BIOMARKERS FOR DISEASE SEVERITY IN CHILDREN INFECTED WITH RESPIRATORY SYNCYTIAL VIRUS (RSV): A SYSTEMATIC LITERATURE REVIEW

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Abstract

Background: The clinical manifestations of RSV infection vary widely from mild, self-limiting illness to severe life-threatening disease. However, there remain key gaps in knowledge of biomarkers to objectively define severe disease and predict clinical outcomes.

Objective: To systematically review identified biomarkers associated with, or predictive of, severe RSV disease in infants and young children.

Methods: A systematic search was performed spanning the period between 1945 to March 2019 on the following databases: Ovid Medline, Embase, Global health, Scopus, and Web of Science. Risk of bias was assessed using the Cochrane tool.

Results: A total of 25,132 abstracts were screened and two review authors independently assessed studies for quality, risk of bias and extracted data. This revealed 111 studies that met the inclusion criteria, of which the majority had a low risk of bias. RSV severity was found to be correlated with reduced T cell and B cell populations, robust innate immunity (*e.g. OLFM4* gene expression in blood), neutrophil mobilisation to the lungs and blood (*e.g. MMP8* and *CXCL8* gene expression in the lungs and/or blood), decreased Th1 innate immunity (*e.g.* decreased IFN-γ cytokine production in the lung) and Th2 shift (*e.g.* increased IL-4:IFN-γ cytokine ratio in the lungs and blood). Microbial exposures in respiratory tract may contribute to neutrophil mobilisation to the lungs of the infants with severe RSV compared with mild RSV disease (*e.g. Haemophilus* presence associated with mucosal IL-8 expression).

Conclusions: Although a wide range of biomarkers have been associated in the literature with RSV disease severity, robust validated biomarkers are lacking. This review illustrates the broad heterogeneity of study designs but also high variability in the definition of “severe” RSV disease.

Hence, prospective studies are required to validate these biomarkers. Additional research investigating epigenetics, metabolomics and microbiome hold promise to reveal novel biomarkers for RSV disease.

Key words: *Respiratory syncytial virus* (RSV); Biomarkers; Bronchiolitis; Lower respiratory tract infection (LRTI); Severe RSV disease; Infant

Introduction

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection (LRTI) in children under-five years old and was responsible for around 33.1 million infections globally in 2015 in this age group[1]. Despite significant efforts over the past decades to develop a better understanding of RSV, there remain significant gaps, including identification of robust molecular markers as predictive tools or biological correlates of disease severity in infants. Host characteristics and microbial exposures during early life may have long-term consequences by altering the immune response and making an infant more susceptible to severe disease. Based on a systematic literature review, we describe the host and microbial factors that have been reported in association with RSV disease severity characteristics, including humoral and cellular immunity, cytokine/chemokine response, genetics, transcriptome, epigenetics, and microbiome.

Methods

Search strategy and selection criteria

A systematic literature review was performed using a combination of search terms ('Human respiratory syncytial virus', 'respiratory syncytial virus' or 'RSV' in human studies) in the Ovid Medline, Embase, Global health, Scopus and Web of Science databases including studies from 1945 to March 2019. No restriction on study design, language or publication were initially applied. Additional articles were identified by scanning of reference lists of identified citations. The inclusion and exclusion criteria (eligibility criteria) of studies are listed in **table 1**. Data selection was performed according to the PRISMA guidelines[2]. The PRISMA flow diagram is shown in **supplementary figure 1**.

Definitions

RSV infection was identified definitively on the basis of a diagnostic test of body fluid including polymerase chain reaction (PCR), viral culture, or antigen test. Biomarkers were defined as any traceable biological parameter/substance that was measurable. Age group inclusion in this study was based on the World Health Organisation (WHO) definition for children; namely, studies including subjects aged 18 years or above were excluded. The control group was defined as a population without RSV infection, without respiratory infection associated with other pathogens or infants without respiratory diseases.

Quality of evidence

Each included study was reviewed by two review authors using the Cochrane tool for quality assessment using Grade Guidelines[3]. The review was registered with PROSPERO (registration

number: CRD42019119615) and conducted according to PRISMA guidelines. The risk of bias result is shown in **supplementary figure 2**.

RSV severity classifications

Throughout the different articles surveyed in this review, an RSV case is defined as 'severe' based on one or more clinical parameters, *e.g.* the 'duration of' and/or the 'need of' one or more of the following parameters: hospitalisation[4,5], oxygen supplementation[6,7], and mechanical ventilation[8,9]. RSV severity scores, such as Wood's Clinical Asthma Score (M-WCAS)[10] or a modified scoring system[11], incorporate different clinical endpoints. Most of these severity scoring systems have, however, been designed for evaluation by medical professionals in a hospital setting where mostly severe disease is observed, but not by parents or to assess mild disease in a community (outpatient) setting. The ReSVinet scale has been proposed to be useful and reliable in paediatric infectious diseases, either recorded by paediatricians or parents[12].

The focus of scoring systems towards more severe disease symptoms poses challenges for biomarker research on diversity in RSV disease severity. Indeed, differences in the classifications of RSV severity may lead to conflicting results. For instance, when infants with LRTI were graded for severity, IL-8 concentrations in blood has been positively associated by a number of studies[10,13–16]. However, a few studies did not confirm the association with RSV severity parameters, even though increased IL-8 was observed between control and RSV patients[4,17]. This difference in the outcome may potentially be attributed to the diversity in RSV severity parameters and to the definition of mild, moderate, or severe RSV disease in different studies. A universally applied scoring system would be beneficial to investigate biomarkers related with RSV disease severity.

In the supplementary tables of this review, we comprehensively summarised the literature findings, *i.e.* associations (positive, negative, or no association when applicable) of the biomarkers and the severity parameters, enabling the reader to balance and judge the underlying evidence. When an association was noted by more than one evidence/study, we listed the corresponding biomarker as

a potential biomarker for RSV severity, regardless of whether the same biomarker led to conflicting data in other studies due to study design limitations.

Risk factors for severe RSV disease in infants

Prematurity

Infants who are born preterm are 3 times more likely to be hospitalised upon RSV infection than are term infants[18]. This may be attributed to differential immune profiles of infants who are born preterm, *e.g.* lower neutrophil proportions[19] or reduced TLR4 surface protein and mRNA expression in monocytes[20]. The reduced *TLR4* mRNA expression has been correlated with decreased IL-1β, IL-6 and TNF-α production in the whole blood *ex-vivo*. Decreased cytokine release leading to supressed innate immunity may create susceptibility to infections of the prematurely born infant[20]. In RSV disease, differential *TLR4* mRNA expression has been also shown between preterm versus term infants with RSV bronchiolitis[21]. Conversely, in preterm infants with RSV bronchiolitis, blood neutrophil *TLR4* mRNA expression was higher compared to term born infants with RSV bronchiolitis despite reduced surface protein levels in blood and bronchoalveolar lavage (BAL), indicating possible impaired TLR4 dependent pathway in preterm infants[21].

Although being born prematurely has been described as an important risk factor for severe RSV, it has been estimated that approximately 80% of RSV-related hospitalisations occur in previously healthy, term born infants[18]. Genetic background and demographics, such as young age and male sex, and external exposures including maternal smoking, absence of breastfeeding, having siblings, and crowding have also been identified as risk factors for RSV-related bronchiolitis[22].

Effect of age

Infants less than 2 months old more frequently suffer from severe RSV infection, comprising 44% of RSV-related hospitalisations[18]. During the first few months of life, maternal antibodies play a role

in protection of infants from bacterial and viral exposures, although this protection is only partial. For instance, infants younger than 3 months were found to display the highest rate of positivity for maternal RSV-specific IgG antibody, however, the avidity of IgG was found to be low compared with older infants[23].

Age-related differences were found also at the transcriptional level in RSV-infected infants. Mejias *et al*. showed that transcriptional profiles of RSV-infected younger infants (< 6 months) have reduced expression of genes related to innate and adaptive immunity when compared with age-matched controls, versus equally ill older infants (6-24 months) when compared with age-matched controls, indicating overall suppressed immunity in younger infants. [24].

In RSV disease, infants' immunity is reported to have a limited T helper 1 (Th1) antibacterial and antiviral response, which is an important host defence system[25]. A T reg and T helper 2 (Th2) skewed response upon RSV infection in infants is assumed to contribute to disease severity and limit recovery[25]. This time window may also predispose the infant to environmental exposures such as permitting the colonisation by commensals in the intestine and respiratory tract. Consequently, Th2 skewed immune response upon RSV infection is particularly important to emphasise when studying the long-term consequences, such as allergy and asthma, of severe RSV infection during infancy.

Host genetics as a potential predictive biomarker

To investigate the effect of host genetics in RSV hospitalisation, Thomsen *et al*. conducted a study with more than 12,000 pairs of twins[26]. They concluded that host genetics contributed up to 16% of the risk for RSV-related hospitalization during infancy, but their study design did not allow identification of the underlying genes[26].

The underlying genetic studies in this systematic review applied a targeted, gene-specific approach. Complementary unbiased genome-wide (*i.e.* whole genome sequencing) studies hold the potential to identify novel genetic biomarkers of severe RSV disease susceptibility. Of note, one reported genome-wide association study (GWAS) in the RSV field focused on predisposition to RSV bronchiolitis rather than the association with RSV disease severity (and without assessing disease severity parameters)[27]. Here we listed potential biomarkers of RSV disease severity assessed by the targeted, gene-specific approach.

Specific genetic polymorphisms in several genes were identified to be associated with predisposition to severe RSV disease[28]. In this review, significant associations of genetic variations with RSV severity were reported in genes involved in Th1 immune response (*e.g. IFNG*)[29,30], cytokines associated with the recruitment of neutrophils (*e.g. CXCL8*)[31] and other pro-inflammatory or anti-inflammatory cytokines *(e.g. IL1RL1, IL-6, IL10)*[30,32,33], surfactant proteins (*e.g. SFTPA2, SFTPD*)[34,35], RSV receptors on respiratory epithelium (*e.g. TLR4* and *CX3CR1)*[7,36,37], cannabinoid receptor (i.e. *CNR2*)[5], and vitamin D receptor (*i.e. GC*)[38]. The list of specific polymorphisms in each gene and associated amino acid and codon change was shown in **supplementary table 1**.

The most biologically plausible evidence from independent studies investigating genetic associations and RSV severity were shown with polymorphisms in *IFNG*[29,30], *IL10*[30,33], and *TLR4*[37] genes.

Biomarkers for severe RSV disease

Investigating biomarkers for severe RSV disease in the respiratory tract

The epithelium in the lung is coated with a layer of viscous mucus covering cilia of the epithelial cells containing mucins. Mucins not only act as a barrier between environmental exposures and the lung epithelium but also regulate the immune response and activate signal transduction pathways[39]. In

transcriptomics analysis, a negative correlation between *TSPAN8* (encoding a cell surface protein TSPAN-8), *MUC13* (encoding to an epithelial cell surface protein MUC-13), *MSP* (immunoglobulin binding factor), and a positive correlation with *CCL7* mRNA levels (a chemokine attracting macrophages and degrading components of extracellular matrix) and RSV severity was shown[40].

RSV-F and RSV-G proteins have been shown to interact with epithelial cell receptors (*e.g.* TLR4 and CX3CR1) and this interaction is related to RSV disease severity. For instance, Zhivaki *et al*. showed that RSV virus infects neonatal-specific regulatory B cells (nBreg) via interaction with the RSV G protein and CX3CR1 receptor on the cell, promoting IL-10 production and downregulating Th1 immunity[41].As shown in supplementary Table 1, single nucleotide polymorphisms (SNPs) in genes encoding RSV receptors (*TLR4* and *CX3CR1*) on respiratory epithelium have been reported[7,36,37]. This may have transcriptional and translational consequences, leading to changes in host immune response upon RSV infection. For instance, lower *CX3CR1* mRNA levels have been reported in infants with prolonged wheezing[42].

RSV infection has been associated with robust innate immunity and neutrophil infiltration[43]. Consistent with this, mRNA expression of nasopharyngeal samples from infants have been characterised by alterations in recruitment of neutrophils. A positive correlation between the mRNA levels of *CXCL8* (a neutrophilic chemotactic factor) levels and RSV disease severity (infants requiring oxygen or mechanical ventilation) has been observed[44]. Although increased concentrations of IL-8 was linked with RSV disease severity[45–47], no significant differences in IL-8 concentrations were found between RSV-induced upper respiratory tract infection (URTI) and bronchiolitis in nasopharyngeal secretions[48,49]. These contradictory results might be attributed to the differences in RSV severity classifications. Although neutrophils aid in clearing the infected cells and inhibiting

viral replication, they release reactive oxygen species (ROS) which potentially also damage the lung epithelium and may predispose infants for subsequent susceptibility to allergy and asthma.

Decreased IFN-γ cytokine levels have been found in respiratory samples from infants with severe RSV disease[6,9,50–53] . Similarly, a negative correlation between IFN-γ mRNA levels and RSV disease severity has been reported in nasopharyngeal samples[54], indicating a supressed type II IFN (IFN-γ) response. A positive association between IL-4:IFN-γ ratio in respiratory samples and gene expression levels has been shown[51], indicating a Th2 shift in the immune response in severe RSV disease. All cytokines associated with RSV disease severity (or those with conflicting evidence for their role) in respiratory samples are listed in **supplementary table 2.**

In nasopharyngeal samples, the association with RSV severity has been found to be negative for IFN-γ[6,9,50–53], and positive for IL-1β[45,47], IL-6[14,17,45,47], IL-8[45–47], and MIP-1β[17,45] cytokines. RSV disease severity has been associated decreased Th1 response and neutrophil recruitment.

Microbiome of the lung

The microbiome refers to the genetic materials of microorganisms in a specific environment (referred to as microbiota). Although still limited, there is growing evidence that suggests an association between the nasopharyngeal microbiome and RSV disease severity and predisposition to severe RSV disease during infancy (**supplementary table 3**)[55].

In a study conducted in Western Australia, it was reported that in children up 2 years old the nasopharyngeal microbiome is composed predominantly of six bacterial genera: *Moraxella* (40.1%), *Streptococcus* (13.3%), *Corynebacterium* (12.1%), *Alloiococcus* (11.1%), *Haemophilus* (8.6%), and

Staphylococcus (4.2%)[56]. An abundance of *Moraxella*, *Streptococcus,* and *Haemophilus* was positively associated with LRTI when compared to URTI in acute respiratory infections including RSV infected infants after adjustments for confounders[56,57]. The data also show that after adjustment to the detected virus, *Streptococcus*, *Haemophilus*, and *Moraxella* microbiome profile groups remained significantly associated with the respiratory symptoms, indicating that both bacteria and viruses may independently contribute to disease[56]. Jiang *et al*. tested nasal aspirates from 608 subjects (< 2 years old) with bronchiolitis and observed that when pathogenic bacteria were present (*e.g*. *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, or *Staphylococcus aureus*), the percentage of neutrophils was higher and the length of hospital stay was longer (4 days vs. 3 days) . In this study, infection with RSV and *Haemophilus influenzae* was found to be an independent risk factor for longer duration of hospitalisation[58]. Consistent with this observation, Suárez-Arrabal *et al*. also observed that infants whose nasopharyngeal microbiome was predominantly colonised with Gram-negative bacteria (*Moraxella catarrhalis, Haemophilus influenzae*) needed a longer duration of oxygen supplementation, had higher plasma IL-6 and IL-8 (but not TNF) levels and higher neutrophil counts when compared with infants whose nasopharyngeal microbiome was predominantly colonised with Gram-positive bacteria (*Staphylococcus aureus, Streptococcus pneumoniae,* β*-hemolytic Streptococcus*)[59]. Consistently, the presence of *Haemophilus* was associated with increased IL-8 chemokine levels, previously also related to RSV disease severity and neutrophil recruitment[60]. In a study by de Steenhuijsen Piters *et al*., an *Haemophilus influenzae* and *Streptococcus* dominated microbiota was associated with RSV infection and related hospitalisation in infants younger than 2 years old[61]. Although no correlation was found between IFN-related genes and the nasal microbiome, *Haemophilus influenzae* and *Streptococcus* dominated microbiota were associated with increased mRNA expression of Toll-like receptor linked genes and by neutrophil and macrophage activation[61]. On the other hand, an

abundance of *Staphylococcus aureus* was negatively associated with RSV infection and related hospitalisation[61]. Brealey *et al*. correlated the detection of *Streptococcus pneumoniae* with RSV disease severity assessed by the RSV severity clinical disease severity score (Woods clinical Asthma Score) in infants younger than 2 years old [62].

Overall, for nasal microbiome data, the association with RSV severity has been found to be positive with an abundance of *Moraxella catarrhalis* [56,57,59], *Haemophilus influenzae* [56–61], and *Streptococcus pneumoniae* [56,57,61,62].

Biomarkers of severe RSV in blood

Biomarkers in blood not only serve as a surrogate for studying lung immunity and biomarkers associated with severe RSV infection in the lung but also provide information about the systemic immune response triggered by the infection.

Studies of cytokine response have shown conflicting evidence, probably due to marked heterogeneity in study design and sample size. Although the data suggest a predominantly decreased *IFN-γ* production in nasal samples, in blood the data are conflicting with either positive association[63], negative association[13,64–66], or a lack of association in several studies (**supplementary table 4**). Although conflicting evidence exists, some data indicate positive associations of other pro- and anti-inflammatory cytokines in blood and RSV disease severity, such as IL-6[4,11,14,15,47], and IL-8[10,13–16], , and negative association of IL-4[13,63], IL-12 and RSV disease severity[8,65], cytokines involved in differentiating T cells into Th2 and Th1 cells. Other cytokines that have been associated with RSV disease severity (or those with conflicting evidence for their role in) in blood are listed in **supplementary table 4.**

Whole blood transcriptomics may provide more robust information on blood cell immunity in severe RSV disease. Mejias *et al*. demonstrated that investigating the transcriptome can assist in discriminating the severity of RSV disease by translating gene sets into a biologically relevant context, such as postulating changes in cellular populations in relations to RSV severity[24]. For instance, RSV severity is associated with the immune dysregulation, *e.g.* overexpression of neutrophil-related gene sets, and inflammation and interferon genes, and suppression of T and B cells-related gene sets[24].

Supplementary figure 3 lists 18 differentially expressed genes that overlap in two studies comparing mild and severe RSV disease [67,68]. The role and function of these overlapping genes is described in the literature and their identification is consistent with the finding of neutrophil recruitment in severe RSV disease[24]. For instance, *MMP8* gene expression has been identified in multiple studies and may regulate neutrophil recruitment from the periphery to the lung[67–69]. Also, elevated *CXCL8* mRNA transcription[44,67] and increased IL-8 cytokine production observed in blood or respiratory samples[10,13–16,45–47] provide supporting evidence for enhanced neutrophil recruitment. These results hold the potential for future diagnostic applications determining severe RSV patient groups[70]. In blood samples, the association with RSV severity has been found to be negative for IFN-y[64,65] and IL-12 [8,65], and positive for cytokines IL-6 [4,14,15,47] and IL-8 [10,13–16] . Additionally, RNA expression of genes involved in neutrophil recruitment (e.g. *MMP8*, *CXCL8, MPO*) were upregulated[44,67–69].

Effect of humoral immunity

Antibodies mediate protection from infection through binding and neutralisation of RSV, therefore they are potentially an important component in protection from severe RSV infection.

In the same age group (<3 months), lower avidity of RSV-specific IgG antibodies was found in RSV-infected infants when compared with healthy controls[71]. However, RSV illness severity was not correlated with several serological characteristics (*i.e.* RSV-IgG antibody titers, avidity of RSV-IgG,

virus neutralising capacity, titers against pre- and postfusion F or G protein ectodomains, and the prefusion F antigenic site Ø)[71]. Although high concentrations of maternal RSV neutralizing antibodies were shown to protect against RSV hospitalisation before 6 months of age, no correlations were found with the severity of illness amongst the infants who were hospitalised[72]. Similarly, no significant difference was observed in RSV antibody concentration as tested by an RSV antibody neutralisation assay when compared between RSV-induced upper and lower respiratory tract infection (< 6 months)[73]. In infants older than 2 years, it was found that the avidity of RSV-specific IgG was lower in infants with RSV-induced LRTI than those with RSV-induced URTI, indicating the protective role of high avidity of RSV-induced IgG in this age group[23].

Interestingly, B cells - as a part of humoral and adaptive immunity - may also play a role in RSV disease severity. B-lymphocyte stimulating factors, such as APRIL (proliferation-inducing ligand), and anti-viral antibodies of IgA and IgM (but not IgG), were associated with better oxygen saturation, indicating a protective role in RSV disease[74]. Capella et al. reported a negative correlation between the concentrations of pre-F and G antibodies (but not post-F antibodies) and clinical severity score in infants younger than 2 years of age[75]. Deleterious effects of IgE have been reported in infants with pneumonia, LRTI, and higher degree of hypoxia[76–78].

In the literature protective effects of RSV-specific IgG[23], pre-F and G antibodies[75], and deleterious effect of IgE antibodies[76–78] were reported. However, The conflicting data may likely be attributed to the small sample size of the included studies, considering other risk factors are also involved (*e.g.* gestational age, birthweight, maternal smoking, siblings, day care). Larger studies are needed to find a difference in outcome.

Conclusions and next steps

The main findings of this review are graphically summarized in **figure 1**. Severe RSV disease is associated with a suppression of T cell, B cell and cytotoxic NK cells, robust innate immunity, and neutrophil mobilisation to the respiratory tract and blood. Neutrophil infiltration to the lung might be mediated through upregulation of *MMP8 and CXCL8* mRNA expression and increased IL-8 cytokine production. Also, peripheral *OLFM4* mRNA expression, as a marker of innate immunity, has been reported as a biomarker to discriminate between mild and severe RSV disease in infants.

Downregulation of IFN-y cytokine in respiratory samples, suppression of T cells and Th2-skewed response have been associated with severe RSV disease. The Th2-skewed innate immune response may permit microbiota colonisation in the lung. Airway microbiome may be affected by, or lead to, recruitment of neutrophils to the lung.

Although published data are conflicting, humoral immunity may also affect the susceptibility to severe infection. Pre-F and G antibodies[75] and RSV-specific IgG[23] may have a protective effect and IgE levels have been reported to increase the risk of RSV severity.

Early reports are available on investigations of epigenetic alterations, such as DNA methylation[45,46] and miRNA[79], and metabolomic biomarkers[80], in relation to RSV severity. As more data will become available, investigations on these endpoints may hold promise for future biomarker research.

It should be noted that the review included biomarkers associated with severe RSV disease, but the robustness of the conclusions may be limited because of large heterogeneity amongst included studies (e.g. definition of “severe RSV” cases, low number of studies per biomarker, varying significance of biomarker data within studies). Therefore, caution is required before drawing

definitive conclusions. Future additional large prospective trials investigating RSV disease severity, combined with biomarker analysis strategies that also include novel and high-dimensional readouts are needed in order to deliver and validate robust biomarkers for disease severity that can bring value to clinical practice.

Notes

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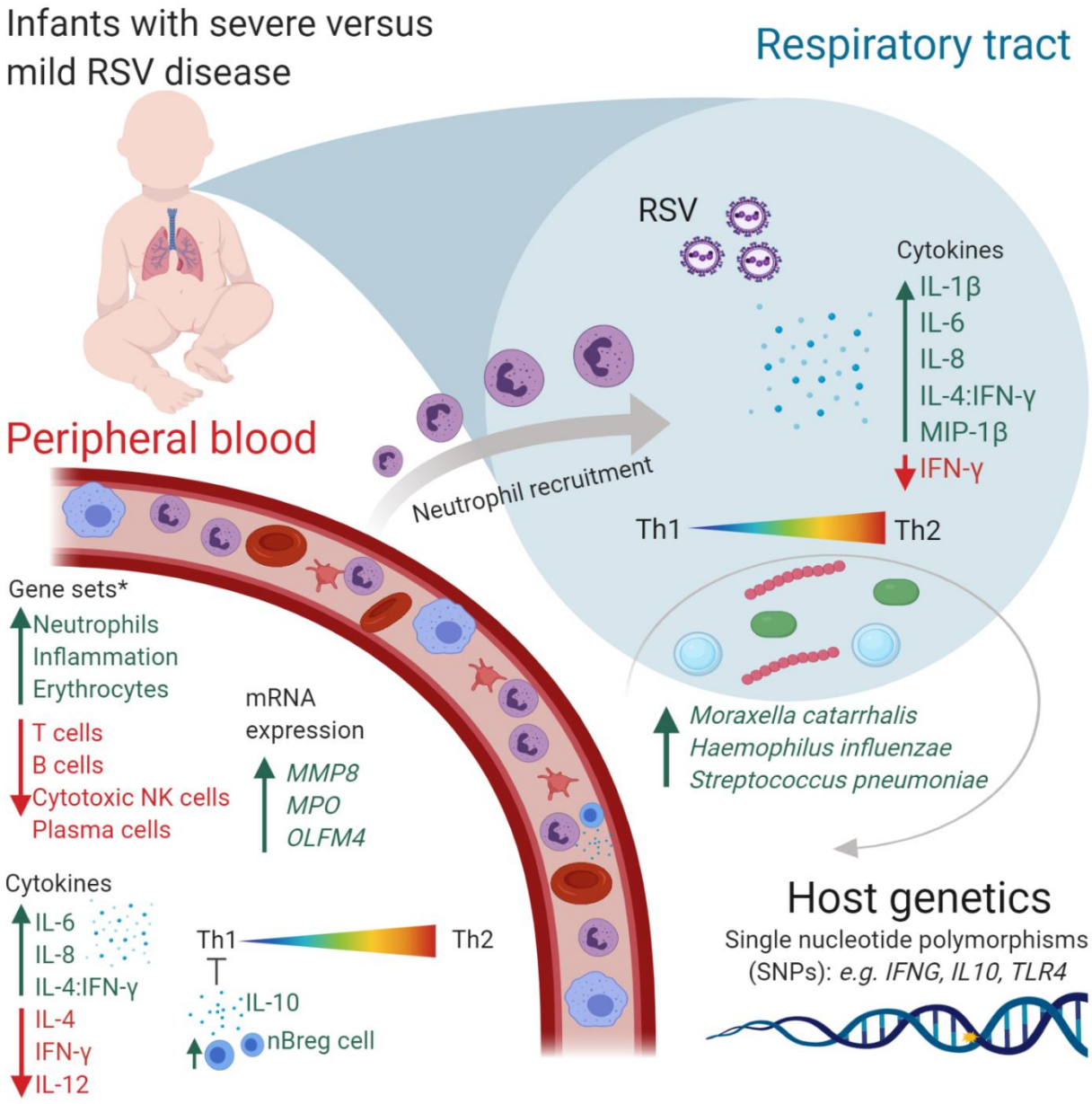
566 Figures and Tables



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568 **Figure 1**: Graphical abstract of the main findings of the systematic literature review on RSV disease severity.
569 The figure distinguishes the proposed biomarkers of severe RSV disease (in comparison to milder disease
570 forms) that can be detected in blood, in respiratory samples and in host DNA. The biomarkers selected for
571 this figure are supported by at least two publication reporting similar results and extrapolated from minimal
572 conflicted evidence. Generated using BioRender.

Table 1: Eligibility criteria

Inclusion	Exclusion
▪ Human RSV studies ▪ Severity of RSV infection assessed ▪ Biological marker investigated ▪ Relation explored between biomarker and severity of RSV infection ▪ Studies written in English, French, Spanish, Italian or Portuguese ▪ Studies including subjects aged below 18 years	▪ Studies in animal models ▪ Studies in cell lines ▪ Studies in adults ▪ Studies focused on treatment, diagnostics or epidemiology of RSV infection ▪ Studies without a clear definition of disease severity ▪ Studies without a definitive RSV diagnosis ▪ Studies on antibodies or viral characteristics. ▪ Literature reviews ▪ Studies written in any language other than those mentioned in the inclusion criteria