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MiR-31-5p: a shared regulator of chronic mucus hypersecretion in asthma and chronic obstructive pulmonary disease

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Abstract

Chronic mucus hypersecretion (CMH) is prevalent in both asthma and chronic obstructive pulmonary disease (COPD) and is associated with more severe symptoms. Recently, we identified several microRNAs (miRNAs) associated with CMH in COPD. In this study, we extended our analysis to CMH in asthma.

Using small RNA sequencing and RNA sequencing profiles of bronchial biopsies from 65 asthmatic patients, we identified 17 miRNAs associated with CMH in asthma. When comparing them to our previously published dataset in COPD, we found a positive association of miR-31-5p with CMH in both diseases. MiR-31-5p was negatively correlated with multiple predicted targets, including *ST3GAL2*, *PITPNM2*, and *ARHGEF15* which were also associated with CMH. Gene set enrichment analysis revealed the enrichment of *MUC5AC*-associated gene set and CMH-associated gene set in COPD among CMH-associated genes in asthma.

Our findings suggest CMH in asthma and COPD share common regulatory mechanisms mediated, at least in part, by miR-31-5p. This study provides novel insights that could contribute to the development of CMH-targeted therapy that efficiently benefits both asthma and COPD patients.

Keywords

chronic mucus hypersecretion, asthma, COPD, microRNA, miR-31-5p

Word count: 1236

To the Editor:

Chronic mucus hypersecretion (CMH) is prevalent in both asthma¹ and chronic obstructive pulmonary disease (COPD)² and is associated with more severe symptoms.²⁻⁵ Mucus is composed of heavily glycosylated proteins called mucins, of which MUC5AC and MUC5B are the main mucins involved in asthma^{6,7} and COPD.^{8,9} As post-transcriptional regulators of gene expression, microRNAs (miRNAs) play a role in various human diseases.¹⁰ Recently, we identified several miRNAs associated with CMH in COPD.¹¹ In the present study, we extended our analysis to CMH in asthma which is important as it would be efficient to develop the same therapy targeting CMH in both diseases if they share common regulatory mechanisms.

Small RNA sequencing and RNA sequencing was performed on bronchial biopsies from 65 asthmatic patients.¹² The patients either had never been treated with inhaled corticosteroid (ICS), had stopped ICS treatment for 6-8 weeks prior to the bronchoscopy, or were current ICS users. The study approach is illustrated in Fig.1A and detailed methods are described in the Online Repository. Patient characteristics are presented in Table E1.

We first compared miRNA profiles between ICS-naïve asthmatic patients with (n=6) and without CMH (n=14) using linear regression adjusting for age, gender, smoking status and library preparation batch. We identified 17 differentially expressed miRNAs associated with CMH (FDR<.05; Fig.1B and E1). In asthmatic patients with CMH, the expression of miR-31-5p, miR-152-3p, and miR-155-5p was higher, while the expression of miR-15b-3p, miR-15b-5p, miR-16-2-3p, miR-25-3p, miR-92a-3p, miR-106b-3p, miR-185-5p, miR-223-3p, miR-3615, miR-423-5p, miR-425-5p, miR-451a, miR-484 and miR-486-5p was lower compared to patients without CMH. The most strongly up-regulated miRNA was miR-31-5p (4.11 fold) and the most strongly down-regulated one was miR-486-5p (9.47 fold) (Table E2). Interestingly, miR-31-5p was previously also found to be associated with CMH in COPD, with the same direction of effect.¹¹ When including the current ICS users in the analysis, none of the miRNAs was significantly associated with CMH, reflecting an evident influence of corticosteroids on expression of these miRNAs.

Next, we identified CMH-associated mRNAs with the same approach. Expression of *MUC5AC*, but not *MUC5B*, was significantly higher in the asthmatic patients with versus those without CMH (P<.0001, Fig.1C). When corrected for multiple tests (FDR<.05), we identified 2 mRNAs associated with CMH, i.e. *Chromosome 3 Open Reading Frame 70 (C3orf70)* and *phosphatidylinositol transfer protein membrane associated 2 (PITPNM2)*. Using Gene Set Enrichment Analysis (GSEA), we assessed whether MUC5AC-associated core genes¹³ and CMH-

associated genes in COPD¹¹ were enriched among the genome-wide mRNAs, ranked based on the strength (fold change) of their association with CMH in ICS-naïve asthma patients as determined by linear regression. As expected, we found significant enrichment ($P<.001$) of both gene sets among the ranked list (Fig.1C-1D), suggesting that MUC5AC is associated with CMH and that CMH in asthma and COPD share common regulatory mechanisms.

We then assessed whether any of the CMH-associated miRNAs were correlated with MUC5AC or MUC5B using matched small RNA-sequencing and RNA-sequencing profiles in all asthmatic patients ($n=65$) (Table E3). There was a significant positive correlation between miR-16-2-3p and MUC5B ($p=0.02$) and a trend of positive correlation between miR-31-5p and MUC5AC ($p=0.06$). Similar to asthma, miR-31-5p was not correlated with MUC5B in the COPD dataset. MUC5AC expression was not measured by the microarray we used for the COPD dataset (Affymetrix HuGene ST1.0v1), however, we previously showed that MUC5AC-associated core genes were enriched among miR-31-5p-correlated genes¹¹ suggesting that miR-31-5p regulates CMH, at least in part, via modulation of MUC5AC synthesis.

Since we found miR-31-5p to be associated with CMH in both asthma and COPD, we assessed correlations between miR-31-5p and genome-wide mRNA expression regardless of their association with CMH using matched small RNA-sequencing and RNA-sequencing profiles in all asthmatic patients ($n=65$). As miRNAs inhibit translation of their mRNA targets partly by inducing mRNA degradation, we expected the mRNA targets of miR-31-5p to be negatively correlated with this miRNA. Spearman's correlation coefficient revealed 890 genes of which expression levels were negatively correlated with miR-31-5p ($FDR<.05$). Among these 890 negatively correlated genes, 48 were predicted miR-31-5p targets according to TargetScan or miRDB or both (Fig.2A). Next, we applied the same approach on our previously published COPD bronchial biopsy dataset¹¹ which resulted in 62 predicted targets negatively correlated with miR-31-5p (Fig.2A). When combining these 48 and 62 predicted targets, we found 17 overlapping negatively correlated predicted targets of miR-31-5p in asthma and COPD (Fig.2A, Table E4). To identify common mechanisms in which these 17 genes may be involved, we performed GO Enrichment Analysis^{14,15} but found no significant enrichment of GO terms.

Interestingly, of these 17 miR-31-5p correlated targets, *ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 2 (ST3GAL2)*, *PITPNM2* and *Rho guanine nucleotide exchange factor 15 (ARHGEF15)* showed significantly lower expression levels in patients with CMH compared to those without CMH in both diseases (Fig.2A and B). When focusing on ICS-naïve group alone,

we found that the negative correlation between miR-31-5p and ARHGEF15 remained nominally significant ($p=.03$), while there was a trend for a negative correlation with ST3GAL2 ($p=.05$) and PITPNM2 ($p=.08$). *ST3GAL2* is a member of sialyltransferases. Previous studies showed that higher sialylation of airway mucins is present in bacterial infected patients with cystic fibrosis and with chronic bronchitis compared to non-infected patients.¹⁶ This sialylation, occurring during mucin biosynthesis, could influence host-microbial interaction during infection.¹⁷ Thus, *ST3GAL2* may play a role in mucin glycosylation and it is worthwhile to investigate if and how this contributes to CMH. Little is known about the function of *PITPNM2*, while *ARHGEF15* is a Rho guanine nucleotide exchange factor specifically expressed in endothelial cells.¹⁸ Notably, all these 17 genes are expressed in both human-derived basal epithelial cells and goblet cells, as assessed in our single-cell RNA sequencing profiles of bronchial brushes from 6 non-asthmatic control donors and 6 asthmatic donors (BioRxiv 527408).¹⁹

It is not yet conclusive whether higher miR-31-5p expression in CMH is a reflection of its stimulatory or protective role on mucus production. Previously, miR-31-5p expression was found to be slightly lower in asthmatic airway epithelial brushings compared to those of healthy controls but CMH status of these patients was not reported.²⁰ Although bronchial biopsies contain various cell types apart from epithelial cells, we found no difference in numbers of inflammatory cells, i.e. T-cells, B-cells, neutrophils, macrophages, eosinophils, and mast cells, between the asthmatic patients with and without CMH. This suggests that these findings were not driven by infiltration of different inflammatory cells.¹² As our data is based on miRNA-mRNA correlations, future studies are warranted to confirm the direct interaction and functional consequences. Furthermore, these miRNAs and mRNAs were identified using a relatively small sample size and the patients with CMH were all males (Table E1). Therefore, similar analyses performed on a larger cohort with equal gender distribution would strengthen these findings. Since these findings are based on association analyses, future functional studies are required to elucidate whether and how these genes and miR-31-5p directly contribute to CMH. Besides, we encourage other researchers who could to replicate these findings using other measurements of CMH, such as overall mucin concentration in sputum which is one of the important markers of CMH.⁹

This is the first study to propose miR-31-5p as a shared regulator of CMH in both asthma and COPD. We identified several mRNAs potentially targeted by miR-31-5p, including *ST3GAL2*, *PITPNM2*, and *ARHGEF15* that were negatively associated with CMH in both diseases. These findings provide novel and important insights on common CMH regulatory mechanisms and could

contribute to the development of CMH-targeted therapy that efficiently benefits both asthma and COPD patients.

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A Conflict of Interest disclosure statement

HT, IMB, AF, WT, MNH, MB, NHTtH, CAB, and IHH report no conflict of interest. MvdB received a research grant for this study from GSK and for other studies from TEVA, Chiesi, Astra Zeneca, and Genentech.

A statement of contribution

HT, AF, WT, MNH, CAB, IHH and MvdB contributed to the study concept and design. NHTtH and MvdB coordinated patient inclusion and data collection. NHTtH performed bronchoscopy. MvdB secured funding for the study. MvdB and IMB organized and performed the RNA- and small RNA-sequencing. MB performed the analysis on the single-cell RNA sequencing data. HT, IMB, CAB, IHH and MvdB analysed and interpreted data. HT drafted the manuscript under

supervision of CAB, IHH and MvdB. HT, AF, WT, MNH, CAB, IHH and MvdB critically read and revised the manuscript. All authors have read, reviewed and approved the final manuscript.

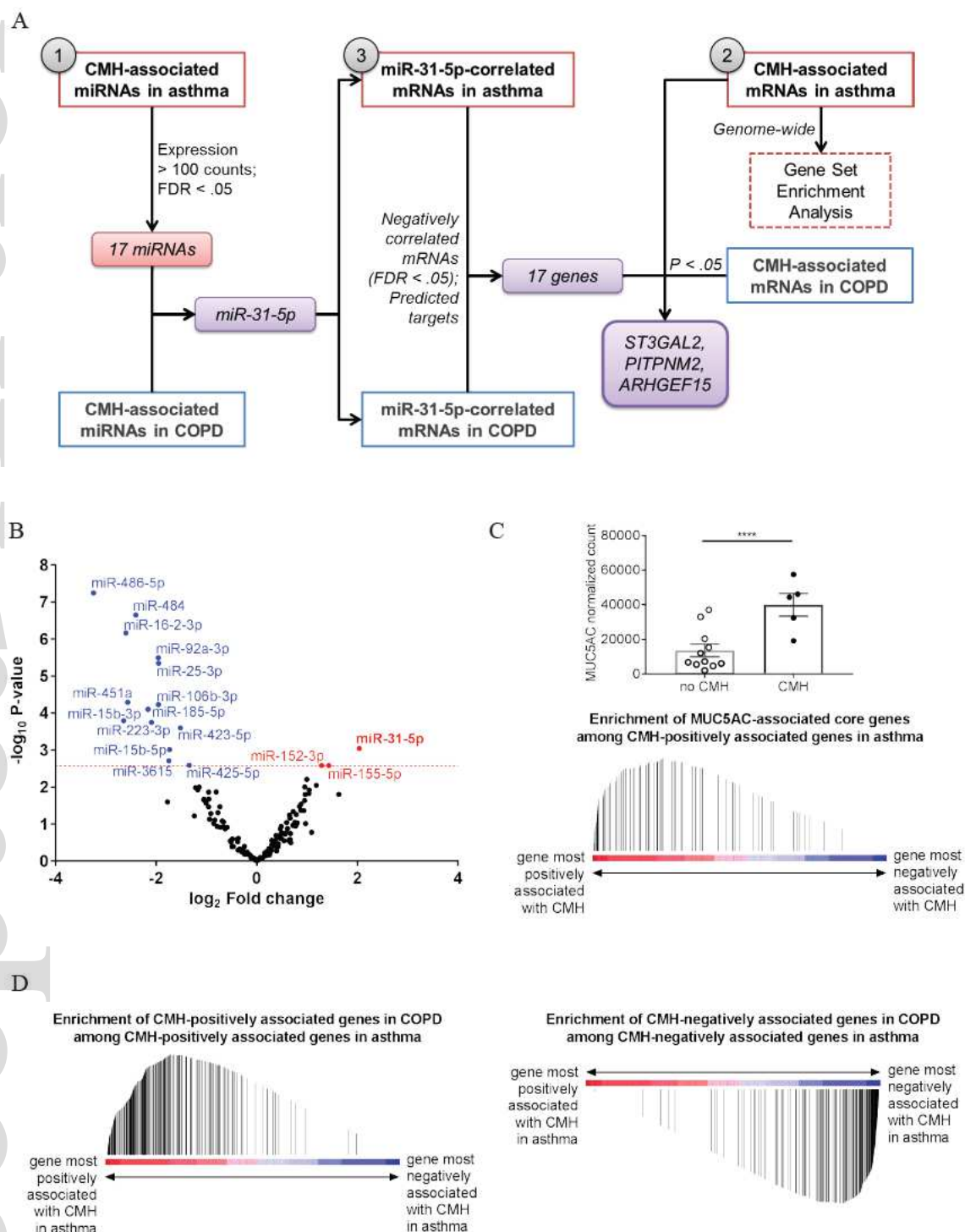


FIGURE 1. MiRNAs and associated genes potentially regulate CMH. A, An overview of the study approach. B, MiRNAs differentially expressed with CMH in asthma. Linear regression was

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associated targets in all asthmatic patients (n=65). Closed circles represent expression in patients who have been or are currently treated with ICS. Open circles represent expression in ICS-naïve group. Statistics shown in the plots (r and p values) were based on all patients. Spearman correlation was performed using R (v3.2.5).

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ONLINE SUPPLEMENTS

METHODS

Patient cohort

Bronchoscopy was performed to collect bronchial biopsies from persistent asthmatic patients at the University Medical Center Groningen between 2002 and 2012. Procedures for sample collection, spirometry, and provocation tests were previously described.¹² These patients either used asthma medication (e.g. inhaled corticosteroid (ICS), beta-agonists and/or other) or experienced asthma symptoms (e.g. wheeze or asthma attacks during the last 3 years). A subset of the patients either have never been treated with ICS, have stopped using ICS for 6-8 weeks prior to the bronchoscopy, or are currently using ICS. Subjects with upper respiratory tract infection or used antibiotics and/or oral corticosteroids within 2 months prior to the study were excluded. The local medical ethics committee approved the study and all patients gave their written informed consent.

MicroRNA (miRNA) and mRNA profiling

Bronchial biopsy specimens were stored in Tissue-Tek (VWR, Radnor, PA, USA) at -80 °C until processing. Total RNA was extracted using AllPrep DNA/RNA/miRNA Universal kit (Qiagen, Venlo, The Netherlands) and the RNA quality was assessed using Nanodrop-1000 and Labchip GX (PerkinElmer, Waltham, MA, USA). Sample randomization was applied to avoid batch effects.

MiRNA expression profiles were obtained from small RNA sequencing. Small RNA library was prepared using NEXTflex Small RNA-Seq Kit v3 (Bioo Scientific Corporation, Austin, TX, USA) according to the manufacturer's protocol. Small RNA fraction was selected using NEXTflex Cleanup Beads and the size distribution of the final libraries were verified using Labchip GX (PerkinElmer, Waltham, MA, USA). The sequencing was performed using an Illumina HiSeq2500 sequencer with default parameters applied (single read 1x50bp). The quality control was performed using FastQC (v0.11.5) and adapter trimming was done using TrimGalore (v0.3.7). The sequencing data was processed using custom scripts documented in the GitHub repository.²¹ The reads were aligned and quantified using miRDeep2 (2.0.0.8)²² with Bowtie (v0.12.7).²³

mRNA expression profiles were obtained from RNA sequencing. RNA library was prepared with TruSeq Stranded Total RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and the Caliper Sciclone NGS Workstation (PerkinElmer, Waltham, MA, USA). The removal of ribosomal RNA was done by Ribo-Zero Gold Kit (Illumina, San Diego, CA, USA). Paired-end sequencing was then performed using an Illumina HiSeq2500 sequencer. Quality control of the raw sequencing data was performed with FastQC (v0.11.3). The reads were aligned to build b37 of the human reference genome using HISAT (v0.1.5) allowing for 2 mismatches.²⁴ The aligned reads were sorted with SAMtools (version 1.2)²⁵ and quantified with HTSeq (v0.6.1p1) using Ensembl release 75 as gene annotation database.²⁶ Quality control of aligned reads was performed using Picard-tools (v1.130). Furthermore, concordance between reported gender and gender-associated gene expression (XIST and Y-chromosomal genes) was assessed and we observed that all samples were concordant.

Identification of miRNAs and mRNAs associated with CMH

The presence of CMH was defined according to the patients' responses to the question: how often did you cough up sputum during the last week?, as part of the Clinical COPD Questionnaires (CCQ).²⁷ Patients who responded never, seldom, or once in a while were categorized as "without CMH" and patients who responded regularly and very often were categorized as "with CMH". No patients included in this study responded mostly or always.

MiRNAs differentially expressed with the presence of CMH were identified using linear regression model adjusted for age, gender, smoking status and library preparation batch. To avoid ICS effects, only miRNA profiles of ICS-naïve asthmatic patients with (n=6) and without (n=14) CMH were included. mRNAs differentially expressed with CMH were identified using the same approach, but only mRNA profiles of 5 patients with CMH and 11 patients with CMH passed the quality control. Expression was normalized using DESeq2 package. RNA transcripts of less than 5 normalized counts in more than 50% of the samples were removed prior to the analyses. Only miRNAs and mRNAs with at least 100 normalized counts on average were reported in this study. Significance was determined by $FDR < .05$. All analyses were performed in R (v3.2.5).

Gene Set Enrichment Analysis (GSEA)

Genome-wide mRNAs were ranked according to the strength of their association with CMH as assessed by the linear regression, from the most positive to the most negative associated mRNAs. GSEA (v2.2.2) was then performed on this ranked list using a MUC5AC-associated core gene set¹³ and CMH-associated gene sets¹¹ previously reported. Enrichment p-value was calculated after 1000 permutations were performed. Significance was determined by $p < .05$.

Identification of miR-31-5p targets in asthma and COPD

Correlations between miR-31-5p expression and genome-wide mRNAs were determined using Spearman's rank correlation coefficient on matched miRNA and mRNA profiles of 65 asthmatic patients regardless of their ICS history. Significance was determined by $FDR < .05$. MiR-31-5p-correlated mRNAs in COPD biopsy dataset¹¹ were identified using the same approach. Next, we compared miR-31-5p-negatively correlated genes identified in both asthma and COPD datasets to miR-31-5p's predicted targets reported on TargetScan (v7.1) and/or miRDB (v5.0). The overlapping genes were considered potential targets of miR-31-5p. We further determined whether any of these genes were significantly associated with CMH in both asthma and COPD using a nominal $p < .05$. The network illustrating these data was created on Cytoscape (v3.4.0). The graphs illustrating correlations between miR-31-5p and selected genes were created on GraphPad Prism (v7).

Table E1 Patient characteristics	ICS-naïve asthmatic group (CMH-associated miRNA and mRNA analyses)		Asthmatic group (miRNA-mRNA correlation analysis)
	no CMH	CMH	
Total patients	14	6	65
Gender (n, male/female)	6/8	6/0*	30/35
Age	50 (29-53)	51 (41-53)	49 (38-55)
Smoking status (n, current-/non-smoker)	8/6	4/2	15/50
Pack-years	7.5 (0.4-13.6)	16.8 (10.1-28.4)	0.2 (0.0-10.0)
BMI	26.8 (23.2-28.9)	28.5 (25.6-30.5)	26.8 (23.8-28.7)
FEV1, % predicted	88.4 (75.5-97.5)	81.5 (78.5-84.9)	85.2 (75.6-96.4)
FEV1/FVC	0.72 (0.62-0.78)	0.7 (0.68-0.76)	0.71 (0.66-0.77)
Atopic (n, yes/no/not available)	11/3/0	3/2/1	48/13/4
Airway hyperresponsiveness (PC ₂₀ AMP mg/ml)	37.6 (13.7-529.3)	58.8 (15.8-86.0)	45.4 (5.9-640.0)
ICS history (n, naive/withdrawn/active)	14/0/0	6/0/0	24/16/25
Inflammatory cell composition (cells/μl)			
CD3+ T-lymphocytes	75.2 (42.2-101.6)	41.4 (33.6-46.4)	-
CD4+ T-lymphocytes	19.3 (7.3-33.2)	8.6 (3.1-12.7)	-
CD8+ T-lymphocytes	14.0 (7.2-27.4)	23.0 (19.6-23.9)	-
CD20+ B-lymphocytes	0.4 (0.2-3.4)	1.9 (0.2-3.5)	-
NP57+ neutrophils	5.8 (1.3-8.8)	12.4 (6.7-16.6)	-
CD68+ macrophages	8.6 (4.1-13.2)	5.5 (3.1-8.6)	-
EPX+ eosinophils	1.9 (0.2-3.4)	0.5 (0.0-0.9)	-
AA1+ mast cells	12.9 (9.5-15.6)	10.6 (10.1-12.3)	-

Data are presented as median (interquartile range) for age, pack-years, body mass index (BMI), forced expiratory volume in 1 second (FEV1), FEV1/forced vital capacity (FVC), provocative concentration causing 20% fall in FEV1 of adenosine-59-monophosphate (PC₂₀ AMP mg/ml), and inflammatory cell composition. ICS is inhaled corticosteroid. Statistical comparisons between CMH and no CMH groups were performed using Mann-Whitney U-test. **P* < .05.

miRNA	Mean count	Fold change	P value	FDR
miR-31-5p	458.74	4.11	9.06E-04	2.63E-02
miR-155-5p	1049.92	2.70	2.64E-03	4.88E-02
miR-152-3p	511.52	2.45	2.64E-03	4.88E-02
miR-425-5p	807.48	-2.53	2.59E-03	4.88E-02
miR-423-5p	1463.75	-2.86	2.53E-04	9.36E-03
miR-15b-5p	2637.20	-3.32	9.78E-04	2.65E-02
miR-3615	101.60	-3.36	1.96E-03	4.43E-02
miR-25-3p	5323.29	-3.87	4.55E-06	3.70E-04
miR-106b-3p	281.44	-3.87	5.95E-05	3.46E-03
miR-92a-3p	27689.78	-3.88	3.18E-06	3.24E-04
miR-223-3p	2149.10	-4.27	1.79E-04	7.28E-03
miR-185-5p	557.90	-4.47	7.98E-05	4.06E-03
miR-484	918.62	-5.30	2.24E-07	4.56E-05
miR-451a	51118.93	-5.92	5.13E-05	3.46E-03
miR-16-2-3p	417.70	-6.07	6.87E-07	9.32E-05
miR-15b-3p	124.79	-6.26	1.62E-04	7.28E-03
miR-486-5p	79385.72	-9.47	5.68E-08	2.31E-05

88 **Table E3 Correlations between CMH-associated miRNAs and *MUC5AC* or *MUC5B***

	MUC5AC			MUC5B	
	$\rho^{\dagger}$	<i>P</i> value [†]		$\rho^{\dagger}$	<i>P</i> value [†]
miR-31-5p	0.2303	0.06		-0.0622	0.62
miR-155-5p	-0.1009	0.42		-0.0521	0.68
miR-152-3p	0.0739	0.56		-0.0422	0.74
miR-425-5p	0.1397	0.27		0.1583	0.21
miR-423-5p	0.1687	0.18		0.0149	0.91
miR-15b-5p	0.0329	0.79		0.1435	0.25
miR-3615	0.1037	0.41		-0.0594	0.64
miR-25-3p	0.0328	0.80		0.1185	0.35
miR-106b-3p	0.0604	0.63		0.0918	0.47
miR-92a-3p	0.1553	0.22		0.0446	0.72
miR-223-3p	0.0058	0.96		-0.0037	0.98
miR-185-5p	0.0489	0.70		0.1378	0.27
miR-484	0.0972	0.44		0.1243	0.32
miR-451a	0.0007	1.00		0.2153	0.09
miR-16-2-3p	-0.0670	0.60		0.2862	0.02
miR-15b-3p	0.0549	0.66		0.1567	0.21
miR-486-5p	0.1101	0.38		0.0623	0.62

89 [†]Statistics from Spearman's correlation performed on the asthmatic cohort (n=65)

90

91 **Table E4 MiR-31-5p-correlated predicted targets**

Gene symbol	Rho [†]	P value [†]	FDR [†]	Predicted by
ARRB1	-0.4998	2.24E-05	2.71E-03	Targetscan
FZD4	-0.4949	2.78E-05	3.00E-03	Targetscan
ST3GAL2 [‡]	-0.4632	1.02E-04	5.66E-03	Targetscan
EBF3	-0.4296	3.55E-04	1.11E-02	Targetscan, miRDB
APBB2	-0.3815	1.71E-03	2.64E-02	Targetscan, miRDB
PRKCB	-0.3747	2.10E-03	2.94E-02	miRDB
TMOD2	-0.3721	2.27E-03	3.05E-02	miRDB
SYDE1	-0.3686	2.52E-03	3.23E-02	Targetscan
KCNN3	-0.3683	2.54E-03	3.25E-02	Targetscan
TNS1	-0.3628	2.98E-03	3.52E-02	Targetscan, miRDB
STARD13	-0.3617	3.07E-03	3.60E-02	Targetscan
ATP8A1	-0.3609	3.14E-03	3.65E-02	Targetscan, miRDB
ZBTB20	-0.3557	3.64E-03	3.96E-02	Targetscan
PITPNM2 [‡]	-0.3506	4.20E-03	4.28E-02	miRDB
ARHGEF15 [‡]	-0.3498	4.28E-03	4.35E-02	Targetscan
SPARC	-0.3441	5.01E-03	4.77E-02	Targetscan, miRDB
SHC4	-0.342	5.30E-03	4.93E-02	miRDB

[†]Statistics from Spearman's correlation performed on the asthmatic cohort (n=65);

[‡]also significantly associated with CMH in asthma and COPD

92

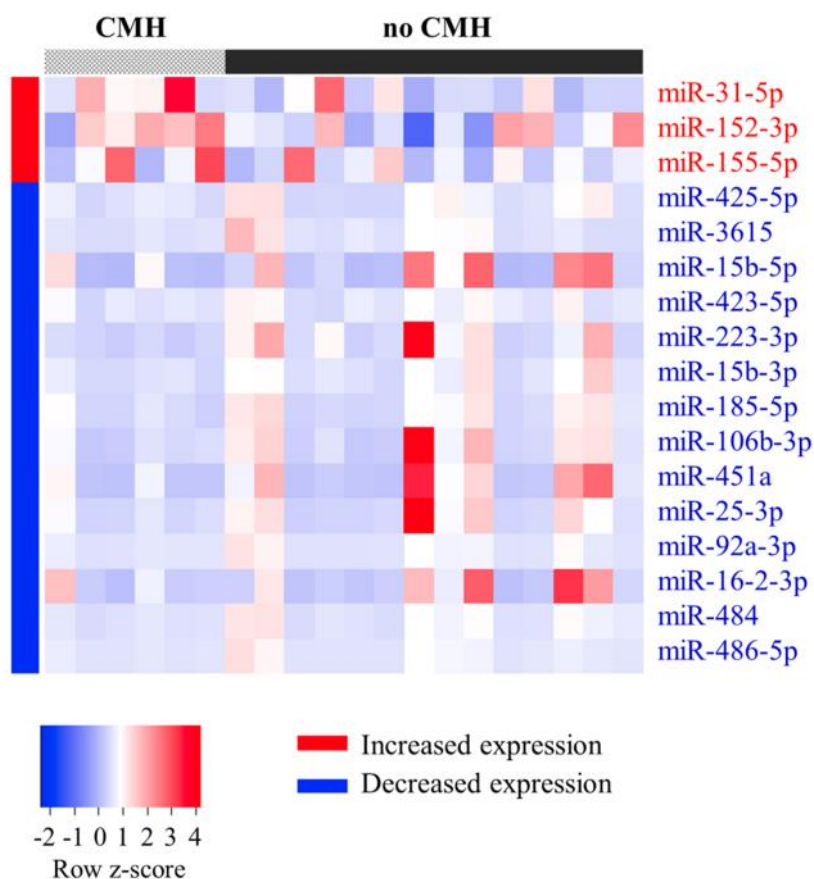


FIGURE E1. Heatmap showing CMH-associated miRNAs in asthma. Only miRNAs of > 100 counts were considered expressed. Linear regression corrected for age, gender and smoking status was performed on the ICS-naïve cohort (n = 20). FDR<.05.

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155