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A Novel Role of Bronchial MicroRNAs and Long Non-coding RNAs in Asthma Remission

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Author's contribution:

The study was designed by MvdB, DSP, NtH, IH, GK, CX and JV. IMB and MMT performed statistical analyses under supervision of MvdB, DSP, VG, CJV, AF and KK. DSP, NtH, WT, MR, IH and MN contributed to data acquisition. All authors contributed to interpretation of the data. IMB drafted the manuscript under supervision of MvdB, DSP, VG and GK. All authors critically reviewed and approved the manuscript.

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To the editor,

Complete asthma remission in adulthood occurs in a minority of patients, but knowledge on its fundamental mechanisms is lacking. We explored underlying mechanisms by investigating bronchial microRNA expression, since microRNAs are increasingly recognized as important regulators of lung development and growth, as well as disease pathogenesis, including Th2-driven airway inflammation(1). We investigated differential microRNA expression among subjects with complete asthma remission, subjects with persistent asthma and healthy controls. Additionally, we integrated these findings with protein-coding- and long non-coding RNA (lncRNA) expression.

All subjects with persistent asthma or complete remission had a doctor's diagnosis of asthma and documented airway hyperresponsiveness (AHR) to histamine in the past. Complete asthma remission was further defined as absence of wheeze or asthma attacks in the last 3 years, no use of inhaled corticosteroids (ICS) or beta-agonists and absence of airway hyperresponsiveness (AHR) and airway obstruction (FEV1>80%). Persistent asthma was defined by doctor diagnosed asthma and documented AHR in the past, and current respiratory symptoms and/or asthma medication use.

Asthma subjects either stopped their inhaled corticosteroids 6-8 weeks before inclusion or did not use ICS at all. They had no exacerbation within 2 months prior to the study. Healthy subjects had no respiratory symptoms and normal pulmonary function. Bronchoscopy was performed 3-6 weeks after inclusion. Total RNA was isolated from bronchial biopsies. During RNA extraction, library preparation and sequencing all samples were equally distributed across all batches to minimize technical variation. Total RNA was isolated from bronchial biopsies, library preparation was done for microRNAs and protein-coding and lncRNAs

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separately and RNA-sequencing was performed. Genes with appreciable expression (for microRNAs: 1 fragment per million in at least one of the clinical groups and for long RNAs: 5 normalized counts in half of the samples) were used for analysis. Differential expression of microRNAs was analyzed with DESeq2 (v1.14.1)(2), adjusting for age, gender, smoking status and library preparation batch and correcting for multiple testing using the Benjamini-Hochberg method. Next, we integrated microRNA-, protein-coding RNA- and lncRNA expression in association with complete remission by performing Bayesian network modeling using the CGBayesnets Package in MATLAB(3). We assessed predictive performance of the network by calculating the Area Under the Receiver Operator Characteristic Curve (AUC) by means of permutation testing (10,000 iterations). Finally, we performed *in-vitro* validation of a top-microRNA by microRNA-transfection of tracheobronchial and 16HBE cells.

Fourteen subjects with complete remission were included, of which 7 (50%) female, 4 (29%) current smoker, mean FEV_1 103% predicted (standard deviation [SD] ±13), median age 48 years (interquartile range [IQR] 36-53) and median time since first asthma attack 45 years (IQR 40-50). Forty-six patients with persistent asthma were included, of which 24 (52%) female, 16 (35%) current smoker, mean FEV_1 84% predicted (SD±16), median age 52 years (IQR 35-57) and median time since first asthma attack 41 years (IQR 23-49). Of all asthma subjects, 28 (61%) did not use ICS at all, while 18 (39%) had stopped their ICS 6-8 weeks before inclusion. Eighty-two healthy controls were included, of which 36 (44%) female, 40 (49%) current smoker, mean FEV_1 101% predicted (SD±12) and median age 42 years (IQR 23-56). All subjects were Caucasian.

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Ten microRNAs were differentially expressed between subjects with complete remission and persistent asthma: 9 upregulated (*miR-320a*, *miR-193a-5p*, *miR-320c*, *miR-4532*, *miR-320d*, *miR-320b*, *miR-423-3p*, *miR-133b* and *miR-3960*) and 1 downregulated (*miR-126-3p*)(Figure 1). Seventy-seven microRNAs were differentially expressed between subjects with complete remission and healthy controls (62 up- and 15 downregulated)(Figure 1). For subjects in complete remission and persistent asthma, we integrated microRNA-, protein-coding RNA- and lncRNA-expression. To this end, expression levels of 518 microRNAs and 22,729 protein-coding RNAs, lncRNAs and pseudogenes were used as input in a Bayesian network analysis. The gene network associated with the binary phenotype complete remission (using persistent asthma as controls) consisted of 24 microRNAs, 20 protein-coding RNAs, 35 lncRNAs and 14 pseudogenes (Figure 2, area under the curve [AUC] 0.99, p=0.0027). Of interest, 6 of the 24 microRNAs in the network were also identified in our differential expression analysis: *miR-126a-3p*, *miR-320a*, *miR-320b*, *miR-320c*, *miR-193a-5p* and *miR-133b*. Only microRNAs and lncRNAs, but not protein-coding RNAs, were directly connected to complete remission. Permutation analysis (100 iterations in which we swapped the phenotype values) showed that our network consistently contained a lower proportion of protein-coding RNAs than what could be expected by chance ($p<0.01$).

Next, we performed *in-vitro* validation of *miR-320d*, one of the top-10 microRNAs upregulated in complete remission compared to persistent asthma. Of interest, in a previous report of our group, *miR-320d* was also found to have anti-inflammatory effects in primary bronchial epithelial cells(4). In tracheobronchial cells and 16HBE cells stimulated with the viral mimic poly-(I:C), *miR-320d* transfection significantly decreased GM-CSF production compared to mimic controls ($p<0.05$).

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We observed a clear signal that expression levels of 10 microRNAs differentiate complete remission from persistent asthma. Moreover, expression changes in microRNAs and lncRNAs, abundantly present in a Bayesian network, were strongly associated with complete remission of asthma. This suggests that non-coding RNAs may be important in complete remission of asthma.

One other group has studied microRNA expression in remission of asthma by performing Bayesian network modeling(5). They identified a network based on serum-RNA profiles of asthmatic children between age 5 and 12 years that was associated with asthma remission at age 14. Although this study differed in many aspects from ours, the importance of microRNAs exhibiting their function by acting in a network is supported by both studies.

Ten microRNAs were differentially expressed between complete remission and persistent asthma. Also, we observed that subjects in complete remission had a distinct bronchial microRNA profile (77 differentially expressed microRNAs) compared to healthy controls. This suggests that subjects in complete remission do not resemble healthy subjects but represent a third, separate molecular state in addition to asthma and healthy. Exploring this phenomenon further can aid in developing new asthma therapies.

Of the differentially expressed microRNAs, *miR-126-3p* has been found to be an agonist of Th2-inflammation, an important mechanism underlying asthma(6). We found *miR-126-3p* downregulated in complete remission compared to persistent asthma, suggesting that this microRNA might play a role in achieving asthma remission. Four members of the *miR-320* family, namely *miR-320a, miR-320b, miR-320c* and *miR-320d*, were upregulated in complete remission compared to persistent asthma. It is suggested that *miR-320* has anti-inflammatory effects (4,7) which is confirmed by our *in-vitro* experiments showing

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decreased GM-CSF production upon poly-(I:C) stimulation in *miR-320d* transfected cells compared to mimic controls. This upregulation of *miR-320* in complete remission may potentially act to suppress inflammatory processes in asthma.

A strength of this study is the careful characterization of the subjects at baseline and at follow-up with standardized questionnaires and extensive pulmonary function tests. A limitation is the lack of a replication cohort for our findings. Also, the presence of only Caucasians in our study population limits generalizability to other groups.

In conclusion, we show that subjects in complete remission of asthma, subjects with persistent asthma and healthy controls differ in their bronchial microRNA expression profiles. Of interest, when integrating microRNA-, protein-coding RNA- and lncRNA expression by performing Bayesian network modeling, we identify a network characteristic of complete remission of asthma in which microRNAs and lncRNAs are abundantly present. Hence, future research should focus on the functional characterization of these non-coding RNAs in asthma remission.

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Figure 1: Heatmap of 83 differentially expressed microRNAs between complete remission and persistent asthma and between complete remission and healthy. miRNA=microRNA.

Figure 2: Bayesian network of microRNAs, protein-coding RNAs, lncRNAs and pseudogenes predicting complete remission. Predictive performance of the network was assessed by calculating the Area Under the Receiver Operator Characteristic Curve (AUC). Statistical significance of the AUC was determined by permutation testing (10,000 iterations) where we compared AUC of our network with the AUCs of the permuted networks. AUC of the network was 0.99 (p=0.0027). Distinct gene types are shown in different colors. Black-lined nodes represent nodes directly connected to complete remission. Diamond-shaped nodes represent microRNAs that were also identified in the differential expression analysis.

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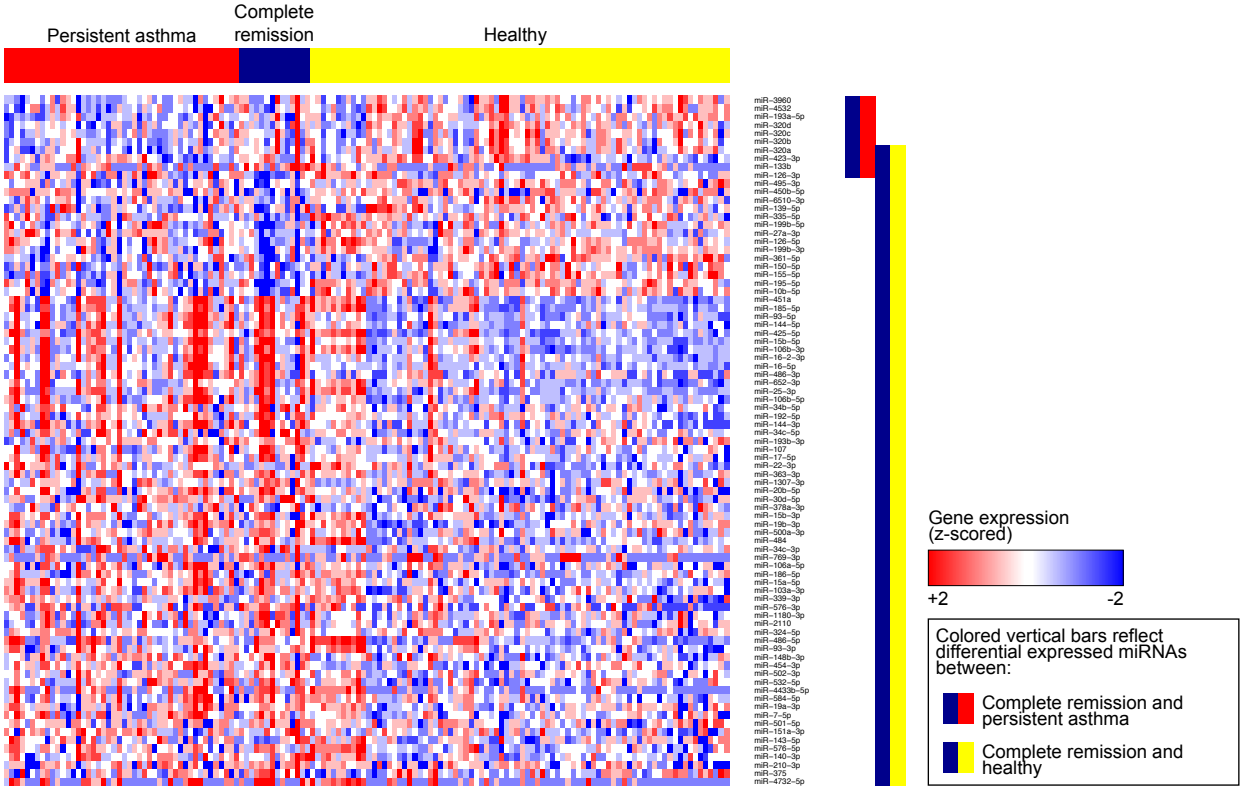

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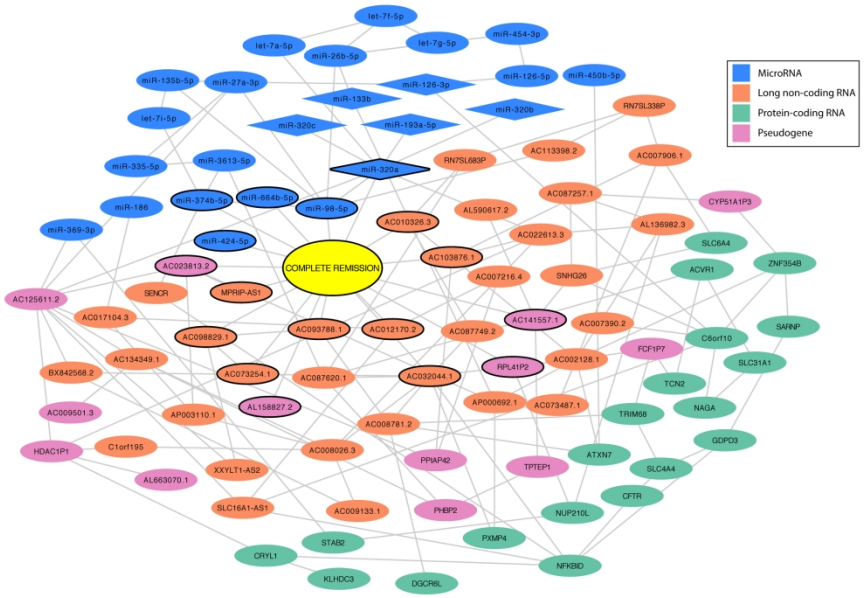

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