BMJ Open Respiratory Research

Nasal microRNA signatures for disease severity in infants with respiratory syncytial virus bronchiolitis: a multicentre prospective study

Michihito Kyo ,[1] Zhaozhong Zhu ,[1] Ryohei Shibata ,[1] Tadao Ooka,[1,2] Jonathan M Mansbach,[3] Brennan Harmon,[4] Andrea Hahn,[4,5,6] Marcos Pérez-Losada,[7] Carlos A Camargo,[1] Kohei Hasegawa[1]

To cite: Kyo M, Zhu Z, Shibata R, *et al*. Nasal microRNA signatures for disease severity in infants with respiratory syncytial virus bronchiolitis: a multicentre prospective study. *BMJ Open Respir Res* 2024;**11**:e002288. doi:10.1136/bmjresp-2023-002288

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/bmjresp-2023-002288).

Received 31 December 2023
Accepted 28 June 2024

For numbered affiliations see end of article.

Correspondence to
Dr Michihito Kyo;
mkyo@hiroshima-u.ac.jp

ABSTRACT

Background Respiratory syncytial virus (RSV) bronchiolitis contributes to a large morbidity and mortality burden globally. While emerging evidence suggests that airway microRNA (miRNA) is involved in the pathobiology of RSV infection, its role in the disease severity remains unclear.

Methods In this multicentre prospective study of infants (aged<1 year) hospitalised for RSV bronchiolitis, we sequenced the upper airway miRNA and messenger RNA (mRNA) at hospitalisation. First, we identified differentially expressed miRNAs (DEmiRNAs) associated with higher bronchiolitis severity—defined by respiratory support (eg, positive pressure ventilation, high-flow oxygen therapy) use. We also examined the biological significance of miRNAs through pathway analysis. Second, we identified differentially expressed mRNAs (DEmRNAs) associated with bronchiolitis severity. Last, we constructed miRNA–mRNA coexpression networks and determined hub mRNAs by weighted gene coexpression network analysis (WGCNA).

Results In 493 infants hospitalised with RSV bronchiolitis, 19 DEmiRNAs were associated with bronchiolitis severity (eg, miR-27a-3p, miR-26b-5p; false discovery rate<0.10). The pathway analysis using miRNA data identified 1291 bronchiolitis severity-related pathways—for example, regulation of cell adhesion mediated by integrin. Second, 1298 DEmRNAs were associated with bronchiolitis severity. Last, of these, 190 DEmRNAs were identified as targets of DEmiRNAs and negatively correlated with DEmiRNAs. By applying WGCNA to DEmRNAs, four disease modules were significantly associated with bronchiolitis severity—for example, microtubule anchoring, cell-substrate junction. The hub genes for each of these modules were also identified—for example, *PCM1* for the microtubule anchoring module, *LIMS1* for the cell-substrate junction module.

Conclusions In infants hospitalised for RSV bronchiolitis, airway miRNA–mRNA coexpression network contributes to the pathobiology of bronchiolitis severity.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Respiratory syncytial virus (RSV) bronchiolitis contributes to a large morbidity and mortality burden globally. Although airway microRNAs (miRNAs) are implicated in the immune responses in RSV infection based on case–control studies by applying microarray or qPCR techniques, no study has investigated airway miRNA signatures and miRNA–messenger RNA (mRNA) interactions to delineate the epigenetic mechanisms in severity in infants with RSV bronchiolitis.

WHAT THIS STUDY ADDS

⇒ By applying a small RNA sequencing, the nasal airway miRNA signatures were associated with bronchiolitis severity and related to the distinct biological pathways. Furthermore, the airway miRNA–mRNA coexpression networks were associated with bronchiolitis severity.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Airway miRNA plays important roles in the pathobiology or severity of RSV bronchiolitis.

BACKGROUND

Bronchiolitis is the most common viral lower respiratory infection in infants.[1] Of the respiratory viruses, respiratory syncytial virus (RSV) is the leading cause of bronchiolitis and contributes to a large morbidity and mortality burden globally.[2 3] Indeed, approximately 5%–13% of infants hospitalised for RSV bronchiolitis undergo mechanical ventilation.[2] While monoclonal antibody[4] and maternal vaccine[5] are effective in preventing infants from medically attended RSV infection, the main therapies for infants with RSV bronchiolitis remain limited to supportive care.[6] Additionally, the pathobiology of bronchiolitis severity remains unclear. Our limited understanding of the disease mechanisms has hindered efforts to develop effective therapies for this large patient population.

MicroRNAs (miRNAs) are small non-coding RNAs that induce messenger RNA

(mRNA) degradation and regulate protein translation by directly binding to their targeted mRNAs. Emerging evidence suggests that airway miRNAs are implicated in the production of inflammatory mediators (eg, nuclear factor-κB, interleukin (IL)-1ß, IL-6 and IL-8)[7–9] and immune cell responses (eg, T cell, macrophage)[10 11] in the airway infected by RSV. Yet, these earlier reports are based on a case–control design by applying microarray or qPCR techniques to characterise miRNAs.[7 9] Despite the clinical and research importance, no study has investigated airway miRNA signatures and miRNA–mRNA interactions to delineate the epigenetic mechanisms in severity in infants with RSV bronchiolitis.

To address this knowledge gap, by applying a small RNA sequencing (RNA-seq), we investigated the airway miRNA signature and miRNA–mRNA network for disease severity in infants hospitalised for RSV bronchiolitis. A better understanding of the role of the airway miRNA would provide insights into the pathobiology of disease severity and facilitate the discovery of a novel therapeutic target for RSV bronchiolitis.

METHODS

Study design, setting and participants

We analysed data from a multicentre prospective cohort study of infants hospitalised for bronchiolitis—35th Multicentre Airway Research Collaboration (MARC-35) study.[12] MARC-35 is coordinated by the Emergency Medicine Network (EMNet, www.emnet-usa.org), an international research collaboration with 247 participating hospitals. The details of the study design, setting, participants, data collection and testing may be found in online supplemental methods.

Briefly, MARC-35 investigators at 17 sites across 14 US states enrolled 1016 infants (age<1 year) who were hospitalised with an attending physician diagnosis of bronchiolitis during 3 bronchiolitis seasons (1 November through 30 April) from 2011 to 2014 (online supplemental table S1). The diagnosis of bronchiolitis was made according to the American Academy of Paediatrics bronchiolitis guidelines, defined as an acute respiratory illness with some combination of rhinitis, cough, tachypnoea, wheezing, crackles or retraction.[13] We excluded infants with pre-existing heart and lung disease, immunodeficiency, immunosuppression or gestational age<32 weeks. All patients were treated at the discretion of the treating physicians. Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research. Of 821 infants hospitalised for RSV bronchiolitis who were enrolled in MARC-35, the current analysis investigated 493 infants with RSV infection who underwent nasal airway small RNA-seq.

Data collection

Clinical data (patients' demographic characteristics, medical history, environmental and family, and details of the acute illness) were collected via structured interview and chart reviews using a standardised protocol.[14] All data were reviewed at the EMNet Coordinating Centre at Massachusetts General Hospital (Boston, Massachusetts, USA). In addition to the clinical data, investigators also collected nasal and nasopharyngeal airway specimens within 24 hours of hospitalisation using a standardised protocol.[12] These specimens underwent (1) viral testing of respiratory viruses (eg, RSV and rhinovirus) using real-time PCR assays, (2) miRNA profiling using small RNA-seq and (3) mRNA profiling using RNA-seq.

Nasal miRNA profiling

The details of RNA extraction, small RNA-seq, quality control and miRNA profiling are described in our previous studies[15 16] and online supplemental methods. Briefly, after total RNA extraction, DNase treatment and rRNA reduction, we used 493 specimens with sufficient RNA quantity and quality to perform small RNA-seq with a NovaSeq6000 (Illumina, San Diego, California, USA) using an S2 50 bp PE Flowcell (Illumina). We estimated miRNA detection and abundance using sMETASeq. Fastq files underwent quality control in Cutadapt and collapsed into unique reads. We mapped trimmed reads against human miRNA sequences from miRBase V.22. Last, we normalised the read count using R *DESeq2* package under default settings.

Nasopharyngeal mRNA profiling

The details of RNA extraction, RNA-seq, quality control and mRNA profiling are described in our previous studies[17] and online supplemental methods. Briefly, after total RNA extraction, DNase treatment and rRNA reduction, we performed RNA-seq with Illumina NovaSeq6000 using an S4 100PE Flowcell (Illumina). All RNA-seq samples had high sequence coverage after quality control. We estimated the transcript abundances with Salmon using the human genome hg38 and the mapping-based mode.

Outcome measures

The clinical outcome of interest was the acute severity of bronchiolitis, defined by the use of respiratory support, defined as the use of high-flow oxygen therapy with admission to the intensive care unit, continuous positive airway pressure ventilation or mechanical ventilation during the hospitalisation.[18]

Statistical analyses

The analytic workflow is summarised in figure 1. First, to investigate the association of nasal miRNA with the risk of respiratory support use, we performed miRNA differential expression analysis using the negative binomial generalised linear model from R *DESeq2* package. In the differential expression analysis, we adjusted for potential confounders (age, sex and prematurity) that were selected based on clinical plausibility and a priori

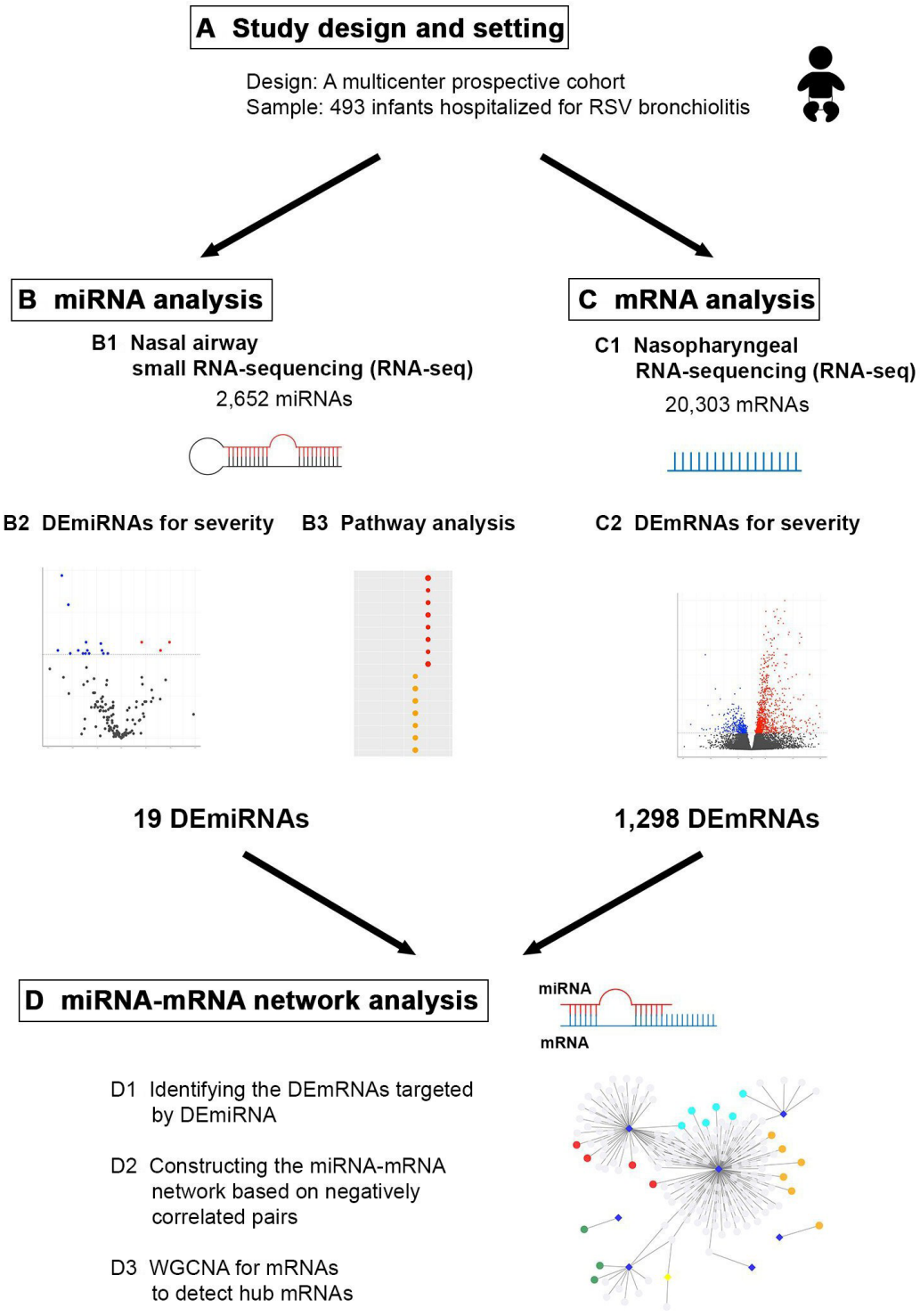

Figure 1 Analytic workflow. (A) The analytic cohort consisted of 493 infants hospitalised for RSV bronchiolitis in a multicentre prospective cohort study—35th Multicentre Airway Research Collaboration. Fastq files underwent quality control and collapsed into unique reads. The trimmed reads were mapped against human miRNA sequences from miRBase V.22. (B) A total of 2652 mature human miRNAs were detected in the nasal airway. In the miRNA analysis, the association of 2652 miRNAs with the risk of respiratory support use was investigated. A total of 19 DEmiRNAs were identified. The pathway analysis using the miRNA data was performed. (C) In the mRNA analysis, the association of 20 303 mRNAs with the risk of respiratory support use was investigated. A total of 1298 DEmRNAs were identified. (D) In the integrated miRNA and mRNA analysis, the miRNA–mRNA coexpression network was constructed, and the hub mRNAs for the corresponding modules were determined by WGCNA. The DEmRNAs targeted by DEmiRNAs were identified using miRNet V.2.0. Next, the miRNA–mRNA coexpression network was constructed based on the negatively correlated pairs. DEmiRNA, differentially expressed miRNA; DEmRNAs, differentially expressed mRNA; miRNAs, microRNA; mRNA, messenger RNA; RSV, respiratory syncytial virus; WGCNA, weighted gene coexpression network analysis.

knowledge.[19 20] We defined differentially expressed miRNA (DEmiRNA) as those miRNAs with a false discovery rate (FDR) of <0.10. We also performed gene set enrichment analysis (GSEA) using the miRNA data between infants with respiratory support use and those without. We referred to the miRPathDB V.2.0 and Gene ontology (GO) biological process database by using R *rbioapi* package.

Second, to investigate the association of nasopharyngeal mRNA with the risk of respiratory support use, we performed mRNA differential expression analysis using the negative binomial generalised linear model. We used the same set of potential confounders for differentially expressed mRNA (DEmRNA) as miRNA analysis.

Third, we identified the DEmRNAs targeted by DEmiRNAs using miRNet V.2.0. We then performed the correlation analysis between DEmiRNAs and DEmRNAs targeted by DEmiRNAs given that the main biological function of miRNAs is to degrade the target mRNAs. We constructed miRNA–mRNA networks based on negatively correlated (Spearman correlation coefficient< −0.10) miRNA–mRNA pairs. Next, we applied weighted gene coexpression network analysis (WGCNA) by using R *wgcna* package to DEmRNAs that are targeted by DEmiRNAs and negatively correlated with DEmiRNAs. To identify biologically meaningful pathways within each of the identified WGCNA modules, we performed functional pathway analysis (over-representation analysis) based on the GO biological process database using R *clusterProfiler* package. Finally, we identified hub mRNAs with module membership of >0.75 for the corresponding modules. In the sensitivity analysis, we repeated miRNA differential expression analysis in infants with solo-RSV infection. We conducted the statistical analysis by using R V.4.1.0 (R Foundation, Vienna, Austria). All p values were two tailed, with p<0.05 considered statistically significant. We accounted for multiple testing using the Benjamini-Hochberg FDR method that allows for the interpretation of statistical significance in the context of multiple hypothesis testing.

RESULTS

Patient characteristics

Of the 821 infants hospitalised for RSV bronchiolitis in the MARC-35 cohort, the current investigation analysed 493 infants who underwent nasal airway small RNA-seq. The analytic and non-analytic cohorts had no significant differences in patient characteristics (p≥0.05; online supplemental table S2). Of infants in the analytic cohort, the median age was 3 months (IQR 2–5 months), 41% were girls, 48% were non-Hispanic white, 22% were non-Hispanic black and 26% were Hispanic. Overall, 72% had solo-RSV infection and 14% had RSV/RV coinfection; 12% underwent respiratory support during the hospitalisation (table 1).

Table 1 Patient characteristics of infants hospitalised for RSV bronchiolitis

Patient characteristics	Overall (n=493)
Demographics	
Age (month), median (IQR)	3 (2–5)
Male sex	289 (59)
Race/ethnicity	
Non-Hispanic white	236 (48)
Non-Hispanic black	108 (22)
Hispanic	129 (26)
Other or unknown	20 (4)
C-section delivery	173 (34)
Prematurity (32–36.9 weeks)	87 (18)
History of eczema	73 (15)
Corticosteroid use during lifetime	71 (14)
Mostly breastfed during 0–2.9 months	220 (48)
Cigarette smoke exposure at home	73 (15)
Maternal smoking during pregnancy	74 (15)
Clinical presentation	
Weight (kg), median (IQR)	6.0 (4.7–7.6)
Respiratory rate (per min), median (IQR)	48 (40–60)
Oxygen saturation at presentation	
<90%	42 (9)
90%–93%	78 (16)
≥94%	361 (75)
Respiratory virus	
RSV solo infection	353 (72)
Rhinovirus coinfection	69 (14)
Other coinfection pathogens*	82 (17)
Laboratory data	
Any IgE sensitisation†	96 (22)
Clinical outcomes	
Respiratory support use‡	57 (12)
Length of hospital stay (days), median (IQR)	2 (1–3)

Data are n (%) of infants unless otherwise indicated. Percentages may not equal 100 because of rounding and missingness.
*Adenovirus, bocavirus, *Bordetella pertussis*, enterovirus, human coronavirus NL63, OC43, 229E or HKU1, human metapneumovirus, influenza A or B virus, *Mycoplasma pneumoniae* and parainfluenza virus 1–3.
†Defined by having one or more positive values for food or aeroallergen-specific IgE at index hospitalisation.
‡Defined as the use of high-flow oxygen therapy and admission to the intensive care unit, continuous positive airway pressure ventilation and/or mechanical ventilation during the hospitalisation.
IgE, immunoglobulin E; RSV, respiratory syncytial virus.

Nasal miRNA signature is related to RSV bronchiolitis severity

The miRNA profiling identified a total of 2652 mature miRNAs. In the differential expression analysis, 19 DEmiRNAs—for example, miR-27a-3p ($\log_2$ FC=−0.54),

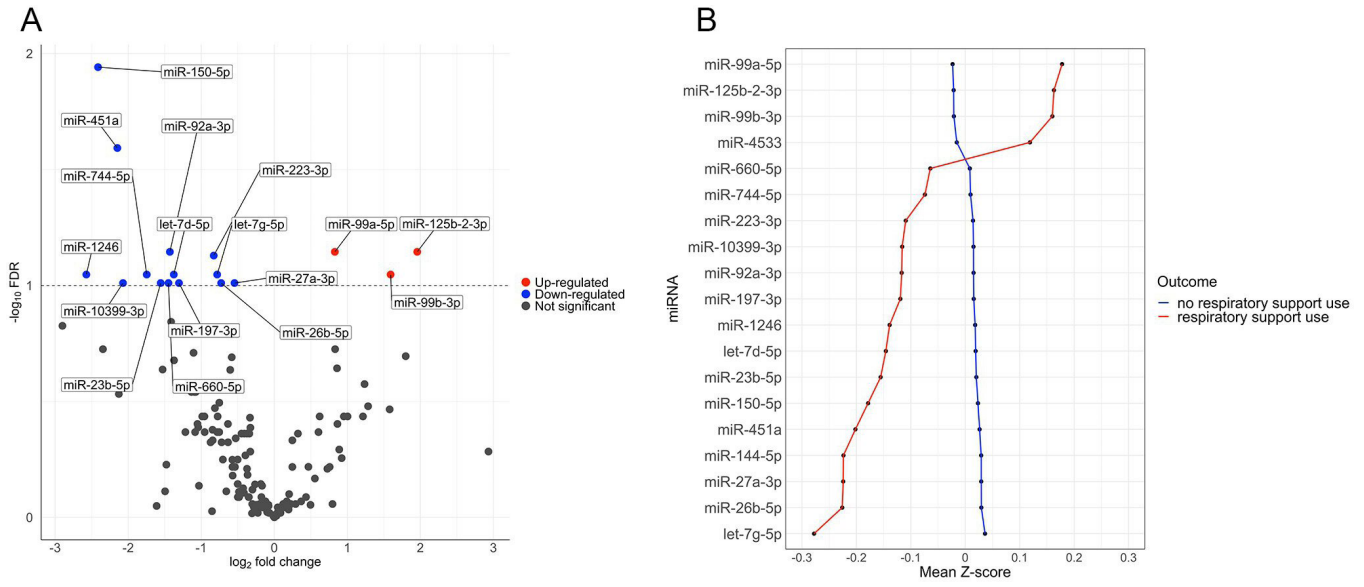

Figure 2 Association of nasal airway miRNAs with disease severity in infants hospitalised for RSV bronchiolitis. (A) The volcano plot shows the association of nasal airway miRNAs with the risk of respiratory support use in infants hospitalised for RSV bronchiolitis. The between-group differences in the expression level were tested by DESeq2 with the negative binomial generalised linear model adjusted for potential confounders, including age, sex and prematurity. The threshold of FDR is <0.10. There were 19 DEmiRNAs. MiR-144-5p had a < −5 log2 fold change and miR-4533 had a >11 log2 fold change; these DEmiRNAs are not shown in the plot. (B) The line plot shows the mean Z score of the 19 DEmiRNAs in infants with respiratory support use and those without. DEmiRNA, differentially expressed miRNA; FDR, false discovery rate; RSV, respiratory syncytial virus.

miR-26b-5p (log_2 FC=−0.73)—were significantly associated with the risk of respiratory support use (FDR<0.10). Of these miRNAs, 4 DEmiRNAs were upregulated, and 15 DEmiRNAs were downregulated in the respiratory support use group (figure 2A,B). In the GSEA by using the GO biological process gene set, 1291 pathways were differentially expressed between infants with respiratory support use and those without (FDR<0.10). In the top 15 upregulated pathways, for example, cell adhesion mediated by integrin, RNA metabolic process and cellular metabolic process pathways (FDR<0.01) were included (figure 3). By contrast, in the top 15 downregulated pathways, apoptotic process-related pathways—for example, apoptotic signalling pathway, negative regulation of cell death (FDR<0.01)—were included; figure 3). In the sensitivity analysis for infants with solo-RSV infection (n=353), 21 DEmiRNAs were significantly associated with the risk of respiratory support use (online supplemental figure S1). Of these, 11 DEmiRNAs were consistent with the DEmiRNAs of the main analysis for infants with RSV infection—for example, miR-223-3p.

Nasopharyngeal mRNA signature is related to RSV bronchiolitis severity

The mRNA profiling identified a total of 20 303 mRNAs. In the differential expression analysis, 1298 DEmRNAs were significantly associated with the risk of respiratory support use (FDR<0.10). Of these DEmRNAs, 966 DEmRNAs were upregulated, and 332 DEmRNAs were downregulated in the respiratory support use group (online supplemental figure S2). In addition, the transcriptomic analytic and non-transcriptomic analytic cohorts did not differ in patient characteristics (p≥0.05; online supplemental table S4)

miRNA–mRNA networks for RSV bronchiolitis severity

Of 1298 DEmRNAs, 190 were targeted by the 19 DEmiRNAs and negatively correlated with the DEmiRNAs. These negatively correlated DEmiRNA–DEmRNA pairs are summarised in figure 4A. Of these DEmiRNA–DEmRNA pairs, for example, miR-27a-3p was negatively correlated with 125 DEmRNAs, and miR-26b-5p was negatively correlated with 56 DEmRNAs. The WGCNA for mRNAs identified six distinct modules. Each of the identified modules was characterised by a distinct host biological pathway (online supplemental table S3 and figure S3). Of these modules, the eigenvalues of 4 modules—for example, cell-substrate junction module, microtubule anchoring module, RNA processing module—were significantly different between infants with respiratory support use and those without (FDR<0.10; figure 4B). The distinct host biological pathways of these significant modules—for example, cell-substrate junction, microtubule anchoring, RNA processing—were consistent with the results of the GSEA of miRNA data—for example, cell adhesion mediated by integrin, apoptotic signalling, RNA metabolic process. WGCNA also identified hub mRNAs for each module (figure 4A). Of these hub mRNAs, some hub mRNAs were included in the gene set of the significantly expressed pathways that characterise each module

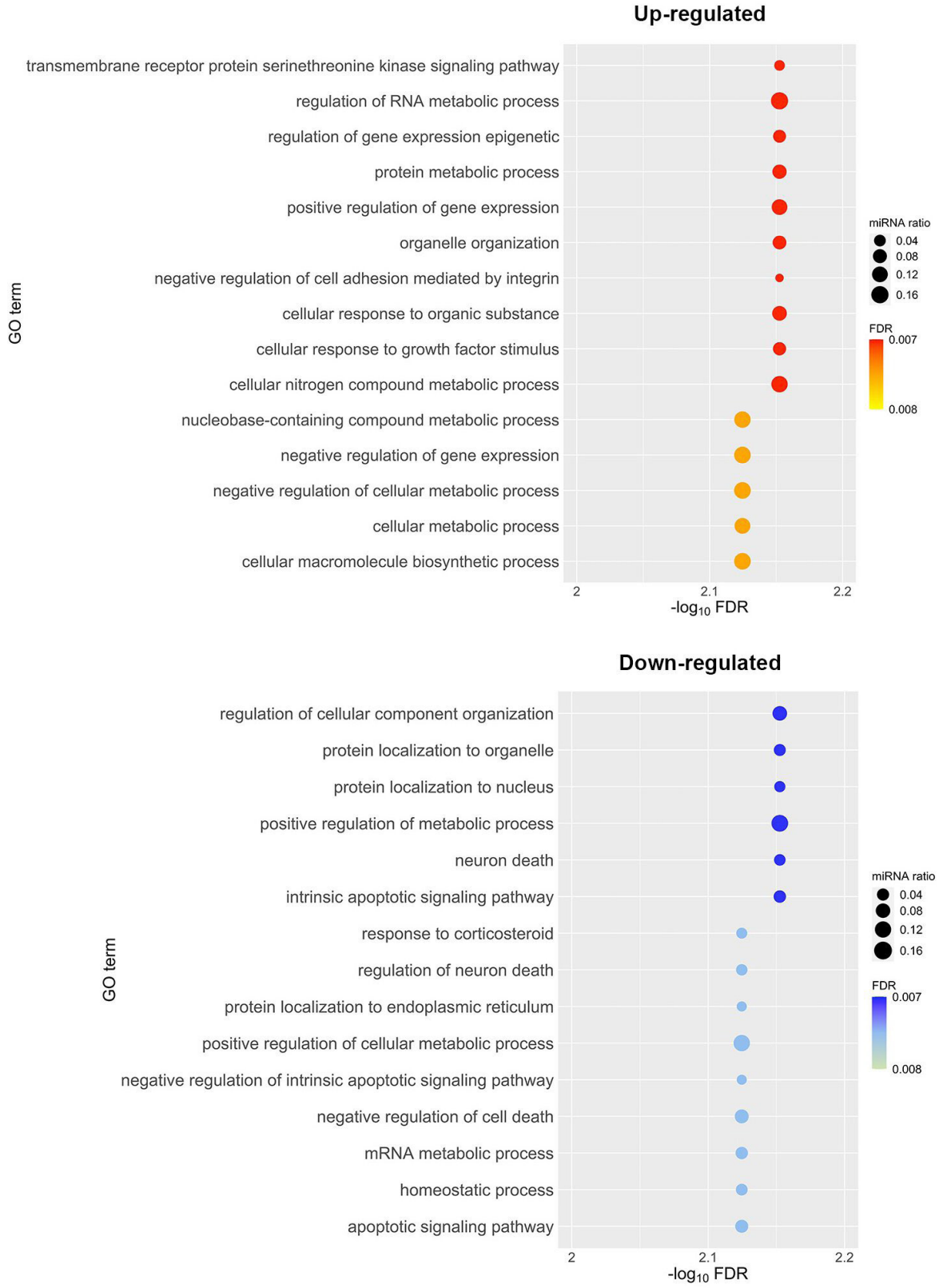

Figure 3 Gene set enrichment analysis of nasal airway miRNA data with regard to the use of respiratory support in infants hospitalised for RSV bronchiolitis. The miRNA pathway analysis in infants hospitalised forRSV bronchiolitis. We used the log2 fold change in miRNAs between patients with respiratory support use and those without for the GSEA based on the GO biological process database. We showed the top 15 upregulated and downregulated pathways with the most significant FDR, with upregulated pathways on the top and downregulated pathways on the bottom. The colour of each dot represents the FDR. The size of each dot represents the proportion of hit miRNA in the corresponding pathway. FDR, false discovery rate; GO, gene ontology; GSEA, gene set enrichment analysis; miRNAs, microRNA; RSV, respiratory syncytial virus.

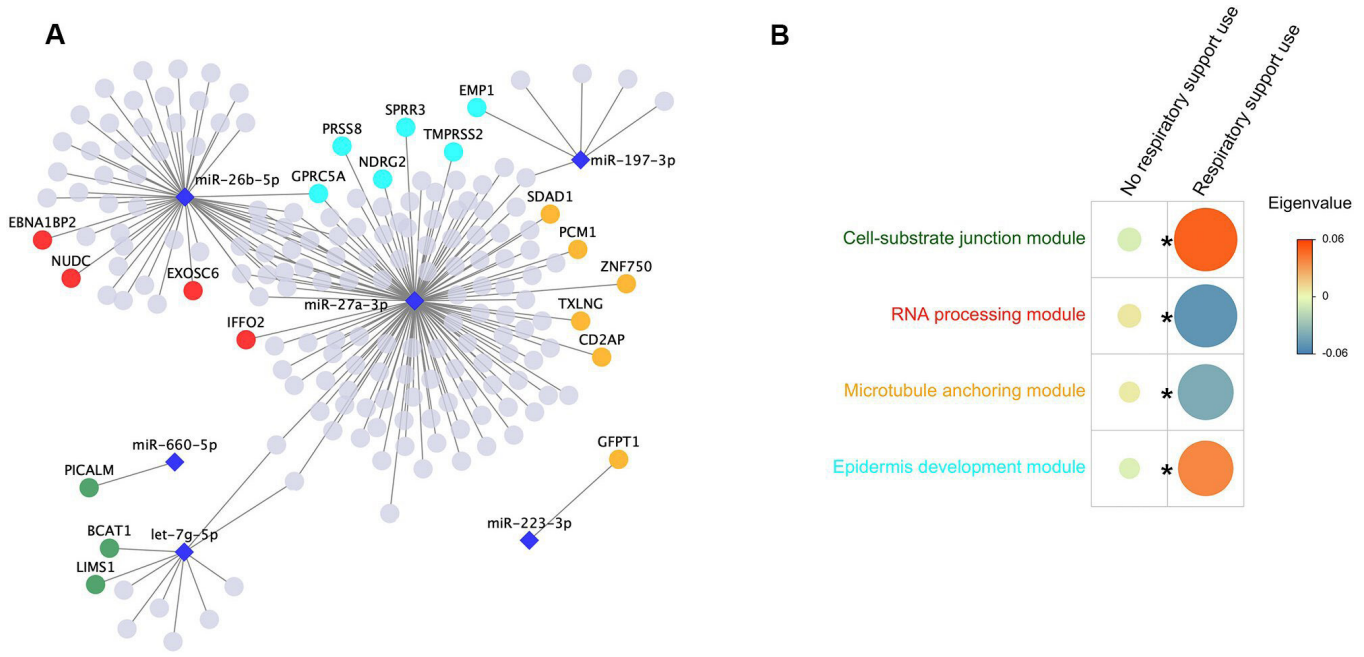

Figure 4 Coexpressed miRNA–mRNA modules and networks with their relationship with the use of respiratory support in infants hospitalised for RSV bronchiolitis. (A) The miRNA–mRNA coexpression networks underlying severity in infants hospitalised for RSV bronchiolitis: The blue diamonds represent differentially expressed miRNAs that were significantly associated with the risk of respiratory support use. The green circles represent hub mRNAs in the cell-substrate junction module. The red circles represent hub mRNAs in the RNA processing module. The orange circles represent hub mRNAs in the microtubule anchoring module. The cyan circles represent hub mRNAs in the epidermis development module. The figure does not show the miRNAs that did not connect to the hub mRNAs and mRNAs that connected to those miRNAs. (B) Heatmap of the median eigenvalues for the corresponding modules in each outcome group: The differentially expressed mRNAs that were targeted and negatively correlated pairs by the differentially expressed miRNAs were used for WGCNA. The six modules and their eigenvalues (the first principal component based on the principal component analysis) for the corresponding modules were identified by WGCNA. We showed the four modules whose eigenvalues were significantly different between the respiratory support use and the non-respiratory support use groups. A two-tailed t-test for eigenvalues of each module between the respiratory support use and the non-respiratory support use groups was performed. The areas of circles and colours represent the median value of the corresponding eigenvalue. *FDR<0.10. FDR is estimated by a two-tailed t-test between the respiratory support use and the non-respiratory support use groups. FDR, false discovery rate; miRNAs, microRNA; mRNA, messenger RNA; RSV, respiratory syncytial virus; WGCNA, weighted gene coexpression network analysis.

(online supplemental table S3). For example, *PCM1* in the microtubule anchoring module was included in the microtubule anchoring pathway; *LIMS1* in the cell-substrate junction module was included in the cell-substrate adhesion and cell-substrate junction assembly pathways; *EBNA1BP2* in the RNA processing module was included in the rRNA processing and non-coding RNA processing pathways. Finally, by mapping the hub mRNAs on the miRNA–mRNA pairs, the miRNA-hub mRNA pairs were identified—for example, miR-27a-3p/*PCM1* in the microtubule anchoring module, let-7g-5p/*LIMS1* in the cell-substrate junction module, miR-26b-5p/*EBNA1BP2* in the RNA processing module (figure 4A).

DISCUSSION

Based on analysis of nasal miRNA data from a multicentre prospective study of infants hospitalised for RSV bronchiolitis, we identified 19 DEmiRNAs (eg, miR-27a-3p, miR-26b-5p) associated with the risk of respiratory support use. We also demonstrated the networks of DEmiRNAs with DEmRNAs (eg, miR-27a-3p/*PCM1* in the microtubule anchoring module, let-7g-5p/*LIMS1* in the cell-substrate junction module, miR-26b-5p/*EBNA1BP2* in the RNA processing module), and its interaction between DEmiRNAs and DEmRNAs contributed to the severity of RSV bronchiolitis. To the best of our knowledge, this is the first investigation that has examined the relationship of the airway miRNA signatures and the miRNA–mRNA networks with disease severity in infants hospitalised for RSV bronchiolitis.

Results in the context

In agreement with the current study, recent research has suggested that miRNAs have an important role in the pathobiology of RSV bronchiolitis. A previous single-centre case–control study (n=42) using microarray for nasal specimens has suggested that 12 miRNAs (eg, miR-125b) are differentially expressed in infants with respiratory tract infection by RSV.[7] Similarly, a previous single-centre case–control study (n=104) using RT-PCR

for nasopharyngeal and peripheral blood samples has reported that miR-140-5p levels are significantly lower in patients with RSV bronchiolitis.[9] Additionally, a previous single-centre case–control study (n=20) using RT-PCR for peripheral blood samples has reported that miR-26b levels are significantly higher and negatively correlated with toll-like receptor 4 expression in infants hospitalised for RSV bronchiolitis.[21] The involvement of miRNAs extends to the pathogenesis of RSV pneumonia. A previous single-centre study (n=46)[22] using RT-PCR for peripheral blood samples with a focus on RSV pneumonia has suggested that 11 miRNAs (eg, miR-125b-5p) are differentially expressed between severe and mild groups, as classified by the British Thoracic Society Guidelines.[23] However, no overlap with the miRNAs identified in the current study was observed, which may be attributed to differences in the samples. The current study—based on the small RNA-seq applied to a large multicentre cohort—builds on these prior reports and extends them by demonstrating the relationship of the airway miRNA–mRNA networks with disease severity among infants hospitalised for RSV bronchiolitis.

Potential mechanisms

The mechanisms underlying the associations of miRNA signatures and miRNA–mRNA network (eg, miR-27a-3p/*PCM1* in the microtubule anchoring module, let-7g-5p/*LIMS1* in the cell-substrate junction module, miR-26b-5p/*EBNA1BP2* in the RNA processing module) with disease severity remain to be elucidated. First, the microtubule is an essential basement to maintain the structure of airway ciliated cells, one of the main components of the airway epithelial cells.[24 25] Experimental studies have shown that the airway ciliated cells are the primary target of RSV infection and decrease following RSV infection in vitro.[26 27] In airway ciliated cells, *PCM1* is involved in the stability of microtubules and is also required for ciliogenesis.[25 28] The high expression of *PCM1* in the current findings may reflect the damage to airway ciliated cells by RSV infection. Recent research has also suggested that miR-27a-3p plays an important role in airway epithelial injury. For example, an experimental study has reported that miR-27a-3p mitigates the apoptosis of alveolar epithelial cells by suppressing reactive oxygen species activation in vitro.[29] These prior studies support our findings that provide new insight into the potential role of miR-27a-3p in airway microtubule anchoring through *PCM1* expression in RSV infection.

Second, the cell-substrate junction regulates the integrity of the airway epithelial barrier.[30] Experimental studies have reported that RSV infection influences tight junction integrity in airway epithelial cells in vivo and in vitro.[30 31] *LIMS1* encodes LIM protein, which modulates the integrin signalling.[32] Given the function of *LIMS1* for cell junction integrity, the highly expressed *LIMS1* in current findings suggest disruption of the airway epithelial barrier in RSV infection. Additionally, a mice model study has shown that the let-7 miRNA family modulates airway inflammation by regulating IL-13 expression that interacts with integrin adhesion complexes in vivo.[33 34] These prior reports support our findings that let-7g-5p is implicated in the airway cell junction through the interaction with *LIMS1*.

Third, RNA processing (eg, alternative splicing, alternative polyadenylation) is implicated in the innate immune response in RSV infection.[35] For example, a previous experimental study has shown that alternative splicing mRNA isoforms are involved in the cell cycle checkpoint and interferon signalling pathway in airway epithelial cells infected by RSV in vitro.[35] *EBNA1BP2* and miR-26b-5p are potential molecules related to RNA processing. For example, an experimental study has shown that *EBNA1BP2* directly binds to *c-Myc*[36] that activates RNA processing, such as splicing, polyadenylation and mRNA capping in vitro.[37 38] Additionally, an experimental study has reported that miR-26b-5p inhibits wingless protein 5a expression through the wingless protein pathway that activates RNA processing (eg, intron splicing, RNA metabolism) in vitro.[39 40] These data support our findings that the interaction between miR-26b-5p with *EBNA1BP2* was related to RNA processing, and its interaction contributed to the pathobiology of disease severity. Notwithstanding the complexity of these potential mechanisms, our miRNA data should advance research into the pathobiology of epigenetic regulation of RSV bronchiolitis in conjunction with the role of DNA methylation in bronchiolitis severity.[41]

Limitations

This study has several potential limitations. First, the current study does not have a non-bronchiolitis cohort. Furthermore, to the best of our knowledge, airway miRNA data from uninfected infants to compare with are not available. Yet, the study objective was not to evaluate the role of the airway miRNAs in developing RSV bronchiolitis (yes vs no) but to investigate its relationship with the disease severity among infants with RSV bronchiolitis. Second, bronchiolitis involves inflammation of both the upper and lower respiratory tracts. Although the current study is based on the miRNA data from nasal specimens, previous research has demonstrated that the data from the upper airway specimens offer a reliable representation of inflammatory profiles in the lower airways.[42] Third, the nasal samples were collected at a single time point. Nonetheless, the findings of this study in the early course of bronchiolitis are clinically and biologically relevant. Fourth, the current study did not have mechanistic experiments to validate the identified miRNA functions. Finally, although the current cohort consisted of racially/ethnically and geographically diverse infants, the inferences may not be generalised beyond infants hospitalised for RSV bronchiolitis. Regardless, our data are directly relevant to tens of thousands of infants hospitalised for RSV bronchiolitis each year.[2]

CONCLUSION

Analysis of the nasal airway miRNA data from a multicentre prospective study of infants hospitalised for RSV bronchiolitis revealed DEmiRNAs associated with bronchiolitis severity. The nasal airway miRNA signatures were also related to the distinct biological pathways (eg, cell adhesion mediated by integrin, RNA metabolic process). Furthermore, the airway miRNA–mRNA coexpression networks, including distinct disease modules and their hub genes, were also associated with bronchiolitis severity. Our data suggest that miRNA plays important roles in the pathobiology of severe RSV bronchiolitis. Our observations advance research into the pathobiological mechanisms of bronchiolitis and facilitate the development of therapies for this clinical population with a large morbidity burden.

Author affiliations
[1]Department of Emergency Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA
[2]Department of Health Science, University of Yamanashi, Kofu, Yamanashi, Japan
[3]Department of Pediatrics, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA
[4]Centre for Genetic Medicine Research, Children's National Hospital, Washington, District of Columbia, USA
[5]Department of Paediatrics, The George Washington University School of Medicine and Health Sciences, Washington, District of Columbia, USA
[6]Division of Infectious Diseases, Children's National Hospital, Washington, District of Columbia, USA
[7]Computational Biology Institute, Department of Biostatistics and Bioinformatics, The George Washington University, Washington, District of Columbia, USA

Acknowledgements We would like to thank the participants, families, staff and investigators who significantly contributed to this cohort study. We also thank Ashley F Sullivan, MPH, MS for her countless contributions, and Dr Ignacio Ramos-Tapia for his assistance with statistical analysis.

Contributors MK carried out the statistical analysis, drafted the initial manuscript and approved the final manuscript as submitted. ZZ, RS and TO carried out the statistical analysis, reviewed and revised the initial manuscript and approved the final manuscript as submitted. JMM, BH and AH collected the study data, reviewed and revised the initial manuscript and approved the final manuscript as submitted. MP-L carried out the bioinformatic analyses of the genomic data, reviewed and revised the initial manuscript and approved the final manuscript as submitted. CAC and KH conceptualised the study, obtained funding, supervised the statistical analysis, reviewed and revised the initial manuscript, approved the final manuscript as submitted and are the guarantors of this publication. All authors read and approved the final manuscript.

Funding This study was supported by grants (UG3/UH3 OD-023253, R01 AI-127507, R01 AI-134940 and R01 AI-137091) from the National Institutes of Health (Bethesda, Maryland, USA). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funding organisation was not involved in the collection, management or analysis of the data; preparation or approval of the manuscript; or decision to submit the manuscript for publication.

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained from parent(s)/guardian(s).

Ethics approval The study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of Partners Human Research Committee (Protocol code 2017P001637, IRB approval date 07/27/2017). The institutional review board at each participating hospital approved the study with written informed consent obtained from the parent or guardian. Written informed consent was obtained from the parent or guardian.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. The RNA-seq profiling data that support the findings of this study are available on the NIH/NIAID ImmPort (https://www.immport.org/shared/study/SDY1883), on reasonable requests from researchers whose work investigates severe bronchiolitis, recurrent wheezing, asthma and related concepts. The data are not available without restriction to be compliant with the informed consent forms of the MARC-35 study and the genomic data sharing plan.

ORCID iDs
Michihito Kyo http://orcid.org/0000-0002-2559-6440
Zhaozhong Zhu http://orcid.org/0000-0001-5662-1541
Ryohei Shibata http://orcid.org/0000-0002-6195-441X

REFERENCES

1 Hasegawa K, Dumas O, Hartert TV, *et al*. Advancing our understanding of infant bronchiolitis through phenotyping and endotyping: clinical and molecular approaches. *Expert Rev Respir Med* 2016;10:891–9.
2 Suh M, Movva N, Jiang X, *et al*. Respiratory syncytial virus is the leading cause of united states infant hospitalizations, 2009-2019: A study of the national (nationwide) inpatient sample. *J Infect Dis* 2022;226:S154–63.
3 Li Y, Wang X, Blau DM, *et al*. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in children younger than 5 years in 2019: a systematic analysis. *Lancet* 2022;399:2047–64.
4 Hammitt LL, Dagan R, Yuan Y, *et al*. Nirsevimab for prevention of RSV in healthy late-preterm and term infants. *N Engl J Med* 2022;386:837–46.
5 Kampmann B, Madhi SA, Munjal I, *et al*. Bivalent prefusion F vaccine in pregnancy to prevent RSV illness in infants. *N Engl J Med* 2023;388:1451–64.
6 Milési C, Baudin F, Durand P, *et al*. Clinical practice guidelines: management of severe bronchiolitis in infants under 12months old admitted to a pediatric critical care unit. *Intensive Care Med* 2023;49:5–25.
7 Inchley CS, Sonerud T, Fjærli HO, *et al*. Nasal mucosal microrna expression in children with respiratory syncytial virus infection. *BMC Infect Dis* 2015;15:150.
8 Thornburg NJ, Hayward SL, Crowe JE. Respiratory syncytial virus regulates human microrna by using mechanisms involving beta interferon and NF-κB. *MBio* 2012;3:e00220-12.
9 Zhang Y, Shao L. Decreased microrna-140-5p contributes to respiratory syncytial virus disease through targeting toll-like receptor 4. *Exp Ther Med* 2018;16:993–9.
10 Simpson LJ, Patel S, Bhakta NR, *et al*. A microrna upregulated in asthma airway T cells promotes TH2 cytokine production. *Nat Immunol* 2014;15:1162–70.
11 Banerjee S, Cui H, Xie N, *et al*. MiR-125a-5p regulates differential activation of macrophages and inflammation. *J Biol Chem* 2013;288:35428–36.
12 Stewart CJ, Mansbach JM, Ajami NJ, *et al*. Serum metabolome is associated with the nasopharyngeal microbiota and disease severity among infants with bronchiolitis. *J Infect Dis* 2019;219:2005–14.
13 Ralston SL, Lieberthal AS, Meissner HC, *et al*. Clinical practice guideline: the diagnosis, management, and prevention of bronchiolitis. *Pediatrics* 2014;134:e1474–502.
14 Stewart CJ, Mansbach JM, Wong MC, *et al*. Associations of nasopharyngeal metabolome and microbiome with severity among

infants with bronchiolitis. A multiomic analysis. *Am J Respir Crit Care Med* 2017;196:882–91.
15 Shibata R, Zhu Z, Ooka T, *et al*. Immunoglobulin E-virus phenotypes of infant bronchiolitis and risk of childhood asthma. *Front Immunol* 2023;14:1187065.
16 Zhu Z, Freishtat RJ, Harmon B, *et al*. Nasal airway microrna profiling of infants with severe bronchiolitis and risk of childhood asthma: A multicentre prospective study. *Eur Respir J* 2023;62:2300502.
17 Zhu Z, Camargo CA, Raita Y, *et al*. Nasopharyngeal airway dual-transcriptome of infants with severe bronchiolitis and risk of childhood asthma: A multicenter prospective study. *J Allergy Clin Immunol* 2022;150:806–16.
18 Kyo M, Zhu Z, Shibata R, *et al*. Respiratory virus-specific nasopharyngeal lipidome signatures and severity in infants with bronchiolitis: A prospective multicenter study. *J Infect Dis* 2023;228:1410–20.
19 Mansbach JM, Piedra PA, Teach SJ, *et al*. Prospective multicenter study of viral etiology and hospital length of stay in children with severe bronchiolitis. *Arch Pediatr Adolesc Med* 2012;166:700–6.
20 Shi T, Vennard S, Mahdy S, *et al*. Risk factors for poor outcome or death in young children with respiratory syncytial virus-associated acute lower respiratory tract infection: A systematic review and meta-analysis. *J Infect Dis* 2022;226:S10–6.
21 Liu S, Gao L, Wang X, *et al*. Respiratory syncytial virus infection inhibits TLR4 signaling via up-regulation of mir-26b. *Cell Biol Int* 2015;39:1376–83.
22 Zhang X, Huang F, Yang D, *et al*. Identification of mirna-mrna crosstalk in respiratory syncytial virus- (RSV-) associated pediatric pneumonia through integrated mirnaome and transcriptome analysis. *Mediators Inflamm* 2020;2020:8919534.
23 Principi N, Esposito S. Management of severe community-acquired pneumonia of children in developing and developed countries. *Thorax* 2011;66:815–22.
24 Kuek LE, Lee RJ. First contact: the role of respiratory cilia in host-pathogen interactions in the airways. *Am J Physiol Lung Cell Mol Physiol* 2020;319:L603–19.
25 Vertii A, Hung H-F, Hehnly H, *et al*. Human basal body basics. *Cilia* 2016;5:13.
26 Villenave R, Thavagnanam S, Sarlang S, *et al*. In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo. *Proc Natl Acad Sci U S A* 2012;109:5040–5.
27 Smith CM, Kulkarni H, Radhakrishnan P, *et al*. Ciliary dyskinesia is an early feature of respiratory syncytial virus infection. *Eur Respir J* 2014;43:485–96.
28 Kim J, Krishnaswami SR, Gleeson JG. CEP290 interacts with the centriolar satellite component PCM-1 and is required for rab8 localization to the primary cilium. *Hum Mol Genet* 2008;17:3796–805.
29 Shang J, Wang L, Tan L, *et al*. MiR-27a-3p overexpression mitigates inflammation and apoptosis of lipopolysaccharides-induced alveolar epithelial cells by targeting FOXO3 and suppressing the activation of NAPDH/ROS. *Biochem Biophys Res Commun* 2020;533:723–31.
30 Smallcombe CC, Linfield DT, Harford TJ, *et al*. Disruption of the airway epithelial barrier in a murine model of respiratory syncytial virus infection. *Am J Physiol Lung Cell Mol Physiol* 2019;316:L358–68.
31 Kast JI, McFarlane AJ, Głobińska A, *et al*. Respiratory syncytial virus infection influences tight junction integrity. *Clin Exp Immunol* 2017;190:351–9.
32 Ripamonti M, Liaudet N, Azizi L, *et al*. Structural and functional analysis of LIM domain-dependent recruitment of paxillin to αvβ3 integrin-positive focal adhesions. *Commun Biol* 2021;4:380.
33 Kumar M, Ahmad T, Sharma A, *et al*. Let-7 microrna-mediated regulation of IL-13 and allergic airway inflammation. *J Allergy Clin Immunol* 2011;128:1077–85.
34 Desai LP, Wu Y, Tepper RS, *et al*. Mechanical stimuli and IL-13 interact at integrin adhesion complexes to regulate expression of smooth muscle myosin heavy chain in airway smooth muscle tissue. *Am J Physiol Lung Cell Mol Physiol* 2011;301:L275–84.
35 Xu X, Mann M, Qiao D, *et al*. Alternative mrna processing of innate response pathways in respiratory syncytial virus (RSV) infection. *Viruses* 2021;13:218.
36 Liao P, Wang W, Shen M, *et al*. A positive feedback loop between EBP2 and c-myc regulates rdna transcription, cell proliferation, and tumorigenesis. *Cell Death Dis* 2014;5:e1032.
37 Zhang Y, Qian J, Gu C, *et al*. Alternative splicing and cancer: a systematic review. *Sig Transduct Target Ther* 2021;6:1–14.
38 Dunn S, Cowling VH. Myc and mrna capping. *Biochim Biophys Acta* 2015;1849:501–5.
39 Jiao X, Lv Q, Cao SN. MicroRNA-26b-5p promotes development of neonatal respiratory distress syndrome by inhibiting differentiation of mesenchymal stem cells to type II of alveolar epithelial cells via regulating wnt5a. *Eur Rev Med Pharmacol Sci* 2019;23:1681–7.
40 Bordonaro M. Crosstalk between wnt signaling and RNA processing in colorectal cancer. *J Cancer* 2013;4:96–103.
41 Zhu Z, Li Y, Freishtat RJ, *et al*. Epigenome-wide association analysis of infant bronchiolitis severity: a multicenter prospective cohort study. *Nat Commun* 2023;14:5495.
42 Poole A, Urbanek C, Eng C, *et al*. Dissecting childhood asthma with nasal transcriptomics distinguishes subphenotypes of disease. *J Allergy Clin Immunol* 2014;133:670–8.