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Respiratory syncytial virus and airway microbiota – A complex interplay and its reflection on morbidity

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Abstract

The immunopathology of respiratory syncytial virus (RSV) infection varies considerably, severe disease occurring only in a minority of the affected children. The variability of the clinical presentation is in part explained by viral and environmental factors but, in infants and young children, disease severity is certainly linked to the physiological immaturity of the innate and adaptive immune system. There is evidence that the maturation of the host immune response is positively influenced by the composition of the nasopharyngeal microbiome that, promoting an efficient reaction, can counteract the predisposition to develop viral respiratory infections and lower the risk of disease severity. However, interaction between the nasopharyngeal microbiota and respiratory viruses can be bidirectional

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since microbial dysbiosis may also represents a reflection of the disease-induced alterations of the local milieu. Moreover, viruses like RSV, can also increase the virulence of potential pathogens in nasopharynx, a main reservoir of bacteria, and therefore promote their spread to the lower airways causing superinfection. Moreover, if negative changes in microbial community composition in early life may constitute a heightened risk towards severe RSV respiratory infection, on the contrary specific groups of microorganisms seem to be associated with protection. A better understanding into the potential negative and positive role of the different nasopharyngeal bacterial species on RSV infection may improve primary prevention and possibly care of this highly contagious disorder.

Key Message: The complex interplay between the members of the nasopharyngeal microbiota and RSV can modulate vulnerability to RSV infection, impact its severity, favor bacterial superinfection and indirectly induce subsequent recurrent wheezing and asthma in later childhood. Better understanding of the respective negative and positive role of the individual components of the nasopharyngeal microbiome on RSV infection and the mechanisms governing the resulting bidirectional interplay microbiome-virus will improve primary prevention, treatment and reduce sequelae of this pernicious virus.

1. INTRODUCTION

Respiratory syncytial virus (RSV) is a seasonal disease which causes an enormous burden on health systems across the world [1]. The spectrum of its clinical manifestations ranges from mild upper airway illness to severe lower respiratory tract infections [1-3]. The mechanisms accounting for the wide variability in the clinical presentation of RSV infection are multifaceted and incompletely understood, however recent insights suggest that modifications of the nasopharyngeal microbiota composition, induced or established by a preceding infection, might be involved. In early life, the baseline components and function of airway immunity is regulated by the local microbial milieu, and distinct microbiota have been viewed as sequelae of specific viral and bacterial lower respiratory tract infections [4-6]. Whether imbalance in the microbial community drives disease pathogenesis or, conversely, reflects disease-induced alterations of the local milieu following respiratory infections is still to be elucidated [5,6]. Modifications of the upper airway microbiota composition appear to be associated with RSV disease severity, but beyond this direct effect there is evidence that RSV can increase the host susceptibility to the large bacterial reservoir of nasopharyngeal pathogens which may consequently spread to the lower airways causing superinfection, at the time of RSV infection [7,8]. In this article the current evidence on the epidemiologic link between RSV and nasopharyngeal microbiome, the mechanisms involved in this bidirectional interplay, and the possible clinical consequences will be reviewed, summarized, and discussed.

2. INFANT NASOPHARYNGEAL MICROBIOME IN HEALTH AND DISEASE

The airways represent a large surface area that interacts with the surrounding environment. A growing body of literature has demonstrated that the nasopharyngeal microbiome, with its mixed microbial communities, is involved in maturation and homeostasis of the host immune response in the upper and lower airways [4-8]. A balanced

competitive/synergistic interplay between commensal and potentially pathogenic taxa, can be viewed as the hallmark of a healthy status. This balance can be disrupted by acquisition of new pathogenic bacteria, by increased virulence or prevalence of potential pathogens, and/or by decreased efficiency of host defenses, the latter being the cause but also the result of dysbiosis [4-6].

2.1 Nasopharyngeal microbiota composition in infancy

In newborns and infants, obligatory nasal breathers, nasopharyngeal airways are both the first line of defense and a main port of entry for bacteria and viruses [9,10]. Therefore, in early life, microbiota composition of nasopharyngeal airways reflects exposures to the surrounding atmospheric environment which, as it shapes host immune responses, ultimately will modulate the predisposition to respiratory disorders [4,10-12]. In an extension of the Childhood Asthma Study in Melbourne, it was shown that nasopharyngeal microbiome in infants has a relatively simple structure, dominated by six clusters: *Moraxella*, *Haemophilus* (Gram negative bacteria), *Staphylococcus*, *Corynebacterium*, *Streptococcus* and *Alloiococcus* (Gram positive bacteria) (figure 1) [13]. Some of these clusters have been identified as potential pathogens or been associated with increased morbidity in different clinical conditions. Healthy nasopharyngeal samples collected around 2 months of age were dominated by *Staphylococcus* and *Corynebacterium*, but the frequency of these microbiome profile groups declined with age and, at 12 months, an increased prevalence of *Moraxella* species (spp) and *Alloiococcus* was detected (figure 1A) [13]. Transient incursions of *Streptococcus*, *Moraxella* and/or *Haemophilus* occurred during acute viral respiratory tract infections, followed by a progressive decline in *Staphylococcus* and *Corynebacterium* spp (figure 1B) [13]. Antibiotic treatments were associated with higher abundances of the potential respiratory pathogens *Haemophilus*, *Streptococcus* and *Moraxella*, with lower abundances of *Corynebacterium* and *Alloiococcus* (figure 1C) [13]. This disruptive microbial dysbiosis may predict subsequent respiratory infections [12,14-16]. Colonization with *Streptococcus*, *Moraxella* or *Haemophilus* spp can be linked to an increased risk for upper and lower respiratory tract infections and asthma during the first years of life, whilst nasopharyngeal microbiota dominated by *Corynebacterium* or *Alloiococcus* clusters appear to be associated with less respiratory morbidity (figure 1D) [13,14]. This putative impact on future respiratory morbidity is buttressed by the Copenhagen Prospective Study on Asthma in Childhood, which showed that more than 20% of 411 infants born to mothers with asthma were colonized by potential pathogens at 1 month of age [17]. Neonatal hypopharyngeal colonization with *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* or a combination of them was associated with persistent wheeze, acute severe exacerbation of wheeze and hospitalization for wheeze, but also with increase in blood eosinophil counts and total IgE at 4 years of age. At 5 years of age, the prevalence of asthma and the reversibility of airway resistance after β_2 -agonist administration were consistently and significantly increased [17]. In these neonates, colonization with *Moraxella catarrhalis* and *Haemophilus influenzae* induced a mixed T helper cell (Th) type 1/Th2 response with high levels of IL-1 β , TNF- α , and macrophage inflammatory protein-1 β , but also IL-4, IL-5, IL-13, eotaxin-1 [18]. Hence, these pathogenic bacterial strains were not innocuous bystanders, but rather inducers of airway mucosal immune-mediated chronic inflammatory responses. In contrast, *Streptococcus pneumoniae* caused a weaker inflammatory

cytokine and chemokine release. The species-dependent differences in these immune profiles indicates that *Moraxella catarrhalis* and *Haemophilus influenzae* colonization in neonates is strongly associated with development of asthma later in childhood, whereas *Streptococcus pneumoniae* showed similar, yet weak, association [18].

Neonates with airway colonization with *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, or combinations thereof, had increased risk of experiencing pneumonia or bronchiolitis within the first 3 years of life, a risk that was lowest for *Streptococcus pneumoniae* alone [19].

2.2 Nasopharyngeal microbiota dysbiosis in preterm infants

Changes in microbial community composition related to early exposure to noxious environmental factors may forebode ominous future consequences and constitute a heightened risk towards severe viral respiratory infections and bacterial superinfections in preterm infants. This can be observed in severely premature infants who face the combined effects of deprivation of the normal intrauterine physical environment, supplemental oxygen with relative hyperoxia, barotrauma from mechanical respiratory support with the added exposure to nosocomial pathogens and to broad-spectrum antimicrobials [16]. In comparison with full term infants, preterm infants show abnormal upper airway immune responses against respiratory viruses, that persisting beyond the neonatal period [16,20]. Perez et al, showed that in preterm infants, during naturally occurring rhinovirus infections, the emergence of a mixed flora, including *Moraxella* and other Gram-negative bacteria (*Burkholderia*, *Neisseria* and *Janthinobacterium*), and the decrease in the Gram-positive *Lactobacillus*, was associated with an abnormal nasal airway immune response to the virus, characterized by increased secretion of Th2 and Th17 cytokines (figure 1E) [16]. This airway microbiome signature has been recently described also in preterm newborns at increased risk of bronchopulmonary dysplasia [16,20]. The presence of within-group dissimilarity, identified also in the nasopharyngeal microbiota of otherwise healthy premature infants, suggests that preterm infants do not develop a stable microbiome in early life. Longitudinal data in this vulnerable group showed that the prematurity-related microbiota profiles persisted during viral infection, played important negative role in modulating airway inflammatory and immune responses, and increased the risk of having at least one pediatric intensive care unit (PICU) admission during the first 2 years of life [16,20].

3. NASOPHARYNGEAL MICROBIOTA COMMUNITY COMPOSITION AND RSV INFECTION

Large epidemiologic studies have demonstrated a relationship between the severity of RSV infection and subsequent recurrent wheezing and asthma into childhood [1-3]. Notably, RSV is not the only virus associated with increased risk of later wheezing but is clearly the most prevalent and best researched of the viruses in infancy [21]. In infants, the severity of the first RSV infection is linked to the viral characteristics and load, but as importantly, to the structural and functional immaturity of the lung and of its host defense mechanisms [3,21]. An impaired response to RSV infection, involving both the innate and the adaptive immunity, results in ineffective excessive inflammatory

response leading to extensive epithelial damage, sloughing off epithelium, mucus production, bronchial obstruction and to long-term changes in neuroimmune control of airway tone [21,22].

3.1 Modulation of RSV infection severity

Shift in microbial composition at the time of viral infection may enhance the severity of RSV infection [7,23,24]. A prospective observational study of 106 children aged 2 years or younger, with a first episode of RSV infection were compared to 26 healthy control subjects [7]. Three groups were evaluated: a) with mild RSV disease (RSV infection), b) with severe RSV disease (RSV hospitalization) and c) healthy controls. Four nasopharyngeal microbiota clusters were identified, characterized by respective enrichment of either *Haemophilus influenzae*, *Streptococcus*, *Moraxella*, and *Staphylococcus aureus*. RSV infection and RSV hospitalization were positively associated with *Haemophilus influenzae* and *Streptococcus* and negatively with *Staphylococcus aureus* abundance (figure 2A) [7]. Rates of PICU admission and length of stay tended to be lower in children within the *Moraxella* cluster and higher abundance of *Moraxella* was observed most often in outpatients [7]. Nasopharyngeal microbiota clusters enriched with *Haemophilus influenzae* and *Streptococcus* at the time of RSV infection were associated with clinically more severe disease and an exaggerated inflammatory host immune response, characterized by overexpression of genes related to toll like receptor (TLR) signaling and neutrophil recruitment and activation [7]. This included overexpression of IFN-related genes, enhanced Toll-like receptor signaling and increased expression of neutrophil- and macrophage-related transcripts [7]. Similarly, in a multicenter U.S.A. study of 1,005 infants hospitalized for bronchiolitis (the MARC-35 study), the odds ratio for PICU admission was higher for subjects with RSV infection and *Haemophilus*-dominant profiles compared with those with profiles dominated by other bacteria, such as *Moraxella* (figure 2A) [25]. Infants with the *Haemophilus*-dominant profile, compared to the others, were older and more likely to have used antibiotics before the index hospitalization and during prehospitalization visits. Overexpression of *Haemophilus* in nasopharyngeal aspirates was also described in a cohort of 54 infants, <6 months of age, hospitalized with RSV infection [26]. In these infants, transcriptome profiles dominated by *Haemophilus* was associated with increase IL-6 and CXCL-8 responses; cytokines that in RSV infection are associated with more severe disease (figure 2A). Indeed in human bronchial epithelial cell cultures pretreated with *Haemophilus influenzae*, the release of IL-6 and CXCL-8 was synergistically increased after RSV, but not after HRV exposure, buttressing the notion that this type of virus-bacterial interplay might be specific for RSV [27]. Further evidence for the ability of the nasopharyngeal microbiota to positively modulate the host immune response in severe bronchiolitis is the demonstration of the presence of specific metabolic profiles related to the microbiome composition and linked to the inflammatory-mediated pathogenesis. In a study on infants <1 year of age, hospitalized with bronchiolitis, the nasopharyngeal airway metabolome profiles and their association with disease severity was evaluated [23]. Among 254 metabolites identified, a panel of 25 showed high sensitivity (84%) and specificity (86%) in predicting the use of positive pressure ventilation (PPV) support. Metagenomic sequencing demonstrated that *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae* dominated the nasopharyngeal airway however, only the relative abundance of *Streptococcus pneumoniae* correlated with the intensity of these selected metabolites to predict

increased risk of PPV support (figure 2A) [23]. Sphingolipid metabolism was the sub-pathway most significantly enriched in infants with PPV use, and in turn, enrichment of sphingolipid metabolites was positively correlated with the relative abundance of *Streptococcus pneumoniae* [23]. In contrast, relative *Moraxella* abundance was negatively correlated with respective intensity of the same metabolites. Sphingolipids are linked to the inflammatory-mediated pathogenesis of airway diseases and play an important role in multiple aspects of viral replication, including the initial infection of mammalian cells, depression of host innate and adaptive immune responses, and assembly and budding of newly synthesized viral components [28]. Thus, specific nasopharyngeal microbiota clusters, like those dominated by *Haemophilus influenzae* and *Streptococcus pneumoniae* can modulate the host immune response against RSV, potentially affecting clinical disease severity. Curiously, *Moraxella* spp, thought to be associated with a general increase in infant morbidity [11,13], appears to have a “protective” effect on RSV bronchiolitis.

3.2 Delayed viral clearance, RSV infection and promotion of recurrent wheezing

Respiratory virus clearance is largely dependent on the interplay between infecting agents and host response initiated by resident respiratory tract cells and innate immune cells and ultimately resolved by adaptive immune cells [29,30]. In infants with RSV infection there is evidence that this interplay may be influenced by nasopharyngeal microbiota composition. The multicenter U.S.A. MARC-35 study reported that among infants hospitalized with RSV bronchiolitis, those with a *Haemophilus*-dominant microbiota profile were more likely than those with a mixed profile to have delayed clearance, after adjustment for 11 factors, including viral load (figure 2B) [25]. In the same study, at follow-up evaluations, recurrent wheezing was detected in 31% of all infants [31]. An increase in relative abundance of *Moraxella* or *Streptococcus* spp, 3 weeks after day 1 of hospitalization, was associated with an increased risk of recurrent wheezing by age 3 years, and with an increased risk of recurrent wheezing and asthma by age 4 years. Of the recurrent wheezers, 74% had nasal swabs positive for RSV and 26% for HRV at time of hospitalization [31]. In a similar study of 74 infants aged 6 months or less hospitalized for severe RSV bronchiolitis, were evaluated and followed until age 3 years [32]. In 35.1% of subjects who later developed recurrent wheezing, a higher relative abundance of *Haemophilus*, *Moraxella* and *Klebsiella* was detected: *Haemophilus* was associated with elevated levels of CXCL8 and, *Moraxella*, of IL-6 and IL-10 in the nasotracheal aspirate (figure 2B) [32]. As part of a multicenter, multiyear U.S.A. study, nasopharyngeal aspirates from children age <2 years were also analyzed at the time of hospitalization [33]. *Haemophilus influenzae* was detected in most RSV-only infected and RSV-HRV-co-infected samples, but not in HRV-only infected samples. In contrast, *Moraxella* spp were detected in co-infections, but not in RSV-only or HRV-only samples, and more commonly in children who were wheezing at admission [33]. It is therefore possible that in the context of bronchiolitis, specific viruses may promote the presence of specific bacterial species or, vice versa, and that perturbations in the microbiome at admission can be associated with acute wheezing. Resolving the conundrum of the differential role of *Moraxella*, as having an apparent protective role on the severity of RSV infection in the acute phase, yet being consistently identified as a risk pathogen for recurrent wheezing, will advance our comprehension on both its role and that of other nasopharyngeal microbial species' in

the course, severity and complications of different respiratory conditions and possibly provide new avenues to ameliorate the long-term respiratory outcomes of infants with severe bronchiolitis.

3.3 RSV infection and reduced risk of recurrent wheezing

A different nasopharyngeal microbiota composition may be associated with a reduced risk of wheeze after RSV infection. A population-based birth cohort of healthy term infants was evaluated during the first RSV acute respiratory infection [34]. Of the 113 children who had 2-year outcome data, 46 had at least one parental reporting a wheezing episode. No association was found between the nasopharyngeal microbiome taxonomic composition, diversity and richness assessed either during the first RSV infection or the subsequent wheeze development. However, detection and relative abundance of *Lactobacillus* in nasopharyngeal aspirate was consistently higher in infants who did not develop wheezing. *Lactobacillus* also ranked first among the different genera in a model distinguishing infant with and without subsequent wheeze [34]. Increasing evidence supports that microbiota dysbiosis in the first years of life may also have a role in the development of allergy and asthma [35]. The Canadian Healthy Infant Longitudinal Development (CHILD) study evaluated gut microbiota of 319 children and showed that infants at risk of asthma exhibited transient gut microbial dysbiosis during the first 100 days of life [36]. In this study, the relative abundance of the bacterial genera *Lachnospira*, *Veillonella*, *Faecalibacterium* and *Rothia* was significantly decreased and was associated with reduced levels of fecal short-chain fatty acids (SCFAs) as well as dysregulation of other enterohepatic metabolites, capable of interacting with host immune cells. In animal models these metabolites have all been shown to protect against airway inflammation through stimulation of regulatory T cells (T_{regs}) and dendritic cells, capable of preventing Th2-type immune responses [37]. These results point to the role of early-life intestinal microbiota in shaping the immune system development. This “gut-lung axis” interaction underlies the notion of use of microbe-based therapies to prevent the development of asthma and other allergic diseases. In a randomized double-blind study conducted in 131 young children (6-24 months old) with at least two wheezing episodes and a first-degree family history of atopic disease, 6 months treatment with oral *Lactobacillus rhamnosus* showed mild effects on reducing allergic sensitization, which persisted 6 months after its cessation, but had no clinical efficacy on asthma-related events [38]. In contrast, in a similar study of 160 children and adolescents (6–18 years old) with asthma diagnosis, 3-month treatment with other species of probiotics, *Lactobacillus paracasei* or *Lactobacillus fermentum*, as well as their combination, decreased IgE levels and improved peak expiratory flow rates and asthma control, as determined by the Childhood Asthma Control Test and Pediatric Asthma Quality of Life Questionnaire scores [39]. Further studies are needed to better substantiate these findings and, importantly, to investigate the potential long-term benefits of some probiotics in infants and young children at high risk for allergic sensitization, recurrent wheezing, and asthma.

4. NASOPHARYNGEAL MICROBIOTA COMMUNITY COMPOSITION AND BACTERIAL SUPERINFECTION DURING RSV INFECTIONS

The interaction between nasopharyngeal microbiota and RSV in children can be bidirectional. As discussed above, specific nasopharyngeal microbiota compositions may modulate RSV severity and its long-term effects but, at the same time, RSV-induced infections can favor potentially pathogenic microorganism growth and activation in the upper airways, thus favoring bacterial superinfections and morbidity [8,40-41].

4.1 Airway epithelial integrity and enhancement of bacterial adhesion and virulence by RSV

RSV infection can promote the invasion of the respiratory epithelium by non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae* through a variety of mechanisms, including upregulation of bacterial adhesion protein expression, loss of epithelial barrier integrity and decrease of bacterial clearance mechanisms [8,42,43]. Two RSV glycoproteins, designated G and F are respectively involved in virus-host cell attachment and cell membrane fusion. Following RSV infection, airway epithelial cells express on their surface the RSV G-protein that also serves as receptor for *Streptococcus pneumoniae* and *Haemophilus influenzae* [8]. Using *in vitro* and lung mouse models of infection, incubation with RSV or purified G protein was shown to increase *Streptococcus pneumoniae* adherence to differentiated ciliated cells of the respiratory epithelium, thus enhancing the severity of the airway infection in animals [44,45]. Also detected in these studies were extensive changes in the pneumococcal transcriptome, with upregulation in the expression of key pneumococcal virulence genes, including those encoding the toxin pneumolysin [44]. Moreover, on the airway epithelium end, RSV infection upregulated the cellular surface expression of other known receptors for *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae*, notably the intercellular adhesion molecule-1 (ICAM-1) and platelet-activating factor receptor [8,46]. Loss of epithelial barrier integrity might further facilitate bacterial superinfection [8]. Indeed, in addition to constituting a selective barrier, the airway epithelium secretes a variety of cytokines and chemokines that promote recruitment and activation of immuno-effector cells and releases antimicrobial peptides that kill inhaled pathogens [21]. Airway epithelial cells are polarized by specialized cell-cell junctional complex, the tight junctions (TJ), that initiate and maintain cell-cell contact and maintain the integrity of the airway epithelial barrier [8,47]. In experiments performed in air-liquid interface cultures of primary human bronchial epithelial cells, reduction of transepithelial electrical resistances with evidence of subtle changes in TJ integrity was detected following RSV infection [48]. Buttressing these findings are studies on BALB/c mice in which intranasal RSV infection induced lung inflammation associated with downregulation of the expression of TJ protein claudin-1 and occluding messenger RNA [49]. In contrast to RSV, influenza virus enhances bacterial adhesion by unmasking cryptic receptors on the epithelial cell surface by promoting cleavage of the terminal sialic residue and exposing sialylated carbohydrate ligands to which *Streptococcus pneumoniae* can bind [8]. In severe infections, influenza virus induces extensive damage to the airway epithelial layer, allowing the physical entry of pathogens and by increasing receptor availability on the basement membrane, providing additional bacterial adherence sites [8]. Information elucidating the complex dialogue between the respiratory microbiota and epithelial cells in the lungs during viral infections is scant [50].

4.2 RSV and bacterial superinfection

In clinical practice, for infants with RSV bronchiolitis the risk of secondary bacterial infections (SBI) is present, and the severity of the resulting bacterial infection appears to parallel that of the RSV infection (figure 3A). The low risk of SBI in mild RSV is borne out in a retrospective cohort study of <8-week-old infants, who presented with fever and a positive RSV antigen test to the Emergency Department at an urban child facility in Nashville, Tenn. [51]. 174 RSV-positive cases were gender- and age-matched to febrile RSV-negative control subjects. Infants with RSV infection were more likely to present with upper respiratory infection symptoms, increased work of breathing, and apnea. However, 2 patients in the RSV group had SBI (both with URT), compared with 22 in the control group (relative risk: 0.009), 17 of which were urinary tract infections (UTI). None of the RSV-positive infants had bacteremia or meningitis. Buttressing these results is a systematic review of 11 studies on in- and out-patients, < 60 to 90 day old, with bronchiolitis, reporting no case of bacteremia or meningitis, yet the rate of UTI resulting slightly higher in the RSV-positive subgroup (5.1% vs 2.0%) compared with the RSV-negative group [52]. These data suggest that, in nontoxic-appearing infants with a positive RSV test, full septic evaluations are unnecessary, however, the finding of a low, but clinically relevant rate of UTI, probably justifies urine work up [51,52]. The need for a complete evaluation for SBI is probably higher in the youngest infants, as suggested in a study of 1,248 febrile infants <60 days of age, from 88 pediatric Emergency Departments in New York [53]. The rate of SBI, was again significantly lower, but still appreciable, in those with RSV infection as compared with those without RSV infection (7.0%, compared with 12.5%). RSV-positive infants had an appreciable rate of UTI (5.4%), a rate that was significantly lower than that of RSV-negative infants (10.1%). No RSV-positive infant had bacterial meningitis and only 1.1% had bacteremia. In a sub-analysis stratified by age, the rate of SBI amongst the RSV positive infants was 10.1% in the younger infant group (<28 days of age), higher as compared to the 5.5% rate of the older (29-60 days of age) infant group, yet this difference did not achieve statistical significance. Since the overall rate of SBI in infants who were <28 days of age did not differ significantly between those who were and those who were not RSV infected, absent RSV infection does not obviate the need for a complete evaluation for SBI in these youngest infants [53]. Conversely, the incidence of SBI and concomitant bacterial pneumonia is more frequently observed in infants with severe RSV bronchiolitis (figure 3B). Out of 127 infants, median age 1.7 months), admitted to PICU to the University Children's Hospital of Zurich for severe RSV bronchiolitis, 57 were mechanically ventilated [54]. Bacterial pneumonia was diagnosed if the tracheal aspirate, obtained within the first hour of intubation, showed significant growth of relevant bacteria and presence of a significant number of leucocytes (both >2+ on a scale from no leucocytes/bacteria to 3+). In the intubated patients, the incidence of bacterial pneumonia was 43.9%, half community acquired and half nosocomial. Tracheal aspirates tested positive for *Moraxella catarrhalis*, *Haemophilus influenzae* type b and *Streptococcus pneumoniae*. Antibiotics were administered over the first two days of hospital admission to a high proportion (57.4%) of infants with negative bacterial cultures. Since clinical signs were not helpful in identifying patients with positive bacterial cultures, the authors conclude that to avoid inappropriate antibiotic prescription, tracheal

aspirates should be investigated microbiologically on a routine basis in mechanically ventilated infants with severe RSV bronchiolitis [54]. These results were confirmed in a study of 165 children (median age 1.6 months) admitted to PICU with RSV bronchiolitis to the Royal Liverpool Children's Hospital [55]. In this study all patients were mechanically ventilated and bacterial co-infection was diagnosed only when pathogen load in bronchoalveolar lavage (BAL) cultures was $>10^5$ colony-forming units (CFU)/mL. BAL samples were positive in 70 (42.4%) children, the bacterial isolate being *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. Children with bacterial co-infection required ventilatory support for longer than those with only RSV. Overall, 45% (74/165) received antibiotics before admission to the PICU. Gender, co-morbidity, origin, prior antibiotics, time on preceding antibiotics, admission oxygen, and ventilation index were not predictive of positive bacterial cultures [55]. Besides concurrent bacterial infections, preterm birth further increases the risk of prolonged hospitalization and PICU admission in infants hospitalized with RSV disease. In a retrospective cohort analysis of the Department of Pediatrics of the Medical University Graz, out of 464 infants (median age 2.75 months) hospitalized due to RSV infection, 42 (9.1%) were born <37 weeks gestational age (median 32, range 26–36) [56]. Admission to intensive care unit occurred in 9 of the 42 preterm (21.4%), compared to 27 of the 422 term (6.4%) infants ($p<0.0001$). Concurrent bacterial infections, diagnosed by either blood or urine cultures, stool culture, tracheal aspirates or smears, were detected in 17 of all cases (3.7%); 4 of 42 in preterm (9.5%) infants, compared to 13 of 422 term (3.1%) infants ($p = 0.017$). Cultures were considered positive if $>10^4$ (CFU)/mL of a single organism was detected, or 3+ bacteria were observed in tracheal aspirates or smears of relevant bacteria (on a scale of no bacteria to 3+). Ten of the 17 infants with positive cultures, including the 4 preterm, were admitted to the ICU. These four preterm infants (gestational age ranging between 31 and 36 weeks) were diagnosed with pneumonia, and isolates were *Streptococcus pneumoniae*, *Chlamydia pneumoniae* and *Streptococcus pneumoniae* with *Haemophilus influenzae*. Mean hospital stay was doubled (22.3 days) in preterm infants with bacterial co-infection compared to ones without it (10.3 days) [56]. Gram-negative pathogen prevalence at nasopharyngeal level and prior antibiotic treatment appear to add to the higher risk. A prospective, observational cohort study, of 136 infants with RSV bronchiolitis enrolled at 24 h of hospitalization, 104 on the ward and 32 in the PICU [57]. As compared to an age-matched group of healthy controls, RSV bronchiolitis infants had more frequent nasopharyngeal colonization with *non-typable Haemophilus influenzae* or *Moraxella catarrhalis* (both Gram-negative bacteria) and *Staphylococcus aureus* and *Streptococcus pneumoniae* (both Gram-positive bacteria). A higher proportion of Gram-negative pathogens was associated with higher blood neutrophil percentages, greater number of nasal wash leukocytes, increased concentrations of plasma IL-8 and IL-6, a trend towards increased disease severity and longer need for oxygen. In this study, 47% of all RSV infants and 75% of those in PICU received antibiotics prior to sample collection. This common use of antibiotic was associated with enhanced disease severity as defined by more prolonged oxygen administration and more frequent radiographic lobar consolidations [57]. The elevated antimicrobial-resistance in bacterial superinfection and the higher risks of enhanced disease severity further highlight the need for microbiological investigation of airway secretion in this patient population. Concomitant bacterial infection can occur even more frequently in older children hospitalized with RSV bronchopulmonary

infections, as shown in a two-center study from Japan [58]. Pathogenic bacteria were isolated from sputum bacterial culture in 43.6% of 188 pediatric inpatient, aged up to 5 years, the three most frequently isolated being *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*. Before admission, 28.7% of the patients received oral antibiotics, most often macrolides and amoxicillin. All *Streptococcus pneumoniae* strains were penicillin G sensitive but 38.9% of the *Haemophilus influenzae* strains isolated were ampicillin-resistant [58]. Finally, the introduction of seven-valent pneumococcal conjugate vaccine (PCV7) was associated with a substantially reduced incidence of vaccine-serotype invasive pneumococcal disease in young children, with declines in hospitalizations for childhood pneumonia (59). Because of the complex interaction between *Streptococcus pneumoniae* and RSV [8,60], a study was performed to evaluate whether changes in pneumococcal disease following the PCV7 introduction could led to variations in RSV epidemic timing and magnitude [61]. Evaluating hospitalization data from community hospitals in 18 U.S.A. states, comparing the 1992/1993 and 2008/2009 seasons, a significant decline in RSV-coded hospitalizations was demonstrated in children aged <1 y and 1–2-year-old following PCV7 introduction in 2000 [61]. The observed decline in RSV hospitalizations could be consistent with the notion that RSV can directly bind to pneumococcal surface proteins and influence the virulence of the bacteria [8,58] and/or that some fraction of the infants diagnosed with RSV have a mixed bacterial–viral etiology [61]. In some instances, co-infection with pneumococcus could increase the severity of an RSV infection and lead to hospitalization, with only RSV infection being diagnosed. In such a scenario, elimination of virulent pneumococci with PCVs would reduce the rate of hospitalizations carrying the diagnosis of RSV. Moreover, the estimates of postvaccine modifications could have been affected by confounding factors, such as by changes in medical practice and in the use of prophylaxis with the anti-RSV humanized monoclonal antibody, palivizumab [61]. We were unable to find similar analyses on the effect of the subsequent introduction of PCV13.

5. NASOPHARYNGEAL BACTERIAL COMMUNITY AS SHIELD AGAINST RESPIRATORY INFECTIONS

Nasopharyngeal bacteria may counteract viral infection and bacterial superinfection. In infant mice, nasal priming with viable *Corynebacterium pseudodiphtheriticum*, a Gram-positive bacterium member of the upper respiratory tract normal microbiota, improved their resistance to primary RSV infection and secondary pneumococcal pneumonia [62]. This resistance was thought to be related to modulation of Toll-like receptor 3 activation, leading to enhanced production of the antiviral cytokines IFN- β and IFN- γ , and of IL-10, that could contribute to restrict the inflammatory reaction and ultimately to limit tissue injury. Improved lung CD3+CD4+IFN- γ +, and CD3+CD4+IL-10+ T cells as well as CD11c+SiglecF+IFN- β + alveolar macrophages numbers were also detected in lungs of treated infant mice, associated with a significant decrease in viral load and lung tissue damage markers. In the same study, priming with viable *Corynebacterium pseudodiphtheriticum* also reduced lung bacterial cell counts and prevented *Streptococcus pneumoniae* dissemination into the blood [62]. Thus, a possible prospective use of these research results could be a seasonal application of a nasal probiotic spray to boost respiratory innate immunity in immunocompetent subjects. Bearing in mind the “gut-lung axis” interaction, one could consider orally given

probiotics or bacterial derived products as alternative options [63,64], the latter being an attractive proposition the theoretical efficacy of which is currently being actively investigated in a large NIH funded study assessing the effect of bacterial lysate to prevent lower respiratory tract infections in children at risk for asthma [65]. A possible approach can be to determine in germ free mice models the effect of orally given probiotics or bacterial derived products on immune maturation and the capacity to mimic the microbiota effect in respiratory virus infection.

6. CONCLUSIONS

The bidirectional interaction between resident nasopharyngeal bacterial communities and RSV can play an important role in modulating the susceptibility to respiratory infections in infants and young children. Early negative changes in microbial community composition, due to exposure to noxious environmental factors, may constitute a heightened risk for severe RSV respiratory infection, delayed viral clearance, increased risk of recurrent wheezing in later life (figure 4A). On the other hand, RSV can enhance bacterial adhesion and virulence, promoting superinfections, whilst enrichment of upper airways microbiota with specific Gram-positive bacterium members may increase resistance to primary RSV infection and secondary pneumococcal pneumonia (figure 4B). The risk of serious bacterial superinfection in infants with RSV infection is low, in those with mild RSV bronchiolitis, but becomes substantial when hospital admission with PICU is required. The elevated antimicrobial-resistance observed during bacterial super infections in these infants mandates careful stewardship and avoidance of inappropriate antibiotic prescription. We all should keep in mind that antimicrobial treatment does not eradicate bacteria from the nasopharynx and that the reduction in carriage is temporary and is followed by a rapid replacement, with overgrowth of more resistant strains, newly acquired or previously masked by other organisms [66]. The finding that specific groups of microorganisms might be associated with protection against RSV infection and reduction of disease severity suggests that studies should be designed to understand the mechanistic insights into the potential positive role of these species in disease prevention. Further studies are also needed to investigate the possible short and long-term benefits of microbiome therapeutic manipulation and immune training with the goal of improving patient outcomes.

CONFLICT OF INTEREST

The author declares no conflicts of interest.

ETHICAL APPROVAL

Because of category of the manuscript, a review, approval from the ethical committee was not necessary.

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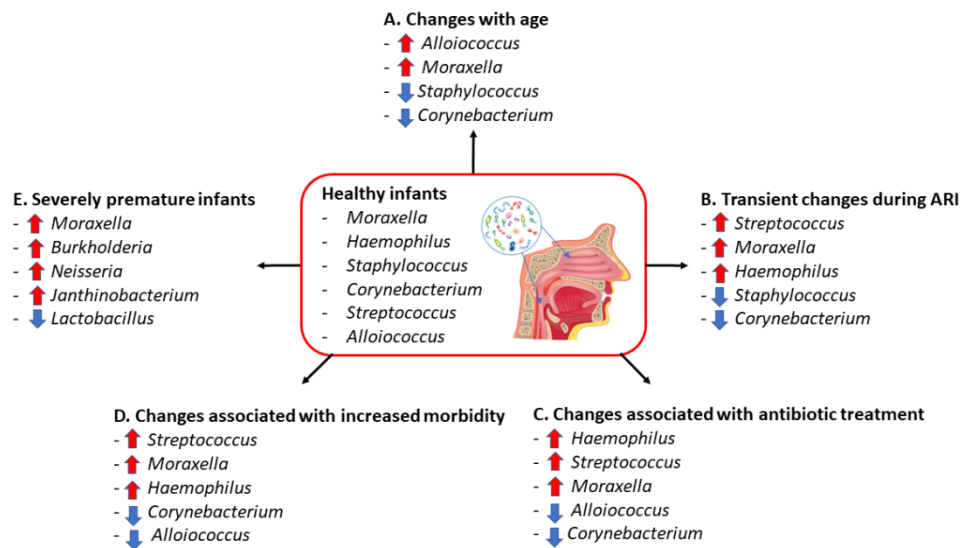
Figure legends

Figure 1. Nasopharyngeal microbiome clusters in healthy infants and changes associated with age, acute respiratory infections (ARI), antibiotic treatment, increase morbidity and severe prematurity.

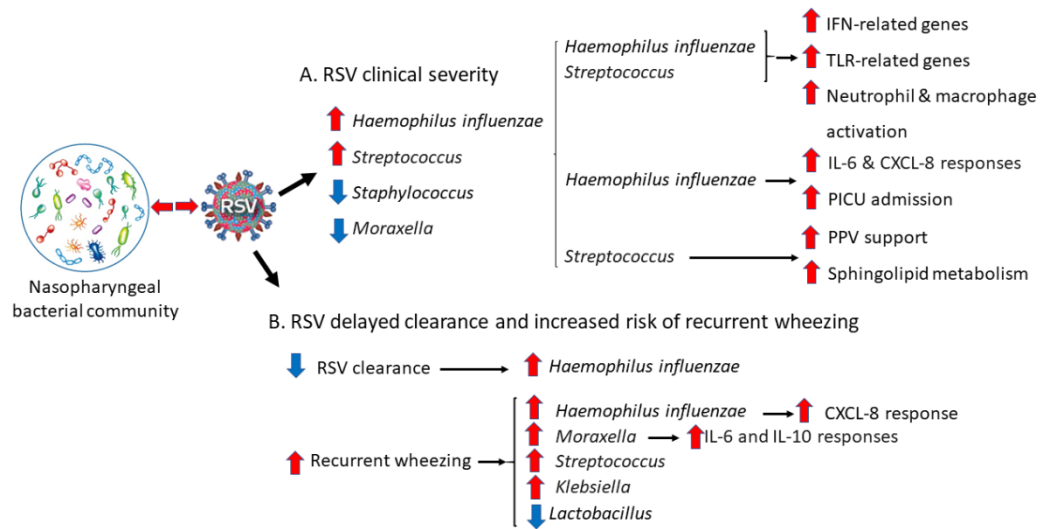
Figure 2. Changes in microbial community composition and their effects on RSV disease severity, delayed viral clearance and increased risk of recurrent wheezing.

Figure 3. The severity of RSV and bacterial superinfection. A. Mild RSV infection and the risks of severe bacterial superinfection and of urinary tract infections. B. Severe RSV infection, pathogens associated with bacterial pneumonia and leading to worsening of RSV infection progression.

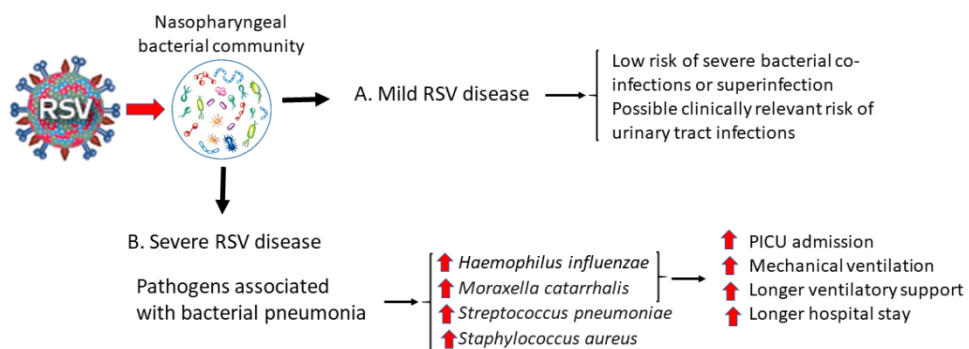
Figure 4. The bidirectional interaction between nasopharyngeal microbiota and RSV infection. A. Early changes in microbial community composition and their effects on RSV infection and recurrent wheezing later in life. B. Enhancement of pathogenic bacterial adhesion and virulence by RSV, may promote bacterial superinfections, whilst upper airways microbiota enrichment with specific commensal bacteria may increase resistance to primary RSV infection and secondary bacterial infections.



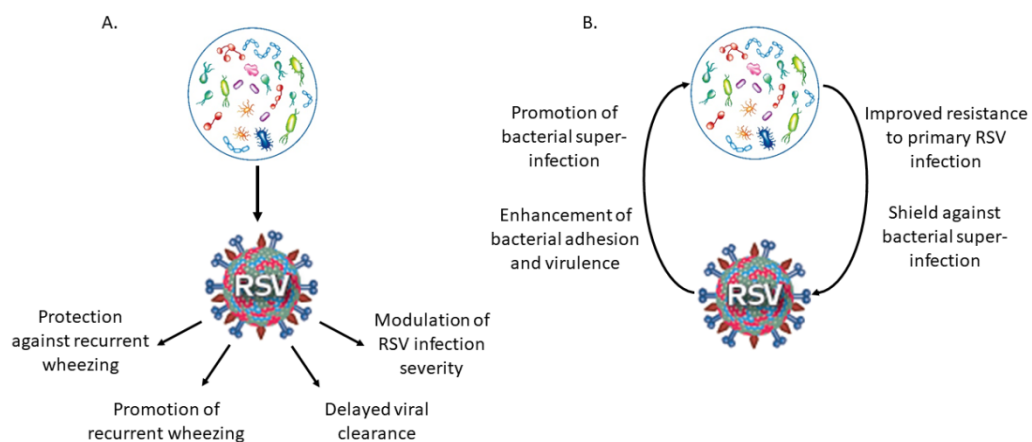
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