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Nasal airway microRNA profiling of infants with severe bronchiolitis and risk of childhood asthma: A multicenter prospective study

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Abstract

Background: Severe bronchiolitis (i.e., bronchiolitis requiring hospitalization) during infancy is a major risk factor for childhood asthma. However, the exact mechanism linking these common conditions remains unclear. We examined the longitudinal relationship between nasal airway miRNAs during severe bronchiolitis and the risk of developing asthma.

Methods: In a 17-center prospective cohort study of infants with severe bronchiolitis, we sequenced their nasal miRNA at hospitalization. First, we identified differentially expressed miRNAs (DEmiRNAs) associated with the risk of developing asthma by age 6 years. Second, we characterized the DEmiRNAs based on their association with asthma-related clinical features, and expression level by tissue and cell types. Third, we conducted pathway and network analyses by integrating DEmiRNAs and their mRNA targets. Finally, we investigated the association of DEmiRNAs and nasal cytokines.

Results: In 575 infants (median age=3 months), we identified 23 DEmiRNAs associated with asthma development (e.g., hsa-miR-29a-3p, FDR<0.10), particularly in infants with respiratory syncytial virus infection ($FDR_{interaction}$<0.05). These DEmiRNAs were associated with 16 asthma-related clinical features (FDR<0.05)—e.g., infant eczema and corticosteroid use during hospitalization. These DEmiRNAs were also highly expressed in lung tissue and immune cells

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(e.g., T_H cells, neutrophils). Third, DEmiRNAs were negatively correlated with their mRNA targets (e.g., hsa-miR-324-3p/*IL13*), which were enriched in asthma-related pathways (FDR<0.05)—e.g., toll-like receptor, PI3K-Akt, and FcεR signaling pathways, and validated by cytokine data.

Conclusion: In a multicenter cohort of infants with severe bronchiolitis, we identified nasal miRNAs during illness that were associated with major asthma-related clinical features, immune response, and risk of asthma development.

Keywords

bronchiolitis; asthma; viral infection; microRNA; RNA sequencing; epigenetics; immunology

INTRODUCTION

Bronchiolitis is the leading cause of infant hospitalizations in the U.S., accounting for ~110,000 hospitalizations (i.e., severe bronchiolitis) annually [1, 2]. Its chronic morbidity burden is also substantial. Of these infants with severe bronchiolitis, approximately 30% develop asthma in childhood [3–8]. Yet, the underlying mechanisms linking these two common conditions remain unclear, and thereby hinder efforts to prevent asthma in this high-risk population.

MicroRNAs (miRNAs) are small non-coding RNAs consisting of 15 to 22 nucleotides. MiRNAs post-transcriptionally regulate gene expression by directly binding to their mRNA targets. Dysregulated expression of miRNAs leads to aberrant immune function [9, 10] and respiratory outcomes [11, 12], such as prevalent asthma [13–22], asthma severity [23, 24], asthma treatment (e.g., corticosteroids) response [25], and asthma remission [26]. Yet, these earlier reports [13, 15–25]—mostly based on a case-control design testing non-airway specimens from a small sample size of *prevalent* adult asthma—have precluded researchers from determining the role of airway miRNAs in *incident* asthma in childhood. Despite the clinical and research significance, no study has investigated miRNA signatures in infants, let alone high-risk infants (i.e., those with severe bronchiolitis), their post-transcriptional regulation of gene expression during the critical period of airway development, and their contribution to the development of asthma.

To address this knowledge gap in the literature, by applying a small RNA sequencing (RNA-seq) approach to a large prospective cohort of infants with severe bronchiolitis , we aimed to identify nasal airway miRNAs that are associated with the development of childhood asthma and to examine their potential mechanisms linking bronchiolitis and asthma.

METHODS

Study Design, Setting, and Participants

The study design and analytic workflow are summarized in Figure 1. We analyzed data from a multicenter prospective cohort study of infants hospitalized for bronchiolitis—the 35th Multicenter Airway Research Collaboration (MARC-35) study [27, 28]. Details of the study design, setting, participants, data collection, testing, and statistical analysis may be found in the Supplementary Methods. At 17 medical centers across 14 U.S. states (Table E1),

MARC-35 enrolled infants (age <1 year) who were hospitalized with an attending physician diagnosis of bronchiolitis during three bronchiolitis seasons in 2011-2014. The diagnosis of bronchiolitis was made according to the American Academy of Pediatrics bronchiolitis guidelines, defined as an acute respiratory illness with a combination of rhinitis, cough, tachypnea, wheezing, crackles, or retraction [29]. We excluded infants with preexisting heart or lung disease, immunodeficiency, immunosuppression, or gestational age of <32 weeks. All infants were treated at the discretion of the treating physicians. Of 921 infants enrolled in the MARC-35 longitudinal cohort, the current study investigated 575 infants who have high-quality nasal small RNA-seq data (Figure E1). The institutional review board at each participating hospital approved the study with written informed consent obtained from the parent or guardian.

Data Collection

Clinical data (study participants' demographic characteristics, and family, environmental, and medical history, and details of the acute illness) were collected via structured interview and chart reviews using a standardized protocol (28, 29). After the index hospitalization for bronchiolitis, trained interviewers began interviewing parents/legal guardians by telephone at 6-month intervals in addition to medical record review by physicians. All data were reviewed at the Emergency Medicine Network Coordinating Center at Massachusetts General Hospital (Boston, MA, USA) [30]. Nasal swab specimens were collected within 24 hours of hospitalization using a standardized protocol [31, 32]. The details of the data collection and measurement methods are described in the Supplementary Methods.

Nasal Small RNA-seq Profiling (for miRNA)—The details of RNA extraction, RNA-seq, and quality control are described in Supplementary Methods. Briefly, after total RNA extraction, we performed small RNA-seq using the PerkinElmer NEXTFLEX® small RNA-seq v3 kit with Unique Dual Indexes (PerkinElmer, Waltham, MA) and sequenced on an Illumina NovaSeq6000 sequencer using an S2 50bp PE Flowcell (Illumina, San Diego, CA). We estimated miRNA detection and abundance using sMETASeq [33]. Fastq files underwent quality control in cutadapt [34] and collapse into unique reads. We mapped trimmed reads against human miRNA sequences from miRBase V22 [35]. We filtered out raw read counts of <15 [25, 36]. Lastly, we normalized the read count by R *DESeq2* package [37] using default settings.

Nasopharyngeal RNA-seq Profiling (for mRNA)—The details of RNA-seq, quality control, and transcriptome profiling are described in our previous studies [38–40] and Supplementary Methods. Briefly, after total RNA extraction, DNase treatment, and rRNA reduction, we performed RNA-seq with Illumina NovaSeq6000 (Illumina, San Diego, CA). All RNA-seq samples had high sequence coverage after quality control. The transcript abundances were estimated with *Salmon* [41] using the human genome (hg38) and the mapping-based mode. A total of 194 infants had both nasal miRNA and nasopharyngeal mRNA data.

Nasal Cytokine Measurement—The details of cytokine measurement and quality control are described in Supplementary Methods. We measured the levels of 10 cytokines

(interferon [IFN]-γ, interleukin [IL]-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, tumor necrosis factor [TNF]-α) in the nasal swab specimens of infants with severe bronchiolitis, using multiplex the Meso Scale Discovery (MSD) electrochemiluminescent V-Plex multiplex immunoassay (Meso Scale Diagnostics, Rockville, MD), on the MESO QuickPlex SQ 120 system (Meso Scale Diagnostics). The samples with cytokine level less than lower limit of detection were removed. A maximum of 503 infants had both nasal miRNA and nasal cytokine data.

Outcome

The outcome of interest was the development of asthma by age 6 years. The definition of asthma was based on a commonly-used epidemiologic definition of asthma [30, 42]—physician diagnosis of asthma by age 6 years, plus either asthma medication use (e.g., albuterol inhaler, inhaled corticosteroids, montelukast) or asthma-related symptoms in the preceding year.

Statistical Analysis

The analytic workflow is summarized in Figure 1. First, to investigate the relationship of the miRNAs with the risk of developing asthma, we performed miRNA differential expression analysis using the negative binomial generalized linear model from *DESeq2* R package [37]. Based on *a priori*-defined hypothesis, we also examined the effect modification by the respiratory syncytial virus (RSV), rhinovirus (RV), RV-A, and RV-C infection on the risk of developing asthma. In the differential expression analysis, we adjusted for potential confounders (i.e., age, sex, number of previous breathing problems, and IgE sensitization) based on *a priori* knowledge and clinical plausibility [30, 39, 43, 44]. We corrected multiple testing using the Benjamini-Hochberg false discovery rate (FDR) method [45]. We defined differentially expressed miRNA (DEmiRNA) as those miRNA significantly associated with asthma development at an FDR<0.10.

Second, we examined the relationship of DEmiRNAs with asthma-related clinical variables [30, 39, 43, 44], including RSV, RSV-A, RSV-B, RV, RV-A, RV-B, RV-C, parental history of asthma and eczema, number of previous breathing problems, infant history of corticosteroids use, eczema, IgE sensitization, blood eosinophil count, need for positive pressure ventilation (PPV), and intensive care use (PPV use and/or intensive care unit adimission). To provide biological insights into the identified nasal DEmiRNAs, we investigated DEmiRNAs across 15 asthma-related tissue types (e.g., adipocyte, lung, lymph nodes, muscle, pancreas, skin) [46] using the **publicly available** human miRNA TissueAtlas data [47]. We also examined DEmiRNAs across six immune cell types [B cell, cytotoxic T (T_C) cell, helper T (T_H) cell, monocyte, natural killer (NK) cell, and neutrophil cell] using **publicly available** single-cell small RNA-seq data from 162 healthy subjects [48]. We normalized the miRNA data using the variance stabilizing transformation approach implemented in *DESeq2* [37].

Third, we identified the mRNAs targeted by DEmiRNAs using DIANA-microT-CDS [49] and conducted a gene-set enrichment analysis to investigate the enrichment of these targeted mRNA in the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using DIANA miRPath v.3.0 [50]. We also performed the

correlation analysis based on upper airway miRNA and mRNA data (Supplementary Methods) given that the main biological function of miRNAs is to degrade the target mRNAs. We constructed network based on negatively correlated (Spearman correlation coefficient<−0.10) miRNA-mRNA pairs and prior knowledge of identified immune-related pathways [13, 20, 24, 51–54]. Finally, we investigated the association of DEmiRNA with ten cytokines using the negative binomial generalized linear model from *DESeq2* R package [37].

RESULTS

Of the 921 infants with severe bronchiolitis enrolled into the MARC-35 longitudinal cohort, the current study focused on 575 infants who had high quality nasal miRNA data. The analytic and non-analytic cohorts did not differ in patient characteristics ($P \geq 0.05$; Table E2), except for RSV and RV-B infection. Among the analytic cohort, the median age was 3 (IQR, 2-6) months, 41% were female, 47% were non-Hispanic White, 28% were Hispanic, and 22% were non-Hispanic Black. Subsequently, 27% developed asthma by age 6 years (Tables 1 and E3). In addition, 76% of infants with RSV infection (vs. 87% with no RSV infection) and 28% with RV infection (vs. 16% with no RV infection) developed asthma. For miRNA profiling, a total of 2,652 human mature miRNAs were identified. Among these, 1,070 miRNAs had raw read counts of more than 15 reads and were included in the subsequent analysis (Figure 2A).

Nasal miRNAs of Infant Bronchiolitis were Associated with Risk of Developing Asthma

A total of 23 DEmiRNAs were significantly associated with asthma risk (FDR<0.10), with 18 DEmiRNAs being upregulated and 5 DEmiRNAs being downregulated (Figure 2B). Of these, hsa-miR-29a-3p was the most significantly associated with the asthma risk ($\log_2$ fold change [FC]=1.44, FDR=5.47×10^{-15}). In the examination of effect modification by virus, 22 of these DEmiRNA had a significant interaction with RSV infection on asthma risk ($FDR_{interaction}$<0.05). In the stratified analysis within infants with RSV infection, 15 miRNAs were associated with a significantly higher risk of developing asthma (FDR<0.05; Figure 2C). Additionally, hsa-miR-22-3p had a significant interaction with RV infection on asthma risk ($FDR_{interaction}$<0.05). Among infants with RV infection, hsa-miR-22-3p was associated with a significantly higher risk of developing asthma ($\log_2$FC=1.98, FDR=5.94×10^{-6}; Figure 2D). However, we did not observe a significant association between miRNAs and asthma development in RV-A or RV-C strata (Figure E2).

DEmiRNAs were Associated with Asthma-related Clinical Features, Tissue Types, and Cell Types

The DEmiRNAs were also associated with major asthma-related clinical features. For example, at the aggregated miRNA level, the DEmiRNAs and non-DEmiRNAs had a difference in the magnitude of association with RSV, RV-A, and RV-B (only 5 children) infection, infant history of eczema and corticosteroids use, serum total IgE level, PPV use, and intensive care use (Figure 3A). At the individual miRNA level, 18 DEmiRNAs were significantly associated with these clinical features (FDR<0.05). For example, hsa-miR-29a-3p was positively associated with infant history of eczema and corticosteroid use,

and hsa-let-7b-5p was negatively associated with serum total IgE level (Figure 3B). In the miRNA tissue expression analysis, the DEmiRNAs had differential expression in lung, spleen, and bone marrow (all FDR<0.001) (Figure 4A). In the examination of the miRNA expression in immune cell types, the expression levels of DEmiRNAs were significantly higher in all immune cells (e.g., T_H cells, neutrophils) than the non-DEmiRNAs (all FDR<0.001) (Figure 4B). Most of the DEmiRNAs were presented commonly in these immune cells (Figure 4C).

Identification of Biological Pathways and Network for Risk of Asthma Development

In the gene-set enrichment analysis by using the GO biological process gene set, 108 pathways were differentially enriched (FDR<0.05; Table E4), including asthma-related pathways—e.g., Fc-epsilon receptor (FcεR), toll-like receptor (TLR), and mitogen-activated protein kinase (MAPK) signaling pathways (Figure 5A). Additionally, in the analysis by using the KEGG gene set, 41 pathways were differentially enriched (FDR<0.05; Table E5), including phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) and MAPK signaling pathways (Figure 5B). The network analysis showed that negatively correlated miRNA-mRNA pairs mapped to the identified immune-related pathways, such as hsa-miR-324-3p/*IL13* for FcεR signaling, hsa-miR-29a-3p/*STAT2* for PI3K-Akt signaling, and hsa-let-7b-5p/*PTPN22* for TLR signaling (Figure 5C). Finally, the DEmiRNAs were significantly associated with cytokines, such as hsa-miR-29a-3p/IFN-γ ($log_2FC=-1.05$, FDR<0.05), and hsa-miR-324-3p/IL-13 ($log_2FC=-1.68$, FDR<0.05) (Figure 5D).

DISCUSSION

By applying a small RNA-seq technique to a multicenter prospective cohort of infants with severe bronchiolitis, we identified nasal miRNAs, their potential contextual functions, and their longitudinal relationship with incident asthma. More specifically, we found 23 miRNAs significantly associated with the risk of developing asthma—e.g., hsa-miR-22-3p with a higher risk of asthma in infants with RSV or RV infection. Furthermore, we also observed that these DEmiRNAs were associated with asthma-related clinical features, such as infant history of eczema and serum total IgE level. Moreover, by using the integrated miRNA and mRNA data, we found that infants who subsequently developed asthma had differentially-enriched pathways—e.g., TLR, PI3K-Akt and FcεR signaling pathways. To the best of our knowledge, this is the first study that has demonstrated the potential role for nasal miRNAs in infants with severe bronchiolitis in the pathobiology of developing asthma.

Results in Context

Concordant with our findings, prior studies have suggested that miRNAs are implicated in asthma pathobiology [13, 15–26]. For example, studies have reported that several miRNAs (e.g., hsa-miR-125b) are associated with adult prevalent asthma and its inflammatory features (e.g., the quantity of eosinophils and neutrophils in blood) [13, 17, 19, 24]. Similarly, the literature has also shown that asthma-related miRNAs are involved in immune-related pathways, such as PI3K-Akt [13], MAPK [15, 52, 53], TLRs [20, 24], and T_H17 [24]. For example, a recent single-center study of 62 adults with asthma reported that—by applying a microarray approach to sputum specimens—a miRNA network was

associated with increased neutrophilic airway inflammation in *prevalent* asthma, and the miRNAs are enriched for TLR signaling pathway [24]. Additionally, in a single-center case-control study of 35 adults with severe asthma, Rupani et al. have reported that—by applying a PCR approach to bronchoalveolar lavage samples—upregulation of three miRNAs reduces TLR7 expression, which drives impaired innate immune responses to RV [20]. While these studies have collectively suggested the role of the miRNAs in prevalent asthma, to date, no study has evaluated the nasal miRNA in infants—let alone in those with severe bronchiolitis—and their contribution to asthma risk. The current study—with a small RNA-seq approach applied to a large multicenter cohort—builds on previous reports and extends them by identifying the pathobiological role of nasal miRNAs in the development of asthma.

Potential Mechanisms

There are several potential mechanisms linking bronchiolitis—by miRNA post-transcriptional regulation—to the subsequent development of asthma. First, the literature has indicated that miRNAs, as mediators between respiratory virus infections and asthma, modulate airway inflammatory processes [11, 55]. Infants with RV bronchiolitis are more likely to develop asthma as compared to infants with RSV bronchiolitis [56]. Growing evidence has shown that both RSV and RV infections are important triggers for perturbations in miRNA expression, which are actively involved in innate immune response, such as TLRs and NFκB signaling pathways [57–59]. The host innate immune response is the first line of defense against all pathogens (e.g., virus). Thornburg et al. have shown that RSV manipulates host cell gene expression through the regulation of miRNA (e.g., hsa-let-7 families) expression related to the TLRs, NF-κB, or interferon (IFN) signaling pathways in human bronchial epithelial cells and monocyte-derived dendritic cells [60]. In addition, in RSV-induced airways inflammation, a population of IFN-γ secreting TC cells potentially attenuates pathogenic T_H2 host response to the RSV G-protein [59]. Of note, our cytokine analysis idenfied that hsa-miR-29a-3p is negatively associated with IFN-γ. This result is consistent with a recent study showing that miRNA-29a-3p upregulation due to pulmonary microbial infection suppresses the immune response by inhibiting IFN-γ expression in T cells, and associated with a higher risk of active and latent pulmonary tuberculosis [61]. Furthermore, studies have reported that miR-22-3p is involved in regulation of asthma-related immune mechanisms (e.g., IFN-γ, NACHT, LRR, and PYD domains-containing protein 3) in both human [15] and mouse [62].

Second, prior research has also showed that miRNAs are involved in the PI3K-Akt signaling pathway, which plays a role in cell proliferation and airway remodelling [63]. For example, Alexandrova et al., by profiling the miRNA expression in bronchial smooth muscle cells from 8 adults with asthma, found that these patients had specific miRNA signatures (e.g., hsa-miR-29a-3p) and that the targeted transcripts were involved in the PI3K-Akt signaling pathway, which plays a role in the airway smooth muscle (ASM) cell growth and proliferation [13]. Consistently, the current study has identified hsa-miR-29a-3p as the top DEmiRNA and had high expression in multiple immune cell types (e.g., T_H cells). A recent study has also found a crosstalk between T_H cells and ASM in pediatric obesity-related asthma [64]. Furthermore, an *in vivo* study using an ovalbumin (OVA)-induced mouse model of allergic asthma has found that mmu-miR-221 modulated airway remodelling via

PI3K-Akt signaling pathway [65]. Indeed, PI3K is required for growth factor-induced cell migration [66], and activation of PI3K can stimulate DNA synthesis and growth, which all promote airway remodelling [67].

Third, while the research has suggested the roles of miRNAs in airway inflammation and remodelling, the role of miRNAs in IgE-mediated asthma remains unclear. IgE-mediated asthma is characterized by the presence of allergen-specific IgE antibodies, which bind to high-affinity FcεR [68]. Multiple studies have reported that RSV and RV infection can activate the FcεR signaling pathway [69, 70], which can trigger allergic airway inflammation [71]. In the current study, among immune-related pathways, we identified FcεR signaling pathway most significantly associated with the risk of asthma development.

Notwithstanding the complexity of these mechanisms, we believe that the identification of the longitudinal relationship between nasal miRNAs in infancy and childhood asthma is important. Of note, evidence has suggested the role of miRNAs in disease prevention, such as cancer [72]. Thus, our findings, in conjunction with the existent literature, should advance research into the development of miRNA-specific strategies for asthma prevention.

Limitations

Our study has several potential limitations. First, bronchiolitis involves inflammation of the lower airways, in addition to the upper airways. Although the current study used the miRNA data from nasal specimens, research has shown that upper airway specimens offer a reliable representation of inflammatory profiles in the lower airways [73]. Additionally, the use of upper airway specimens is practical because lower airway sampling (e.g., bronchoscopy) would be invasive in young infants. Second, the nasal specimens were obtained at a single time point. While longitudinal molecular data would also be informative, the study objective was to investigate the role of miRNA at the time of bronchiolitis in asthma development. Third, nasal specimens during respiratory infection might have been contaminated with blood immune cells. Accordingly, the miRNA profiles may have partially reflected those of these cells. Accordingly, it is likely that the miRNAs partially reflect the infiltration of blood immune cells. Fourth, our study does not have data on specific RSV genotype variants, while research has suggested different RSV genotype variants may be related with altered functions and/or immunogenicity, potentially leading to an impact on disease severity [74]. Fifth, it is possible that asthma diagnosis (by age 6 years) may have been misclassified and that some children are going to develop asthma at a later age. To address these points, the cohort is currently being followed up to age 9 years. Sixth, the current study does not have mechanistic experiments to validate the identified miRNA functions. Yet, our independent nasal cytokine data has partially validated the miRNA functions, which are consistent with the literature. This study derives well-calibrated hypotheses that facilitate future experiments. Seventh, our data are limited to investigating the effect modification of respiratory infection at infancy on miRNA and asthma development. It is possible that prenatal risk factors (e.g., vertical transmission) [75] may have affected the relationship between miRNA and asthma development. Eighth, we have used miRNA data of publicly available tissue and blood immune cells data from subjects without asthma to investigate the expression of nasal miRNAs from the current study. Although these results elucidate the

role of miRNAs in tissue and cell-specific manner, the interpretation of these results requires careful interpretation. Lastly, despite the study sample consisting of racially/ethnically- and geographically-diverse infants, our inferences must be cautiously generalized beyond infants with severe bronchiolitis. Nonetheless, our data remain directly relevant for the 110,000 infants hospitalized yearly in the U.S [2].

Conclusions

In conclusion, by applying small RNA-seq approach to a multicenter cohort of infants with severe bronchiolitis, we found a complex interplay between nasal miRNA, virus, asthma risk factors, and their longitudinal relationship with asthma development. For example, RSV or RV infection modifies the effect of miRNAs on asthma development. Additionally, the data suggest that these miRNAs play key roles in mechanisms relating to asthma, such as innate immunity, airway remodelling, and IgE regulation. For clinicians, our findings provide an evidence base for the early identification of high-risk infants during a critical period of airway development—early infancy. For researchers, these observations should facilitate 1) further understanding of the interplay between virus and host, and their contribution to asthma; 2) further investigations into the development of miRNA-targeted strategies for asthma prevention [11] in infants with severe bronchiolitis—a population with substantial morbidity burden.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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Conflict of interests statement:

Dr. Zhu reports grants from National Institutes of Health during the conduct of the study. Dr. Hahn reports personal fees from Johnson and Johnson, outside the submitted work. Dr. Teach reports grants from National Institutes of Health during the conduct of the study. Dr. Hasegawa reports grants from National Institutes of Health during the conduct of the study; grants from Novartis, outside the submitted work. Dr. Camargo reports grants from National Institutes of Health during the conduct of the study. All other authors have indicated that they have no financial relationships relevant to this article to disclose.

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Take home message:

In infants with severe bronchiolitis, we found a complex interplay of nasal miRNA with virus, asthma risk factors, and their longitudinal relationship with asthma development, facilitating development of miRNA-targeted strategies for asthma prevention.

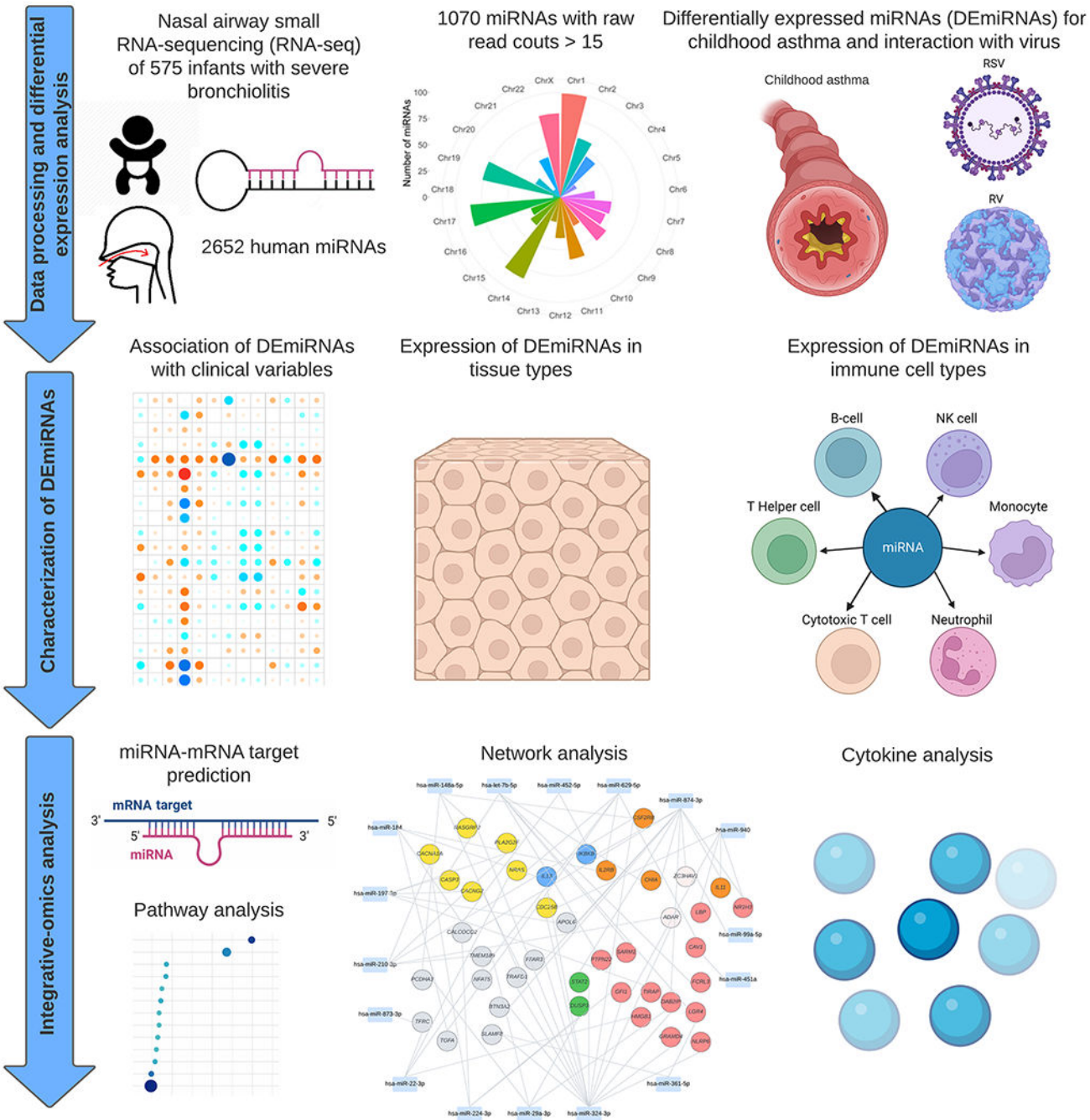

Figure 1. Study design and analytic workflow

The analytical cohort consists of 575 infants hospitalized for bronchiolitis (severe bronchiolitis) in a multicenter prospective cohort study—the 35th Multicenter Airway Research Collaboration (MARC-35). Fastq files underwent quality control and collapse into unique reads. The trimmed reads were mapped against human miRNA sequences from miRBase V22. A total of 2652 human mature miRNAs were detected. Raw read counts of <15 were filtered out, leading to 1070 high-quality miRNAs for downstream analysis. In Aim 1, the association of 1070 miRNAs with the risk of developing asthma was examined.

Effect modification by respiratory syncytial virus (RSV) and rhinovirus (RV) infection on the miRNA-asthma relationship was also investigated. A total of 23 differentially expressed miRNAs (DEmiRNAs) were identified. In Aim 2, the association of DEmiRNAs with asthma-related clinical variables was determined. The between-tissue and -immune cell types expression of the DEmiRNAs were also examined. In Aim 3, the mRNAs targeted by DEmiRNAs were identified using DIANA-microT-CDS and the association of biological pathways with the asthma risk was examined by performing the pathway analyses. The DEmiRNA-mRNA target network was constructed based on negatively correlated pairs.

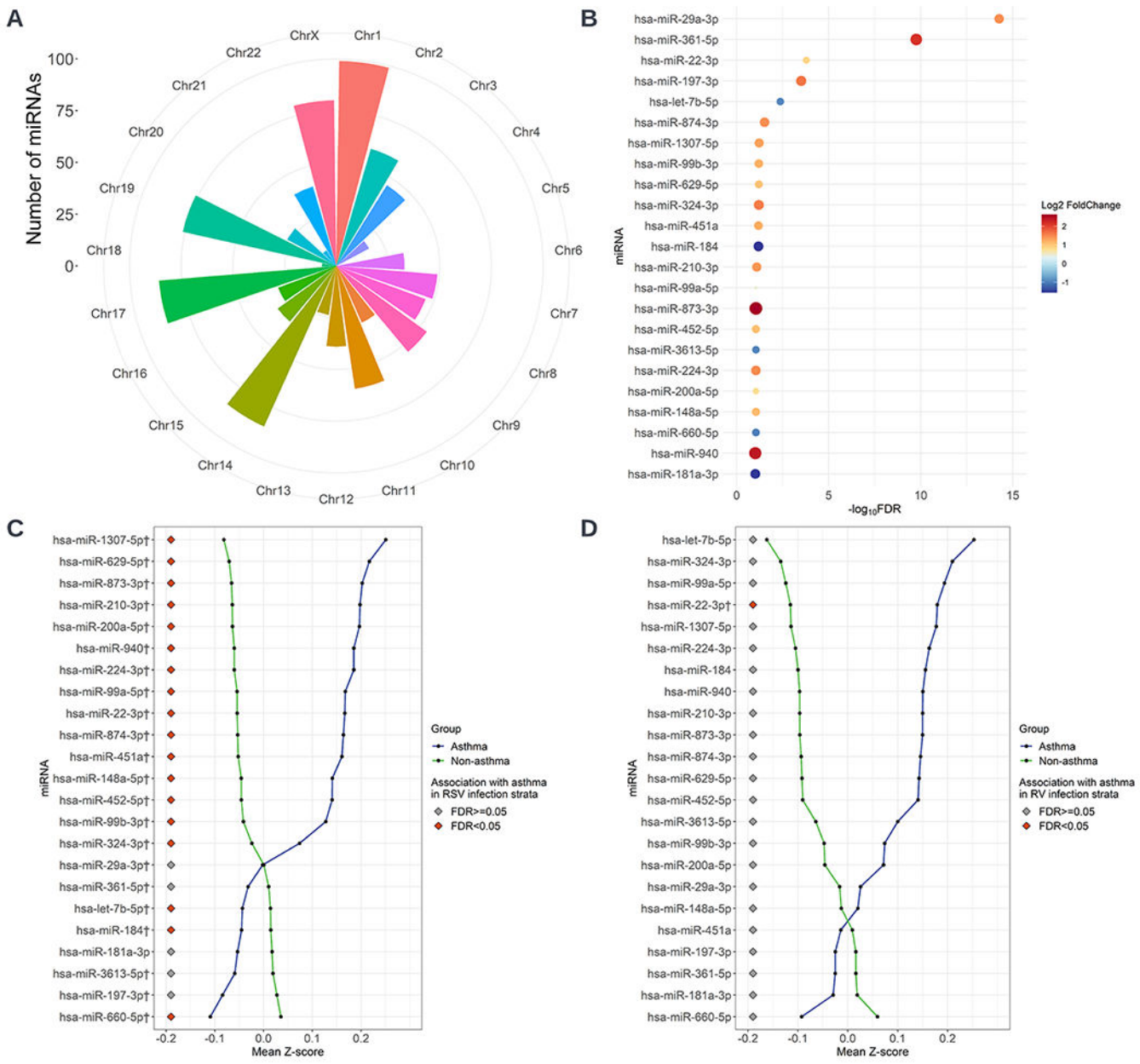

Figure 2. Association of nasal airway miRNAs in infants with bronchiolitis with risk of developing asthma .

A. The plot included 1070 miRNAs after quality control and showed the genome-wide distribution of the number of miRNAs in each chromosome. **B.** The plot shows the association of the 23 DEmiRNAs in the nasal airways with asthma risk. The between-group differences in the expression level were tested by *DESeq2* with the negative binomial generalized linear model adjusted for potential confounders, including age, sex, number of previous breathing problems, and IgE sensitization. **C.** The plot shows the RSV infection-stratified analysis for the association of the 23 DEmiRNAs with the risk of developing asthma. The *DESeq2* models adjusted for potential confounders, including age, sex, number of previous breathing problems, and IgE sensitization. **D.** The plot shows the RV infection-

stratified analysis for the association of the 23 DEmiRNAs with the risk of developing asthma. The *DESeq2* models adjusted for potential confounders, including age, sex, number of previous breathing problems, and IgE sensitization. Abbreviations: FDR, false discovery rate; RSV, respiratory syncytial virus; RV, rhinovirus.

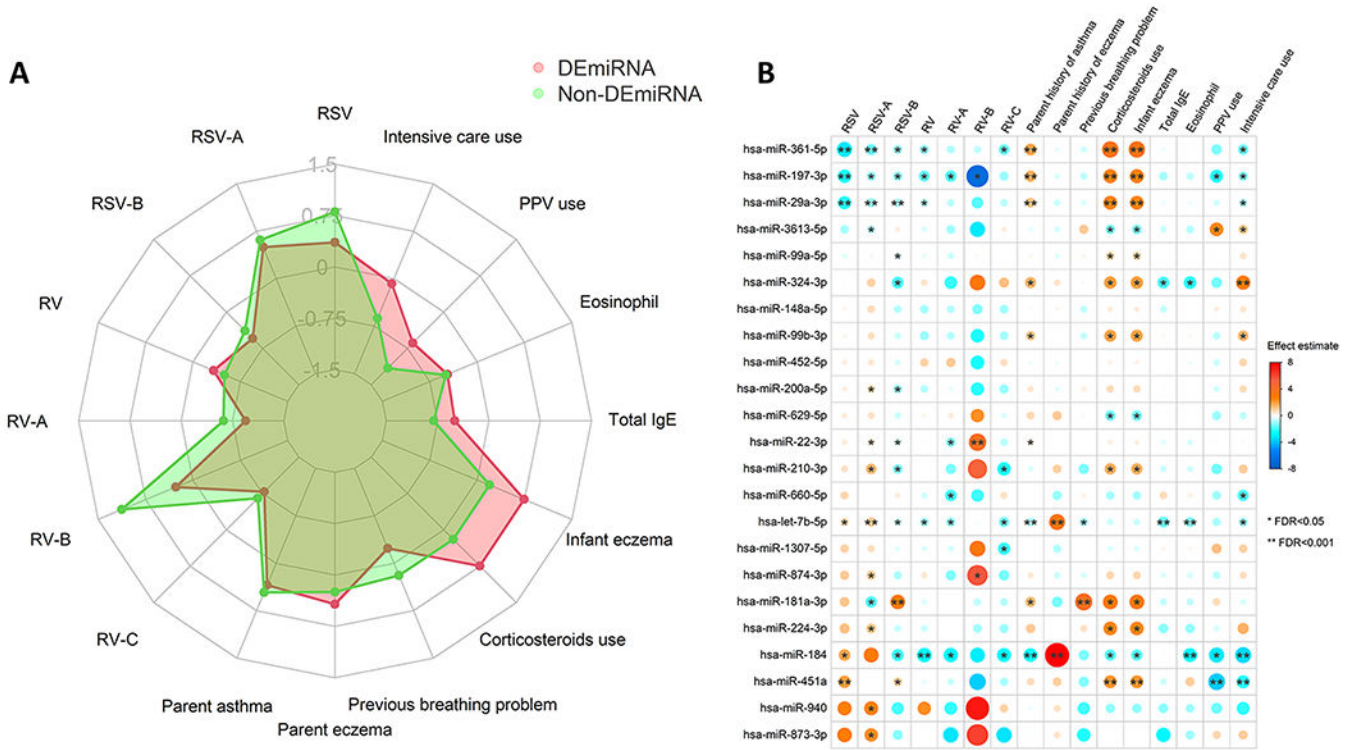

Figure 3. Association of nasal airway DEmiRNAs in infants with bronchiolitis with asthma-related clinical variables.

A. Association between the DEmiRNAs or non-DEmiRNAs and that of 16 asthma-related clinical variables in infants with severe bronchiolitis. The red web denotes the average effect estimates of the aggregated DEmiRNAs for the 16 clinical variables. The green web denotes the average effect estimates of the aggregated non-DEmiRNAs for the 16 clinical variables. **B.** The relationship of the 23 DEmiRNAs with the 16 asthma-related clinical variables was examined by the *DESeq2* negative binomial generalized linear model adjusting for age and sex. The blue-to-red gradient in the heatmap denotes the magnitude and direction of the associations. The size of the dot denotes the magnitude of the associations. Abbreviations: FDR, false discovery rate; IgE, immunoglobulin E; PPV, positive pressure ventilation; RSV, respiratory syncytial virus; RSV-A, respiratory syncytial virus A; RSV-B, respiratory syncytial virus B; RV, rhinovirus; RV-A, rhinovirus A; RV-B, rhinovirus B; RV-C, rhinovirus C.

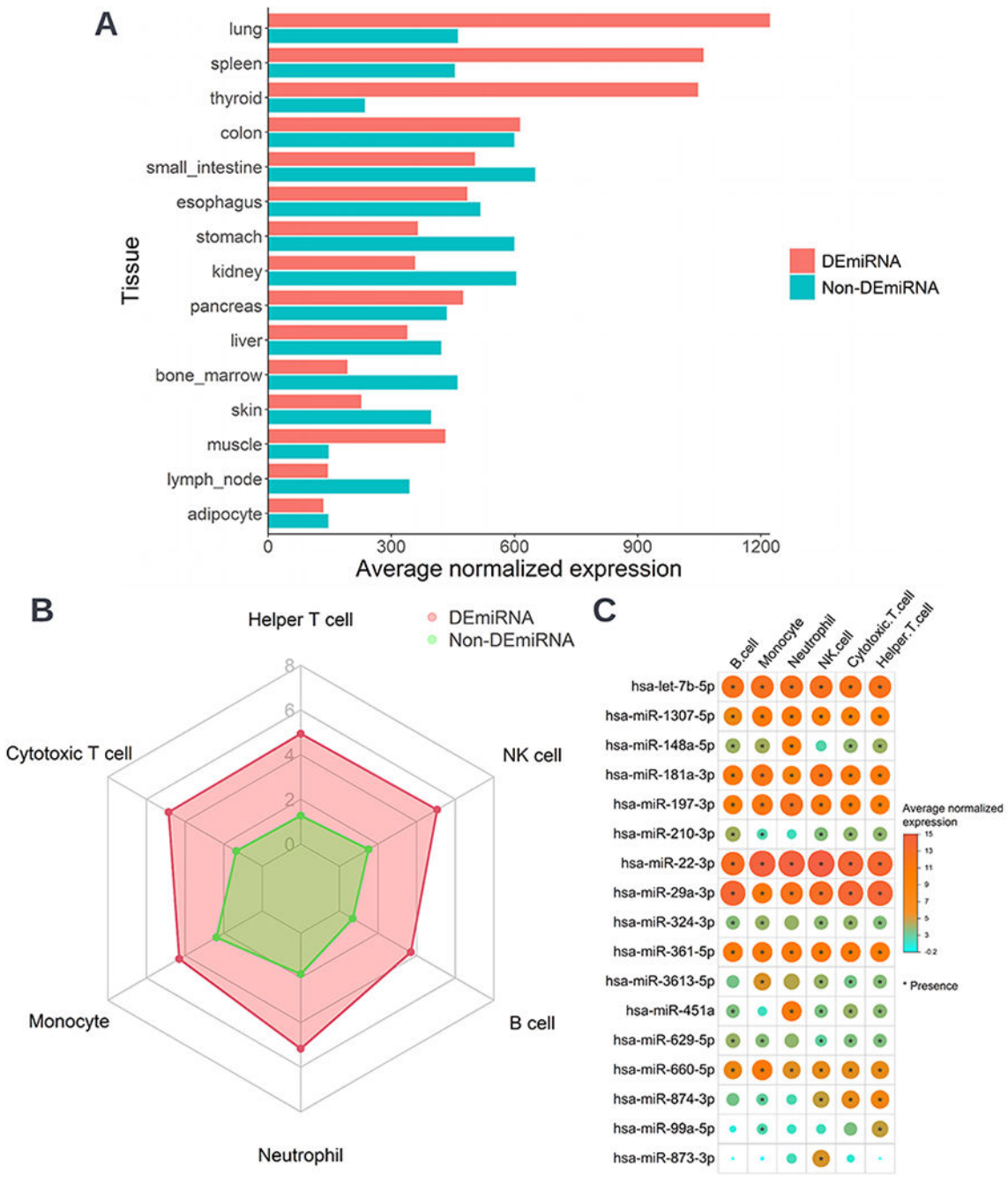

Figure 4. Expression of nasal airway DEmiRNAs in infants with bronchiolitis with tissue and immune cell types.

A. Average normalized expression level of the aggregated DEmiRNAs or non-DEmiRNAs in 15 asthma-related tissues. The between-group differences in the expression level were tested by the Wilcoxon rank-sum test. The between-group differences for all tissues were significant (FDR<0.001). **B.** Average normalized expression level of the aggregated DEmiRNAs or non-DEmiRNAs in six blood immune cell types, including B cell, cytotoxic T cell, helper T cell, monocyte, natural killer cell, and neutrophil cell. The between-group

differences in the expression level were tested by the Wilcoxon rank-sum test. The between-group differences for all cell types were significant (FDR<0.001). **C.** Normalized expression level of the 17 DEmiRNAs in six blood immune cell types. A asterisk denotes the presence of the miRNAs in the cell types. Only the miRNAs which were detected (expression value > 5) in at least 85% of the samples in at least one of the blood cell type were considered as being present. Abbreviation: NK, natural killer.

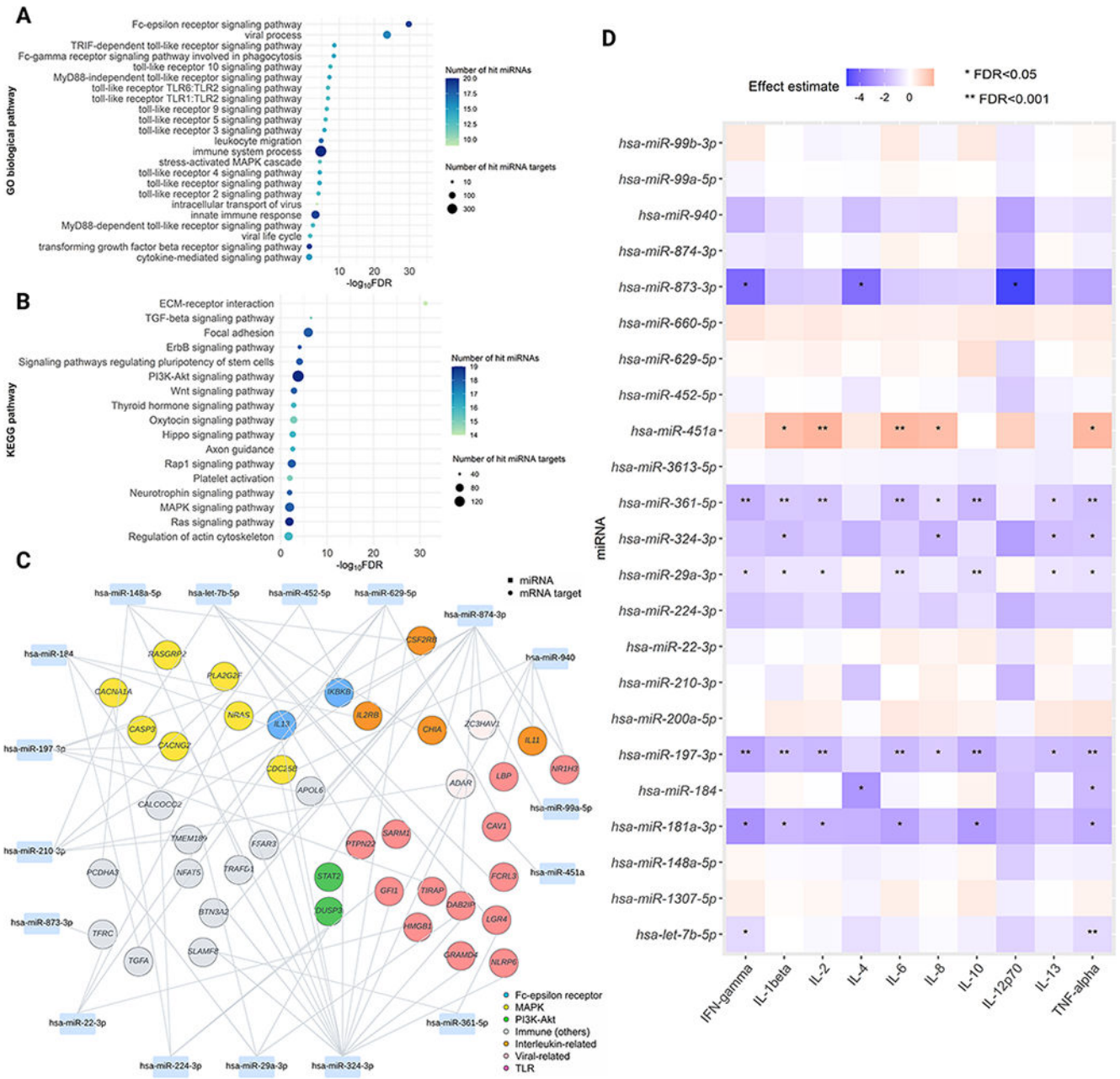

Figure 5. Nasal airway DEmiRNAs pathways and risk of developing asthma

A. Functional enrichment analysis using GO biological process gene sets We identified 108 differentially-enriched pathways (FDR<0.05) associated with the asthma risk. Of these, we selected immune-related pathways to visualize the plot. **B.** Functional enrichment analysis using KEGG gene sets We identified 41 differentially-enriched pathways (FDR<0.05) associated with the asthma risk. Of these, we selected non cancer-related pathways to visualize the plot. **C.** DEmiRNA-mRNA target network A rectangle denotes miRNA, a circle denotes mRNA target. mRNA targets that are mapped within major immune-related pathways are highlighted in various colors. In this network, we only included mRNA targets

that are negatively correlated (Spearman correlation coefficient<−0.1) with DEmiRNAs and have immune-related functions based on prior knowledge. **D.** Association of the 23 DEmiRNAs with the ten nasal cytokines. The blue-to-red gradient in the heatmap denotes the magnitude and direction of the associations. Abbreviations: FDR, false discovery rate; IFN, Interferon; IL, interleukin; MAPK, Mitogen-activated protein kinase; PI3K-Akt, phosphatidylinositol 3−kinase-protein kinase B; TLR, Toll-like receptor; TNF, Tumor necrosis factor.

Table 1.

Baseline characteristics and clinical course of 575 infants hospitalized for bronchiolitis

Characteristics	Overall (n=575)
Demographics	
Age (month), median (IQR)	3 (2–6)
Female sex	234 (41)
Race/ethnicity	
Non-Hispanic White	268 (47)
Non-Hispanic Black	126 (22)
Hispanic	162 (28)
Other or unknown	19 (3)
Prematurity (32–36.9 weeks)	96 (17)
Birth weight (kg), median (IQR)	3.29 (2.90–3.60)
Mode of birth (cesarean delivery)	194 (34)
Previous breathing problems before the index hospitalization *	
0	469 (82)
1	87 (15)
2	19 (3)
Previous ICU admission	7 (1)
History of eczema	85 (15)
Lifetime antibiotic use	170 (30)
Ever attended daycare	143 (25)
Cigarette smoke exposure at home	87 (15)
Maternal smoking during pregnancy	87 (15)
Parental history of asthma	186 (32)
Parental history of eczema	110 (19)
Clinical presentation	
Weight at presentation (kg), median (IQR)	6.07 (4.70–7.60)
Respiratory rate at presentation (per minute), median (IQR)	48 (40–60)
Oxygen saturation at presentation	
<90%	49 (9)
90–93%	87 (16)
≥94%	427 (76)
Blood eosinophilia (≥4%)	52 (10)
IgE sensitization (%)	125 (22)
Length of hospitalization (day), median (IQR)	2 (1–3)
Corticosteroid use during hospitalization	71 (12)
Respiratory virus	
RSV infection	485 (84)
RSV-A	351 (61)
RSV-B	137 (24)
RV infection	110 (19)

Characteristics	Overall (n=575)
RV-A	50 (9)
RV-B	5 (1)
RV-C	53 (9)
Chronic clinical outcome	
Childhood asthma[†]	156 (27)

Abbreviations: ICU, intensive care unit; IgE, immunoglobulin E; IQR, interquartile range; RSV, respiratory syncytial virus; RV, rhinovirus.

Data are no. (%) of infants unless otherwise indicated. Percentages may not equal 100, because of rounding and missingness.

[*]Defined as an infant having a cough that wakes him or her at night or causes emesis, or when the child has wheezing or shortness of breath without cough.

[†]Asthma was defined as physician-diagnosis of asthma by age 6 years, plus either asthma medication use (e.g., albuterol inhaler, inhaled corticosteroids, montelukast) or asthma-related symptoms in the preceding year.