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Identification of asthma-associated microRNAs in bronchial biopsies

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Shareable abstract (@ERSpublications)
Using small RNA sequencing on bronchial biopsies of asthma patients and healthy controls, this study identified microRNAs associated with clinical and inflammatory features of asthma that are potential regulators of asthma-associated gene expression https://bit.ly/3rZXMDf

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Abstract

Background Changes in microRNA (miRNA) expression can contribute to the pathogenesis of many diseases, including asthma. We aimed to identify miRNAs that are differentially expressed between asthma patients and healthy controls, and explore their association with clinical and inflammatory parameters of asthma.

Methods Differentially expressed miRNAs were determined by small RNA sequencing on bronchial biopsies of 79 asthma patients and 82 healthy controls using linear regression models. Differentially expressed miRNAs were associated with clinical and inflammatory asthma features. Potential miRNA–mRNA interactions were analysed using mRNA data available from the same bronchial biopsies, and enrichment of pathways was identified with Enrichr and g:Profiler.

Results In total, 78 differentially expressed miRNAs were identified in bronchial biopsies of asthma patients compared with controls, of which 60 remained differentially expressed after controlling for smoking and inhaled corticosteroid treatment. We identified several asthma-associated miRNAs, including miR-125b-5p and miR-223-3p, based on a significant association with multiple clinical and inflammatory asthma features and their negative correlation with genes associated with the presence of asthma. The most enriched biological pathway(s) affected by miR-125b-5p and miR-223-3p were inflammatory response and cilium assembly/organisation. Of interest, we identified that lower expression of miR-26a-5p was linked to more severe eosinophilic inflammation as measured in blood, sputum as well as bronchial biopsies.

Conclusion Collectively, we identified miR-125b-5p, miR-223-3p and miR-26a-5p as potential regulators that could contribute to the pathogenesis of asthma.

Introduction

Asthma is a chronic inflammatory airway disease characterised by airway obstruction and airway hyperresponsiveness resulting in shortness of breath, cough, wheezing and chest tightness. Around 300 million people worldwide are affected by asthma [1]. Although asthma symptoms can be controlled by inhaled corticosteroids (ICS) and long-acting β-agonists, there is no cure available and optimal asthma control cannot be obtained in all patients with currently available treatments [1]. Therefore, there is a need

to better understand the pathogenesis of asthma to identify new therapeutic targets and improve the management of asthma patients.

Genetics, epigenetics and microRNAs (miRNAs) are involved in the pathogenesis of asthma [2]. miRNAs are small noncoding RNA molecules that target specific mRNAs leading to their degradation or translational repression. Several miRNAs can target the same mRNA and each miRNA can regulate hundreds of mRNAs [3]. Therefore, miRNAs have the potential to regulate many cellular functions, including inflammation, cell differentiation and cell death [4]. miRNAs can provide novel targets for new therapeutic treatment strategies, because miRNAs are small, highly conserved and have a specific sequence. As an example, blocking the function of miR-155 with an miR-155 antagomir has been shown to overcome cancer cell drug resistance in a mouse model [5].

Several groups have investigated miRNA profiles in bronchial biopsies, brushings, blood and sputum of asthma patients compared with healthy controls [6–11]. However, generally small cohorts were used and most studies were based on microarray technology, which is less sensitive and specific compared with sequencing. In this study, we performed small RNA sequencing with matched RNA sequencing in the same bronchial biopsies of 79 well-characterised asthma patients and 82 matched healthy individuals. We aimed to identify miRNAs involved in the pathogenesis of asthma. We focused on differentially expressed miRNAs and miRNA–mRNA correlations that are associated with clinical and inflammatory asthma features.

Material and methods

Subject characteristics

Asthma patients (n=79) had a doctor's diagnosis of asthma and presence of bronchial hyperresponsiveness to AMP or histamine. Healthy subjects (n=82) had normal pulmonary function, normal spirometry with forced expiratory volume in 1 s (FEV_1) >80% predicted and FEV_1/forced vital capacity (FVC) greater than the lower limit of normal, absence of reversibility (FEV_1 % pred to salbutamol <10%), no bronchial hyperresponsiveness (provocative concentration causing a 20% fall in FEV_1 (PC_{20}) methacholine >16 $mg \cdot mL^{-1}$), and no respiratory symptoms [12]. Patients were included from three studies [12, 13], all carried out in the University Medical Center Groningen (Groningen, The Netherlands) between 2001 and 2012, and approved by the local Medical Ethics Committee (METc 2009/007, 2001/074 and 2004/271). All subjects provided written informed consent.

Clinical measurements

Sputum induction, blood sampling, lung function and bronchial biopsies were performed in all subjects as previously described [12].

RNA isolation

Total RNA was extracted from frozen bronchial biopsies using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. Quality of RNA was assessed using the NanoDrop 1000 and Labchip GX platform (PerkinElmer, Waltham, MA, USA). Total RNA was extracted at the same time, and asthma patients and controls were evenly distributed over batches of 12 samples based on disease/control status, sex, age and smoking status.

miRNA sequencing

Small RNA sequencing library preparation was performed using the NEXTFLEX Small RNA-Seq Kit V3 (Bioo-Scientific, Austin, TX, USA). Clean-up beads were used to eliminate the small RNA fraction and quality control of the RNA library was checked using LabChip GX (Perkin Elmer, Waltham, MA, USA). Sequencing was performed using the HiSeq 2500 system (Illumina, San Diego, CA, USA). Quality control of raw RNA sequencing data was performed using FastQC (version 0.11.5) and quality control of raw reads of adapter sequences was done using TrimGalore (version 0.3.7). NEXTFLEX Small RNA-Seq Kit V3 processing was done using custom scripts documented in the GitHub repository. Alignment and quantification were performed using miRDeep2 (version 2.0.0.8) with Bowtie (version 0.12.7). During library preparation and sequencing, the samples were evenly distributed over the batches based on disease/control status, sex, age and smoking status.

mRNA sequencing

Sample preparation and mRNA sequencing were performed as previously described [14]. Library preparation for mRNA and miRNA sequencing was performed at the same time for asthma patients and healthy controls, and samples were evenly distributed over the batches.

Statistical analyses of small RNA sequencing data

The (small) RNA sequencing data analyses were performed with R (version 3.5.3). miRNAs with an average read count <100 in asthma patients or healthy controls were filtered out. Differential expression of microRNAs was analysed with DESeq2 (version 1.24.0) adjusting for sex, age, smoking and library batch, and corrected for multiple testing using the Benjamini–Hochberg false discovery rate (FDR); FDR adjusted p-values <0.05 were considered as statistically significant. A heatmap was generated and ordered from highest to lowest negative fold change miRNA in asthma patients compared with healthy controls. To exclude potential effects of ICS and smoking, a sensitivity analysis was performed in a subset of asthma patients (n=30) compared with healthy controls (n=42) excluding participants that smoked or used ICS.

Linear regression was performed to associate differentially expressed miRNAs with relevant clinical and inflammatory features of asthma: FEV_1 % pred, PC_{20} AMP, eosinophil and neutrophil counts in blood and tissue, and percentages of eosinophils and neutrophils in sputum in all asthma patients adjusting for age, sex, smoking status and ICS treatment.

To assess for miRNA–mRNA correlations, expression levels were log_2 transformed into counts per million (logCPM). Pearson's correlations for miRNA–mRNA were calculated for miRNAs with one or more associations with asthma features and genomewide gene expression data available in matched subjects (FDR adjusted p-values <0.05 were considered statistically significant). Negatively correlated genes were checked for their association with asthma using the same statistical model in matched biopsies; these were defined as asthma genes. For those miRNAs, we checked for experimentally validated target genes in miRTarBase (version 7.0) [15].

Gene Ontology and pathway analyses

For each miRNA, the negatively or the positively and negatively correlated asthma-associated genes were analysed with Enrichr [16, 17] and g:Profiler [18] to identify enriched biological processes (Gene Ontology (GO) Biological Process 2018 in Enrichr and GO:BP releases/2020-12-15 in g:Profiler) and Reactome pathways (Reactome_2016 in Enrichr and Reactome_2020-12-15 in g:Profiler).

Results

Patients characteristics

The clinical characteristics of 79 asthma patients and 82 healthy controls are shown in table 1. There were no significant differences between asthma patients and healthy controls with respect to sex and pack-years

TABLE 1 Characteristics of asthma patients and healthy controls

	Including smokers and ICS treatment		Excluding smokers and ICS treatment	
	Asthma (n=79)	Healthy controls (n=82)	Asthma (n=30)	Healthy controls (n=42)
Age (years)	50 (38–56)	42 (23–56)*	53 (36–58)	38 (22–58)
Female/male[#]	39/40	36/46	19/11	19/23
Smoking status[#]		*		
Nonsmoker	38 (48)	42 (51)	30 (100)	42 (100)
Ex-smoker	22 (28)	0 (0)		
Current smoker	19 (24)	40 (49)		
Pack-years	11 (7–27)	16 (4–29)		
FEV_1 (% pred)	82±17	101±12*	85±15	102±14*
FEV_1/FVC	70±11	79±6*	71±10	80±7*
Atopy (yes/no/NA)[#]	58/17/4	30/51/1*	21/7/2	19/22/1*
PC_{20} AMP ($mg \cdot mL^{-1}$)[¶]	39 (0.02–640)	543 (33–640)*	22 (0.02–640)	626 (270–640)*
PC_{20} methacholine ($mg \cdot mL^{-1}$)[¶]		38.9 (19.6–39.2)		38.6 (19.6–39.2)
ICS therapy (yes/no)[#]	33/46	0/82*	0/30	0/42
ICS dose ($\mu g \cdot day^{-1}$)[+]	500 (250–800)			

Data are presented as median (interquartile range), n, n (%) or mean±SD, unless otherwise stated. ICS: inhaled corticosteroid; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; NA: not available; PC_{20}: provocative concentration causing a 20% fall in FEV_1. [#]: Fisher's exact test was performed; [¶]: geometric mean with range; [+]: beclomethasone equivalent. The Mann–Whitney U-test was performed, unless otherwise stated. *: p<0.05 *versus* asthma.

of smoking. Asthma patients had significantly higher age, were less often current smokers, had a lower FEV_1 % pred and FEV_1/FVC, and had more severe bronchial hyperresponsiveness to AMP.

Differentially expressed miRNAs in asthma patients compared with healthy controls

Of the 1860 miRNAs that were detected in bronchial biopsies, 136 miRNAs remained after filtering for low abundance. Of these 136, 78 miRNAs were differentially expressed in asthma patients compared with healthy controls: 48 having higher expression and 30 having lower expression in asthma (FDR adjusted p-value <0.05) (figure 1a). miR-451a had the highest fold change, whereas miR-125b-5p had the lowest negative fold change in asthma. A volcano plot illustrates all differentially expressed miRNAs, with the top five most significant highest and lowest miRNAs indicated (FDR adjusted p-value <0.05) (figure 1b). To assess whether smoking and ICS treatment influenced the 78 differentially expressed miRNAs, we conducted a sensitivity analysis on a subgroup of individuals that did not smoke and did not use ICS. Of the 78 miRNAs that were differentially expressed between asthma patients and healthy controls in the complete cohort, 60 miRNAs remained significant in the same direction in the sensitivity analysis, showing that our findings are robust (FDR adjusted p-value <0.05) (figure 1c). Supplementary tables S1 and S2 present the full lists of significantly differentially expressed miRNAs in asthma patients compared with healthy controls, including and excluding smokers and ICS-treated patients.

Expression of 25 differentially expressed miRNAs is associated with asthma features

To determine whether the 60 differentially expressed miRNAs in bronchial biopsies are relevant in asthma, we investigated the association of differentially expressed miRNAs with FEV_1 % pred, PC_{20} AMP, eosinophil and neutrophil counts in blood and tissue, and percentages of eosinophils and neutrophils in sputum of all asthma patients. We observed that 25 out of the 60 differentially expressed miRNAs were associated with one or more clinical and inflammatory feature(s). Figure 2 illustrates the association between miRNAs and features of asthma with T-values of the linear regression ($p<0.05$). Higher miR-21-5p expression in biopsies had the most significant association with more neutrophils in blood, and it was also associated with more neutrophils and eosinophils in tissue. Furthermore, miR-26a-5p had three significant associations with asthma features: lower miR-26a-5p expression was associated with higher eosinophil levels in blood, sputum and tissue. Moreover, higher expression levels of miR-223-3p were linked to higher eosinophils and neutrophils in tissue. Additionally, we observed that lower miR-125b-5p levels, the lowest negative fold change miRNA in asthma, were associated with lower FEV_1 % pred and higher neutrophil levels in blood. For miR-451a, the miRNA with the highest fold change in asthma, we did not observe any associations with asthma features in our study.

Identification of key asthma-associated miRNA–mRNA targets

To investigate potential interactions between miRNA and mRNA expression, we correlated expression levels of the 25 miRNAs that were associated with one or more clinical and inflammatory asthma features with mRNA sequencing data available from the same biopsies from all asthma patients and healthy controls. Hereby, we focused on the negatively correlated genes. The expression of 23 of the 25 miRNAs was negatively correlated with mRNA expression of one or more genes (table 2). Thereafter, we investigated which of the negatively correlated genes were also associated with the presence of asthma. For miR-199b-5p, miR-223-3p, miR-199b-3p, miR-142-5p, miR-181b-5p, miR-195-5p and miR-125b-5p, we observed that ∼10% of the negatively correlated genes were also associated with asthma. A full list of negatively correlated genes per miRNA is provided in supplementary table S3. As the expression of these seven miRNAs (miR-199b-5p, miR-223-3p, miR-199b-3p, miR-142-5p, miR-181b-5p, miR-195-5p and miR-125-5p) was associated with one or more clinical and inflammatory asthma features and ∼10% of their negatively correlated genes were associated with asthma genes, we selected those miRNAs as our candidate asthma miRNAs. For the five lower expressed miRNAs in asthma in our study, *i.e.* miR-199b-5p, miR-199b-3p, miR-181b-5p, miR-195-5p and miR-125b-5p, we observed that the genes *HBA1* and *RGS18* had the most significant negative correlation (figure 3a and b). *HBA1* and *RGS18* expression levels were higher in bronchial biopsies of asthma patients compared with healthy controls. Furthermore, for the two higher expressed asthma miRNAs in our study, *i.e.* miR-223-3p and miR-142-5p, we observed that *GREM2* and *RPS3AP5* were the most significant negatively correlated genes (figure 3c and d). *GREM2* expression levels were lower in bronchial biopsies of asthma patients compared with healthy controls, while no expression differences were found for *RPS3AP5*. In addition, among the negatively correlated asthma-associated genes we identified three validated target genes for miR-199b-5p (*AGTRAP*, *RNF11* and *SNTB1*), one validated target gene for miR-125b-5p (*S100A8*) and 13 validated target genes for miR-223-3p (*ARL8B*, *ARTN*, *CAPRIN1*, *CDS1*, *CHMP2B*, *CHUK*, *MSMO1*, *NSUN3*, *PDZD8*, *SECISBP2L*, *TWF1*, *WASL* and *ZBTB18*).

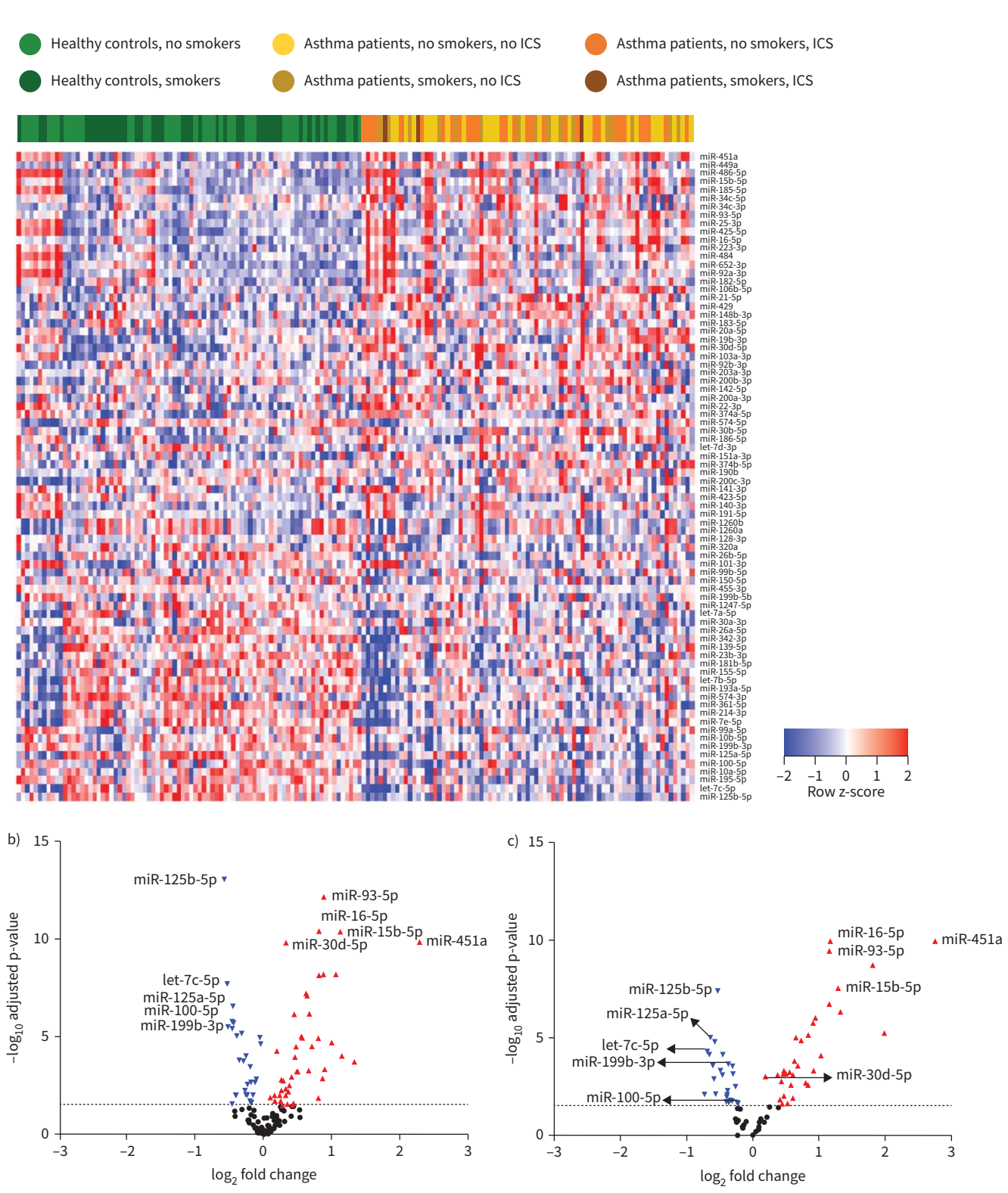

FIGURE 1 MicroRNAs (miRNAs) that were differentially expressed in bronchial biopsies of asthma patients compared with bronchial biopsies of healthy controls. a) Heatmap showing 78 differentially expressed miRNAs in asthma patients (n=79) compared with healthy controls (n=82). b) Volcano plot showing differentially expressed miRNAs (false discovery rate (FDR) adjusted p<0.05) between asthma patients and healthy controls including smokers and inhaled corticosteroid (ICS) treatment. c) Volcano plot showing the differentially expressed miRNAs (FDR adjusted p<0.05) between asthma patients (n=30) and healthy controls (n=42) excluding smokers and ICS treatment. The top five upregulated (red) and downregulated (blue) miRNAs are indicated. The dotted line indicates the significance level.

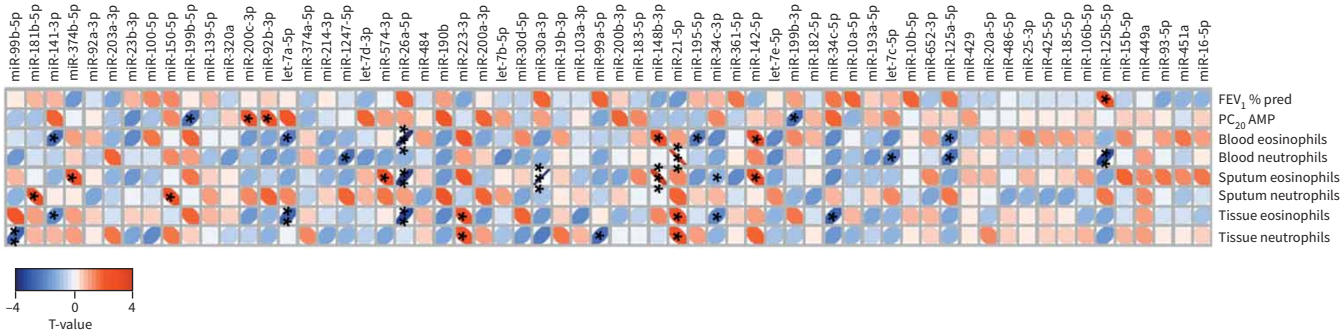

FIGURE 2 Linear regression between differentially expressed microRNAs (miRNAs) in bronchial biopsies and clinical parameters. Positive associations are displayed in red and negative associations are displayed in blue (n=79, all asthma patients). FEV_1: forced expiratory volume in 1 s; PC_{20}: provocative concentration causing a 20% fall in FEV_1. *: p<0.05; **: p<0.01; ***: p<0.001.

Enrichment of biological processes and pathways in negatively correlated asthma genes

To identify the potential mechanisms underlying the involvement of our seven key candidate asthma miRNAs in disease pathogenesis, pathway enrichment analyses were performed on the negatively correlated asthma-associated genes, whereby we included all asthma patients and healthy controls. GO revealed in total 187 biological processes and 212 Reactome pathways that were significantly enriched among the negatively correlated mRNA transcripts (FDR adjusted p-value <0.05). The most significant biological process for miR-199b-5p and miR-223-3p was "cilium assembly", for miR-181b-5p, miR-199b-3p and miR-195-5p "neutrophil degranulation", and for miR-125b-5p "inflammatory response".

TABLE 2 Differentially expressed microRNAs (miRNAs) in asthma patients compared with healthy controls that have a correlation with clinical and inflammatory asthma features: negatively correlated genes per miRNA

	Differentially expression asthma *versus* healthy controls[#]	Negatively correlated genes (FDR p<0.05) (n)[¶]	Negatively correlated asthma genes (n)[+]	Negatively correlated asthma genes (%)[§]
miR-199b-5p	−0.40	1773	920	51.89
miR-223-3p	0.80	8764	2641	30.13
miR-199b-3p	−0.37	165	33	20.00
miR-142-5p	0.68	1198	236	19.70
miR-181b-5p	−0.23	115	22	19.13
miR-195-5p	−0.49	310	54	17.42
miR-125b-5p	−0.53	202	20	9.90
miR-148b-3p	0.56	471	40	8.49
miR-30a-3p	−0.46	571	37	6.48
miR-99b-5p	−0.19	16	1	6.25
miR-34c-5p	1.03	92	2	2.17
miR-21-5p	0.47	509	9	1.77
miR-26a-5p	−0.26	516	9	1.74
miR-92b-3p	0.61	598	2	0.33
miR-125a-5p	−0.64	6417	21	0.33
let-7c-5p	−0.69	848	2	0.24
miR-200c	0.42	5255	6	0.11
let-7a-5p	−0.39	6789	1	0.01
miR-99a-5p	−0.30	1	0	0
miR-150-5p	−0.36	13	0	0
miR-1247-5p	−0.73	6	0	0
miR-347b-3p	0.47	1	0	0
miR-34c-3p	0.92	126	0	0

[#]: fold change between asthma patients *versus* healthy controls (excluding subjects that smoked or used inhaled corticosteroid); [¶]: negatively correlated miRNA–mRNA expression (all asthma and healthy subjects); [+]: overlap between negatively correlated miRNA–mRNA expression and genes that are differentially expressed in asthma patients; [§]: percentage of negatively correlated asthma-associated genes. FDR: false discovery rate.

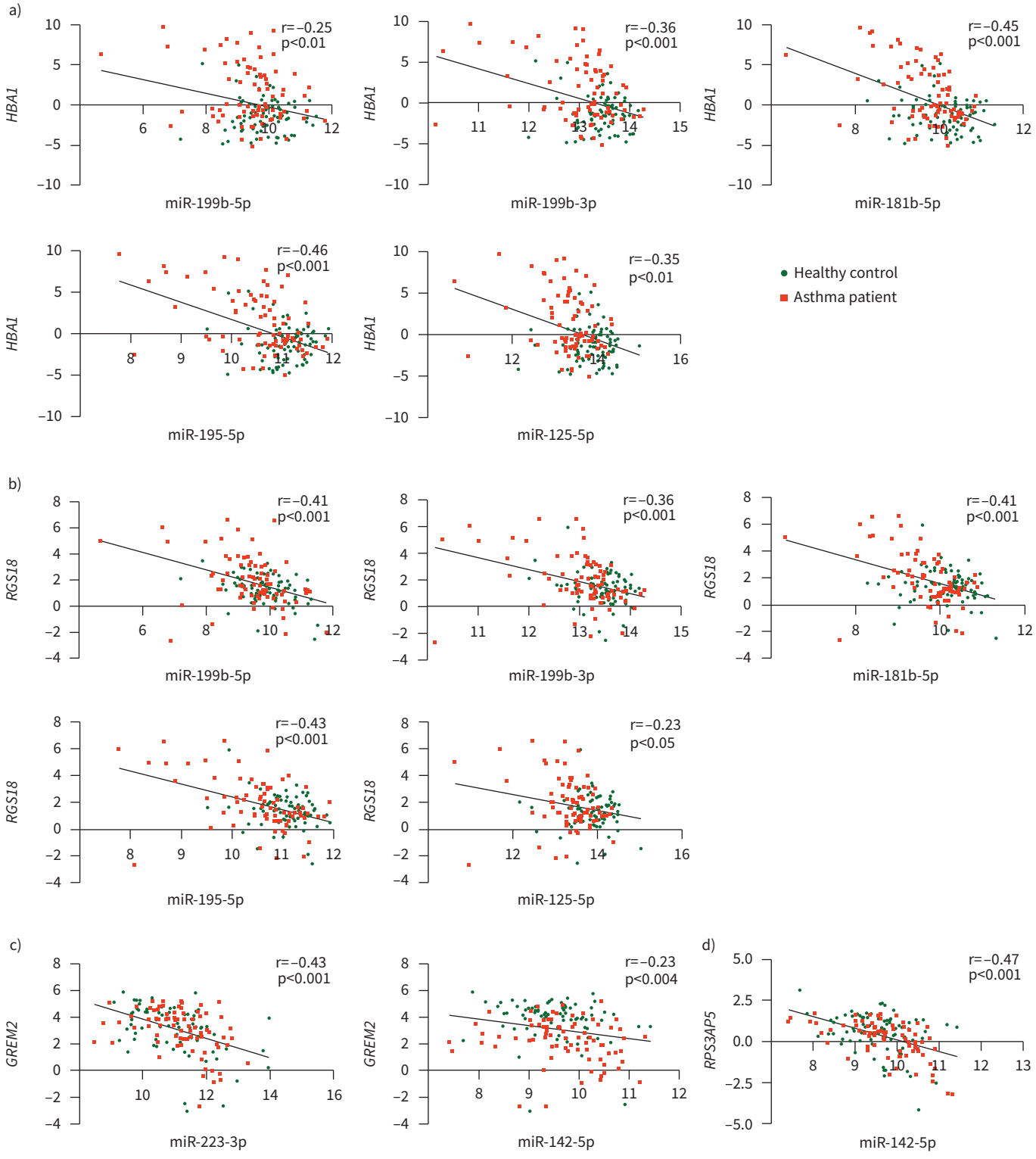

FIGURE 3 Correlation between asthma-associated genes and microRNA (miRNA) expression. Pearson's correlation coefficient (r) between a) *HBA1*, b) *RGS18*, c) *GREM2* and d) *RPS3AP5* expression correlated with miRNA expression in all subjects (n=79 asthma patients and n=82 healthy controls). The *x*- and *y*-axes represent normalised read counts.

For miR-142-5p, no significant enrichment was found using Enrichr; however, using g:Profiler, four biological processes were obtained. The most significant biological process for miR-142-5p was "protein transport along microtubules". The top five biological processes and Reactome pathways for each of the

six asthma miRNAs are displayed in tables 3 and 4, respectively; a list of all biological processes and Reactome pathways is provided in supplementary tables S4 and S5. Additionally, we also performed pathway enrichment analyses on positively and negatively correlated asthma-associated genes. Using Enrichr, in total 56 biological processes and 51 pathways were significantly enriched among the positively and negatively correlated genes. The most significant biological process for miR-199b-5p and miR-223-3p remained "cilium assembly", for miR-199b-3p "potassium ion transport", for miR-142-5p "neutrophil degranulation", and for miR-181b-5p, miR-195-5p and miR-125b-5p no significantly enriched biological processes were found. A full list of all biological processes and Reactome pathways identified by Enrichr and g:Profiler is provided in supplementary tables S6 and S7.

Discussion

In this study, we identified 78 differentially expressed miRNAs in bronchial biopsies of asthma patients compared with healthy controls. Associations between miRNA expression and clinical and inflammatory asthma features identified seven candidate "asthma miRNAs", *i.e.* miR-199b-5p, miR-223-3p, miR-199b-3p, miR-142-5p, miR-181b-5p, miR-195-5p and miR-125b-5p, that were significantly associated with one or more clinical and inflammatory asthma features as well as asthma-associated gene expression. The biological pathways affected by these miRNAs included cilium assembly/organisation, neutrophil activation/degranulation and inflammatory response. Of interest, we identified that lower miR-26a-5p expression was strongly related to more eosinophilic inflammation.

TABLE 3 Top five Gene Ontology (GO) biological processes of negatively correlated asthma genes per microRNA

	Adjusted p-value
miR-199b-5p	
Cilium assembly (GO:0060271)	1.21E-17
Cilium organisation (GO:0044782)	1.23E-17
Plasma membrane bounded cell projection assembly (GO:0120031)	1.19E-13
Organelle assembly (GO:0070925)	1.64E-11
Intraciliary transport (GO:0042073)	4.19E-09
miR-223-3p	
Cilium assembly (GO:0060271)	3.88E-11
Organelle assembly (GO:0070925)	4.60E-09
Plasma membrane bounded cell projection assembly (GO:0120031)	1.15E-08
Intraciliary transport involved in cilium assembly (GO:0035735)	5.37E-08
Intraciliary transport (GO:0042073)	2.55E-07
miR-199b-3p	
Neutrophil degranulation (GO:0043312)	1.66E-08
Neutrophil activation involved in immune response (GO:0002283)	1.66E-08
Neutrophil-mediated immunity (GO:0002446)	1.66E-08
Inflammatory response (GO:0006954)	2.06E-04
Positive regulation of endopeptidase activity (GO:0010950)	3.73E-02
miR-181b-5p	
Neutrophil degranulation (GO:0043312)	1.75E-06
Neutrophil activation involved in immune response (GO:0002283)	1.75E-06
Neutrophil-mediated immunity (GO:0002446)	1.75E-06
Inflammatory response (GO:0006954)	3.20E-04
miR-195-5p	
Neutrophil degranulation (GO:0043312)	4.39E-09
Neutrophil activation involved in immune response (GO:0002283)	4.39E-09
Neutrophil-mediated immunity (GO:0002446)	4.39E-09
Inflammatory response (GO:0006954)	4.14E-05
Cellular defence response (GO:0006968)	1.45E-03
miR-125b-5p	
Inflammatory response (GO:0006954)	2.14E-04
Neutrophil degranulation (GO:0043312)	2.14E-04
Neutrophil activation involved in immune response (GO:0002283)	2.14E-04
Neutrophil-mediated immunity (GO:0002446)	2.14E-04
Positive regulation of endopeptidase activity (GO:0010950)	7.56E-03

TABLE 4 Top five Reactome pathways of negatively correlated asthma genes per microRNA

	Adjusted p-value
miR-199b-5p	
Assembly of the primary cilium: *Homo sapiens* (R-HSA-561783)	9.30E-16
Intraflagellar transport: *Homo sapiens* (R-HSA-5620924)	2.70E-11
Organelle biogenesis and maintenance: *Homo sapiens* (R-HSA-1852241)	7.81E-11
Cargo trafficking to the periciliary membrane: *Homo sapiens* (R-HSA-5620920)	8.92E-05
BBSome-mediated cargo-targeting to cilium: *Homo sapiens* (R-HSA-5620922)	1.10E-02
miR-223-3p	
Assembly of the primary cilium: *Homo sapiens* (R-HSA-5617833)	2.52E-13
Membrane trafficking: *Homo sapiens* (R-HSA-199991)	1.36E-12
Organelle biogenesis and maintenance: *Homo sapiens* (R-HSA-1852241)	9.38E-12
Vesicle-mediated transport: *Homo sapiens* (R-HSA-5653656)	5.57E-10
Intraflagellar transport: *Homo sapiens* (R-HSA-5620924)	3.04E-09
miR-181b-5p	
Immune system: *Homo sapiens* (R-HSA-168256)	1.88E-02
Immunoregulatory interactions between a lymphoid and a nonlymphoid cell: *Homo sapiens* (R-HSA-198933)	4.09E-02

The most significant lower expressed miRNA in asthma was miR-125b-5p, which was associated with more severe airflow obstruction and increased blood neutrophil counts. Enrichment of biological pathways revealed that miR-125b-5p is linked to inflammatory responses. Previously, Liu *et al*. [19] showed that miR-125b-5p is also lower expressed in sputum of childhood asthma patients *versus* controls, especially in those with more severe eosinophilic inflammation. Furthermore, in current smokers with or without chronic obstructive pulmonary disease, lower levels of miR-125b-5p were also linked with more airflow obstruction [20]. In our study, miR-125b-5p still remained lower expressed in asthma in the sensitivity analysis excluding smokers and patients that used ICS, indicating that in our cohort smoking had no major effect on the expression of miR-125b-5p. Additionally, functional studies demonstrated that intranasal administration of miR-125b can attenuate asthma features by reducing interleukin (IL)-4 and IL-13 levels, goblet cell differentiation, and mucus production in a murine asthma model [19].

Since asthma patients had higher miR-223-3p levels, and those levels were associated with higher tissue eosinophils and neutrophils and negatively correlated with asthma-associated genes, we suggest that miR-223-3p could play a role in the pathogenesis of asthma. In line with this, several studies showed higher levels of miR-223-3p in bronchial airway epithelial cells and sputum of asthma patients, especially in severe (neutrophilic) asthma patients [10, 21]. Furthermore, several studies demonstrated that overexpression of miR-223-3p reduced pro-inflammatory responses, while depletion of miR-223-3p enhanced inflammatory responses (reviewed in [22]). In our study, higher levels of miR-223-3p in biopsies were strongly associated with lower *GREM2* expression. *GREM2*, which encodes for gremlin-2 and is lower expressed in asthma patients, is an antagonist of bone morphogenetic proteins [23]. Gremlin-2 is involved in abnormal tissue damage and repair responses, including lung fibrosis [24]. Additionally, the most significantly enriched biological process in our study for miR-223-3p is cilium assembly/organisation. Cilium assembly/organisation is important for the formation of cilia on bronchial epithelia cells, leading to mucociliary function and clearance of mucus, and preventing infections and inflammation [25]. We also observed that miR-223-3p is enriched in several neutrophilic processes, which is in line with previous studies [10, 21]. However, in our study these processes do not belong to the top 50 most significantly enriched biological pathways, which could be due to the differences in sources (biopsies *versus* sputum) and patient cohorts (mild to moderate *versus* severe asthma patients). Overall, this indicates that miR-223-3p may contribute to aberrant airway inflammation and repair responses by regulating (among others) *GREM2* expression, suggesting a mechanistic role of miR-223-3p in asthma pathogenesis.

In addition, we identified miR-199b-5p, miR-181b-5p, miR-199b-3p and miR-195-5p as key candidate asthma miRNAs, since these miRNAs are associated with clinical and inflammatory asthma features and can play a potential role as regulators of asthma-associated gene expression. In this study, lower levels of miR-199b-5p, miR-181b-5p, miR-199b-3p and miR-195-5p were associated with higher levels of *HBA1* in bronchial biopsies of asthma patients. Erythrocyte precursors are the major producer of *HBA1*, which encodes for the α subunit of haemoglobin [26]. Additionally, lung epithelial cells can produce the haemoglobin α subunit [27]. As yet, little is known about the role of *HBA1* in clinical and inflammatory

features of asthma. Furthermore, enrichment analyses with the negatively correlated asthma-associated genes of these miRNAs (miR-199b-5p, miR-181b-5p, miR-199b-3p and miR-195-5p) revealed that miR-199b-5p was involved in cilium assembly/organisation, and miR-181b-5p, miR-199b-3p and miR-195-5p were involved in neutrophil activation/degranulation. Regarding neutrophil activation/degranulation, neutrophilic inflammation is linked to severe, steroid-resistant asthma patients and to smoking asthmatic subjects [28, 29]. Using both negatively and positively correlated asthma-associated genes for enrichment analyses, it remains that miR-199b-5p was involved in cilium assembly/organisation. However, for miR-181b-5p and miR-195-5p no significant biological processes were found, and miR-199b-3p was revealed to be involved in potassium ion transport. Future functional studies should help to elucidate the role of these miRNAs in cilium organisation/assembly, neutrophil activation/degranulation and potassium ion transport.

Another miRNA of interest was miR-26a-5p. The expression of miR-26a-5p was lower in asthma patients and associated with more severe eosinophilic inflammation as measured in blood, sputum as well as bronchial biopsies. Other studies have reported that miR-26a was lower expressed in bronchial epithelial brushings, serum and exosomes of bronchoalveolar lavage fluid derived from asthma patients compared with controls [8, 30, 31]. Furthermore, a previous study linked lower miR-26a expression in serum of asthma patients to lower levels of FEV_1 % pred [31]; however, we did not observe this association for miR-26a-5p in bronchial biopsies. An *in vivo* mice study showed that miR-26a/b regulates allergic inflammation by reducing the levels of cyclooxygenase-2 [32]. While the expression of cyclooxygenase-2 is increased in airways of asthma patients [33], we did not observe differences in cyclooxygenase-2 expression in bronchial biopsies of asthma patients compared with healthy controls and also found no correlation between miR-26a-5p and cyclooxygenase-2. Altogether, these data indicate that there might be a potential role for miR-26a-5p in the pathogenesis of asthma, especially eosinophilic asthma.

Of interest, miR-451a was the miRNA with the highest fold change in bronchial biopsies of asthma patients, while no correlation was found with clinical features of asthma. This may suggest that the association of this miRNA with asthma is not driven by any of the well-known asthma features assessed in our study and may point towards a different underlying mechanism. Previous studies have shown decreased miR-451a expression in peripheral blood lymphocytes from asthmatic children [34] and that *in vivo* overexpression of miR-451a inhibits airway remodelling by targeting cadherin-11 [35], suggesting a potential role in (controlling) airway remodelling.

One of the main strengths of this study is the size of the cohort and the careful characterisation of the subjects, which made it possible to make the association of miRNAs with clinical and inflammatory asthma features. Moreover, we had matched miRNA and mRNA data available from the same bronchial biopsies. Therefore, we could directly correlate global miRNA and gene expression levels to identify miRNAs involved in the regulation of gene expression changes in asthma. One limitation of this study is the lack of suitable replication datasets to validate the differentially expressed miRNAs in asthma and their association with gene expression. There are only a few studies that have determined miRNA expression in asthma patients; however, to the best of our knowledge, no miRNA–mRNA data are available of bronchial biopsies.

In conclusion, we have profiled miRNA expression in bronchial biopsies from asthma patients and controls, and identified several candidate “asthma miRNAs”, including miR-223-3p and miR-125b-5p, that were associated with multiple clinical and inflammatory asthma features and their negatively correlated genes are linked with the presence of asthma. Furthermore, miR-26a-5p is linked with inflammatory asthma features, especially eosinophilic inflammation. Therefore, these miRNAs are important candidates for future studies to unravel their mechanistic role in the pathogenesis of asthma.

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