


ORIGINAL ARTICLE

Epigenetic regulation of inflammation by microRNAs in post-infectious bronchiolitis obliterans

Ruth P Duecker¹ , Ines De Mir Messa², Silviya-Pera Jerkic¹, Annalena Kochems¹, Gabriele Gottwald¹, Antonio Moreno-Galdó^{2,3}, Martin Rosewich¹, Lucia Gronau¹, Stefan Zielen¹, Andreas Geburtig-Chiocchetti⁵, Hermann Kreyenberg⁴ & Ralf Schubert¹

¹Division for Allergy, Pneumology and Cystic Fibrosis, Department for Children and Adolescence, Goethe University, Frankfurt, Germany

²Allergy, Pulmonology and Cystic Fibrosis Section, Department of Pediatrics, Vall d'Hebron Hospital Universitari, Universitat Autònoma de Barcelona, Barcelona, Spain

³CIBER of Rare Diseases (CIBERER), Instituto de Salud Carlos III (ISCIII), Madrid, Spain

⁴Division for Stem Cell Transplantation and Immunology, Department for Children and Adolescence, Goethe University, Frankfurt, Germany

⁵Department of Child and Adolescent Psychiatry, Psychosomatics and Psychotherapy, Goethe University, Frankfurt, Germany

Correspondence

RP Duecker, Division for Allergy, Pneumology and Cystic Fibrosis, Department for Children and Adolescence, Goethe University, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany.
E-mail: ruthpia.duecker@kgu.de

Received 27 March 2021;
Revised 14 November 2021 and
3 February 2022;
Accepted 4 February 2022

doi: 10.1002/cti2.1376

Clinical & Translational Immunology
2022; 11: e1376

Abstract

Objectives. Post-infectious bronchiolitis obliterans (PiBO) is a rare, chronic disease initiated by severe infection and followed by perpetuating inflammation and obliteration of the small airways. MicroRNAs (miRNAs) have been proposed to play a central role as epigenetic regulators, which control resolution and prevent the uncontrolled progress of inflammation. The aim of this study was to define biomarkers on the level of post-transcriptional gene regulation in order to characterise PiBO. **Methods.** A total of 39 patients with well-defined PiBO and 31 controls from two centres, Barcelona, Spain, and Frankfurt, Germany, were analysed by next-generation sequencing (NGS). The evaluation of the biological targets of the miRNAs was performed by pathway enrichment analysis and protein–protein interaction network analysis respectively. **Results.** Patients with PiBO had significantly lower lung function values and increased airway inflammation in induced sputum as indicated by total cell counts, neutrophils, IL-1 β , IL-6, IL-8 and TGF- β compared to controls. Next-generation sequencing analysis revealed a total of 22 dysregulated miRNAs, which passed significance threshold for $P_{adj} \leq 0.001$ with 17 being upregulated and 5 being downregulated. Of these dysregulated miRNAs, miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p were further validated using qRT-PCR. Interestingly, these miRNAs are functionally implicated in cytokine–cytokine receptor interaction, TGF- β signalling and FoxO signalling pathway and significantly correlated with lung function values (FEV1). **Conclusion.** Our results demonstrate an aberrant miRNA expression profile in PiBO, which impacts pathways responsible for the regulation of inflammation and fibrosis. The defined miRNAs are useful

biomarkers and should be assessed as potential target in the field of miRNA therapeutics.

Keywords: fibrosis, inflammation, microRNA, post-infectious bronchiolitis obliterans

INTRODUCTION

Post-infectious bronchiolitis obliterans (PiBO) is a rare, chronic pulmonary disease characterised by inflammation and fibrous wall thickening of the bronchioles, narrowing their lumens and restricted air circulation, which emerges after severe pulmonary infection.^{1,2} Although the pathogenesis of PiBO is still largely unclear, the lack of resolution of the ongoing inflammation seems to be an important part of the disease process. A pathomechanism with a predominant neutrophilic inflammation in the bronchioles together with increased concentrations of inflammatory cytokines has been proposed.^{3,4} This inflammation stimulates the migration of fibroblasts and myofibroblasts into the luminal exudate, resulting in intraluminal granular tissue formation. Thus, the new tissue formation can lead to a complete occlusion of the small airways.⁵ Recent observations in induced sputum of PiBO patients demonstrated ongoing neutrophilic inflammation without termination.⁶ This unresolved inflammation in PiBO and the identification of endogenous control mechanisms in the inflammatory response is of critical importance.

Studies in other immune-mediated lung diseases such as asthma, cystic fibrosis, idiopathic interstitial pneumonia or COPD suggest an epigenetic dysregulation of the immune response to external and internal stimuli.⁷ The post-transcriptional regulation by microRNAs (miRNAs) is a central epigenetic mechanism.⁸ miRNAs are a highly conserved group of small (22–25 nucleotides), relatively stable, non-coding RNA molecules, which control gene expression primarily via mRNA silencing.⁹ Dysregulation of miRNA expression leads to aberrant gene expression and might be responsible for pathological conditions.¹⁰ In this regard, miRNA expression profiles might have the potential as biomarkers in inflammatory lung diseases.

However, so far, only limited data about miRNA expression in PiBO exist. Therefore, the aim of the present study was to investigate the regulation of inflammation and fibrotic processes in the context

of miRNA expression. A study cohort of Spanish and German patients with PiBO was investigated to overcome biases in regional and lifestyle factors. Clinical, laboratory and computational analyses were performed to identify relevant miRNAs, which have the potential as biomarkers in PiBO and which should be investigated as targets for new therapies.

RESULTS

In total, 39 patients with PiBO and 31 controls from the Children's Hospitals in Barcelona and Frankfurt were enrolled into the study (Table 1). No differences were found in age or gender between the sites. Two patients from the Frankfurt cohort had to be excluded from the NGS analysis. No blood for NGS was available from one patient. One patient sample had to be removed from the data analysis because of too-low reads.

Pulmonary function

As expected, measurement of lung function parameters showed a significant reduction of FVC [patients (F) 76.3 (range 54.5–94.2%; $P < 0.001$); patients (B) 80.6 (range 49.3–98.4%; $P < 0.01$); controls 90.8 (range 73.9–102.7)], FEV1 [patients (F) 59.8 (range 40.2–96.9%; $P < 0.001$); patients (B) 62.0 (range 29.03–99.0%; $P < 0.001$); controls 97.4 (range 73.5–116.4%)], FEV1/FVC [patients (F) 0.7 (range 0.4–1.0%; $P < 0.001$); patients (B) 0.7 (range 0.4–1.0%; $P < 0.001$); controls 0.9 (range 0.7–1.0%)] and FEF75 [patients (F) 25.1 (range 9.2–195.7%; $P < 0.01$); patients (B) 52.1 (range 7.1–97.9%; $P < 0.001$); controls 107.4 (range 41.3–184.7%)] among patients with PiBO compared to controls (Table 1, Supplementary tables 3–5). This was true for patients from the Frankfurt cohort as well as for patients from Barcelona.

Bronchial inflammation

To assess airway inflammation, induced sputum was analysed for inflammatory cells and mediators in the Frankfurt patient and control group. In the

Table 1. Lung function of patients with post-infectious bronchiolitis obliterans from Frankfurt (F) and Barcelona (B) compared to controls

	Controls	Patients (F)	Patients (B)
N	31	19	20
Age (years)	14 (7 to 28)	13 (6 to 28)	13 (6 to 20)
Sex (m/f)	19/12	13/6	11/9
FVC (%)	90.8 (73.9 to 102.7)	76.3*** (54.5 to 94.2)	80.6** (49.3 to 98.4)
FVC (z score)	-0.77 (-2.23 to 0.24)	-2.04***(-3.84 to -0.46)	-1.68** (-4.11 to -1.30)
FEV1 (%)	97.4 (73.5 to 116.4)	59.8*** (40.2 to 96.9)	62.0*** (29.3 to 99.0)
FEV1 (z score)	-0.54 (-2.20 to -1.41)	-3.42*** (-4.86 to -0.25)	-3.08*** (-5.64 to -0.08)
FEV1/FVC	0.9 (0.7 to 1.0)	0.7*** (0.4 to 1.0)	0.7*** (0.4 to 1.0)
FEV1/FVC (z score)	0.33 (-2.26 to 2.49)	-2.19*** (-4.45 to 2.19)	-3.58*** (-4.50 to 1.42)
FEF75 (%)	107.4 (41.3 to 184.7)	25.1** (9.2 to 195.7)	52.1*** (7.1 to 97.9)
FEF75 (z score)	0.21 (-2.30 to 2.01)	-3.20*** (-5.03 to 2.07)	-1.70*** (-5.30 to 0.1)

Values are expressed as percentage and z score (median and range). FEF, forced expiratory flow; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant to controls.

patient group, neutrophils and inflammatory cytokines such as IL-1β, IL-6 and IL-8 were significantly increased compared to controls (Figure 1a–e). In addition, TGF-β, which is an essential cytokine for the induction of the fibrotic response, was significantly increased in induced sputum of PiBO patients (Figure 1f).

miRNA expression

Differences in the global miRNA expression between 37 PiBO patients and 31 controls from

Barcelona and Frankfurt were successfully assessed. Analysis revealed ~158 Mio reads with an average read count per sample of 2 323 748 reads per miRNA. Overall, 877 miRNAs were detected in at least one peripheral blood sample, of which 288 had 5 or more reads in each sample and 538 miRNAs were detected with 5 or more reads in at least 50% of the samples. The groups showed no differences (ANOVA *P*-values > 0.1) with respect to age, sex, site, RNA quality and read depth.

Statistical analysis revealed a total of 22 dysregulated miRNAs passing the significance

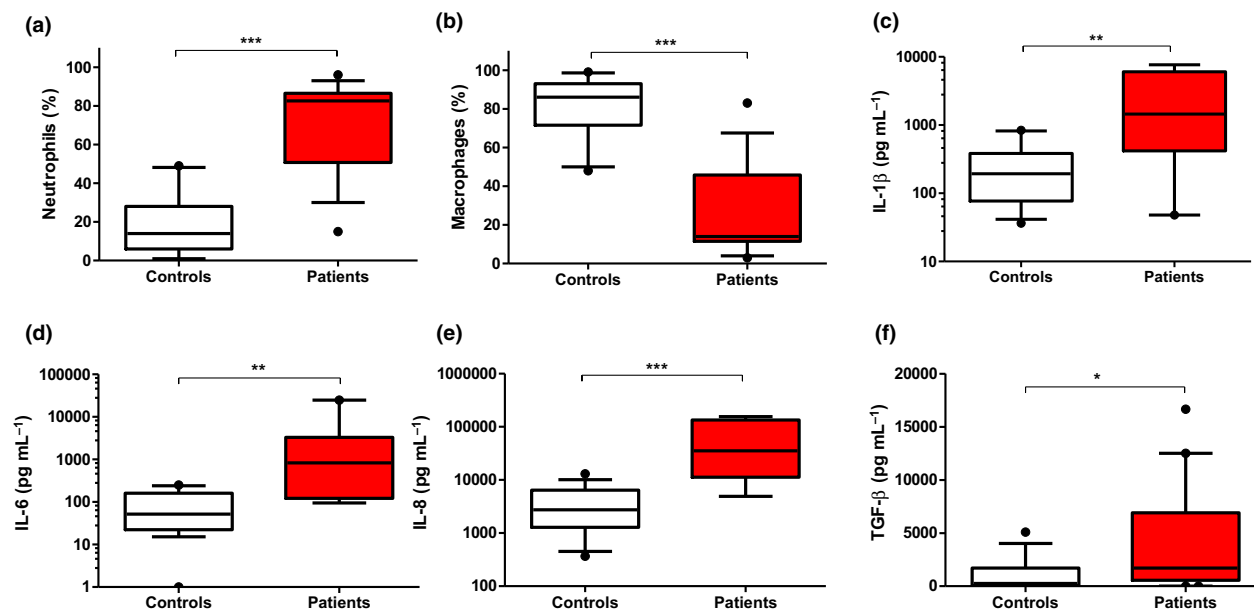


Figure 1. Bronchial inflammation. (a) Neutrophils and (b) macrophages as well as the inflammatory cytokines (c) IL-1β, (d) IL-6, (e) IL-8 and (f) TGF-β were analysed in induced sputum from patients with PiBO (*n* = 14) and healthy controls (*n* = 13) by cell differentiation and cytometric bead array, respectively. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

threshold of $P_{adj} \leq 0.001$ (195 miRNAs for $P < 0.05$), of which 17 were upregulated and 5 downregulated (Figure 2a, Supplementary table 2). Of these dysregulated miRNAs, miR-1287-5p, miR-335-5p, miR-5695, miR-30b-5p, miR-186-5p, miR-190b, let-7f-1-3p, miR-30c-5p, miR-26a-2-3p, miR-4677-3p and miR-199a-5p share dysregulated expression after separate analyses of the two centres (Figure 2b; Supplementary figure 1a and b). Of these, four miRNAs were successfully confirmed by TaqMan qPCR namely miR-335-5p, miR-30b-5p, miR-186-5p and miR-30c-5p (Figure 2c–f). These four were considered in the further computational analysis. The expression levels for miR-1287-5p, let-7f-1-3p and miR-199a-5p only showed a tendency between patients and controls, and no differences were found for miR-5695, miR-26a-2-3p and miR-4677-3p (Supplementary figure 1c–h). Expression of miR-190b could not be detected using TaqMan in most of the samples.

KEGG pathway analysis

To identify the contribution of the miRNAs to biological processes, KEGG pathway enrichment analysis has been performed, and targets of the individual miRNAs were assessed. In total, four miRNAs of interest were predicted to be involved in 34 pathways including cancer, cell signalling, cell proliferation and apoptosis, cell–cell interaction; cytokine–cytokine receptor interaction ($P < 0.05$); and TGF- β signalling pathway ($P < 0.05$) and target 3775 genes (Figure 3). Shared targets for miR-186-5p and miR-30b/c-5p were ST3GAL5, LRRC3C, PLXNA1 and LPCAT1.

Single analysis of the individual miRNAs concentrating on inflammatory processes revealed that 61 target genes of miR-335-5p are strongly involved in cytokine–cytokine receptor interaction ($P < 0.0009$), 20 target genes of miR-186-5p are strongly involved in the TGF- β signalling pathway ($P < 0.0002$), and 18 target genes of miR-30b-5p and miR-30c-5p are strongly involved in the FoxO signalling pathway ($P < 0.0095$) respectively (Table 2; Supplementary figure 2).

Protein–protein interaction in inflammation

Based on the KEGG analysis, we used the STRING web server to visualise the network of the protein–protein interaction of the miRNA targets involved in cytokine–cytokine receptor interaction,

TGF- β signalling pathway and FoxO signalling pathway (Figure 4). Network analysis showed a strong interaction of the target proteins between the pathways (PPI enrichment P -value $< 1.0^{-16}$). Three targets, TGFB2, TGFBR1 and TGFBR2, were located in all three pathways; IL-6 and EGFR were located in cytokine–cytokine receptor interaction; and 11 targets, ACVR1, ACVR2B, AMHF, BMP2, BMPR1A, BMPR1B, BMPR2, GDF5, INHBA, INHBB and INHBE, were located in cytokine–cytokine receptor interaction and in the FoxO signalling pathway. Most important, we identified the inflammatory cytokines IL-1A, IL-6 and CXCL8 (IL-8) as well as TGFB2.

Knockdown of miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p enhances IL-6 protein levels in A549 cells

To demonstrate the functional output in altering protein expression and in order to validate the role of miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p as regulators of IL-6 expression, we measured the secretion of IL-6 in a lung epithelial cell inflammation system. As shown in Figure 5a, an increase of IL-6 secretion of A549 cells was evident after the separate inhibition of miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p and after the inhibition of all four miRNAs at every level of CM stimulation. Combination of these experiments comparing fold change of IL-6 secretion of the inhibited miRNAs with the respective CM stimulation showed that IL-6 expression after inhibition of the four miRNAs separately as well as in the knockdown of all four miRNAs simultaneously was significantly increased (miR-335-5p, 1.23 ± 0.04 ; miR-186-5p, 1.23 ± 0.04 ; miR-30b-5p, 1.34 ± 0.08 ; miR-30c-5p, 1.22 ± 0.05 ; all miRNAs inhibited together, 2.46 ± 0.43 ; $P < 0.05$; Figure 5b).

miRNA expression of miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p in induced sputum

miRNA expression in induced sputum samples of PiBO patients was compared with miRNA profile of age-matched healthy individuals (Figure 6a). miR-335-5p and miR-186-5p were significantly upregulated in sputum of PiBO patients compared to controls (miR-335-5p controls 1.74 ± 0.1 , PiBO 2.36 ± 0.22 , miR-186-5p controls 637.5 ± 120.5 , PiBO 1849 ± 503.8 ; $P < 0.05$). No significant

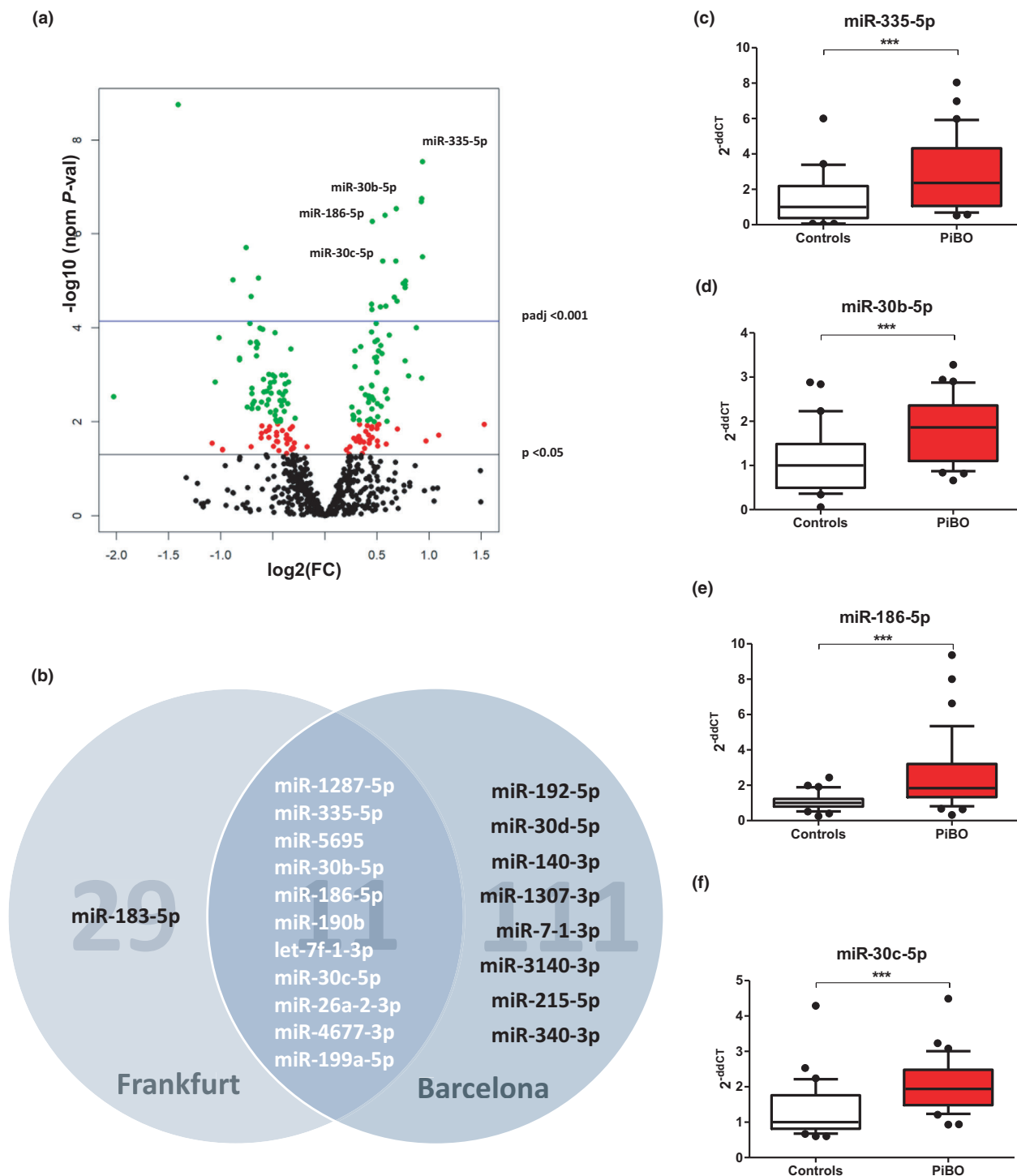


Figure 2. Global miRNA expression of PiBO patients ($n = 36$) and controls ($n = 30$). **(a)** Volcano plot showing 877 detected miRNAs, of which 195 were significant dysregulated at nominal level $P < 0.05$ and 22 were significant with a $\text{Padj} < 0.001$. **(b)** Intra-centre evaluation of the dysregulated miRNAs. Intersections shows miRNAs with $\text{Padj} < 0.05$ in the individual analysis of the respective centre. Only these miRNAs were used for further analysis. **(c–f)** Expression levels of miR-335-5p, miR-30b-5p, miR-186-5p and miR-30c-5p confirmed by TaqMan qPCR analysis. $***P < 0.001$.

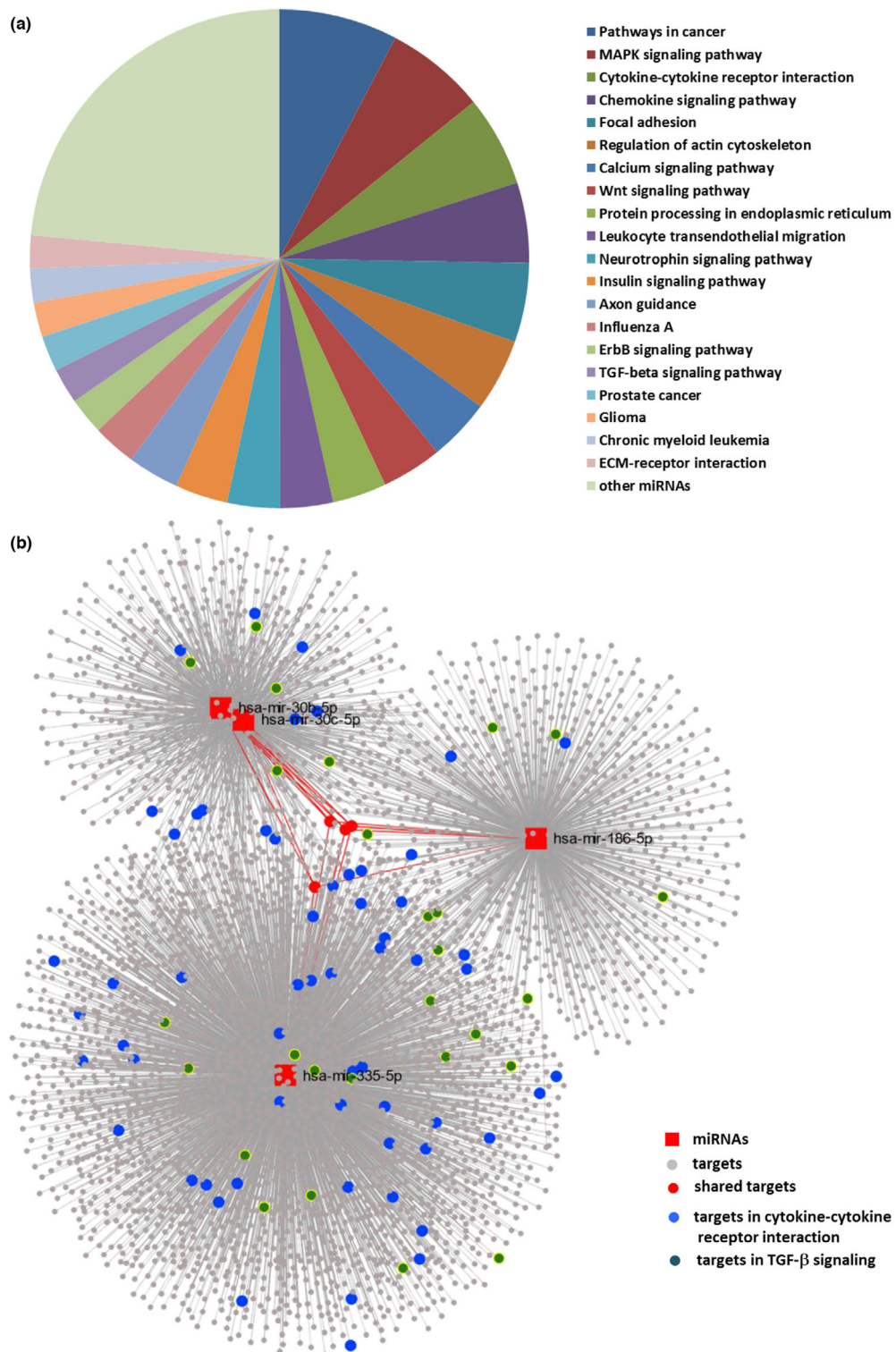


Figure 3. Interaction of the dysregulated miRNAs. **(a)** Circle chart of Kyoto encyclopaedia of genes and genomes (KEGG) pathway analysis based on microRNA signature was performed with miRNet (miRTarBase v8.0) for all the identified miRNAs together. **(b)** Dissected miRNA–target interactions and functional associations through network-based visual analysis (miRNet). miR-335-5p, miR-30b-5p, miR-186-5p and miR-30c-5p regulate 3775 genes, four of which are shared targets (red dots). Targets involved in cytokine–cytokine receptor interaction (blue dots) and in TGF- β signalling (green dots) are shown.

Table 2. miRNA targets in inflammation

miRNA	Targets database	KEGG pathway	P-value	Genes
miR-335-5p	TarBase v7.0	Cytokine–cytokine receptor interaction	0.0009	61
miR-186-5p	microT-CDS (5.0)	TGF-beta signalling pathway	0.0002	20
miR-30b-5p	microT-CDS (5.0)	FoxO signalling pathway	0.0095	18
miR-30c-5p	microT-CDS (5.0)	FoxO signalling pathway	0.0095	18

differences could be detected for miR-30b-5p and miR-30c-5p.

Expression of miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p correlates with the lung function parameter FEV1

Next, we performed correlation analyses to investigate the relationship between circulating miRNA expression in the blood and lung function parameter such as forced expiratory volume in 1 s (FEV1). Expression of miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p significantly correlated with FEV1 (miR-335-5p $P < 0.001$, $r = -0.4345$, miR-186-5p $P < 0.01$, $r = -0.3582$, miR-30b-5p $P < 0.001$, $r = -0.4322$, miR-30c-5p $P < 0.01$, $r = -0.3990$, Figure 6b) of PiBO patients and healthy controls.

DISCUSSION

A potent role of miRNAs in the regulation of resolution circuits of inflammation has been proposed.¹¹ MicroRNAs are regulatory factors involved in most biological processes and have to be considered to play a key role in the development and manifestation of inflammatory lung diseases, such as CF, IPF, COPD, PiBO and asthma.¹² Unresolved neutrophilic inflammation also contributes to the chronicity of lung disease in PiBO.^{4,6,13} This prompted us to use global NGS to get deeper insights into post-transcriptional regulation of gene expression in patients with PiBO. As described, PiBO patients exhibited impaired pulmonary function parameters as it has been described previously.^{13–15} Airway inflammation in induced sputum was confirmed by increased neutrophils and pro-inflammatory cytokines such as IL-1 β , IL-6 and IL-8 in German samples.⁴ In addition, we detected increased TGF- β levels in the sputum of the patients pointing to fibrotic processes in the bronchial tissue.¹⁶

In accordance with increased levels of neutrophils and pro-inflammatory cytokines such as IL-1 β , IL-6 and IL-8 in induced sputum, our

analysis identified four aberrant miRNAs, miR-335-5p, miR186-5p, miR-30b-5p and miR-30c-5p, which are involved in inflammatory and fibrotic processes such as cytokine–cytokine receptor interaction, TGF- β and FoxO signalling.

The role of these miRNAs in the regulation in inflammatory processes was confirmed by our *in vitro* experiments, showing that knockdown of miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p enhances IL-6 protein levels in stimulated A549 cells. In their function as post-transcriptional gene regulators, miRNAs inhibit the translation or transcription of inflammatory target mRNAs. The downregulation of these miRNAs leads to an increase in protein synthesis. In their study, Long *et al.*¹⁷ impressively demonstrated that upregulation of miR-335 has protective effects against sepsis-induced myocardial injury and that downregulation reverses the protective effect. Several studies have connected miR-335-5p to the activation of pathways, which are directly related to inflammation and the TGF- β pathway.^{18–20} This is highly supported by our laboratory and computational findings, which show the involvement of miR-335-5p in the cytokine and chemokine network, which interacts closely with the TGF- β pathway and reflects the bronchial inflammatory and fibrotic processes in PiBO. Our analysis demonstrated that miR-335-5p induces the secretion of various pro-inflammatory chemokines/cytokines including IL-1 α , IL-6 and IL-8 as well as targeting members of the TGF- β signalling such as TGF β 2, TGF β 1 and TGF β 2. In this regard, upregulation of miR-335-5p reduced inflammation in a chronic rhinosinusitis mouse model.¹⁹ In addition, confirmation of modelling pathway analyses was proved by Du *et al.*,²⁰ who showed that miR-335-5p inhibits TGF- β 1-induced epithelial–mesenchymal transition in non-small-cell lung cancer via ROCK1. Beside the tight connection of miR-335-5p and the TGF- β pathway, we also found ROCK1 as part of the protein–protein interaction.

Expression of miR-335-5p has been found to be decreased in parenchymal lung fibroblasts from current smokers and to be reduced in the serum from patients with Parkinson's disease, investigating miRNAs targeting cell death and/or inflammation pathways.^{18,21} In contrast, in our present study miR-335-5p expression was elevated in blood samples from PiBO patients compared to controls. This fact might be because of the nature, quality and duration of the inflammation in PiBO, which drives the expression of miR-335-5p without significant effect on the resolution of inflammation. However, the high expression of miR-335-5p qualifies this specific miRNA as a potential marker for inflammation in PiBO.

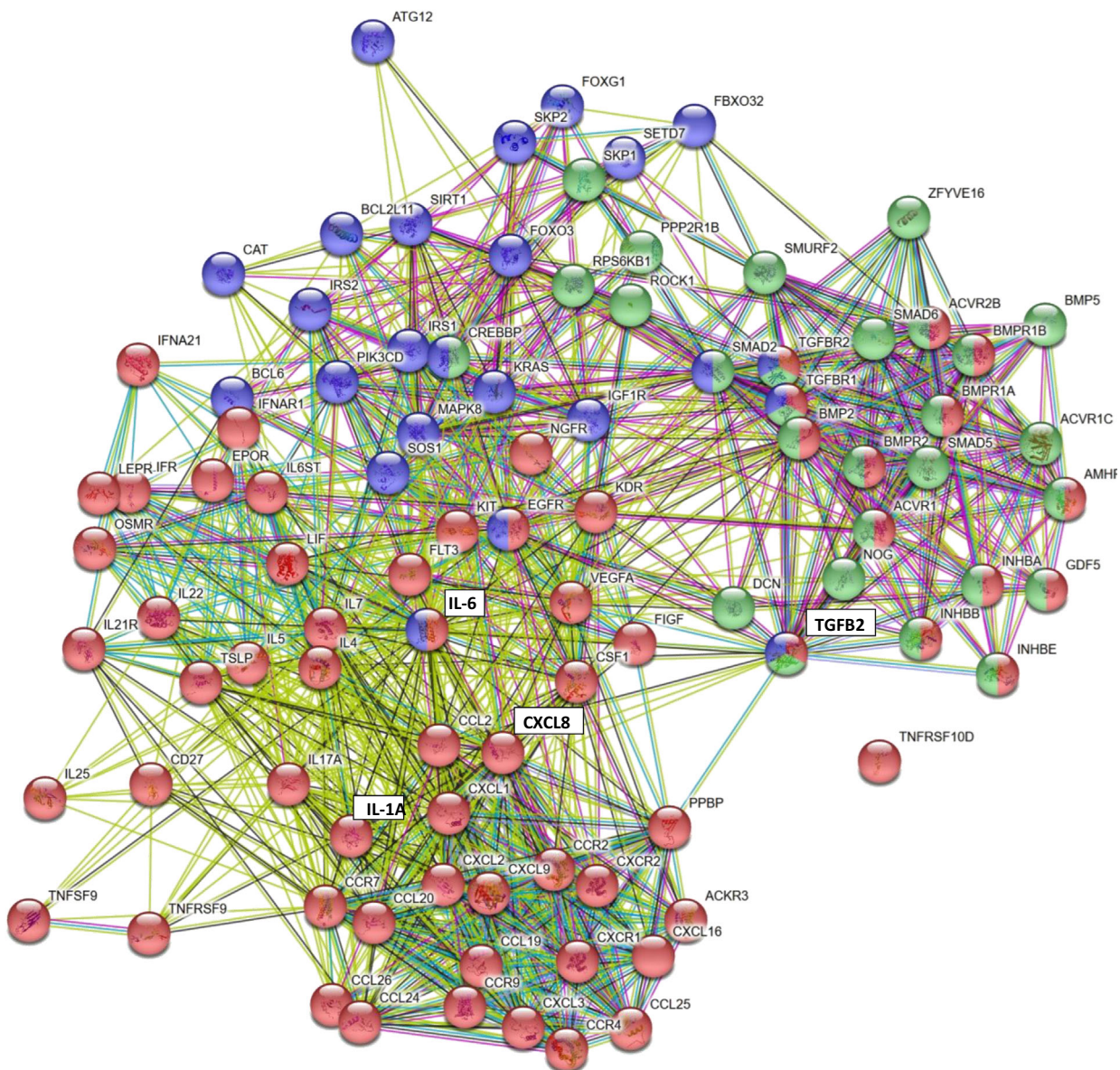
Beside miR-335-5p, NGS analysis also showed a highly significant differential expression of miR-186-5p and of the miR-30 family members, miR-30b-5p and miR-30c-5p. miR-186-5p has been shown to impact regulation of proliferation and apoptosis strongly and has been proposed to be involved in the pathogenesis of COPD.^{22,23} To our knowledge, this is the first study, which connects directly increased miR-186-5p expression in association with fibrotic processes and TGF- β signalling. TGF- β controls proliferation, differentiation and other functions including inflammation and fibrosis.²⁴ Dysregulation of the TGF- β pathway results in activation of myofibroblasts, excessive production of extracellular matrix (ECM) and inhibition of ECM degradation.²⁵ Early extracellular matrix changes and changes in VEGF/VEGFR2 axis have been demonstrated to be associated with the development of bronchiolitis obliterans syndrome (BOS) after lung transplantation or hematopoietic stem cell transplantation.^{26,27} Like in BOS, patients with PiBO undergo fibrotic processes after lower respiratory tract infection, which is in line with our findings of TGF- β concentrations in induced sputum in our patient cohort.²⁸ Moreover, VEGF A was found to be a target of miR-335-5p. Interestingly, we identified a group of bone morphogenetic proteins (BMPs) and their receptors, ACVR1, ACVR2B, BMP2, BMPR1A, BMPR1B and BMPR2, located in cytokine-cytokine receptor interaction and the FoxO signalling pathway, which are also members of the transforming growth factor- β (TGF- β) family.²⁹ Alteration in BMP-signalling has been linked to immune regulation, autoimmunity and infection diseases.³⁰ Moreover, BMPs such as TGF- β 1 and FGF- β 2 as well as BMP-4 and BMP-7 are

increased in obliterative airway remodelling in transplanted and non-transplanted lungs.³¹ Thus, our findings support the hypothesis that BMPs are important players in the pathomechanism of airway remodelling.

Further impact on inflammation and on TGF- β signalling, controlling the expression of SMAD2 and TGF β R1, has been described for the miR-30 family.^{32,33} Increased miR-30b expression inhibits endothelial cell capillary morphogenesis through upregulated TGF- β 2 expression.³⁴ In addition, miR-30c attenuates atrial fibrosis via inhibition of proliferation, differentiation, migration and collagen production by targeting TGF β R2.³⁵ Our KEGG pathway analysis revealed that upregulated miR-30b and miR-30c were highly associated with the FoxO signalling pathway. FoxO family members have been increasingly recognised to be involved in chronic inflammation and immune homeostasis.³⁶ FOXO1 transcription factors affect a number of cell types that are important in the host immune response including the mucosal dermis, macrophages and neutrophils.³⁷ Overexpression of FOXO1 enhances neutrophil-mediated inflammation by increasing inflammatory expression of TNF- α and IL-1 β . Recent studies have been shown that FOXO1 is involved in cystic fibrosis-related diabetes.³⁸ Accordingly, the network analysis of miR-30 targets shows TGF- β 2, TGF β R1 and TGF β R2, which connects cytokine-cytokine receptor interaction and the TGF- β pathway. The data are underlined by the fact that the shared targets for miR-186-5p and miR-30b/c-5p, LPCAT1, LRR3C, PLXNA1 and ST3GAL5 are also involved in inflammatory and fibrotic processes respectively.³⁹⁻⁴²

Dysregulated miRNAs have been identified as biomarkers for various inflammatory lung diseases.¹² Although several miRNAs, miR-21, miR-29a, miR-103 and miR-191, have been suggested as biomarkers for BOS, the significance of circulating miRNAs for the diagnosis PiBO is still unclear.^{43,44} Our study confirms some of the dysregulated miRNAs in BOS, such as miR-29a, miR-146a, miR-151, miR-181b and others, but clearly highlighted miR-335-5p, miR186-5p, miR-30b-5p and miR-30c-5p as possible new biomarkers for PiBO. In addition, the strong correlations between the expression of these miRNAs and FEV1 underline their role as new biomarkers in the identification of PiBO patients.

In conclusion, our study identifies four miRNAs, namely miR-335-5p, miR-186-5p, miR-30b-5p and miR-30c-5p, which impact pathways responsible for the regulation of inflammation and fibrosis in



- Cytokine-cytokine receptor interaction [hsa04060](#)
- TGF-beta signaling pathway [hsa04350](#)
- FoxO signaling pathway [hsa04068](#)

Figure 4. Interaction of miRNA targets. STRING diagram of target protein-protein interaction of the miR-335-5p target proteins in cytokine-cytokine receptor interaction (red bullets), miR-186-5p protein targets in the TGF-β signalling pathway (green bullets) and miR-30b/c-5p target proteins in the FoxO signalling pathway (blue bullets). Lines indicate interaction evidence.

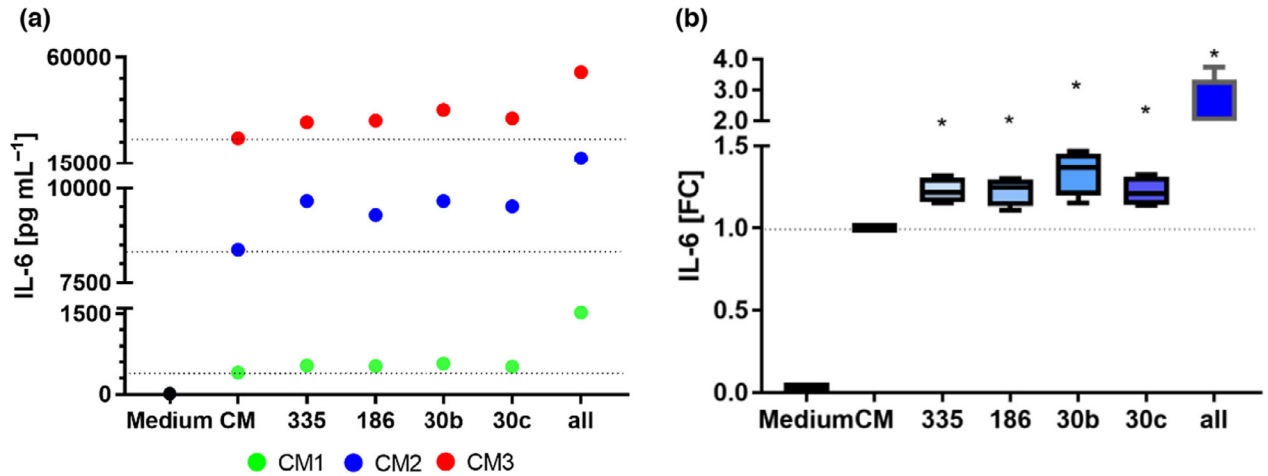


Figure 5. Inhibition of hsa-miR-335-5p, hsa-miR-186-5p, hsa-miR-30b-5p and hsa-miR-30c-5p via transfection with corresponding anti-miRs in A549 cells. **(a)** IL-6 expression was assessed in A549 cells after 24-h stimulation with three different cytokine mixtures (CM1, CM2 and CM3) and inhibition of the four miRNAs using cytometric bead array against positive control (CM; $n = 3$). **(b)** IL-6 expression is illustrated as Fold Change combining all three CM stimulations together after inhibition of the four miRNAs separately and taken all four miRNAs together ($n = 3$).

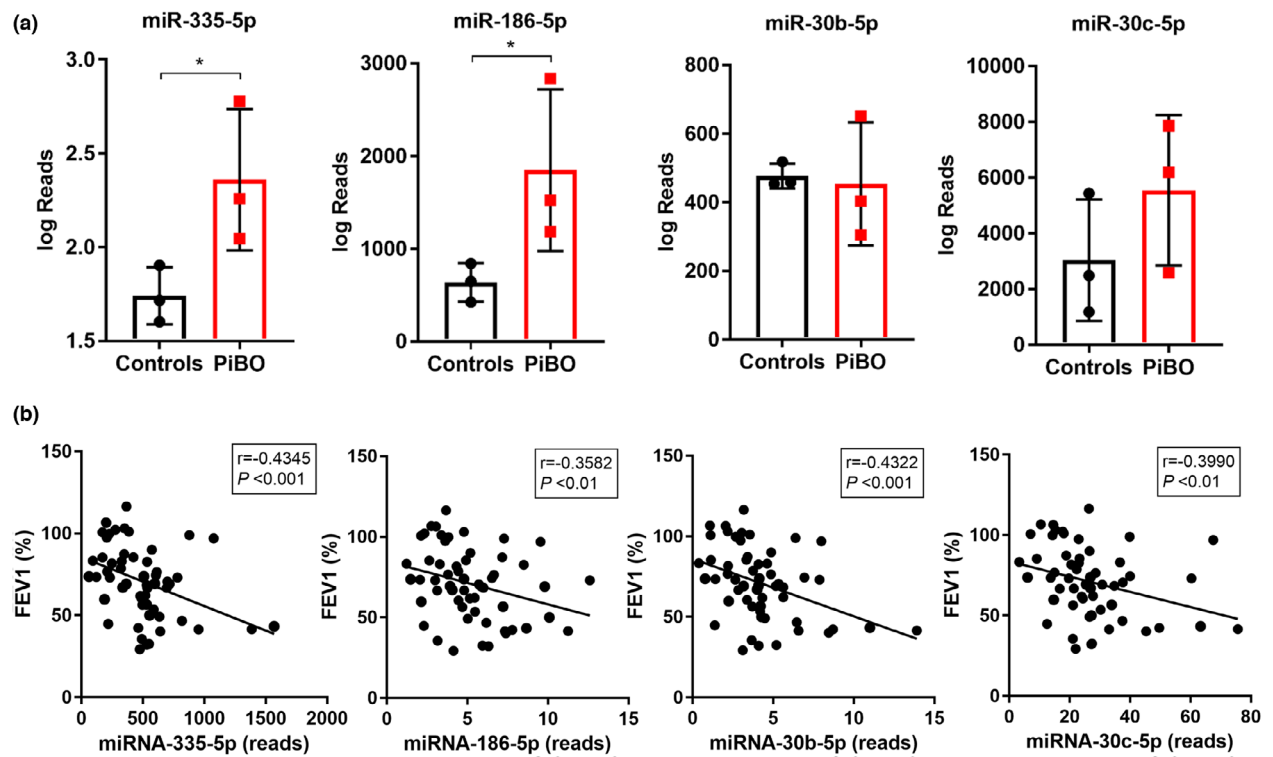


Figure 6. **(a)** NGS in samples of induced sputum of PiBO patients compared to control samples ($n = 3$) showing the reads for hsa-miR-335-5p, hsa-miR-186-5p, hsa-miR-30b-5p and hsa-miR-30c-5p. **(b)** Correlations between expression (reads) of verified miRNAs and the lung function parameter FEV1 analysed in peripheral blood of PiBO patients compared to healthy controls. $*P < 0.05$.

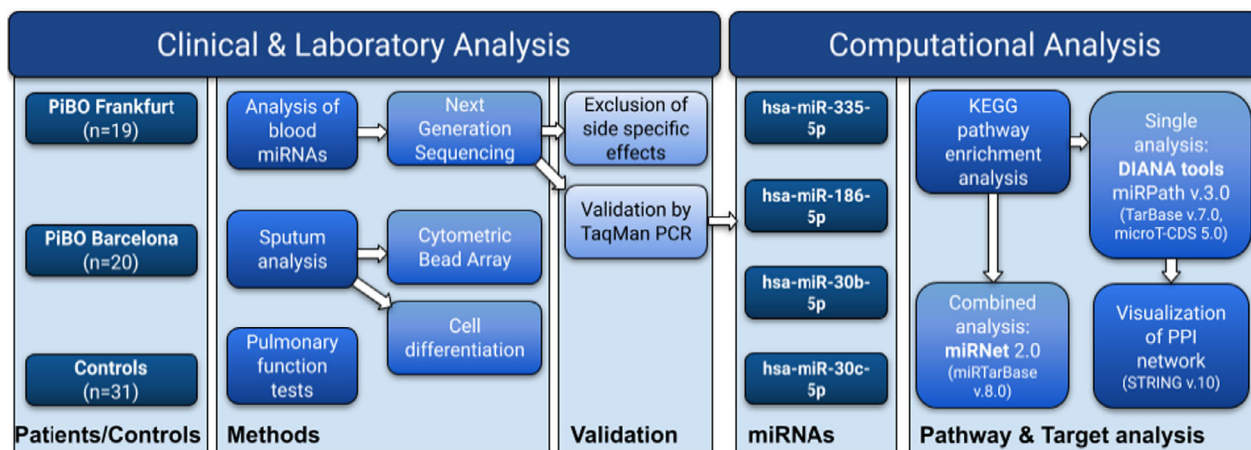


Figure 7. Overview of the study design. Clinical, laboratory and computational analysis, which were performed in the present study.

PiBO. The identified miRNAs should be considered as novel biomarkers, which can support disease diagnosis and amplify treatment options. Moreover, we consider these newly identified miRNAs as subjects for further investigations, with promising potential in the field of miRNA therapeutics.

METHODS

Study design

Figure 7 gives an overview of the study design, which consists of clinical and laboratory as well as computational analyses. Clinical and laboratory analysis included lung function tests, cell differentiation and cytokine analysis in induced sputum, and miRNA analysis in the peripheral blood by next-generation sequencing (NGS). A systematic bias in miRNA regulation because of regional and lifestyle factors was excluded by using two study cohorts from Germany and Spain, respectively, and data were validated by TaqMan PCR. Combined KEGG pathway enrichment analysis of all the identified miRNAs was performed in total (combined analysis) to detect common targets and in individual miRNAs (single analysis) to detect miRNA specific targets. Finally, identified targets were visualised in a protein–protein interaction (PPI) network.

Patients

Patients with PiBO and controls were recruited from the Vall d’Hebron Hospital Universitari, Barcelona, Spain, and from the Department for Children and Adolescents, Goethe University Frankfurt, Germany, from October 2015 to April 2019. The diagnosis of PiBO was made according to National Institutes of Health Clinical Centre (NIH-CC), and new onset of changes on HRCT including mosaic pattern, hyperinflation and bronchial wall thickening.⁴⁵

Prior to the start of the study, the subjects or their legal guardians had to sign the informed consent form. The study was approved by the Ethics Committee of the Goethe University Frankfurt (166/16) and is registered by ClinicalTrials.gov Identifier: NCT02627833.

Blood samples using the PAXgene Blood RNA System for miRNA were collected and all patients performed lung function tests. Induced sputum was collected and processed from the Frankfurt patient cohort and stored until analysis (Figure 7).

Pulmonary function tests

Baseline lung function tests were assessed at both centres using the same body plethysmograph (VIASYS Healthcare GmbH, Hoechberg, Germany). The following parameters have been recorded: FVC, FEV1, FEV1/FVC and FEF. Reversibility was defined as positive with a post-bronchodilator change of $\geq 12\%$ and 200 mL.

Sputum cell differentiation and inflammatory mediators

Sputum cell differentiation⁴⁶ and analysis of inflammatory cytokines/chemokines by cytometric bead array were performed as described previously.⁴⁷ Samples were measured with a BD FACS Array™ cytometer (BD Biosciences-Pharmingen, USA). FCAP Array Software (BD Biosciences-Pharmingen) was used to analyse the data.

miRNA sequencing and analysis

The PAXgene Blood miRNA Kit (Qiagen, Hilden, Germany) was used to extract total RNA, including miRNA, from peripheral blood and sputum samples according to the manufacturer’s recommendations. Nanodrop Lite spectroscopy (Thermo Scientific, Dreieich, Germany) was used to determine RNA concentration, and the Agilent RNA 6000 Nano kit and Agilent 2100 Bioanalyzer was used to determine RNA integrity (RIN) (Agilent Technologies, Santa

Carla, CA, USA). The QIAseq miRNA Library Kit was used to create miRNA libraries (Qiagen). Finally, cDNA concentrations were determined using the Qubit dsDNA Assay Kit, Qubit Assay Tubes and a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Dreieich, Germany). DNA quality was verified using Agilent High Sensitivity DNA Reagents with High Sensitivity DNA Chips and an Agilent 2100 Bioanalyzer (Agilent Technologies). Libraries were frozen at -20°C until miRNA sequencing.

Next-generation sequencing was performed using MiSeq Reagent Kit v3, PhiX Sequencing Control v3 and MiSeq™ Desktop Sequencer (Illumina Inc., San Diego, USA). Data were preprocessed using the Qiagen Web Portal service, which converts coverage files into raw count matrices (<https://geneglobe.qiagen.com/de/analyze>). Differential expression analysis was performed in RStudio 1.2.1335 (<https://cran.r-project.org/>) as previously described.⁴⁸ Fdr correction was applied to each miRNA that passed the DESeq quality threshold, and miRNAs were considered to be < 0.05 with Paje. miRNAs with $P_{adj} < 0.001$ were used for further analysis.

miRNA validation by TaqMan qPCR

Next-generation sequencing-based expression levels of the following miRNAs were validated by TaqMan qPCR (TaqMan® Advanced miRNA Assays; Thermo Fisher): hsa-miR-1287-5p, hsa-miR-335-5p, hsa-miR-5695, hsa-miR-30b-5p, hsa-miR-186-5p, hsa-miR-190b, hsa-7f-1-3p, hsa-miR-30c-5p, hsa-miR-26a-2-3p, hsa-miR-4677-3p and hsa-miR-199a-5p as described previously.⁴⁸ miR-24-3p was used as endogenous control based on low variance and high abundance over control samples (Supplementary table 2). cDNA preparation was performed by extending 5 ng t-RNA at the 3' end of mature transcripts through poly (A) addition. The 5' ends were extended by adapter ligation, followed by universal reverse transcription and amplification using the TaqMan® Advanced miRNA cDNA Synthesis Kit and a thermal cycler (GeneAmp Cyclor PCR Systems 9700 v3.12., Thermo Fisher). The thermocycler programme for RT cycles was programmed according to the manufacturer's instructions. For qPCR, 5 μL of a 1:10 (v/v, in TE buffer) diluted cDNA was introduced to the oligonucleotide. StepOnePlus Real-Time PCR Systems and StepOnePlus™ software were used to perform qPCR (Thermo Fisher Scientific). Evaluation was performed by the $2^{-\Delta\Delta C_t}$ method in Expression Suite software v1.1 (Fisher Scientific).

Pathway enrichment analysis of target genes and protein–protein interaction network

Kyoto encyclopaedia of genes and genomes (KEGG) pathway analysis based on microRNA signature was performed with miRNet (miRTarBase v8.0) for all the identified miRNAs together and with DIANA-mirPath v.3.0 to detect targets of individual miRNAs.⁴⁹ For DIANA-mirPath v.3.0, three different algorithms, namely, TarBase v.7.0, microT-CDS and TargetScan 7.0, were manually screened for pathways involved in inflammation and targets were selected according to the highest *P*-value. Targets of

the candidate miRNAs in relation to inflammation revealed cytokine–cytokine interaction, TGF- β signalling pathway and FoxO signalling pathway. Targets were grouped together, and analysis of networks was conducted using the software tool String v.10 (<https://string-db.org/>) with standard parameters for visualising target interaction networks.

Knockdown of hsa-miR-335-5p, hsa-miR-186-5p, hsa-miR-30b-5p and hsa-miR-30c-5p in A549 cells

Human lung epithelial cell line A549 was cultured with 10% foetal calf serum (FCS) (Sigma-Aldrich, Taufkirchen, Germany) and 1% penicillin–streptomycin (Life Technologies, Darmstadt, Germany). Cells were seeded at 2×10^6 cells at 37°C in a humidified atmosphere containing 5% CO_2 . For *in vitro* experiments, A549 cells were detached from tissue culture flasks by accutase™ cell isolation solution (Sigma-Aldrich Chemie). 25 000 cells per 96 wells were seeded in medium without penicillin/streptomycin.

Cells were stimulated with a pro-inflammatory cytokine mixture (CM) containing 4 U mL^{-1} IFN- γ , 0.5 U mL^{-1} IL-1 β and 0.2 ng mL^{-1} TNF- α (CM1); 40 U mL^{-1} IFN- γ , 5 U mL^{-1} IL-1 β and 2 mL^{-1} TNF- α (CM2); and 400 mL^{-1} IFN- γ , 50 U mL^{-1} IL-1 β and 20 ng mL^{-1} TNF- α (CM3) (Peprotech, Hamburg, Germany). For miRNA knockdown, cells were reversely transfected with either miVana anti-hsa-miR-335-5p, anti-hsa-miR-186-5p, anti-hsa-miR-30b-5p and anti-hsa-miR-30c-5p (Thermo Fisher) at 20 nM, using Lipofectamine RNAiMAX (1:1 v/v; Invitrogen, Karlsruhe, Germany) in Opti-MEM® media (Thermo Fisher). miVana miRNA Inhibitor Negative Control (Thermo) was used as a negative control (in Figure not shown) and the sole cytokine mixture stimulation (CM) without miRNAs served as positive control. The effect of transfection of miRNA inhibitors on IL-6 protein was assessed at 48 h after transfection via cytometric bead array.

Statistics

Expression levels of miRNAs, determined by qPCR, are presented as relative quantification (RQ) of gene expression, whereas the rest of the data are presented as median (10–90 percentile). All experiments have been carried out at least three times. Differences between groups were determined by one-way ANOVA (multiple groups) with *post hoc* Bonferroni analysis. The comparison of the two groups was performed by Mann–Whitney *U* test. For the correlation analysis, we used the Spearman test. Calculations were performed with RStudio 1.2.1335 (<https://cran.r-project.org/>) or GraphPad Prism 7 software (GraphPad software, La Jolla, California) and Excel (Microsoft Office, München, Germany). Statistical significance was defined as $P < 0.05$.

Sample size estimation

Sample size calculations were based on previous results published by our group.⁶ In this study, 20 children and adolescents with PiBO were compared with 20 age-matched controls. As described, we found significant differences in

lung function parameters as well as neutrophils and cytokines in induced sputum.⁶ Since no reports on miRNAs in relation to inflammation in PiBO are available, we assumed that two cohorts with 20 patients for the measurement of miRNAs are sufficient for an explorative investigation.

ACKNOWLEDGMENTS

The authors thank Katrin Krug und Petra Schoen for their excellent technical assistance. This project was supported by the Starke Lunge Foundation. Starke Lunge is a non-profitable foundation registered in Germany (Regierungspräsidium Darmstadt: AZ: I 1325d 04/11(6)-78), which supports research projects in paediatric rare lung diseases, specifically in BO. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

Dr Duecker reports grants from Starke Lunge Foundation, during the conduct of the study. Dr de Mir Messa reports grants from Starke Lunge Foundation, during the conduct of the study; personal fees from GSK, other from Novartis and other from Aldo Union, outside the submitted work. Dr Moreno-Galdó reports grants from Starke Lunge Foundation, during the conduct of the study; personal fees from AbbVie, personal fees from AbbVie, personal fees from Sanofi, other from Novartis, other from AbbVie and other from Actelion outside the submitted work. Dr Rosewich reports grants from Starke Lunge Foundation, during the conduct of the study; other from Engelhard Arzneimittel, other from GSK Arzneimittel GmbH and other from Bencard GmbH, outside the submitted work. Dr Zielen reports grants and personal fees from bene-Arzneimittel GmbH, grants and personal fees from Biotest GmbH, grants from Vifor Pharma Deutschland GmbH, grants from ALK Arzneimittel, personal fees from Novartis GmbH, grants and personal fees from Böhlinger Ingelheim, personal fees from Lofarma GmbH, personal fees from IMS HEALTH GmbH & Co. OHG, personal fees from GSK, personal fees from Stallergen, personal fees from Procter and Gamble, personal fees from Allergopharma GmbH, personal fees from Engelhard Arzneimittel GmbH, personal fees from AstraZeneca, personal fees from Sanofi-Aventis GmbH, personal fees from Allergy Therapeutics and personal fees from Aimmune Therapeutics, outside the submitted work. Dr Schubert reports grants from Starke Lunge Foundation, during the conduct of the study. All other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

AUTHOR CONTRIBUTION

Ruth Pia Duecker: Data curation; Formal analysis; Investigation; Methodology; Software; Visualization; Writing – original draft. **Ines De Mir Messa:** Data curation; Formal analysis; Investigation; Resources; Writing – review & editing. **Silvija Jerkic:** Data curation; Formal analysis; Investigation; Methodology; Supervision; Visualization; Writing – review & editing. **Annalena Kochems:** Data curation; Investigation;

Methodology; Writing – review & editing. **Gabriele Gottwald:** Data curation; Formal analysis; Writing – review & editing. **Antonio Moreno:** Data curation; Investigation; Writing – review & editing. **Martin Rosewich:** Data curation; Investigation; Methodology; Writing – review & editing. **Lucia Gronau:** Data curation; Formal analysis; Methodology; Visualization; Writing – review & editing. **Stefan Zielen:** Conceptualization; Funding acquisition; Investigation; Project administration; Resources; Supervision; Writing – review & editing. **Andreas Geburtig-Chiocetti:** Data curation; Formal analysis; Visualization; Writing – review & editing. **Hermann Kreyenberg:** Data curation; Formal analysis; Methodology; Visualization; Writing – review & editing. **Ralf Schubert:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Software; Supervision; Visualization; Writing – original draft; Writing – review & editing.

REFERENCES

1. Kurland G, Michelson P. Bronchiolitis obliterans in children. *Pediatr Pulmonol* 2005; **39**: 193–208.
2. Colom AJ, Teper AM. Post-infectious bronchiolitis obliterans. *Pediatr Pulmonol* 2019; **54**: 212–219.
3. Cazzato S, Poletti V, Bernardi F *et al.* Airway inflammation and lung function decline in childhood post-infectious bronchiolitis obliterans. *Pediatr Pulmonol* 2008; **43**: 381–390.
4. Rosewich M, Zissler UM, Kheiri T *et al.* Airway inflammation in children and adolescents with bronchiolitis obliterans. *Cytokine* 2015; **73**: 156–162.
5. Smith KJ, Fan LL. Insights into post-infectious bronchiolitis obliterans in children. *Thorax* 2006; **61**: 462–463.
6. Eckrich J, Herrmann E, Voss S *et al.* Short-term variation of lung function and airway inflammation in children and adolescents with bronchiolitis obliterans. *Lung* 2016; **194**: 571–579.
7. Liu Y, Li H, Xiao T *et al.* Epigenetics in immune-mediated pulmonary diseases. *Clin Rev Allergy Immunol* 2013; **45**: 314–330.
8. Guo H, Ingolia NT, Weissman JS *et al.* Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 2010; **466**: 835–840.
9. Cai Y, Yu X, Hu S *et al.* A brief review on the mechanisms of miRNA regulation. *Genomics Proteomics Bioinformatics* 2009; **7**: 147–154.
10. Alipoor SD, Adcock IM, Garsen J *et al.* The roles of miRNAs as potential biomarkers in lung diseases. *Eur J Pharmacol* 2016; **791**: 395–404.
11. Recchiuti A, Serhan CN. Pro-resolving lipid mediators (SPMs) and their actions in regulating miRNA in novel resolution circuits in inflammation. *Front Immunol* 2012; **3**: 298.
12. Stolzenburg LR, Harris A. The role of microRNAs in chronic respiratory disease: recent insights. *Biol Chem* 2018; **399**: 219–234.
13. Jerkic SP, Michel F, Donath H *et al.* Calprotectin as a new sensitive marker of neutrophilic inflammation in patients with bronchiolitis obliterans. *Mediators Inflamm* 2020; **2020**: 4641585.

14. Colom AJ, Maffey A, Garcia Bournissen F et al. Pulmonary function of a paediatric cohort of patients with postinfectious bronchiolitis obliterans. A long term follow-up. *Thorax* 2015; **70**: 169–174.
15. Jerkic S-P, Brinkmann F, Calder A et al. Postinfectious bronchiolitis obliterans in children: diagnostic workup and therapeutic options: a workshop report. *Can Respir J* 2020; **2020**: 5852827.
16. Ellsner A, Jaumann F, Dobmann S et al. Elevated levels of interleukin-8 and transforming growth factor-beta in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome: proinflammatory role of bronchial epithelial cells. *Munich Lung Transplant Group. Transplantation* 2000; **70**: 362–367.
17. Long X, Huang Y, He J et al. Upregulation of miR-335 exerts protective effects against sepsis-induced myocardial injury. *Mol Med Rep* 2021; **24**: 806.
18. Ong J, van den Berg A, Faiz A et al. Current smoking is associated with decreased expression of miR-335-5p in parenchymal lung fibroblasts. *Int J Mol Sci* 2019; **20**: 5176.
19. Gu X, Yao X, Liu D. Up-regulation of microRNA-335-5p reduces inflammation via negative regulation of the TPX2-mediated AKT/GSK3 β signaling pathway in a chronic rhinosinusitis mouse model. *Cell Signal* 2020; **70**: 109596.
20. Du W, Tang H, Lei Z et al. miR-335-5p inhibits TGF- β 1-induced epithelial-mesenchymal transition in non-small cell lung cancer via ROCK1. *Respir Res* 2019; **20**: 225.
21. Oliveira SR, Dionísio PA, Correia Guedes L et al. Circulating inflammatory miRNAs associated with Parkinson's disease pathophysiology. *Biomolecules* 2020; **10**: 945.
22. Feng H, Zhang Z, Qing X et al. miR-186-5p promotes cell growth, migration and invasion of lung adenocarcinoma by targeting PTEN. *Exp Mol Pathol* 2019; **108**: 105–113.
23. Li R, Xu F, Wu X et al. CUL1-mediated organelle fission pathway inhibits the development of chronic obstructive pulmonary disease. *Comput Math Methods Med* 2020; **2020**: 5390107.
24. Stewart AG, Thomas B, Koff J. TGF- β : master regulator of inflammation and fibrosis. *Respirology* 2018; **23**: 1096–1097.
25. Kramer EL, Clancy JP. TGF- β as a therapeutic target in cystic fibrosis. *Expert Opin Ther Targets* 2018; **22**: 177–189.
26. Müller C, Andersson-Sjöland A, Schultz HH et al. Early extracellular matrix changes are associated with later development of bronchiolitis obliterans syndrome after lung transplantation. *BMJ Open Respir Res* 2017; **4**: e000177.
27. Larsson-Callerfelt A-K, Müller C, Andersson-Sjöland A et al. VEGF synthesis and VEGF receptor 2 expression in patients with bronchiolitis obliterans syndrome after lung transplantation. *Respir Med* 2020; **166**: 105944.
28. Yu J. Postinfectious bronchiolitis obliterans in children: lessons from bronchiolitis obliterans after lung transplantation and hematopoietic stem cell transplantation. *Korean J Pediatr* 2015; **58**: 459–465.
29. Gomez-Puerto MC, Iyengar PV, García de Vinuesa A et al. Bone morphogenetic protein receptor signal transduction in human disease. *J Pathol* 2019; **247**: 9–20.
30. Sanjabi S, Oh SA, Li MO. Regulation of the immune response by TGF- β : from conception to autoimmunity and infection. *Cold Spring Harb Perspect Biol* 2017; **9**: a022236.
31. Jonigk D, Theophile K, Hussein K et al. Obliterative airway remodelling in transplanted and non-transplanted lungs. *Virchows Arch* 2010; **457**: 369–380.
32. Zhou T, Chen Y-L. The functional mechanisms of miR-30b-5p in acute lung injury in children. *Med Sci Monit* 2019; **25**: 40–51.
33. Braun J, Hoang-Vu C, Dralle H et al. Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas. *Oncogene* 2010; **29**: 4237–4244.
34. Howe GA, Kazda K, Addison CL. MicroRNA-30b controls endothelial cell capillary morphogenesis through regulation of transforming growth factor- β 2. *PLoS One* 2017; **12**: e0185619.
35. Xu J, Wu H, Chen S et al. MicroRNA-30c suppresses the pro-fibrogenic effects of cardiac fibroblasts induced by TGF- β 1 and prevents atrial fibrosis by targeting TGF β RII. *J Cell Mol Med* 2018; **22**: 3045–3057.
36. Peng SL. Forkhead transcription factors in chronic inflammation. *Int J Biochem Cell Biol* 2010; **42**: 482–485.
37. Graves DT, Milovanova TN. Mucosal immunity and the FOXO1 transcription factors. *Front Immunol* 2019; **10**: 2530.
38. Smerieri A, Montanini L, Maiuri L et al. FOXO1 content is reduced in cystic fibrosis and increases with IGF-I treatment. *Int J Mol Sci* 2014; **15**: 18000–18022.
39. Dukhinova M, Kuznetsova I, Kopeikina E et al. Platelets mediate protective neuroinflammation and promote neuronal plasticity at the site of neuronal injury. *Brain Behav Immun* 2018; **74**: 7–27.
40. Söderman J, Berglind L, Almer S. Gene expression-genotype analysis implicates GSDMA, GSDMB, and LRR3C as contributors to inflammatory bowel disease susceptibility. *Biomed Res Int* 2015; **2015**: 834805.
41. Tomati V, Pesce E, Caci E et al. High-throughput screening identifies FAU protein as a regulator of mutant cystic fibrosis transmembrane conductance regulator channel. *J Biol Chem* 2018; **293**: 1203–1217.
42. Cheng S, Chen H, Wang A et al. Lentiviral vector-mediated delivery of lysophosphatidylcholine acyltransferase 1 attenuates airway inflammation in ovalbumin-induced allergic asthmatic mice. *Asian Pac J Allergy Immunol* 2015; **33**: 320–329.
43. Budding K, Rossato M, van de Graaf EA et al. Serum miRNAs as potential biomarkers for the bronchiolitis obliterans syndrome after lung transplantation. *Transpl Immunol* 2017; **42**: 1–4.
44. Xu Z, Yang W, Steward N et al. Role of circulating microRNAs in the immunopathogenesis of rejection after pediatric lung transplantation. *Transplantation* 2017; **101**: 2461–2468.
45. Walther S, Rettinger E, Maurer HM et al. Long-term pulmonary function testing in pediatric bronchiolitis obliterans syndrome after hematopoietic stem cell transplantation. *Pediatr Pulmonol* 2020; **55**: 1725–1735.

46. Koc-Günel S, Schubert R, Zielen S *et al.* Cell distribution and cytokine levels in induced sputum from healthy subjects and patients with asthma after using different nebulizer techniques. *BMC Pulm Med* 2018; **18**: 115.
47. Eickmeier O, Huebner M, Herrmann E *et al.* Sputum biomarker profiles in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) and association between pulmonary function. *Cytokine* 2010; **50**: 152–157.
48. Fussbroich D, Kohnle C, Schwenger T *et al.* A combination of LCPUFAs regulates the expression of miRNA-146a-5p in a murine asthma model and human alveolar cells. *Prostaglandins Other Lipid Mediat* 2020; **147**: 106378.
49. Vlachos IS, Zagganas K, Paraskevopoulou MD *et al.* DIANA-miRPath v3.0: deciphering microRNA function

with experimental support. *Nucleic Acids Res* 2015; **43**: W460–W466.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.



This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.