiviral therapy available for the treatment of RSV infections. Its use is highly limited to life-threatening RSV LRTIs in immunocompromised patients. Safety and financial concerns often overshadow the potential benefits of ribavirin, limiting its routine clinical use. In addition to its high cost and complex

delivery system, the efficacy of ribavirin has been controversial as shown in a number of studies with conflicting results[120-122]. Early diagnosis and antiviral treatment are essential for developing novel RSV antiviral drugs as demonstrated by the nebulized therapeutic nanobody ALX-0171, which is directed against the F protein. In a phase II trial, ALX-0171 reduced viral replication but did not improve clinical symptoms in infants with RSV bronchiolitis[123]. Future home-based point-of-care tests may offer parents a solution to diagnose RSV on the first day of symptoms, in time to seek care and obtain antiviral treatment. Similar to oseltamivir treatment for influenza, it is likely that antivirals probably need to be administered within 24–48 h after onset of symptoms to improve the outcome of RSV LRTI during infancy[124]. An optimal strategy for RSV treatment might comprise administration of antivirals within 24–48 h after onset of symptoms in combination with immunomodulatory therapy (Fig. 5). RSV causes direct cytopathology ('fire damage') during the initial phase of viral replication by epithelial destruction of the small airways[100]. The host immune response to RSV ('damage from fighting fire') is a secondary reaction by neutrophils and other innate immune cells to protect the airways, but at the same time causing collateral tissue damage. In addition to damage to epithelial cells, neutrophils contribute to airway obstruction by inducing mucus production. Mucus forms a protective barrier to viral infection by limiting access of viral particles to the pulmonary epithelium[100]. The result of virus-mediated pathology and immunopathology defines the course of disease. If early antiviral treatment is not possible, immunomodulatory treatment, such as combined neutrophil-regulating therapeutic agents, under an umbrella of antivirals might be an effective alternative to ameliorate the outcome of RSV infection[125]. The rationale for the relative timing is that during the early course of RSV infection, virus replication is the leading principle that could still be interrupted by antiviral treatment. However, the subsequent immune cascade that results in clinical disease may be impossible to interrupt[124]. The outcome of RSV may be improved by host-targeted interventions. Local or systemic glucocorticosteroids are not effective[126]. Being the dominant cell type, neutrophils are important targets for intervention. Chemotaxis, phagocytosis, radical oxygen species production and NET formation are neutrophil-related pathways that might be blocked under an umbrella of antivirals to decrease disease severity[100].

Most RSV antiviral drugs currently in clinical development are fusion inhibitors. Fusion inhibitors target viral epitopes or cell receptors to prevent viral binding, fusion and entry. One antiviral, ziresovir (AK0529), is close to licensure in China after positive results from a phase III trial were announced in April 2022 (company press release)[15]. In the AIRFLO trial, ziresovir significantly reduced bronchiolitis symptoms ($P = 0.002$) and viral load ($P = 0.006$) in hospitalized infants after 2 days of treatment[127]. Sisunatovir (RV5421) is another fusion inhibitor that has progressed into phase IIa after a successful human challenge study[128]. EDP-938, which is a non-fusion inhibitor that modulates the N protein, has been recently studied in a phase II trial following a successful human experimental challenge study[129]. Recently, EDP-938 failed in a phase IIb trial[130]. However, the evaluation of EDP-938 in high-risk popu-

lations in clinical studies is still ongoing as EDP-938 demonstrated good antiviral activity in vivo and a favourable safety profile (company press release)[130]. EDP-323 is a novel oral L-protein inhibitor that potently blocks RSV replication in preclinical models. This promising non-nucleoside inhibitor recently moved into a phase I trial. First results are expected in the second quarter of 2023 (unpublished data; see ref. 131).

Fig. 5 | Combined antiviral and immunomodulatory therapy for the treatment of respiratory syncytial virus infections.



Respiratory syncytial virus (RSV) causes direct damage ('fire damage') early on during infection by causing inflammation of the small airways. The host immune response to RSV ('damage from fighting fire') is a secondary reaction by neutrophils and other innate immune cells to protect the airways, but at the same time causing collateral tissue damage. In addition to damage to epithelial cells, neutrophils contribute to airway obstruction by inducing mucus production. Mucus forms a protective barrier to viral infection by limiting access of viral particles to the pulmonary epithelium. This is often associated with the onset of symptoms and patients seeking medical care. The combination of virus-mediated pathology and immunopathology defines the course of disease. An optimal strategy for RSV treatment might comprise administration of antivirals within 24–48 h after onset of symptoms in combination with immunomodulatory therapy.

After initial promising steps in clinical development, the fusion inhibitor rilematovir has recently been suspended in a phase III trial, potentially because it was not sufficiently effective in clinical trials[132]. Similarly, the development of lumicitabine (ALS-8176), a nucleoside analogue, was also discontinued in 2018 because of toxicity concerns in phase IIb trials (company press release)[133]. Two novel nucleoside analogues are currently being investigated as inhibitors of RSV polymerases. Molnupiravir (EIDD-2801/MK-4482-017) is being tested

in a phase IIa controlled human infection model study[134] and 4′ fluorouridine (EIDD-2749) showed oral efficacy against RSV in in vivo preclinical studies[135]. At the moment, only one antiviral seems to have finished clinical development successfully, but more detailed trial data are needed before the broad introduction of ziresovir is feasible.

Candidate RSV entry inhibitors are at risk to lose therapeutic benefit because of rapidly emerging viral resistance. An effective mechanism of secondary RSV resistance has been reported in which escape mutations accumulate in genes encoding RSV F microdomains that govern the structural stability of the pre-F complex. Resistance against entry inhibition may affect the impact of viral entry inhibitors currently considered for clinical use, and therefore a proactive design for future RSV drug discovery campaigns is required[136].

Preventive strategies

We have reviewed the mAb and vaccine pipeline previously[137]. We have complemented this work with this summary of recent developments and challenges that have arisen from the different prevention programmes (Fig. 6). Thirty years ago, the first approach towards passive immunization for prevention of RSV LRTI was RSV-IVIG (RespiGam), an intravenous administration of polyclonal gamma globulins[138,139]. RSV-IVIG was voluntarily withdrawn from the market in 2004 due its contraindication in children with hemodynamically significant heart disease because of safety concerns[140]. The development of humanized antibodies against the RSV surface glycoproteins moved forward with the intention to increase specificity and to improve potency compared to polyclonal RSV antibodies. In 1998, palivizumab (Synagis, Astra- Zeneca) was approved for high-risk children. Real-world evidence has confirmed trial evidence that palivizumab immunoprophylaxis is associated with low incidence of severe RSV infection[141,142]. Palivizumab is an IgG1 mAb directed to antigenic site II of both pre-F and post-F forms; it has a half-life of 28 days and so is administered monthly during the RSV season. Owing to high costs, palivizumab is only used in high-risk preterm infants in HIC settings[143]. Thus, the search for more potent, less expensive and longer-lasting mAbs continued. Increased efforts in identifying the structure of the F protein have resulted in the identification of potent neutralizing epitopes, including antigenic sites Ø and IV[144]. Motavizumab was the first second-generation mAb derived from palivizumab, also targeting site II of the F protein. Although motavizumab looked promising in clinical trials, its development was discontinued after a negative FDA evaluation because of mild skin reactions and lack of superiority over palivizumab[145]. The development of suptavumab was discontinued after an unsuccessful phase III trial because of resistance due to a mutation in the antibody-binding epitope in all RSV-B strains[146]. Recently, nirsevimab has been approved for prevention of RSV in infants[12]. Nirsevimab (MEDI8897) has high neutralizing activity and extended half-life compared to palivizumab because of its YTE amino acid substitutions. Nirsevimab targets the conserved antigenic site Ø of the F protein, which is unique to pre-F and more sensitive to neutralization than antigenic site II, which is targeted by palivizumab and motavizumab[147]. Nirsevimab

showed 70% and 78% efficacy against medically attended RSV LRTI and RSV hospitalization, respectively, in preterm infants with gestational ages between 29 and 34 weeks[148]. These results were expanded through a trial in late-preterm infants ≥35 weeks gestational age and term infants showing similar efficacy rates of 75% and 62%, respectively[149]. The safety profile of nirsevimab is comparably favourable to that of palivizumab (company press release)[150]. In May 2022, a pre-specified pooled analysis of phase IIb and IIII data demonstrated 79.5% efficacy against medically attended LRTI including hospitalizations (company press release)[151] followed by a positive assessment by the EMA. Another mAb candidate that binds to site Ø is RSM01, currently in phase I clinical development by the Bill and Melinda Gates Medical Research Institute (Gates MRI), a non-profit organization aiming to develop affordable prophylaxis for LMICs.

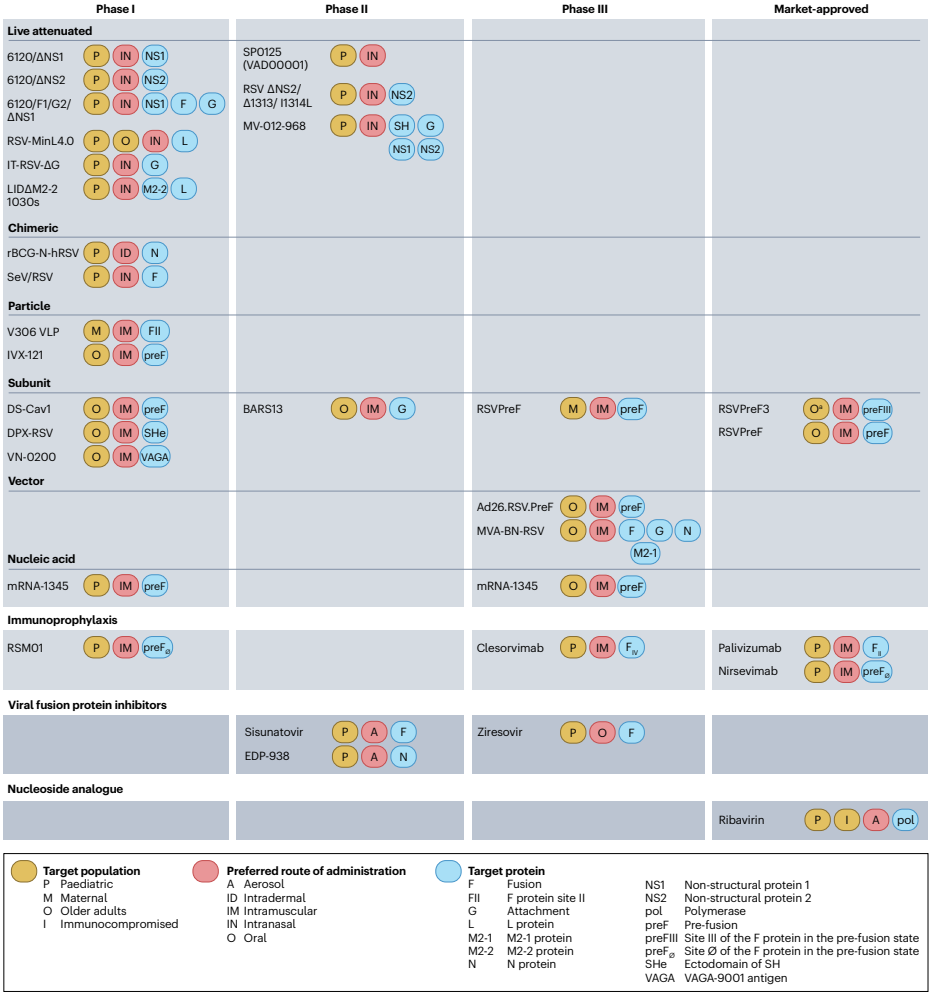
Clesrovimab (MK-1654) is a promising late-stage mAb and shares the YTE mutation with nirsevimab, but binds to the highly conserved site IV of the F protein. Clesrovimab is derived from a human clone without additional mutations made to the antibody-binding site (unpublished data; see ref. 152). An in vitro study showed high potency against RSV-A and RSV-B clinical isolates and low threshold to resistance, but efficacy is largely unknown with only phase I adult data published to date[153,154]. A phase Ib/IIa study showed 80.6% efficacy against medically attended RSV LRTI (unpublished data; see ref. 152). Clesrovimab is currently being studied in a phase III trial in children with enrolment of its final participant during the summer of 2025 (ref. 155).

The development of viral escape is a potential risk of the widespread use of mAbs[156,157]. In vitro data showed that mAb-resistant mutants may develop under selective pressure with nirsevimab but without a significant impact on viral replication. In addition to the development of viral escape mutants after the introduction of a mAb, subpopulations may exist with natural resistance (natural polymorphisms) as was reported for palivizumab[158]. In 2017, the clinical development of suptavumab (REGN2222) directed against antigenic site V of the F protein was discontinued after a phase III trial in 18 countries globally. A single spontaneous mutation in the suptavumab-binding epitope of RSV-B isolates (L127Q and S137L) resulted in a 2-amino acid substitution that resulted in complete resistance[146]. For nirsevimab, it was recently demonstrated that escape mutants were rare and have not increased over time[159]. A combination of mAbs could be a risk mitigation strategy to prevent the development of resistance but, thus far, no company has started to develop such a strategy.

To date, 22 vaccine candidates are in clinical development using six different approaches: live attenuated, recombinant vector, subunit, particle-based, chimeric and nucleic acid-based[160]. Understanding modern-day vaccines requires use of lessons learned from past failures. A disastrous clinical trial of an inactivated RSV vaccine study conducted among infants in the 1960s showed that the vaccine did not reduce RSV infection rates. Vaccine recipients were more likely to be hospitalized for RSV bronchiolitis, and two children even died[160]. The vaccine induced exaggerated inflammatory responses, which led to more severe bronchiolitis

caused by aberrant cell-mediated immune responses during subsequent natural infection[92,161]. As vaccine development picked up speed, some late-phase clinical trials were halted[162]. This includes a maternal F protein recombinant nanoparticle vaccine that was discontinued after a phase III trial as it failed to reach its primary end point[163,164]. Development of a subunit maternal vaccine was halted for safety reasons, which have not yet been made available (company press release[165]).

Fig. 6 | Preventive and therapeutic pipeline for the treatment of respiratory syncytial virus infections.



Vaccine candidates, monoclonal antibodies and antivirals currently in clinical trials are categorized in the following categories as of 1 June 2023 (ref. 160): live attenuated vaccines, chimeric vaccines, particle vaccines, subunit vaccines, vector vaccines, nucleic acid vaccines, immunoprophylaxis, viral fusion protein inhibitors and nucleoside analogues. Preventive measures are shown in light grey, therapeutics in dark grey. aRSVPreF3 has been halted for the maternal population (M) and has only been approved for older adults (O). The target protein for VAD00001 is not known.

Four vaccine candidates are currently in phase III clinical trials for the older adult and maternal populations. The development of subunit vaccines is most advanced. These vaccines induce high levels of neutralizing antibodies with a favourable pre-F to post-F ratio. Post-F antibodies may sterically hinder binding of neutralizing pre-F antibodies[104]. The FDA has approved two subunit vaccines for RSV for older adults after positive results from two different international phase III trials (company press release[166])[61], a milestone that has eluded vaccine developers for almost 60 years. The RSVPreF3 vaccine prevented RSV-related LRTI disease in adults ≥60 years of age and had an acceptable safety profile[167]. This is the first RSV vaccine to be approved anywhere in the world (company press release[9])[8]. The bivalent RSVPreF vaccine prevented RSV-associated LRTI and acute respiratory infection in adults ≥60 years of age, and no safety concerns were identified[61]. RSVPreF was approved recently by the FDA for prevention of LRTI of RSV in older adults (company press release)[10]. The same pre-F subunit vaccine has also been tested in a phase III maternal vaccination trial, which demonstrated that the vaccine administered during pregnancy was effective against RSV-associated LRTI in infants, without any safety concerns[13]. The RSVPreF vaccine for pregnant individuals is awaiting approval, likely coming this summer. A limitation of the maternal vaccination strategy is that it has reduced benefit for premature infants as antibody transfer only reaches peak levels towards the end of the third trimester. Two vector candidates are in phase III clinical development for older adults. The Ad26.RSV.preF/RSV preF protein combination vaccine elicits both humoral and cellular immune responses without requiring an adjuvant. Although currently in phase III trials, the previous phase II trials demonstrated protection in a human challenge model, with an acceptable safety profile[168,169]. The phase IIb CYPRESS study demonstrated durable vaccine efficacy of >70% for Ad26.RSV. preF/RSV preF over three RSV seasons in the prevention of RSV LRTI in older adults (unpublished data; see refs. 170,171). Preliminary data suggest that the antibody response of the combination vaccine was more polyclonal than that for Ad26.RSV.preF or RSV preF protein alone (unpublished data; see ref. 172). MVA-BN-RSV uses a non-replicating modified vaccinia Ankara poxvirus vector to express the F, G, N and M2 proteins. The vaccine induced a robust cellular immune response and is well tolerated[173-175]. mRNA-1345 is a lipid nanoparticle-encapsulated mRNA-based vaccine encoding the RSV F glycoprotein stabilized in the pre-F conformation. The technology is leveraging the success of the COVID mRNA vaccination programmes. Early in 2022, a phase III trial to evaluate mRNA-1345 trial was initiated (company press release)[176,177]. Recently, mRNA-1345 demonstrated vaccine efficacy of 83.7% against RSV lower respiratory tract disease, defined by at least two symptoms (such as cough and fever), in older adults, with no safety concerns[14]. Live attenuated vaccine (LAV) candidates have the potential of inducing durable neutralizing antibodies comparable to the primary response to RSV[178]. LAVs are in early-stage trials. Most LAVs have been attenuated by deleting one or more genes, including the genes encoding SH, NS2, M2-2 and G. Advantages of LAVs include safety, needle-free administration in the form of alternative cutaneous or mucosal

immunization, and a broad immune response[179,180]. The durability of vaccine-mediated protection is uncertain. Therefore, older adults may need annual vaccination, and pregnant people are likely to require vaccination during every pregnancy. Extension of ongoing pivotal trials will define the duration of protection of vaccines submitted for licensure. Although a handful of vaccines are currently in late-stage clinical development, vaccines may not always be effective in immunocompromised individuals. And although the outcome of RSV infection in these patients may not always be unfavourable, treatment options are needed for these populations.

OUTLOOK

The unmet need for safe and effective RSV interventions has led to the development of several promising candidates, including mAbs, vaccines and antivirals. Combined paediatric and maternal strategies may be considered for the prevention of RSV infections for infants. To prevent escape mutations, a mAb cocktail targeting different epitopes, such as nirsevimab and clesrovimab, might be considered[146]. The EMA has approved nirsevimab, which offers prevention of RSV infections for all infants and not only targeting those at risk[12]. Following large-scale RSV infection prevention in infants, the possibility of indirect protection of elderly individuals through herd protection should be studied. Continuous monitoring of global RSV strains and their sequence variability is important for the detection of new strains and possibly antigenic changes or escape mutants that may affect the effectiveness of prophylaxis. Although LMICs have a higher RSV burden than HICs, most have limited RSV surveillance to inform future immunization programmes[181]. The World Health Organization (WHO) has now established RSV surveillance using the existing Global Influenza Surveillance and Response System (GISRS) platform[182]. Current candidate mAbs are targeted primarily for HICs, unless solutions such as tiered pricing will be considered. Given the rapid development of both RSV mAbs and maternal vaccination, LMICs may consider whether and how they can introduce RSV immunization within the next few years. A less costly approach for LMICs could be found in RMS01, a mAb targeting site Ø of the pre-F of RSV[183]. Gavi, the Vaccine Alliance, included maternal vaccines and RSM01 as part of its 2018 vaccine investment strategy and may support these products subject to licensure in LMICs[184].

A final challenge arising from the changes in RSV epidemiology due to the COVID-19 pandemic is the timing of introduction of immunization programmes. It is unclear whether mAbs with extended half-life should be administered at birth or at the start of the expected RSV season. Passive immunization of newborn infants may be performed seasonally in countries with active RSV surveillance programmes and defined patterns[185]. The universal roll-out of nirsevimab may result in a delayed exposure to RSV infection in newborn infants similar to what we have seen during the COVID-19 pandemic: symptomatic infection is delayed

beyond the first year after birth. Although seasonality of RSV informs a critical component of the nirsevimab immunization strategy, nirsevimab enables the protection of infants during their window of vulnerability. Despite its extended half-life, nirsevimab is not expected to protect infants beyond their first RSV season. Administration of nirsevimab should therefore be carefully timed so that its protection period covers the complete RSV season. Modelling studies have suggested that in countries where RSV causes annual epidemics, seasonal immunization with mAbs would be more cost-effective than year-round immunoprophylaxis shortly after birth. However, implementing seasonal immunization programmes will be challenging if RSV activity remains unpredictable. We expect RSV transmission will soon return to the seasonality seen before the COVID-19 pandemic[186]. As maternal vaccines provide protection for several months after birth, this implies that year-round vaccination may be the best strategy, but this requires further evaluation[185].

Recently, concerns were raised about the association of the pre-F vaccines with the induction of Guillain–Barré syndrome in clinical trials, albeit yet unproven to be vaccine-associated and at very low frequency. RSV vaccines have had enormous difficulties in reaching safe and effective endpoints, and the current optimism and excitement might need to be tempered in view of the current FDA announcements[187].

In conclusion, the landscape of RSV therapeutics has improved substantially since the discovery of the pre-F conformation of the RSV F protein. A mAb for all infants and two vaccines for older adults have just approached market access and are likely to be followed by approval of other vaccines for use in older adults and pregnant people. Other mAbs and RSV vaccines will soon follow thereafter. Although the development of RSV treatment is lagging, one fusion inhibitor may soon be licensed following successful clinical development. With exciting times ahead of us, we can now hopefully make a big step towards reducing the global burden of RSV.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

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Chapter 3

The Burden of Respiratory Syncytial Virus in Healthy Term-Born Infants in Europe: A Prospective Birth Cohort Study

"RESCEU"

The Lancet Respiratory Medicine, 2022

"Look at the stars
look how they shine for you
and everything you do
yeah, they were all yellow

I swam across
I jumped across for you
oh, what a thing to do
'cause you were all yellow."

— Yellow, Coldplay

The burden of respiratory syncytial virus in healthy term-born infants in Europe: a prospective birth cohort study

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SUMMARY

Background Respiratory syncytial virus (RSV) is a major cause of hospitalisation in infants. The burden of RSV infection in healthy term infants has not yet been established. Accurate health-care burden data in healthy infants are necessary to determine RSV immunisation policy when RSV immunisation becomes available.

Methods We performed a multicentre, prospective, observational birth cohort study in healthy term-born infants (!37 weeks of gestation) in five sites located in different European countries to determine the health-care burden of RSV. The incidence of RSV-associated hospitalisations in the first year of life was determined by parental questionnaires and hospital chart reviews. We performed active RSV surveillance in a nested cohort to determine the incidence of medically attended RSV infections. The study is registered with ClinicalTrials.gov, NCT03627572.

Findings In total, 9154 infants born between July 1, 2017, and April 1, 2020, were followed up during the first year of life and 993 participated in the nested active surveillance cohort. The incidence of RSV-associated hospitalisations in the total cohort was 1·8% (95% CI 1·6–2·1). There were eight paediatric intensive care unit admissions, corresponding to 5·5% of 145 RSV-associated hospitalisations and 0·09% of the total cohort. Incidence of RSV infection in the active surveillance cohort confirmed by any diagnostic assay was 26·2% (24·0–28·6) and that of medically attended RSV infection was 14·1% (12·3–16·0).

Interpretation RSV-associated acute respiratory infection causes substantial morbidity, leading to the hospitalisation of one in every 56 healthy term-born infants in high-income settings. Immunisation of pregnant women or healthy term-born infants during their first winter season could have a major effect on the health-care burden caused by RSV infections.

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Research in context

Evidence before this study We searched PubMed, using the terms "RSV" or "respiratory syncytial virus", "hospitalisations", and "infant" or "first year of life", on May 31, 2022, for studies published between Jan 1, 1993, and May 31, 2022, with no language restrictions. The results, 4957 articles, included mostly retrospective analyses of RSV-coded hospitalisations from health registries or prospective studies conducted in a single country. These studies emphasised the large morbidity and mortality burden in young children associated with RSV. In a systematic review and meta-analysis from The Lancet, RSV was estimated to be associated with 3·6 million hospitalisations for acute lower respiratory infections and 101 400 in-hospital or out-of-hospital deaths in children younger than 5 years, annually, worldwide. A gap exists in the knowledge of the RSV burden in healthy term infants, the largest population of RSV-infected infants. We identified ten birth cohort studies that reported RSV-associated hospitalisation in infants with estimates varying between 0·6% and 5%. These birth cohorts had relatively small sample sizes with 156 to 1143 participants, and only five included only healthy term-born children. The reliability and the precision of these estimates can be improved by large prospective birth cohorts conducted in multiple countries. Several maternal vaccines and passive immunisation against RSV are currently at advanced stages of clinical development or under review for licensure. To decide how these new prevention strategies should be included in national vaccination programmes, precise estimates of the health-care burden of RSV infections in the first months of life are required.

Added value of this study The RESCEU birth cohort study is the largest multicentre prospective birth cohort that evaluated the incidence of RSV-associated hospitalisations and medically attended acute respiratory infections. It was designed to provide a precise and up-to-date estimate of the total RSV incidence and health-care burden in Europe. Almost 10 000 participants were enrolled in five European countries and 97% were successfully followed up during the first year of life. To estimate the incidence of medically attended RSV infections, we actively followed up a nested cohort of approximately 1000 participants. The incidence of RSV-confirmed hospitalisations in the first year of life was 1·8% (95% CI 1·6–2·1). About half of hospitalisations for respiratory tract infection in the first year of life were associated with RSV. The majority (57·9%) of RSV-associated hospitalisations occurred in children younger than 3 months. The incidence of medically attended RSV infections was 14·1% (12·3–16·0).

Implications of all the available evidence This study provides the precise estimates of the health-care burden of RSV required to decide on future RSV immunisation programmes. The health-care burden of RSV among healthy infants is considerable in Europe, with one in 56 healthy term-born infants hospitalised for RSV infection annually. As the incidence of severe RSV infection is highest in the first months of life, maternal vaccination as well as passive infant immunisation could have a major effect on the health of healthy term infants.

INTRODUCTION

Respiratory syncytial virus (RSV) causes a substantial burden of disease in infants worldwide with an estimated annual mortality of 101 400 in children younger than 5 years.[1] Although more than 97% of RSV-attributable deaths occur in low-income and middle-income countries, the health-care burden of RSV infection in high-income countries is considerable, with an estimated annual hospitalisation rate of three per 1000 children younger than 5 years in the USA.[2] Passive immunisation against RSV with palivizumab is available for high-risk groups, including premature infants and children with congenital heart disease or bronchopulmonary dysplasia. Because the majority of children hospitalised with RSV have no pre-existing conditions, a high morbidity is seen in infants younger than 6 months despite the availability of palivizumab.[2] Various maternal vaccine and passive immunisation trials, which aim to protect all infants in the first months of life, are currently in phase 3 or submitted for regulatory approval.[3–5] Expectations are that within 1–3 years one or several of these products will be approved by regulatory authorities and governments will have to decide whether these newly available prevention strategies should be implemented into their national immunisation schedule.[6] Accurate information about RSV health-care burden in healthy infants is essential for decision makers to evaluate the health and economic benefit of these new prevention strategies.

Most large studies that aimed to determine RSV-associated hospitalisation rates in young children included children with comorbidities, were country-specific, and partly based on estimates instead of actual numbers.[2,7,8] Birth cohort studies estimate disease incidence more accurately, but previous prospective birth cohorts in healthy infants were relatively small (158–1143 participants) and done in one centre or country, restricting generalisability.[9–18] To our knowledge, the largest prospective birth cohort determining RSV burden was a South African, single-centre study that reported 54 RSV-associated hospitalisations in 1143 children (17% with comorbidity) in the first 2 years of life.[13] To prepare for the introduction of RSV immunisation, the Respiratory Syncytial virus Consortium in Europe (RESCEU) international consortium was funded by the EU Commission to obtain accurate data on the incidence and long-term consequences of RSV infection in healthy term infants.

The primary objective of this study was to determine the incidence of medically attended and hospitalised RSV-associated respiratory infections in healthy term infants in Europe. Secondary objectives included estimating the incidence of symptomatic RSV infections, the incidence of all-cause respiratory infections, and the proportion of respiratory infections attributable to RSV.

METHODS

Study design

The study design and protocol have been described previously.19 In short, healthy term-born infants were enrolled at birth between July 1, 2017, and July 31, 2020, in five sites each located in a di!erent European country representing western, northern, and southern Europe (Spain, Finland, England, Scotland, and the Netherlands). Children born at 37 weeks or more of gestation with no evidence of significant cardiovascular, respiratory, renal, gastro intestinal, haematological, neurological, endocrine, immunological, musculo skeletal, oncological, or congenital disorders were considered healthy term-born.[18] All participating children were followed up for at least 1 year. Children diagnosed with comorbidities later were not systematically excluded. We used parental question naires to screen for hospi talisation for acute respiratory infection (ARI) during the first year of life at the age of 1 year. Hospital records, including RSV testing results, were retrospec tively assessed in case of hospitalisation for ARI. All partici pating hospitals tested for RSV during the RSV season as part of standard care and were situated in a distinct geographical area to ensure that children were preferen tially referred to that hospital if inpatient care was needed. For infants whose parents did not complete the 1-year questionnaire, hospital records were screened for ARI hospitalisations within the first year of life in participating hospitals.

At enrolment at all five sites, participants of the birth cohort were also invited to participate in a nested cohort (referred to as active surveillance cohort). Participants of the birth cohort and the active surveillance cohort were recruited on a voluntary basis and therefore were a convenience sample of term-born children living in the catchment area of the sites. To obtain a cohort with evenly distributed months and years of birth over the recruitment period, sites were instructed to recruit 15–20 participants per week, including two participants in the active surveillance cohort. Enrolment in the active surveillance cohort continued until the planned sample size was reached in each site (200 per site). Infants were actively followed up until their first birthday during the RSV seasons of 2017–18, 2018–19, and 2019–20. Between Oct 1 and May 1 (or longer if RSV was still circulating), parents were contacted weekly to report ARI symptoms of their child. In case of an ARI, a study visit was planned within 72 h of notification to obtain a nasal swab for RSV testing. Parents completed a diary with respiratory symptoms and health-care usage for 14 days after symptoms onset.18 Written or electronic informed consent was obtained from the parents of all study participants.

RSV detection in active surveillance cohort

At all sites, a nasal sample was collected during each ARI episode by using minitip flocked swabs (FLOQSwab, Copan Diagnostics, California, USA), and directly stored in viral transport medium (MicroTest M4RT [Remel, 3 mL]). All samples were stored at –80°C. After the end of the study, all samples were tested with in-house RSV quantitative reverse transcription

PCR (RT-qPCR; appendix p 2).[20,21] In addition, a point of care test (POCT, Alere i RSV assay [Alere, Waltham, MA, USA]) was performed at the time of sample collection at the three sites in Spain, England, and the Netherlands. If the infant had an RSV-positive ARI episode, POCT was not performed during further ARIs. An RSV-positive ARI episode was defined as a positive test result from either in-house RT-qPCR or POCT, or both.

Outcomes and statistical analysis

An ARI episode was defined as the onset or worsening of any of the following symptoms for at least 1 day: runny or blocked nose, coughing, wheezing, or dyspnoea.[19] Episodes were associated with RSV if a POCT or in-house PCR test was positive for RSV. Samples taken more than 10 days after onset were excluded from analysis. Medically attended ARI were defined as ARI episodes with at least one visit to a health-care provider (outpatient clinics, emergency department visits, general practitioner visits) or hospitalisation. RSV-associated hospitalisations, RSV-associated ARI, and medically attended RSV-associated ARI were reported as incidence (ie, the proportion of infants experiencing the event at least once during their first year of life) and as incidence rate per 1000 infant-months (number of events per 1000 infant-months of follow-up). The use of incidence rates in addition to incidence was pre-defined in the statistical analysis plan to account for possible variation in follow-up time due to early dropouts of participants and for participants experiencing outcomes more than once (appendix pp 17–44). Wheezing during the first year of life was defined as at least one wheezing episode reported by parents in the 1-year questionnaire.

Statistical analyses were performed according to the predefined statistical analysis plan (appendix pp 17–44). For sample size calculation of the total cohort, a yearly incidence of hospitalisations of 0·7% was assumed on the basis of previous literature.[2,22] A sample size of 8700 would produce a two-sided 95% Clopper-Pearson CI with a half-width of 0·2% for this incidence. If accounting for 10% loss to follow-up 10 000 infants were to be included.[19] Similarly, a sample size of 1000 infants was estimated for the active surveillance cohort, which would produce a two-sided 95% Clopper-Pearson CI with a half-width of 2%, for an assumed incidence of medically attended ARI of 10%.[2,9,22] Baseline characteristics and clinical parameters were summarised by frequency and percentage for categorical variables and mean (SD) or median (IQR) for continuous variables. Baseline characteristics were compared between groups using χ^2 tests for categorical variables, Student's *t* tests for normally distributed continuous variables and Mann-Whitney U tests for not normally distributed continuous variables. RSV status was assumed negative when hospitalisation occurred outside of the RSV season. RSV status of hospitalisations during the RSV season and ARI in the active surveillance cohort with invalid or missing RSV test results were imputed using multiple imputation based on site, sex, age, and meteorological season at time of hospitalisation or ARI. Any missing observations for medical attendance of ARIs was subsequently imputed using the same set of predictors to which RSV status was added. Imputation yielded ten

complete datasets for each of the two cohorts. After imputation, pooled 95% Wilson-score CIs were calculated for the proportion of infants with at least one RSV-associated hospitalisation or ARI in the first year. Incidence rates were calculated together with 95% CIs based on a Poisson distribution and compared between subgroups of infants using Poisson generalised linear models. Statistical analyses were performed using SPSS (version 26) and R statistical software (version 3.5.1).

The study was approved by the Institutional Review Board of the University Medical Center Utrecht (ref 17/069), National Health Service National Research Ethics Service Oxfordshire Committee A (ref 17/SC/0335) and South East Scotland Research Ethics Committee (ref 17/SS/0086), the Ethics Committee of the Hospital District of Southwest Finland (ref 17201), and Hospital Clínico Universitario de Santiago de Compostela (ref 2017/175).

This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline for cohort studies (appendix pp 11–16). The study is registered with ClinicalTrials.gov, NCT03627572.

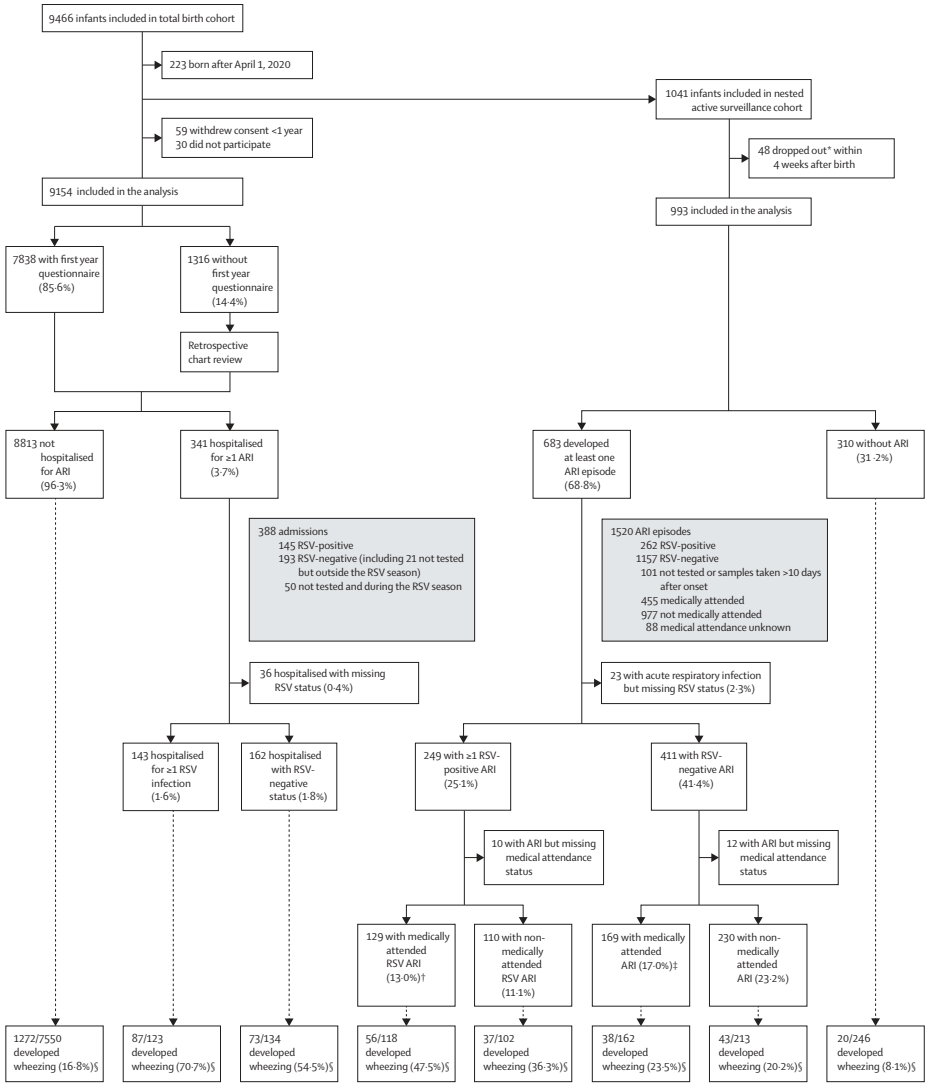
Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report or the decision to submit for publication.

RESULTS

Between July 1, 2017, and July 31, 2020, 9466 healthy term infants were recruited at birth, of whom 9154 (96·7%) were included in the primary analysis (figure 1). Because of the COVID-19 pandemic, 223 infants born after April 1, 2020, were excluded as RSV was not circulating during their first year of life. Between Sept 1, 2017, and Nov 30, 2019, 1041 infants were enrolled in the active surveillance cohort and 993 (95·4%) who participated for at least 4 weeks were included in the analysis (figure 1). Five deaths occurred in study participants, none was related to RSV. There was substantial and expected variation in baseline characteristics between countries (table 1). Non-exhaustively, the most common ethnic origin was according to country geographical location, smokers in the family were more common in Spain, and maternal vaccination was almost never reported in the Netherlands where it was not recommended at the time. Compared with the rest of the cohort, participants of the active surveillance cohort more frequently reported maternal vaccination against influenza or pertussis, multiple births, a family history of atopy, and parental university level of education, whereas parental smoking and parental origin from northwest Europe were reported less frequently; they also had fewer siblings and were born later in the year than other participants.

Figure 1: Flow chart of participants in RESCEU birth cohort study for total cohort and active surveillance cohort



ARI=acute respiratory infection. RSV=respiratory syncytial virus. *Did not continue with active surveillance. †Including 16 RSV admissions (also counted in RSV admissions). ‡Including seven ARI admissions (also counted in RSV-negative admission). §Number of children with wheezing of total number of children with known wheezing status.

Table 1: Baseline characteristics of participants by recruitment sites based on participants with available information

	Total cohort						Active surveillance cohort					
	Scotland	England	Spain	Finland	Netherlands	All	Scotland	England	Spain	Finland	Netherlands	All
Total number of participants	2130	1972	1080	2093	1879	9154	203	198	205	200	187	993
Follow-up time (infant-months)	25 498	23 458	12 949	25 119	22 484	109 507	2408	2288	2404	2384	2245	11 729
Pregnancy												
Vaccination*	1815/2127 (85%)	1766/1947 (91%)	632/1037 (61%)	933/2072 (45%)	626/1827 (34%)	5772/9010 (64%)	188/203 (93%)	183/196 (93%)	118/200 (59%)	129/198 (65%)	57/186 (31%)	675/983 (69%)
Influenza	1444/2127 (68%)	1417/1947 (73%)	294/1037 (28%)	931/2072 (45%)	25/1827 (1%)	4111/9010 (46%)	154/203 (76%)	142/196 (72%)	37/200 (19%)	128/198 (65%)	5/186 (3%)	466/983 (47%)
Pertussis	1742/2127 (82%)	1672/1947 (86%)	597/1037 (58%)	3/2072 (<1%)	617/1827 (34%)	4631/9010 (51%)	181/203 (89%)	179/196 (91%)	113/200 (57%)	1/198 (1%)	55/186 (30%)	529/983 (54%)
Smoking during pregnancy	153/2129 (7%)	102/1954 (5%)	109/1049 (10%)	112/2086 (5%)	65/1827 (4%)	541/9045 (6%)	9/203 (4%)	10/198 (5%)	18/204 (9%)	13/198 (7%)	4/186 (2%)	54/989 (5%)
Birth												
Month of birth*												
Oct–Dec	509/2130 (24%)	437/1972 (22%)	285/1080 (26%)	435/2093 (21%)	518/1879 (28%)	2184/9154 (24%)	30/203 (15%)	26/198 (13%)	69/205 (34%)	38/200 (19%)	62/187 (33%)	225/993 (23%)
Jan–March	658/2130 (31%)	565/1972 (29%)	254/1080 (24%)	324/2093 (15%)	612/1879 (33%)	2413/9154 (26%)	32/203 (16%)	28/198 (14%)	33/205 (16%)	58/200 (29%)	63/187 (34%)	214/993 (22%)
April–June	468/2130 (22%)	543/1972 (28%)	157/1080 (15%)	615/2093 (29%)	310/1879 (16%)	2093/9154 (23%)	70/203 (34%)	60/198 (30%)	29/205 (14%)	68/200 (34%)	29/187 (16%)	256/993 (26%)
July–Sept	495/2130 (23%)	427/1972 (22%)	384/1080 (36%)	719/2093 (34%)	439/1879 (23%)	2464/9154 (27%)	71/203 (35%)	84/198 (42%)	74/205 (36%)	36/200 (18%)	33/187 (18%)	298/993 (30%)
Male sex	1108/2130 (52%)	1036/1944 (53%)	555/1080 (51%)	1093/2093 (52%)	933/1869 (50%)	4725/9116 (52%)	106/203 (52%)	108/197 (55%)	107/205 (52%)	106/200 (53%)	85/187 (45%)	512/992 (52%)
Female sex	1022/2130 (48%)	908/1944 (47%)	525/1080 (49%)	1000/2093 (48%)	936/1869 (50%)	4391/9116 (48%)	97/203 (48%)	89/197 (45%)	98/205 (48%)	94/200 (47%)	102/187 (55%)	480/992 (48%)

Table 1: Baseline characteristics of participants by recruitment sites based on participants with available information (*continued*)

	Total cohort						Active surveillance cohort					
	Scotland	England	Spain	Finland	Netherlands	All	Scotland	England	Spain	Finland	Netherlands	All
Multiple birth*	52/2120 (2%)	65/1940 (3%)	36/1080 (3%)	17/2093 (1%)	27/1872 (1%)	197/9105 (2%)	18/203 (9%)	5/195 (3%)	7/205 (3%)	2/200 (1%)	6/187 (3%)	38/990 (4%)
Caesarean delivery*	927/2126 (44%)	742/1941 (38%)	238/1080 (22%)	293/2091 (14%)	409/1872 (22%)	2607/9110 (29%)	83/203 (41%)	76/197 (39%)	65/205 (32%)	28/200 (14%)	44/187 (24%)	296/992 (30%)
Birth weight <2500 g	40/2092 (2%)	54/1938 (3%)	27/1080 (3%)	22/2091 (1%)	26/1831 (1%)	169/9032 (2%)	4/201 (2%)	6/197 (3%)	9/205 (4%)	3/200 (2%)	3/183 (2%)	25/986 (3%)
Antibiotics <72 h post-partum	8/2130 (<1%)	146/1972 (7%)	6/1080 (1%)	6/1080 (5%)	41/1879 (2%)	305/9154 (3%)	0/203	14/198 (7%)	1/205 (<1%)	8/200 (4%)	14/198 (1%)	24/993 (2%)
Intention to breastfeed*	1681/2129 (79%)	1659/1947 (85%)	739/1051 (70%)	2025/2082 (97%)	1368/1826 (75%)	7472/9035 (83%)	182/203 (90%)	182/198 (92%)	146/205 (71%)	196/198 (99%)	154/186 (83%)	860/990 (87%)
Family												
Any siblings	924/2130 (43%)	979/1959 (50%)	549/1055 (52%)	1103/2091 (53%)	892/1849 (48%)	4447/9084 (49%)	104/203 (51%)	89/198 (45%)	99/205 (48%)	95/200 (48%)	118/186 (63%)	505/992 (51%)
Number of siblings*	1 (1–2)	1 (1–2)	1 (1–1)	1 (1–2)	1 (1–2)	1 (1–2)	1 (1–1)	1 (1–1)	1 (1–1)	1 (1–1)	1 (1–2)	1 (1–1)
Siblings in daycare or primary school	799/2130 (38%)	817/1959 (42%)	474/1055 (45%)	849/2091 (41%)	823/1849 (45%)	3762/9084 (41%)	92/203 (45%)	70/198 (35%)	87/205 (42%)	70/200 (35%)	106/186 (57%)	425/992 (43%)
Smokers in the family*	320/2129 (15%)	274/1947 (14%)	299/1050 (28%)	261/2085 (13%)	301/1826 (16%)	1455/9037 (16%)	15/203 (7%)	20/198 (10%)	58/204 (28%)	23/198 (12%)	21/186 (11%)	137/989 (14%)
Mother	83/2129 (4%)	47/1947 (2%)	61/1050 (6%)	50/2085 (2%)	59/1826 (3%)	300/9037 (3%)	5/203 (2%)	2/198 (1%)	8/204 (4%)	4/198 (2%)	1/186 (1%)	20/989 (2%)
Father	265/2129 (12%)	218/1947 (11%)	254/1050 (24%)	235/2085 (11%)	257/1826 (14%)	1229/9037 (14%)	12/203 (6%)	5/198 (8%)	50/204 (25%)	21/198 (11%)	19/186 (10%)	117/989 (12%)
Other family member	21/2129 (1%)	45/1947 (2%)	34/1050 (3%)	0/2085	26/1826 (1%)	126/9037 (1%)	1/203 (<1%)	4/198 (2%)	6/204 (3%)	0/198	1/186 (1%)	12/989 (1%)

Table 1: Baseline characteristics of participants by recruitment sites based on participants with available information (*continued*)

	Total cohort						Active surveillance cohort					
	Scotland	England	Spain	Finland	Netherlands	All	Scotland	England	Spain	Finland	Netherlands	All
Smoking in the house	29/2129 (1%)	15/1947 (1%)	41/1050 (4%)	9/2085 (<1%)	4/1826 (<1%)	98/9037 (1%)	1/203 (<1%)	4/198 (2%)	6/204 (3%)	0/198	1/186 (1%)	12/989 (1%)
Family history of atopy*	1568/2129 (74%)	1409/1951 (72%)	578/1037 (56%)	1319/2079 (63%)	1292/1831 (71%)	6166/9027 (68%)	163/203 (80%)	150/198 (76%)	121/203 (60%)	132/198 (67%)	142/186 (76%)	708/988 (72%)
Siblings use or used respiratory medicine	172/2130 (8%)	212/1959 (11%)	112/1055 (11%)	167/2091 (8%)	198/1849 (11%)	861/9084 (9%)	11/203 (5%)	15/198 (8%)	17/205 (8%)	10/200 (5%)	28/186 (15%)	81/992 (8%)
Ethnic origin of the mother*												
Northwest Europe	1643/2124 (77%)	1473/1952 (75%)	31/1048 (3%)	2029/2086 (97%)	1416/1827 (78%)	6592/9037 (73%)	146/203 (72%)	143/198 (72%)	9/205 (4%)	195/198 (98%)	163/186 (88%)	656/990 (66%)
Southern Europe	94/2124 (4%)	46/1952 (2%)	943/1048 (90%)	10/2086 (<1%)	29/1827 (2%)	1122/9037 (12%)	11/203 (5%)	6/198 (3%)	179/205 (87%)	0/198	4/186 (2%)	200/990 (20%)
Other	393/2124 (19%)	453/1952 (23%)	106/1048 (10%)	54/2086 (3%)	434/1827 (24%)	1440/9037 (16%)	46/203 (23%)	49/198 (25%)	17/205 (8%)	5/198 (3%)	20/186 (11%)	137/990 (14%)
Ethnic origin of the father*												
Northwest Europe	1664/2124 (78%)	1475/1952 (76%)	35/1048 (3%)	1979/2086 (95%)	1414/1827 (77%)	6567/9037 (73%)	155/203 (76%)	156/198 (79%)	9/205 (4%)	192/198 (97%)	165/186 (89%)	677/990 (68%)
Southern Europe	79/2124 (4%)	53/1952 (3%)	946/1048 (90%)	13/2086 (1%)	23/1827 (1%)	1114/9037 (12%)	7/203 (3%)	3/198 (2%)	181/205 (88%)	0/198	2/186 (1%)	193/990 (19%)
Other	387/2124 (18%)	444/1952 (23%)	99/1048 (9%)	101/2086 (5%)	442/1827 (24%)	1473/9037 (16%)	41/203 (20%)	39/198 (20%)	15/205 (7%)	8/198 (4%)	20/186 (11%)	123/990 (12%)
Highest level of education of the mother*												
Secondary or vocational school	780/2125 (37%)	743/1954 (38%)	540/1049 (51%)	721/2085 (35%)	580/1826 (32%)	3364/9039 (37%)	36/203 (18%)	40/198 (20%)	103/205 (50%)	62/198 (31%)	46/186 (25%)	287/990 (29%)
University of (applied) sciences	1336/2125 (63%)	1202/1954 (62%)	471/1049 (45%)	1315/2085 (63%)	1230/1826 (67%)	5554/9039 (61%)	167/203 (82%)	158/198 (80%)	94/205 (46%)	133/198 (67%)	140/186 (75%)	692/990 (70%)
Highest level of education of the father*												

Table 1: Baseline characteristics of participants by recruitment sites based on participants with available information (*continued*)

	Total cohort						Active surveillance cohort					
	Scotland	England	Spain	Finland	Netherlands	All	Scotland	England	Spain	Finland	Netherlands	All
Secondary or vocational school	1000/2102 (48%)	917/1928 (48%)	685/1037 (66%)	982/2059 (48%)	732/1818 (40%)	4316/8944 (48%)	58/202 (29%)	67/197 (34%)	138/203 (68%)	90/197 (46%)	68/185 (37%)	421/984 (43%)
University of (applied) sciences	1096/2102 (52%)	1001/1928 (52%)	253/1037 (24%)	986/2059 (48%)	1063/1818 (58%)	4399/8944 (49%)	143/202 (71%)	129/197 (65%)	54/203 (27%)	101/197 (51%)	116/185 (63%)	543/984 (55%)
Employment of the mother before birth												
Full-time	1384/2129 (65%)	1250/1954 (64%)	619/1045 (59%)	1432/2084 (69%)	763/1827 (42%)	5448/9039 (60%)	140/203 (69%)	142/198 (72%)	109/205 (53%)	137/198 (69%)	83/186 (45%)	611/990 (62%)
Part-time	519/2129 (24%)	511/1954 (26%)	168/1045 (16%)	264/2084 (13%)	902/1827 (49%)	2364/9039 (26%)	51/203 (25%)	48/198 (24%)	38/205 (19%)	29/198 (15%)	93/186 (50%)	259/990 (26%)
Employment of the father before birth												
Full-time	1933/2129 (91%)	1843/1954 (94%)	955/1045 (91%)	1827/2084 (88%)	1520/1827 (83%)	8078/9039 (89%)	193/203 (95%)	187/198 (94%)	187/205 (91%)	164/198 (83%)	150/186 (81%)	881/990 (89%)
Part-time	82/2129 (4%)	48/1954 (2%)	37/1045 (4%)	74/2084 (4%)	244/1827 (13%)	485/9039 (5%)	3/203 (1%)	8/198 (4%)	7/205 (3%)	8/198 (4%)	31/186 (17%)	57/990 (6%)

Data are n/N (%), and median (IQR). *p<0·05 total active surveillance versus total passive (without active) cohort.

We observed 388 ARI hospitalisations (figure 1 and 2, appendix pp 3–4). Of these, 145 (37·4%) were positive for RSV, 193 (49·7%) were negative or occurred outside the RSV season, and 50 (12·9%) occurred during the RSV season but were not tested for RSV (and status was imputed). Among 145 RSV-associated hospitalisations, RSV was detected during admission by hospital laboratory PCR tests in 71 (49·0%) and by POCT in 67 (46·2%). The test used was not documented for seven RSV-associated hospitalisations. Overall, 143 (1·6%) children were hospitalised with confirmed RSV, including two who were admitted twice with RSV. After imputing missing RSV test results, the incidence of RSV-associated hospitalisation was 1·8% (95% CI 1·6–2·1), corresponding to an RSV-associated hospitalisation incidence rate of 1·6 per 1000 infant-months (1·3–1·8; table 2). RSV-associated hospitalisation incidence in countries varied between 1·1% (0·7–1·5) in Finland and 2·5% (1·8–3·4) in Spain (table 3). RSV-associated hospitalisation incidence rate was higher in children born in autumn (2·6 per 1000 infant-months, 2·0–3·3) than in children born in winter (1·1 per 1000 infant-months, 0·8–1·6, Bonferroni adjusted p=0·002) and spring (0·8 per 1000 infant-months, 0·5–1·3, Bonferroni adjusted p=0·001; table 3, appendix p 10). RSV-associated hospitalisation incidence rate was highest in 2017–18 (2·7 per 1000 infant-months, 1·9–4·0) when the proportion of participating children younger than 6 months was high, and lowest in 2019–20 (1·5 per 1000 infant-months, 1·1–1·8; table 3).

Out of 145 RSV-associated hospitalisations, 84 (57·9%) were in children younger than 3 months (appendix p 5, 10). In that age group, incidence of RSV-associated hospitalisations peaked at 1 month to less than 2 months of age (appendix p 10). Median duration of hospitalisation was 3 days (range 1–19 days, IQR 2–5 days). Hospi-talisations lasted longer in Spain (median 6 days, IQR 5–6 days) than in the Netherlands (median 3 days, IQR 2–6 days; p<0·003), Finland (median 2 days, IQR 1–4 days), England (median 3 days, IQR 2–4 days), and Scotland (median 2 days, IQR 1–3 days; p<0·001). Duration of hospitalisation and other measures of severity were not found to be associated with the incidence rate of RSV-associated hospitalisations. Length of hospitalisation was longer in infants younger than 3 months when compared with infants aged 6 months to younger than 12 months (p=0·004), but not when compared with infants aged 3 months to younger than 6 months (p=0·27). Eight of 145 RSV-associated hospitalisations (5·5%) led to admission to the paediatric intensive care unit (0·09% of total cohort [n=9154 infants]), and three (2%) required mechanical ventilation (0·03% of total cohort). Six of eight infants admitted to the intensive care unit were aged younger than 3 months (median age 1 month). Any respiratory support was more frequently used in RSV-positive than RSV-negative hospitalisations (77 [53·1%] of 145 vs 45 [23·3%] of 193, p<0·001). Coinfections with other respiratory viruses were tested as part of routine care in 85 (58·6%) and found in 34 (23·4%) of 145 RSV-associated hospi talisations. Rhinovirus was most frequently co-detected. In RSV-negative hospi-talisations, rhinovirus, influenza, and parainfluenza were the three most prevalent viruses (appendix p 5).

Table 2: Incidence and incidence rates of RSV-associated ARI, medically attended ARI, and hospitalised ARI in the first year of life

	RSV incidence after imputation*†	RSV incidence before imputation‡	Cohort size or person-time	Number of hospitalisations or number of ARI episodes	Number of RSV-positive (observed)
RSV-associated hospitalisation in total cohort					
Incidence§	1 8% (1·6–2·1)	1 6% (1·3–1·8)	9154 infants	341 infants hospitalised	143 infants with RSV-associated hospitalisation
Incidence rate per 1000 infant-months	1 6 (1·3–1·8)	1 3 (1·1–1·6)	109 507 infant months	388 hospitalisations	145 RSV-associated hospitalisations
Medically attended RSV-positive ARI in active surveillance cohort					
Incidence§	14 1% (12·3–16·0)	13 0% (11·0–15·2)	993 infants	683 infants with ARI	129 infants with medically attended RSV-associated ARI
Incidence rate per 1000 infant-months	12 1 (10·2–14·3)	11 2 (9·3–13·3)	11 728 infant months	1520 ARI	131 medically attended RSV-associated ARI
RSV-positive ARI in active surveillance cohort					
Incidence§	26 2% (24·0–28·6)	25 1% (22·4–27·9)	993 infants	683 infants with ARI	249 infants with RSV-associated ARI
Incidence rate per 1000 infant-months	23 7 (21·0–26·7)	22 3 (19·7–25·2)	11 728 infant months	1520 ARI	262 RSV-associated ARI

ARI=acute respiratory infection. RSV=respiratory syncytial virus. *Missing RSV status imputed using multiple imputation based on site, sex, age, and meteorological season at time of hospitalisation or ARI, and missing medical attendance imputed using site, sex, age, meteorological season at time of hospitalisation or ARI and RSV status (observed or imputed). †Outcomes that required imputations included: 50 hospitalisations with missing RSV status, 166 ARI episodes with missing RSV status or missing medical attendance status, and 101 ARI episodes with missing RSV status. ‡Assuming all missing outcomes were negative. §Incidence as proportion of infants experiencing the event at least once during their first year of life. ¶Incidence rate as number of events per 1000 infant-months of follow-up.

We registered 1520 ARI episodes in 993 infants in the active surveillance cohort (figure 1, 2). A nasal swab was collected during 1442 (94·9%) episodes. Missed episodes was the main reason for not collecting a swab. 23 samples collected later than 10 days after start of symptoms were excluded. Most samples (87·7%) were collected within 7 days after the start of symptoms. In total, 262 (18·5%) of 1419 episodes were positive for RSV in 249 infants (figure 1). Among the 840 episodes tested by PCR and POCT, RSV was detected only by POCT in five (0·6%).

Table 3: Incidence and incidence rates after imputation for missing RSV test results and missing medical attendance status of RSV-associated hospitalised ARIs, medically attended ARIs, and ARIs by age group, according to season, recruitment site, cohort, and season of birth

	RSV-associated hospitalised ARI				Medically attended RSV-associated ARI				RSV-associated ARI			
	<3 months	3 to <6 months	6 to <12 months	<12 months	<3 months	3 to <6 months	6 to <12 months	<12 months	<3 months	3 to <6 months	6 to <12 months	<12 months
RSV incidence proportion												
Overall	0·97% (0·82–1·16)	0·49% (0·38–0·63)	0·39% (0·29–0·52)	1·80% (1·58–2·05)	3·39% (2·56–4·49)	4·55% (3·55–5·80)	6·32% (5·13–7·77)	14·07% (12·31–16·03)	5·05% (4·01–6·33)	9·29% (7·84–10·97)	12·61% (10·93–14·51)	26·22% (23·95–28·63)
Site												
Scotland	1·15% (0·83–1·60)	0·47% (0·28–0·79)	0·73% (0·48–1·10)	2·31% (1·83–2·92)	1·48% (0·59–3·64)	5·72% (3·55–9·11)	6·75% (4·30–10·45)	13·74% (10·17–18·31)	3·50% (1·91–6·33)	12·69% (9·17–17·3)	13·60% (9·88–18·43)	29·21% (24·05–34·97)
England	1·03% (0·71–1·51)	0·71% (0·44–1·14)	0·43% (0·23–0·81)	1·97% (1·50–2·57)	2·58% (1·26–5·20)	5·05% (2·97–8·46)	3·03 % (1·48–6·09)	10·4% (7·18–14·84)	3·99% (2·21–7·11)	9·95% (6·89–14·15)	7·61% (4·93–11·55)	20·51% (15·96–25·94)
Spain	1·20% (0·77–1·88)	1·00% (0·60–1·65)	0·28% (0·11–0·69)	2·48% (1·81–3·40)	6·00% (3·77–9·43)	6·65% (4·27–10·21)	5·35% (3·22–8·76)	17·71% (13·65–22·65)	7·71% (5·10–11·49)	11·15% (7·98–15·37)	11·80% (8·50–16·16)	29·56% (24·49–35·19)
Finland	0·62% (0·4–0·97)	0·24% (0·12–0·49)	0·19% (0·08–0·44)	1·05% (0·74–1·49)	1·00% (0·33–2·98)	1·01% (0·33–2·99)	4·95% (2·95–8·19)	6·90% (4·48–10·49)	1·00% (0·33–2·98)	2·51% (1·23–5·07)	7·07% (4·62–10·68)	10·50% (7·45–14·61)
Netherlands	0·97% (0·65–1·43)	0·26% (0·12–0·57)	0·25% (0·11–0·56)	1·47% (1·07–2·03)	6·04% (3·73–9·63)	4·28% (2·43–7·43)	11·66% (8·32–16·10)	21·98% (17·38–27·39)	9·25% (6·30–13·38)	10·16% (7·08–14·38)	23·32% (18·6–28·81)	42·19% (36·35–48·26)
RSV incidence rate per 1000 infant-months												
Overall	3·26 (2·63–4·04)	1·67 (1·23–2·27)	0·65 (0·45–0·92)	1·56 (1·33–1·82)	11·69 (8·34–16·38)	15·21 (11·28–20·52)	10·77 (8·36–13·88)	12·11 (10·24–14·34)	17·55 (13·34–23·1)	31·69 (25·76–38·98)	22·81 (19·16–27·17)	23·70 (21·02–26·73)
Site												
Scotland	3·88 (2·60–5·80)	1·55 (0·82–2·92)	1·21 (0·73–2·00)	1·96 (1·48–2·61)	4·95 (1·60–15·35)	19·10 (10·63–34·32)	11·47 (6·62–19·87)	11·75 (8·06–17·12)	11·70 (5·58–24·56)	44·82 (30·18–66·56)	24·77 (16·78–36·56)	26·52 (20·54–34·25)
England	3·46 (2·20–5·45)	2·56 (1·47–4·47)	0·72 (0·34–1·51)	1·87 (1·38–2·55)	8·61 (3·58–20·71)	17·00 (8·89–32·54)	5·04 (2·09–12·10)	8·98 (5·69–14·18)	13·31 (6·44–27·51)	34·07 (21·68–53·55)	12·99 (7·63–22·1)	18·39 (13·4–25·23)

Table 3: Incidence and incidence rates after imputation for missing RSV test results and missing medical attendance status of RSV-associated hospitalised ARIs, medically attended ARIs, and ARIs by age group, according to season, recruitment site, cohort, and season of birth (*continued*)

	RSV-associated hospitalised ARI				Medically attended RSV-associated ARI				RSV-associated ARI			
	<3 months	3 to <6 months	6 to <12 months	<12 months	<3 months	3 to <6 months	6 to <12 months	<12 months	<3 months	3 to <6 months	6 to <12 months	<12 months
Spain	4·01 (2·33–6·90)	3·34 (1·81–6·14)	0·46 (0·15–1·44)	2·07 (1·41–3·03)	20·11 (11·37–35·55)	22·22 (12·92–38·24)	8·93 (4·80–16·61)	15·09 (10·82–21·06)	27·46 (16·81–44·88)	37·28 (24·56–56·59)	20·58 (13·77–30·75)	26·49 (20·63–34·03)
Finland	2·07 (1·20–3·56)	0·80 (0·33–1·92)	0·31 (0·1–0·9)	0·87 (0·57–1·33)	3·34 (0·84–13·35)	3·35 (0·84–13·41)	8·24 (4·37–15·52)	5·79 (3·40–9·85)	3·34 (0·84–13·35)	8·38 (3·49–20·14)	11·78 (6·98–19·89)	8·81 (5·74–13·51)
Netherlands	3·23 (2·02–5·18)	0·86 (0·33–2·27)	0·40 (0·14–1·15)	1·23 (0·83–1·81)	21·93 (12·46–38·57)	14·27 (7·14–28·54)	20·28 (13·43–30·64)	19·20 (14·21–25·93)	32·63 (20·57–51·77)	33·90 (21·62–53·15)	44·48 (33·62–58·85)	38·89 (31·49–48·02)
Season												
2017–18	3·90 (2·51–6·08)	2·49 (1·21–5·09)	0*	2·71 (1·85–3·98)	15·01 (7·81–28·86)	11·98 (4·49–31·94)	0*	12·05 (7·00–20·75)	20·75 (11·75–36·67)	18·08 (8·03–40·72)	0*	17·15 (10·79–27·26)
2018–19	3·17 (2·30–4·38)	1·41 (0·83–2·41)	0·90 (0·50–1·62)	1·76 (1·38–2·25)	8·36 (4·75–14·71)	9·79 (5·50–17·46)	10·37 (6·64–16·19)	9·60 (7·12–12·95)	12·10 (7·56–19·38)	20·32 (13·60–30·37)	21·30 (15·62–29·05)	18·19 (14·67–22·55)
2019–20	3·03 (2·10–4·36)	1·79 (1·17–2·76)	0·74 (0·47–1·15)	1·45 (1·14–1·83)	14·90 (8·66–25·64)	21·24 (14·44–31·24)	12·65 (9·26–17·29)	15·06 (12·04–18·83)	24·32 (15·89–37·22)	46·16 (35·79–59·54)	27·20 (21·99–33·66)	31·25 (26·81–36·42)
Cohort												
Cohort A	2·92 (1·48–5·77)	2·45 (1·13–5·29)	0·72 (0·27–1·91)	1·71 (1·08–2·69)								
Cohort P without cohort A	3·30 (2·63–4·14)	1·57 (1·13–2·19)	0·64 (0·44–0·93)	1·54 (1·30–1·82)								
Sex												
Female	3·16 (2·31–4·32)	1·44 (0·90 –2·30)	0·55 (0·32–0·93)	1·42 (1·13–1·80)	10·68 (6·45–17·71)	11·37 (6·94–18·63)	11·49 (8·07–16·37)	11·26 (8·77–14·46)	17·39 (11·66–25·92)	28·39 (20·73–38·89)	23·99 (18·8–30·61)	23·43 (19·71–27·84)
Male	3·38 (2·53–4·51)	1·89 (1·24–2·88)	0·74 (0·47–1·17)	1·69 (1·37–2·08)	12·65 (8·05–19·86)	18·82 (12·87–27·52)	10·09 (7·04–14·48)	12·92 (10·31–16·19)	17·73 (12·08–26·03)	34·16 (25·81–45·21)	21·72 (16·98–27·78)	23·82 (20·16–28·14)
Season of birth†												

Table 3: Incidence and incidence rates after imputation for missing RSV test results and missing medical attendance status of RSV-associated hospitalised ARIs, medically attended ARIs, and ARIs by age group, according to season, recruitment site, cohort, and season of birth (*continued*)

	RSV-associated hospitalised ARI				Medically attended RSV-associated ARI				RSV-associated ARI			
	<3 months	3 to <6 months	6 to <12 months	<12 months	<3 months	3 to <6 months	6 to <12 months	<12 months	<3 months	3 to <6 months	6 to <12 months	<12 months
Spring	0·47 (0·15–1·45)	0·77 (0·31–1·95)	1·02 (0·56–1·83)	0·82 (0·51–1·31)	0*	6·15 (2·45–15·40)	18·52 (12·77–26·86)	10·72 (7·60–15·12)	0*	16·71 (9·70–28·77)	42·87 (33·49–54·87)	25·43 (20·31–31·83)
Summer	1·55 (0·86–2·80)	4·24 (2·92–6·15)	0·29 (0·10–0·82)	1·60 (1·18–2·16)	8·17 (3·90–17·14)	36·82 (25·64–52·88)	2·03 (0·65–6·30)	12·32 (9·01–16·83)	14·99 (8·66–25·95)	78·13 (61·17–99·79)	4·92 (2·39–10·15)	25·81 (20·85–31·95)
Autumn	8·53 (6·60–11·04)	1·35 (0·70–2·61)	0·17 (0·04–0·65)	2·57 (2·03–3·25)	31·56 (20·95–47·55)	11·37 (5·69–22·73)	1·48 (0·37–5·91)	11·55 (8·19–16·27)	46·95 (33·56–65·67)	17·83 (9·98–31·88)	4·22 (1·90–9·40)	18·41 (13·99–24·23)
Winter	2·03 (1·18–3·48)	0·15 (0·02–1·05)	1·17 (0·7–1·95)	1·13 (0·78–1·62)	7·23 (2·71–19·29)	0*	25·22 (17·40–36·55)	14·41 (10·17–20·41)	7·23 (2·71–19·29)	0*	46·33 (35·24–60·90)	24·97 (19·17–32·51)
Birthweight												
<2500 g	5·78 (1·86–17·91)	0*	0*	1·49 (0·48–4·63)	0*	38·45 (11·15–132·56)	6·94 (0·98–49·29)	13·42 (4·87–36·98)	0*	72·07 (30·01–173·09)	7·44 (1·05–52·97)	22·04 (9·96–48·75)
≥2500 g	3·18 (2·55–3·96)	1·69 (1·25–2·30)	0·66 (0·47–0·95)	1·55 (1·32–1·82)	12·04 (8·59–16·88)	14·72 (10·77–20·12)	10·94 (8·47–14·13)	12·16 (10·25–14·43)	18·10 (13·75–23·82)	30·54 (24·63–37·87)	23·17 (19·43–27·62)	23·73 (21·01–26·81)

Data are % (95% CI) or incidence rate (95% CI). ARI=acute respiratory infection. A=active surveillance. P=passive surveillance. RSV=respiratory syncytial virus. *Incidence rate estimated as 0, 95% CI not determined because of 0 cases. †Season of birth was defined as: spring from March 21 to June 20, summer from June 21 to Sept 20, autumn from Sept 21 to Dec 20, and winter from Dec 21 to March 20.

RSV-A was detected in 142 (54·2%) of RSV-associated ARI and RSV-B in 111 (42·4%). One sample was positive for both RSV-A and RSV-B. RSV subtype was unknown for ten ARI episodes: five were only tested by POCT, four were only tested in hospital as part of routine care, and for one the RSV subtype could not be determined. Information about medical attendance was available for 1432 (94·2%) episodes. For 1353 (89·0%) ARI episodes both RSV and medical attendance status were available. Medical attendance was reported in 131 (52·2%) of 251 RSV-positive ARI, which was more frequent than in RSV-negative ARI (298 [27·0%] of 1102, p<0·001).

After imputing missing RSV test results, the incidence of medically attended RSV-associated ARI was 14·1% (95% CI 12·3–16·0) with an incidence rate of 12·1 per 1000 infant-months (10·2–14·3; table 2). The incidence of RSV-associated ARI overall was 26·2% (24·0–28·6) with an incidence rate of 23·7 per 1000 infant-months (21·0–26·7). Incidence rate of RSV-associated ARI and medically attended RSV-associated ARI were similar for infants younger than 6 and those aged 6 months and older (table 3). The incidence rates for RSV-associated ARI and medically attended RSV-associated ARI episodes were highest in the Netherlands (38·9 per 1000 infant-months [31·5–48·0] and 19·2 per 1000 infant-months [14·2–25·9], respectively) and lowest in Finland (8·8 per 1000 infant-months [5·7–13·5] and 5·8 per 1000 infant-months [3·4–9·9] respectively, Bonferroni adjusted p<0·05; table 3).

Information on wheezing was available for 7838 children whose parents completed the 1-year question naire (85·6% of the 9154 participants), which included 7807 participants of the total cohort with complete information on hospitalisations for ARI and 841 participants of the active surveillance cohort with complete information on ARI episodes (figure 1). Wheezing was reported in 87 (70·7%) of 123 infants admitted with RSV. Wheezing was less frequent in infants hospitalised for RSV-negative ARI only (73 [54·5%] of 134, p=0·008) and in infants never admitted for an ARI (1272 [16·8%] of 7550, p<0·001, figure 1). In the active surveillance cohort, wheezing was reported for 56 (47·5%) of 118 infants with medically attended RSV-associated ARI and 37 (36·3%) of 102 infants with non-medically attended RSV-associated ARI (p=0·09). This occurrence was more frequent than in children who had no ARI (20 [8·1%] of 246, p<0·001 and p<0·001), had medically attended RSV-negative ARI (38 [23·5%] of 162, p<0·001 and p=0·03) or had non-medically attended RSV-negative ARI (43 [20·2%] of 213, p<0·001 and p=0·002). When adjusted for family history of atopy and smoking household members at birth, the di!erence in wheezing between RSV-positive and RSV-negative or no ARI remained significant (p=0·003 and p<0·001 for hospitalisations, p<0·001 and p<0·001 for medically attended ARI, and p=0·002 and p<0·001 for non-medically attended ARI).

DISCUSSION

To our knowledge, this is the first international birth cohort study powered to accurately estimate the health-care burden of RSV in healthy term-born infants. Our results showed an incidence of RSV-associated hospi-talisation of 1·8% in the first year of life. Almost half of all ARI hospitalisations in the first year of life were RSV-associated. The burden of RSV-associated hospi-talisation was highest in infants younger than 3 months with an incidence rate of 3·3 per 1000 infant-months. Children born in autumn had a significantly higher risk of hospitalisation than children born in other seasons. One quarter of infants experienced an RSV-associated ARI, of which half were medically attended. Wheezing during the first year of life was associated with RSV hospitalisation, medically attended RSV-associated ARI, and overall RSV-associated ARI.

Our findings are consistent with previous literature. Although not a birth cohort study, a study conducted in the USA reported an incidence of RSV-associated hospitalisations of 1·7% in infants younger than 6 months (1·5% in our study), and 0·5% in infants aged 6 to younger than 12 months (0·4% in our study).[2] The higher admission rate in infants younger than 6 months reported by Hall and colleagues[2] might be related to the 35% of higher-risk infants included. In our study, incidence of RSV-associated hospitalisations per country varied between 1·1% and 2·5%, which was in line with previous findings from these countries.[9,11,18,22] In other birth cohort studies, RSV-associated hospitalisation incidence in the first year of life varied between 0·6% and 5%. Some studies also included high-risks infants (appendix p 6).[10,12–17] The two largest birth cohort studies in healthy term-born infants showed an incidence of RSV-associated hospitalisations of 1·9% in an Indian birth cohort of 310 infants and 1% in 298 infants of a Dutch birth cohort.[9,14]

Wheezing in the first year of life was associated with RSV infection irrespective of severity. The association between severe RSV infections and wheezing has been described earlier.[23] Whether this is also associated with development of childhood asthma remains unclear, as well as whether RSV immunisation will prevent wheezing during later childhood.[24] Intervention studies are required to define the causal association between RSV infection during infancy and wheezing in healthy term-born infants.

The major strength of our study is the prospective design with the power to accurately estimate RSV incidence in European countries over several seasons. We used active surveillance to capture mild RSV disease to provide a precise estimate of total RSV incidence and disease burden. Follow-up rates were high with collection of swabs in 95% of reported ARI episodes and more than 85% completion of the 1-year questionnaire in the total cohort. In addition to parental report, we screened the study participants' hospital charts to ensure no ARI hospitalisation was missed. This study also has limitations. First, in 50 of 388 ARI hospitalisations during the RSV season, no RSV test was performed. When using a cohort study design with RSV testing results as primary outcome, missing test results will systematically

lead to an underestimation of true incidence if assumed negative. To avoid this systematic bias, primary outcomes were reported after using multiple imputation for missing RSV test results and medical attendance status. As the proportion of missing information was small, using multiple imputation resulted in a small increase in incidence compared with estimating incidence assuming all cases with missing RSV status were RSV-negative. Two of the five sites did not use POCT, which could have led to underestimating incidence in those countries; however, that e!ect was probably small. Of 840 episodes tested by PCR and POCT, five (0·6%) were detected by POCT only. Assuming a similar rate, two additional RSV cases would have been detected by POCT among the 415 episodes tested by PCR only at the sites not using POCT. Second, data on coinfection with other respiratory viruses were scarce. Third, the participants in the study might not be representative of the country population and not all countries in Europe were represented. The education level of participants, especially in the active surveillance cohort, was high with 70% of mothers reporting university education and is therefore not necessarily representative of the whole population. Lower socio-economic status and younger age of the mother have been reported as risk factors for RSV-associated hospitalisation in infancy.[25] Other risk factors like parental smoking were less frequently reported by active surveillance cohort participants than the rest of the study population. This could have resulted in an under-estimation of RSV incidence in the study population compared with the country population and in the active cohort compared with the entire cohort. Although children with evidence of significant comorbidities at birth were excluded, we cannot rule out that a minority of participants had comorbidities diagnosed later in life. Fourth, it is possible that we missed ARI episodes despite weekly contacts with parents during the period of active surveillance (October to May, or longer if RSV was still circulating). We cannot rule out that some participants could have stopped reporting ARI of their children, which could result in underestimating incidence rate and would be more pronounced in the older infants. However, participation to the 1-year questionnaire was 89% in the active surveillance cohort, suggesting a high retention rate. ARI episodes occurring outside of the active surveillance period would not have been captured, which probably contributed to the finding of 31% of active cohort participants with no ARI in the first year of life. However, it is unlikely that those uncaptured ARI episodes were associated with RSV infection. Fifth, the COVID-19 pandemic impacted RSV incidence in 2020. The 2019–20 RSV season was virtually finished in the participating countries when the COVID-19 pandemic started, except for Finland, where the usual continuation of the RSV outbreak into late spring was abruptly terminated because of the COVID-19 pandemic.[26,27] The COVID-19 pandemic might have contributed to the lower incidence of RSV-associated hospitalisations, medically attended ARIs, and RSV-associated ARIs in the study in Finland. Participants born after April 1, 2020, were excluded as RSV did not circulate during their first year of life. Follow-up time after Nov 1, 2020, represented less than 3% of total follow-up time of the cohort and concerned only participants aged 6 months or older. Sixth, health-care burden does not reflect the total

burden of RSV. Health-care burden is key information to estimate economic and societal burden, and the incidence of medically attended and hospitalised RSV infections is expected to be a major part of the health-care burden in Europe where RSV-related deaths are rare. Overall, study limitations have possibly resulted in a modest under estimation of actual RSV burden.

In conclusion, the health-care burden of RSV in healthy term-born infants in Europe is considerable with an incidence of RSV-associated hospitalisation of 1·8% in the first year of life, which means that one in 56 healthy term-born infants is hospitalised with RSV annually. Because the highest burden is seen in infants in their first months of life, maternal vaccination and passive immunisation could have a profound impact on the RSV burden.

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Contributors

JGW, AP, TH, SC, FMT, MS, and LJB designed the study. JGW, RZ, MvH, TH, SC, MS, SC, FMT, KK, SD, HR, ADU, and TON collected data. JGW, MB, PvdV, and LJB analysed and interpreted data. JGW wrote the first draft. AP, TH, SC, FMT, MS, RZ, MvH, KK, SD, HR, ADU, BR, and TON reviewed and commented on the manuscript. JGW and MB accessed and verified the data. JGW and LJB were responsible for the decision to submit the manuscript.

Declaration of interests

LJB has regular interaction with pharmaceutical and other industrial partners. He has not received personal fees or other personal benefits. UMCU has received major funding (>€100 000 per industrial partner) for investigator initiated studies from AbbVie, MedImmune, AstraZeneca, Sanofi, Janssen, Pfizer, MSD, and MeMed Diagnostics. UMCU has received major funding for the RSV GOLD study from the Bill & Melinda Gates Foundation. UMCU has received major funding as part of the public private partnership IMI-funded RESCEU and PROMISE projects with partners GSK, Novavax, Janssen, AstraZeneca, Pfizer, and Sanofi. UMCU has received major funding by Julius Clinical for participating in clinical studies sponsored by MedImmune and Pfizer. UMCU received minor funding (€1000–25 000 per industrial partner) for consultation and invited lectures by AbbVie, MedImmune, Ablynx, Bavaria Nordic, MabXience, GSK, Novavax, Pfizer, Moderna, Astrazeneca, MSD, Sanofi, Genzyme, and Janssen. LJB is the founding chairman of the ReSViNET Foundation. SC has provided consultancy or investigator roles in relation to product development for Ablynx, Janssen, MedImmune, AstraZeneca, Pfizer, GSK, Vertex, AbbVie, Valneva, Fibrogen, and Boehringer Ingelheim, with fees paid to the University of Edinburgh. FM-T reports honoraria from GSK group, Pfizer, Sanofi Pasteur, MSD, Seqirus, Biofabri, and Janssen for taking part in advisory boards and expert meetings and for acting as a speaker in congresses, outside the submitted work; and principal investigator-role in randomised controlled trials for GSK, Pfizer, Sanofi Pasteur, MSD, Seqirus, Biofabri, Janssen, Ablynx, Gilead, Regeneron, Roche, Abbott, Novavax, and MedImmune, with honoraria paid to his institution. MDS acts as an investigator on behalf of the University of Oxford on research studies funded by vaccine manufacturers including GSK, Janssen, MCM vaccines, Novavax, AtraZeneca, and Pfizer. MDS was an National Institute for Heath and Care Research (NIHR) Senior Investigator and received salary support from the NIHR Oxford Biomedical Research Centre during the course of this work. MDS is currently an employee of Moderna. SBD had received honoraria from MSD and Sanofi Pasteur for taking part in advisory boards and has provided consultancy or investigator roles in relation to product development for Janssen, AstraZeneca, Pfizer, Valneva, MSD, and Sanofi Pasteur with fees paid to St George's University of London. TH has received honoraria for lectures or participation in advisory boards or data monitoring committees from Janssen, Sanofi Pasteur, Enanta, and MSD. BR is a full-time employee of the GSK group and holds shares and restricted shares in the GSK group as part of their employee remuneration. AJP is currently Chair of the Department of Health Social Cares Joint Committee on Vaccination and Immunisation and was previously a member of WHO Scientific Advisory Group for Emergencies and chair of the European Medicine's Agency Scientific Advisory Group on Vaccines. All other authors declare no competing interests.

Data sharing

The anonymised data of the RESCEU birth cohort study will be made available for research purposes after the end of the long-term follow-up. The data will be stored on the Elixir data platform at https://elixir-europe. org/platforms/data. Requests to access the data should be sent via Elixir to the RESCEU consortium.

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Chapter 4

A Systematic Review on Global Respiratory Syncytial Virus Genetic Data: Identification of Knowledge Gaps

Reviews in Medical Virology, 2021

"Play is the highest form of research."
— Einstein, genius

A systematic review on global RSV genetic data: Identification of knowledge gaps

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ABSTRACT

Respiratory syncytial virus (RSV) is a major health problem. A better understanding of the geographical and temporal dynamics of RSV circulation will assist in tracking resistance against therapeutics currently under development. Since 2015, the field of RSV molecular epidemiology has evolved rapidly with around 20–30 published articles per year. The objective of this systematic review is to identify knowledge gaps in recent RSV genetic literature to guide global molecular epidemiology research. We included 78 studies published between 2015 and 2020 describing 12,998 RSV sequences of which 8,233 (63%) have been uploaded to GenBank. Seventeen (22%) studies were performed in low- and middle-income countries (LMICs), and seven (9%) studies sequenced whole-genomes. Although most reported polymorphisms for monoclonal antibodies in clinical development (nirsevimab, MK-1654) have not been tested for resistance in neutralisation essays, known resistance was detected at low levels for the nirsevimab and palivizumab binding site. High resistance was found for the suptavumab binding site. We present the first literature review of an enormous amount of RSV genetic data. The need for global monitoring of RSV molecular epidemiology becomes increasingly important in evaluating the effectiveness of monoclonal antibody candidates as they reach their final stages of clinical development. We have identified the following three knowledge gaps: whole-genome data to study global RSV evolution, data from LMICs and data from global surveillance programs.

KEYWORDS

molecular epidemiology, monoclonal antibodies, respiratory syncytial virus, RSV, systematic review

LIST OF ABBREVIATIONS

F protein	fusion protein
LMICS	low- and middle-income countries
RSV	respiratory syncytial virus
mAb	monoclonal antibody
WG	whole genome

1 | INTRODUCTION

Respiratory syncytial virus (RSV) is a global cause of morbidity and mortality in children under the age of five, predominantly in low- and middle-income countries (LMICs).[1,2] Although RSV has been recognised as a major health problem, there are no licensed vaccines available.[3] Prevention of severe RSV illness is limited to an approved monoclonal antibody (mAb) administration of palivizumab.[4] Although effective in high-risk populations, there is a critical need for affordable mAbs for healthy infants to reduce severe disease. More than 20 vaccine candidates and mAbs are currently in clinical development.[5] The main target for vaccine and mAb development, the fusion (F) protein, is characterised by a high genetic and antigenic stability.[6] The F protein has a pre- and post-fusion conformation and contains six antigenic sites (Ø -V) that are key epitopes for prophylactic neutralising of mAbs. With promising F targeting mAbs on the horizon, it is likely that they will replace palivizumab in the near future. Two next-generation candidate mAbs with prolonged half-life are nirsevimab (targeting site Ø) and MK-1654 (targeting site IV), which are in phase 3 and phase 2b development, respectively.[7,8] With both mAbs in the final stages of clinical development, global monitoring of RSV resistance development grows more important.[9] Although the binding epitopes are known to be conserved, mutations may emerge and spread.[10] The emergence of escape variants may result in prophylaxis resistance, as seen in the suptavumab trial. This phase 3 trial was discontinued due to a lack of efficacy caused by two amino acid substitutions (L172Q and S173L) in the binding epitope of all circulating RSV B strains. These escape mutants had emerged globally over three RSV seasons and were identified during F sequences analyses.[11]

Previously, only Zhu et al. had reviewed viral neutralisation susceptibility for nirsevimab-related amino acid substitutions in strains collected from 1956 to 2014.[12,13] In addition, Mas et al. evaluated the variability of F sequences until 2017, but this review lacks an analysis of mAb binding epitopes.[14] Despite these efforts, potential escape mutants have not been reviewed systematically and gaps in the recent knowledge remain with respect to recent polymorphisms. In addition to detecting resistance against therapeutics, understanding the molecular epidemiology of RSV is fundamental for elucidating temporal and spatial dynamics, which can help inform surveillance efforts and guide interventions in future epidemics.

As the molecular epidemiology of RSV is a rapidly evolving research field, we provide a comprehensive overview of literature to date. This systematic review aimed to identify knowledge gaps in recent RSV genomic literature required to study global RSV evolution and transmission patterns and, at the same time provide guidance for monitoring next generation mAbs before and after licensure.

2 | METHODS

2.1 | Search strategy and selection criteria

This review was registered at PROSPERO (International Prospective Register of Systematic Reviews) under registration number CRD42021237337. We have followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) reporting guidelines and the Cochrane Handbook.[15,16]

We searched for studies of any design and in any setting that included sequencing of human RSV samples. We searched MEDLINE and Embase for studies published from 1 January 2015 until 1 December 2020. The search included the terms 'RSV' and 'sequences' (see Table S1 for full search). The search strategy was not limited by language. Case reports were excluded. Reference lists of included studies were assessed for additional relevant studies. We classified LMICs based on the World Bank list of economies from 2020.[17]

We selected studies describing the RSV genotype distribution and/or the amino acid polymorphisms in the F protein as we felt that articles with these criteria describe complete and high-quality data. Genotype distribution was defined as the number of individually reported genotypes for RSV A and/or RSV B. Amino acid polymorphisms in the F protein were reported as the amino acid change, neutralising capacity, number and percentage, country and year. Studies without genotype data or amino acid polymorphisms in the F protein were excluded. Antibody binding sites of RSV F were defined using the mAb binding epitopes Ø, II, IV and V described in the original publications: aa 62-69, 196-212 for nirsevimab,[12,13] 254-277 for palivizumab,[11] 426-44[7] for MK-16547 and 161-182 for suptavumab.[11]

2.2 | Data collection

Titles and abstracts were screened and full texts were reviewed by three authors (ACL, ERH and BK) independently, using the web app Rayyan.[18] Endnote X9 (Thomson Reuters) was used to upload full text articles.[19] Disagreements between reviewers were resolved by consensus. Data were extracted by two authors (ACL, ERH) and checked by the third author (BK). We extracted data for: first author, year of publication, study period, country, study design, study population, age, main finding, sequenced gene(s), sequencing platform, reference strains, availability of data in public domain and quantitative outcomes on subtyping, genotyping and clinic. The overall quality of the studies included was independently assessed by two authors (ACL, ERH) using the Critical Appraisal Skills Program (CASP) checklist for cohort studies.

2.3 | Data analysis

We performed a descriptive analysis to describe the number of RSV positives, generated sequences, genes, genotypes, and availability of data in the public domain. Maps and Gantt charts were made in R[20] 3D structures of the prefusion and postfusion RSV F protein were generated with BioRender.com using 4MMT[6] and 3RRR.[21]

3 | RESULTS

We identified 78 studies from 43 countries across six continents in 89 articles for our systematic review (Figures 1 and 2, Tables S2 and S3). Seventy-seven (99%) studies were observational in nature; one (1%) interventional trial was identified.[11,22] No studies were excluded based on lack of quality.

A total of 223,857 samples were identified of which 59,721 (32%) were RSV positive (Table S4). 12,994 sequences were successfully generated of which 8,233 (63%) were uploaded into GenBank. Overall, seven studies (9%) sequenced full genomes accounting for 593 whole genome (WG) sequences and 71 studies (91%) sequenced individual genes. Of these individual genes, 65 studies (92%) sequenced the attachment (G) gene, 22 (31%) the F gene, and 2 (3%) the SH gene. 75 (96%) studies involved children, mainly under the age of five years. Only a few studies (n = 3, 4%) focused on adults. The time from sample collection to publication ranged from 1 to 9 years with an average of 3 years.

FIGURE 1 Study selection

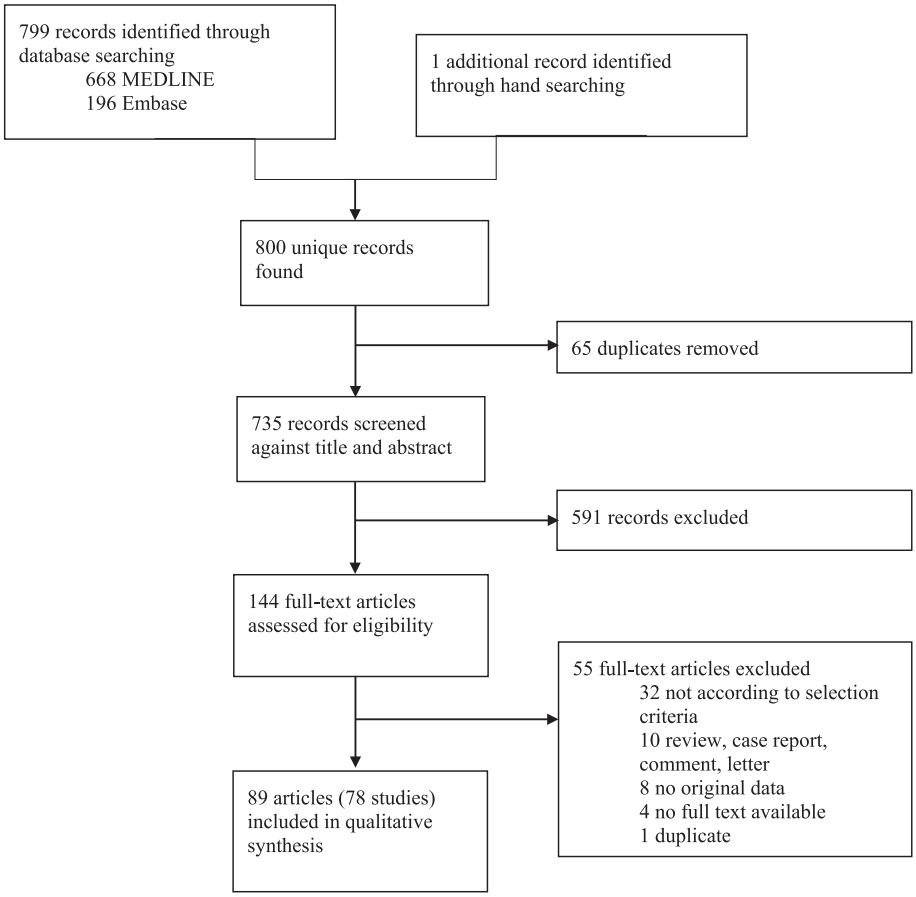
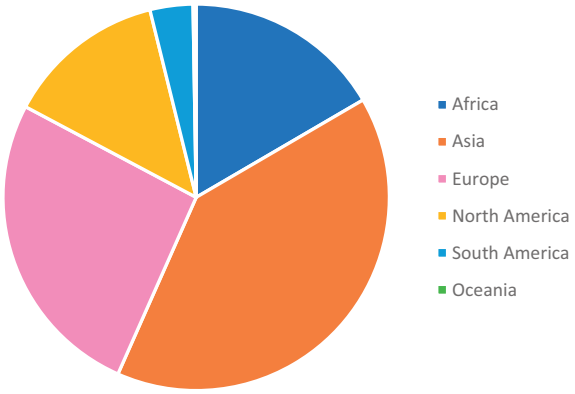


FIGURE 2 Continental distribution of sequences (n = 6 continents)



3.1 | International studies

From 78 studies included, 75 (96%) were performed in a single country. One study had analysed sequences from two countries (the UK, Spain).[23] Only two studies with patients from ≥5 continents were included in this systematic review.[11,24] Country of origin from the sequences (n = 47) from the suptavumab trial could not be analysed as this information was not provided in the manuscript.[11] Tabor et al. have published data (n = 476 RSV positives; n = 410 successful sequences) from 2017 to 2018 from Spain, the UK, the Netherlands, Finland, Japan, South Africa, Brazil and Australia.[24]

3.2 | LMICs

LMICs have contributed to global data with 17 studies (21.8%), including 7,084 RSV positive subjects (15%) (Table S4). In total, 266 WG sequences (4%) derived from LMICs: Philippines (n = 13), Kenya (n = 184) and Vietnam (n = 69). A large proportion of LMIC sequence data (43%) were published by Kenyan researchers who conducted a longitudinal surveillance study and a community outpatient study.[25–30]

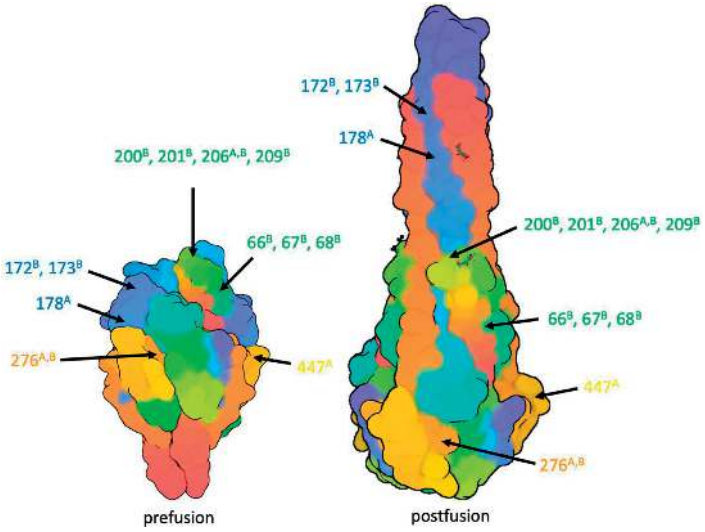
3.3 | Natural polymorphisms in the F protein

Out of 22 studies sequencing the F gene, WG sequences included, 12 studies (55%) described the amino acid diversity in the binding epitopes of RSV A F (n = 2,474) and RSV B F (n = 3,238) for mAbs in clinical development (Figure 3). F sequences originated from Africa (Kenya, South Africa), Asia (China, Japan, Philippines, Iran, Lebanon), Europe (The Netherlands, The UK, Spain, Finland), South America (Brazil), North America (the USA), and Oceania (Australia), and were collected from 2005 to 2018.

All amino acid changes found in literature were reported per country and study period (Figure 3, Table S4). Most of the changes identified in the antibody binding sites have not been reported in literature before. Known detected resistanceassociated mutations include

L172Q (*967/1,401*; South Africa 2015–2017, the USA, 2015–2017, global 2015–2018, Korea 2009–2015, Kenya 2015–2016) and **S173L** (*948/1,409*; South Africa 2015–2017, global 2015–2018, the USA 2015–2017, Philippines 2015–2016, Korea 2009–2015, Kenya 2015–2016) for suptavumab. The number of suptavumab escape mutants (L172Q and S173L) increased to almost 100% in 2017–2018 (Table S4). Changes associated with partial resistance for nirsevimab were **E66K** (*27/27*; Iran 2015–2016), **K68N** (*210/787*; Kenya, the USA 2016–2017), **N201S** (*8/376*; South Africa 2015/2017, The Netherlands 2017–2018, Korea 2009–2015), **Q209K** (*183/1,007*; China 2014–2016, South Africa 2015, The Netherlands 2017– 2018, Korea 2009–2015, Philippines 2014–2016, the USA 2015–2017) and **Q209L** (*88/856*; Brazil 2017–2018, the USA 2015–2017). For palivizumab, **S276N** (*377/535*; South Africa 2017–2018, Korea 2009–2015, China 2014–2016, Iran 2015– 2016, Lebanon 2016–2017) was found in RSV A strains. S276N for RSV B samples in the palivizumab binding site was not associated with resistance. No changes associated with resistance were detected for MK-1654.

FIGURE 3 Amino acid polymorphisms detected at ≥10% frequency (Table S4) are highlighted with arrows. Previously defined mAb binding sites are delineated in colour (green = nirsevimab, orange = palivuzumab, blue = suptavumab, yellow = MK-1654). A and B superscripts denote subtype A and B, respectively

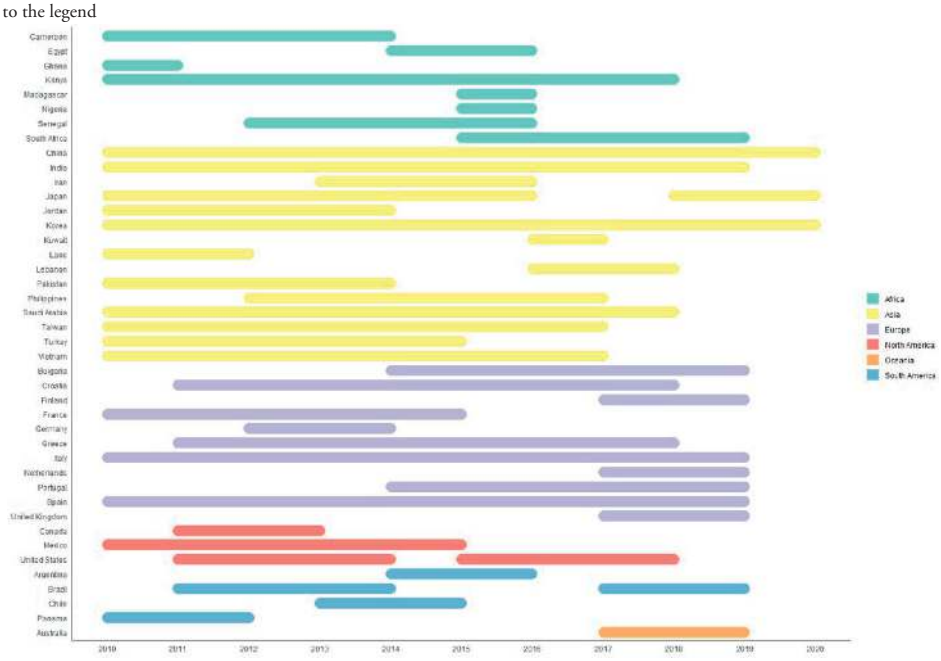


3.4 | Geographical and temporal distribution

While sequences were obtained in all continents, most sequences were derived from China (n = 1,514; 11.6%) followed by Kenya (n = 1,360; 10.5%) and the USA (n = 1,153; 8.9%) (Figure 4). Temporal distribution is shown in Figure 5. Although the INFORM study[24] has contributed to samples in the 2017-2018 season, data from 2015 to 2019 were lacking for multiple countries. Especially in Africa and South America recent data were rare.

FIGURE 4 Geographic distribution of all sequences (n = 43 countries)

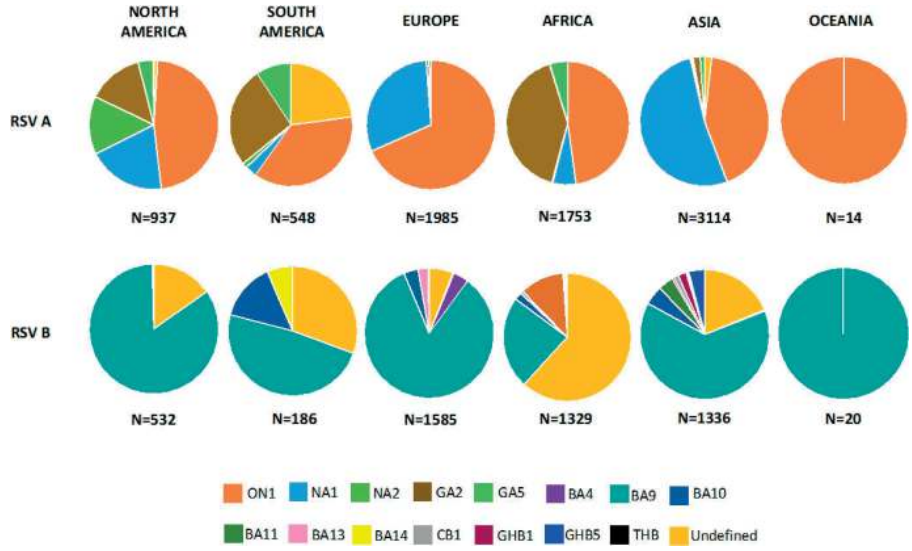
FIGURE 5 Temporal distribution of recent sequences. The bars show the time period of sample collection. Countries included in this review are shown on the y-axis. The time line from 2010–2020 is shown on the x-axis. The continents are coloured according to the legend



3.5 | Genotype distribution

Individually reported genotypes per study were summarised to give an overall impression of the genotype distribution in literature per continent from 2015–2020 (Figure 6). Ontario (ON) 1 and Buenos Aires (BA) 9 were the most reported genotypes for RSV A and B, respectively.

FIGURE 6 Reported RSV A and RSV B genotype distribution (2015–2020). RSV, respiratory syncytial virus



4 | DISCUSSION

This systematic review aimed at identifying knowledge gaps about RSV molecular epidemiology in order to guide future research. While most of the reported amino acid changes have not been tested in neutralization assays, changes associated with partial resistance were only reported in a limited amount for nirsevimab and palivizumab. The suptavumab binding site was associated with high levels of complete resistance for RSV B strains. We have identified a time lag between data collection and publication, as it took the authors of the articles included on average 3 years to publish their sequencing data. This indicates a delay of recent sequences.

This review of available data has identified three knowledge gaps in the field of molecular epidemiology of RSV. First, only a few studies performed WG sequencing. The availability of whole-genome sequences is important for both mAb as well as vaccine development as sequencing data increase the opportunity of the identification of novel vaccine candidates.[31] In more detail, full genomes are needed for two reasons. The first reason is the higher chance of detecting variation in WG compared to individual genes which makes WG sequences essential for reconstructing relatedness. The second reason for the added value of WG sequences is the possibility of amino acid changes outside G, and even outside F, that could have an impact on replication rate and capacity. Currently, data are lacking for genome regions other than the G gene.[32] While the F gene is the most important target for mAb development, only a few studies have sequenced it. The G gene is considered to have the highest genetic diversity. As this confers higher resolution for phylogeny-based reconstructions compared to

other genes, the G gene has been the preferred target for sequencing. This is reflected in the fact that nearly all of the included studies (65/78) focused on the G gene. Although F gene sequences were derived from five continents, non-wild type amino acid polymorphisms in F were found in only 14 countries indicating that many countries remain without data. Mas et al. analysed amino acid variability within the F protein from sequences uploaded in GenBank. F sequences originated from 19 countries. Although a high number of changes were identified, the authors showed that a certain level of variation is natural within evolution. They suggest prospective sample collection to identify potential evolutionary changes driven by passive immunisation programs.[14]

Second, most patients came from China, Kenya and the USA (n = 21,589; 45%). Interestingly, only 7,084 patients (14.8%) were recruited in LMICs, while the burden of RSV disease is disproportionally high in these countries. More than 90% of ALRIs and 99% of RSV related childhood mortality occur in these settings. Furthermore, 58% of deaths in LMICs occur in children aged younger than six months.[1,33] Global numbers on RSV positive children in LMICs are underestimated due to limited access to care. The number of RSV positive children in LMICs reported in this systematic review is not representative for all LMICs as most of the samples derived from a large study in Kenya. LMICs should be considered for RSV research, and included in global studies. Ideally, sampling efforts should focus on places with high burden.[1] Because we lack the data on the global burden of RSV, we used a high population number (>50,000,000) as a proxy for a high burden. LMICs without sequencing data fulfilling this criterion include Indonesia, Nigeria, Bangladesh, Ethiopia, Congo, and Thailand should be prioritised for RSV studies. Sampling was not only uneven in space, but also in time. The temporal distribution showed a lack of recent sampling efforts. In addition to the lack of LMIC data, our review identified specific geographical gaps of sampling. While sequencing data from countries with large populations (China, India, the USA) were available, data from other large populations, for example, Russia with a current population over 145,000,000, would be valuable in a global context.

Third, ongoing molecular surveillance is crucial with regard to the introduction of next generation mAbs. We should learn from past failures like the suptavumab trial.[11] Current programs include the WHOGlobal RSV Surveillance program[34] (Mozambique, Sierra Leone, South Africa, Argentina, Brazil, Canada, Chile, Egypt, Russia, the UK, India, Thailand, Australia, Mongolia) and the OUTSMART program (the USA).[35] We demonstrated that almost all globalRSVBstrains collected between 2015 and 2018 reported amino acid mutations in the suptavumab target epitope sequence. In order to learn from past failures continued monitoring should take place on a global scale.

To the best of our knowledge, this is the first systematic review evaluating the molecular epidemiology of RSV worldwide. There are limitations to this systematic review. Although we attempted to correct for overlapping data in articles that described the same study population, some overlap may remain. Another limitation was that clinical data including age,

sex, comorbidity, and disease severity, were lacking in a considerable number of papers. In addition, we only included articles when authors described polymorphisms in the F protein or when genotype data were shown. A further limitation is that the genotype classification as reported by individual papers is error-prone. Ramaekers et al. described the challenges in identifying RSV genotypes from sequencing data. Neither the hypervariable region (HVR) 2 fragment nor the G gene contain sufficient phylogenetic signal to define genotypes. Therefore, a new classification procedure has been proposed based on full genomes.[36] We recommend that future studies perform WG sequencing and apply the new genotype criteria to determine genotypes. Next to the limitation of the genotype classification, we did not correct for studies that focused on a specific RSV subtype or genotype. We only showed an overall coverage of genotypes as reported in literature. Because reporting bias may have occurred, the genotype distribution could be different on population level. An additional limitation was that this review focused on genetic data in published articles and not on the total number of RSV sequences uploaded in GenBank. A final limitation is that confirmation bias could have impacted our review, as the amino acid changes that have been reported could be an overestimation. More papers could have investigated these changes, but did not find any and therefore did not report on them.

In conclusion, with this systematic review we would like to provide guidance to the scientific community for future RSV research. Sampling effort is crucial in LMICs and other countries with high burden and/or high population to timely detect the development of resistance to RSV therapeutics. Global WG surveillance studies are required to ultimately understand the mechanisms underlying RSV global circulation patterns.

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CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

The study team conceived the project. ACL, ERH and BK were the review authors. ACL drafted the first version of the manuscript. All authors contributed and endorsed the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Chapter 5

How Viral Sequence Analysis May Guide Development of Respiratory Syncytial Virus Monoclonal Antibodies

Clinical Infectious Diseases, 2020

"The loco that you make me
it is just un poco crazy
the sense that you're not making
the liberties you're taking
leaves my cabeza shaking
you are just
un poco loco
un poquititi-ti-ti-ti-ti-ti-ti-ti-ti-ti-ti-to loco."
— Coco, Disney film

How Viral Sequence Analysis May Guide Development of Respiratory Syncytial Virus Monoclonal Antibodies

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Clinical Infectious Diseases® 2021;73(11):e4409–10

Keywords. respiratory syncytial virus; monoclonal antibodies; clinical trial.

Respiratory syncytial virus (RSV) is a seasonal respiratory virus that effects mainly young children [1]. Palivizumab has been licensed since 1998 in over 50 countries worldwide to prevent severe RSV infection in infants with prematurity or congenital heart disease. Although safe and effective, it has some limitations as it is only available for high-risk infants and costly and invasive because of monthly injections. The landscape of RSV-preventive drug candidates exists of more than 60 vaccines and monoclonal antibodies (mAbs) [2, 3]. Currently, 7 mAbs are under development, of which 6 are targeting the fusion (F) protein [4]. Two candidates have progressed to clinical development with nirsevimab (MEDI8897) as the most advanced candidate underway in phase 3.

Simões and colleagues performed a global, randomized, double-blind, placebo-controlled phase 3 trial evaluating the efficacy and safety of suptavumab. They randomized 1154 preterm infants to receive either 1 or 2 doses of suptavumab or placebo. The study was conducted in 18 countries over 3 RSV seasons (2015–2017). The primary endpoint was the proportion of individuals with medically attended RSV infection. The authors showed that suptavumab did not prevent hospitalized or outpatient RSV infections. The majority of breakthrough infections occurred within the first 60 days after dose administration, suggesting that breakthrough infection was not due to subtherapeutic serum levels of suptavumab. Suptavumab failed because of a spontaneous broad introduction of a newly circulating strain of RSV B. Clinical trial samples showed good sensitivity of RSV A strains but complete resistance to RSV B strains to suptavumab. Consequently, suptavumab prevented about 60% of RSV A infections, but was completely ineffective against RSV B. The authors are commended for presenting their data in so much detail, which not always happens in negative drug trials, leading to publication bias, redundant research efforts, and unnecessary patient exposures.

Suptavumab (REGN2222) is a latestage RSV mAb candidate directed against the antigenic site V of the F protein of RSV. Suptavumab promised greater clinical efficacy compared with palivizumab, as preclinical studies predicted it to be 10- and 5-fold more potent in vitro in RSV A and RSV B neutralization, respectively. To our knowledge, there are no in vivo data in the public domain. Simões and colleagues [5] found no suptavumab epitope changes for RSV A when comparing their F protein sequences with reference sequences from 4 years before. As predicted, suptavumab prevented RSV A infections with a single dose or with 2 doses. While the study was not powered to demonstrate separate efficacy for RSV A and RSV B, it is remarkable that suptavumab was effective for RSV A, demonstrating proof-of-concept that suptavumab failed directly due to the spontaneous RSV B escape mutant.

Although the article basically describes a negative trial [6], it feeds the conversation about mAb epitopes and viral escape mutants. An important lesson that Simões and colleagues [5] provide us with in their paper is that RSV genome sequences are important during clinical development of mAbs. In the absence of sequencing data of circulating RSV strains, monoclonal antibody escape mutants (MARMs) were not identified before or during the study period. After completing the study, Simões and colleagues [5] sequenced the F protein of 137

RSV isolates, resulting in 47 high-quality sequences of which 13 were derived from RSV A and 34 from RSV B. While the sequences of the suptavumab epitope in all RSV A isolates were similar to reference strains from 2013, substitutions in 2 amino acid positions (L172Q and S173L) were observed in all RSV B isolates. These mutations have been described before but had been found to be rare (<1%). The RSV B strains with these mutations were first reported to emerge in 2015 in China and later (2017) also in the United States. In the current study, the mutated strains of RSV B globally emerged over the 3 RSV seasons of the study period (2015–2017). Gaps in our knowledge remain with regard to the cause of mutations in the F protein. We know that the RSV B genome is more diverse compared with the RSV A genome [7]. Suptavumab escape does not seem to be the driving mechanism of escape mutants in this study, as the isolates from the placebo group showed the same mutations. Taken together, it remains unclear what caused the new RSV B strain to develop at the exact moment this phase 3 trial was performed.

Simões and colleagues [5] show in the current paper that a single spontaneous mutation may have high impact on antibody binding, and therefore clinical efficacy. Here, MARMs against site V developed spontaneously and not under pressure of suptavumab. As 1 or more prophylactic mAbs are likely to be licensed in the next 5–10 years, it is important to take action to prevent recurrence of mAb failure during clinical development or after large-scale implementation. To this end, several strategies may be considered. First and foremost, it is important to develop mAbs against highly conserved epitopes to minimize the risk of spontaneous MARMs to develop. Second, as the authors suggest, we may consider a cocktail of nonoverlapping mAbs to prevent induction of escape mutants. Although conceptually interesting, there are several hurdles, including regulatory challenges to develop cocktails of RSV mAbs. Third, continuous monitoring of the molecular evolution of RSV is a realistic approach to timely detection of MARMs. If escape mutants are picked up, this may lead to interruption of clinical development programs, and thereby prevent unnecessary patient exposures and high costs of phase 3 clinical trials. Following future introduction of RSV mAbs, surveillance programs are needed to pick up induced or spontaneous escape mutants. Taken together, the current study demonstrates that palivizumab will probably be replaced by next-generation mAbs in the coming years, but that the molecular evolution of RSV is complex and a critical factor to be recognized.

Note

Potential conflicts of interest. L. J. B. has regular interaction with pharmaceutical and other industrial partners. He has not received personal fees or other personal benefits. University Medical Center Utrecht (UMCU) has received major funding (>€100 000 per industrial partner) for investigator-initiated studies from AbbVie, MedImmune, Janssen, Pfizer, the Bill and Melinda Gates Foundation, and MeMed Diagnostics. UMCU has received major cash or in-kind funding as part of the public– private partnership Innovative Medicines Initiative

(IMI)-funded Respiratory Syncytial virus Consortium in EUrope (RESCEU) project from GSK, Novavax, Janssen, AstraZeneca, Pfizer, and Sanofi. UMCU has received major funding by Julius Clinical for participating in the International Network For Optimal Resistance Monitoring (INFORM) study sponsored by MedImmune. UMCU has received minor funding for participation in trials by Regeneron and Janssen from 2015–2017 (total annual estimate <€20 000). UMCU received minor funding for consultation and invited lectures by AbbVie, MedImmune, Ablynx, Bavaria Nordic, MabXience, Novavax, Pfizer, Janssen (total annual estimate <€20 000). L. J. B. is the founding chairman of the ReSViNET Foundation. A. C. L. reports no potential conflicts. Both authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Chapter 6

Global Molecular Diversity of Respiratory Syncytial Virus

"INFORM"

BMC Infectious Diseases, 2019

"Ik heb het nog nooit gedaan
dus ik denk dat ik het wel kan."
— Astrid Lindgren, Pippi Langkous

Global molecular diversity of RSV – the "INFORM RSV" study

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Infectious Diseases (2020) 20:450

ABSTRACT

Background: Respiratory syncytial virus (RSV) is a global cause of severe respiratory morbidity and mortality in infants. While preventive and therapeutic interventions are being developed, including antivirals, vaccines and monoclonal antibodies, little is known about the global molecular epidemiology of RSV. INFORM is a prospective, multicenter, global clinical study performed by ReSViNET to investigate the worldwide molecular diversity of RSV isolates collected from children less than 5 years of age.

Methods: The INFORM study is performed in 17 countries spanning all inhabited continents and will provide insight into the molecular epidemiology of circulating RSV strains worldwide. Sequencing of > 4000 RSV-positive respiratory samples is planned to detect temporal and geographical molecular patterns on a molecular level over five consecutive years. Additionally, RSV will be cultured from a subset of samples to study the functional implications of specific mutations in the viral genome including viral fitness and susceptibility to different monoclonal antibodies.

Discussion: The sequencing and functional results will be used to investigate susceptibility and resistance to novel RSV preventive or therapeutic interventions. Finally, a repository of globally collected RSV strains and a database of RSV sequences will be created.

Keywords: Respiratory syncytial virus, Next generation sequencing, Temporal and geographical diversity, Molecular epidemiology, Monoclonal antibodies, Vaccines

ARTICLE SUMMARY

Strengths

- INFORM RSV is large enough to identify drivers of spatial and temporal distribution.
- Sequencing platform was selected based on a comparative pilot study.
- RSV is cultured to translate genotype to function.
- INFORM RSV is collaborating with others including researchers from the UEDIN, WHO and NIH.

Limitations

- Clinical information is limited, no follow-up data available.

BACKGROUND

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infections in children worldwide [1]. While most children infected with RSV suffer from runny noses, coughing and wheezing, RSV infection can escalate to bronchiolitis, pneumonia and even death [2]. Globally in 2015, 48,000–74,500 children under the age of 5 years died with RSV in-hospital, predominantly in low- and middle-income countries [2].

Although RSV is recognized as a global health problem, there is no licensed vaccine currently available anywhere in the world. Efforts to develop a vaccine initially failed in the 1960s when the first vaccine candidate, a formalin-inactivated vaccine, did not protect against RSV in children but instead induced exacerbated lung disease after subsequent RSV exposure requiring hospitalization and causing death [3, 4]. The potential risk of enhanced disease has hampered vaccine development such that, even after more than 50 years of effort, no vaccine is available yet. An alternative approach for prevention of RSV disease is passive immunization with monoclonal antibodies (mAbs). RSV-IGIV (RespiGam), an intravenous immunoglobulin containing high titers of RSV neutralizing antibodies, was initially approved in 1995 as a passive immunization strategy but was discontinued in 2003 after its replacement by the more potent mAb palivizumab (humanised mAb that targets the RSV fusion (F) protein) [5]. Palivizumab is the only currently approved prophylaxis and its use is limited to high-risk infants (premature, heart and lung disease, Down syndrome) in high-income countries [3]. These data demonstrate that neutralizing Abs are efficient in preventing RSV disease and that antibody levels correlate with RSV disease prevention. The development of suptavumab (REGN2222), another mAb targeting the RSV F protein as a preventive strategy for use in preterm infants was discontinued in 2017 as it failed to meet the primary endpoint of preventing medically-attended RSV infections [6, 7]. A promising mAb candidate currently in clinical development is nirsevimab (MEDI8897), which targets the prefusion form of RSV

F protein [8]. With a higher potency and extended half-life as compared to palivizumab, nirsevimab holds promise for protecting from RSV-associated lower respiratory disease for all infants entering their first RSV season and highrisk infants entering their first and second RSV seasons [7, 8].

Future clinical use of therapeutics, vaccines and mAbs to prevent RSV raises concerns about the emergence of local resistant strains [9, 10]. Therefore, RSV global surveillance is required. The Observational US Targeted Surveillance of Monoclonal Antibody Resistance and Testing of RSV (OUTSMART-RSV) surveillance program characterized circulating RSV strains in the U.S. during the 2017–18 season [11]. RSV strains that are resistant to palivizumab were found to be rare [10]. The frequency of natural resistance-associated polymorphisms for nirsevimab was also low (in vitro < 1%). However, the degree to which the acquisition of resistance will impact the effectiveness of current and future RSV therapeutics on a global scale remains unclear. To date, mAb-resistant mutants (MARMs) have not been thoroughly studied worldwide and little is known about the prevalence of naturally occurring resistant RSV strains either. The International Network For Optimal Resistance Monitoring of RSV (INFORM RSV) study will therefore prospectively describe the molecular epidemiology of RSV by monitoring temporal and geographic distribution of whole viral genome sequences. In addition to monitoring, we will construct a large repository of RSV sequence derived from a diverse geographic location. In the present article, we describe the methodology of the INFORM RSV study.

Study objectives

Primary objective

To investigate the molecular diversity of RSV isolates recovered from a global population of children less than 5 years of age over a five-year period.

Secondary objectives

1. To evaluate the prevalence of strains with polymorphisms in the binding regions for RSV mAbs
2. To compile a repository for RSV sequences
3. To perform functional virology studies
4. To test for susceptibility of newly identified RSV strains to RSV mAbs
5. To establish natural molecular evolution of RSV genomes before the widespread use of RSV mAbs or vaccines

METHODS

Study design

INFORM RSV is a global clinical study initiated in 2017 by AstraZeneca to prospectively analyze RSV strains collected from children < 5 years of age. Collaborators were identified via the Respiratory Syncytial Virus Network (ReSViNET; www.resvinet.org). The ReSViNET Foundation is the international leading non-profit organization committed to reducing global burden of RSV infection. In the INFORM RSV study, RSV positive nasal samples will be collected from subjects as part of routine clinical care at local clinical sites and shipped to the laboratory of the University Medical Centre Utrecht (UMCU), the Netherlands, for sequencing and culturing.

In the INFORM RSV study, the goal is to collect and sequence approximately 4000 RSV positive respiratory samples during a 5-year period (2017–2022), which correlates to 50 or 100 samples per site per year (Additional files, Table 1). At the time of writing, the INFORM RSV study has been ongoing for 2 years and is currently conducted in 17 countries at 18 sites (Fig. 1). We aim to expand to other countries where disease burden studies are ongoing. To ensure both seasonal and geographical diversity, we endeavor to collect 10–20 samples per site per month, over the ~ five-month RSV season, which is on average 5 months long. If the site is able to collect more than the required number of samples, a subset will be randomly selected. Viral genomic sequencing will be performed on all samples by NGS using RT-PCR amplified cDNAs at the UMCU laboratory. To study molecular resistance, a subset of strains (~ 10%) will be randomly selected and cultured to evaluate functional susceptibility to antiviral drugs being developed, and viral fitness of RSV variant with drug binding site changes or dominant changes in non-drug binding site.

Table 1 Eligibility criteria for the INFORM RSV study

Inclusion Criteria	Exclusion Criteria
Age < 5 years	Use of palivizumab or experimental medication for RSV
Confirmed RSV positive diagnosis	
Written informed parental consent	

Study participants

Children are eligible to participate in the study if they meet all the inclusion criteria (Table 1). Children can participate in the study when they fulfill all the following criteria: (1) under the age of 5 years at time of sampling, (2) admitted to the hospital or visiting the outpatient clinic, (3) tested positive for RSV or suspected to have RSV infection when RSV testing is not standard practice. Suspicion of RSV is defined by respiratory tract infection (RTI) symptoms. In instances where testing for RSV is not a standard of care, the informed consent procedure is performed before sample collection for study purposes. Lower and upper RTIs

Fig. 1 Countries participating in the INFORM RSV study.

Red – Start in 2017; Blue – Start in 2018; Yellow – Start in 2019. The figure was created by ACL using Maptive (https://www.maptive.com)

are not differentiated. Signed and dated written informed consent is obtained from parent(s)/ legal representative(s) in accordance with the INFORM RSV study protocol, the International Conference on Harmonization Guideline on Good Clinical Practice E6 (ICH-GCP) and applicable national and international regulatory requirements including the Declaration of Helsinki. Children who meet the exclusion criteria of using preventive or treatment medication for RSV e.g. palivizumab, ribavirin or an experimental RSV mAb or vaccine will be excluded from participation.

Sample collection, storage and shipment

After informed consent is obtained, nasopharyngeal samples are collected using flocked swabs and placed in Copan Universal Transport Medium (UTM). When patients are ventilated, bronchial aspirates are collected in Copan UTM. Samples are stored locally at − 80 °C. When − 80 °C storage is unavailable, samples can be stored at − 20 °C before shipment to the UMCU laboratory. Samples are preferably stored in the original container and labeled with unique barcode provided by the UMCU corresponding to the INFORM RSV code. Samples are shipped frozen on dry ice to the UMCU laboratory for sequencing and culturing after each season.

Nucleic acid extraction and RSV subtyping

Nucleic acids are extracted from 250 to 500 µL of RSV positive nasal specimens using the MagNA Pure 96 DNA and Viral NA Large Volume kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids are eluted in 50 µL elution buffer. RSV subtyping and quantification is performed by multiplexed TaqMan RT-PCR analysis of the RSV N gene using RSV-A and RSV-B specific primer/ probe mixes. The TaqMan RT-PCR reactions are performed on a StepOnePlus System (Applied Biosystems) in 10 µL total volume, including 1 µL of nucleic acids, TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific), 900 nM RSV-A forward primer (5′ AGATCA ACTTCTGTCATCCAGCAA 3′), 900 nM RSV-A reverse primer (5′ TTCTGCACATCATAATTAGGAG TATCAAT 3′), 300 nM RSV-B forward primer (5′ AAGATGCAAATCATAAATTCACAGGA 3′), 300 nM RSV-B reverse primer (5′ TGATATCCAGCATCTTTA AGTATCTTTATAGTG 3′), 58.3 nM RSV-A probe (5′ CACCATCCAACGGAGCACAGGAGAT 3′, 5′6-FAM/ ZEN/3′IBFQ), and 66.7 nM RSV-B probe (5′ TTCCCT TCCTAACCTGGACATAGCATATAACATACCT 3′, 5′ JOE NHS/ZEN/3′ IBFQ) (Integrated DNA Technologies). Cycling conditions are 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s.

RT-PCR amplification of RSV genomes and next generation sequencing

Upon RSV subtyping, the appropriate primer pairs are used to reverse transcribe and PCR amplify the four overlapping RSV genome fragments by using the Super- Script IV One-Step

RT-PCR System (Invitrogen, CA) in a 9800 Fast thermal cycler (Applied Biosystems). The four overlapping genome fragments together comprise of the full RSV genome encompassing all viral genes, yet lacking the far 3′ and 5′ genome termini. Degenerate bases are used in places of genetically variable bases across RSV-A and RSV-B strains when necessary (Table 2). Cycling conditions are 55 °C for 10 min and 98 °C for 2 min, followed by 40 cycles of 98 °C for 10 s, 61 °C for 10 s and 72 °C for 3 min. Amplicons are verified on 1% agarose gels, pooled in equimolar amounts, and purified from 1% agarose gel using the GeneJet PCR Purification Kit (Thermo Fisher Scientific). The purified amplicons are then quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequently, the normalized PCR products are subjected to Next Generation Sequencing (NGS) library construction using the Nextera XT DNA Library Prep Kit according to the manufacturer's protocol (Illumina). Illumina sequencing adapters and barcodes are added to the tagmented DNA via PCR amplification using unique custom oligo sequences (Integrated DNA Technologies). Subsequently, the DNA is purified and size-eselected using 0.6 X volume of Ampure XP reagent (Beckman Coulter, Inc.) according to the manufacturer's protocol. Next, the purified DNA is quantified using the QuantiT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) and mixed in equimolar amounts. Sequencing is performed on the Illumina NextSeq500 platform (Illumina, Inc), generating paired-end 150 bp reads.

Table 2 Primers used in this study to amplify overlapping RSV genome fragments

Primer	Sequence (5′-3′)
RSVA-fragment 1-Fw	AAAAATGCGTAC**W**ACAAACTTGC
RSVA-fragment 1-Rev	GTTGGTCCTTGGTTTTGGAC
RSVA-fragment 2-Fw	CACAGTGACTGACAACAAAGGAG
RSVA-fragment 2-Rev	GCTCATGGCAACACATGC
RSVA-fragment 3-Fw	CGAGGTCATTGCTTGAATGG
RSVA-fragment 3-Rev	CACCACCACCAAATAACATGG
RSVA-fragment 4-Fw	AGGGTGGTGTCAAAAACTATGG
RSVA-fragment 4-Rev	ACGAGAAAAAAAGTGTCAAAAACT
RSVB-fragment 1-Fw	AAAAATGCGTACTACAAACTTGC
RSVB-fragment 1-Rev	TTGTGCTTGGCTTGTTGTTC
RSVB-fragment 2-Fw	AAGGGTTAGCCCATCCAA**M**C
RSVB-fragment 2-Rev	TGCTAAGGCTGATGTCTTTCC
RSVB-fragment 3-Fw	GTCCTCGTCTGA**R**CAAATTGC
RSVB-fragment 3-Rev	TAGGTCCTCTTTCACCACGAG
RSVB-fragment 4-Fw	GAGGGATCCACAGGCTTTAGG
RSVB-fragment 4-Rev	ACGAGAAAAAAGTGTCAAAAACT

RSV genome assembly and annotation

Assembly of the sequencing reads into complete genomes is performed with AstraZeneca's Next- Generation Sequencing Microbial Surveillance Toolbox (NGS-MSTB) – a fully automated distributed pipeline implemented with a Common Workflow Language (CWL), and with a user interface based on the Galaxy bioinformatics workbench [12]. The main processing step is a targeted de-novo assembly using Ariba [13] with AstraZeneca's customized assembly protocol tuned for robustness in the presence of mixed viral subpopulations and very high coverage variability. This is followed by post-assembly filtering of the low-abundance poorly assembled quasi-species. The pipeline creates a Web report with quality control metrics and genome browser views at the contig and individual read levels. A manuscript with detailed description of the assembly pipeline and its opensource release is in preparation.

The assignment of RSV subtypes is performed during the assembly process and the assignment of RSV genotypes is performed by phylogenetic clustering using a reference database of previously described genotypes [14].

To determine the polymorphisms in the F protein binding regions of RSV mAbs, the gene sequences are translated into amino acid sequences, aligned against reference sequences (NL13 strains), and the amino acid changes are recorded.

RSV culture

Frozen respiratory samples stored in UTM are thawed, combined 1:1 with DMEM (Dulbecco's Minimal Essential Medium; Lonza) supplemented with 5% FBS and 100 µg/ml Normocin (InvivoGen), and subsequently filtered through a 0.45 µm filter. The filtrate is used to infect HEp-2 cells (60% confluent) in T25 flasks for 1 h at 33 °C and 5% CO2. The supernatant is replaced with fresh DMEM supplemented with 5% FBS and 100 µg/ml Normocin and placed back into the 33 °C humidified, 5% CO2 incubator. The viral culture is harvested upon reaching approximately 70% cytopathic effect (CPE) by centrifugation at 247×g for 10 min and combining the supernatant with 50% sucrose in dPBS (sterile filtered). The viruses are stored in 1 ml aliquots at − 80 °C.

Data collection and management

Data is recorded on an electronic sample reporting form (SRF) (Table 3). SFRs from all sites are uploaded to a central database (eCASTOR) by Julius Clinical after which the clinical data are merged with the sequencing data. To ensure subject anonymity only a unique subject number and the age in months will be entered. Data will be locked after each season.

Outcomes

Primary endpoint

RSV sequences from a global population of hospitalized children.

Secondary endpoints

1. Total number of RSV A and B subtypes and related genomes and the association of these subtypes with patient characteristics (Table 3)
2. Homology of the F gene from wild-type circulating RSV to that of reference strains
3. The total number of RSV strains with polymorphisms in RSV mAbs binding regions or antigenic sites of RSV F protein

Table 3 Patient variables in the electronic case record form

Variables	Description
Site ID	
Study ID	
Country	
Visiting date	
Age	Age in months
Gender	Male / Female
Length of stay	< 24 h / > 24 h / Outpatient
Referring department	Paediatric Intensive Care Unit / General Paediatric Ward / Outpatient clinic (including Emergency Department)
RSV detection method	PCR / molecular point-of-care-test
RSV subtype	A / B
Storage temperature	-20 °C / -80 °C
Gestational age at birth	Calculated duration of pregnancy in weeks
Severe comorbidity	Congenital heart disease / Hematological malignancies / Neurological disease / Bronchopulmonary dysplasia / Other (specified in provided space)
Breastfeeding	Yes (exclusive) / No / Partial
Day care attendance	Yes / No
Current hay fever, asthma and/or eczema in either parent	Yes / No
Smoking in household	Yes / No
Other children in household under the age of 6	Yes / No

Sample size calculation

The minimal number of samples needed for this study is 2500. The sample size will result in precise frequency estimates of RSV A and B subtypes as well as polymorphisms. The width of the 95% confidence interval (CI) will be no larger than 4%. In extremely low or high prevalence (e.g. < 7.5% or > 92.5%) the width of the 95% CI will be less than 2%. This study is also well powered to detect differences in the prevalence of subtypes (RSV A vs B). An estimate of the mean prevalence of RSV A (two-thirds) was derived from the study by Zhu et al. [10]. The INFORM RSV study has at least 90% power to detect a difference in the prevalence of subtypes between groups of 7% at an alpha of 0.05 (e.g. 70% RSV A in males

vs. 63% RSV A in females), and at least 90% power to detect an effect size of 0.08 using a 4 degrees of freedom chi-square test. This means, for example, that this study can detect a difference in the distribution of RSV subtypes if the prevalence of RSV A across the sites were approximately as follows: 58, 62, 66, 70, and 74%. These sample size calculations were conducted in PASS software, using the two-sided CIs for single proportions with the simple asymptotic method with continuity correction and a chi-square test power analysis. Although 2500 samples are sufficient to detect the desired effect, and based on the minimal invasiveness for INFORM participants, the study will expand and add more countries to maximize insight in geographic and temporal diversity.

DISCUSSION

In the INFORM RSV study, RSV isolates are subject to RSV subtyping and viral genome analysis. The main purpose of the project is to secure RSV samples to monitor RSV strains for changes in key epitopes recognized by mAbs. RSV is a member of the human orthopneumoviridae family [15], which are RNA viruses and therefore prone to genomic mutations. The possibility of immunological escape or viral resistance from mAbs approved or under development is a potential concern. In fact, a previous study performed by Regeneron (NCT02325791) to evaluate the efficacy and safety of suptavumab for the prevention of medically attended RSV infection in preterm infants failed to meet its predefined efficacy endpoint based on its reduced efficacy against RSV B strains [16]. The reduced RSV B efficacy was due to a twoamino acid change at positions 172 and 173 in the antigenic site V region of the F protein, the epitope of suptavumab, which reduced susceptibility to suptavumab neutralization in vitro. It is therefore important that clinical studies involving anti-RSV F mAbs monitor for amino acid substitutions in antigenic binding regions of RSV isolates from subjects experiencing virologic failure, and to assess the impact of these changes on phenotypic susceptibility and viral fitness.

A key challenge for the INFORM RSV study is temporal diversity, as the timing of RSV outbreaks differs by season and location around the world. Another challenge is how to best integrate and interpret whole genome sequences in relation to clinical variables. To overcome this challenge, bioinformaticians from Astra- Zeneca, UMCU and Julius Clinical are working closely together to develop an integrated database and a robust pipeline to characterize the thousands of RSV sequences that will be generated.

In summary, this global prospective study aims at monitoring the molecular epidemiology of RSV to ensure that already approved therapeutics and those in development will be effective against currently circulating strains worldwide. The study has the potential to provide valuable information for vaccines, monoclonal antibodies and therapeutic drugs in development and will contribute to creating an international RSV repository.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10. 1186/s12879-020-05175-4.

Abbreviations

RSV: Respiratory syncytial virus; INFORM RSV: International Network for Optimal Resistance Monitoring of RSV; mAb: Monoclonal antibody; NA: Not applicable; SRF: Sample reporting form; CI: Confidence interval; F: Fusion

Acknowledgements

Not applicable.

Patient and public involvement

· At what stage in the research process were patients/public first involved in the research and how?

Answer: We have an active patient advisory board that has been part of the research team from the start. This includes prioritizing research questions, designing the study and involvement of knowledge transfer of study results.

· How were the research question(s) and outcome measures developed and informed by their priorities, experience, and preferences?

Answer: Research questions and outcomes were developed by the UMC Utrecht and approved by the patient advisory board.

· How were patients/public involved in the design of this study?

Answer: See above.

· How were they involved in the recruitment to and conduct of the study?

Answer: Patients have not been involved in recruitment other than optimizing the patient information.

· Were they asked to assess the burden of the intervention and time required to participate in the research?

Answer: See above.

· How were (or will) patients and the public be involved in choosing the methods and agreeing?

Answer: See above.

· Plans for dissemination of the study results to participants and wider relevant communities?

Answer: See above.

Authors' contributions

All named authors in this article participated in the INFORM study. All authors read and approved the final manuscript. ACL, AG, TH, RTS, PR, FMT, MN, MH, CK, MB, RC, JP1*, JP2*, MPH and JGW were involved in patient recruitment. RJL, AE, MCV and FEJC performed laboratory analyses. ACL, CN, HJ, DET, AT, AR, MEA, DW, LKT, MTE and LJB contributed to the design of the work, analysis and/or interpretation of data. ACL wrote the manuscript. * JP1 is corresponding to Jesse Papenburg, chosen because this name is furthest up in the author list.

Funding

The INFORM study received funding from AstraZeneca. The protocol has been developed by the authors. AstraZeneca has access to all data except viral sequences outside the RSV F and G genes. Data analysis is done by the principle investigator (Louis J Bont).

Availability of data and materials

As the current manuscript describes the study protocol and no other data, we do not have any raw data to share at the moment.

Ethics approval and consent to participate

The INFORM study has been approved by the ethic committees of all 18 participating sites:

- The Netherlands: The Medical Research Ethics Committee of the UMC Utrecht (reference number WAG/mb/17/016170).
- Italy: Ethics Committee for Clinical Testing of the Province of Padova of the Padova Hospital (no. 345 of 27/10/2016).
- Russia: The Department for Science, Innovation Development and Management of Health and Biological Risks, Ministry of Health of the Russian Federation.
- Germany: Ethics Committee of the Medical Faculty of the Philipps University Marburg.
- France: Ethics Committee Southwest and Overseas of the Créteil Intercommunal Hospital Centre (ID-RCB No.: 2018-A02360–55 (file 1–18-73).
- Spain: Ethics Committee for Research Santiago-Lugo of the Hospital Centre University of Santiago (registration code 2017/397).
- South Korea: Medical Research Committee of the Seoul National University Hospital.
- Finland: Ethics Committee of the Hospital District of Southwest Finland, Turku.
- Australia: Human Research Ethics Committee of the Perth Children's Hospital.
- Brazil: The Research Ethics Committee of the Centro INFANT at Pontificia Universidade Catolica de Rio Grande do Sul (opinion number 2,569,872).
- Canada: Hamilton Integrated Research Ethics Board of the McMaster University.
- Canada: Research Ethics Board of the McGill University Health Centre.

- South Africa: Human Research Ethics Committee of the University of the Witwatersrand Johannesburg (no. M170966).
- Japan: Research Ethics Committee of the Fukushima Medical University (no. 29212).
- The United Kingdom: Health Research Authority of the King's College Hospital (no. 17/EM/0469).
- Taiwan: Mackay Memorial Hospital Institutional Review Board (no. 19MMHIS171e).
- Chile: Ethics Committee for Research on Human Subjects of the Faculty of Medicine, University of Chile.
- Mexico: Ethics Committee of the University Autónoma De Nuevo León, Faculty of Medicine.

Written informed consent was obtained from parent(s)/legal representative(s) of all children participating in the study.

Consent for publication

Not applicable.

Competing interests

FMT and JP are members of the editorial board of BMC Infectious Diseases. MCN has received grant funding from AstraZeneca. JP has received consulting/speaker fees/honoraria from AbbVie, Seegene Canada and Cepheid, and research grant funding outside of the current work from AbbVie, BD Diagnostics, AstraZeneca, Sanofi Pasteur, Hoffmann-La Roche and Janssen Pharmaceutical. LJB has not received personal fees or other personal benefits. UMCU has received funding from Abbvie, AstraZeneca, Janssen, the Bill and Melinda Gates Foundation, Nutricia (Danone) and MeMed Diagnostics. UMCU has received major cash or in kind funding as part of the public private partnership IMI-funded RESCEU project from GSK, Novavax, Janssen, AstraZeneca, Pfizer and Sanofi. UMCU has received major funding by Julius Clinical for participating in the INFORM study sponsored by AstraZeneca. UMCU has received minor funding for participation in trials by Regeneron and Janssen from 2015 to 2017. UMCU received minor funding for consultation and invited lectures by AbbVie, AstraZeneca, Ablynx, Bavaria Nordic, MabXience, Novavax, Pfizer, Janssen. LJB is the founding chairman of the ReSViNET Foundation. Nirsevimab development is jointly funded by AstraZeneca and Sanofi Pasteur.

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Chapter 7

Global Molecular Epidemiology of Respiratory Syncytial Virus from 2017-2018

"INFORM"

Journal of Clinical Microbiology, 2020

"Ga je mee verdwalen
ik weet de weg."
— Loesje

Global Molecular Epidemiology of Respiratory Syncytial Virus from the 2017–2018 INFORM-RSV Study

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ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection among infants and young children, resulting in annual epidemics worldwide. INFORM-RSV is a multiyear clinical study designed to describe the global molecular epidemiology of RSV in children under 5 years of age by monitoring temporal and geographical evolution of current circulating RSV strains, F protein antigenic sites, and their relationships with clinical features of RSV disease. During the pilot season (2017–2018), 410 RSV G-F gene sequences were obtained from 476 RSVpositive nasal samples collected from 8 countries (United Kingdom, Spain, The Netherlands, Finland, Japan, Brazil, South Africa, and Australia). RSV B (all BA9 genotype) predominated over RSV A (all ON1 genotype) globally (69.0% versus 31.0%) and in all countries except South Africa. Geographic clustering patterns highlighted wide transmission and continued evolution with viral spread. Most RSV strains were from infants of <1 year of age (81.2%), males (56.3%), and patients hospitalized for >24 h (70.5%), with no differences in subtype distribution. Compared to 2013 reference sequences, variations at F protein antigenic sites were observed for both RSV A and B strains, with high-frequency polymorphisms at antigenic site Ø (I206M/Q209R) and site V (L172Q/S173L/K191R) in RSV B strains. The INFORM-RSV 2017–2018 pilot season establishes an important molecular baseline of RSV strain distribution and sequence variability with which to track the emergence of new strains and provide an early warning system of neutralization escape variants that may impact transmission or the effectiveness of vaccines and MAbs under development.

KEYWORDS evolution, genetic variation, molecular epidemiology, resistance, respiratory syncytial virus, surveillance

Respiratory syncytial virus (RSV) is the leading cause of acute lower respiratory tract infection (LRTI) among infants and young children worldwide (1, 2). Most infections occur seasonally during the winter months in temperate regions, but with greater variability throughout the year in the tropics (3, 4). In 2015, RSV was associated with 33.1 million episodes of LRTI, 3.2 million RSV-related hospital admissions, and 118,000 deaths in children less than 5 years of age, predominantly in developing countries (2). Although prematurity and congenital lung or heart conditions are well-known risk factors for severe RSV LRTI, characterized by bronchiolitis and pneumonia, all children are at risk for RSV LRTI with primary RSV infection during infancy (2, 5).

Prevention of RSV LRTI in all infants is a major public health priority; however, despite many years of attempted vaccine development, there are no licensed vaccines (6). While palivizumab (Synagis) is the only approved passive monoclonal antibody approach for prophylaxis of RSV disease, it is recommended for use only with high-risk infants and children (7). Because there is no approved RSV prophylaxis for the broader population of healthy infants, more than 20 vaccine candidates and monoclonal antibodies (MAbs) are currently in clinical development (8). The most advanced candidate is nirsevimab—a potent, extended half-life MAb recently shown to significantly reduce medically attended RSV LRTI and hospitalization throughout the RSV season in healthy preterm infants in a phase 2b trial (9).

RSV is a nonsegmented, single-stranded, negative-sense RNA *Orthopneumovirus* belonging to the *Pneumoviridae* family (10). The attachment (G) and fusion (F) surface glycoproteins mediate viral entry and are both important antigenic targets for virus-neutralizing antibodies. Based on the genetic variability of the second hypervariable 2 region (HVR2) of the G gene, RSV strains are classified into subtype A or B and further characterized into different genotypes (11). In contrast, the F protein exhibits relative genetic and antigenic stability (12), making it a major target for vaccine and MAb development. The extracellular region of the mature F protein is a trimer of F_1 and F_2 subunits produced by cleavage of an inactive precursor F_0 and exists in prefusion and postfusion conformations. Six antigenic sites (Ø and I to V) have been identified in prefusion and/or postfusion F proteins (13) with target epitopes for prophylactic neutralizing MAbs, including: palivizumab (site II), nirsevimab (site Ø), suptavumab (site V), and MK-1654 (site IV) (14).

As RSV immunization candidates reach the final stages of clinical development, the need for global monitoring of RSV molecular epidemiology becomes increasingly important to ensure their effectiveness during licensure and use. While prophylactic approaches invariably rely on conservation of neutralizing epitopes, RSV replication is inherently error-prone, resulting in natural polymorphisms (15). Selective immune pressure may further result in the emergence and spread of neutralization escape variants, allowing for immune and/or prophylaxis resistance. Finally, evolutionary dynamics of RSV genotypes may correlate with transmission between seasons (16) and disease severity among patient types (17).

The International Network For Optimal Resistance Monitoring of RSV (INFORM-RSV) study was established to describe global molecular epidemiology of RSV by monitoring temporal and geographical distribution of current circulating strains, with a focus on antigenic site changes that may confer selective advantages in transmission or resistance. Here, we describe geographic, demographic, and clinical distribution of RSV strains and sequence diversity of G genes and F proteins collected from mostly hospitalized infants in 8 countries across 4 continents during the pilot 2017–2018 RSV season.

MATERIALS AND METHODS

Study design.

INFORM-RSV is a prospective, multicenter, global molecular epidemiology study to investigate temporal and geographic diversity of RSV isolates collected from children less than 5 years of age who are admitted to the hospital or visiting the outpatient clinic and are not using preventive or treatment medication for RSV. Over the course of a 5-year period (2017–2022), 10 to 20 RSV-positive nasal samples will be collected per month per site each RSV season. Informed consent is obtained from parent(s)/legal representative(s) in accordance with the International Conference on Harmonization Guideline on Good Clinical Practice E6 (ICH-GCP) and applicable national and international regulatory requirements (18).

Sample collection.

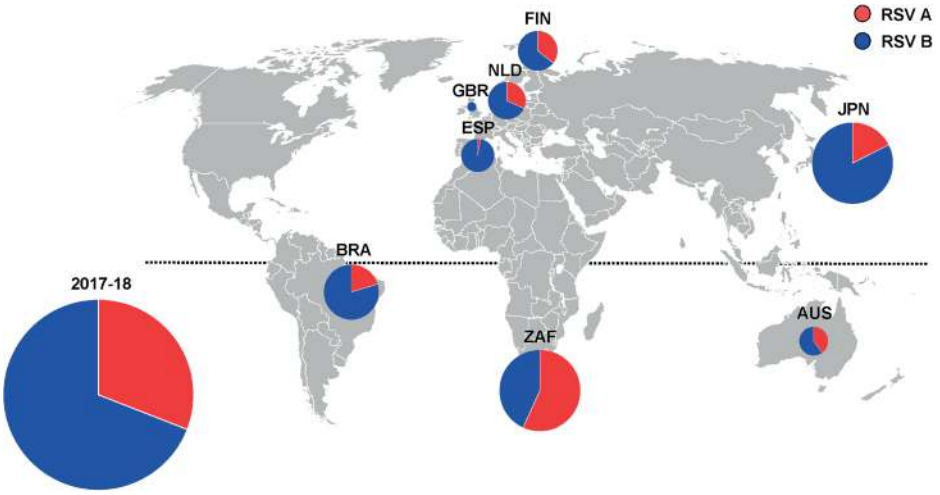
The INFORM-RSV study was initiated in 2017–2018 in 8 countries (United Kingdom [GBR], Spain [ESP], The Netherlands [NLD], Finland [FIN], Japan [JPN], Brazil [BRA], South Africa [ZAF], and Australia [AUS]) with an aim to expand to other countries where disease burden studies are ongoing (Fig. 1). RSV-positive nasal samples were collected in Universal Transport Medium from hospital-based laboratories as part of routine clinical care or specifically for research purposes and shipped to the University Medical Centre Utrecht for sequencing. Individual patient data collected included: location, sample date, age, gender, referring department, and length of hospital stay (18).

RNA extraction, subtyping, RSV genome amplification, and next-generation sequencing.

Nucleic acids were extracted from RSV-positive nasal specimens using the MagNA Pure LC kit (Roche Diagnostics, Mannheim, Germany) as previously described (18). RSV subtyping and quantification were performed by multiplexed TaqMan RT-PCR analysis of the RSV N gene using RSV A and RSV B specific primer/probe mixes. Subsequently, subtype-specific RT-PCR was performed using the SuperScript IV one-step RT-PCR system (Invitrogen, Carlsbad, CA USA) to amplify 4 overlapping fragments covering the full RSV genome.

The resultant 3.5 to 5.0 kb amplicons were pooled, purified from 1% agarose gels, used to construct libraries by means of the Nextera XT DNA Library Prep kit, and sequenced on a NextSeq 500 system (Illumina, San Diego, CA USA) (18).

FIG 1 Geographic distribution of RSV A (n = 128) and RSV B (n = 283) subtypes, 2017–2018 (n = 8 countries).



Overall size of the pies is proportional to the number of RSV isolates and the segments of the pies are proportional to the frequency of subtype A (red) and subtype B (blue) (Table 1). Northern hemisphere: GBR, United Kingdom (n = 2); ESP, Spain (n = 36); NLD, The Netherlands (n = 43); FIN, Finland (n = 45); JPN, Japan (n = 91). Southern hemisphere: BRA, Brazil (n = 64); ZAF, South Africa (n = 95); AUS, Australia (n = 34). (The figure was created with Microsoft PowerPoint.)

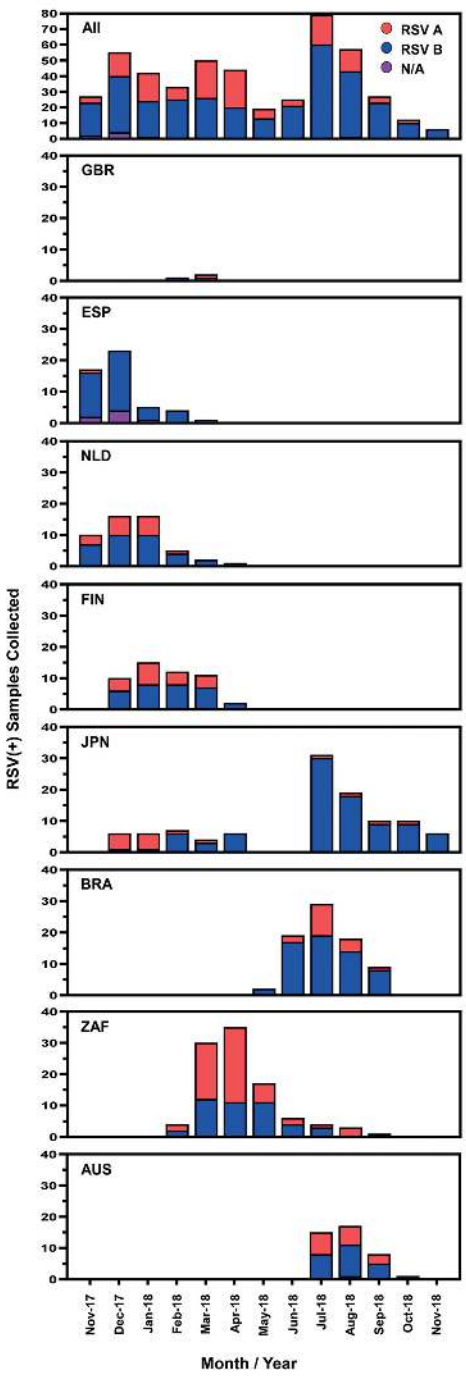
Sequence assembly and genotyping analysis.

Assembly of next-generation sequencing (NGS) reads into RSV G-F contigs was performed using AstraZeneca's open-source NGS-Microbial Sequencing Toolbox, as previously described (18, 19). Alignment of RSV G HVR2 and full-length nucleotide sequences was performed in MUSCLE and evolutionary analyses of full-length RSV G sequences were conducted in MEGA7. Assignment of RSV genotypes was performed by phylogenetic clustering of RSV G HVR2 nucleotide sequences using a previously described 2014 reference database (11).

Amino acid sequence variation analysis of RSV F proteins.

The RSV A and RSV B F sequences in FASTA format were translated into amino acid sequences and aligned against reference F sequences derived from year 2013 Netherlands RSV A/13-005275 (GenBank accession no: KX858757) and RSV B/13-001273 (GenBank accession no: KX858756) reference strains, respectively. Amino acid variation per position was determined and reported from pairwise alignments as previously described (18).

FIG 2 Monthly collection of RSV-positive(+) samples by country and overall number of RSV(+) detected, collected, and isolated/sequenced for RSV G-F gene analysis.



RSV Subtype	RSV(+) Detected	RSV(+) Collected (%)	RSV(+) Isolated (%)
Total	1835	476 (25.9)	410 (86.1)
RSV A	-	142	127
RSV B	-	326	283
N/A	-	8	-

Total	244	3 (1.2)	2 (66.7)
RSV A	-	1	0
RSV B	-	2	2
N/A	-	0	-

Total	232	50 (21.6)	36 (72.0)
RSV A	-	1	1
RSV B	-	42	30
N/A	-	7	-

Total	58	50 (86.2)	43 (86.0)
RSV A	-	16	13
RSV B	-	33	30
N/A	-	0	-

Total	136	50 (36.8)	45 (90.0)
RSV A	-	19	16
RSV B	-	31	29
N/A	-	0	-

Total	106	105 (99.0)	91 (86.7)
RSV A	-	16	16
RSV B	-	89	75
N/A	-	0	-

Total	180	77 (42.8)	64 (83.1)
RSV A	-	17	13
RSV B	-	60	51
N/A	-	0	-

Total	450	100 (22.2)	95 (95.0)
RSV A	-	56	54
RSV B	-	44	41
N/A	-	0	-

Total	429	41 (9.6)	34 (82.9)
RSV A	-	16	14
RSV B	-	21	20
N/A	-	1	-

Structural visualization of RSV F protein antigenic sites.

The 3D structures of prefusion and postfusion RSV F protein trimers were visualized with PyMOL molecular Graphics System, v2.2.2 (Schrödinger, LLC) using PDB 5UDE (12) and PDB 3RRR (20), respectively. Antigenic sites were defined using the six antibody epitopes (Ø and I to V) previously described (13).

Statistical analyses.

A two-sided Fisher's exact test was used to assess statistical significance of global subtype distribution among demographic categories and to compare proportions of amino acid changes between antigenic sites.

RESULTS

Geographic and demographic distribution of RSV A and B subtypes and genotypes.

Between November 2017 and November 2018, 1,835 nasal samples tested RSV-positive among participating sites in 8 countries. Among the RSV-positive detections, 476 (25.9%) nasal samples were collected for inclusion in the INFORM-RSV study. The frequency and monthly pattern of RSV-positive samples collected from each country are shown in Fig. 2. Delayed study initiation resulted in fewer than the targeted 50 RSV-positive samples collected in 5 of the 8 countries. With some exceptions, the peak period for RSV-positive sample collection occurred from December to January and July to August in northern and southern hemisphere countries, respectively. Sequencing and assembly of full-length RSV G-F sequences was successful for 410 of the 476 (86.1%) RSV-positive samples, with even distribution between northern (52.9%; 217 of 410) and southern (47.1%; 193 of 410) hemispheres. The remaining 66 of 476 (13.9%) RSV-positive nasal samples failed sequencing due to unsuccessful RT-PCR amplification, insufficient sequencing depth, or low read quality. Among the 410 RSV strains with G-F sequence data, 127 (31.0%) were subtype A and 283 (69.0%) were subtype B. Overall, the proportion of RSV subtypes differed by country ($P = 0.001$), as RSV B was more prevalent than RSV A in 7 of 8 countries studied, with the exception being South Africa (Fig. 1 and Table 1). Finally, genotype determination revealed that all RSV A strains were of the Ontario 1 (ON1) genotype and all RSV B strains were of the Buenos Aires 9 (BA9) genotype.

Distribution of RSV strains by gender, age, and length of hospital stay was also determined. The median age of RSV-positive individuals was 5 months (interquartile range [IQR], 2 to 9 months) and 81.2% (333 of 410) were aged less than 1 year; 56.3% (231 of 410) were males; and 70.5% (289 of 410) were hospitalized for ≥24 h. RSV isolates from outpatients, characterized by a length of hospital stay of <24 h, were mostly derived from 3 countries

TABLE 1 Frequency of RSV A (n = 127) and RSV B (n = 283) subtypes by demographic and clinical characteristics and country

Country[a]	No. Total	Gender		Age			Length of Stay		Referring dept.[b]			
		(%) No. male (%)	No. female (%)	No. <1 yr (%)	No. 1 to <2 yrs (%)	No. 2 to <5 yrs (%)	No. ≥24 h (%)	No. <24 h (%)	No. ER/ED (%)	No. PICU (%)	No. PW (%)	No. other (%)
All	410	231	179	333	59	18	289	121	25	39	74	272
RSV A	127 (31.0)	82 (35.5)	45 (25.1)	104 (31.2)	21 (35.6)	2 (11.1)	88 (30.4)	39 (32.2)	3 (12.0)	8 (20.5)	19 (25.7)	97 (35.7)
RSV B	283 (69.0)	149 (64.5)	134 (74.9)	229 (68.8)	38 (64.4)	16 (88.9)	201 (69.6)	82 (67.8)	22 (88.0)	31 (79.5)	55 (74.3)	175 (64.3)
GBR	2	0	2	2	0	0	2	0	0	1	1	0
RSV A	0	0	0	0	0	0	0	0	0	0	0	0
RSV B	2 (100.0)	0	2 (100.0)	2 (100.0)	0	0	2 (100.0)	0	0	1 (50.0)	1 (50.0)	0
ESP	36	20	16	31	3	2	31	5	5	0	0	31
RSV A	1 (2.8)	1 (5.0)	0	1 (3.2)	0	0	1 (3.2)	0	0	0	0	1 (3.2)
RSV B	35 (7.2)	19 (95.0)	16 (100.0)	30 (96.8)	3 (100.0)	2 (100.0)	30 (96.8)	5 (100.0)	5 (100.0)	0	0	30 (96.8)
NLD	43	23	20	40	1	2	39	4	1	22	19	1
RSV A	13 (30.2)	7 (30.4)	6 (30.0)	12 (30.0)	1 (100.0)	0	11 (28.2)	2 (50.0)	0	4 (18.2)	8 (42.1)	1 (100.0)
RSV B	30 (69.8)	16 (69.6)	14 (70.0)	28 (70.0)	0	2 (100.0)	28 (71.8)	2 (50.0)	1 (100.0)	18 (81.8)	11 (57.9)	0
FIN	45	27	18	45	0	0	4	41	0	0	0	45
RSV A	16 (35.6)	11 (40.7)	5 (27.8)	16 (35.6)	0	0	1 (25.0)	15 (36.6)	0	0	0	16 (35.6)
RSV B	29 (64.4)	16 (59.3)	13 (72.2)	29 (64.4)	0	0	3 (75.0)	26 (63.4)	0	0	0	29 (64.4)
JPN	91	43	48	46	33	12	43	48	5	0	54	32
RSV A	16 (17.6)	8 (18.6)	8 (16.7)	6 (13.0)	9 (27.3)	1 (8.3)	2 (4.7)	14 (29.2)	0	0	11 (20.4)	5 (15.6)
RSV B	75 (82.4)	35 (81.4)	40 (83.3)	40 (87.0)	24 (72.7)	11 (91.7)	41 (95.3)	34 (70.8)	5 (100.0)	0	43 (79.6)	27 (84.4)
BRA	64	42	22	59	5	0	44	20	14	13	0	37
RSV A	13 (20.3)	9 (21.4)	4 (18.2)	12 (20.3)	1 (20.0)	0	7 (15.9)	6 (30.0)	3 (21.4)	2 (15.4)	0	8 (21.6)
RSV B	51 (79.7)	33 (78.6)	18 (81.8)	47 (79.7)	4 (80.0)	0	37 (84.1)	14 (70.0)	11 (78.6)	11 (84.6)	0	29 (78.4)
ZAF	95	55	40	83	11	1	92	3	0	0	0	95
RSV A	54 (56.8)	35 (63.6)	19 (47.5)	47 (56.6)	6 (54.5)	1 (100.0)	52 (56.5)	2 (66.6)	0	0	0	54 (56.8)
RSV B	41 (43.2)	20 (36.4)	21 (52.5)	36 (43.4)	5 (45.5)	0	40 (43.5)	1 (33.3)	0	0	0	41 (43.2)
AUS	34	21	13	27	6	1	34	0	0	3	0	31
RSV A	14 (41.2)	11 (52.4)	3 (23.1)	10 (37.0)	4 (66.7)	0	14 (41.2)	0	0	2 (66.7)	0	12 (38.7)
RSV B	20 (58.8)	10 (47.6)	10 (76.9)	17 (63.0)	2 (33.3)	1 (100.0)	20 (58.8)	0	0	1 (33.3)	0	19 (61.3)

[a]GBR, United Kingdom; ESP, Spain; NLD, The Netherlands; FIN, Finland; JPN, Japan; BRA, Brazil; ZAF, South Africa; AUS, Australia.

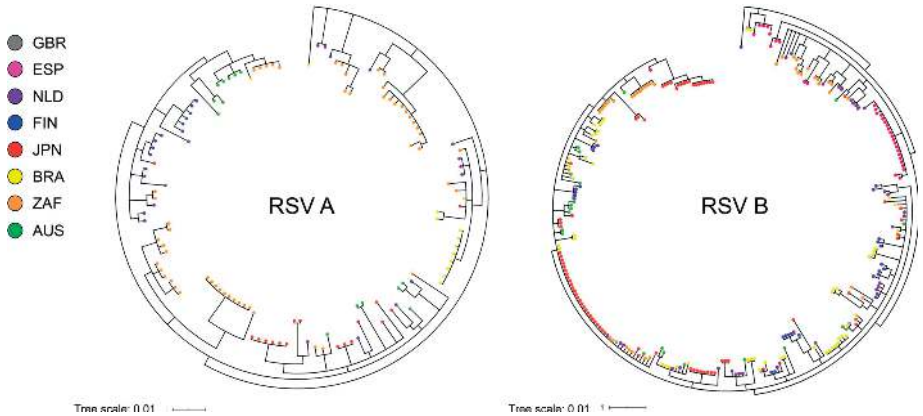
[b]ER/ED, emergency room/department; PICU, pediatric intensive care unit; PW, pediatric ward; Other, other/undefined location.

(Finland, Japan, and Brazil) and accounted for 29.5% (121 of 410) of the total. Stratification by referring department revealed that most RSV isolates came from other/undefined locations (66.3%; 272 of 410), followed by the pediatric ward (PW) (18.0%; 74 of 410), emergency room/department (ER/ED) (6.1%; 25 of 410), and pediatric intensive care unit (PICU) (9.5%; 39 of 410) (Table 1). Overall, RSV B was more frequent than RSV A in all categories and there were no significant differences in the global proportion of subtypes by age group (P = 0.141) or length of hospital stay (P = 0.722). While a significantly higher proportion of RSV B cases were observed globally in females compared to males (P = 0.0311), no gender differences were observed within individual countries.

Global analysis of RSV genetic variability.

To understand genetic variability of the 2017–2018 RSV strains, we performed a phylogeographic analysis of G gene sequences by country. Within both RSV A (all ON1 genotype) and RSV B (all BA9 genotype) phylogenies, some sequences clustered within a country, suggesting microevolution, while other clusters contained sequences from multiple countries (Fig. 3). These data show that RSV A ON1 and RSV B BA9 strains from 2017–2018 were genetically diverse by geographic locale, consistent with wide transmission and continued evolution.

FIG 3 RSV A ON1 (n = 127) and RSV B BA9 (n = 283) G-based clades by country.

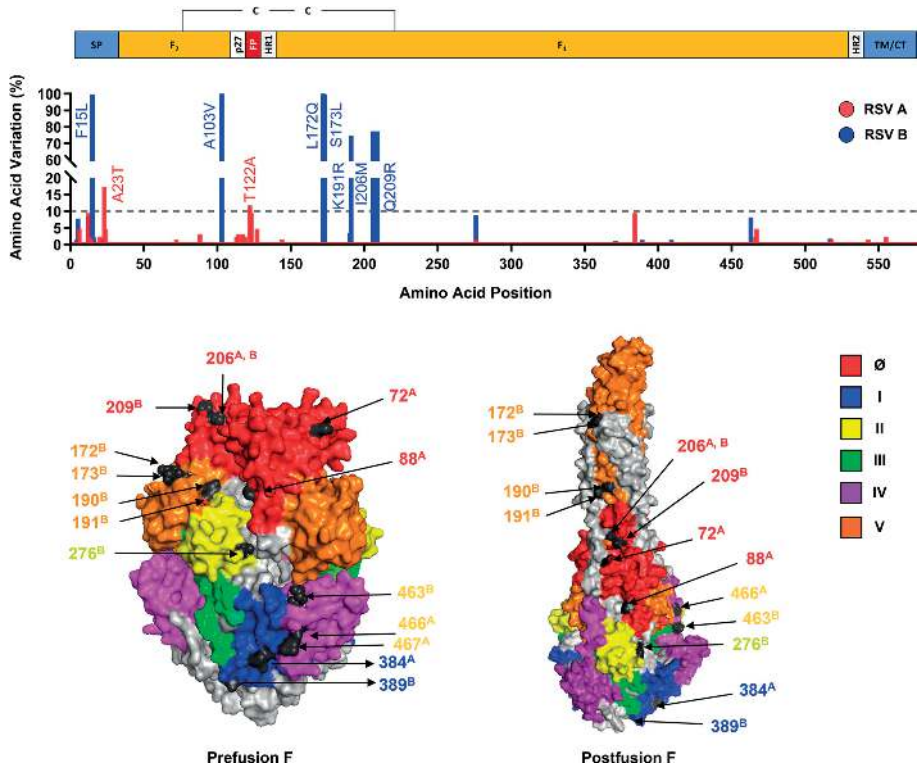


Evidence for evolution of the RSV F protein.

To assess recent evolution of the fusion protein, 2017–2018 RSV A F and B F protein sequences were compared to year 2013 RSV A/13-005275 and RSV B/13-001273 reference strains, respectively. Overall, diversity of RSV F sequences was low, with mostly conserved amino acid changes detected at 45 of 574 positions (7.8%) in RSV A F and at 62 of 574 positions (10.8%) in RSV B F (Fig. 4). Only 2 amino acid changes in RSV A F were highly

polymorphic: A23T (17.3%) in the signal peptide and T122A (11.8%) in the fusion peptide. In contrast, 7 amino acid changes in RSV B F were detected in a majority of sequences as follows: F15L (99.6%) in the signal peptide, A103V (100%) in F2, and L172Q (100%), S173L (99.6%), K191R (74.2%), I206M (77.4%), and Q209R (76.3%) in F1.

FIG 4 Individual frequency and structural location of amino acid polymorphisms in RSVAF(n = 127) and RSVBF(n = 283) protein sequences.



(Top) Major structural features of full-length RSV F protein (amino acids [AA] 1–574), including the extracellular region (F2:AA 24 –109 and F1: 137-524); SP, signal peptide; p27 peptide; FP, fusion peptide; HR, heptad repeats; and TM/CT, transmembrane/carboxy terminus. (Middle) Linear plot of individual amino acid variation frequency in full-length RSV A F (red) and RSV B F (blue) protein sequences compared to year 2013 RSV A/13-005275 and RSV B/13-001273 reference strains, respectively. Amino acid polymorphisms detected at ≥10% frequency (Table 2) are denoted. (Bottom) Proximal locations of amino acid polymorphisms in antigenic sites of mature prefusion and postfusion RSV F protein trimers. Previously defined antigenic sites (Ø and I to V) (13) are delineated in color. Amino acid positions at which polymorphisms were detected at ≥1% frequency (Table 2) are highlighted in black with adjoining arrows. A and B superscripts denote subtype A and B, respectively.

Amino acid variation was further examined in each antigenic site (Ø and I to V) by geography (Table 2) and depicted on prefusion and postfusion F protein trimer structures (Fig. 4). No statistical differences in the global proportion of amino acid changes were observed between antigenic sites (data not shown) and some changes occurred in both RSV A F and B F at the same positions (Y33, I206, S255, and S276). Overall, 11 amino acid changes were

TABLE 2 Global frequency of amino acid polymorphisms in antigenic sites of RSV A F (n=127) and RSV B F (n=283) protein sequences and countries of detection

		RSV A (n = 127)			RSV B (n = 283)		
Site	Amino acid positions[a]	Change[b]	No. (%)	Country[c]	Change[b]	No. (%)	Country[c]
Ø	62–96; 195–227	T72A	2 (1.6)	ESP,NLD	K68R	1 (0.4)	AUS
		N88T	4 (3.1)	FIN	K68Q	1 (0.4)	JPN
		I206T	1 (0.8)	FIN	N201S	1 (0.4)	NLD
					I206M	**219 (77.4)**	**All**
					Q209K	1 (0.4)	NLD
					Q209L	2 (0.7)	BRA
					Q209R	**216 (76.3)**	**All**
I	27–45; 312–318; 378–389	Y33H	1 (0.8)	ZAF	Y33F	1 (0.4)	ZAF
		I384T	12 (9.4)	ZAF	P312H	1 (0.4)	NLD
					S380N	2 (0.7)	BRA
					L381I	1 (0.4)	FIN
					S389F	1 (0.4)	ZAF
					S389P	3 (1.1)	BRA
II	254–277	S255N	1 (0.8)	ZAF	S255G	1 (0.4)	ESP
		S276N	1 (0.8)	ZAF	M264I	1 (0.4)	FIN
		S276R	1 (0.8)	FIN	K272N	1 (0.4)	BRA
					L273I	1 (0.4)	ZAF
					S276N	25 (8.8)	BRA,ESP,FIN,GBR,NLD
III	46–54; 301–311; 345–352; 367–378				L303I	2 (0.7)	NLD
					I305T	1 (0.4)	ESP
					V349A	1 (0.4)	JPN
					N371S	3 (1.1)	JPN
IV	422–471	S425T	1 (0.8)	ZAF	K433R	1 (0.4)	AUS
		S466N	3 (2.4)	ZAF,NLD,JPN	L462Q	1 (0.4)	NLD
		L467I	6 (4.7)	ZAF,BRA	E463D	24 (8.5)	BRA,ESP,FIN,NLD
V	55–61; 146–194; 287–300				**L172Q**	**283 (100.0)**	**All**
					S173L	**282 (99.6)**	**All**
					K176R	1 (0.4)	AUS
					V179I	1 (0.4)	JPN
					S190N	10 (3.5)	BRA,ESP,FIN,JPN
					K191R	**210 (74.2)**	**All**
					V300I	1 (0.4)	FIN

[a]Amino acid positions that define antigenic sites Ø and I–V (13).

[b]Amino acid changes compared to year 2013 reference sequences; high-frequency polymorphisms (≥10%) are indicated in boldface type.

[c]GBR, United Kingdom; ESP, Spain; NLD, The Netherlands; FIN, Finland; JPN, Japan; BRA, Brazil; ZAF, South Africa; AUS, Australia.

detected in 4 of 6 antigenic sites for RSV A F, with frequencies ranging from 0.8 to 9.4%, and 32 amino acid changes were detected in 6 of 6 antigenic sites for RSV B F, with frequencies ranging from 0.4 to 100.0%. Only 5 of the 32 antigenic site changes in RSV B F were highly polymorphic and detected in all countries: I206M (77.0%) and Q209R (76.3%) in site Ø and L172Q (100.0%), S173L (99.6%), and K191R (74.2%) in site V. With few exceptions, antigenic site changes of intermediate polymorphic frequency (≥1% and <10%) were detected in multiple countries. These results indicate that F protein sequences and antigenic sites from 2017–2018 were generally well-conserved compared to year 2013 reference strains, although RSV B strains exhibited greater variability.

DISCUSSION

RSV A and B cocirculate during seasonal epidemic periods with alternating patterns of predominance over time (21). However, little is known about temporal evolution of RSV strains, global spread of unique genotypes, or how these factors relate to disease severity. Also important to the development of vaccines and MAbs is the need to identify and track patterns of F protein antigenic site changes, which may confer selective advantages in transmission or resistance. The INFORM-RSV study aims to describe global molecular evolution and epidemiology of RSV by prospectively monitoring temporal and geographical distribution of currently circulating strains. At the time of writing, the INFORM-RSV study has been ongoing for 3 years and is currently being conducted in 17 countries across 5 continents. The results herein provide baseline information on RSV strain distribution associated with different clinical parameters of disease severity and genetic variation of RSV G and F from 8 countries (GBR, ESP, NLD, FIN, JPN, BRA, ZAF, and AUS) across 4 continents during the 2017–2018 pilot season.

Genomic variation and evolutionary dynamics of RSV may affect its geographic, demographic, and clinical transmission behavior with important implications. During the INFORM-RSV 2017–2018 season, RSV B predominated over RSV A in all countries except South Africa, which may be attributed to virulence and local spread of RSV A strains specific to South Africa. Recent reports from North America (USA, 2015–2017 [22, 23]; Mexico, 2003–2015 [24]), Africa (ZAF, 2015–2017 [25]); [Kenya, 2000–2012 (26)], Asia (China, 2007–2015 [21]), and Australia (AUS, 2010–2016 [27]) describe alternating periodicity of RSV subtype prevalence, dominated by RSV A ON1 and RSV B BA9 genotypes. Consistent with these reports, RSV A ON1 and RSV B BA9 were the predominant genotypes of circulating RSV strains during the 2017–2018 RSV season. Geographic clustering patterns further suggest RSV transmission is characterized by continued genotype diversification during local spread and global dissemination.

Because the impact of viral factors on clinical parameters of disease severity has remained inconclusive (28), it was important to understand the distribution of RSV strains among demographic and clinical characteristics. Ultimately, most RSV strains were collected from hospitalized male infants aged less than 1 year, consistent with estimates of incidence and hospitalization rates (29), known risk factors, and the anatomic nature of shorter and narrower airways in infant males who are more likely to develop bronchial obstruction due to RSV infection (5). Unfortunately, the outpatient burden of RSV on health care resources has not been well defined (1, 2, 30) and few INFORM-RSV countries collected RSV-positive samples from outpatients who were medically managed without hospital admission. While hospital-based laboratory data on RSV infections may markedly underestimate the global burden of RSV disease, nevertheless, we observed no significant or meaningful differences in subtype/genotype distribution on clinical features of disease severity as assessed by gender, age group, or length of hospital stay.

The RSV F protein has historically been relatively well conserved, yet continues to evolve (12, 31). To that end, data from the INFORM-RSV 2017–2018 pilot season establishes an important molecular baseline of RSV F protein sequence and antigenic site variation from which to track frequency, geography, and evolutionary trajectory of potential neutralization escape variants as an early warning for vaccines and MAbs in development. Although the observed variability of the 2017–2018 RSV F sequences was low, with no differences in the proportion of amino acid changes between antigenic sites, the frequency and geographical distribution of some variants suggest a recent positive selection of favorable amino acid changes. Indeed, RSV B strains containing Q209R (site Ø) and L172Q/S173L (site V), first reported in China (2014 –2016) (32), have recently emerged as dominant variants, with the addition of the I206M (site Ø) and K191R (site V) changes detected in the United States (2015–2019) (22, 33). These additional changes are possibly due to natural selective pressure from maternal or host neutralizing antibodies. Since site Ø and V elicit the greatest frequency of high-potency antibodies (34) in a structural area requiring a great deal of flexibility (13), these sites may tolerate greater amino acid variation than others. Additional, less frequent amino acid changes detected during the INFORM-RSV 2017–2018 study were frequent enough to be resampled in multiple countries but have yet to spread globally.

While the impact that widespread use of anti-RSV F MAbs will have on the emergence and transmission of resistant variants is unknown, these variants may also arise naturally in the absence of drug selection pressure. To date, palivizumab resistanceassociated polymorphisms have been rarely observed in circulating RSV strains (35). Consistent with these reports, the restricted use of palivizumab (Synagis) (7), and the growth disadvantage of resistant variants in the absence of palivizumab selective pressure (36), we observed no known palivizumab target site II polymorphisms among 2017–2018 RSV strains. Also consistent with the rapid emergence and outgrowth of a RSV B strains containing L172Q/S173L in the United States (2015–2019) (22, 33), these nonconservative polymorphisms in suptavumab target site V

were detected in 100% of global 2017–2018 RSV strains and coincide with clinical resistance and the recent failure of suptavumab to reduce overall RSV hospitalizations or outpatient LRTI in preterm infants in a phase 3 trial (6, 37). Finally, conservative I206M/Q209R polymorphisms in nirsevimab target site Ø were detected in 77% of RSV B strains but have been shown to retain susceptibility to neutralization by nirsevimab (38). Accordingly, despite the recent emergence of these polymorphisms, nirsevimab significantly reduced medically attended RSV LRTI in healthy preterm infants in a recent Phase 2b trial (9).

There are some limitations to the INFORM-RSV study. Key challenges to temporal analyses between geographies include adequate country representation and timing of RSV epidemics by season and location. Although low rates of RSV A and B coinfection (<2%) have been reported (22, 39), the use of subtype-specific primers/probes in the INFORM-RSV study did not permit detection of RSV A and B coinfection. Data on patients' viral load are unavailable and therefore additional phylodynamic evolutionary and viral spread analyses are not possible. Since our data are heavily weighted toward infants with severe RSV disease that required hospitalization, we do not know about trends and molecular analyses of RSV from children who were medically managed as outpatients or were asymptomatic and did not seek medical attention. Our use of a 2014 RSV G HVR2 reference database (11) to genotype contemporary isolates has limitations as RSV continues to evolve. Accordingly, an extensible, centralized, curated, open database of reference sequences is needed to standardize genotyping and allow comparability across studies. Finally, future phenotypic susceptibility data would help to understand the functional impact of F protein antigenic site changes against anti-RSV F MAbs.

The strength of the INFORM-RSV study is reflected in its prospective design to characterize temporal and geographic trends in RSV diversity and to progress for several years with widespread global participation. Historically, RSV molecular epidemiology studies have been retrospective, focused exclusively on G gene diversity, and/or have been limited by geographical and low sampling effort constraints (15, 26, 40, 41). While global RSV surveillance is conducted by the European Influenza Surveillance Network (4) and the World Health Organization (42), none provide subtype differentiation or sequence analyses when reporting patterns of circulation. Findings from the INFORM-RSV study may have important implications in understanding the impact of RSV evolution on transmission, pathogenesis, and prophylaxis effectiveness. Tracking the frequency, recurrence, and distribution of amino acid changes that may confer selective advantages is a key focus of INFORM-RSV. Recent strains and dominant genotypes have genetic differences from the prototype virus strain used in most vaccine research (43). Since antigenic site changes could alter viral antigenicity for vaccines and affect their susceptibility to MAbs, novel agents for prophylaxis cannot afford to miss their contemporary targets when they are eventually deployed.

In conclusion, ongoing surveillance of global molecular epidemiology of RSV is important for detecting the emergence and spread of new strains, predicting their clinical impact,

and providing an early warning system of antigenic changes that may affect the effectiveness of vaccines and MAbs. To that end, the INFORM-RSV 2017–2018 pilot season establishes an important molecular baseline of RSV strain distribution and sequence variability among hospitalized infants from which to investigate temporal and geographic relationships in the years ahead.

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We declare that the planning, conduct, and reporting from this study was in line with the Declaration of Helsinki, as revised in 2013. The Medical Research Ethics Committee of the UMC Utrecht confirmed in their letter of 31 May 2017 (reference number WAG/mb/17/016170) that the Medical Research Involving Human Subjects Act (WMO) does not apply to the present study and therefore an official approval of this study by the MREC UMC Utrecht was not required under the WMO. Informed consent was obtained from parent(s) or legal representative(s) prior to sample collection in accordance with the International Conference on Harmonization Guideline on Good Clinical Practice E6 (ICH-GCP) and applicable national and international regulatory requirements.

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Chapter 8

Nirsevimab Binding Site Conservation in Respiratory Syncytial F Glycoprotein and Lack of Escape Variants Worldwide Since 1956: An Analysis of Observational Study Sequencing Data

"INFORM"

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"Life is like the surf,
so give yourself away like the sea."
— Y Tu Mamá También, film

Nirsevimab binding-site conservation in respiratory syncytial virus fusion glycoprotein worldwide between 1956 and 2021: an analysis of observational study sequencing data

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SUMMARY

Background Nirsevimab is an extended half-life monoclonal antibody to the respiratory syncytial virus (RSV) fusion protein that has been developed to protect infants for an entire RSV season. Previous studies have shown that the nirsevimab binding site is highly conserved. However, investigations of the geotemporal evolution of potential escape variants in recent (ie, 2015–2021) RSV seasons have been minimal. Here, we examine prospective RSV surveillance data to assess the geotemporal prevalence of RSV A and B, and functionally characterise the effect of the nirsevimab binding-site substitutions identified between 2015 and 2021.

Methods We assessed the geotemporal prevalence of RSV A and B and nirsevimab binding-site conservation between 2015 and 2021 from three prospective RSV molecular surveillance studies (the US-based OUTSMART-RSV, the global INFORM-RSV, and a pilot study in South Africa). Nirsevimab binding-site substitutions were assessed in an RSV microneutralisation susceptibility assay. We contextualised our findings by assessing fusion-protein sequence diversity from 1956 to 2021 relative to other respiratory-virus envelope glycoproteins using RSV fusion protein sequences published in NCBI GenBank.

Findings We identified 5675 RSV A and RSV B fusion protein sequences (2875 RSV A and 2800 RSV B) from the three surveillance studies (2015–2021). Nearly all (25 [100%] of 25 positions of RSV A fusion proteins and 22 [88%] of 25 positions of RSV B fusion proteins) amino acids within the nirsevimab binding site remained highly conserved between 2015 and 2021. A highly prevalent (ie, >40·0% of all sequences) nirsevimab binding-site Ile206Met:Gln209Arg RSV B polymorphism arose between 2016 and 2021. Nirsevimab neutralised a diverse set of recombinant RSV viruses, including new variants containing binding-site substitutions. RSV B variants with reduced susceptibility to nirsevimab neutralisation were detected at low frequencies (ie, prevalence <1·0%) between 2015 and 2021. We used 3626 RSV fusion-protein sequences published in NCBI GenBank between 1956 and 2021 (2024 RSV and 1602 RSV B) to show that the RSV fusion protein had lower genetic diversity than influenza haemagglutinin and SARS-CoV-2 spike proteins.

Interpretation The nirsevimab binding site was highly conserved between 1956 and 2021. Nirsevimab escape variants were rare and have not increased over time.

Funding AstraZeneca and Sanofi.

RESEARCH IN CONTEXT

Evidence before this study

We searched PubMed on Jan 13, 2023, for published research articles using the search terms "respiratory syncytial virus" AND "antibody prophylaxis" AND "viral escape" without any date limits or language restrictions. Results were complemented by a Google search using the same search terms. We found multiple papers describing the influence of individual and co-occurring amino-acid substitutions on virus neutralisation by several anti-respiratory syncytial virus (RSV) fusion protein monoclonal antibodies in vitro (eg, palivizumab, suptavumab, and nirsevimab). The prevalence of an RSV B strain containing Ile206Met:Gln209Arg polymorphisms in the nirsevimab binding site has increased in RSV seasons between 2016 and 2018. Although previous studies assessed the influence of nirsevimab binding-site substitutions on variants identified from 1956 to 2014, comprehensive data evaluating the influence of binding-site substitutions from recent (ie, 2015–2021) RSV seasons were absent.

Added value of this study

We present complementary analyses using prospective (n=5675) and retrospective (n=3626) fusion-protein sequence data to show nirsevimab binding-site conservation since 1956. Using our prospective dataset, we show a high degree of amino-acid conservation within the nirsevimab binding site in recent RSV seasons (2015–2021). Shannon entropy analyses of the fusion protein show that amino-acid variability is predominately concentrated in regions outside of the nirsevimab binding site. Using recombinant RSV viruses in a microneutralisation assay, we found that nirsevimab has broad neutralising activity against binding-site substitutions identified in recent RSV seasons. The prevalence of the nirsevimab binding site Ile206Met:Gln209Arg RSV B polymorphism increased in successive RSV seasons between 2016 and 2020. We observed that nirsevimab-neutralisation escape variants were rare (ie, prevalence <1%) in circulating RSVs between 2015 and 2021. We contextualise these findings with additional analyses using our retrospective dataset of RSV fusion-protein sequences published to the National Center for Biotechnology Information GenBank between 1956 and 2021. We show the genetic stability of the RSV fusion protein compared with envelope glycoproteins from other contemporary respiratory viruses. Additional amino-acid variation and Shannon entropy analyses reveal a high degree of nirsevimab binding-site conservation from 1956 to 2021.

Implications of all the available evidence

Despite the emergence of new RSV B polymorphisms (eg, Ile206Met:Gln209Arg), RSV A and B nirsevimab binding sites remain generally conserved. Nirsevimab retains its activity against the common co-occurring Ile206Met:Gln209Arg polymorphisms and rarer substitutions. Individual nirsevimab binding-site substitutions might incur a reduction in viral fitness that is potentially compensated for by additional substitutions. Nirsevimab-neutralisation escape variants were rare and have not increased in successive RSV seasons between 2015 and 2021. As RSV continues to evolve, ongoing surveillance of circulating variants is necessary to detect molecular changes over time and their effect on susceptibility to nirsevimab neutralisation.

INTRODUCTION

Respiratory syncytial virus (RSV) is a leading cause of childhood acute lower respiratory tract infection, with seasonal RSV disease epidemics resulting in substantial global morbidity and considerable burden on health-care systems.[1,2] Studies of RSV seasonality in temperate climates have previously observed annual RSV epidemics during the winter months.[3] However, the public-health measures implemented in response to the COVID-19 pandemic (eg, social distancing, lockdowns, and face mask mandates) have altered the seasonality of RSV epidemics between 2020 and 2022.[4,5] RSV seasonality is less predictable in subtropical regions, necessitating vigilant RSV surveillance programmes.[3] Although both RSV A and B subtypes can cocirculate during an epidemic season, usually one subtype is predominant.[3,6]

The RSV virion comprises a lipid bilayer with three externally exposed transmembrane glycoproteins: the small hydrophobic protein, the attachment glycoprotein, and the fusion protein.[6,7] Although the attachment glycoprotein and fusion protein are known to elicit protective neutralising responses following infection, the fusion protein is highly conserved and possesses essential functions for host-cell entry, making it a prime target for the development of vaccines and therapeutic monoclonal antibodies.[6] The mature fusion protein is a homotrimer comprising extracellular F1 and F2 subunits (F2 amino acid residues 26–109 and F1 137–574) resembling the core structures of other class I viral fusion proteins (eg, influenza virus haemagglutinin and SARS-CoV-2 spike protein).[7] The RSV fusion protein comprises six antigenic sites (Ø, I–V) and has been identified as having pre-fusion and post-fusion conformations.[6,7] Antigenic sites Ø and V are found only in the pre-fusion conformation, while sites I–IV are present in both the pre-fusion and the post-fusion conformation.[6,7]

Until late 2022, the only approved preventative measure for RSV disease was immunoprophylaxis with the humanised murine anti-RSV fusion protein monoclonal antibody palivizumab.[6–8] However, due to the cost of monthly injections, policy makers have restricted

palivizumab use to high-risk infants with underlying lung or heart disease, despite evidence of a considerable RSV burden among all newborns.[9–11]

This unmet need for RSV prophylaxis for all infants led to the development of new anti-RSV fusion protein monoclonal antibodies such as nirsevimab, a recombinant human IgG1 κ monoclonal antibody that binds the F1 and F2 subunits of the pre-fusion RSV fusion protein at a highly conserved discontinuous neutralising epitope in site Ø (amino-acid residues 62–69 for F2 and 196–212 for F1) to block viral entry into host cells.[12] Nirsevimab was optimised from a precursor anti-RSV IgG1 monoclonal antibody (D25), which was selected from memory B cells of human donors through functional screening.[12] The fragment crystallisable region of nirsevimab has been enhanced with a Met252Tyr:Ser254Thr:Thr256Glu modification to prolong serum half-life in vivo and enables a single intramuscular injection of nirsevimab to confer protection for an entire RSV season (ie, around 150 days or 5 months after dose).[12–14]

Prophylaxis approaches invariably rely on the conservation of neutralising epitopes. Although the RSV fusion protein shows little antigenic drift as an immune evasion strategy, RSV's RNA-dependent replication cycle is inherently error prone.[6,7] Previous studies have shown low genetic diversity and a high degree of conservation of amino acids within the nirsevimab binding site between the years of 1956 and 2014.[12,15] However, investigations of geotemporal evolution and transmission patterns of potential escape variants in recent (ie, 2015–2021) RSV seasons have been hindered by a paucity of up-to-date prospective genomic data, minimal sequencing data, and restrictive geographical coverage among individual RSV surveillance programmes.

In this Article, we use prospective RSV molecular epidemiology data from 17 countries across five continents to examine the geotemporal prevalence of RSV A and B and the conservation of the nirsevimab binding site in recent RSV seasons (ie, 2015–2021). We functionally assessed the effect of amino-acid substitutions in the nirsevimab binding site (including the prevalent Ile206Met:Gln209Arg substitution in RSV B) on recombinant-RSV neutralisation by nirsevimab. Finally, we contextualise these findings by examining the genetic diversity of the fusion protein relative to other class I viral fusion glycoproteins using sequences published in the National Center for Biotechnology Information (NCBI) GenBank and the Global Initiative on Sharing Avian Influenza Data (GISAID) and evaluate nirsevimab binding-site conservation between 1956 and 2021.

METHODS

Prospective RSV molecular epidemiology studies

In this study, we used data from the OUTSMART-RSV,[16,17] INFORM-RSV,[18,19] and South African pilot[20] RSV surveillance studies, covering 17 countries, to assess nirsevimab binding-

site conservation. These studies were initiated to prospectively evaluate the conservation of residues in the nirsevimab binding site, establish a molecular baseline of RSV fusion-protein sequence variability, and track the prevalence of variants with nirsevimab and palivizumab binding-site substitutions, including neutralisation escape variants (appendix p 3).[16–20] Data for these studies were gathered between Feb 4, 2015, and Dec 3, 2021. Informed consent and ethical approval were not directly required for this study, but were obtained for the three surveillance studies in line with country-specific regulations.

RSV-positive nasal samples (confirmed by a RSV diagnostic test) and anonymised demographic data were collected from participants seeking medical attention for a respiratory infection as part of routine clinical care in inpatient and outpatient settings. Nucleic acid extraction, RSV fusion protein and glycoprotein next-generation sequencing, sequence assembly, and sequence-annotation procedures have been previously described.[16,18,20] Assemblies were validated and annotated by visual inspection before sequence analysis.

Assignment of RSV subtypes and reference strains

Assignment of RSV subtypes was done during the assembly process based on the RSV glycoprotein hypervariable region 2 (HVR2) alignment against reference sequences derived from the 2013 RSV reference strains (Netherlands RSVA/13-005275 GenBank accession number KX858754.1; RSV B/13-001273 GenBank accession number KX858755.1). Alignment of RSV attachment glycoprotein-HVR2 nucleotide sequences, Netherlands RSV attachment-glycoprotein gene-reference sequences, and a reference sequence database of 11 RSV A genotypes and 23 RSV B genotypes was done using MUSCLE with the UPGMA clustering method in Molecular Evolutionary Genetics Analysis (version 10).[21] The R (version 4.1.2) spline function package was used to do a cubic spline interpolation of sample counts for RSV subtype and hemisphere over the study years examined (ie, 2015–2021).

RSV fusion protein amino-acid sequence variation analysis

RSV A and B fusion-protein gene sequences in FASTA format were translated to amino-acid sequences and aligned against the Netherlands RSV A and RSV B 2013 reference strains to assess amino-acid variation (RSV A/13- 005275 GenBank accession number KX858757.1; RSV B/13-001273 GenBank accession number KX858756.1). The frequency of amino-acid variation for the RSV fusion protein was calculated by dividing the number of sequences containing amino-acid substitutions by the number of sequences in the RSV A or RSV B subtype sets and comparing the percentages of amino-acid substitutions at 5% and 20% variation cut-offs. Following visualisation (appendix p 3) amino-acid residues were colour coded according to percentage sequence conservation.

Shannon entropy is a measurement of amino-acid diversity.[22–24] Shannon entropy analyses were done to assess regions of amino-acid variability for RSV fusion proteins using the Shannon Entropy-One tool through the HIV database.[25] Regions of RSV fusion protein were

defined as amino-acid residues 62–96 and 195–227 for site Ø and 254–277 for site II. Non-extracellular regions of RSV fusion protein were defined as amino-acid residues 1–23 for signal peptide, 110–136 for p27, and 525–574 for transmembrane and intracellular domains.[6,7]

Assessment of genetic variability in prospective molecular epidemiology studies

The 2875 RSV A fusion-protein and 2800 RSV B fusion-protein sequences collected in the prospective molecular epidemiology studies between 2015 and 2021 were compared collectively and temporally to the 2013 Netherlands reference strains. Substitutions in the extracellular region of the mature RSV fusion protein (amino-acid residues 24–109 and 137–524) detected with at least 10% prevalence within an RSV season or at least 3·0-fold increase of 1% or more from the previous RSV season were also included in the phenotypic assessment. The inverse of the categories used to describe amino-acid sequence conservation were used to describe the prevalence of amino-acid substitutions in RSV seasons between 2015 and 2021 (ie, rare <1% of sequences, low 1–2%, moderate 3–39%, and high ≥40%).

RSV microneutralisation susceptibility assay

The effect of nirsevimab binding-site substitutions on the in-vitro potency of nirsevimab neutralisation was assessed using recombinant RSV variants (appendix p 3) in a validated RSV microneutralisation assay at Viroclinics Biosciences (Rotterdam, Netherlands). Nirsevimab and palivizumab were serially diluted in 96-well plates. A fixed concentration of recombinant RSV reference strain and recombinant RSV variant test virus was added at a median tissue culture infectious dose of 50–1700 per well and incubated for 1 h followed by the addition of HEp-2 cells. After 5 days of incubation at 37°C RSV-infected cells were fixed and stained with an anti-RSV monoclonal antibody and a horseradish peroxidase-labelled detection antibody. Tetramethylbenzidine substrate was added and the optical density (450 nm) was measured.

Half-maximal inhibitory concentrations (IC^{50}) were calculated using 4 parameter logistic regression non-linear curve fitting and were compared with the IC^{50} values of the recombinant RSV A and recombinant RSV B reference strains on the same plate to establish the fold-change in IC^{50} values. Based on statistical power to detect IC^{50} fold-change values relative to reference strains, shifts in neutralisation susceptibility of at least 5·0-fold can be detected with 99·2% confidence. Reduced susceptibility to nirsevimab or palivizumab neutralisation was defined as a change in IC^{50} values of 10·0 fold or more.

Phylogenetic analysis of RSV, influenza, and SARS-CoV-2 viral fusion proteins

We analysed the RSV A and B fusion protein sequences (RSV A n=2024, RSV B n=1602; appendix p 4) published in NCBI GenBank to estimate the overall genetic diversity of RSV fusion between 1956 and 2021. Sequences of other class I viral fusion proteins (influenza

A/H1 [2009–2019], H3 [2003–2019], and SARS-CoV-2 spike [2019–2022]) published by GISAID were used as controls and were randomly subsampled to have similar total numbers to RSV (3000 for each virus).[26] Phylogenetic trees were generated using FastTree (version 2.1.11) using the Jones-Taylor-Thorton substitution model within Geneious Prime (version 2020.0.5) and visualised using Interactive Tree of Life (version 6).

Statistical analysis

Descriptive pre-planned comparative statistical testing was not a component of the three-constituent prospective molecular epidemiology studies described in this analysis. Statistical methods for Shannon entropy comparisons are available in the appendix (p 3).

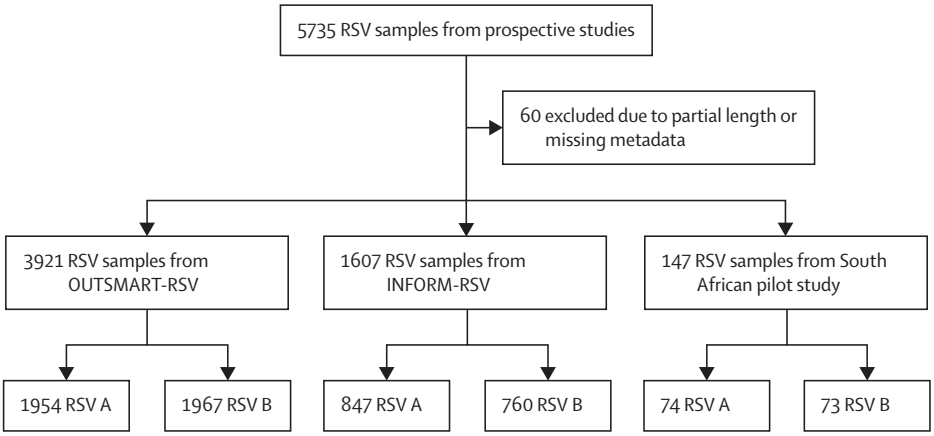
Role of the funding source

The sponsors of the study supported study design, data collection, data analysis, data interpretation, and writing of this report in collaboration with external authors.

RESULTS

A total of 7547 RSV-positive samples with associated demographic and geographical data were collected during the prospective molecular epidemiology studies between Feb 4, 2015, and Dec 3, 2021. Sequencing and assembly of RSV attachment glycoprotein HVR2 fusion protein sequencing was successful for 5735 RSV-positive samples. 60 samples were removed as they had a partial fusion-protein sequence length or missing metadata, leaving a total of 5675 (75%) of 7547 for fusion protein sequence analysis (figure 1).

Figure 1: RSV A and RSV B samples from the three prospective surveillance studies



INFORM-RSV=International Network For Optimal Resistance Monitoring of RSV. OUTSMART-RSV=The Observational United States Targeted Surveillance of Monoclonal Antibody Resistance and Testing of RSV. RSV=respiratory syncytial virus.

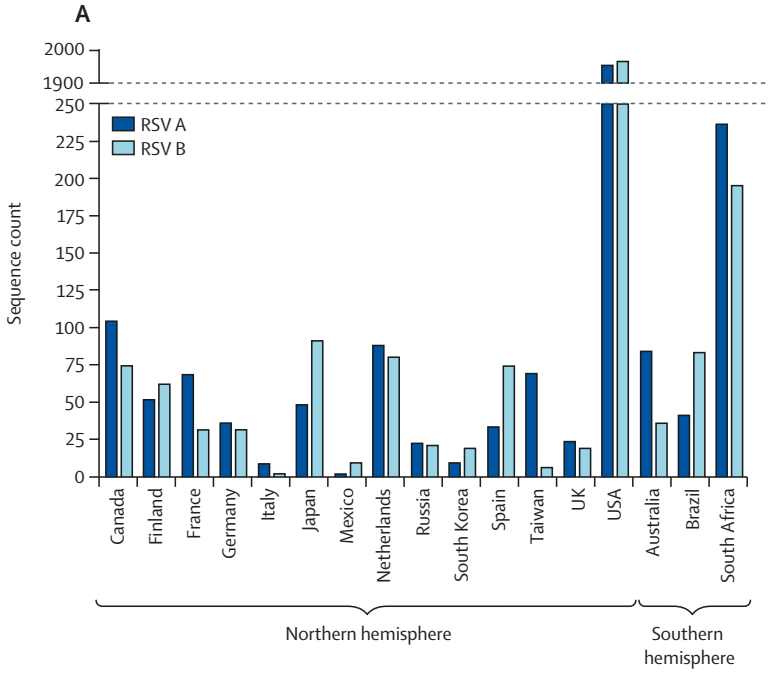
Between 2015 and 2021, RSV A-positive and RSV B-positive samples were obtained from 14 countries across the northern hemisphere and three countries across the southern hemisphere (figure 2A). RSV A and B cocirculated globally, with RSV A predominant during 2015, 2019, and 2020, and RSV B predominant between 2016 and 2018 in both hemispheres (figure 2B). Frequencies of RSV B were increased compared with RSV A in 2021.

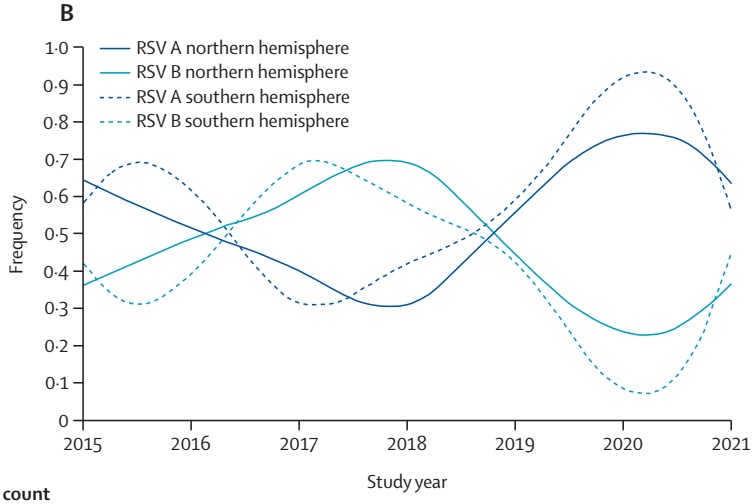
Overall, the genetic diversity of RSV fusion-protein sequences has remained low since 2015 with 554 (97%) of 574 amino-acid residues in the RSV A fusion protein and 550 (96%) of 574 amino-acid residues in the RSV B fusion protein displaying more than 99% conservation across RSV seasons and hemispheres (appendix p 7). Single amino-acid substitutions were observed in at least 10% of RSV sequences between 2015 and 2021 (appendix pp 3, 8).

The nirsevimab binding site was highly conserved (>98·82%) at all positions of the RSV A fusion protein (figure 3A) and in 22 (88%) of 25 positions (ie, excluding amino-acid residues 191, 206, and 209) of the RSV B fusion protein (figure 3B). Analysis of the three variable residues in RSV B revealed more than 98% sequence conservation at amino-acid residue 191. The two remaining residues had more than 31% sequence conservation due to the emergence and prevalence of the Ile206Met:Gln209Arg combination of polymorphisms among current circulating RSV B strains. Among isolates with genotype data available (appendix p 11), BAIX was overwhelmingly the most frequent RSV B genotype, suggesting the Ile206Met:Gln209Arg substitutions emerged within BAIX rather than in a separate genotype. The palivizumab binding site displayed a similarly high degree of conservation (>99%) at all 14 positions of site II in the RSV A fusion protein (figure 3A) and the RSV B fusion protein (figure 3B). Subsequent Shannon entropy analyses showed that amino-acid variability was predominantly concentrated in the non-extracellular regions of the RSV A fusion protein (figure 3C).

Nirsevimab retained its neutralisation activity against recombinant RSVs with highly prevalent fusion-protein substitutions identified in RSV strains circulating between 2015 and 2021 (appendix p 8). Recombinant RSV variants with reduced susceptibility to nirsevimab included Lys68Glu (12·6-fold) in RSV A (figure 4A, appendix pp 9–10, 12–13), and Lys68Gln (369·5-fold) and Asn201Thr (>405·7-fold) in RSV B (figure 4B, appendix pp 9–10). Fold changes in IC^{50} values were similar to those observed for known RSV B nirsevimab-neutralisation escape variants Lys68Asn (29·9-fold) and Asn201Ser (126·7-fold). Each of these binding-site substitutions were rare (<1%) in circulating RSVs between 2015 and 2021.[15]

Figure 2: Geographical diversity and temporal prevalence of RSV A and RSV B samples collected during prospective molecular epidemiology studies between 2015–2021

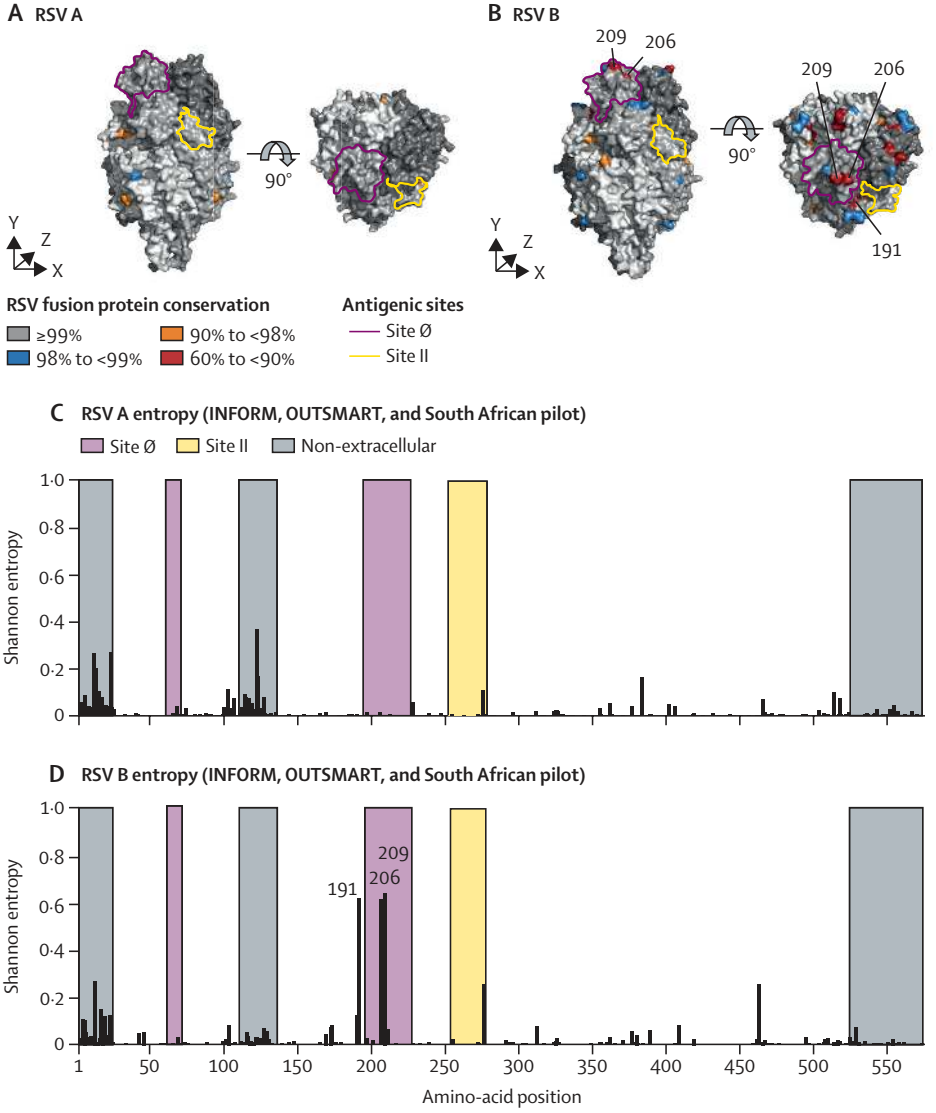




Sequence count							
RSV A northern hemisphere	23	313	365	358	747	626	82
RSV B northern hemisphere	13	296	555	797	585	193	47
RSV A southern hemisphere	29	30	15	81	113	35	58
RSV B southern hemisphere	21	19	33	112	82	3	44

(A) Geographical distribution of RSV A and RSV B isolates obtained in the northern and southern hemispheres during prospective molecular epidemiology studies. (B) Temporal prevalence of RSV A and B by season and hemisphere. RSV=respiratory syncytial virus.

Figure 3: Nirsevimab and palivizumab binding-site conservation in RSV A and RSV B samples collected during prospective molecular epidemiology studies between 2015 and 2021



(A) Percentage conservation of amino-acid residues in RSV A fusion-protein sequences. (B) Percentage conservation of amino-acid residues in RSV B fusion-protein sequences. Degree of shading denotes percentage of sequence conservation. Purple and yellow outlines denote sites on one of the three constituent protomers.

(C) Variation of Shannon entropy and position in RSV A fusion-protein sequences. (D) Variation of Shannon entropy and position in RSV B fusion-protein sequences. Shannon entropy (random data=1, deterministic data range 0–1) was used at each amino-acid position across the fusion-protein envelope glycoprotein. Amino-acid residues (AA) with Shannon entropies >0·4 are annotated. Antigenic sites I and III–V are not shown.

Site Ø=AA 62–96 and 195–227; site II=AA 254–277; non-extracellular regions: signal peptide=AA 1–23; p27=AA 110–136; transmembrane and intracellular domains=AA 525–574; nirsevimab binding site=AA 62–69 and 196–212; palivizumab binding site=AA 262–275. INFORM=International Network For Optimal Resistance Monitoring. OUTSMART=Observational United States Targeted Surveillance of Monoclonal Antibody Resistance and Testing. RSV=respiratory syncytial virus.

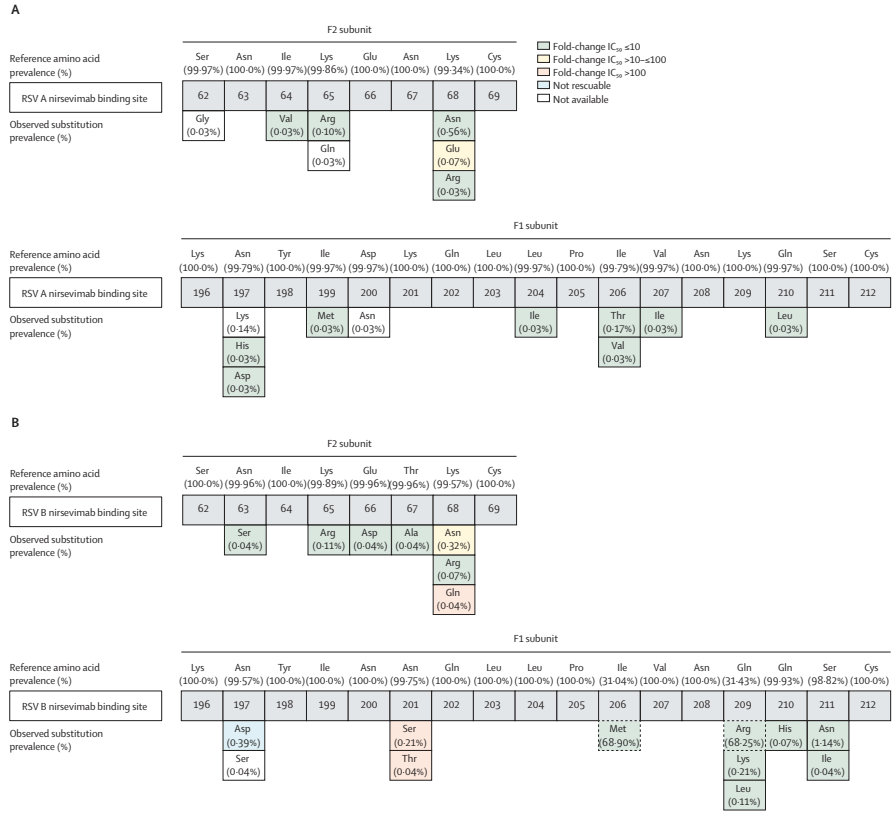
Table: Prevalence and influence of co-occurring RSV B fusion protein substitutions on susceptibility to nirsevimab and palivizumab neutralisation

	Frequency* (n=2800)	IC_{50}, fold change†		IC_{50}, µg/mL	
		Nirsevimab	Palivizumab	Nirsevimab	Palivizumab
F1 protein domain					
Nirsevimab binding site					
Ile206Met:Gln209Leu	2 (0·1%)	4·5	3·6	6·0	292·6
Ile206Met:Gln209Arg	1843 (65·8%)	0·2	1·3	0·4	79·8
Ile206Met:Gln209Arg:Ser211Ile	1 (<0·1%)	1·8	2·6	2·9	169·5
Ile206Met:Gln209Arg:Ser211Asn	32 (1·1%)	0·5	3·7	0·9	204·4
Asn197Asp:Ile206Met:Gln209Arg	11 (0·4%)	0·6	1·9	1·3	167·1
Asn197Ser:Ile206Met:Gln209Arg	1 (<0·1%)	1·6	2·5	2·3	240·0
Asn201Ser:Gln209Lys	2 (0·1%)	0·8	3·4	1·2	247·4
Asn201Thr:Ile206Met:Gln209Arg	1 (<0·1%)	>417·8	3·2	>600·0	335·8
Nirsevimab and palivizumab binding site					
Ile206Met:Gln209Arg:Lys272Asn	1 (<0·1%)	0·6	>306·5	1·0	>20 000·0
Ile206Met:Gln209Arg:Lys272Gln	1 (<0·1%)	0·2	>269·0	0·4	>20 000·0
Ile206Met:Gln209Arg:Lys272Arg	1 (<0·1%)	NA	NA	NA	NA
Ile206Met:Gln209Arg:Leu273Ile	1 (<0·1%)	0·3	2·2	0·4	175·3
Ile206Met:Gln209Arg:Met264Ile	1 (<0·1%)	0·4	2·9	0·6	234·8
F1 and F2 protein domain					
Extracellular domain					
Ala103Val:Leu172Gln:Ser173Leu	587 (21·0%)	0·7	1·4	1·3	87·2
Nirsevimab binding site					
Ala103Val:Leu172Gln:Ser173Leu: Lys191Arg: Ile206Met:Gln209Arg	1421 (50·8%)	0·3	2·5	0·4	156·6
Lys65Arg:Ile206Met:Gln209Arg	1 (<0·1%)	0·5	2·4	0·9	165·0
Lys68Asn:Ile206Met:Gln209Arg	8 (0·3%)	NA	NA	NA	NA
Lys68Gln:Ile206Met:Gln209Arg	1 (<0·1%)	46·4	1·6	91·7	127·9
Lys68Arg:Ile206Met:Gln209Arg	2 (0·1%)	0·3	1·1	0·4	90·9
Asn63Ser:Ile206Met:Gln209Arg	1 (<0·1%)	0·6	3·7	1·1	227·1
Thr67Ala:Ile206Met:Gln209Arg	1 (<0·1%)	1·1	7·1	1·8	574·6

Data are n (%) unless otherwise specified. F1=subunit, extracellular region, amino-acid residues (AA) 137–524. F2=subunit, extracellular region, AA 24–109. Nirsevimab binding site=AA 62–69 and AA 196–212. Palivizumab binding site=AA 262–275. IC_{50}=half-maximal inhibitory concentration. NA=not available. RSV=respiratory syncytial virus. *Global prevalence, based on the ratio of RSV attachment glycoprotein HVR2 fusion-protein sequences containing fusion-protein substitutions (full and mixtures) to all RSV B sequences collected (N=2800). †Fold change in IC_{50} of monoclonal antibodies required for a 50% reduction in infection compared with the wild-type reference strain tested in parallel on the same plate in a validated recombinant RSV neutralisation-susceptibility assay.

Nirsevimab had a 5·0-fold reduction in neutralisation of recombinant RSV B when it had an Ile206Met substitution. However, this substitution has rarely been found in the absence of Gln209Arg since 2015 (18 of 2800, 0·64% prevalence); nirsevimab shows numerically

Figure 4: Nirsevimab-neutralisation potency against RSV A and B with site Ø amino-acid substitutions identified during prospective molecular epidemiology studies between 2015 and 2021



Any amino-acid substitutions >10% in any given year or more than a 3-fold change year-to-year over 1% of collected RSV A (A) or RSV B (B) sequences. The prevalence of individual nirsevimab binding-site substitutions in RSV A and B is outlined in the appendix (pp 9–10). Nirsevimab reference potency for RSV A=0·6–3·4 ng/mL; RSV B=1·0–3·3 ng/mL. Dashed outlines indicate a prevalence of >40%. IC_{50}=half-maximal inhibitory concentration. RSV=respiratory syncytial virus.

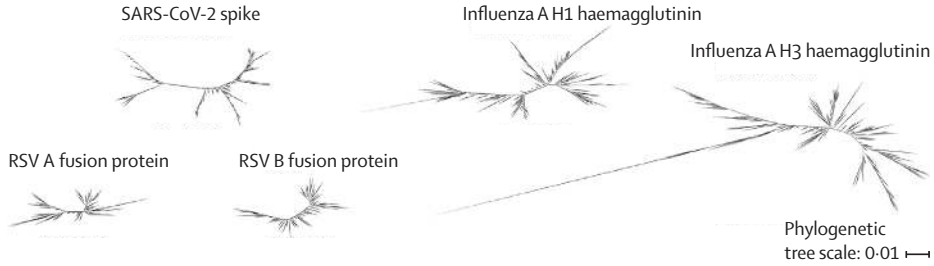
increased potency against Gln209Arg and the co-occurring Ile206Met:Gln209Arg polymorphism (table; appendix pp 14–15). The rare Lys68Gln:Ile206Met:Gln209Arg (112 of 2800, 0·04% prevalence, 46·4-fold susceptibility change) and Asn201Thr:Ile206Met:Gln209Arg (112 of 2800, 0·04% prevalence, >417·8-fold susceptibility change) substitutions in RSV B were the only recombinant RSV with co-occurring binding-site substitutions that have reduced susceptibility to nirsevimab.

Substitutions known to be associated with palivizumab resistance (eg, Lys272Met, Lys272Thr, and Ser275Phe in RSV A)[8] have been rare (<0·05%) among recent circulating RSV strains (2015–2021) and have not persisted between successive RSV seasons. Nirsevimab retained its neutralisation activity against all recombinant RSVs with palivizumab resistance-associated substitutions in RSV B. These data highlight an important absence of cross-resistance between palivizumab and nirsevimab. The geotemporal prevalence of RSV

A or RSV B strains containing nirsevimab or palivizumab binding-site substitutions has been rare (<1%) in every RSV season between 2015 and 2021 (figure 4; table; appendix pp 9–10, 12–15), with the exception of the Ile206Met:Gln209Arg substitution, which has become prevalent in at least 66% of RSV B strains in this time.

Phylogenetic analyses of RSV fusion, SARS-CoV-2 spike, and influenza haemagglutinin sequences obtained from NCBI GenBank and GISAID[27] revealed that RSV A and RSV B fusion proteins have lower genetic diversity than other class I viral fusion proteins (figure 5). This high degree of RSV fusion-protein sequence conservation was reflected in lower amino-acid residue variability for both RSV A and RSV B (figure 6A). 13 (2·3%) of 574 of RSV A and 11 (1·9%) of 574 of RSV B fusion protein amino acids were shown to vary with more than a 5% variability cut-off compared with 4·4% observed in SARS-CoV-2 spike and influenza H1N1 and 7·2% in influenza H3N2 haemagglutinin. Similar reduced trends for RSV A and RSV B versus the other class I viral fusion proteins were observed at a variability cut off of more than 20%. We confirmed higher genetic diversity for RSV glycoproteins than RSV fusion proteins (appendix p 5).

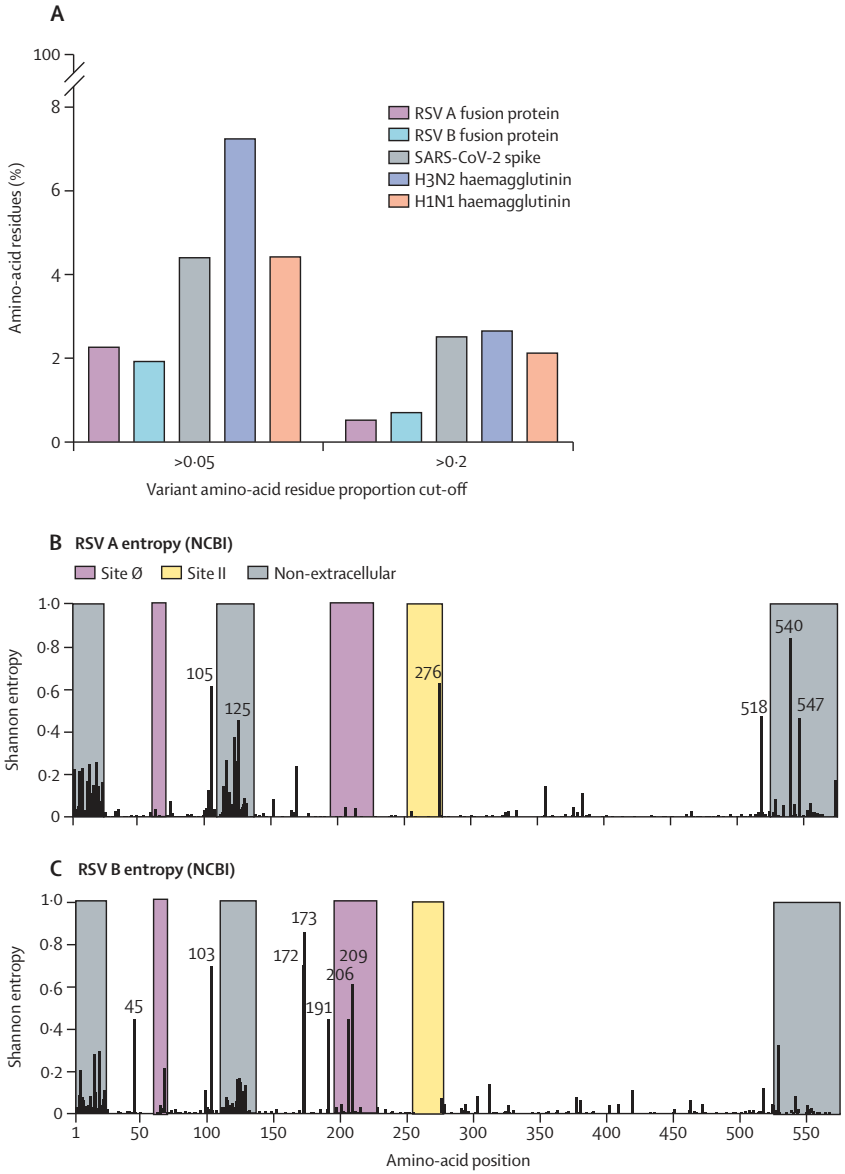
Figure 5: Evolutionary distances of RSV, SARS-CoV-2, and influenza class I viral fusion proteins



Phylogenies generated from genetic sequences of RSV A fusion protein (sequence count=2024; year range 1956–2021), RSV B fusion protein (sequence count=1602; year range 1962–2021), influenza A H1 (sequence count=3000; year range 2009–2019), H3 haemagglutinin (sequence count=3000; year range 2003–2019), and SARS-CoV-2 spike proteins (sequence count=3000; year range 2019–2022) obtained from NCBI and GISAID databases. Distance of individual phylogenetic trees represents the overall diversity of glycoprotein sequence. Scale bar indicates the horizontal scale derived from the dissimilarity metric. GISAID=Global Initiative for Sharing Avian Influenza Database. NCBI=National Center for Biotechnology Information. RSV=respiratory syncytial virus.

Subsequent analyses of Shannon entropy showed that RSV fusion protein amino-acid variability was predominantly concentrated in the non-extracellular regions of RSV A (figure 6B) and RSV B (figure 6C) fusion proteins. RSV A had low (Shannon entropy <0·1) sequence variability within site Ø and moderate (Shannon entropy 0·3–0·6) sequence variability at one amino acid within site II corresponding to amino-acid residue 276, a known site of palivizumab resistance.[8] Median Shannon entropy for RSV A and B fusion proteins were significantly lower compared with influenza H3N2 ($p \leq 0{\cdot}0001$) and H1N1 (RSV A $p=0{\cdot}0341$; RSV B $p=0{\cdot}0242$; appendix p 6). Median Shannon entropies for RSV A site Ø were also lower compared with the SARS-CoV-2 spike receptor-binding domain ($p=0{\cdot}0001$) and head

Figure 6: Analyses of RSV fusion protein amino-acid variability in NCBI GenBank sequences (1956–2021)



(A) Amino-acid variation frequency (%) at 5% and 20% amino-acid variability cut-offs among RSV A and RSV B fusion protein, SARS-CoV-2 spike, and influenza H1N1 and H3N2 envelope glycoproteins based on reference sequences obtained from NCBI GenBank (1956–2021) and GISAID databases. Site of amino-acid variation in RSV A (B) and RSV B (C) fusion-protein sequences as identified by Shannon entropy. Shannon entropy (random data=1, deterministic data range 0–1) was used at each amino-acid position across the fusion protein envelope glycoprotein. Amino-acid residues (AA) with Shannon entropies >0·4 are annotated. Site Ø=AA 62–96 and 195–227. Site II=AA 254–277. Non-EC regions: signal peptide=AA 1–23; p27=AA 110–136; transmembrane and intracellular domains=AA 525–574; nirsevimab binding site=AA 62–69 and 196–212; palivizumab binding site=AA 262–275. GISAID=Global Initiative for Sharing Avian Influenza Database. NCBI=National Center for Biotechnology Information. RSV=respiratory syncytial virus.

region of influenza H3N2 and H1N1 (p≤0·0001; appendix p 6). Median Shannon entropies for RSV B site Ø were lower compared with the head region of influenza H3N2 (p=0·0052; appendix p 6). RSV B had moderate variability within site Ø and comparatively higher variability than RSV A's site Ø due to the emergence of the Ile206Met:Gln209Arg polymorphism in RSV seasons between 2016 and 2021 (figure 6B and 6C), consistent with the prospective analysis (figure 3C and 3D).

DISCUSSION

RSV is a leading cause of morbidity in infants aged between 0 and 60 months with global annual costs from hospital admission alone at over £2 billion.[1,27,28] Development of a paediatric RSV vaccine has been a longstanding public-health priority but progress has been challenging due to inadequate adaptive immune responses from the developing immune system, particularly within the first 6 months of life when RSV burden is at its greatest.[1,2,29] Immunoprophylaxis can provide direct virus neutralisation to those without sufficient immune responses suggesting that long-acting monoclonal antibodies could be beneficial as vaccine surrogates.[12] However, prophylaxis approaches rely on the conservation of neutralising epitopes. As shown by the emergence of a suptavumab-neutralisation-resistant RSV B variant during the 2015 RSV season, vigilant molecular surveillance programmes are needed to monitor the emergence of polymorphisms conferring reduced neutralisation potency to maintain monoclonal antibody effectiveness between successive RSV seasons.[17,18,30] Here we have demonstrated the conservation of the nirsevimab binding site in RSV fusion protein from 1956 to 2021.

Within our analysis of 5675 prospective attachment glycoprotein HVR2 fusion-protein sequences, we observe clear geotemporal patterns with successive RSV A and RSV B predominance between RSV seasons across both hemispheres, with low genetic diversity within the nirsevimab and palivizumab binding sites. The Ile206Met:Gln209Arg polymorphism in the nirsevimab binding site of RSV B emerged in 2015 and became globally dominant with a prevalence of 1843 (65·82%) of 2800 among RSV B strains observed between 2015 and 2021. We show that nirsevimab effectively neutralises a diverse panel of the recombinant RSV variants identified in prospective surveillance studies, including the highly prevalent site Ø Ile206Met:Gln209Arg polymorphism. The frequency of nirsevimab-neutralisation escape variants in naturally circulating viruses is rare (<1%) and has not persisted or increased in frequency with successive RSV seasons. Importantly, the in-vitro selected nirsevimab-neutralisation escape RSV B variants Lys68Asn and Asn201Ser have rarely been identified (<0·5%) among the strains circulating between 2015 and 2021.[12,15]

Of note, co-occurrence of nirsevimab binding-site substitutions appears to differentially affect their influence on nirsevimab neutralisation (eg, Asn201Ser vs Asn201Ser:Gln209Lys).

We hypothesise that these differential influences on nirsevimab-neutralisation susceptibility could be the result of steric effects, based on previous observations of individual substitutions conferring neutralisation resistance when co-occurring with another substitution by altering the polarity of the nirsevimab binding site.[15] These observations underscore the value of phenotyping all binding-site substitutions identified in RSV molecular surveillance programmes including in the context of co-occurring substitutions. Similarly, individual substitutions could incur reductions in viral fitness that are compensated for by additional substitutions, thereby influencing the selection pressure in vivo and enabling the co-occurring substitution to become more prevalent than the constituent single substitutions over time.

Analyses of RSV fusion-protein sequences published in NCBI GenBank (1956–2021) show low genetic diversity of the RSV fusion protein compared with class I viral fusion proteins from other respiratory viruses assessed in 2003, between 2009 and 2019, and between 2019 and 2021. Of the variable regions in the RSV fusion protein, Shannon entropy analyses show the conservation of antigenic sites Ø and II compared with the moderate sequence diversity observed in non-extracellular regions, suggesting that both neutralising epitopes are stable. The RSV fusion protein had less amino-acid variation than the attachment glycoprotein. Collectively, these analyses support the hypothesis that the RSV fusion protein is not prone to substantial antigenic drift as an immune-evasion strategy and show that the nirsevimab binding site was highly conserved between 1956 and 2021.

Limitations to the prospective analysis include a potential under-representation of circulating RSVs in the southern hemisphere due to a lower number of countries profiled and over-representation of samples collected in the USA and infants with severe RSV disease who required hospital visit. Additionally, the public-health measures implemented to mitigate the COVID-19 pandemic altered RSV seasonality between 2020 and 2021 and, consequently, our samples might under-represent RSV A and B in circulation in some territories. Despite these limitations, this analysis represents a substantial addition to publicly available RSV fusion sequences (3626 NCBI GenBank 1956–2021 vs 5675 deposited to GenBank with this submission). Although the binding-site substitutions highlighted in our analysis show reduced susceptibility to nirsevimab neutralisation by an in-vitro microneutralisation assay, we are unable to assess whether these are neutralisation escape variants in vivo. We were unable to assess the influence of nirsevimab selection pressure on RSV evolution in this dataset. We acknowledge that widespread nirsevimab use might exert increased evolutionary pressure on RSV. However, it is not possible to predict population-level effects, particularly focused on a specific age group, but ongoing RSV surveillance with nirsevimab roll out is planned. Limitations to the historical analysis of RSV fusion diversity (1956–2021) include the low number of samples submitted between 1956 and 2000, that a high proportion of the available sequences were collected from 2010, and the clustering of RSV isolates collected in certain geographical regions (ie, USA, Europe, Kenya).

In summary, these findings show a high degree of conservation within the nirsevimab binding site between 1956 and 2021. Based on these findings, a single intramuscular injection of nirsevimab is expected to neutralise more than 99% of current circulating RSV strains, resulting in enduring protection from RSV disease through 150 days post-dose, the duration of a typical RSV season (ie, around 5 months).[13,14] The potential emergence of nirsevimab-neutralisation escape variants must continue to be closely monitored as part of ongoing RSV-surveillance studies. These results are consistent with our previous report that nirsevimab exhibits highly potent and broad antiviral activity against a diverse panel of naturally occurring RSV A and RSV B variants.[12,15] This study provides strong evidence that substitutions in the nirsevimab binding site are rare and are unlikely to affect the efficacy of nirsevimab.

Contributors

DW, ACL, RJL, CM, MEA, EJK, MTE, DET, and LJB conceptualised the analysis. EB, HC, EHC, RC, DMD, AG, TH, MH, CK, LK-T, FM-T, AHMdLS, MAP, JP, JMP, PR, RTS, and CV were the trial site investigators and gathered the data. DW, RJL, CM, VG, EJK, and KMT verified the data. AstraZeneca and DW, ACL, RJL, CM, MEA, BA, AAA, TB, ATC, VG, MCN, KMT, MTE, DET, and LJB analysed the data. Data were interpreted by all the authors. All authors had full access to the analyses and data and granted their final approval of the paper before submission. All authors had final responsibility for the decision to submit for publication. The first draft of the manuscript was written under the direction of the authors by a medical writer funded by the study sponsors. All authors reviewed and provided substantive revisions to subsequent drafts, and all authors approved the final draft and the decision to submit for publication.

Declaration of interests

DW, CM, BA, AAA, TB, VG, EJK, KMT, and MTE are current employees of and hold stock or stock options in AstraZeneca. MEA and DET are former employees of and hold stock or stock options in AstraZeneca. EB has received honoraria from AstraZeneca and Sanofi for lectures, presentations, speaker bureaus, manuscript writing, or educational events. TH has participated in Data Safety Monitoring Boards and ad-hoc advisory boards for Sanofi, Data Safety Monitoring Boards for Enanta, and ad-hoc advisory boards for Janssen and has received honoraria from Janssen and Merck, Sharp & Dohme (MSD) for lectures at academic meetings. CK has received honoraria from F Hoffmann-La Roche for lectures. FM-T's research activities are supported by grants from Instituto de Salud Carlos III (grant numbers: PI16/01569, PI19/01090, PI22/00406, and CB21/06/00103), GEN-COVID (grant number: IN845D 2020/23), and Grupos de Referencia Competitiva (grant number: IIN607A2021/05), and he has received additional grants to his institution from AstraZeneca and Sanofi; he also received honoraria from GlaxoSmithKline, Pfizer, Sanofi Pasteur, MSD, Seqirus, Biofabri, and Janssen for taking part in advisory boards and expert meetings and for acting as speaker in

congresses outside the scope of the submitted work, in addition to travel support from GlaxoSmithKline, MSD, Pfizer, and Sanofi. FM-T has also participated in advisory boards for Pfizer, MSD, Sanofi, and GlaxoSmithKline, and in Data Safety Monitoring Boards for Biofabri; is a member of The European Technical Advisory Group of Experts—WHO Europe and the Spanish Paediatric Infectious Diseases Society; and has also acted as principal investigator in randomised controlled trials for AstraZeneca, Biofabri Seqirus, GlaxoSmithKline, Janssen, MSD, Novavax, Novartis, Pfizer, Roche, Regeneron, and Sanofi Pasteur with honoraria paid to his institution. MCN has received grants from the Bill & Melinda Gates Foundation, European and Developing Countries Clinical Trials, Pfizer, and Sanofi Pasteur; honoraria from Sanofi for lectures presentations, speakers' bureaus, manuscript writing or educational events; payment for expert testimony from Pfizer and Sanofi Pasteur; and is a board member for Gavi vaccine alliance. JMP has received payments to his institution from the Canadian Paediatric Review and Yearly RSV Coordinators Workshop and is an unpaid co-chair of the Ontario Immunization Advisory Committee. PR has received investigator-initiated research grants to his institution from MSD and has received institutional funding from GlaxoSmithKline for local and international lectures and from AstraZeneca, GlaxoSmithKline, MSD, Sanofi, and Pfizer for participation in advisory boards. RTS has received payment or honoraria for lectures for AstraZeneca, Pfizer, and Sanofi Pasteur. CV has received payment or honoraria for lectures from AstraZeneca. LJB has not received personal fees or other personal benefits from pharmaceutical companies. His institution, University Medical Center Utrecht (UMCU), has received major funding (>€100 000 per industrial partner) from AbbVie, AstraZeneca, Sanofi, Janssen, Pfizer, MSD, and MeMed Diagnostics for investigator-initiated studies. UMCU has received major funding for the RSV-GOLD study from the Bill & Melinda Gates Foundation. UMCU has received major funding as part of the public private partnership IMI-funded RESCEU and PROMISE projects with partners GlaxoSmithKline, Novavax, Janssen, AstraZeneca, Pfizer, and Sanofi Pasteur. UMCU has received major funding by Julius Clinical for participating in clinical studies sponsored by AstraZeneca and Pfizer. UMCU has received minor funding (€1 000–25 000 per industrial partner) for consultation and invited lectures by AbbVie, AstraZeneca, Ablynx, Bavaria Nordic, MabXience, GlaxoSmithKline, Novavax, Pfizer, Moderna, AstraZeneca, MSD, Sanofi, Genzyme, and Janssen. LJB is the founding chairman of the ReSViNET Foundation. All other authors declare no competing interests.

Data sharing

Data underlying the findings described in this manuscript can be obtained in accordance with AstraZeneca's data sharing policy described at https://astrazenecagrouptrials.pharmacm.com/ST/Submission/ Disclosure. Data for studies directly listed on Vivli can be requested through Vivli at https://www.vivli.org. Data for studies not listed on Vivli could be requested through Vivli at https://vivli.org/members/enquiries-about-studies-not-listed-on-the-vivli-platform/. 410 of the 5675 sequences reported in the manuscript were submitted to NCBI GenBank in

support of a previous analysis of the INFORM-RSV study (NCBI PopSet accession number: 2120396151).19 The remaining 5265 sequences described in this analysis are available from NCBI GenBank upon publication of this manuscript (NCBI GenBank accession numbers: OQ275548–OQ280812; appendix pp 16–102).

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Chapter 9

The Genomic Evolutionary Dynamics and Global Circulation Patterns of Respiratory Syncytial Virus

"INFORM"

Nature Communications (provisionally accepted)

"That something is difficult must be a reason the more for us to do it."
— Rainer Maria Rilke, Austrian poet

The Genomic Evolutionary Dynamics and Global Circulation Patterns of Respiratory Syncytial Virus

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ABSTRACT

Respiratory syncytial virus (RSV) is a leading cause of acute lower respiratory tract infection in young children and the second leading cause of infant death worldwide. While global circulation has been extensively studied for respiratory viruses such as seasonal influenza, and more recently also in great detail for SARS-CoV-2, a lack of global multi-annual sampling of complete RSV genomes limits our understanding of RSV molecular epidemiology. Here, we capitalise on the genomic surveillance by the INFORM-RSV study and apply phylodynamic approaches to uncover how selection and neutral epidemiological processes shape RSV diversity. Using complete viral genome sequences, we show similar patterns of site-specific diversifying selection among RSVA and RSVB and recover the imprint of non-neutral epidemic processes on their genealogies. Using a phylogeographic approach, we provide evidence for air travel governing the global patterns of RSVA and RSVB spread, which results in a considerable degree of phylogenetic mixing across countries. Our findings highlight the potential of systematic global RSV genomic surveillance for transforming our understanding of global RSV spread.

INTRODUCTION

With the recent approval of the first-ever RSV vaccines and the monoclonal antibody (mAb) nirsevimab for the prevention of RSV in all infants[1], our understanding of the global transmission dynamics of RSV becomes increasingly important. An important unsolved question is to what extent RSV epidemics are fueled by local persistence from a previous epidemic versus that of viral seeding from other geographic areas. A better understanding of the global circulation dynamics and local persistence is crucial for RSV surveillance and prevention.

Viral genetic sequence data may offer valuable information to aid in testing predictors of spread and to empirically develop and validate epidemiological models. A challenge for reconstructing viral spread through space and time from genetic data has been the lack of a systematic and comprehensive global sampling of whole genomes from circulating RSV lineages. Current such sampling efforts include the global multiyear multicentre INFORM-RSV study and the Global RSV Surveillance Programme of the World Health Organization (WHO). The INFORM-RSV study combines large-scale full genome sequencing and a global coverage over multiple RSV seasons to provide a molecular reference of RSV strains and sequence variability[2]. The best way of mapping genomic evolutionary dynamics of RSV is by analysing nucleotide substitutions of the complete genome. Previously selective pressure analyses with samples from the 2001-2011 time period showed that RSV genes consist predominantly of negatively selected and neutrally evolving sites. Only the G gene encoding for the surface glycoprotein G stood out in terms of detectable positive selection[3]. The primary role of the *G protein* is to attach virions to cell *surfaces* through interaction with host cell attachment factors[1,4]. The genetic factors that impact the replacement dynamics remain poorly understood and a full-genome perspective on the adaptive evolution of RSV is needed to reveal which other genomic variations affect the fitness of strains.

While sequencing efforts have been implemented on a large scale for SARS-CoV-2, systematic sequencing of RSV is still at an early, small scale stage. For respiratory viruses such as seasonal SARS-CoV-2 and influenza, human air-based travel (flight) has been shown to be an important driver of global circulation[4-8]. Air travel may also shape seasonal RSV dynamics. RSV molecular epidemiology data from Kenya showed that several new variants are introduced every epidemic season[9-15]. The interspersed nature of sequences from Kilifi and other parts of Kenya indicates a degree of mixing of lineages, which in turn suggests that air travel may be an important driver of spread. However, the global circulation patterns of RSV have remained unexplored. Therefore, we integrated human movement patterns with whole genome sequences from RSV samples that were collected in 17 countries worldwide over three RSV seasons (2017-2020) prior to the COVID-19 pandemic. Travel restrictions due to COVID-19 have not affected the current analysis.

RESULTS

Circulating genotypes

We obtained 1,282 complete RSV genome sequences collected over a period of three years from 17 countries worldwide enrolled in the INFORM-RSV study. We complemented these sequences with 1,180 publicly available sequences from NCBI GenBank sampled within the same time interval. All RSVA and RSVB genomes in the genotyping datasets cluster among strains that were typed as A23 and B6. For this reason, the genotyping alignments were appended with strains of genotype A22 (RSVA) and B5 (RSVB) that served as outgroups for rooting the maximum likelihood (ML) trees. Applying previously established genotyping criteria show that genotypes A23 and B6, from which the currently circulating strains have evolved, can be reclassified into a set of 25 RSVA and 2 RSVB genotypes (Fig.1). Variants with a duplication in the G gene have emerged[16]. These variants appear to have a fitness advantage[17] and have started to replace previously circulating strains. This observation is reflected in our data, as 100% of the sequenced RSVA and RSVB isolates carry these duplications.

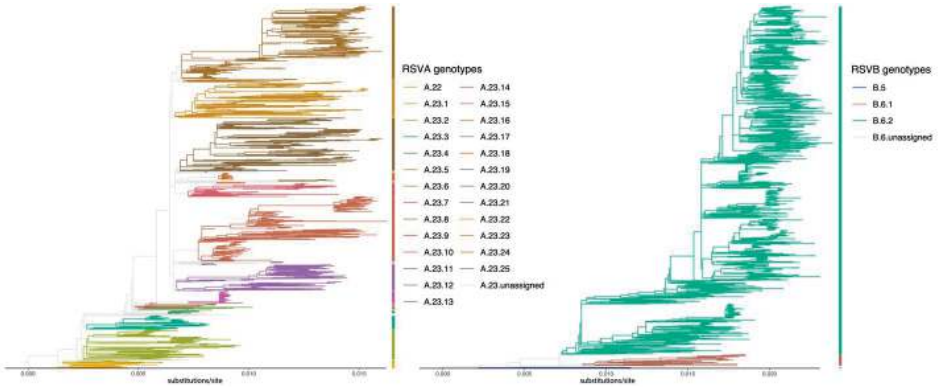

Figure 1: Maximum likelihood reconstructions of RSVA (1,482 genomes/taxa; 2006-2020) and RSVB (1,543 genomes/taxa; 1997-2020) complete genome phylogenies and genotypes identification. Lineages that are not assigned to a genotype are shown in light grey. The SH-aLRT and UFB support values for the genotypes are provided in Supplementary Table S1.

Comparable site-specific diversifying selection in RSVA and RSVB

To identify positively selected sites in the coding genes of the RSV genome, we employ three different methods (FUBAR, MEME, and RC, cfr. Methods) that aim to capture different aspects of site-specific selection and report sites that were identified by at least two of these methods. Using this approach, we identify 28 positively selected amino acid sites in RSVA. Of these, 21 are located in the G protein, one in the F protein, and six in the L protein. Eight of the G protein sites and one L protein site are supported by all three methods. We obtain a similar number ($n = 26$) and distribution of positively selected sites in RSVB, with 18 sites in

the G protein, two in the F protein, and six in the L protein. Eight of the G protein sites and one F protein site are supported by all three methods. Three of the positively selected sites are identified at the exact same amino acid position in the G protein of RSVA and RSVB (amino acid positions 136, 274, 310). However, the amino acid position on the linear protein sequence for RSVA may not necessarily be the same as for RSVB in the protein crystal structure. Substitutions in positions under positive selection are found on different branches of phylogeny, which is consistent with the expectation under diversifying selection (Figure S1 and S2).

Both RSVA and RSVB genealogies are shaped by non-neutral population turnover

RSV evolution may be shaped by selection for variants with higher replicative fitness and variants that evade host immune responses[18]. The latter is indicated by the site-specific selection analyses that identify the G gene as the major target of diversifying selection[3,18]. However, earlier testing has found that only RSVB tree shapes inferred from complete genome data deviate from what we expect under neutrality[3,18]. Now that considerably more complete genome data are available, we revisit the genealogical testing using posterior predictive simulation[19]. We employ the genealogical Fu and Li statistics as well as a trunk length proportion statistic as tree shape statistics (see Methods). We plot bivariate distributions for these statistics based on the genealogies inferred from the genomic data and the equivalent genealogies simulated under neutrality accommodating for potentially complex histories of population size change (Fig.S3). Both RSVA and RSVB show significant deviations from neutrality, with a more pronounced deviation for RSVB as compared to RSVA.

Global RSV circulation patterns are shaped by human air travel

To explore the factors that shape RSV global circulation, we apply a Bayesian phylogeographic approach that models the movement of virus lineages between a set of discrete locations[20]. This process is generally parameterized in terms of transition rates for all pairs of locations. Here, we use an extension of the discrete phylogeographic model that parameterizes these transition rates as a function of a number of potential predictors[5]. This generalized linear model (GLM) parameterization allows estimating the contribution of each predictor to the spatial diffusion as a coefficient (on a log scale). In addition, the model includes boolean indicator variables that determine the in- or exclusion of predictors allowing to estimate their inclusion probability. Here, we report the posterior distribution of the product of the log coefficient and inclusion probability for each predictor; positive estimates indicate a positive association between predictors and diffusion intensity while the opposite is true for negative estimates. As predictors, we consider human air travel, population size, geographic distances, and latitude differences (see Methods). Our analyses consistently support human air travel as a strong predictor of RSV global spread at both the country (strongly positive estimates,

Fig.2) and continental level (Fig. S4) for RSVA and RSVB separately, as well as for a model applied to both RSVA and RSVB data sets combined. The support for air travel is robust to the inclusion of sample sizes as predictors. Other candidate predictors occasionally find support, but not consistently so, suggesting that these other predictors could for example be attributed to sampling variability. For instance, the human population size at the origin location is estimated to have a negative log coefficient for its effect size in the RSVB analyses. This may be explained by the fact that the most populous countries, such as China and India, are represented by only a few genomes that are distributed as singletons in the phylogeny, thereby resulting in an underestimation of their potential role as origin locations in the global circulation dynamics. In fact, these two locations specifically have been shown to be important for persistence and global dissemination of seasonal influenza viruses[4]. Therefore, better global coverage will be needed to characterise the role of undersampled countries in RSV circulation and how they may relate to demographic characteristics.

While the phylogeographic data sets include genomes sampled between 2012 and 2020, the INFORM-RSV study contributes to the most recent years (2017-2020) of sampling. To determine how these data contribute to predictor support, we also apply a time-inhomogeneous GLM-diffusion model distinguishing between the five most recent years and the 5-year time period before that (Fig.S5). This illustrates that the support for air travel is consistently found for the recent time period whereas this is less convincing or less consistent across analyses in the earlier time period. This demonstrates how systematic global sampling contributes to the opportunity to identify meaningful patterns of RSV spatial spread.

Phylogeographic reconstructions indicate extensive geographic mixing

RSV spread by air travel offers the opportunity for substantial geographic mixing of viral lineages between locations. To assess geographic mixing, we use recently proposed entropy-based phylogeographic summaries for the genome sampling in the most recent pre-pandemic INFORM-RSV season (2019-2020). Specifically, we summarise normalised entropy measures or the phylogeographic clustering by country, reflecting the degree of phylogenetic interspersion of country-specific lineages (Fig.3), and the number of unique lineages associated with each country circulating at the start of the most recent RSV season (see Supplementary Files S1 and S2 for the MCC summary trees from the evolutionary reconstructions underlying these inferences). Some countries have different results for RSVA versus RSVB, which could be explained by the fact that whether a lineage grows to be a persistent one is a stochastic event even if particular countries would be more prone to persistent circulation. This normalised entropy ranges between 0, reflecting no intermixing of viruses from different countries, and 1, reflecting a clustering that is randomised with respect to country of sampling.

For RSVA, we infer relatively high entropy estimates, with 13 out of 15 estimates above 0.8. For the Netherlands for example, we estimate entropy of 0.88 [95% highest posterior density interval (HPD) 0.82,0.94] and 10 [95% HPD 8,12] unique lineages circulating at the

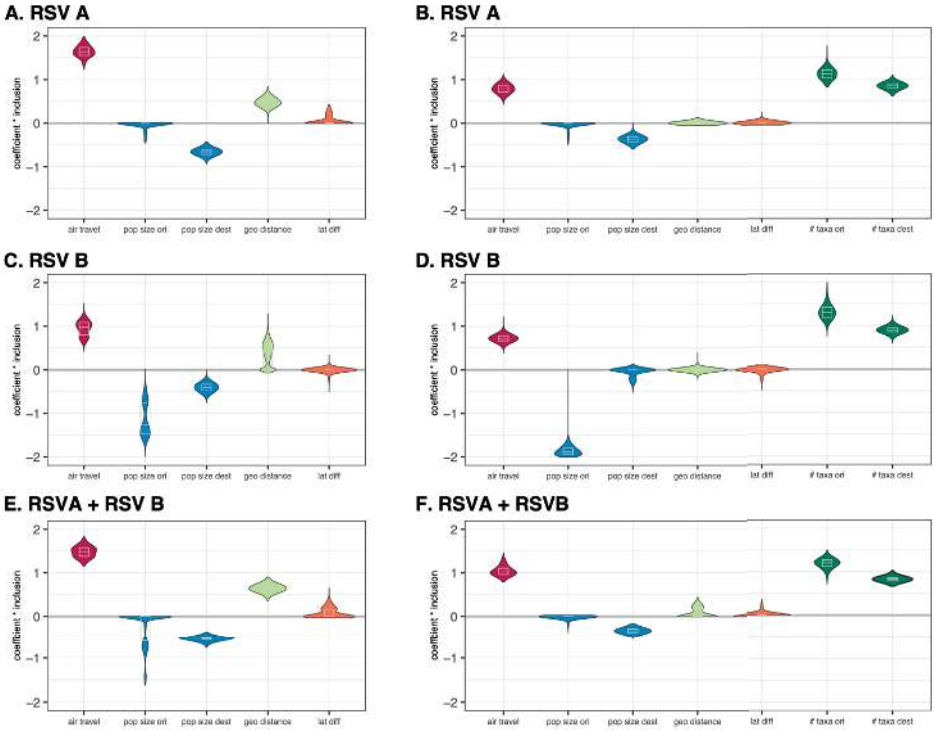

Figure 2: Posterior estimates of time-homogeneous predictor contributions to RSV diffusion between countries. The predictors include the number of passengers travelling by air between each pair of countries represented in the data set (air travel, in dark red), population size at the origin and destination location (pop size ori & pop size dest, in blue), geographic distance (geo distance, in light green), absolute differences in latitude (lat diff, in dark orange) and sample sizes at the origin and destination locations (# taxa ori & # taxa dest, in dark green). The Y-axis represents the product of the coefficient (on a log scale) and the inclusion probability for the predictors (coefficient * Inclusion). (A-B: RSVA. C-D: RSVB. The plots on the left and right distinguish between analyses without and with sample size predictors respectively. E and F summarize the estimates for a single GLM-diffusion model applied to the combined RSVA and RSVB data sets at the country level. The grey boxes in the violin plots represent the median and quantile estimates.

start of the most recent season (2019-2020), which together are represented by 23 sampled genomes in the final season. With an entropy estimate of 0.33 [95% HPD 0.28,0.38], South Africa appears to be an exception to the pattern of relatively extensive mixing. While we estimate a substantial number of unique South African lineages at the start of the final season (26 [95% HPD 21,30]), there is also a substantial degree of clustering of the 58 genomes sampled from that season, with 50 out of 58 samples belonging to a large South African cluster including also samples from the previous season (Fig.S6). Similarly high entropies are estimated for RSVB in most countries. While two more mean estimates fall below 0.8, their credible intervals are broad. Although the mean entropy estimate for South Africa is also < 0.8 for RSVB, the deviation from countries with high entropy values is far more limited. Overall, these estimates suggest a substantial global geographic mixing of both RSVA and RSVB.

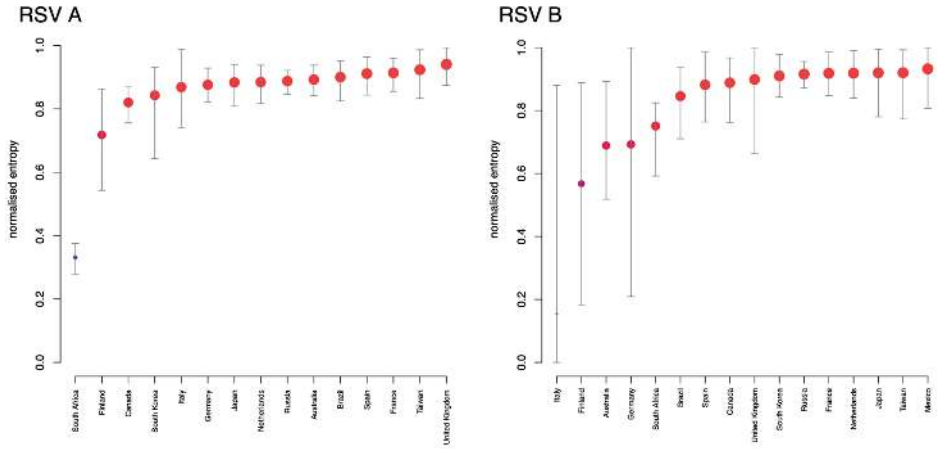

Figure 3: Posterior estimates of the normalised entropy for RSVA and RSVB phylogeographic clustering by country during the most recent season (2019-2020) of INFORM-RSV sampling. The normalised entropy ranges between 0 (~no mixing of lineages by country) and 1 (~random mixing with respect to country). Circles and error bars refer to the mean and 95% Highest Posterior Density (HPD) interval of the normalised entropy estimates respectively. The size of the circles is proportional to what fraction of the highest mean estimate each average estimate represents. The same is indicated by the colors of the circles, which range from blue for an average estimate that represents 0% of the highest value to bright red for the highest mean estimate.

DISCUSSION

Optimised surveillance and prevention of RSV infection at a global scale relies on our understanding of its spread. Here, we combine existing RSV genomic data and new full genomes from a systematic global sampling effort with empirical data on human mobility, demography and a proxy for synchronicity of RSV seasonality to evaluate which factors shape global RSV circulation. We show that air travel predicts global RSV spread, similar to what has been demonstrated for influenza H3N2[5,8], influenza H1N1[4], and recently SARS-CoV-2[6]. Additional sampling efforts (including those within the framework of the ongoing INFORM-RSV study) are expected to generate more densely sampled genomic data. This will increase the resolution of phylogeographic reconstructions and it will likely allow testing predictors at other spatial scales where other forms of mobility could also shape RSV circulation. Understanding RSV spread is also important in the light of monitoring for escape mutations to emerging prophylactic approaches to RSV, as our findings show these have the potential to spread rapidly on a global scale.

Human air travel increases the likelihood of infectious diseases spreading rapidly between countries and continents[21]. We speculate that air traffic could be a mechanism of RSV transmission. It is still unclear how patients acquire viral respiratory disease in the context of air travel, and the prevalence of RSV in airplane passengers has not been studied. Previous research showed that almost one-half of all patients with clinical symptoms upon travel turn were infected with respiratory viruses[22,23]. Other evidence suggests that SARS-CoV-2

is transmitted during air travel[24,25]. Global concerns such as the emergence of Ebola Virus Disease in West Africa[26] and novel SARS-CoV-2 variants[27] have already led to a number of protocols implemented at airports of departure or arrival (e.g. testing, genomic surveillance, quarantines, etc.). As global connectivity has increased, so has the potential for RSV to spread across countries. Before the COVID-19 pandemic, over four billion passengers travelled by airplane annually and this number is likely to double by 2036. We expect the main mechanism of global spread to be spread at the country of arrival, mostly due to travelers infected in the community and bringing the infection from a seeding area where the epidemic is ongoing to the destination country. We show that seasonal RSV epidemics are likely fueled by many independent introductions. However, the exact source locations cannot be identified with our data..

Our reconstructions provide some evidence of local RSV persistence in South Africa. These data build on earlier evidence of clustering and strong selective pressure for both RSVA and RSVB in South Africa[28]. RSV clustering in South Africa resembles data on influenza A which persisted in West Africa for almost two years[29]. Extensive spatial mixing of influenza A by air travel was observed in West Africa, perhaps because of its relatively lower connection within the global air transportation network. The climatic variability may also have contributed to the influenza persistence generating temporal overlap among epidemics[29].

Currently, several genotype definitions are used in parallel and there is no universal approach to classify virus genetic diversity[30]. Therefore, genotyping based on complete genome sequences, instead of genotyping based on nucleotide sequence variability of subgenomic regions (mostly the G gene), can improve the RSV surveillance field by providing a more coherent classification. By focusing on active virus lineages and those spreading to new locations, this universal nomenclature would assist in tracking and understanding the patterns and determinants of the global spread of RSV. For SARS-CoV-2, a similarly proposed nomenclature represents an important asset to the field[31]. We hope that our study will motivate large-scale implementation of whole genome sequencing for RSV surveillance.

Site-specific selection analyses identified the G gene as the main target of diversifying selection. When compared to influenza with its ladder-like phylogeny and strong turnover, positive selection for RSV is less strong. Our results confirmed that the RSV genome is largely conserved, with the exception of the highly variable G gene. We have identified different positions under selective pressure for RSV A and B. This is the first report on positive selection on the L gene at amino acid position 146, 624, 1725, 1748, 2111, and 2113 for RSVA and 560, 1712, 1718, 1719, 1759 and 2019 for RSVB, which may represent epitopes under pressure of adaptive immunity[32]. Immunological studies are required to confirm adaptive immune responses are developed during RSV infections against these epitopes on the L gene.

Strengths of this study are the sample size, the use of complete genomes, and a broad geographic coverage over a period of many years. Another strength is that our study only included prepandemic RSV sequences and mobility data, as COVID-19 drastically impacted

human air travel. An important limitation of our study is lack of data from most of the African continent, as well as from specific large countries including China and India. Additionally, the sample size within countries was too small to explain short-distance spread of RSV. Broader and denser coverage is likely to reveal additional predictors at different scales of transmission.

RSV research and therapeutics are rapidly advancing with the recent approval of nirsevimab and two vaccine for older adults, which might be shortly followed by the approval of a maternal vaccine[1]. Surveillance of RSV may be particularly important in the wake of these vaccines, given the potential for increased immunologic pressure on RSV F. The integration of epidemiological and phylogenetic approaches has received great attention for other viruses because of its potential to uncover mechanisms of pathogen emergence, evolution, and spread. By capturing the spatial spread of RSV, our reconstructions of spatial evolutionary history shed light on viral persistence and transmission dynamics. We demonstrate that the use of human air travel data together with viral genetic data provides a powerful model to describe global spread of RSV. This work also provides a baseline of RSVA and RSVB genome evolution before the widespread use of immunisation programmes, and the new genome data will constitute a key resource for further extensive research in the field of RSV epidemiology.

Ethical approval and consent

We declare that the planning, conduct, and reporting from this study was in line with the Declaration of Helsinki, as revised in 2013. Informed consent was obtained from parent(s) or legal representative(s) prior to sample collection in accordance with the International Conference on Harmonization Guideline on Good Clinical Practice E6 (ICH-GCP) and applicable national and international regulatory requirements.

The INFORM-RSV study has been approved by the ethics committees of all 18 participating sites: The Netherlands: The Medical Research Ethics Committee of the UMC Utrecht (reference number WAG/mb/17/016170); Italy: Ethics Committee for Clinical Testing of the Province of Padova of the Padova Hospital (no. 345 of 27/10/2016); Russia: The Department for Science, Innovation Development and Management of Health and Biological Risks, Ministry of Health of the Russian Federation; Germany: Ethics Committee of the Medical Faculty of the Philipps University Marburg; France: Ethics Committee Southwest and Overseas of the Créteil Intercommunal Hospital Centre (ID-RCB No.: 2018-A02360–55 (file 1– 18-73); Spain: Ethics Committee for Research Santiago-Lugo of the Hospital Centre University of Santiago (registration code 2017/397); South Korea: Medical Research Committee of the Seoul National University Hospital; Finland: Ethics Committee of the Hospital District of Southwest Finland, Turku; Australia: Human Research Ethics Committee of the Perth Children's Hospital; Brazil: The Research Ethics Committee of the Centro INFANT at Pontificia Universidade Catolica de Rio Grande do Sul (opinion number 2,569,872); Canada: Hamilton Integrated Research Ethics Board of the McMaster University; Canada: Research Ethics Board of the McGill University Health Centre; South Africa: Human Research Eth-

ics Committee of the University of the Witwatersrand Johannesburg (no. M170966); Japan: Research Ethics Committee of the Fukushima Medical University (no. 29212); The United Kingdom: Health Research Authority of the King's College Hospital (no. 17/EM/0469); Taiwan: Mackay Memorial Hospital Institutional Review Board (no. 19MMHIS171e); Chile: Ethics Committee for Research on Human Subjects of the Faculty of Medicine, University of Chile; Mexico: Ethics Committee of the University Autónoma De Nuevo León, Faculty of Medicine.

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Author contributions

ACL and LJB conceived the research. ACL and LJB drafted the manuscript with substantial help of BV and PL. BV and PL performed data analyses along with ACL. All authors discussed the results and contributed to the revision of the final manuscript. All authors approved the final version of the manuscript and accept responsibility for the data therein.

Declaration of interests

LJB has regular interaction with pharmaceutical and other industrial partners. He has not received personal fees or other personal benefits. UMCU has received major funding (>€100,000 per industrial partner) for investigator initiated studies from AbbVie, MedImmune, Janssen, the Bill and Melinda Gates Foundation, Nutricia (Danone) and MeMed Diagnostics. UMCU has received major cash or in kind funding as part of the public private partnership IMI-ffunded RESCEU project from GSK, Novavax, Janssen, AstraZeneca, Pfizer and Sanofi. UMCU has received major funding by Julius Clinical for participating in the INFORM-RSV study sponsored by AstraZeneca and Sanofi. UMCU has received minor funding for participation in trials by Regeneron and Janssen from 2015-2017 (total annual estimate less than €20,000). UMCU received minor funding for consultation and invited lectures by AbbVie, MedImmune, Ablynx, Bavaria Nordic, MabXience, Novavax, Pfizer, Janssen (total annual estimate less than €20,000). LJB is the founding chairman of the ReSViNET Foundation. PL and MAS acknowledge support from the European Union's Horizon 2020 research and innovation programme (grant agreement no. 725422-ReservoirDOCS), from the Wellcome Trust through project 206298/Z/17/Z and from the NIH grant R01 AI153044. PL acknowledges support From the Research Foundation - Flanders ('Fonds voor Wetenschappelijk Onderzoek - Vlaanderen', G0D5117N and G051322N) and from the European Union's Horizon 2020 project MOOD (grant agreement no. 874850). DW and EJK are employees of AstraZeneca.

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METHODS

Clinical samples

The INFORM-RSV study is a prospective, multiyear, multicentre, global clinical study enrolling children with medically-attended RSV infection under the age of 5 years. Details about the study design and protocol have been previously described[2]. In summary, RSV positive nasal samples were collected from November 2017 to March 2020 at 18 hospitals in 17 countries globally. Whole genome sequencing was performed at the UMC Utrecht using the Illumina NextSeq 500 platform (details have been published a separate methodology paper[2]) and annotated with sampling data and country. Whole genome sequences derived from the first three seasons of the INFORM-RSV study are available at GISAID.

Data set compilation

Sequence data on the F protein of RSVA and RSVB from the INFORM-RSV study have previously been published[33]. However, the current data represent the first whole genome sequences which were complemented with a selection of publicly available RSV sequences downloaded from NCBI GenBank on April 21st 2021. These were first size-selected (only those of length without N >= 10k bases were kept for further analyses, n = 2865/27417 or 10.4%) and typed as RSVA or RSVB. After alignment with MAFFT v.7.475[34] and manual verification using AliView v.1.26[35], RDP5[36] was used to clean the RSV A and RSVB alignments from putative recombinant sequences. Next, only sequences with known country of sampling and sampling date known up to the year or more precise were retained for further analyses. The resulting alignment served to obtain a maximum likelihood tree with branch support estimated with the SH-aLRT test[37] as implemented in IQtree v.2.1.2[38]. From this tree, a well-supported subtree containing all INFORM-RSV sequences was selected for downstream analyses (Figures S7 and S8).

Circulating genotypes

We investigated whether the additional genomic diversity from the INFORM samples warrants a reclassification. For this we adhered to the RSV type-specific patristic distance thresholds suggested by Ramaekers et al[30] but assess clade support with the computationally more efficient SH-aLRT and UFB branch support tests, and require minimal support values of 80 (SH-aLRT) and 90 (UFB). The criteria for genotype delineation put forward by Ramaekers et al[30] involve a patristic distance and a clade support threshold. This definition implies that genotypes form monophyletic clades in which a limited number of genetic differences has accrued. It can therefore be anticipated that, as evolution continues, a clade that was formerly classified as a single genotype can diversify into a set of new genotypes.

TempEst v.1.5.3[39] was used to identify sequences that represented outliers in a regression of root-to-tip divergence as a function of sampling time. To this end, an operational definition of outliers was used: outliers were defined as sequences for which the residual of the regression of root-to-tip genetic distance against sampling time falls outside the 99% credible interval of residuals, which was derived using the CODA R package[40,41]. 13 outliers were removed from the RSVA and RSVB data sets. This increased the correlation between the root-to-tip distance and sampling time from 0.94 to 0.95 for RSVA and from 0.79 to 0.83 for RSVB. Likewise, the R^2 of the regression increased from 0.89 to 0.91 for RSVA and from 0.63 to 0.70 for RSVB. The resulting data sets, with 1213 taxa for RSVA and 1223 taxa for RSVB, were used for phylogeographic reconstruction and genotype classification[30]. For the latter, a maximum likelihood tree was estimated using IQtree[38] with ModelFinder[42] and branch support was evaluated with the SH-aLRT and ultra-fast bootstrapping (UFB) procedures. Genotypes were called using an in-house developed R[41] script that capitalises on several packages (treeio, phytools, geiger).

A down-sampled data set was created for site-specific selective pressure analyses. For this, within-country transmission networks were downsized to a randomly chosen taxon according to a two-step procedure. First, within-country transmission networks were identified as clades with perfect SH-aLRT support for which all taxa were from the same country[43] based on a midpoint rooted maximum likelihood tree (obtained with IQtree v.2.1.2[38]) from the phylogeo-datasets. Next, this reduced data set was used for estimating time-calibrated evolutionary histories with the Bayesian Evolutionary Analysis by Sampling Trees software (BEAST v1.10)[44] along with the high-performance BEAGLE v.3.2.0 library for computational efficiency[45]. The RSVA and RSVB data sets were equipped with the same evolutionary models. To capture the nucleotide substitution process while allowing for differences between the coding and non-coding genome regions, a General Time Reversible (GTR) model with Γ-distributed among site rate variation[46,47] was specified for either region. The estimated rate of evolution was informed by the amount of evolution that accrued over the sampling time differences, and the rate was allowed to vary among lineages through a relaxed clock model with lognormally distributed branch rates[48]. The demographic history was modelled with the flexible skygrid tree prior[49] with changes in the relative genetic diversity over time allowed at 6-month intervals between January 1st 2020 and January 1st 2005. Within country transmission chains were now identified as clades of taxa from the same country with perfect posterior support.

Phylogeographic inference

Time-calibrated evolutionary histories were estimated from the phylogeography data sets using the Bayesian Evolutionary Analysis by Sampling Trees software (BEAST v1.10)[44] along with the high-performance BEAGLE v.3.2.0 library for computational efficiency[45]. The same models as for identifying within-country transmission networks (see above) were specified. Mixing and convergence properties of the Markov Chain Monte Carlo simulation were inspected using Tracer v1.7[53]. Maximum Clade Credibility (MCC) summary trees were obtained with TreeAnnotator (distributed with BEAST v.1.10) and visualised in FigTree v.1.4[50]. Continuous parameter estimates are summarised as means and 95% highest posterior density intervals (95% HPDs).

Generalized linear mixed model

To test for predictors of the global spatial diffusion process, we applied a generalized linear model (GLM) parameterization of the discrete phylogeographic model[5]. Briefly, this model parameterises the log transition rates between pairs of locations as a function of potential predictors. Each predictor is associated with an estimable log effect size and inclusion probability. We reported the posterior estimates for the product of these parameters for our analyses. We applied this model both at the country and the continental level and employ a set of

1000 time-scaled trees sampled evenly throughout the post-burning posterior as empirical tree distributions for both RSVA and RSVB.

For the reconstruction at continental level, taxa were assigned to Africa, Asia, Europe, North America, Oceania or South America based on the WHO region classification. Specifically, taxa from the Sub-Saharan Africa and Northern Africa regions were categorized as African. Taxa from the Western, Central, Southern Eastern and South-Eastern Asia regions were categorized as Asian. Taxa from the Caribbean, Central and Northern America regions were categorized as North American. South American countries were categorized as South American. Countries from Melanesia, Micronesia, Polynesia together with Australia and New Zealand were categorized as Oceania. Taxa from Eastern, Western, Northern and Southern Europe were binned as European.

As predictors, we included passenger fluxes (i.e. the number of passengers travelling by air between countries and continents provided by the International Air Transport Association (IATA)[51] for the period 2019-2020), population size (for 2019)[52] at the origin and destination location, geographic distance and absolute difference in latitude (as proxy for synchronicity in northern or southern hemisphere transmission). For the geographic distances and absolute latitude differences, latitude and longitude coordinates representing the countries' midpoints were downloaded from the Dataset Publishing Language as provided by Google[53]. Geographic distances were calculated using the Haversine formula. At the continental level, we used data for the countries from which genome samples are included in the analyses. In additional analyses, we assessed the sensitivity of predictor support with respect to sampling heterogeneity by also including sample size at the origin and destination location as potential predictors. Analyses were performed for both RSVA and RSVB separately, but we also ran the inference applying a single GLM-diffusion model to both data sets to examine the shared signal in both. Finally, for the country-level analyses we also applied a time-inhomogeneous version of the model[54] partitioning the evolutionary history in an epoch before and after 5 years since the most recent sampling time. These analyses were performed to examine which time period was informing the predictor support.

Posterior summaries of geographic mixing

To quantify the degree by which RSV clustering is structured by country, we used a normalised entropy measure recently proposed by Lemey et al (2021)[6]. We focused on the most recent season (2019-2020) because the phylogenetic clustering of these samples and their degree of phylogenetic interspersion is expected to be maximally informed by the INFORM-RSV sampling during the two previous seasons. For each country, we considered a time interval that encompasses the sampling from that recent season and goes back to the end of the previous season for that country. The start and end months of RSV seasons were determined by the relative infection intensities per month for each country. In these time intervals, we summarised the times associated with contiguous partitions of a tree estimated to be in

each country. Based on these time estimates we computed a normalised Shannon entropy for each country:

$$-\frac{1}{ln(n)}\sum_{i}^{n} \quad p_i ln(p_i)$$

Where p_i is the proportion of time associated with that country for partition i of the tree, and n represents the number of partitions for that country in the tree. In case all genomes sampled during the most recent season in a specific country would form a single cluster (partition) in the phylogeographic tree, the entropy measure is expected to be ≈ 0. When none of the genomes from the same country would cluster together, and hence are interspersed with genomes from other countries, the measure is expected to be ≈ 1. We used this measure to summarize the posterior distribution of phylogeographic reconstructions for the analysis with a single time-inhomogeneous GLM-diffusion model shared by both RSVA and RSVB (without sample size predictor). To aid interpretation of the entropy measures, we also summarized the number of unique lineages circulating in each country at the start of the most recent season. Multiple branches associated with the same country sharing a common ancestor with that country state after the end of the previous season are considered to constitute a single unique lineage[6]. We also attempted to summarize whether these unique lineages represented new introductions or persisting lineages since the end of the previous season for each country[6], but this results in uninformative estimates because of an insufficiently dense sampling each season and lack of global coverage. Specifically, lineages from the last season often coalesced with other lineages earlier than the previous season, biasing the estimates towards persistence.

Identification of positively selected sites

Following recommendations by Kosakovsly Pond and Frost (2005)[29], we identified positively selected sites using different complementary approaches. Specifically, we employed the fast unconstrained Bayesian approximation (FUBAR) and the mixed effects model of evolution (MEME) approach implemented in HyPHy and the renaissance counting (RC)[55] approach implemented in BEAST. For FUBAR, we used the variational Bayes approximation and the default threshold of a posterior probability > 0.9 for sites to be identified as subject to diversifying positive selection. For MEME, we used the default *p*-value threshold of 0.1 for testing for selection and we restrict the test to internal branches. For RC, we specified a skygrid coalescent prior, an uncorrelated relaxed clock model, and a GTR model for each codon position. We considered sites to be positively selected if the site-specific empirical Bayes estimate of the nonsynonymous to synonymous rate ratio (dN/dS) results in a lower 95% HPD interval boundary that is larger than 1 and if the mean dN/dS estimate is larger than 1.5. We only reported sites as positively selected if they are identified by at least two of the three approaches used.

Genealogical neutrality tests

To evaluate whether RSV evolution adheres to neutral evolution, we employed a model-based Bayesian procedure that distinguishes between the effects of demography from the effects of selection[19]. Specifically, we employed the posterior distribution from the genealogical inference produced by BEAST and perform posterior predictive simulation of genealogies under neutral coalescent models accounting for potentially complex demographic histories. For the latter, we adopt the skygrid coalescent model. For posterior predictive simulation under this model, we fit skew normal distributions to the estimates of the interval-specific population sizes and use these in an MCMC simulation procedure. By comparing the genealogical shapes of the inferred tree distribution to that obtained by the posterior predictive simulation using summary statistics, we tested for significant departures from neutral evolution. Here we used two genealogical summary statistics: i) the genealogical Fu and Li statistic (DF), which compares the length of terminal branches to the total length of the coalescent genealogy[19], and ii) the ratio of the trunk (or backbone) length over the entire tree length. The concept of a trunk, representing the lineage(s) that persist(s) through time, has frequently been used in characterization of the viral population turnover dynamics[56,57], with viruses like human seasonal influenza that experience strong selective pressure to escape antibody responses showing pronounced trunk and short-lived side branches.

Data availability

Alignments, predictor data and BEAST XML files used for this work are publicly available on GitHub (https://github.com/bramvrancken/RSV_INFORM.git). Whole genome sequences are publicly availabe on GISAID.

SUPPLEMENTARY FIGURES

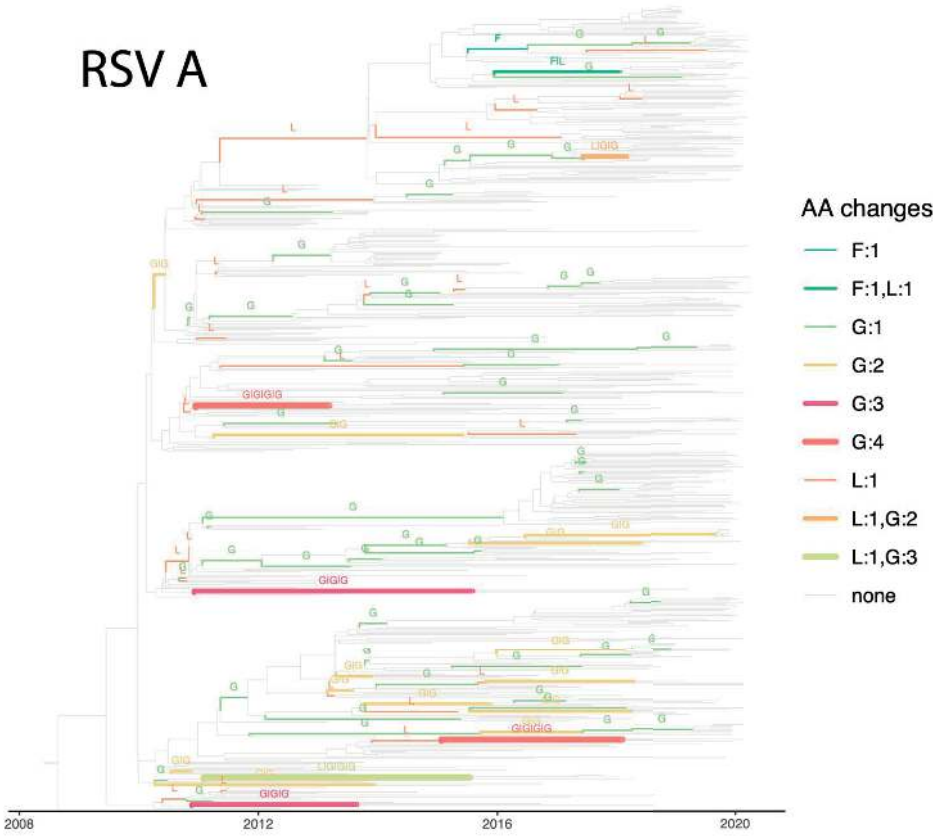

Figure S1: Phylogenetic summary of the amino acid substitutions at positively selected sites (as supported by at least two of these methods) for RSVA. The thickness of each branch in the Maximum Clade Credibility (MCC) tree corresponds to the number of AA changes that occurred over that branch as indicated in the legend. Branch colours identify the number of AA changes and affected protein(s), with correspondence as in the legend. The number of AA changes and affected protein(s) are also indicated next to the relevant lineage. Lineages that did not accommodate a nonsynonymous change are depicted in light grey.

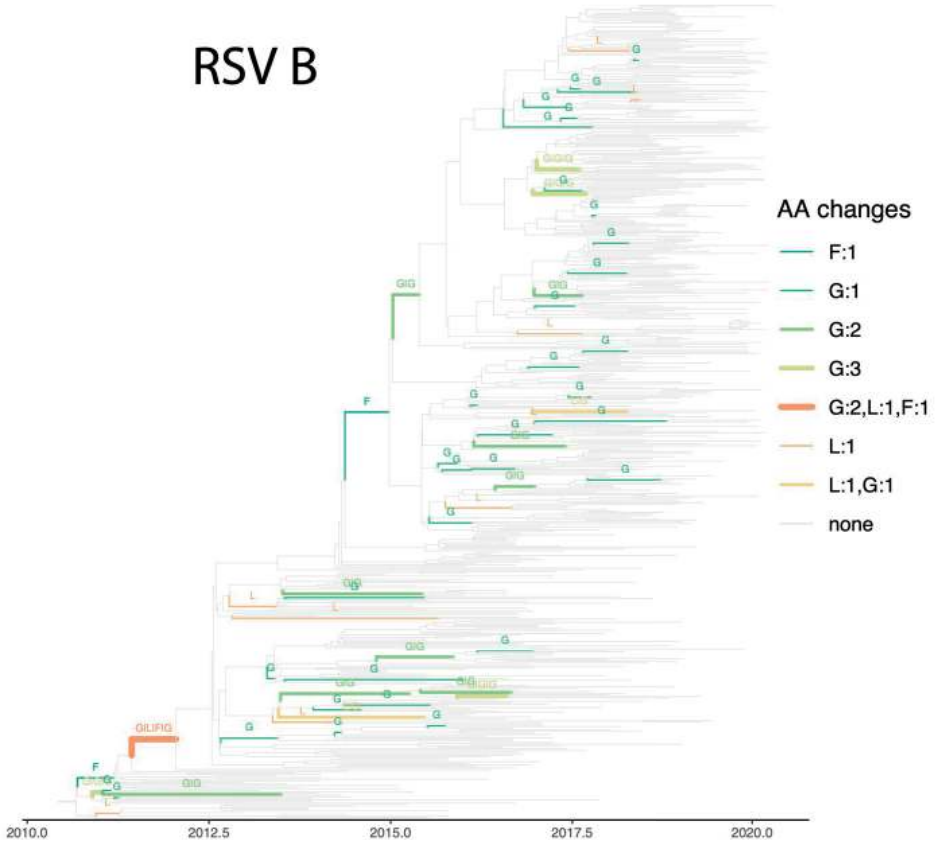

Figure S2: Phylogenetic summary of the amino acid substitutions at positively selected sites (as supported by at least two of these methods) for RSVB. The thickness of each branch in the Maximum Clade Credibility (MCC) tree corresponds to the number of AA changes that occurred over that branch as indicated in the legend. Branch colours identify the number of AA changes and affected protein(s), with correspondence as in the legend. The number of AA changes and affected protein(s) are also indicated next to the relevant lineage. Lineages that did not accommodate a nonsynonymous change are depicted in light grey.

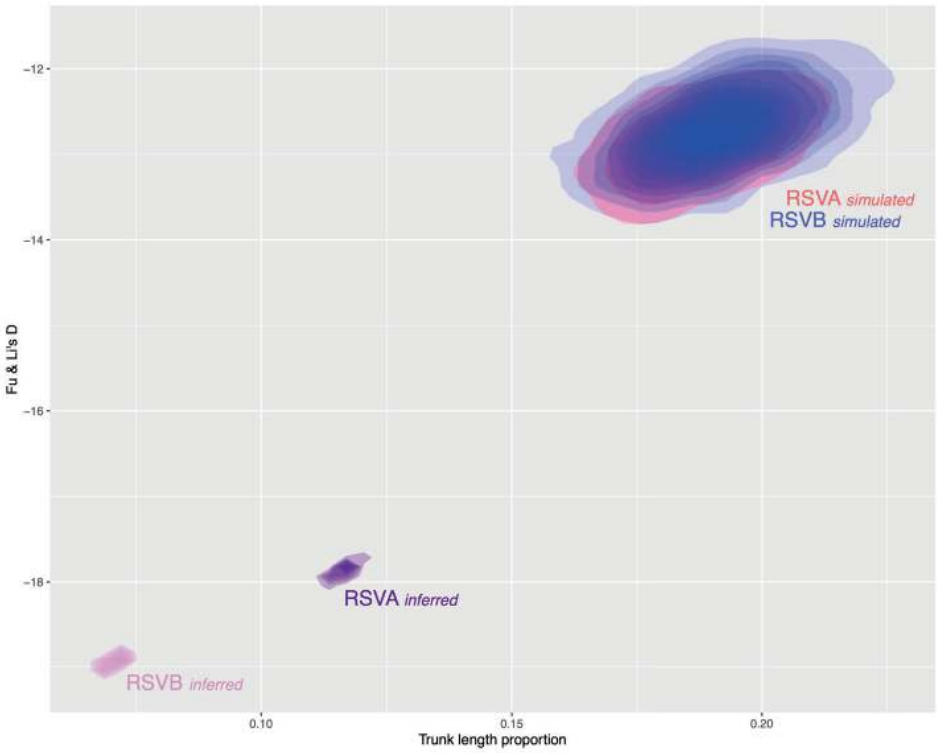

Figure S3: Comparison of tree shape statistics derived from inferred and simulated RSV A and B genealogies. Contours filled with colors of different intensity represent the 2D density estimates of the tree shape statistics. Darker colors correspond to higher densities. The colors of the labels indicate which contours pertain to which analysis.

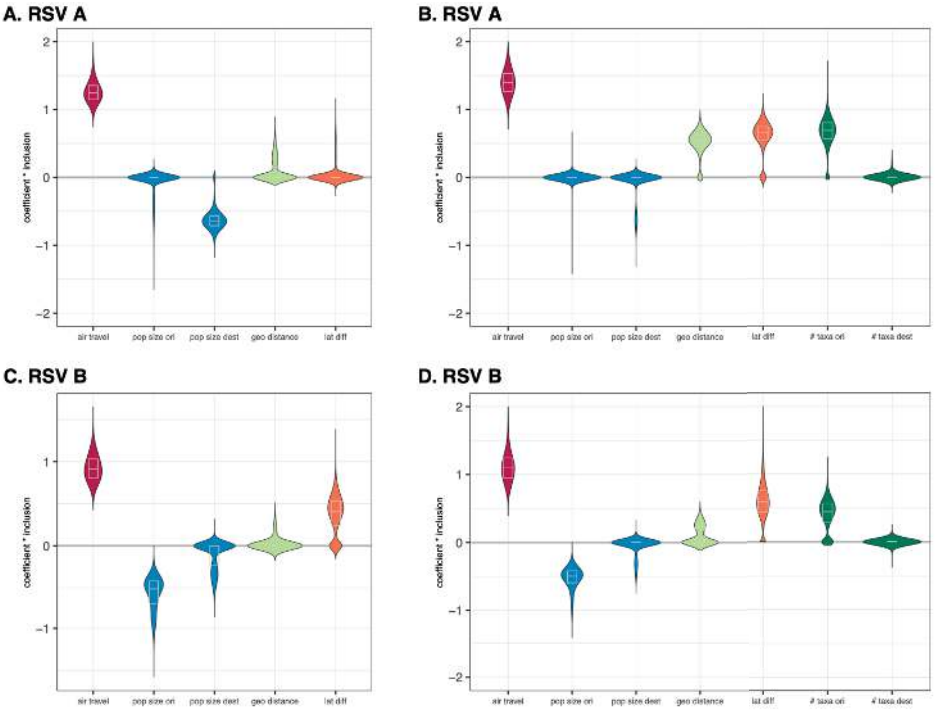

Figure S4: Posterior estimates of time-homogeneous predictor contributions to RSV diffusion at the continent level. The predictors include the number of passengers travelling by air between each pair of continents represented in the data set (air travel, in dark red), population size at the origin and destination location (pop size ori & pop size dest, in blue), geographic distance (geo distance, in light green), absolute differences in latitude (lat diff, in dark orange) and sample sizes at the origin and destination locations (# taxa ori & # taxa dest, in dark green). The Y-axis represents the product of the coefficient (on a log scale) and the inclusion probability for the predictors (coefficient * Inclusion). A-B: RSVA. C-D: RSVB. The plots on the left and right distinguish between analyses without and with sample size predictors respectively. The grey boxes in the violin plots represent the median and quantile estimates.

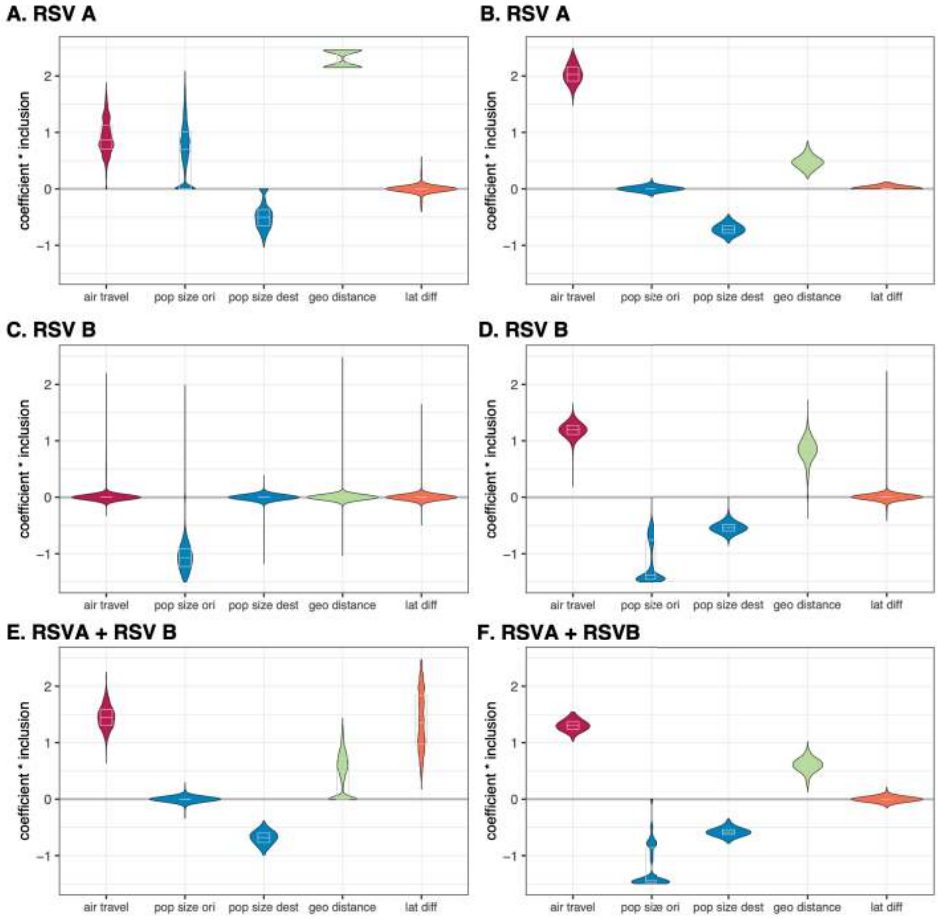

Figure S5: Posterior estimates of time-inhomogeneous predictor contributions to RSV global diffusion. The predictors include the number of passengers travelling by air between each pair of countries represented in the data set (air travel, in dark red), population size at the origin and destination location (pop size ori & pop size dest, in blue), geographic distance (geo distance, in light green), absolute differences in latitude (lat diff, in dark orange). The Y-axis represents the product of the coefficient (on a log scale) and the inclusion probability for the predictors (coefficient * Inclusion). A and B: RSVA estimates at the country level. C and D: RSVB estimates at the country level. E and F summarise the estimates for a single GLM-diffusion model applied to the combined RSVA and RSVB data sets at the country level. The plots on the left and right distinguish between estimates before and after 5 years prior to the most recent sampling time and are derived from a single epoch GLM-diffusion model. The grey boxes in the violin plots represent the median and quantile estimates.

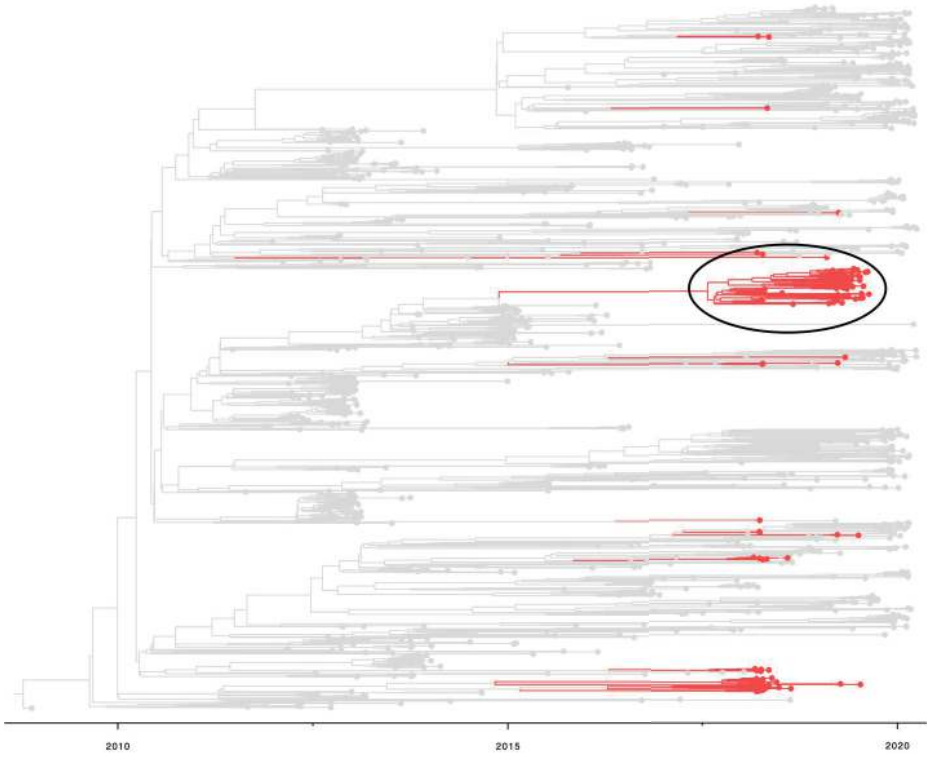

Figure S6: Unique clustering of South African lineages at the start of the 2019-2020 season (black ellipse).

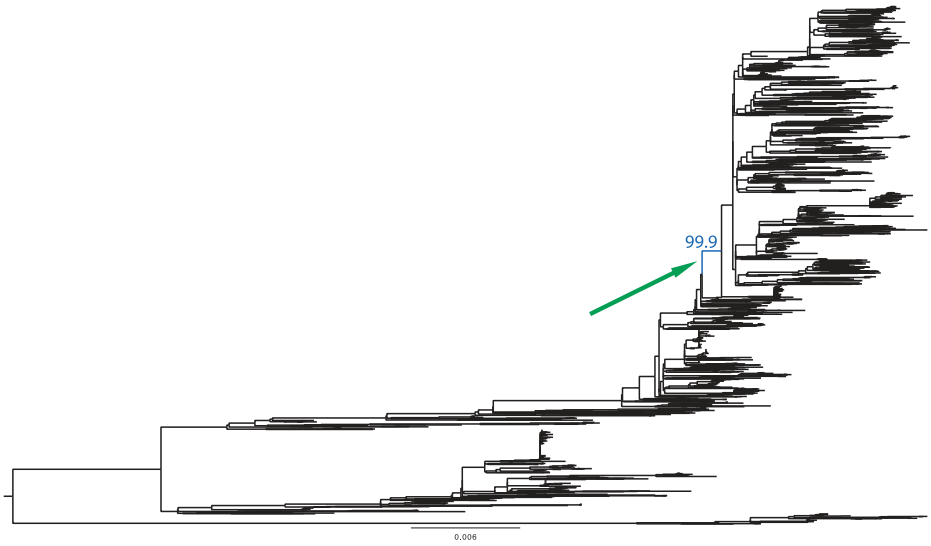

Figure S7: Midpoint rooted ML tree for RSV A. The highly supported branch that is basal to the well supported clade containing all INFORM sequences is highlighted in blue and indicated by a green arrow. The SH-aLRT support is indicated next to the branch.

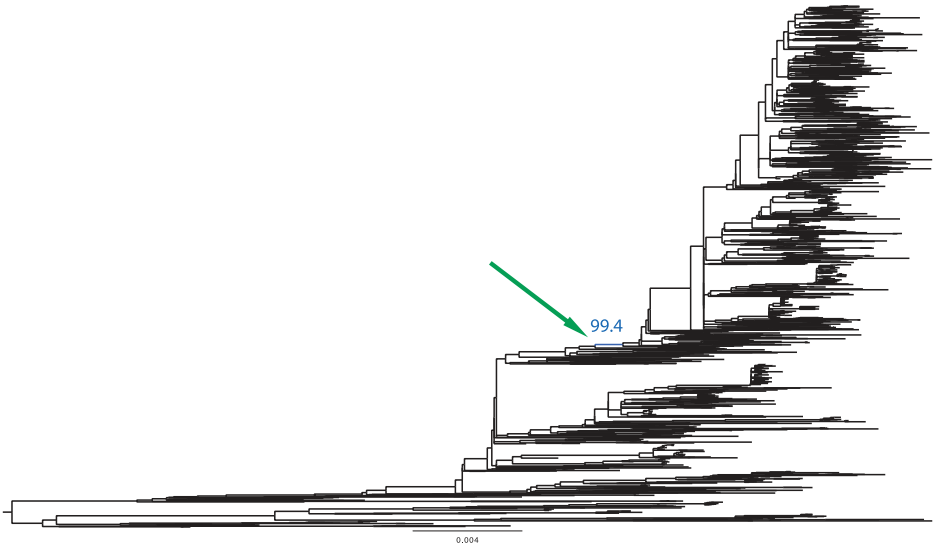

Figure S8: Midpoint rooted ML tree for RSV B. The highly supported branch that is basal to the well supported clade containing all INFORM sequences is highlighted in blue and indicated by a green arrow. The SH-aLRT support is indicated next to the branch.

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Chapter 10

Lethal Respiratory Syncytial Virus in Zambia is Sensitive to Long-Acting Monoclonal Antibodies

"ZPRIME"

The Pediatric Infectious Diseases Journal, 2023

"You miss 100% of the shots you don't take."
— Michael Scott, The Office

Lethal Respiratory Syncytial Virus in Zambia Is Sensitive to Longacting Monoclonal Antibodies

The Pediatric Infectious Disease Journal

To the Editors:

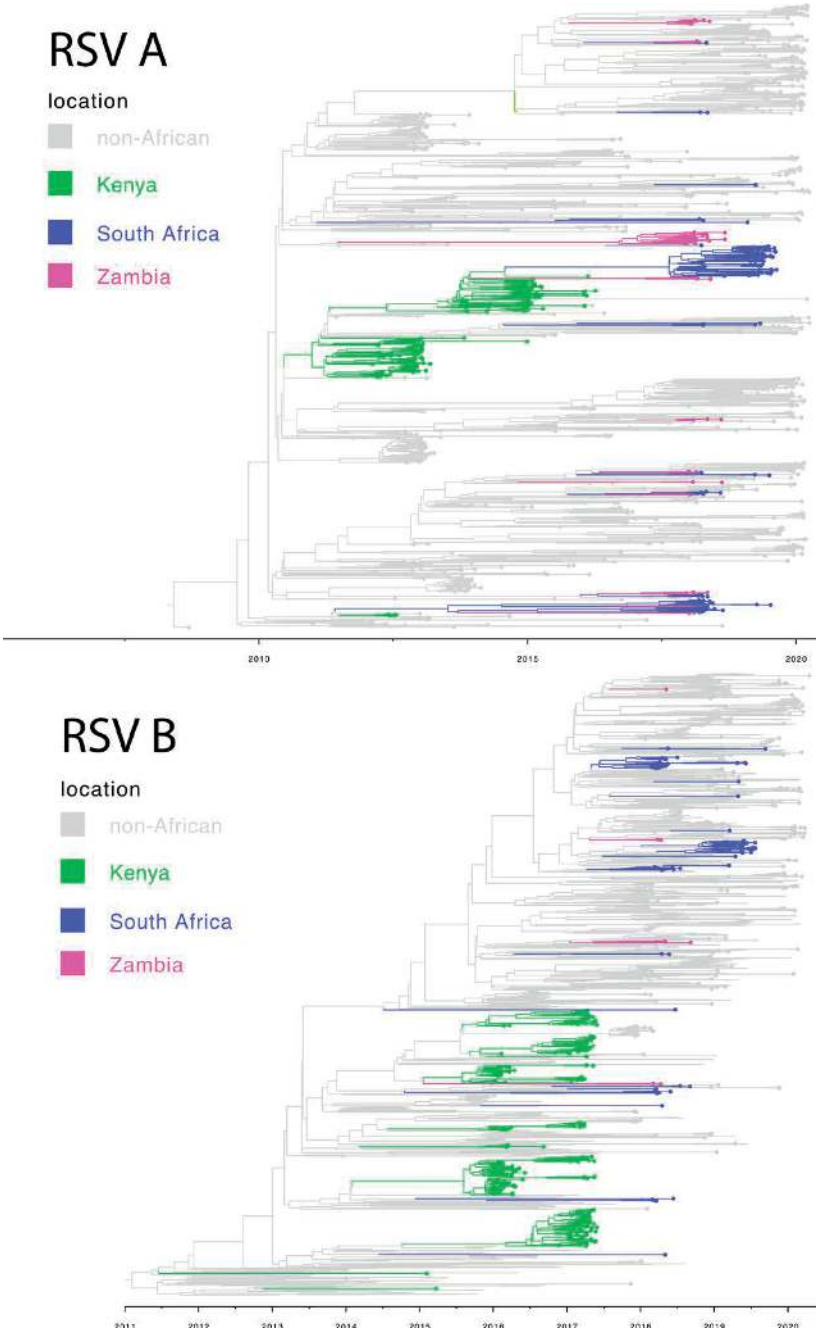
Respiratory syncytial virus (RSV) is the most common cause of severe lower respiratory tract infection in the first 6 months of life with more than 97% of mortality occurring in low- and middle-income countries (LMICs).[1] RSV mortality data from these geographic regions are limited, and if available, they mainly reflect in-hospital deaths resulting in an underestimate of the global burden of fatal RSV. Because of poor access to healthcare and low-quality healthcare, a sizable proportion of RSV-related deaths among infants in LMICs occurs in the community. For most infectious diseases, including influenza, genetic diversity of viruses affects mortality risk.+

Previously, we demonstrated that RSV is a major cause of overall infant mortality in Zambia.[3] In the Zambia Pertussis and RSV Infant Mortality Estimation (ZPRIME) study, we measured facility and community RSV deaths among infants in Lusaka, Zambia through a systematic postmortem surveillance project at the University Teaching Hospital morgue. Between August 2017 and 2020, we found that RSV was present in 7% of all deceased infants and 32% of the RSV+ infant deaths occurred in the community. RSV deaths were concentrated in infants younger than 3 months and in infants from densely populated Lusaka townships.

The key distinguishing feature of the ZPRIME study, compared with most studies that have measured the impact of RSV, is that all the participants were deceased, and therefore represented the most extreme of infection outcomes. We aimed to establish whether fatal RSV infection is related to specific RSV genetic sequences, or they could reflect nonvirologic factors such as the vulnerability of the infant population and/or ease of access to supportive medical care. To test the former hypothesis, we performed whole-genome sequencing as described previously[4] on a subset of nasopharyngeal samples (n = 116) collected under the ZPRIME study resulting in 71 full-genome RSV sequences (success rate of 62.2%). Of these 71 sequences, 62 were subtyped RSV-A and 9 RSV-B. We complemented ZPRIME sequences with publicly available sequences from other African countries (South Africa and Kenya) and with not yet published sequences generated by the INFORM study from 17 countries globally.[4] We inferred phylogenetic trees and the migration history for both subtypes in a Bayesian framework (Fig. 1).[5,6]

Here, we demonstrate that infants in Zambia are dying of RSV linked to diverse viral strains that are intermixed across the globe. Clusters of Zambian RSV sequences obtained from postmortem samples were identified throughout the phylogenetic trees, making it highly unlikely that there was a virologic factor involved in mortality (Fig. 1). In terms of global diversity of RSV, we found no single lineage specific for Zambia: Zambian sequences cluster with sequences from elsewhere. Zambian sequences are closely related to South African sequences and to a lesser extent to Kenyan sequences, indicating that RSV strains cocirculate within Africa. We therefore suggest that there does not appear to be anything distinct about the Zambian RSV strains per se compared with other African locations. We found limited local persistence of RSV within African countries, as sequences from African countries also cluster

FIGURE 1. Phylogenetic reconstruction of ZPRIME postmortem sequences, and sequences from other African and non-African locations.



Tips and internal branches are colored according to the most probable reconstructed ancestral state (location). The correspondence between the colors and locations is as in the legend.

with those obtained at non-African locations. We did not find evidence of molecular nirsevimab resistance among the RSV strains from the ZPRIME study. The Ile206Met:Gln209Arg polymorphism in the nirsevimab binding site of RSV B became globally dominant with a prevalence of 1843 of 2800 (65.8%) among RSV B strains observed between 2015 and 2021.[7] This RSV B polymorphism was also highly prevalent (7/9 sequences; 77.8%) in Zambia between 2017 and 2020. In sum, the newly obtained lineages suggest that Zambian RSV is typical of global RSV.

Using analysis of viral genetics, we found no evidence supporting viral genetic risk to mortality. This finding is important for understanding the impact of RSV on infant deaths in Africa. RSV in Zambia seems entirely typical. Mortality may not be virus-related, but explained by the poor healthcare system, population (within-host diversity) or both. To date, host factors for RSV mortality have been poorly defined. Our virologic sequence study showed no substantial differences in RSV sequences from Zambia as compared with elsewhere. We therefore conclude that the fatal outcomes in these cases are not explained by genetic factors, but more likely nonvirologic factors, such as challenges in timely access to supportive care as we have documented previously,[8] limited availability of supportive treatments at facilities or intrinsic vulnerabilities in the Zambian infant population. Mutation analysis of the nirsevimab binding site showed that currently available immunoprophylaxis strategies may be effective to prevent RSV mortality in LMICs.

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L.J.B. has regular interaction with pharmaceutical and other industrial partners. He has not received personal fees or other personal benefits. UMCU has received major funding (>€100,000 per industrial partner) for investigator initiated studies from AbbVie, MedImmune, Janssen, the Bill and Melinda Gates Foundation, Nutricia (Danone) and MeMed Diagnostics. UMCU has received major cash or in kind funding as part of the public private partnership IMI-ffunded RESCEU project from GSK, Novavax, Janssen, AstraZeneca, Pfizer and Sanofi. UMCU has received major funding by Julius Clinical for participating in the INFORM-RSV study sponsored by AstraZeneca and Sanofi. UMCU has received minor funding for participation in trials by Regeneron and Janssen from 2015- 2017 (total annual estimate less than €20,000). UMCU received minor funding for consultation and invited lectures by AbbVie, MedImmune, Ablynx, Bavaria Nordic, MabXience, Novavax, Pfizer, Janssen (total annual estimate less than €20,000). L.J.B. is the founding chairman of the ReSViNET Foundation. Since April 1, 2021, L.J.B. has been given a new position as medical scientific division manager of the Children's Division of the Wilhelmina Children's Hospital in Utrecht.

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All 71 ZPRIME sequences reported in the manuscript were submitted to GISAID with the following accession numbers:

hRSV/A/Zambia/Lusaka-4082/2018, hRSV/A/Zambia/Lusaka- 1708/2018, hRSV/A/Zambia/Lusaka-1925/2018, hRSV/A/ Zambia/Lusaka-3230/2019, hRSV/A/Zambia/Lusaka-3099/2018, hRSV/A/Zambia/Lusaka-2617/2019, hRSV/A/Zambia/Lusaka- 1210/2019, hRSV/A/Zambia/Lusaka-2615/2019, hRSV/A/Zambia/ Lusaka-3935/2018, hRSV/A/Zambia/Lusaka-1937/2018, hRSV/ A/Zambia/Lusaka-4073/2018, hRSV/A/Zambia/Lusaka-1948/ 2018, hRSV/A/Zambia/Lusaka-4520/2019, hRSV/A/Zambia/ Lusaka-4593/2018, hRSV/A/Zambia/Lusaka-1129/2018, hRSV/ B/Zambia/Lusaka-5070/2019, hRSV/A/Zambia/Lusaka-2141/2019, hRSV/A/Zambia/Lusaka-3103/2018, hRSV/A/Zambia/Lusaka-2057/2018, hRSV/A/Zambia/Lusaka-2133/2019, hRSV/A/Zambia/ Lusaka-4089/2018, hRSV/A/Zambia/Lusaka-1983/2018, hRSV/A/ Zambia/Lusaka-3117/2018, hRSV/A/Zambia/Lusaka-2557/2019, hRSV/A/Zambia/Lusaka-1103/2018, hRSV/A/Zambia/Lusaka- 3922/2018, hRSV/A/Zambia/Lusaka-3924/2018, hRSV/A/ Zambia/Lusaka-2074/2018, hRSV/B/Zambia/Lusaka-3221/2019, hRSV/A/Zambia/Lusaka-3195/2019, hRSV/B/Zambia/Lusaka-1207/2019, hRSV/A/Zambia/Lusaka-1932/2018, hRSV/A/Zambia/ Lusaka-3145/2018, hRSV/A/Zambia/Lusaka-1122/2018, hRSV/ A/Zambia/Lusaka-3239/2019, hRSV/A/Zambia/Lusaka-3204/ 2019, hRSV/A/Zambia/Lusaka-3215/2019, hRSV/B/Zambia/ Lusaka-5051/2019, hRSV/A/Zambia/Lusaka-3934/2018, hRSV/A/ Zambia/Lusaka-4508/2018, hRSV/B/Zambia/Lusaka-1212/2019, hRSV/A/Zambia/Lusaka-3939/2018, hRSV/A/Zambia/Lusaka- 3938/2018, hRSV/B/Zambia/Lusaka-3219/2019, hRSV/A/Zambia/ Lusaka-1970/2018, hRSV/A/Zambia/Lusaka-1139/2018, hRSV/ A/Zambia/Lusaka-4654/2019, hRSV/A/Zambia/Lusaka-3210/2019, hRSV/A/Zambia/Lusaka-1238/2019, hRSV/A/Zambia/Lusaka- 1953/2018, hRSV/A/Zambia/Lusaka-1119/2018, hRSV/A/Zambia/ Lusaka-4627/2018, hRSV/A/Zambia/Lusaka-4113/2019, hRSV/ A/Zambia/Lusaka-1112/2018, hRSV/B/Zambia/Lusaka-1201/2019, hRSV/A/Zambia/Lusaka-4652/2019, hRSV/B/Zambia/Lusaka- 3224/2019, hRSV/B/Zambia/Lusaka-3209/2019, hRSV/A/ Zambia/Lusaka-1218/2019, hRSV/A/Zambia/Lusaka-4116/2019, hRSV/A/Zambia/Lusaka-3923/2018, hRSV/A/Zambia/Lusaka- 1704/2018, hRSV/A/Zambia/Lusaka-1117/2018, hRSV/A/Zambia/ Lusaka-5034/2019, hRSV/A/Zambia/Lusaka-1105/2018, hRSV/ A/Zambia/Lusaka-1936/2018, hRSV/A/Zambia/Lusaka-1126/2018, hRSV/A/Zambia/Lusaka-4573/2018, hRSV/A/Zambia/Lusaka- 1200/2019, hRSV/A/Zambia/Lusaka-3102/2018, hRSV/A/ Zambia/Lusaka-1197/2019.

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PART II

Product Development of Biomarkers

Chapter 11

Saliva as an Alternative to Nasopharyngeal Swabs for Detection of Respiratory Syncytial Virus in Infants

"FRIENDS"

Unpublished Data

"I began to realize how important it
was to be an enthusiast in life
if you are interested in something
no matter what it is
got at it fullspeed
embrace it with both arms
hugh it
love it
and above all
become passionate about it
lukewarm is no good."
— Roald Dahl, author

Saliva as an Alternative to Nasopharyngeal Swabs for Detection of Respiratory Syncytial Virus in Infants

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ABSTRACT

Background. Globally, respiratory syncytial virus (RSV) causes a significant burden of acute lower respiratory tract infection and is a major cause of morbidity and mortality, especially in infants. With RSV vaccines progressing through clinical trials, large-scale post-vaccine surveillance studies will be required. Saliva offers an accessible and equitable sample method which may contribute to more sensitive diagnostic methods as well as sustainable surveillance approaches, especially in low-resource and remote settings.

Methods. Here, we enrolled infants admitted to the Wilhelmina Children's Hospital (Utrecht, the Netherlands) with medically-attended respiratory infection, to compare the collection burden and sensitivity for the detection of RSV in saliva as compared to the gold-standard nasopharyngeal swab (NPS). Saliva was tested using an RNA-extraction-free method, widely deployed during the COVID-19 pandemic for the detection of SARS-CoV-2.

Results. To date, 69 infants have been enrolled in this ongoing study; 47 (68.1%) tested positive for RSV of which 31 sample sets have been analysed. Of these 31 sample sets, 22 (71.0%) samples had adequate saliva volume for testing. With NPS as gold standard, we compared the number of infants positive by saliva vs. NPS. Of 22 RSV positive infants by NPS, 14 (63.6%) tested positive in saliva. Discomfort was significantly higher for NPS versus saliva collection ($p<0.001$).

Conclusion. We observed a modest reduction in the sensitivity of saliva for the detection of RSV detection in infants, as compared to NPS. Saliva testing offers an accessible and equitable testing solution for improving RSV diagnosis and for surveillance following vaccine implementation. The benefits of this may prove even greater in low-resource and remote settings.

INTRODUCTION

Respiratory syncytial virus (RSV) causes severe disease in the very young, elderly, and in high-risk groups. Worldwide, there are an estimated 33 million cases of acute lower respiratory tract infection (ALRI), 3.4 million ALRI hospitalisations, and >200,000 deaths associated with RSV in children <5 years old, each year.[1] RSV infection in childhood is associated with subsequent wheezing and asthma.[2] The world's first RSV vaccine and mAb for the protection of all infants have just been approved.[3] Critical to the development and evaluation of these prophylactics are robust diagnostic tools. Previous diagnostic studies have demonstrated that improved sampling methods may contribute to more accurate and faster RSV diagnosis.[4-6]

Currently, testing nasopharyngeal swabs (NPS) with RT-qPCR is considered the gold standard for the diagnosis of RSV. Although sensitivity is high, NPS comes with relative sampling burden for the patient, time interval between sample collection and PCR test result, and high personnel and resource expenses. In addition to an increasing testing aversion to swabs, these drawbacks of NPS may present an obstacle to the frequent testing of infants. Several studies have shown that saliva may offer an alternative, non-invasive sample type for virus testing which overcomes some of the hurdles of NPS. In their recent review, Laxton *et al*. identified that paired-sample studies found high concordance (93-100%) between saliva and nasopharyngeal specimens for the detection of respiratory pathogens.[8] More specifically, a recent study demonstrated a sensitivity of 70% for saliva versus 51% for NPS in adults.[7]

Building upon the benefits of saliva collection, SalivaDirect, a saliva-based, RNA-extraction-free PCR test was developed and widely deployed during the COVID-19 pandemic to address the limitations of traditional sampling and molecular testing for SARS-CoV-2.[8] Owing to its success, it was expanded for the detection of RSV, substituting in the RSV primer/probe sequences published by Fry *et al*.[9] In this assay, saliva is tested almost directly in RT-qPCR, removing the need for the most resource-demanding step of pathogen detection: RNA extraction. While SalivaDirect has not been clinically validated in case of RSV infection, and while challenges in saliva collection from infants persist, the expansion of this method for the successful detection of RSV could support large-scale and frequent clinical and community sampling. This would have important implications for research, for example the use in large trials related to the introduction of novel vaccinations strategies for RSV. Therefore, in this study we evaluated the potential of saliva for the detection of RSV in infants with medically-attended respiratory infection (MARI).

METHODS

Study design

We conducted a single-centre observational cohort study at the Wilhelmina Children's Hospital. The study was approved by the institutional board of the University Medical Centre Utrecht, the Netherlands (21/617).

Study population

This study included infants under 12 months of age at enrolment with MARI. We excluded infants with exposure to ribavirin, palivizumab or an RSV investigational vaccine. Paired NPS and saliva samples were obtained by trained study personnel. Sampling occurred as soon as possible, but no later than 72 hours after hospital admission for acute RTI or 96 hours after onset of illness for those not admitted.

Sample collection

Three upper respiratory tract samples were collected in the following sequence: (1) a raw saliva sample, with a minimal desired volume of 0.5 mL using a bulb transfer pipette; (2) a raw saliva sample using an ORACOL, sponge-based collection device placed in the mouth for 1-2 minutes; and (3) a NPS stored in universal transport media (UTM). To prevent mixing with feeding, samples were taken at least 30 minutes after feeding. Sampling was repeated in case of macroscopic contamination with milk or other parts of food.

If at least 0.5 ml of raw saliva could not be collected on the first sampling attempt with the bulb pipette, we waited 1-5 minutes for more saliva to be produced or we stimulated saliva production with the infant's pacifier. If at least 0.6 ml of saliva was collected, 0.1 ml was transferred to a tube UTM for sequencing purposes. If possible, an additional saliva sample was collected with the ORACOL swab also for sequencing purposes.

Saliva samples and NPS were stored at room temperature until frozen at -80°C within 5 hours of sample collection.

Burden assessment of sample collection

Burden assessment was conducted by a trained member of the study team during sample collection in the order as described above (bulb pipette, ORAOCOL, NPS). Discomfort for all procedures was assessed in all infants by measuring cry duration (seconds), the Face, Legs, Activity, Cry and Consolability (FLACC) score (score 0-10), and the Oucher pain scale. The FLACC Scale incorporates five areas: facial expression; leg movement; activity; cry; and consolability, each scored with 0 to 2 points. OUCHER is a visual analogue scale comprising six standardised photographs of a child's facial expression or a numerical scale ranging from 0-10. We also scored video recordings of at least 20 participants by a trained researcher blinded to the procedure.

RNA-extraction-free detection of RSV in saliva

Saliva samples were thawed on ice. For samples collected using the ORACOL device, saliva was eluted following manufacturer's instructions. Saliva samples were then processed by the standard SalivaDirect workflow.[10-11] Briefly, proteinase K was added to 50 µl of saliva, mixed thoroughly by vortexing, then incubated for 5 minutes at 95°C to inactivate the proteinase K, before being tested in multiplex RT-qPCR for RSV-specific gene targets.

Whole genome sequencing of RSV-positive samples

From RSV-positive infants, a subset of NPS and saliva samples were sequenced by NextSeq500 as previously described.[12] An additional subset of NPS and saliva samples were also sequenced using Minion sequencing to compare accuracy and costs between both techniques for future large-scale use.[13] Rates of successful sequencing as well as nucleotide order were compared for saliva versus NPS.

Clinical data

A questionnaire capturing clinical and demographic data was filled out by the study team after sample collection. The following variables were collected: age (months), sex (male/female), symptom onset (days), hospital admission (date), length-of-stay (LOS; days), location of recruitment (PICU/ward/Emergency Department), ventilation (yes/no), other children in household <6 years (yes/no), gestational age (weeks), breastfeeding (yes/no), daycare attendance (yes/no), smoking in the household (yes/no), and allergies parents (yes/no).

Statistical analysis

The primary goal of this study was to compare the sensitivity of saliva to NPS. The predefined sample size of 100 infants was based on the expectation that 45 infants would be RSV-positive. Descriptive statistics were performed in SPSS (version 25.0.0.02). An estimate was considered statistically significant at $p<0.05$.

RESULTS

Study population

Since September 2021, 69 infants have been enrolled in the study. Median age was 2 months [IQR 1-4] and 63.8% were male. Of these, 47 (68.1%) tested positive for RSV. Only infants which were originally diagnosed with NPS through routine hospital diagsosis were included in the study. No infants were excluded.

RSV detection in NPS vs. saliva

To date, 31 sample sets have been shipped to the Yale School of Public Health for analysis of which 22 (71.0%) samples had adequate saliva volume for testing. Of those 22 saliva samples, 14 (63.6%) tested positive for RSV, despite all 22 infants receiving a diagnosis of RSV when their NPS was tested at time of hospital admission. In all 22 cases, a paired NPS was collected at the same time as the saliva sample and tested with the study protocol. Of those, only 17 (72.7%) tested positive for RSV. Additionally, of the 9 saliva samples that were of inadequate volume for testing, 2 (22.2%) of their paired NPS tested negative for RSV, despite having tested positive upon hospital admission.

Assessing burden of NPS and saliva collection

We evaluated the discomfort caused by the collection of NPS (n=66) and saliva (n=68) samples (Table). Discomfort measures, including cry duration, OUCHER pain score and FLACC score, were significantly higher for the collection of NPS as compared to saliva ($p<0.0001$). When comparing the two saliva collection devices, the ORACOL device showed less discomfort compared to the pipette method although this was not significant ($p=0.06$).

Table: Discomfort of NPS and saliva collection in 69 infants with medically-attended respiratory infection

Study Specimens	N	Cry duration in seconds (mean, SD)	OUCHER pain score range 0-10 (mean, SD)	FLACC score range 0-10 (mean, SD)
NP Swab	66	8 (11.3)	5.7 (3.2)	4.8 (3.6)
Saliva pipette	68	4 (11.1)	3.0 (2.5)	2.8 (2.7)
Saliva ORACOL	68	1 (2.9)	2.5 (2.6)	2.1 (2.7)

DISCUSSION

This chapter presents the preliminary data of the FRIENDS study. In this study, we evaluated the sensitivity of saliva as a sample type for the detection of RSV in infants hospitalised with MARI. As compared to diagnosis using a NPS at time of hospital admission, we noted a modest reduction in the diagnostic sensitivity of saliva collected within 72 hours of hospital admission. However, we also observed a decrease in the number of infants testing positive for RSV with a subsequent NPS collected during the same period. Recognising that preliminary numbers are low an expanded investigation into this is warranted; recent evidence has demonstrated that saliva may even be more sensitive for RSV detection than NPS.[7] Moreover, we observed a significantly reduced sampling burden for saliva versus NPS. Combined with its non-invasive collection and low-resource requirement relative to swabbing, saliva holds potential as an alternative sample type for large-scale surveillance studies. When coupled with a streamlined, RNA-extraction free PCR test, testing becomes even more efficient (the

turnaround time for the SalivaDirect test can be anywhere from *4 to 24 hours after lab receipt of samples* 4 hours) and relatively low-cost ($1.21-$4.39/sample in reagent costs).[10]

A challenge that has limited saliva collection from infants is the minimal saliva volume generally available from young infants. Moreover, there are no established standards for this. Most kits and diagnostic tests recommend to collect ≥1 mL saliva while on average, it is only possible to collect ≤50 μL of saliva from neonates at each sampling event. Additionally, saliva is virtually absent in sedated, intubated patients. This presents an additional challenge for burden assessment, as it results in an absence of most measurable clinical parameters. In an effort to remedy this, our clinical study team has started to measure changes in heart rate and blood pressure, and plans to implement a learning review of research assistants to ensure sufficient training has been received to properly interpret burden assessment scores. Owing to the limited recommendations available, in this first study to explore the detection of RSV in clinial saliva samples from infants with MARI, we evaluated different saliva collection methods. Results to date indicate that the bulb pipette as a viable option for collecting a sufficient sample volume and for sensitive virus detection, probably because it collects raw – thus undiluted - saliva. One limitation of this approach however, was the time this required to achieve the sufficient volume. The ORACOL device differs in its collection approach, being sponge-based. While we observed less collection discomfort using the ORACOL, this wasn't significantly different to that of the bulb pipette and once transported to the lab, removal of saliva from the sponge proved difficult in incidences of low sample volume. Further work in this study will evaluate the Saletto collection device,[14] which while also sponge-based, it has a colour change indication technology alerting the user when enough volume of sample has been collected. While it has already been validated for children, we will be the first to evaluate its use in infants.

We are also working to evaluate whole-genome sequencing of RSV from paired saliva and NPS samples. The ability to sequence RSV from different specimen types can further remove the dependence on NPS, thereby improving surveillance capacity, resulting in an increased number of full genomes available for monitoring seasonal epidemics. Moreover, since saliva may not only be a promising sample type for RSV diagnosis but also for other respiratory viruses,[8] we will also explore saliva samples collected in this study for the detection of non-RSV respiratory viruses (data not included in this chapter).

Our preliminary findings provide support for the potential of saliva specimens in the diagnosis of RSV infection. The evidence for saliva as a reliable and cost-effective sample type for SARS-CoV-2 diagnostics and surveillance is strong and continues to grow. With further validation for RSV, widespread implementation of saliva sampling could also transform RSV diagnosis and surveillance in infants. Saliva testing could also be proposed as a potential method to monitor vaccine and mAb effectiveness. A less resource-intensive sample type could also reduce the coverage gap between lower- and higher-resource countries. LMICs face many barriers in RSV testing and substantial inequities in global access to vaccines/

mAbs. Testing saliva reduces the reliance on swabs and the necessary specialized infrastructure and resources necessary for this; saliva can overcome the lack of trained technicians to perform PCR and healthcare personnel to collect NPS in LMIC settings, making it a more sustainable option in many settings.[15] Additional applications of saliva may include microbiome, antibody and metabolic testing. Looking to the future, the potential applications of saliva range from clinical diagnostics to post-vaccine and mAb disease burden and immunity surveillance.

DECLARATIONS

Conflict of Interest

LJB has regular interaction with pharmaceutical and other industrial partners. He has not received personal fees or other personal benefits. University Medical Centre Utrecht (UMCU) has received major funding (>€100,000 per industrial partner) for investigator-initiated studies from AbbVie, MedImmune, AstraZeneca, Sanofi, Janssen, Pfizer, MSD and MeMed Diagnostics. UMCU has received major funding for the RSV GOLD study from the Bill and Melinda Gates Foundation. UMCU has received major funding as part of the public–private partnership Innovative Medicines Initiative (IMI)-funded RESCEU and PROMISE projects with partners GSK, Novavax, Janssen, AstraZeneca, Pfizer and Sanofi. UMCU has received major funding from Julius Clinical for participating in clinical studies sponsored by MedImmune and Pfizer. UMCU received minor funding (€1,000–25,000 per industrial partner) for consultation and invited lectures by AbbVie, MedImmune, Ablynx, Bavaria Nordic, MabXience, GSK, Novavax, Pfizer, Moderna, AstraZeneca, MSD, Sanofi and Janssen. LJB is the founding chairman of the ReSViNET Foundation. Since April 1, 2021, LJB has been given a new position as medical scientific division manager of the Children's Division of the Wilhelmina Children's Hospital in Utrecht. The other authors do not report any conflict of interest. ALW has received consulting and/or advisory board fees from Pfizer, Merck, Diasorin, PPS Health, Co-Diagnostics, and Global Diagnostic Systems for work unrelated to this project, and is Principal Investigator on research grants with Pfizer, Merck, Flambeau Diagnostics, Tempus Labs, and The Rockefeller Foundation to Yale University. DMW has received consulting fees from Pfizer, Merck, GSK, Affinivax, and Matrivax for work unrelated to this project and is Principal Investigator on research grants and contracts with Pfizer and Merck to Yale University.

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Authors' Contributions

ACL, DMW, ALW and LJB had the idea and initiated the study. ACL and ALW wrote the study protocols. ACL, OMA, MvW, HvZ and JGW managed the study and collected the data. ACL, OMA, RJL and DY-C were responsible for and performed the assays. ACL, OMA and RJL analysed and interpreted the data. ACL and ALW drafted the manuscript. All authors amended and commented on the final manuscript.

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Chapter 12

Natural Variability of TRAIL, IP-10, and CRP in Healthy Adults

"HERACLES"

Submitted Manuscript

"We don't make mistakes, just happy accidents."
— Bob Ross, painter

Natural Variability of TRAIL, IP-10, and CRP in Healthy Adults – the "HERACLES" study

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ABSTRACT

A novel host-protein score (called MMBV) helps to distinguish bacterial from viral infection by combining the blood concentrations of three biomarkers: tumour necrosis factor related apoptosis inducing ligand (TRAIL), interferon gamma induced protein 10 (IP-10), and C-reactive protein (CRP). These host biomarkers are differentially expressed in response to bacterial versus viral acute infection. We conducted a prospective study, with a time series design, in healthy adult volunteers in the Netherlands. The aim was to determine the variability, of TRAIL, IP-10, and CRP and the MMBV score in healthy adults across time. Up to six blood samples were taken from each healthy volunteer over a period of up to four weeks. In 77 healthy participants without recent or current symptoms, MMBV scores were bacterial in 1.3% and viral (or other non-infectious etiology) in 93.5% of participants. There was little variation in the mean concentrations of TRAIL (74.5 pg/ml), IP-10 (113.6 pg/ml) and CRP (1.90 mg/L) as well as the MMBV score. The variability of biomarker measurement was comparable to the precision of the measurement platform for TRAIL, IP-10 and CRP. Our findings establish the mean values of these biomarkers and MMBV in healthy individuals and indicate little variability between and within individuals over time, supporting the potential utility of this novel diagnostic to detect infection-induced changes.

INTRODUCTION

A novel host-response based score was previously shown to differentiate between bacterial and viral infections in children with respiratory tract infections (RTI) and fever without source[1,2] and in adults with lower respiratory tract infections[3]. This blood-based score (MeMed BV®, MMBV) integrates concentrations of three host biomarkers: tumour necrosis factor related apoptosis inducing ligand (TRAIL), interferon gamma induced protein 10 (IP-10), and c-reactive protein (CRP). While CRP has been extensively studied as a biomarker that is induced in bacterial infections in febrile patients, the evidence relating to TRAIL expression and infection is relatively new[4]. Its concentration increases in viral infection and decreases in bacterial infection[2]. New host-proteins that are up-regulated in viral infections may be an innovative complement to bacterially induced proteins in current clinical use[1]. The expression dynamics of individual biomarkers in healthy subjects have not been reported. In the present study we examined the natural variability of TRAIL, IP-10, and CRP expressed in the healthy individual across time.

The Hospital Employees Response Ante COVID-19 Listed Early Symptoms (HERACLES) study aimed at detecting viral infections including COVID-19 during the pre-symptomatic phase. However, none of the study participants were detectably infected with any respiratory viruses during the study period. The lack of infections enabled the present study, where we determined the natural variability of TRAIL, IP-10, and CRP in healthy adults across time.

METHODS

Study design and participants

All adult hospital staff employed at the Wilhelmina Children's Hospital with high exposure to COVID-19 were invited to participate in the study. Employees who considered themselves to have any contact with patients in relation to their work were defined as having high exposure. Invitations for participation in the study were posted in newsletters and sent out individually to all employees.

Participants were excluded in case of a previous episode of acute RTI in the past two weeks or at time of enrolment. Other exclusion criteria were: previously proven COVID-19 infection, a proven or suspected HIV, HBV, or HCV infection, active malignancy, current treatment with immune-suppressive or immune-modulation therapies, and severe illnesses that affect life expectancy and quality of life (other than suspected COVID-19 infection).

Sample collection

Multiple blood samples were collected prospectively from subjects over a period of up to four weeks during the first COVID-19 wave (Figure S1). Blood was collected by trained study

personal up to six times within the study period and/or when respiratory symptoms occurred (Figure S2). When respiratory symptoms were reported by the participant to the study team, additional blood samples and respiratory samples were collected on three subsequent days after onset of symptoms. During every blood draw, a serum sample and an RNA sample were collected which were stored at -80C and -20C, respectively. Respiratory samples (nasopharyngeal and oropharyngeal) were collected and placed in Universal Transport Medium (UTM) when participants showed symptoms. Respiratory samples were taken using flocked swabs by qualified staff members and were stored at -80C until analysis. PCR analysis for 18 respiratory viruses (adenovirus, bocavirus, coronavirus (229E, HKU1, NL63, OC43 and SARS-CoV2), human metapneumovirus (hMPV), influenza virus type A, influenza virus A(H1N1)pdm09, influenza virus type B (influenza virus), parainfluenza virus types 1 through 4 (PIV1-4), RSV types A and B (RSV), rhinovirus and enterovirus) was performed for all symptomatic participants[5].

Study procedure

Study visits took place six times for a period up to four weeks with a minimum of 48 hours and a maximum of 7 days between blood draws (Figure S3). At 21 days after the sixth sample, a seventh sample was taken for serologic assessment. Nasal congestion, defined as the blockage of nasal passages, was examined by asking the participants to close one nostril and breath through the other, and vice versa.

At enrolment, data on demographics, medical history, medication and possible COVID-19 exposure were collected through a questionnaire. When symptoms were notified during one of the visits, physical examination took place including measuring temperature, heart rate, respiratory rate, and saturation. In addition, a respiratory sample was collected. Symptomatic subjects were followed for three subsequent days taking blood samples and respiratory samples. After the third visit, participants were excluded from further participation. Symptomatic participants could be sampled at home according to the hospital policy at that moment.

Blood samples of all participants were also tested for SARS-CoV-2 IgG antibodies by the Afinity IgG ELISA. Samples with IgG ratios >1.4 were considered to be positive. Two samples from two individuals after a proven COVID-19 infection served as controls in the serology analysis.

For this study of biomarker variability in healthy subjects, subjects were excluded if there was a suspicion of infection, as indicated by one or more of the following: positive serology results, clinical symptoms, or a change in MMBV score across the time course.

MeMed BV® (MMBV, MeMed, Israel) tests were conducted using blood samples of healthy participants. The tests were run on MeMed Key® (MeMed, Israel) a multi-purpose immunoassay analyser for quantitative diagnostic immunoassays that provides MMBV results in 15 minutes, and is therefore designed for on-site diagnostics.

Statistical analysis

Two to three MMBV measurements were performed per time point and the average was employed to calculate the coefficient of variation (CV) for each subject across their time course. Note that the lowest measurement for a biomarker is the value of the limit of quantitation, LoQ, which were established in Hainrichson et al. 2022 as follows: 15 pg/ml for TRAIL, 100 pg/ml for IP-10 and 1 mg/L for CRP[6]. When comparing natural variability to the precision of MeMed Key, the precision for measurements close to the LoQ was employed for IP-10 and CRP.

Ethical considerations

This study was approved by the Medical Research Ethics Committee of the UMC Utrecht (IRB number 20-206/D). Informed consent procedures followed in compliance with UMC Utrecht guidelines. Virologic and serologic results were shared with participants after complete analysis.

RESULTS

Clinical results

Between April 14 and May 22, 2020, 294 healthcare workers (HCWs) were enrolled in the study of which 291 (99%) participants provided a first blood sample (Figure S2 & S3). A total of 286 (98%) participants completed the final study visit. The median age was 44 years (range 18-65) and almost all (92.4%) participants were female (Table S1). Out of 286 participants, 9 had possible serology findings and 17 had clinical symptoms; the remaining 260 were considered as healthy subjects.

Healthy subjects

Here we present the natural variability of infection biomarkers in 77 healthy subjects. Samples from every enrolled male (n=22) and a randomly selected subset of females (n=55) were measured across all time points (3-6). In healthy individuals without current or recent symptoms 93.5% of MMBV results were within the “viral (or other non-infectious)” range (Figure S4).

In these 77 healthy participants without any symptoms during sample collection, mean biomarker concentrations for TRAIL, IP-10, and CRP were 74.5 pg/ml (standard deviation (SD) 18.3), 113.6 pg/ml (SD 27.0) and 1.90 mg/L (SD 2.3), respectively (Figure 1). Healthy biomarker variability across time was not significantly different from the precision of the measurement platform[6] (Figure S5.)

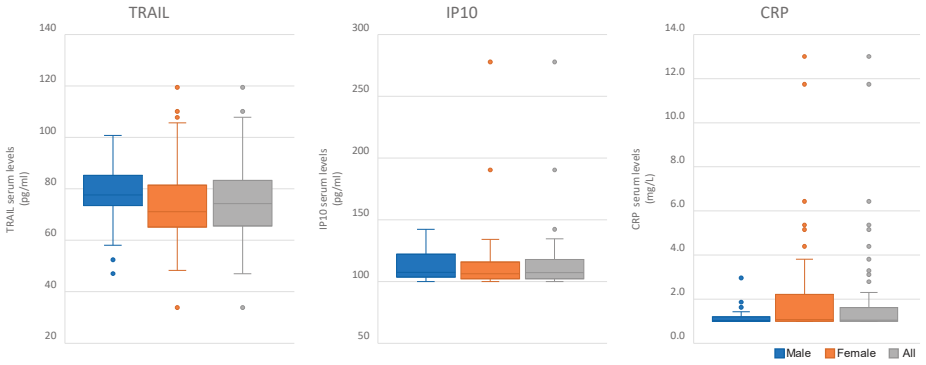

Figure 1. Healthy biomarker concentrations in male (n=22), female (n=55) and all participants with sequential MMBV measurements (n=77). Each data point is the average of a subject's time course.

Symptomatic subjects

Of the 286 HCWs who completed the study, 17 (5.9%) developed RTI symptoms during the study period. RTI episodes in all participants were characterised by mild symptoms, including a runny/blocked nose, minimal coughing, sore throat, headache, muscle pain, or fatigue. Temperature was marginally increased in two participants (38.0°C and 38.1°C). All symptomatic participants were negative for 18 respiratory viruses tested for by PCR, including SARS-CoV-2. Of 17 symptomatic participants, 8 had been diagnosed with hay fever or allergies previously. All seven blood samples from two of the HCWs showed SARS-CoV-2 antibodies, indicating that they entered the study with antibodies.

DISCUSSION

We conducted a prospective study over a two-month period in HCWs in a children's hospital in the Netherlands. With the HERACLES study, we present a baseline of host biomarker dynamics in 77 healthy adults. Our findings provide mean values of TRAIL, IP-10 and CRP and the MMBV score in healthy individuals and indicate little variability between and within individuals over time. This finding supports the potential utility of this novel diagnostic in detecting acute infection-induced changes.

To the best of our knowledge, this is the first study examining inflammatory host biomarkers in healthy adults. There are some limitations to our study. Although we received ethical approval within a short time (few days), the study started after the peak of the first wave in the Netherlands (Figure S2). This could explain why we did not detect any PCR-confirmed SARS-CoV-2 infection in the HCWs. Although this clinical study did not achieve its objective of capturing the dynamic expression of the biomarkers during natural SARS-CoV-2 infection, we consider the dynamics of the biomarkers in healthy individuals to be a valuable

baseline finding. A strength of the HERACLES study is that 291 HCWs were successfully recruited at a challenging time for global healthcare. The low SARS-CoV-2 incidence among the HCWs at a pediatric facility could reflect its low incidence among children. Other studies have reported similar infection rates[7].

In conclusion, the dynamics of novel and traditional host proteins in a large sample size of healthy subjects contributes to our understanding of the healthy baseline of these host biomarkers. A translational benefit of this finding is that these biomarkers may serve to detect early infection with viruses such as RSV and SARS-CoV-2. Future challenge studies are warranted to explore this further.

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CONTRIBUTORS

ACL, KOK, TG, and LJB contributed to the study design. ACL and EH contributed to data collection. ACL, TG, and LJB contributed to the data analysis. All authors discussed the results and contributed to the revision of the final manuscript.

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SUPPLEMENTARY MATERIAL

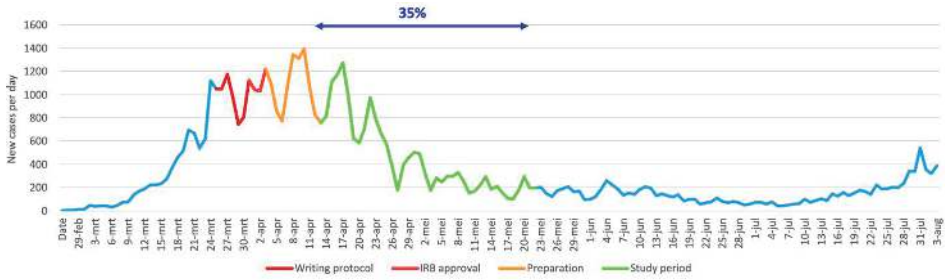

Figure S1. HERACLES study period as part of the first wave of COVID-19 in the Netherlands. The blue line is based on national data by the National Institute for Public Health and Environment.

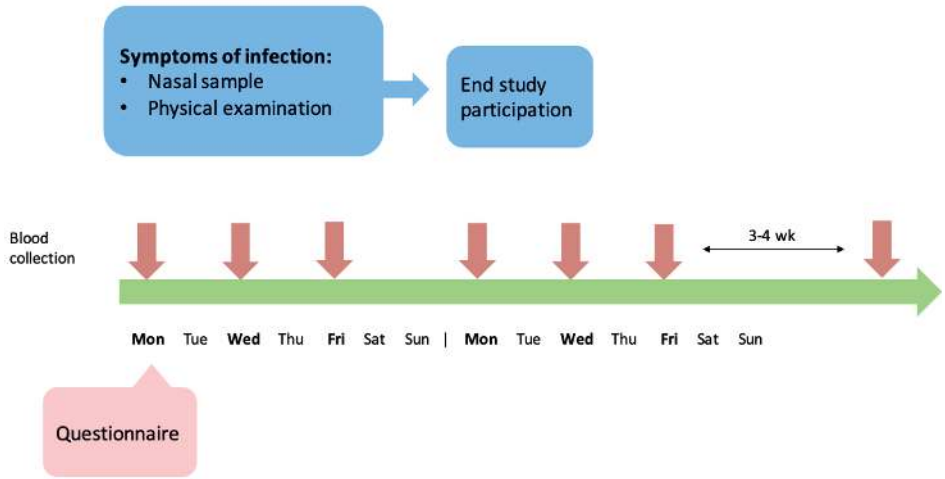

Figure S2. Example of sample collection, adjusted to work schedule of the healthcare worker.

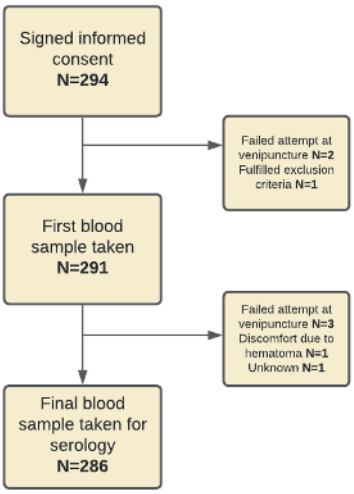

Figure S3. Study recruitment.

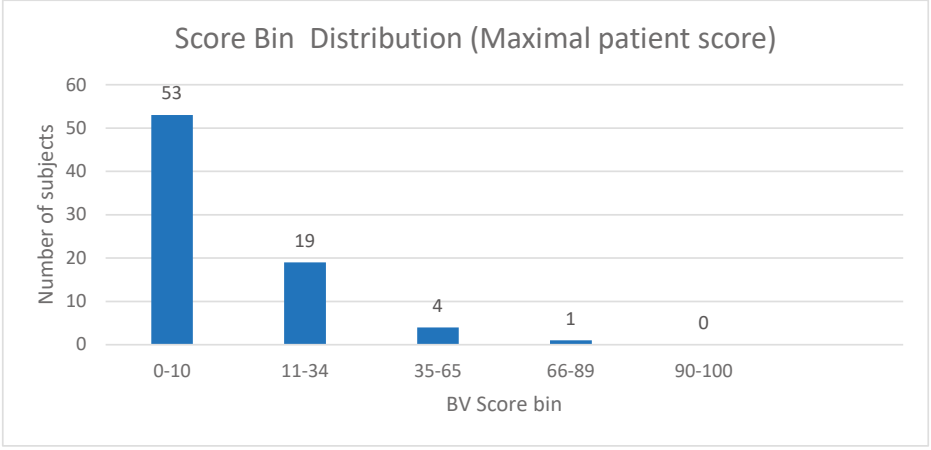

Figure S4. Healthy subject MMBV score distribution. Score = computational integration of TRAIL, IP-10 and CRP using proprietary algorithm. Each data point is the maximal score for a subject across their time course. Scores ranging 0-34 indicate a viral infection (or other non-bacterial etiology), 35-65 are equivocal and 66-100 indicate a bacterial infection (or co-infection)

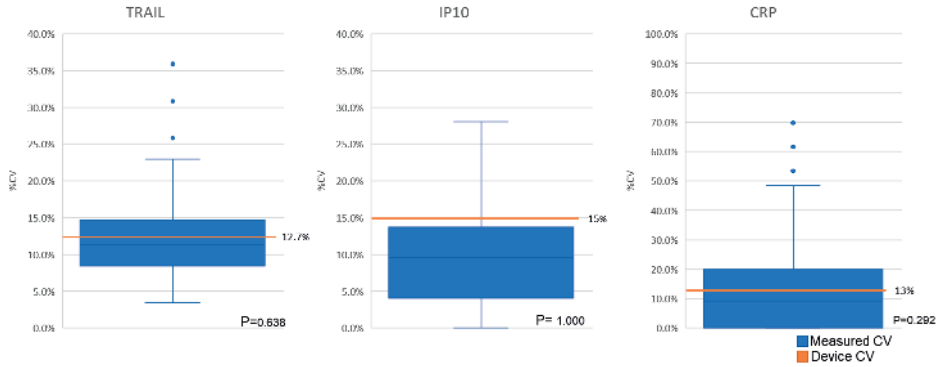

Figure S5. The coefficient of variance (CV) of biomarker concentrations in healthy participants (n=77). The orange line represents previously reported measurement platform precision. Each data point is the CV of a subject's time course. p values denote the results of a statistical test comparing the mean CV in this study to the measurement platform CV.

Table S1. Baseline characteristics of healthcare workers

	Participants (N=291)
Sex	
Female	269 (92.4%)
Male	22 (7.6%)
Age, years (median, range)	44 (18-65)
Profession	
Nurse	166 (57.0%)
Assistant nurse	38 (13.1%)
Physician	29 (10.0%)
Midwife	20 (6.9%)
Administrative staff	12 (4.1%)
Nursing specialist / Physician assistant	8 (2.7%)
Other	18 (6.2%)
Employment	
Fulltime	91 (31.3%)
Parttime	200 (68.7%)
Hay fever	
Yes	100 (34.4%)
No	191 (65.6%)
Travel outside the Netherlands during past month	
Yes	7 (2.4%)
Germany	1 (14.3%)
Spain	1 (14.3%)
Nepal	1 (14.3%)
South Africa	1 (14.3%)
Austria	1 (14.3%)
Servia	1 (14.3%)
No	284 (97.6%)
Environmental exposure	
Yes	12 (4.1%)
Proven COVID-19	1 (8.3%)
Not tested for COVID-19	11 (91.7%)
No	279 (95.9%)

Chapter 13

Clinical and Viral Factors Associated With Disease Severity and Subsequent Wheezing in Infants With Respiratory Syncytial Virus Infection

"RESCEU"

Journal of Infectious Diseases, 2022

"It had long since come to my attention that people of accomplishment rarely sat back and let things happen to them. They went out and happened to things."
— Leonardo da Vinci, architect

Clinical and Viral Factors Associated With Disease Severity and Subsequent Wheezing in Infants With Respiratory Syncytial Virus Infection

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Respiratory syncytial virus (RSV) causes substantial morbidity and mortality in infants and young children worldwide. Here we evaluated host demographic and viral factors associated with RSV disease severity in 325 RSV-infected infants under 1 year of age from 3 European countries during 2017–2020. Younger infants had a higher clinical severity (ReSViNET) score and were more likely to require hospitalization, intensive care, respiratory support, and/or mechanical ventilation than older infants (,3 months vs 3 to ,6 months and 3 to ,6 months vs ≥6 months). Older age (≥6 months vs ,3 months), higher viral load, and RSV-A were associated with a greater probability of fever. RSV-A and RSV-B caused similar disease severity and had similar viral dynamics. Infants with a more severe RSV infection, demonstrated by having a higher ReSViNET score, fever, and requiring hospitalization and intensive care, were more likely to have developed subsequent wheezing at 1 year of age.
Clinical Trials Registration. NCT03756766.

Keywords. respiratory syncytial virus; disease severity; wheezing; subgroup; viral load.

Human respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection (LRTI) in infants and young children. The annual RSV epidemic is responsible for an estimated 33 million episodes of LRTI, 3 million hospitalizations, and up to 75 000 in-hospital deaths globally in children under 5 years of age [1]. Infants under 3 months of age, those born before 30 weeks' gestation, and those with cardiopulmonary disease are at higher risk of severe RSV infection than healthy term infants [2].Despite the impact of the disease, there is no licensed vaccine or effective treatment for RSV disease, and the standard of care remains supportive management of respiratory compromise.

RSV is classified into 2 subgroups, A and B. Both subgroups cocirculate during annual epidemics with alternating patterns of subgroup dominance [3]. The majority of studies have shown that RSV-A causes more severe infection than RSV-B [4, 5], although a few studies have shown the opposite [6] or similar disease severity with both subgroups [7, 8]. Similarly, there is conflicting evidence on the association between RSV viral load and disease severity [9–12].

There is evidence of the association between RSV-associated LRTI in early childhood and the development of recurrent wheeze and asthma in later childhood up to early adulthood [13–16]. A double-blind, placebo-controlled, randomized trial showed that prophylaxis with palivizumab (an anti-RSV monoclonal antibody) in otherwise healthy preterm infants born at 33–35 weeks' gestation reduced the number of days of recurrent wheeze in the first year of life [17]. A follow-up study of the same cohort, however, did not find a significant difference in the incidence of asthma nor any changes in lung function at 6 years of age [18]. Another double-blind, placebo-controlled, randomized trial showed no effect of prophylaxis with motavizumab (another anti-RSV monoclonal antibody) in healthy term infants on rates of wheeze during the first 3 years of life [19]. Further studies are required to establish the causal link between RSV infection and subsequent wheeze and asthma, and the potential of preventative or therapeutic interventions [20].

In this study, we prospectively enrolled RSV-infected infants in the Netherlands, Spain, and the United Kingdom between 2017 and 2020. We sought to explore the host demographic and viral factors associated with the clinical characteristics of RSV infection and subsequent wheeze. In doing so, we could advance our understanding of RSV infection and help identify target populations where trials of therapeutic and preventative measures could be directed.

METHODS

Study Design and Clinical Data Collection

Infants with primary RSV infection under 1 year of age were prospectively enrolled in a clinical study from communities and hospitals in the Netherlands, Spain, and the United Kingdom during the 2017–2020 RSV seasons (from October to April). This study is 1 of 4 clinical

studies in the Respiratory Syncytial Virus Consortium in Europe (RESCEU) project and has been described in detail previously (ClinicalTrials.gov identifier: NCT03756766) [21]. Infants who were previously healthy and those with preexisting medical conditions (including prematurity, defined as being born before 37 weeks' gestation) were eligible for inclusion in this study. The goal was to recruit 500 previously healthy infants and 50 infants with preexisting medical conditions. Exclusion criteria included infants who had received antiviral medication to treat RSV infection (eg, ribavirin), human immunoglobulin, or monoclonal antibodies (including palivizumab), infants who had been exposed to an RSV investigational vaccine or medication, and infants who had received steroids or montelukast within 7 days of enrolment in the study. These medications may affect the symptom presentation, so infants who had received any of them were excluded from this study. RSV infection was confirmed using point-of-care testing on the Alere i RSV assay (Abbott) in a community setting or by routine tests (eg, rapid antigen detection or polymerase chain reaction [PCR]) at a central laboratory in a hospital setting.

A nasopharyngeal swab was collected from each participant within 96 hours of symptom onset or 48 hours of admission to the hospital. In addition, hospitalized participants had daily nasopharyngeal swabs collected, where possible, until hospital discharge. Swabs were immersed in M4RT transport medium after collection, aliquoted, and frozen at −80°C until use.

Demographic and clinical information was gathered through initial screening, medical record review, and a 14-day online diary completed from the time of enrolment by the parents. Clinical severity was evaluated using the following criteria: (1) the ReSViNET score [22]; (2) presence of fever; and requirement for (3) hospitalization, (4) intensive care (ie, admission to a high dependency unit or an intensive care unit), (5) respiratory support, and/or (6) invasive mechanical ventilation. The ReSViNET score takes 7 clinical parameters into account, including feeding intolerance, medical intervention, respiratory difficulty, respiratory frequency, apnea, general condition, and fever. Total scores range from 0 to 20, with higher scores indicating more severe disease. Fever was defined as at least 1 episode of a rectal or tympanic temperature of 38°C or above during the acute course of the infection.

Additional clinical information was gathered using parental questionnaires when the participants were 1 year of age. This included the occurrence of subsequent wheezing and other respiratory symptoms during the period between the RSV infection and the first birthday.

The study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by the relevant ethics committees at each site: the Medical Ethical Committee, University Medical Center Utrecht (No. 17/563) in the Netherlands; Comité de Ética de la Investigación de Santiago-Lugo (No. 2017/395) in Spain; and the Health Research Authority (No. 231136) and South Central and Hampshire A Research Ethics Committee (No. 17/SC/0522) in the United Kingdom. The parents or guardians of all participants provided written, informed consent.

Viral Load Measurement and Typing

Viral load and RSV subgroup were determined by quantitative reverse transcription PCR (RT-qPCR). RT-qPCR was performed at GlaxoSmithKline (protocol proprietary). The primers of this duplex RT-qPCR assay targeted the N gene for both RSV-A and RSV-B. The limit of detection was 304 copies/ mL for RSV-A and 475 copies/mL for RSV-B.

Samples with undetectable viral load were removed from analyses. To compare viral load data between participants, either initial or peak viral load was used. For participants with serial swabs collected, initial viral load was defined as the viral load of the first collected sample with detectable viral load, and peak viral load was defined as the maximum viral load detected among all available samples. For participants who had only 1 viral load data point, it was used to represent both initial and peak viral load. When there was not enough sample for RT-qPCR or the viral load was below the limit of detection, subgroup information was gathered from a previously described viral whole-genome sequencing study in the same cohort [23].

Statistical Analyses

Pearson correlation analyses were used to evaluate the correlation between variables. Mann-Whitney *U* or Kruskal-Wallis tests were used to compare continuous variables between groups. $\chi 2$ tests with Yates correction were employed for contingency analyses, and Fisher exact tests were used when the expected value for a cell was less than 5. The rate of viral load change was determined by linear regression of viral load on the days after symptom onset, where samples with undetectable viral load were removed from the regression analysis.

When comparing clinical outcome variables between different groups of participants,multiple linear regression (for continuous outcome variables),multiple logistic regression (for dichotomous outcome variables), and proportional odds ordered logistic regression (for ordered outcome variables) were used to adjust for covariates (eg, participant age, gestational age, sex, etc.). Likelihood ratio tests were used to evaluate the effect of age or country on the goodness of fit of models including other covariates. A post hoc adjustment for multiple comparisons with the Benjamini-Hochberg procedure was applied to determine false discovery rate-corrected *Q* values in all these comparisons of clinical outcomes between participants. Analysis of covariance (ANCOVA) tests were performed to compare the rates of viral load decline between different groups of participants.

All statistical analyses were performed using R (version 4.1.1) [24]. A *P* value, or *Q* value in the case of multiple comparisons, of less than .05 was considered to indicate statistical significance.

RESULTS

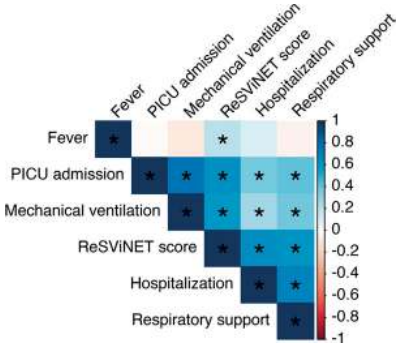
Study Population

A total of 325 RSV-infected infants were enrolled from the Netherlands, Spain, and the United Kingdom during 2017–2020 (Supplementary Table 1). Infants enrolled in the Netherlands were younger and had a higher proportion requiring intensive care and mechanical ventilation than those enrolled in the other 2 countries (median age, 2.5 months; interquartile range [IQR], 1.4–5.7months vs 3.5 months; IQR, 1.7–7.5 months; intensive care, 42% vs 16%; mechanical ventilation, 44% vs 12%; Supplementary Table 2).

Excluding 5 infants without available age information, the median age of the 320 infants was 3.0 months (IQR, 1.5–6.6 months). Females accounted for 42% of the infants. Eight percent of the infants were born preterm; 31% of these preterm infants were born before 32 weeks' gestation, all of whom were enrolled in the United Kingdom. Of the 325 infants, 44 (14%) had comorbidities, including prematurity with or without bronchopulmonary dysplasia, ventricular septal defect, congenital hypothyroidism, wheeze, or other congenital abnormalities. The demographic characteristics of these infants are shown in Table 1 and Supplementary Table 2.

The associations between disease severity and demographic features (age, gestational age, sex, comorbidity) were evaluated using different clinical outcomes, including ReSVi-NET score, presence of fever, and the need for hospitalization, intensive care, respiratory support, or mechanical ventilation, after adjusting for these demographic variables and RSV subgroup. These clinical outcomes were positively correlated with each other, except fever (Figure 1). While older infants had a lower ReSViNET score and were less likely to require hospitalization, intensive care, respiratory support, and/or mechanical ventilation, they were more likely to have fever than younger infants (Table 1).

Figure 1. Correlations between clinical outcome variables.



Pearson correlation analyses were used to evaluate the correlations between these variables. Pearson correlation coefficients for these correlations are coded according to the color scale. Significant correlations are marked with stars. All significant correlations had a *P* value of ,.001. The ReSViNET score accounts for 7 clinical variables: feeding intolerance, medical intervention, respiratory difficulty, respiratory frequency, apnea, general condition, and fever. Abbreviation: PICU, pediatric intensive care unit.

Table 1. Characteristics of the RSV-Infected Infants, Stratified by Age Group (n=320)[a]

Characteristic	<3 Months (n=160)	3 to <6 Months (n=63)	≥6 Months (n=97)	*P* Value	*Q* Value
Demographic features					
Gestational age, wk					
Median (IQR)	39.0 (38.0–40.1)	39.6 (38.1–40.3)	39.9 (38.8–40.7)	.012	
Distribution				.990	
<32	4/160 (3)	1/62 (2)	3/96 (3)		
32 to <37	9/160 (6)	3/62 (5)	6/96 (6)		
≥37	147/160 (92)	58/62 (94)	87/96 (91)		
Female sex	57 (36)	25 (40)	50 (52)	.041	
Weight, total No.	158	59	94		
Mean±SD, kg	4.5±1.0	6.7±0.9	8.4±1.3	2.7×10^{-49}	
Comorbidity	21 (13)	6 (10)	17 (18)	.338	
Virological features					
Subgroup A	73/147 (50)	35/61 (57)	41/78 (53)	.597[b]	
Peak viral load, total No.	137	56	77		
Mean±SD, $\log_{10}$ copies/mL	6.6±1.4	6.8±1.3	7.1±1.3	.109[c]	
Clinical features[d]					
ReSViNET score					
Mean±SD	10.3±4.6	8.9±4.7	7.0±4.2	2.1×10^{-8}	4.8×10^{-8}
Distribution				1.2×10^{-6}	1.6×10^{-6}
0–7	49/155 (32)	26/61 (43)	51/90 (57)		
8–13	62/155 (40)	23/61 (38)	31/90 (34)		
14–20	44/155 (28)	12/61 (20)	8/90 (9)		
Fever	34/156 (22)	21/61 (34)	44/90 (49)	5.2×10^{-4}	5.2×10^{-4}
Hospitalization	141/158 (89)	45/62 (73)	47/93 (51)	1.2×10^{-9}	4.3×10^{-9}
PICU admission	61/158 (39)	13/62 (21)	12/93 (13)	4.5×10^{-6}	5.3×10^{-6}
Respiratory support	126/148 (85)	37/55 (67)	34/81 (42)	6.5×10^{-13}	4.5×10^{-12}
Mechanical ventilation	55/148 (37)	10/55 (18)	5/81 (6)	5.6×10^{-7}	9.8×10^{-7}

Unless otherwise specified, data are shown as No. (%) or No./total No. (%) if there are missing data. Percentages may not total 100 due to rounding.

Abbreviations: IQR, interquartile range; PICU, pediatric intensive care unit; RSV, respiratory syncytial virus.

[a]Five infants without available age information were excluded from this table.

[b]Multiple logistic regression was used to adjust for sampling season. A likelihood-ratio test was used to assess the effect of age on the goodness of fit of the models.

[c]Multiple linear regression was used to adjust for days between symptom onset and sample collection. A likelihood-ratio test was used to assess the effect of age on the goodness of fit of the models.

[d]Multiple linear regression, ordered logistic regression, and multiple logistic regression were used to adjust for gestational age, sex, comorbidity, and viral subgroup when comparing different clinical features between the age groups. Likelihood-ratio tests were used to assess the effect of age on the goodness of fit of the models. Infants 3 to <6 months old had a similar rate of fever to that of infants ,3 months old and infants ≥6 months old. Infants ,3 months old and infants 3 to <6 months old had a similar distribution of the ReSViNET score. All other pairwise comparisons of the clinical features between the 3 age groups were significantly different.

Preterm and term infants did not show any significant difference in these clinical outcomes. Similarly, male and female infants had similar disease severity in all tested clinical outcomes. Infants with any preexisting medical condition had a higher ReSViNET score (mean, 12.0 [SD 4.8] vs 8.6 [SD 4.5]; Q= 0.044) and were more likely to require respiratory support (odds ratio [OR], 4.9; 95% confidence interval [CI], 1.5–19; Q=0.049) than those without comorbidity.

RSV Subgroup and Viral Load

Of the 292 infants for whom RSV subgroup information was available, 151 (52%) were infected with RSV-A, 140 (48%) with RSV-B, and 1 was coinfected with RSV-A and RSV-B. The subgroup information of 14 infants was gathered from sequencing results due to lack of RT-qPCR data. RSV-B was the predominant circulating subgroup during the 2017– 2018 RSV season, accounting for 67% (38/57) of the isolates, whereas RSV-A dominated the 2019–2020 season, accounting for 64% (79/123) of the infections. During the 2018–2019 season, RSV-A and RSV-B were cocirculating with similar prevalence. The incidences of the 2 subgroups in each country and season are shown in Table 2. RSV-A-infected and RSV-B-infected infants had similar demographic and clinical features in our dataset, except infants infected with RSV-A were more likely to have fever than those infected with RSV-B (OR, 1.8; 95% CI, 1.1–3.2; P=.029; Table 3). However, this difference was not significant after correction for multiple comparisons.

Table 2. Incidences of the 2 RSV Subgroups in Each Country and Each Season

Season and Country	RSV-A (n=151)	RSV-B (n=140)	Mixed (n=1)	Unknown[a] (n=33)
2017–2018	19	38	0	0
Netherlands	15	36	0	0
Spain	0	0	0	0
United Kingdom	4	2	0	0
2018–2019	53	58	1	11
Netherlands	13	23	0	4
Spain	6	4	0	6
United Kingdom	34	31	1	1
2019–2020	79	44	0	22
Netherlands	28	17	0	1
Spain	14	5	0	0
United Kingdom	37	22	0	21

[a]Samples from 25 participants were not tested by quantitative reverse transcription PCR.
Samples from 8 participants were tested, but viral load was under the limit of detection.

Table 3. Characteristics of the RSV-Infected Infants by RSV Subgroup

Characteristic	RSV-A (n=151)	RSV-B (n=140)	*P* Value	*Q* Value
Demographic features				
Age, mo				
Median (IQR)	3.1 (1.5–6.4)	2.8 (1.5–6.1)	.689	
Distribution, No./total No. (%)			.595	
<3	73/149 (49)	74/137 (54)		
3 to <6	35/149 (23)	26/137 (19)		
≥6	41/149 (28)	37/137 (27)		
Gestational age, wk				
Median (IQR)	39.4 (37.9–40.3)	39.3 (38.3–40.1)	.715	
Distribution, No./total No. (%)			.436	
<32	3/150 (2)	5/140 (4)		
32 to <37	11/150 (7)	6/140 (4)		
≥37	136/150 (91)	129/140 (92)		
Female sex, No. (%)	61 (40)	64 (46)	.426	
Weight, total No.	151	132		
Mean±SD, kg	6.1±1.9	6.0±2.0	.836	
Comorbidity, No. (%)	17 (11)	21 (15)	.440	
Virological feature				
Peak viral load, total No.	139	136		
Mean±SD, $\log_{10}$ copies/mL	6.9±1.3	6.7±1.4	.183[a]	
Clinical features[b]				
ReSViNET score				
Mean±SD	8.8±4.3	9.1±5.0	.730	0.851
Distribution, No./total No. (%)			.918	0.918
0–7	63/149 (42)	57/137 (42)		
8–13	60/149 (40)	49/137 (36)		
14–20	26/149 (17)	31/137 (23)		
Fever, No./total No. (%)	54/150 (36)	34/137 (25)	.029	0.203
Hospitalization, No. (%)	114 (75)	99 (71)	.151	0.352
PICU admission, No. (%)	34 (23)	44 (31)	.114	0.352
Respiratory support, No./total No. (%)	93/133 (70)	88/127 (69)	.366	0.512
Mechanical ventilation, No./total No. (%)	31/133 (23)	38/127 (30)	.325	0.512

Percentages may not total 100 due to rounding.

Abbreviations: IQR, interquartile range; PICU, pediatric intensive care unit; RSV, respiratory syncytial virus.

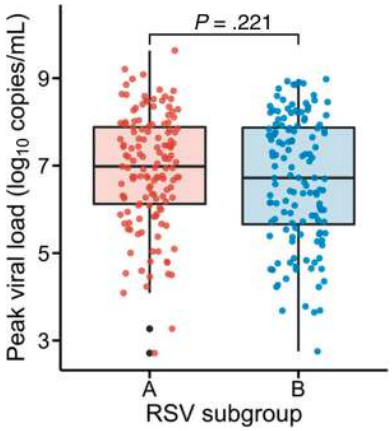
[a]Multiple linear regression was used to adjust for days between symptom onset and sample collection.

[b]Multiple linear regression, ordered logistic regression, and multiple logistic regression were used to adjust for age, gestational age, sex, and comorbidity when comparing different clinical features between the subgroups.

Nasopharyngeal viral load data were available for 278 infants. Among them, 77 had multiple days of viral load data during hospitalization (mean, 3.6 [SD 2.0] days). Combining all 483 viral load data points from the 278 infants, samples collected within 7 days of symptom onset had mean viral load of 6.4 (SD 1.5) log10 copies/mL. This figure decreased to 4.6 (SD 1.5) log10 copies/mL for samples collected between 8 and 14 days of symptom onset. No sample collected after 16 days of symptom onset had detectable viral load.

Samples generating the initial and peak viral loads were collected mean 3.9 (SD 1.7) and 4.1 (SD 1.8) days after symptom onset, respectively. RSV-A and RSV-B samples had similar peak viral load (Figure 2 and Table 3). After adjusting for age, gestational age, sex, comorbidity, RSV subgroup, and days between symptom onset and sample collection, infants with a febrile RSV infection had a higher peak viral load than those with an afebrile RSV infection (7.1 [SD 1.2] vs 6.6 [SD 1.4] $\log_{10}$ copies/mL, P=.042; Table 4). However, this difference became insignificant after correction for multiple comparisons (Table 4) or excluding infants with only 1 swab collected (Supplementary Table 3). Supplementary Figure 1 shows the probability of fever increasing with age, viral load, and RSV-A infection. Peak viral load did not correlate with the ReSViNET score, the need for hospitalization, intensive care, respiratory support, and/or mechanical ventilation after adjusting for the same covariates (Table 4 and SupplementaryTable 3). Similar results were seen when using initial viral load in the above-mentioned comparisons (data not shown).

Figure 2. Distributions of peak viral loads for RSV-A and RSV-B.



Samples generating the peak viral loads were collected at mean 4.0 (SD 1.8) and 4.1 (SD 1.8) days after symptom onset for RSV-A and RSV-B, respectively. A Mann-Whitney *U* test was used to evaluate the significance of the difference in peak viral load between the 2 subgroups. The center line of each box denotes the median; box limits, the first and third quartiles; whiskers, the highest and lowest values within 1.5 times the interquartile range from the box limits; and outlying points, outliers. The *P* value is shown above the boxplot. Abbreviation: RSV, respiratory syncytial virus.

Table 4. Associations Between Clinical Variables and Peak Viral Load in RSV-Infected Infants (n=275)[a]

Variable	Number of Infants	Peak Viral Load, Mean±SD	Days Since Symptom Onset	*P* Value	*Q* Value
ReSViNET score				.246	0.493
0–7	115	6.7±1.4	3.7±1.5		
8–13	101	6.8±1.3	4.3±1.9		
14–20	54	6.8±1.3	4.5±1.9		
Fever				.042	0.251
No	190	6.6±1.4	4.1±1.8		
Yes	81	7.1±1.2	4.1±1.8		
Hospitalization				.456	0.547
No	75	6.8±1.5	3.3±1.3		
Yes	200	6.8±1.3	4.3±1.8		
PICU admission				.331	0.496
No	200	6.8±1.4	4.0±1.8		
Yes	75	6.8±1.3	4.3±1.9		
Respiratory support				.717	0.717
No	74	7.0±1.4	3.5±1.4		
Yes	172	6.7±1.3	4.4±1.9		
Mechanical ventilation				.133	0.400
No	180	6.8±1.4	4.1±1.8		
Yes	66	6.9±1.3	4.3±1.9		

Abbreviations: PICU, pediatric intensive care unit; RSV, respiratory syncytial virus.

[a]Ordered logistic regression or multiple logistic regression was used to adjust for age, gestational age, sex, comorbidity, RSV subgroup, and days between symptom onset and sample collection when comparing viral load between infants with different clinical features.

RSV viral dynamics were analyzed using serial viral load data from 77 hospitalized infants. Overall, viral load decreased by 59% daily (95% CI, 48%–67%) between 3 and 9 days after symptom onset (Supplementary Figure 2). Infants in different age groups or severity groups had similar rates of decrease in viral load. In addition, infants infected with RSV-A and RSV-B had similar rates of viral clearance (Supplementary Table 4).

Respiratory Sequelae at 1 Year of Age

Among the 325 infants, 165 (51%) had 1-year follow-up data on respiratory sequelae. Association analysis of clinical features and sequelae (wheezing episodes since infection) was performed using this cohort (Table 5). The median age at the time of the RSV infection of infants who subsequently developed wheezing in this study was 3.7 months (IQR, 1.3–6.2 months), while infants who did not subsequently develop wheezing had a median age of 4.3 months (IQR, 1.5–7.3 months) when infected. Hospitalization showed the greatest influence on the development of subsequent wheezing ($Q = 2.8\times10^{-4}$) after adjusting for age, gestational age, sex, comorbidity, and viral subgroup, while high ReSViNET scores, fever, and

requirement of intensive care were also significantly associated with wheezing development (Q=0.007, 0.014, and 0.014, respectively; Table 5).

DISCUSSION

In this study, we found that chronological age was significantly associated with severe RSV disease. Younger infants were more likely to require hospitalization, intensive care, respiratory support, and/or mechanical ventilation, while older infants were more likely to have fever. While both RSV subgroups had similar viral dynamics, RSV-A and high viral load were associated with a higher probability of fever than RSV-B and low viral load. Infants who had a high ReSViNET score, fever, or required hospitalization or intensive care were more likely to develop subsequent wheezing at 1-year follow-up.

We demonstrated a positive relationship between the probability of fever and infant age. Elevated levels of interferon-α (IFN-α) and IFN-γ have been known to cause fever, based on results of IFN trials [25]. Studies have also shown that decreased levels of type I IFN (particularly IFN-α1) and IFN-γ in peripheral blood and the nasopharynx are associated with more severe RSV disease in children under 2 years of age [12, 26]. In addition, 1 study showed that RSV induced lower levels of IFN-α in infants than in children 12 months to ,5 years old [27], suggesting that younger infants may have lower levels of IFN than older infants at acute RSV infection. Altogether, IFN levels may explain why younger infants have more severe disease but a lower incidence of fever than older ones. Blood, stool, urine, and respiratory microbiome swabs were collected in this study to investigate the immunological factors determining disease severity. Results of these analyses will be published separately.

RSV-A was more likely to cause a febrile infection than RSV-B in our cohort, while other clinical outcomes were not significantly different between the 2 subgroups. Previous studies have shown conflicting results on the association between RSV subgroup and disease severity [4–8], but none have included fever as an outcome variable. From our previous sequencing study [23], we have shown that all of the RSV strains isolated in this study were genotype ON1 (RSV-A) and BA (RSV-B), which are the current dominant strains worldwide [28–30].

In our dataset, viral load positively correlated with the probability of fever, but not with other clinical outcome measures. Studies enrolling previously healthy RSV-infected children under 2 years of age had contrasting results. One showed that viral load positively correlated with the duration of hospitalization and the requirement for intensive care and mechanical ventilation [9], while the other showed that a higher initial viral load was associated with milder disease in terms of the need for hospitalization and intensive care [31]. These studies did not evaluate the association between viral load and the possibility of fever [9, 31]. Furthermore, we did not find the rate of viral clearance associated with any of the tested clinical

variables. However, delayed viral clearance has been shown in children requiring a longer duration of hospitalization and those requiring intensive care [9, 31].

It is worth noting that studies on the association between RSV disease severity and viral factors (subgroup and viral load) have yielded conflicting results [4–12, 31]. The inconsistency is likely due to differences in study populations, definitions of disease severity, and/or genotypes of the infecting viruses. Our study is robust insofar as it included infants with a wide range of disease severities and from 3 different countries, albeit limited to Europe. We also included infants who were previously healthy and those who had comorbidities. Furthermore, we used several clinical outcome measures to account for the variability in disease presentation. Individual variation in patient characteristics (particularly immunological characteristics) is a potential cause of the difficulties in robustly assessing the relationship between viral load and disease severity. It may be possible to control for immunological variation using a biomarker thatmeasures immunological predisposition towards more severe RSV disease to produce a more accurate assessment.

In our dataset, having more severe disease, demonstrated by high ReSViNET scores, fever, and requiring hospitalization or intensive care, was associated with subsequent wheezing. The fact that multiple clinical variables correlated with wheezing suggests a complex origin for wheezing. Further follow-up of these children up to 3 years of age is ongoing and will reveal if wheezing is transient or persistent throughout early childhood. Prospective controlled trials including interventions, such as vaccines or monoclonal antibodies, may be required to definitively characterize the relationship between RSV disease and respiratory sequelae.

Our study represents one of the most comprehensive datasets evaluating the associations between host demographic and viral factors and RSV disease severity and sequelae. Our findings deepen our understanding of the risk factors for severe RSV disease and subsequent wheezing, and identify the target populations for therapeutic and preventative measures, particularly antivirals, vaccines, and monoclonal antibodies, in latestage clinical trials.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Chapter 14

General Discussion

"Look, I'm jazzing!"
— Soul, Disney film

IT TAKES TWO TO TANGO

During the course of my PhD studies I have collaborated with various industry partners in respiratory syncytial virus (RSV) research. These collaborations have enabled me to understand the importance in connecting academia and industry. The topic of public private partnerships (PPP) is such a vast and exciting area. I have recently discussed developments in RSV interventions[1]; and because it is of great personal interest to me, the discussion of my Discussion will fully focus on the ethical dilemmas and considerations in PPPs which are largely based on personal experience. Of note, the Discussion was written prior to my transition to a major pharmaceutical company.

Theoretical background of PPPs

This is an exciting time for RSV research. With the recent approval of the first-ever RSV vaccines and monoclonal antibody (mAb) for all infants, we are on the cusp of major advances in the prevention of RSV disease. Of these RSV vaccines, many are a result of joint research between academia and industry. The rationale is that *these PPPs overcome challenges in financing, implementation and delivery of infrastructure and public services*, based on the assumption that the private sector brings additional finance and competence and that private companies are inherently more efficient than the public sector in delivering high-quality public services. While the concept of PPPs appears straightforward, the implementation may face multiple challenges. PPPs are complex as the value for money is often ethically questioned and the procurement processes can be lenghty because of legal and regulatory barriers. Lack of transparency and accountability may be the top barrier of PPPs, however, legal barriers including approval and permits and law and regulation changes may also slow down the process. With RSV prevention within reach and the potential risk of PPPs creating complex challenges, *we explore how PPPs co-create value in RSV research.*

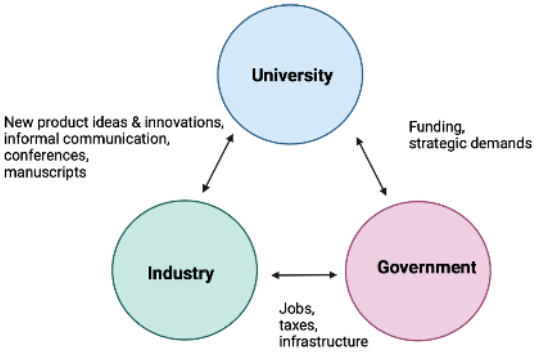
Theoretical framework of PPPs

The theoretical foundation of PPPs in medical research orginates from various fields, including public health, economics and management. Several theoretical perspectives inform and guide the establishment and functioning of PPPs. Here are some key theoretical foundations relevant to PPPs in medical research:

1. *Triple Helix Model*: The Triple Helix model, developed by Etzokowitz and Leydesdorff[2], describes the interaction between academia, industry and governments as key actors in fostering innovation and economic development[2] (Figure 1). This theory suggests that successful innovation ecosystems require strong collaborations between these sectors. Etzkowitz and Leydesdorff argue that the initial role of universities is to provide education. The linear model of innovation is an early model designed to understand the relationship of science and technology breaking it down to a three-step process: (1) invention,

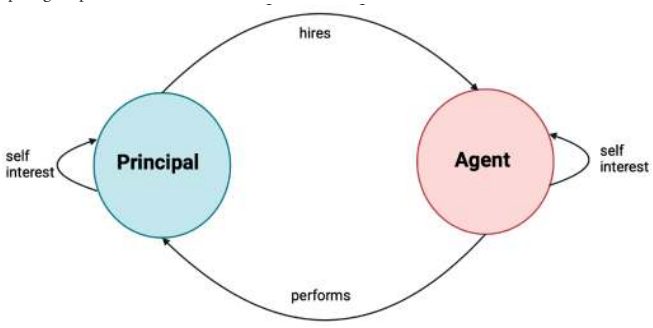
(2) innovation, (3) diffusion. It hypothesizes scientific research as the basis of innovation which eventually leads to economic growth. From the perspective of the linear model of innovation, universities provide basic research that the industry translates into commercial products. Knowledge transfer between university and industry also occurs through informal communication, conferences and publishing co-created manuscripts. PPPs can be seen as practical manifestation of the Triple Helix Theory as it helps to understand the commercialization of research outcomes.

Figure 1: The triple helix model of university-industry-government relations.



2. *Principal-Agent Theory*: The Principal-Agent Theory focuses on the relationship between a principal (university) and an agent (industry) and the challenges of aligning their interests and goals (Figure 2). The most cited reference to this theory comes from Jensen and Meckling[3]. A dilemma arises when the agent represents the principal given their interest differ. Through the lens of the Principal-Agent Theory, PPPs are challenging as the agent's self-interested goals such as profit maximization do not necessarily co-incide with principal's societal interest. This theory can help in understanding how contractual agreements and performance incentives can be designed to ensure that the academic and industry partners work towards shared objectives and maximize the benefits of the partnership.

Figure 2: The principal-agent problem.



3. *Public Value Theory:* The Public Value Theory, orginally developed by Moore[4], emphasizes the importance of public sector actions in creating value for society. In the context of medical research, it highlights the role of PPPs in generating societal benefits, such as equitable access to healthcare innovations and cost-effective healthcare solutions. This theory describes the need for the partnerships to align its activites with broad public interest and values.
4. *Resource Dependence Theory:* The Resource Dependence Theory, developed by Pfeffer and Salancik[5], argues that organisations form partnerships to access resources they lack internally. In medical research, this theory suggests that PPPs are formed to leverage the unique capabilities and resources of both academia and industry. Academia may provide research infrastructures, expertise, and access to patient populations, while the industry brings in funding, commercialization expertise and market access.

These theoretical foundations provide a framework for understanding the motivations, dynamics and outcomes of PPPs in medical research. They help guide the design, implementation, and evaluation of such collaborations, facilitating the effective integration of university and industry efforts to address healthcare challenges and improve patient care.

What true collaboration looks like

Everyone celebrates innovation as novel drugs and devices are there to improve patient care. Since the onset of the COVID-19 crisis *it has become clear that having PPPs is vital in ensuring a successful product*. While there is no argument on the crucial role of industry in bringing new products to patient care, there is discussion on possible biases in research that is sponsored by industry.

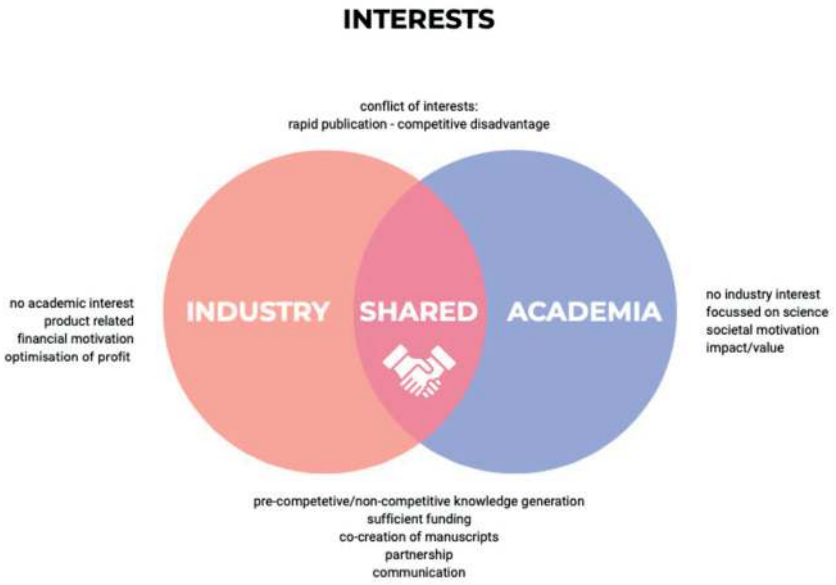
The creative ideas that spark clinical development derive from various sources. Academia have no monopoly; many outstanding ideas come directly from industry. *Academic curiosity drives innovation* which can catalyse industry discoveries, while *industry ensure new products reach the market*. I have summarised different and shared interests between industry and academic environments in Figure 3.

Several PPP initiatives have been highly successful. A variety of pharmaceutical companies have successfully developed COVID-19 vaccines in less than 12 months, which is an extraordinary achievement, given it typically takes a decade or longer to develop new vaccines (Figure 4).

One of the most successful stories specific to RSV vaccine development is that of Barney Graham and his team at the National Institute of Allergy and Infectious Diseases (NIAID) who contributed to the development of fusion glycoprotein vaccines[16]. The discovery and stabilisation of the pre-fusion confirmation provided a new target for vaccines and mAbs as pre-fusion specific antibodies are more potent than post-fusion antibodies in protection against lower respiratory infection[16]. While first described in 2013, Pfizer's older adult vac-

cine just received FDA approval after positive phase III trials[1]. Pfizer has pledged for more equal access to RSV vaccines. Their maternal RSV vaccine is expected to be approved by the FDA for use in pregnant women in August and Pfizer has recently received a $28 million grant from the Bill and Melinda Gates Foundation to support the launch of this maternal RSV vaccine in LMICs[19]. The vaccine will need a different delivery system to be used in LMICs, including alternative packaging and syringes. Preparations for those modifications are already in production and will hopefully be made readily available in the near future.

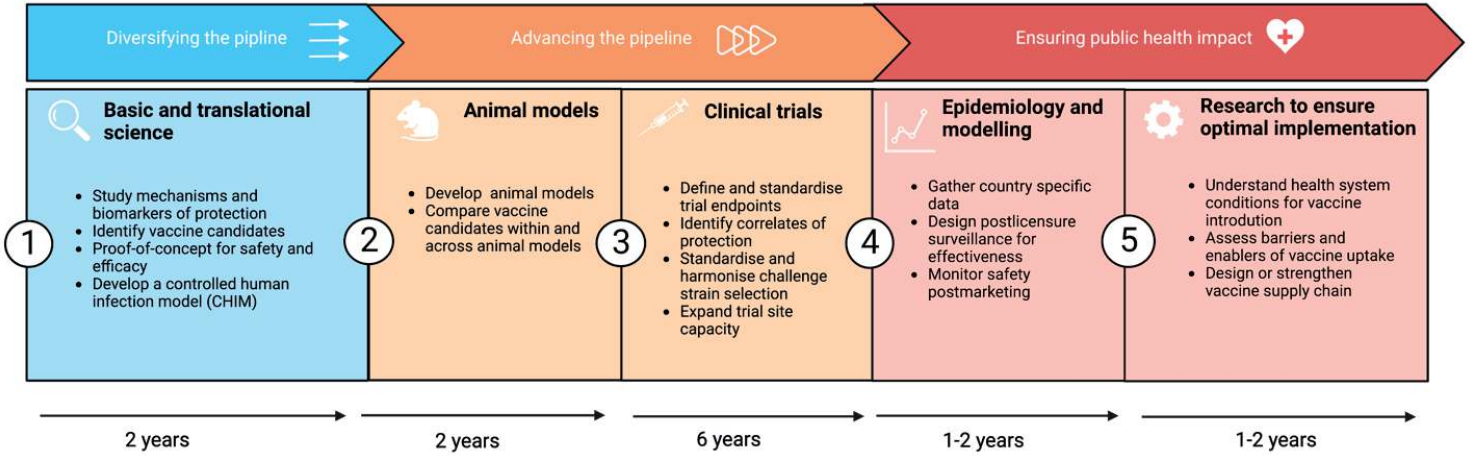
Figure 3: Interests of industry, academia, and combined.



Navigating conflict of interest with transparency

In the past 10 years, almost all RSV antivirals with the exception of zirezovir (AK0529), EDP938 and sisunatovir (RV521) have been discontinued during clinical development while results have not been published[1]. The antiviral drug rilematovir has been suspended in a phase III trial and lumicitabine has been discontinued in a phase IIb trial both without any scientific publications explaining the circumstances or company statements[1,12]. If results were published, there was often a large delay in publication, as seen with the resistance against the monoclonal antibody suptavumab (REGN2222), which ran a phase III trial from 2015-2017 but only published the results in 2021, 4 years after its conclusion[13]. These observations indicate a complete failure in transparency, in which participants would not have participated in the trial if they had known that trial results would not be made available immediately depending on the outcome of the study. Ethical committees have often limited leverage ensuring

Figure 4: Translational vaccine pipeline.



publication of study results. Publishing results in peer-reviewed journals and posting results to the Clinical Trial Group website should be standard practice for those working in this field. However, the probability a given study achieved public disclosure of results varied significantly as a function of funding source and phase development: studies were less likely to be published if they were at phase II, randomised, involved only adult subjects, and had smaller sample size[14,15]. *Ultimately, one may argue academia and industry have different incentives to comply*. These differing incentives may lead to reporting of phase II versus phase III-IV trials by industry versus academics. Many industry-sponsored phase II trials fail to publish results timely as they are only published after licensure: e.g. Saito & Gill research indicated studies took longer to be published and were indeed less likely to be published if funded solely by industry[14]. It is therefore not unreasonable to deduce that the decision to publish, or more importantly not to publish, is frequently driven by commercial considerations. This is made all the more necessary when one considers the clandestine effort to shroud irrefutable evidence which has already been gathered and proven as referenced previously.

However, the stakes are high and trust may not be enough. *Whether intentionally or unconsciously, conflicts of interest may occur at any time at both academic and industry-funded scientific work.* Ensuring that government decisions are not influenced by public officials' private interests is an ongoing concern. Managing conflict of interest requires a balance: a strict approach to controlling private interests may conflict with other rights, be unworkable or deter experienced and competent candidates from entering public service. For example, ZonMw, one of the major health research funding sources in the Netherlands, only invite academics free of any conflict of interest to their Veni, Vidi or Vici grant committees. This may result in a lack of competent candidates, indirectly giving academics with a "conflict of disinterest" a voice. Ideally, researchers with sufficient expertise and a powerful network should be part of such committees which will create a potential but not necessarily an actual conflict of interest. There is a distinction made between conflicts of interest as either actual or potential. An actual conflict of interest involves a direct conflict between academic duties and responsibilities and existing private interests. A potential conflict of interest arises where an academic has private interests that could conflict with their official duties in the future. *Transparency around conflict of interest is key*: What constitutes a conflict of interest? Could it be tied to stock shares, personal financial gains, or even incentives at both individual and departmental levels? The potential scenarios are multifaceted.

The general public may argue that the involvement of pharmaceutical companies corrupts medical science. At the same time clinician scientists need approval for research funding from the hospital board which is a key mechanism protecting against potential corruption in working with industry. In case of patient studies, the Institutional Research Board (IRB) also assesses carefully whether the role of industry is acceptable from a ethical perspective. Recently, the Dutch news described a situation of cardiologists receiving millions of euros from the industry without the hospital's permission, including cardiologists involved in re-

search at academic hospitals[17]. While the cardiologists explained that the money received was used for research purposes, the lack of transparency turned out a major issue for all involved in this case. This example shows that financial compensation should be balanced and that transparency should always be a key element at the centre of decision making in collaboration agreements with companies.

Public opinion

Critics argue that private sector involvement may prioritise commercial interests over public health goals, potentially leading to high prices, limited access or and inadequate focus on vaccines for diseases that primarily affected disadvantaged populations. Therefore, many people emphasize the importance of transparency and accountability in PPPs. They advoduce for clear guidelines, ethical practices and safeguards to ensure public interests are protected, intellectual property rights are managed responsibly and access to vaccines is equitable.

The general public's perception of vaccine uptake can vary depending on numerous factors, including cultural, socioeconomic and individual beliefs[18]. It is important to note that public opinion can be diverse and evolve over time. Many individuals have a positive attitude towards vaccine uptake and view newly introduced vaccines as essential tools for preventing diseases and protecting public health. They believe in the scientific evidence supporting vaccines and trust in the rigorous approval processes and regulatory agencies responsible for ensuring their safety and efficacy. Vaccine hesitancy refers to a spectrum of attitudes ranging from mild concerns to outright refusal of vaccines. Some individuals may have concerns about vaccine safety, efficacy or potential side effects. Factors contributing to vaccine hesitancy can include misinformation, misconceptions, fear, lack of trust in health authorities or previous negative experiences. Another concern raised by many is vaccine equity: the importance of vaccine distribution and uptake especially in vulnerable communities. They advocate for ensuring access to vaccines for all regardless of socioeconomic status, race or geographic location. Concerns about vaccine equity may arise from historical disparities, unequal healthcare access and structural inequalities. Efforts to address these concerns and prioritise equity can garner public support and enhance vaccine uptake.

Ensuring equitable access to vaccines and addressing issues of affordability, availability and distribution is vital for building trust. If PPPs are perceived as favouring profit motives or excluding vulnerable populations, it can undermine public trust and raise concerns about fairness. To better understand the current public opinion on PPPs, it would be necessary to consult surveys, polls and other forms of data collection that provide insights from the general public or specific interest groups.

An introspective journey

Personal success factors

Trust is the foundation of any partnership, and PPPs are no exception. Communication is another critical component of PPPs. *Trust and communication are therefore integral to the sucesss of PPPs* (Table 1). They create an environment conducive to collaboration, allow for effective decision-making, manage risks and conflicts, and enhance accountability and transparency. By fostering trust and promoting open communication, PPPs can realize their potential to deliver sustainable and mutually beneficial outcomes. Additionally, *team members on successful PPP projects would "fail fast": they admit failures when they occur, and they subsequently move quickly to correct them.* In this way, failures are used as opportunities to strengthen their commitment to the partnership. The word "partner" truly must connote the message: "we are all in the same team", which is a sentiment that no contract can ever convey.

Table 1: Opportunities and challenges in personal PPP experiences.

		Mechanism	**Personal experience**
Success factors	Mutual trust	Having built professional relationships with industry partners shows that trust is at the core of any successful collaboration.	Having initiated shared moments during General Assembly Meetings and conferences, especially the 'informal' meet ups (coffees, dinners etc), has contributed to the strong relationships.
	Shared goals	With shared goals but different focus academia and industry can offer their own expertise in sample and data-analysis.	In the INFORM-RSV study, AstraZeneca focused on nirsevimab binding site analysis, while we (UMCU/KU Leuven) focused on global dynamics of RSV. Both are important for RSV spread. Importantly, both goals can be reached by obtaining data that can be used for both studies simultaneously.
	High quality output	Having published in peer-reviewed journals.	Joint publications in respected peer-reviewed journals.
Obstacles	Legal barriers	Legal activities performed within academia are traditionally slow, while turn-around time is an important factor for both academia and industry.	In our Inno4Vac collaboration it took over one year to obtain the necessary legal and IRB approval for sharing RSV samples.
	Open access	Openly sharing research data ensures a greater level of reproducibility in clinical research.	INFORM-RSV whole genome sequencing data still have not entered the public domain.
	Profit-maximization vs. societal impact	Different strategies lead to different priorities and subsequent actions.	Different views in study site selection for clinical studies (LMIC vs HIC).

Abbreviations: HIC, high-income countries; LMIC, low-and-middle-income countries; IRB, Institutional Review Board

Success can be defined as a situation where the project is completed on time and on budget, and with all participants being happy with how the study was conducted. Projects which have an explicit plan of the goals to be met *and* which keep the working relationships of all parties strong throughout the process have a much higher likelihood of success.

An additional factor contributing to success is the specialized expertise that both academia and industry bring to the table when striving for *shared goals*. The expertise outlined in this thesis acknowledges the distinct proficiencies of academia and industry, which synergistically complemented each other in the realm of data analysis.

Both academia and industry were in the lead writing distinct manuscripts, *always embracing a co-creative process*. Clear authorship policies were established at the start of the projects. There were instances where being the first author of manuscripts resulting from extensive collaborative endeavors led me to perceive receiving more academic recognition than warranted (e.g. INFORM-RSV); nonetheless, there were also moments when I sensed being attributed with less acknowledgment than merited (e.g. RESCEU). This experience has underscored the importance of duly acknowledging middle authors. Overall, PPPs mirror the ethos of reciprocal exchange similar to academic collaboration.

Personal barriers

The primary hurdle that comes to light within my PPP experience in the presence of legal barriers. The Inno4Vac project's protracted timeline serves as an exemplar of this issue. Despite the well-defined objectives, parental consent, and prompt sample distribution by the laboratory, the exhaustive process of gaining approval from the legal department, implementing quality control measures, and navigating the IRB consumed such a substantial amount of time that it extended over a year from the project's commencement before we finally obtained the samples in our possession. Legal activities performed within the academic set up of an institute or any university are traditionally slow. However, it is evident that factors contributing to this delay encompass the defensive nature of the legal system.The Inno4Vac collaboration made us reflect on research ethics, wondering whether the speed could be increased by overcoming legal barriers. *A stable regulatory framework that can be easily enforced is essential.* A short turn-around time for contracts and financing is desirable for both academia and industry. Factors that decrease the turn-around time in academia include increased privacy legislation, which is often country-dependent. Usually, academia and industry operate from different law systems (e.g. Europe versus United States) and working with standardised templates for academia-industry collaboration contracts (Confidential Disclosure Agreements, Data Transfer Agreements, Material Transfer Agreements) on the academic side could speed up the process. Another possible solution to overcome these delays is for academia to speed up the process by not bringing all contracts to the legal department for review, but instead by working with standard operation procedures and templates for low-risk research. In addition to legal review, ethical review often slows down the process on the academic side. A risk-

based limited evaluation process with short timelines may be useful for the ethical approval of low-risk studies.

Open access science is a public health good. The demand that publicly funded scientific research should be freely available to the public and beyond academia has become increasingly important, however, for the INFORM-RSV study the whole-genome sequencing data have still not entered the public domain.

Another barrier in PPPs is the different motivation of industry and academia. Are our efforts to prevent RSV driven solely by societal motivation, or is our ultimate goal based on financial motivation? The latter complicates the delivery of RSV vaccines and mAbs to resource poor countries. *There is a mismatch between disease burden and the financial resources devoted to health research*. Low-and-middle-income countries (LMICs) suffer badly from global health inequity defined by the 10/90 gap whereby 90% of the world's investment in health research addresses only 10% of the global health problems[20]. The vaccine industry aims at growing 10% each year, addressing the question of altruistic motives. While one may argue about different motivations e.g. societal versus financial, the ultimate motivation aligns: the development of preventive or therapeutic strategies.

The harmonious dance between academia and industry

Is industry-funded RSV research on balance beneficial? Clearly it is. Ultimately, the positives of PPPs in RSV research heavily outweigh the negatives. The question is not whether there may be conflicts of interest for – there are. Rather, the goal is to work around those and achieve a fair, transparent, unbiased, and scientifically valid result that benefits all. We cannot pretend that the public sector owns public health: promoting clarity and transparency in clinical research is an intrinsic public health good and a shared responsibility of both academia and industry. *While pure academic research is an important adjuvant to industry-supported development, it cannot replace the focus or scope of industry funding.*

The crux of the matter is clear: academia and industry must collaborate closely in the pursuit of RSV prevention. The evidence resoundingly underscores the need for a more synergistic partnership, one that reaps the results and upholds the standards demanded by the public. Moving forward, it holds immense value to explore potential barriers unique to specific countries or regions in the realm of PPPs. Local conditions can substantially influence community and public support, as well as the transparent processes involved. This avenue of investigation could unveil insights that further refine the alignment of PPPs with societal needs and expectations, strengthening the collective efforts to combat RSV.

In the graceful choreography uniting academia and industry, the orchestrated symphony of PPPs unveils not just scientific advancements, but also weaves a melodic tapestry of collaborative determination that forges a future fortified against RSV.

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Chapter 15

Appendices

"I see all the colours in you."
— Multicolour, Son Mieux

BIOGRAPHY

Annefleur Christel Langedijk, who made her debut on the 7th of May 1992, rocked her early years in the vibrant town of Hoorn, Netherlands. Upon completing her high school education (Gymnasium), where she held a strong passion for both philosophy and biology (successfully merging both subjects to secure the prize for the best high school research project by delving into the scientific basis of acupuncture), it was only natural that she pursued her studies in Biomedical Sciences, Medicine, and Infectious Diseases in Amsterdam. Right from the start, Annefleur found herself captivated by infectious diseases, with a particular emphasis on tropical medicine (which might have been influenced by her travels to Africa). Little did she know, this rollercoaster ride catapulted her into not one, but two thrilling internships at the Centre for Tropical Medicine and Travel Medicine of the Academic Medical Centre in Amsterdam. Her research has led to significant changes in both national pneumococcal and rabies vaccination policies. Annefleur has published the manuscripts she authored during her university studies in high impact journals.

Fresh out of the master's degree oven in 2017, Annefleur wasted no time diving headfirst into the world of graduate studies as a PhD candidate in the RSV Research Group at the Wilhelmina Children's Hospital in Utrecht. Annefleur not only participated in the Training Upcoming Leaders in Paediatrics (TULIPS) programme but also sprinkled her own unique charm on the ESCMID Study Group for Respiratory Viruses (ESGREV). One of Annefleur's proudest achievements is the HERACLES study, a remarkable journey she embarked upon during the vibrant momentum of the COVID-19 pandemic. Leading an ensemble of 30 students, nurses, and doctors, she orchestrated this remarkable expedition. With the fervour of a caffeine-powered whirlwind, Annefleur poured her heart and soul into crafting the protocol, securing the green light from the ethical board in just three days. The team's relentless pursuit saw them gather an astonishing seven blood samples per individual from an impressive cohort of 330 healthcare workers, all within a two-week span. Embedded in her heart are the memories of indulging in peanut butter sandwiches within the charming embrace of the Wilhelmina Children's Hospital garden. And let's not overlook the tradition of rewarding the record amount of successful blood draws with a collection of dinosaur stickers – because who knew extracting blood could turn into a dino-themed achievement hunt. Annefleur is a

blue-sky thinker and dreams big. Her long-held dream of being published in Nature became a reality in 2023.

After completing the final chapter of her thesis, Annefleur embarked on a backpacking journey across Central America before returning to her new role as a postdoctoral researcher within the same research group. While delving into public-private partnerships as a component of her thesis, Annefleur's curiosity about a career in the pharmaceutical industry was piqued. In the summer of 2023, she embraced an opportunity and assumed the role of a medical advisor specializing in RSV at a world-renowned pharmaceutical company. She sees herself as a bridge builder between academia and industry and is eagerly anticipating the opportunity to further her contributions in the field of RSV.

During her free time, Annefleur finds joy in the art of surfing, a passion that also doubles as a metaphor for life's ebbs and flows, encapsulating the idea that "it comes in waves". She is a fan of hiking through the great outdoors, shaking it up with African dance moves, diving into books, and penning whimsical poems. Annefleur is often described by friends and family as delightfully quirky. Her vibrant and sunny disposition radiates a yellow personality, bringing the warmth of a summer sunflower wherever she goes.

Annefleur firmly lives by the motto: "If everything is under control, you are going too slow" (courtesy of the race car maestro Mario Andretti). Therefore, she enjoys a dynamic life always doing many things at the same time. While working as a PhD candidate, she also acquired proficiency in conversational Spanish, developed skills in playing the ukulele, and engaged in regular yoga practice. Throughout the past six years, Annefleur's life has taken her through different cities, across several unique homes, and on adventures that have spanned the world. In the here and now, she calls Amsterdam home, sharing her world with her beloved Robin.

And as the chapters of her journey unfold, if you ever spot a yellow butterfly fluttering by, know that Annefleur's heart flutters along with it. For these delicate wings and their enchanting dance remind her that the most wonderful journeys are often painted with unexpected and colourful moments.

PHD PORTFOLIO

Conferences

2023	SalivaDirect Conference; New Orleans, USA (oral)
2023	European Congress of Clinical Microbiology & Infectious Diseases; Copenhagen, Denmark (chair)
2023	European Congress of Clinical Microbiology & Infectious Diseases (meet-the-expert) virtual (oral)
2023	RSV Vaccines for the World; Lisbon, Portugal (oral)
2022	Zambia Health Research Conference; Lusaka, Zambia
2022	ESCMID webinar "The Interplay of Respiratory Viruses and COVID-19: Re-emergence of Respiratory Viral Seasonality and the New Normal After the Pandemic"; virtual (oral)
2022	International RSV Symposium; Belfast, UK (poster)
2022	European Respiratory Symposium; Barcelona, Spain (oral)
2022	European Congress of Clinical Microbiology & Infectious Diseases; Lisbon, Portugal)
2022	European Society for Paediatric Infectious Diseases; virtual (poster)
2020	Virology Africa; Cape Town, South Africa (oral)
2019	RESCEU Annual Meeting; Utrecht, Netherlands (poster)
2019	RSV Vaccines for the World; Ghana, Africa (oral)
2019	National Institute of Health meeting on RSV Genomic Diversity and the Development of a Globally Effective RSV Intervention; Bethesda, USA
2018	International RSV Symposium; Asheville, USA (poster)
2018	RESCEU Annual Meeting; Oxford, UK
2017	RSV Vaccines for the World; Malaga, Spain (poster)

Teaching

2019-2021	Global Health and Tropical Medicine Course; Utrecht, Netherlands
2020	Supervised Training in professional Attitude, Research and Teaching (START class) (sixth year of medical school); Utrecht, Netherlands
2019	Epidemiology (sixth year of medical school); Utrecht, Netherlands
2018	Infection and Immunity (first year of medical school); Utrecht, Netherlands

Supervision

2018-2023	Provided student supervision for several clinical studies involving Safia Laqqa, Sanne van der Pol, Aria Atash, Michelle van Wijk, Anna Vera Verschuur, Marin Bont, Victor Kroon, Lieke van der Kam, Trisja Boom, Daphne van Meerwijk, Renske Bijl and Merlijn Hamel

2022	Research internship and master thesis, Michelle van Wijk (Pharmacology, Utrecht University)
2021	Research internship and master thesis, Eline Harding (Medicine, Utrecht University)
2021	Literature research for honours programme, Burak Konya (Medicine, Utrecht University)
2019	Bachelor thesis, Puck Bemelmans (Pre-Med, Utrecht University College)

Registrations

2018	Basic Course for Clinical Investigators (BROK)
2017	Registered epidemiologist

Courses

2022	European Society of Clinical Microbiology & Infectious Diseases (ESCMID) Summer School; Rome, Italy
2018	Summer School Global Child Health; Utrecht, Netherlands
2018	Global Health Writing Retreat; Utrecht, Netherlands
2018	Molecular Epidemiology of Infectious Diseases; Utrecht, Netherlands
2017	Stress Management, Utrecht University, Utrecht, Netherlands

Other experiences

2022	Visit to Lusaka, Zambia with the purpose of initiating an RSV project at the NICU
2022-2023	ESCMID Study Group for Respiratory viruses (*ESGREV)* member
2019-2022	Several visits to Leuven University, Belgium for collaboration on the INFORM-RSV study
2018-2020	TULIPS PhD Curriculum. *TULIPS* (Training Upcoming Leaders in Paediatric Science) is a national *PhD curriculum* for clinician scientists in the field of Child Health
2019	RESCEU annual conference organisation; Utrecht, Netherlands
2018	Public-private partnership experience at AstraZeneca; Washington DC & San Francisco, USA

Grants

2020	€50.000 collaboration grant UMC Utrecht – KU Leuven for the INFORM-RSV study
2019	RSV Vaccines for the World travel grant; Accra, Ghana
2018	International RSV Symposium travel grant; Asheville, USA

Peer review

Lancet Infect Dis, Lancet Resp Med, ARJCCM, Clin Infect Dis, J Infect Dis, BMC Ped, Clin Microbiol Infect, Sci Rep

NEDERLANDSE SAMENVATTING (DUTCH SUMMARY)

1.1 RSV-infectie en de ontwikkeling van nieuwe producten

Respiratoir syncytieel virus (RSV) is een belangrijke oorzaak van kindersterfte. Meer dan 97% van deze sterfgevallen komt voor in landen met een laag en middeninkomen (LMIC), waar sterfte buiten het ziekenhuis aanzienlijk is. In landen met een hoog inkomen (HIC) is RSV een grote oorzaak van ziekenhuisopname bij zuigelingen: één op de 56 gezonde aterm geboren zuigelingen wordt opgenomen vanwege een RSV-infectie. De last van RSV beperkt zich niet tot zuigelingen; het wordt ook erkend als een probleem bij ouderen, vergelijkbaar met de last van influenza. De last van RSV-ziekte kan zelfs groter zijn dan die van influenza bij ouderen die in het ziekenhuis zijn opgenomen. Ondanks de aanzienlijke impact verloopt de ontwikkeling van antivirale behandelingen voor RSV, afgezien van ribavirine, langzaam. Op dit moment blijft de voornaamste benadering gericht op ondersteunende zorg, waarbij interventies als zuurstofsuppletie en ventilatie op basis van intensive care worden toegepast.

Het huidige landschap binnen RSV-onderzoek wordt gekenmerkt door opmerkelijke vooruitgang: recentelijk zijn twee vaccins goedgekeurd voor gebruik bij oudere volwassenen, één voor zwangere vrouwen en een monoklonaal antilichaam (mAb) voor alle zuigelingen. In klinische onderzoeken vertoonde het vaccin van GSK een werkzaamheid van 83% tegen lagere luchtweginfecties (LRTI) veroorzaakt door RSV, en het vaccin van Pfizer toonde zelfs een werkzaamheid van 87%. Bovendien is het vaccin van Pfizer ook aangewezen voor gebruik bij zwangere vrouwen, met een werkzaamheid van 82%. De mAb nirsevimab, ontwikkeld door AstraZeneca en Sanofi, bleek voor 75% effectief in het voorkomen van LRTI bij zuigelingen die medische aandacht nodig hadden. De brede implementatie van RSV profylaxe benadrukt de noodzaak van actieve surveillance. Een dergelijke surveillance is essentieel, niet alleen om het wereldwijde effect van deze interventies in de loop van de tijd te begrijpen, maar ook voor de snelle identificatie van virale escape-mutanten ten opzichte van nieuwe producten.

Een uitgebreidere introductie van dit onderwerp wordt geboden in **Hoofdstuk 2**.

1.2 Biomarkers van infectie

De ontwikkeling van veilige en effectieve mAbs en vaccins vraagt om biomarkers die gerelateerd zijn aan diagnose. De diagnostische waarde van slijmvlies- en serum-biomarkers heeft steeds meer aandacht gekregen. Bovendien is het identificeren van virale biomarkers die milde van ernstige RSV-infectie kunnen onderscheiden essentieel voor de ontwikkeling van interventies voor RSV. Echter, de ontwikkeling van diagnostiek duurt lang:

- De academische wereld bouwt een wetenschappelijke basis voor (niet-inferieure) hoge prestaties (3-5 jaar).
- Innovatieve bedrijven nemen het idee over en werken aan een commercieel product (3-5 jaar).

- Klinische validatie door zowel de academische wereld als de industrie en regelgevende goedkeuring (1-3 jaar).
- Het commerciële product wordt overgenomen door een grote biotechnologische onderneming (1-2 jaar).
- Integratie in regelgevende en klinische richtlijnen (1-2 jaar).

1.2.1 Speeksel als bron voor onderzoek naar biomarkers van respiratoire virussen - de FRIENDS studie

Nauwkeurige diagnostische testen en nog specifieker eerlijke toegang tot testen zijn van essentieel belang voor de preventie van RSV. De huidige gouden standaard voor RSV-diagnose is RT-PCR van neuswatten. Het gebruik van speeksel voor RSV-detectie heeft de potentie om barrières geassocieerd met neuswatten weg te nemen. Speeksel heeft een hoge sensitiviteit en specificiteit en is bewezen minder invasief dan neuswatten voor het detecteren van *Streptococcus pneumoniae* en SARS-CoV-2. De afnameprocedure is niet alleen niet-invasief, maar ook eenvoudig en goedkoop. We zijn het project Finding Respiratory viruses In Diagnostic Saliva (FRIENDS) gestart om een speekseltest voor RSV-diagnose te ontwikkelen met behulp van de huidige SARS-CoV-2-speekseltest. Naast het bewijzen van het principe van virale detectie in speeksel, streven we ernaar om afnamemethodes te evalueren, aangezien het verzamelen van speekselmonsters bij zuigelingen extra uitdagingen met zich meebrengt.

1.2.2 Biomarkers van bacteriële co-infectie - de HERACLES studie

Onze onderzoeksgroep heeft eerder een op drie eiwitten gebaseerde test onderzocht om bacteriële van virale infectie te onderscheiden bij kinderen met luchtweginfecties, en heeft de diagnostische waarde van de biomarkers CRP, TRIAL en IP-10 in deze populatie aangetoond. Tijdens de beginperiode van de COVID-19-pandemie besloten we onze ervaring te benutten door ook normale waarden van deze drie biomarkers bij gezonde individuen te verstrekken. Dit was een uitdagende periode van mijn promotieonderzoek, aangezien alle RSV-studies werden stopgezet door de medisch-ethische commissies als gevolg van de COVID-19-pandemie. We maakten van een negatieve situatie een positieve en startten het HERACLES-project in samenwerking met MeMed.

1.2.3 Virale biomarkers van RSV-infectie tijdens de kindertijd – de rol van virale load

Het RESCEU (REspiratory Syncytial virus Consortium in Europe) project heeft een duurzame en multidisciplinaire gemeenschap van belanghebbenden gecreëerd om een infrastructuur te bieden voor toekomstige onderzoeken naar RSV-vaccins en therapieën. Het is essentieel om de populaties te identificeren voor onderzoeken naar therapeutische en preventieve maatregelen. Deze studie is 1 van de 4 klinische onderzoeken in het RESCEU-project en onderzoekt de demografische en virale factoren die geassocieerd zijn met de klinische kenmerken van RSV-

infectie. Er is tegenstrijdig bewijs over de associatie tussen de RSV virale load en de ernst van de ziekte: de meerderheid van eerder uitgevoerde onderzoeken heeft een positieve relatie gerapporteerd, wat aangeeft dat een hoge virale load mogelijk geassocieerd is met ernstige ziekte. Hoewel dit intuïtief te begrijpen is, is het noodzakelijk om prospectief monsters te verzamelen van eerder gezonde zuigelingen gedurende meerdere jaren en in meerdere landen.

1.3 Verkennen van gezamenlijk succes

Ik begon mijn promotieonderzoek met een eenvoudig project (INFORM-RSV) om de moleculaire epidemiologie van RSV-infectie wereldwijd te definiëren. Gedurende mijn promotie heb ik een aantal andere projecten geïnitieerd en bijgedragen, die me hebben geholpen meer te leren over epidemiologie, virologie en immunologie. Deze projecten maken nu deel uit van mijn proefschrift. Alle studies hadden één ding gemeen: ze waren allemaal een vorm van publiek-private samenwerkingsprojecten (PPP). Nu, aan het einde van mijn tijd als promovendus, zie ik dat PPP het belangrijkste onderliggende thema van mijn proefschrift is, dat me heeft geïnspireerd, mijn denken als wetenschapper heeft beïnvloed en me naar mijn volgende baan heeft geleid.

1.3.1 PPP is een vorm van co-creatie

Wie betaalt voor het proces om een ruw creatief idee om te zetten in een goedgekeurd product? Het zijn noch academische instellingen noch de overheid; onderzoek wordt grotendeels ondersteund door de industrie. De industrie vertrouwt op academische onderzoekers voor de meeste productontwikkeling en heeft de expertise, het netwerk en de patiëntenpopulatie van clinici nodig. Ongeveer een kwart van de academische onderzoekers in biomedisch onderzoek heeft financiering ontvangen van de industrie. PPP's creëren een samenwerkingsomgeving om maximale interdisciplinaire expertise te benutten tussen academia en industrie, om gezamenlijk waarde te creëren door cruciale factoren zoals dialoog en transparantie aan te raken. In 2011 heeft de Vaccin Alliantie Gavi met succes een lagere prijs onderhandeld voor menselijk papillomavirusvaccins via een PPP in LMICs en meer recentelijk heeft de COVID-19-pandemie unieke omstandigheden gecreëerd voor PPP's, wat waardevolle lessen heeft opgeleverd voor andere ziekteverwekkers zoals RSV. PPP's zijn populair geworden in het RSV-veld, met de Bill & Melinda Gates Foundation en Gavi die werken aan het verbeteren van de toegang tot RSV-vaccins en mAbs in LMICs.

1.3.2 Verschillende vormen van PPP

Klinisch onderzoek kan op drie manieren gefinancierd worden: (1) de afdeling van de onderzoeker ondersteunt het onderzoek; (2) de onderzoeker kan samenwerken met een bedrijf dat interesse heeft in het product of concept; of (3) een studie kan ondersteund worden door een onafhankelijke publieke organisatie zoals het Rijksinstituut voor Volksgezondheid en Milieu of een stichting. Alle drie vormen zijn "onderzoeker-geïnitieerd onderzoek", wat betekent dat

de academische onderzoeker de controle heeft over de studie. Het andere model is dat een bedrijf een studie initieert, financiert en beheert, wat wordt aangeduid als een "gesponsorde klinische trial". Dit verschilt duidelijk van onderzoeker-geïnitieerd onderzoek, omdat het sponsorende bedrijf betrokken is bij de ontwikkeling van het protocol, de uitvoering van de studie en de voorbereiding van manuscripten. Dit maakt door de industrie gefinancierd onderzoek cruciaal voor de ontwikkeling van nieuwe producten. Zonder gesponsorde klinische trials zou academisch onderzoek stagneren met weinig innovatie. Sommigen beweren zelfs dat innovatie niet zou plaatsvinden zonder financiering door de industrie. De artikelen die zijn opgenomen in dit proefschrift zijn het resultaat van verschillende PPP's binnen het RSV-veld, waarbij alle samenwerkingen voor PhD-onderzoek worden samengevat in de onderstaande tabel.

Tabel: PPP's tijdens mijn promotietraject.

Studienaam / samenwerking	**Studiedoel**	**Industriepartner**	**Academische partner**
INFORM	Het begrijpen van de wereldwijde verspreiding van RSV en het tijdig ontdekken van escapemutatenten.	AstraZeneca	>17 universiteiten, KU Leuven, UMC Utrecht
HERACLES	Het onderzoeken van de dynamieken van de host respons op virale luchtweginfectie in gezonde individuen.	MeMed	UMC Utrecht
FRIENDS	Het detecteren van RSV in speekselmonsters vs. neuswatten in zuigelingen.	Merck	Yale Universiteit, UMC Utrecht
RESCEU	Het identificeren van host en virale biomarkers van ernstige RSV ARTI in zuigelingen.	AstraZeneca, GSK, Janssen, Novavax, Pfizer, Sanofi (IMI)	>5 universiteiten, UMC Utrecht, RIVM (IMI)
HARTI (geen data opgenomen in dit proefschrift)	Het beter begrijpen van de evolutie van de oppervlakte eiwitten van RSV.	Janssen	UMC Utrecht
BRICE (geen data opgenomen in dit proefschrift)	Het schatten van de ziekte-ernst van ernstige RSV ALRI in kinderen ≤2 jaar.	Merck	Lokale ziekenhuizen in Engeland, Frankrijk, Duitsland, Spanje, Italië en het UMC Utrecht
Inno4Vac RSV challenge model validatie (geen data opgenomen in dit proefschrift)	Het identificeren van de benodigde inoculatiedosis om RSV-infectie met een nieuwe stam te induceren, gebruikmakend van RSV-isolaten van het UMC Utrecht.	Sanofi, GSK (IMI)	Universiteit Hannover, RIVM, Imperial College London, UMC Utrecht, RIVM (IMI)

Afkortingen: GSK, GlaxoSmithKline; IMI, Innovative Medicines Initiative; RIVM, Rijksinstituut voor Volksgezondheid en Milieu

1.4 Opzet van dit proefschrift

Het overkoepelende doel van dit promotieonderzoek is om de implicaties en uitdagingen te identificeren en aan te pakken die gepaard gaan met de ontwikkeling van RSVproducten, met

als primaire doel de vooruitgang in de ontwikkeling van veilige en effectieve interventies ter bevordering van de volksgezondheid.

1.4.1 Deel I. Productontwikkeling van RSV immunoprofylaxe

We hebben een state-of-the-art review geschreven waarin nieuwe RSV-interventies worden beschreven, inclusief de ontwikkeling van immunoprofylaxe, in **Hoofdstuk 2**.

Gegevens over de ziektelast bij gezonde zuigelingen zijn nodig om RSV-vaccinatiebeleid te bepalen. In **Hoofdstuk 3**, schatten we het aantal RSV-geassocieerde ziekenhuisopnames in het eerste levensjaar om de uitrol van mAbs en vaccins te begeleiden.

Met de goedkeuring van een mAb voor alle zuigelingen en de allereerste RSV-vaccins is de behoefte aan wereldwijde monitoring van RSV steeds belangrijker geworden bij het evalueren van de effectiviteit van die mAbs en vaccins. In **Hoofdstuk 4**, streven we ernaar om kennislacunes in recente RSV-literatuur te identificeren om de wereldwijde evolutie- en transmissiepatronen van RSV te bestuderen en tegelijkertijd begeleiding te bieden voor monitoring van mAbs voor en na verlening van de vergunning.

In **Hoofdstuk 5**, bouwen we voort op de bevinding van een enkele spontane mutatie die de antilichaambindingscapaciteit van het mAb suptavumab negatief beïnvloedde. We stellen dat palivizumab waarschijnlijk zal worden vervangen door mAbs van de volgende generatie in de komende jaren en dat de moleculaire evolutie van RSV complex is.

Om deze kennisklovens te overbruggen, is de INFORM-RSV-studie (International Network for Optimal Resistance Monitoring of RSV) gestart om de dynamiek van wereldwijde RSV-transmissie beter te begrijpen en tijdig mAb-resistentiemutaties te detecteren. INFORM-RSV is de grootste klinische studie ter wereld die momenteel circulerende RSV-stammen bij kinderen onder de 5 jaar monitort. We beschrijven het studieontwerp in **Hoofdstuk 6.**

In **Hoofdstuk 7**, analyseren we samples uit het eerste INFORM-RSV-seizoen (2017-2018), dat een belangrijke moleculaire basislijn van RSV-stamverdeling en sequentievariatie vaststelt.

De geotemporele evolutie van potentiële escape-varianten in recente RSV-seizoenen is nog niet grondig onderzocht. Daarom beoordelen we in **Hoofdstuk 8** de nirsevimab-bindingsplaatsconservering op basis van de nieuwste prospectieve surveillancerapporten, inclusief INFORM-RSV.

Surveillance en preventie van RSV op mondiaal niveau steunen sterk op het begrip van de verspreiding van RSV. Door fylodynamische benaderingen toe te passen, onthullen we hoe selectie en neutrale epidemiologische processen RSV-diversiteit vormgeven en onderzoeken we de dynamiek van wereldwijde RSV-circulatie en de drijvende factor ervan in **Hoofdstuk 9**.

In **Hoofdstuk 10**, onderzoeken we of fatale RSV-infecties in Zambia te wijten kunnen zijn aan genetische verschillen in de virale stammen, of dat ze niet-virologische factoren zoals slechte toegang tot ondersteunende medische zorg kunnen weerspiegelen.

1.4.2 Deel II. Productontwikkeling van biomarkers

In **Hoofdstuk 11**, onderzoeken we of speeksel een alternatieve diagnostische methode kan zijn. Uiteindelijk zal detectie in speeksel grootschalige en frequente klinische en gemeenschapsonderzoeken praktischer maken.

In **Hoofdstuk 12**, evalueren we een nieuwe bloedtest die bacteriële van virale infectie kan onderscheiden door concentraties van drie host biomarkers te evalueren. Onze bevindingen bieden normale waarden bij gezonde individuen.

In **Hoofdstuk 13**, evalueren we demografische en virale factoren die geassocieerd zijn met de ernst van RSV-ziekte bij zuigelingen jonger dan 1 jaar uit 3 Europese landen. Deze resultaten verdiepen het begrip van risicofactoren en identificeren doelpopulaties voor therapeutische en preventieve maatregelen.

In **Hoofdstuk 14**, bekijken we de lessen die zijn geleerd uit het werk in dit proefschrift. We reflecteren op persoonlijke ervaringen met PPP.

PUBLICATIONS IN THIS THESIS

1. **Langedijk AC,** Bont LJ. Respiratory syncytial virus infection and novel interventions. Nat Rev Microbiol. 2023 Jul 12. doi: 10.1038/s41579-023-00919-w. Epub ahead of print. PMID: 37438492.

2. Wildenbeest JG, Billard MN, Zuurbier RP, Korsten K, **Langedijk AC**, van de Ven PM, Snape MD, Drysdale SB, Pollard AJ, Robinson H, Heikkinen T, Cunningham S, O'Neill T, Rizkalla B, Dacosta-Urbieta A, Martinón-Torres F, van Houten MA, Bont LJ; RESCEU Investigators. The burden of respiratory syncytial virus in healthy term-born infants in Europe: a prospective birth cohort study. Lancet Respir Med. 2023 Apr;11(4):341-353. doi: 10.1016/S2213-2600(22)00414-3. Epub 2022 Nov 10. PMID: 36372082; PMCID: PMC9764871.

3. **Langedijk AC**, Harding ER, Konya B, Vrancken B, Lebbink RJ, Evers A, Willemsen J, Lemey P, Bont LJ. A systematic review on global RSV genetic data: Identification of knowledge gaps. Rev Med Virol. 2022 May;32(3):e2284. doi: 10.1002/rmv.2284. Epub 2021 Sep 20. PMID: 34543489; PMCID: PMC9285027.

4. **Langedijk AC**, Bont LJ. How Viral Sequence Analysis May Guide Development of Respiratory Syncytial Virus Monoclonal Antibodies. Clin Infect Dis. 2021 Dec 6;73(11):e4409-e4410. doi: 10.1093/cid/ciaa944. PMID: 32640025.

5. **Langedijk AC**, Lebbink RJ, Naaktgeboren C, Evers A, Viveen MC, Greenough A, Heikkinen T, Stein RT, Richmond P, Martinón-Torres F, Nunes M, Hosoya M, Keller C, Bauck M, Cohen R, Papenburg J, Pernica J, Hennus MP, Jin H, Tabor DE, Tovchigrechko A, Ruzin A, Abram ME, Wilkins D, Wildenbeest JG, Kragten-Tabatabaie L, Coenjaerts FEJ, Esser MT, Bont LJ. Global molecular diversity of RSV - the "INFORM RSV" study. BMC Infect Dis. 2020 Jun 26;20(1):450. doi: 10.1186/s12879-020-05175-4. PMID: 32591017; PMCID: PMC7316634

6. Tabor DE*, Fernandes F*, **Langedijk AC***, Wilkins D, Lebbink RJ, Tovchigrechko A, Ruzin A, Kragten-Tabatabaie L, Jin H, Esser MT, Bont LJ, Abram ME; INFORM-RSV Study Group. Global Molecular Epidemiology of Respiratory Syncytial Virus from the 2017-2018 INFORM-RSV Study. J Clin Microbiol. 2020 Dec 17;59(1):e01828-20. doi: 10.1128/JCM.01828-20. PMID: 33087438; PMCID: PMC7771447
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