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Identification of a plasma miRNA biomarker-signature for allergic asthma: a translational approach

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ABSTRACT

Background

Asthma is a heterogeneous chronic disease with different phenotypes and treatment responses. Thus, there is a high clinical need for molecular disease biomarkers to aid in differentiating these distinct phenotypes. As MicroRNAs (miRNAs), that regulate gene expression at the post-transcriptional level, are altered in experimental and human asthma, circulating miRNAs are attractive candidates for the identification of novel biomarkers.

This study aimed to identify plasmatic miRNA-based biomarkers of asthma, through a translational approach.

Methods

We pre-screened miRNAs in plasma samples from two different murine models of experimental asthma (ovalbumin and house dust mite); miRNAs deregulated in both models were further tested in a human training cohort of 20 asthma patients and 9 healthy controls. Candidate miRNAs were then validated in a second, independent group of 26 asthma patients and 12 healthy controls.

Results

10 miRNA ratios consisting of 13 miRNAs were differentially regulated in both murine models. Measuring these miRNAs in the training cohort identified a biomarker signature consisting of 5 miRNA ratios (7 miRNAs). This signature showed a good sensitivity and specificity in the test cohort with an area under the receiver operating characteristic curve (AUC) of 0.92. Correlation of miRNA ratios with clinical characteristics further revealed associations with FVC % predicted, and oral corticosteroid or antileukotriene use.

Conclusion

Distinct plasma miRNAs are differentially regulated both in murine and human allergic asthma and were associated with clinical characteristics of patients. Thus, we suggest that miRNA levels in plasma might have future potential to subphenotype patients with asthma.

INTRODUCTION

Asthma is characterized by chronic inflammation of the airways associated with airway hyperresponsiveness and airflow obstruction that is mostly reversible. Diagnosis can be challenging, especially at preschool age and in elderly patients. In addition, asthma is now recognized as a heterogeneous disease encompassing several phenotypes, which can be defined in terms of both clinical and molecular characteristics (1). Hence, molecular biomarkers that could differentiate patient subgroups are of high clinical interest for future development of targeted therapies.

MicroRNAs (miRNAs) are evolutionary conserved short (20-22 nts) non-coding RNAs that regulate gene expression by promoting messenger RNA degradation or inhibiting protein translation. They can be found extracellularly in biofluids such as plasma in several forms,

e.g. enclosed in extracellular vesicles or apoptotic bodies, bound to protein-miRNA complexes, or pure due to passive cell lysis (reviewed in (2)). These extracellular miRNAs are attractive candidates for biomarker development as they are highly stable in various body fluids, which are readily accessible and can be easily assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Circulating miRNAs have already been studied as biomarkers in cancer (3), and in complex chronic diseases such as cardiovascular disease and type II diabetes (4). More recently, deregulation of miRNAs in airways has been shown in asthma – in both human (5,6) and murine models (7). However, few studies have investigated circulating miRNAs as biomarkers in asthma (8–11). In contrast to cancer, where tumour-specific miRNAs may be produced and released into body fluids in high levels by proliferating and lysing tumour cells, changes in the miRNA profile in asthma may be more subtle. Therefore, we here aimed in a first step to identify a set of plasma miRNAs that can differentiate patients with allergic asthma from healthy controls, and if so test whether they are related to clinical parameters.

Thus, plasma miRNA profiles from two different murine models for experimental asthma were used as a pre-screening tool to identify candidate miRNAs. These candidate miRNAs were then investigated for their suitability as biomarkers in plasma samples from human asthma patients and healthy controls (termed training cohort) by applying a regularised logistic regression model. The resulting model included several microRNAs was then tested for its ability to predict the correct disease status pilot study (asthma vs control) in a second group of asthma patients and healthy controls (termed test cohort).

MATERIALS AND METHODS

Animal studies

Female 6-8 week old Balb/c mice (Charles River, Sulzfeld, Germany) were housed in individually ventilated cages in a specific pathogen-free facility with a 12 h day-night cycle and provided with standard rodent chow and water *ad libitum*. The study was conducted under German federal guidelines for the use and care of laboratory animals, and was approved by the Government of the District of Upper Bavaria. For a detailed description of the methods and phenotypical characterization of the mouse models please refer to the methods section in this article's online Supplement and (12).

Plasma collection

In mice, blood was collected from the orbital sinus of anaesthetised animals using a micro-haematocrit glass capillary into EDTA-tubes 24 h after the last scheduled aerosol challenge. Plasma was subsequently obtained by serial centrifugation at 1200 x g and 3000 x g for 10 min.

RNA extraction and quality control

Haemolysis is known to alter miRNA profiles significantly (13). Therefore, a quality threshold for haemolysis was established by correlating absorption of plasma at 415 nm (Soret peak of haemoglobin) with the levels of miR-451/16, which are highly expressed in red blood cells. Plasma absorption at 415 nm was measured with a NanoDrop® ND-1000 (NanoDrop Technologies, Erlangen, Germany). Only samples with an absorbance value <2.5 were used for further processing, samples with absorbance value >2.5 were excluded from analysis.

RNA extraction from 50 µl of plasma was performed using miRCURY RNA Isolation Kit (Biofluids, Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. All RNA samples were electrophoresed on a Small RNA chip (2100 Bioanalyser, Agilent Technologies, Santa Clara, USA) to confirm the presence of small RNAs and absence of ribosomal RNA (rRNA) peaks, indicative of cellular contamination, in eluted RNA. Samples were excluded from further analysis if rRNA was present or if qRT-PCR efficiency was low. To control for qRT-PCR inhibitors, we analysed the PCR efficiency of a U6 small RNA spiked-in to all RNA samples and to clean PCR water prior to the RT reaction. Only samples showing a similar amplification efficiency of the U6 spike-in, defined as a difference $<2C_p$ compared to the water control, were considered for further analyses (Figure S1). This threshold for quality control was chosen according to the manufacturer's suggestions (Exiqon QC-PCR Manual, accessible at: <https://www.exiqon.com/ls/Documents/Scientific/QC-PCR-Panel-Manual.pdf>). Please refer to this article's online supplement for a detailed scheme of the miRNA profiling workflow and quality controls (Supplementary Fig. S1).

miRNA profiling and single qRT-PCR assay in mice

Alterations of plasma miRNA expressions were analysed by comparing asthmatic (OVA/OVA) with control mice (PBS/ OVA) and sensitised mice (OVA/ PBS), as well as HDM-treated mice with PBS controls in the HDM model. Six high quality RNA samples per group were screened using Serum/Plasma Focus microRNA PCR Panels (Exiqon, Vedbaek, Denmark) which allow quantification of 179 distinct miRNAs. A total of 4 µl of eluted mouse RNA and 2 µl of U6 spike-in were used as a template in 20 µl Universal-RT product (Universal cDNA-synthesis kit, Exiqon, Vedbaek, Denmark). PCR panels were prepared and run according to

the manufacturer's instructions on a LightCycler 480 II (Roche, Germany). The programme entailed: 1) denaturation: 10 min at 95°C; 2) amplification: 45 cycles at 95°C for 10 sec and 60°C for 1 min, ramp-rate 1.6°C/s, optical read; 3) melting curve analysis.

For single miRNA expression analysis, cDNA synthesis was transcribed as above. cDNA was diluted 1:40 in low-binding tubes (DNA LoBind Tube, Eppendorf, Hamburg, Germany), and 4µl of diluted cDNA were added to the PCR Mix containing 5µl of 2x SybrGreenMaster (Exiqon, Vedbaek, Denmark) and 1µl of microRNA-LNA-Primer-Set (Exiqon).

Analysis of plasma panels

The raw cycle threshold (Ct) values and melting temperatures (T_m) were calculated using LightCycler96 software version 1.0 with automatic settings for baseline. The Ct threshold was set to Ct>35. Melting curve analysis was performed by visual inspection to exclude samples with multiple peaks, followed by calculation of T_m ranges of each assay excluding assays with ranges >0.8. Only miRNA assays with valid values in at least 3 samples per experimental group were included in the further analyses (Fig. S1).

Human study

Between June 2014 and April 2015 consecutive patients aged > 18 years with a diagnosis of allergic asthma who, as part of their regular follow-up, were visiting the respiratory medicine practice at Munich-Pasing (used as the "training cohort" for biomarker signature development) or our outpatient asthma clinic at the University Hospital Munich (used as the "test cohort" for biomarkers developed in the training cohort) were asked to participate in

the study. Asthma patients were included in the study when they had a history of typical symptoms of asthma such as wheeze, shortness of breath, chest tightness and cough with variation over time and in intensity and variable expiratory airflow limitation documented by an abnormal spirometric test (either positive bronchodilator reversibility or positive metacholine challenge test) plus positive skin prick testing. Subjects without a current or previous asthma diagnosis, without any symptoms or indications of any current disease, and who were not taking any current medication were recruited as controls. We also excluded anyone with clinical or laboratory signs of acute infection. Age, sex and smoking status was recorded from all participants. In asthma patients, data from lung function measurements, FeNO, blood eosinophils and Asthma Control Test (ACT score) were obtained as part of the clinical routine. Asthma severity was assessed retrospectively by required controller medication, as defined in the GINA guidelines that was current at the time of study (14). Included patients and control subjects gave written informed consent. The study was approved by the local Institutional Review Board (No. 315-14). Plasma was obtained from 5 ml peripheral blood (EDTA) by serial centrifugation at 1200 x g for 10min, then 3000 x g for 10 min and stored at -80°C until further processing for measurement of miRNA and total IgE. RNA extraction from 200 µl of plasma was performed using miRCURY RNA Isolation Kit – Biofluids (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. Quality controls and qRT-PCR were performed using the same methodology as with the murine samples.

Statistical Analysis

For all tests statistical significance was considered to be $p < 0.05$. Please refer to this article's online supplement for a detailed description of the statistical analyses. Briefly, analyses of murine phenotypic data were performed using Prism (GraphPad Software, La Jolla, USA)

with two-way ANOVA and post-hoc Bonferroni-tests or t-tests as appropriate. The cluster analysis of murine miRNA profiles were generated with Genex 5 (MultiD Analyses), average linkage and Euclidean distances were used. R (Version 3.0.1) was used for all other analyses.

RESULTS

Plasma miRNA profiling in an OVA model of allergic asthma.

Out of 179 distinct miRNAs analysed on the panels, a total of 108 miRNAs were detectable in murine plasma and passed quality control in more than 4 samples per group which ensured eligibility for statistical testing. Hierarchical cluster analysis was performed on the 18 data sets using Ct' values of all 108 miRNAs for which expression was observed, showing that the expression of these miRNAs was linked to the experimental groups (Fig. 1A). Next, we analysed miRNA expression using Global Mean Normalisation (GMN); 32 miRNAs were significantly altered in OVA/ OVA mice compared to PBS/ OVA controls (Fig. 1B). Of these, three miRNAs (miR-103, miR-24 and miR-342-3p) were also significantly different between OVA/ OVA and OVA/ PBS mice.

Plasma miRNA profiling in a HDM model of allergic asthma.

In the HDM model, a total of 109 miRNAs were detectable and passed quality control in more than 4 samples per group. Hierarchical cluster analysis on the 12 data sets using Ct' values of all expressed miRNAs demonstrated differential expression of these miRNAs between the experimental groups (Fig. 1C), with 10 miRNA being significantly regulated between HDM and PBS treated mice (Fig. 1D).

Candidate miRNA-ratio selection and validation in murine models

The relative quantities (RQ) of the miRNAs showed a high variability between different animals (Supplemental Fig. S2, upper panel). To obtain normal distribution and increase screening sensitivity, we calculated miRNA ratios by dividing each RQ one-by-one by all the other RQs. The overall distribution of thus obtained miRNA ratios was comparable in all animals (Supplemental Fig. S2, lower panel). Inferential statistics were then performed on these miRNA ratios.

15 miRNA ratios that were significantly regulated in the same direction in “asthmatic” mice in both models (HDM and OVA/ OVA), but not in ‘atopic’, sensitised only, animals (OVA/PBS) compared to the respective healthy controls (PBS and PBS/ OVA) were chosen as initial candidates (Fig. 2A). Two further miRNA ratios were significantly regulated between “asthmatic” (OVA/ OVA) and sensitised mice (OVA/ PBS) in the OVA model as well as between “asthmatic” and control mice in both models were also included. MiRNA ratios differentiating sensitized animals (OVA/ PBS) from healthy controls (PBS/ OVA) were excluded (Fig. 2A). The remaining 18 miRNA ratios were then validated by qRT-PCR in an independent set of samples from both murine models, which confirmed the regulation of 10 miRNA ratios consisting of 13 miRNAs in both models (Fig. 2B, Fig. S3). Therefore, these 13 miRNAs were chosen for further validation in the human samples.

Validation in adults with allergic asthma

To assess if the 13 miRNA identified in our murine models also differentiate human asthma patients from healthy non-asthmatic subjects, we first checked their expression levels (see Supplemental Fig. S4A for heatmaps) in a training cohort that consisted of 20 patients with

asthma, together with 9 control subjects (Table 1). Subsequent hierarchical clustering analysis indicated a close linkage of miRNA ratios to the same diagnostic group (asthma or control) (Fig. 3A). Next, training a regularised logistic regression model on data from the training cohort resulted in a model with five miRNA ratios (miR-21-5p/miR-15a-5p, miR-27a-3p/miR-15a-5p, miR-29c-3p/miR-15a-5p, miR-223-3p/miR-425-5p, miR-15a-5p/miR-342-3p) as shown in Table 2.

This model was then applied to the so-called test cohort - a second and independent human cohort of 26 patients with asthma and 12 controls (Table 1). Similar to the training cohort, hierarchical clustering of the miRNA expression profiles (see heatmap in Supplemental Fig. S4B) differentiated asthma patients from controls (Fig. 3B), and the resulting ROC curve had an AUC of 0.9167 demonstrating a good sensitivity and specificity of the model to indicate the presence of asthma (Fig. 3C).

To investigate if the five miRNA ratios would potentially be able to stratify distinct patient subphenotypes, we next tested in the combined training and the test cohort whether they showed differences according to clinical patient characteristics. Here we found that miR-15a-5p/miR-342-3p was inversely correlated to FVC % predicted (Fig. 4A, Supplementary Table S2). Furthermore, patients taking oral steroids had significantly lower values for miR-15a-5p/miR-342-3p, and significantly higher values for miR-21-5p/miR-15a-5p, miR-27a-3p/miR-15a-5p and miR-29c-3p/miR-15a-5p compared to those not on oral steroids (Fig. 4B). Patients receiving antileukotriene therapy showed decreased values for miR-223-3p/miR-425-5p (Fig. 4C). For the remaining categorical and numerical variables no significant

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differences or correlations were observed for any of the five ratios (Supplementary tables S2 and S3). Of note, no significant alterations were found in sensitised controls that had a history of allergic rhinitis compared to those that were not (Fig. 4D), demonstrating the specificity of the miRNA ratios for asthma.

DISCUSSION

In this study, we have successfully identified miRNAs in plasma that are deregulated in two models of murine experimental asthma. In a translational approach, we could further show that these plasmatic miRNAs are differentially expressed in patients with allergic asthma compared to healthy individuals, both with and without sensitization to aeroallergens, and correlate with clinical characteristics. Thus, we speculate that miRNAs in plasma could potentially be used as a diagnostic biomarker.

As asthma is a very heterogeneous disease, and subphenotypes could react different to therapy, there is a high clinical need for a novel tool to differentiate patients into different subgroups. However, screening for a clinically relevant biomarker in patients with asthma usually requires a large and well-characterised human study sample as confounders such as comorbidities, medications (15,16) or physical activity (17) can influence circulating miRNA profiles. To avoid this both time and cost consuming approach, we aimed to pre-identify a miRNA-based biomarker for allergic asthma in two different murine models of experimental asthma (HDM and OVA). In this regard, murine models are relevant tools, as miRNAs and their targets are highly conserved between human and mice (18), while mouse models offer the possibility of high standardisation. By pre-screening for deregulated miRNAs in mouse models of allergic asthma we might have overlooked candidates that are regulated in

human allergic asthma only, and not in the mouse models. However, our intention in this study was not to provide completeness, but to test if our approach yields candidate biomarkers that are clearly linked to allergic airway disease. The identified miRNAs were then successfully confirmed in a pilot approach by using a training and a test cohort of patients with asthma and healthy controls. As we have obtained promising results in this pilot study with rather low patient numbers we suggest that the identified five miRNA ratios should be tested in larger, and more heterogeneous patient cohorts in the future, especially with regards to correlations with clinical markers and sub-phenotyping. Yet, it should be noted that the observed differences in individual miR-ratios were rather small and therefore a combination of five miR-ratios was necessary to obtain a reliable biomarker with good sensitivity and specificity.

The OVA model used in this study consists of two separate phases, a systemic sensitisation followed by an aerosol challenge which allows controlling for allergic sensitisation without asthma symptoms. In contrast, the HDM model has the advantage of employing a relevant aeroallergen, and exclusively uses the natural nasal route for allergen exposure. Hence, the HDM model is considered more realistic for modelling of the human disease (19). However, it does not allow to control for sensitisation only, as sensitisation and challenge occur simultaneously via inhalation. Therefore, we chose to use both models to take advantage of both characteristics. Additionally, we expected that using overlapping miRNAs from both models would increase reliability and generalisability of our results.

We have shown that the calculation of miRNA ratios is a suitable tool for internal normalisation of samples while increasing sensitivity of screening. Numerous different approaches have been employed for normalisation in miRNA research. When studying

cellular miRNAs, small nuclear or ribosomal RNAs may be used as reference (20). However, in extracellular fluid, no such generally applicable reference small RNAs are present and alternative approaches are necessary. When measuring a large number of miRNAs, as in profiling of samples with PCR panels or miRNA arrays, global mean normalisation can be used. However, this is not possible when measuring only selected miRNAs. Inserting artificial “spike-in” miRNAs is often used to overcome this problem, but this does not take into account deviations in sampling and quality of samples (20). This may lead to a substantial bias, especially when quality controls for cell lysis and cellular contents are not performed as different amounts of total, intact miRNA may be present in the same sample volume. We therefore used calculation of miRNA-ratios as a tool for normalization and conducted two quality control steps to exclude cellular contamination and haemolysis. Another advantage of this approach is the increase of screening sensitivity by combining regulations in numerator and denominator of the ratio.

To translate our findings to human asthma, we first used a training cohort which included mostly young or middle-aged adults with mild-to-moderate asthma. The test cohort was, on average, older and included patients with severe disease. Nevertheless, the classification model derived from the training cohort had a good sensitivity and specificity for indicating the presence of asthma in the test cohort. Thus, we estimate that our biomarkers are generally indicative for allergic asthma.

We found associations of miRNA ratios included in the classification model with the clinical parameters FVC % predicted, oral corticosteroid use and antileukotriene treatment. Recently, Kho et al. found an association of several circulating miRNAs with lung function

measures suggesting that they could be markers of disease severity (11). Additionally, oral corticosteroid treatment has been shown to modulate circulating miRNAs in healthy individuals (16), with miR-27a being induced by steroid treatment, consistent with our finding. These associations suggest that various factors may influence miRNA regulation, and thus a very thorough characterisation of subjects and conditions is necessary to avoid confounding results. However, these findings also point to the possible utility of using miRNA-based markers for disease sub-phenotyping, if sufficiently large and well-characterised cohorts are used.

Some of the miRNAs identified here, such as miR-21 and miR-223, have already been implicated in the pathogenesis of asthma (7,21–23) or even as biomarker of the disease (miR-21) (8,10,24), while others such as miR-27a emerged as new candidates. A few studies have recently screened circulating miRNAs as biomarkers for asthma (8,10), with partially overlapping but also varying results compared to our study. These variations may be due to differences in screening strategies and clinical characteristics of the patient cohorts, but also less stringent strategies for normalisation and intensive sample quality controls as applied in this study.

In summary, by applying a translational approach we identified a signature that was able to distinguish human asthmatics from non-asthmatic subjects with good sensitivity and specificity and was further related to clinical characteristics of asthma. These results create the prerequisites for the development of miRNAs biomarker signatures for deeper sub-phenotyping of asthmatic patients in larger studies which could facilitate precision medicine approaches in the future.

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AUTHOR CONTRIBUTIONS

S.K-E. conceived the manuscript, designed and supervised the study and had the primary responsibility for writing; K.M. and J.G. performed and analyzed the experiments and wrote the manuscript; F.A., P.N., and S.B., designed and conducted the animal studies; AT, RF, JB, NK recruited and treated the patients and critically revised the manuscript. LK, NM, FT performed the biostatistical analyses. All authors contributed to writing of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing conflicts of interest in relation to this study.

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- Accepted Article
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- Accepted Article
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TABLES

Table 1: Patient Characteristics of the training and test cohort

	TRAINING COHORT		TEST COHORT	
	Asthmatic	Healthy Control	Asthmatic	Healthy Control
Total Number –n	20	9	26	12
Females -n (%)	8 (40)	5 (65)	17 (65)	11 (92)
Age - yr (mean±SD)	39.6 ± 19.3	36.0±11.9	50.5±18.3	32.6 ± 6.2
Smoker -n (%)				
Current	2 (10)	2 (22)	2 (8)	1 (8)
Former	4 (20)	3 (33)	12 (46)	1 (8)
Never	14 (70)	4 (44)	12 (46)	10 (83)
Sensitiation –n (%) *	20 (100)	1 (11)	26 (100)	4 (33)
Polyvalent	9 (45)	1 (11)	17 (65)	1 (8)
Pollen	15 (75)	1 (11)	19 (73)	1 (8)
HDM	8 (40)	1 (11)	11 (42)	3 (25)
Animal dander	8 (40)	0	14 (53)	0
Moulds	1 (5)	0	4 (15)	0
Allergic rhinitis	8 (40)	1 (11)	17 (65)	4 (33) **
Asthma Severity -n (%) #				
Mild	13 (65)		9 (35)	
Moderate	6 (30)		6 (23)	
Severe	1 (5)		11 (42)	
Current asthma treatment – n (%)				
None	3 (15)		2 (8)	
Inhaled corticosteroids	13 (65)		24 (92)	
Dosage: low/ medium/ high	6/ 6/1		9/8/7	
Oral corticosteroids	0		8 (31)	
Inhaled β2-agonist +	17 (85)		24 (92)	
Inhaled anticholinergic	2 (10)		7 (27)	
antileukotrien (Montelukast)	0		5 (19)	
Anti-IgE (Omalizumab)	0		2 (8)	
Degree of Control -n (%) ++				
Controlled	13 (65)		10 (38)	
Partially Controlled	2 (10)		6 (23)	
Not controlled	5 (25)		10 (38)	
Spirometry - mean±SD +++				
FEV1 -% predicted	88.0±17.5		80.6±25.6	
FEV1/ FVC	0.78±0.12		0.69±0.14	
Biomarkers – median (min; max)				
FeNO –ppb	25 (5; 152)		42 (6; 21) ***	
Total IgE - kU/μl	191.5 (27.2;		310.5 (12.8;	

1253)	2480) ***
	329 (49; 897)
	##

* Assessed by Prick-Testing at the time point of first presentation to our facility.

** Control subjects reported a history of AR, but no current symptoms or medications.

Assessed retrospectively by necessary medication as defined by GINA guidelines (25).

+ + Either regular use of short acting β 2-agonist > 2x/week, or use of long acting β 2-agonist

++ As defined by GINA guidelines (14)(25)(29)(28)(28)(28)(29).

+++ pre-bronchodilator

*** Data available from 15 patients.

Data available from 24 patients.

Table 2

miRNA-ratio	effect coefficient (exp(beta_i))
miR-21-5p/ miR-15a-5p	0.1350375
miR-27a-3p/ miR-15a-5p	0.7792143
miR-29c-3p/ miR-15a-5p	0.2216006
miR-223-3p/miR-425-5p	1.2330062
miR-15a/ miR-342-3p	2.4039645

Table 2 Result of the regularized logistic regression: effect coefficients

FIGURES & LEGENDS

Figure 1 Profiling of circulating microRNAs in an ovalbumin model and HDM model.

A. Cluster analysis of miRNA profiles obtained from plasma of ovalbumin mouse model.

Clustering generated with Genex 5 (MultiD Analyses AB, settings: Average linkage, Euclidean

distance). **B** Table of most significantly regulated miRNAs in the ovalbumin mouse model

($p < 0.05$ for OVA/ OVA vs PBS/ OVA); bold: significantly regulated in both, Asthma vs Control

and Asthma vs Atopy. **C.** Cluster analysis of miRNA profiles obtained from plasma of HDM

mouse model. Clustering generated with Genex 5 (MultiD Analyses AB, settings: Average

linkage, Euclidean distance). **D** Table of most significantly regulated miRNAs in the HDM

mouse model ($p < 0.1$ for HDM vs PBS).

Figure 2 Candidate miRNA-ratio selection and validation in murine models.

A Venn-diagram (left) and table (right) illustrating the selection of candidate miRNA-ratios

by statistical analysis using data from both animal models. **B** miRNA-ratios that were

validated using samples from independent experiments of the same animal models.

Figure 3 Validation of miRNA ratios in human cohorts and biomarker performance.

A + B cluster dendrograms based on the miRNA ratios of individual samples, (**A**) for the

training cohort and (**B**) for the test cohort. (Clustering generated in R, ward.D2, Euclidean

distance). **C** Receiver operating characteristics (ROC) curve for the test cohort depicting the

values for the false positive rate versus the true positive rate as a measure for the accuracy

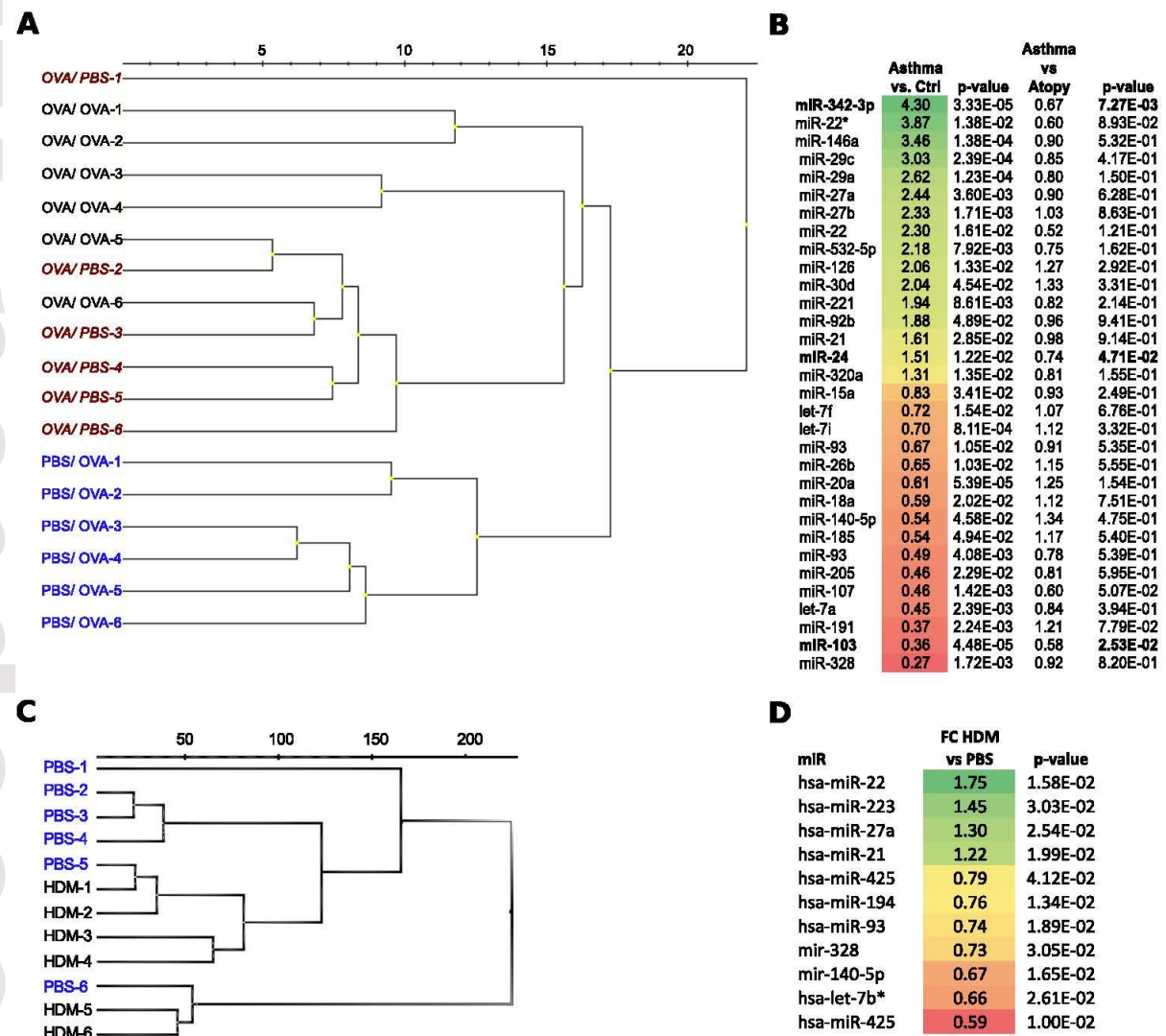
of the model shown in Table 2. AUC = Area under the ROC curve. **D** candidate miRNA-ratios

evaluated in human cohorts (left) and adjusted p-values comparing asthma patients and

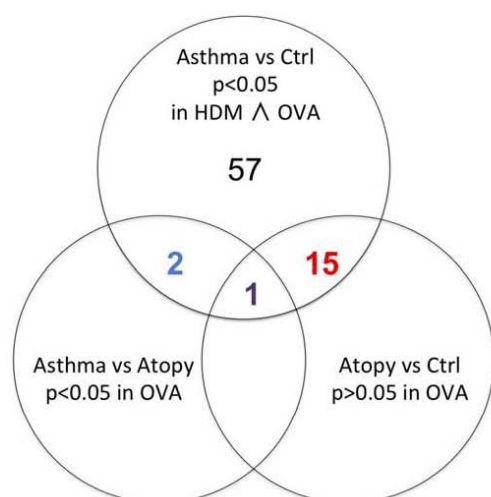
controls (right)

Figure 4: Correlation of biomarker miRNA ratios with phenotypical data.

A Correlation of miR-15a/ mir342-3p with forced vital capacity (FVC) % predicted in patients with asthma ($r = -0.402$; $p = 0.034$). **B** Association of miRNA ratios and current oral corticosteroid therapy **C** Association of miRNA ratios and current antileukotriene therapy **D** miRNA ratios in patients with asthma and control subjects depending on history of allergic rhinitis. * = $p < 0.05$; ** = $p < 0.01$.



A

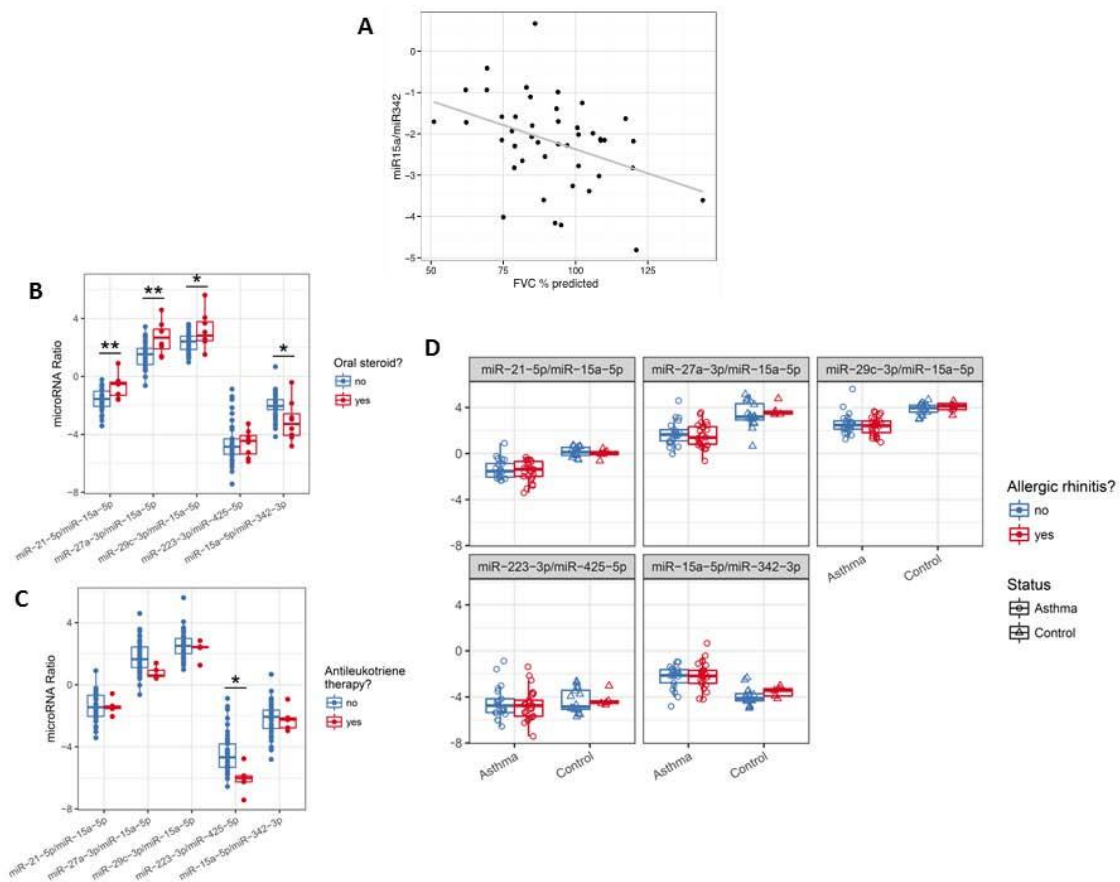
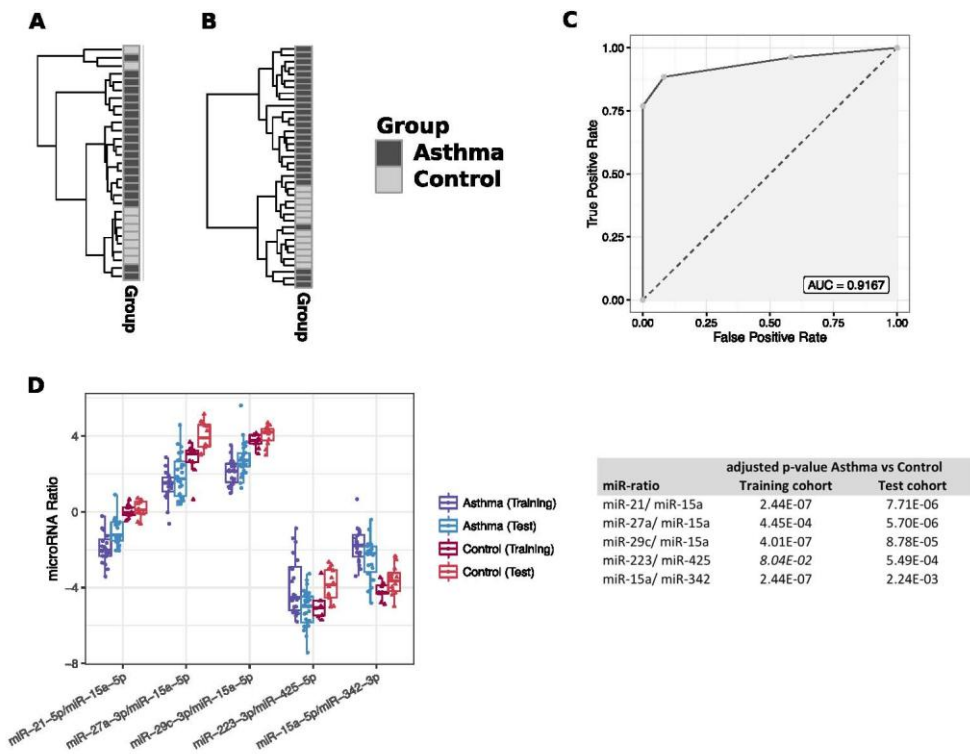


B

miR Ratios validated ($p < 0.05$)

miR-425-5p / miR-223-3p
miR-223-3p / miR-93-5p
miR-223-3p / miR-15a-5p
miR-130a-3p / miR-29c-3p
miR-342-3p / miR-320a
miR-320a / miR-27a-3p
miR-320a / miR-29a-3p
miR-342-3p / miR-130a-3p
miR-425-5p / miR-21-5p
miR-342-3p / miR-140-3p

miR Ratios	P_{HDM}	P_{OVA}
$P_{HDM} < 0.05 \wedge P_{OVA} < 0.05 \wedge P_{Atopy\ vs.\ Ctrl} > 0.05$		
miR-130a-3p / miR-27a-3p	0.045	0.014
miR-18a-5p / miR-27a-3p	0.004	0.023
miR-425-5p / miR-27b-3p	0.008	0.030
miR-18a-5p / miR-27b-3p	0.006	0.023
miR-18a-5p / miR-22-3p	0.021	0.009
miR-27b-3p / miR-320a	0.044	0.025
miR-25-3p / miR-27b-3p	0.036	0.048
miR-425-5p / miR-27a-3p	0.002	0.038
miR-18a-5p / miR-29c-3p	0.027	0.004
miR-27a-3p / miR-23a-3p	0.023	0.033
miR-27a-3p / miR-320a	0.033	0.035
miR-25-3p / miR-27a-3p	0.020	0.045
miR-23a-3p / miR-21-5p	0.024	0.039
miR-26b-5p / miR-30c-5p	0.011	0.024
miR-21-5p / miR-15a-5p	0.018	0.009
$P_{HDM} < 0.05 \wedge P_{OVA} < 0.05 \wedge P_{OVA\ vs.\ Atopy} < 0.05$		
miR-148b-3p / miR-29a-3p	0.006	0.0002
miR-342-3p / miR-223-3p	0.015	0.001
$P_{HDM} < 0.05 \wedge P_{OVA} < 0.05 \wedge P_{Atopy\ vs.\ Ctrl} > 0.05 \wedge P_{OVA\ vs.\ Atopy} < 0.05$		
miR-140-3p / miR-27a-3p	0.047	0.039



Identification of a plasma miRNA-signature as biomarker for allergic asthma: a translational approach

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Short title: Allergic asthma miRNA -signature in plasma

Key words: animal models, asthma, pneumology

METHODS

Induction of allergic airway inflammation

Female 6 to 8 weeks old Balb/c mice were sensitised and challenged with ovalbumin (OVA) as previously described (1). In short, mice were sensitised with five intraperitoneal (i.p.) injections of 1 µg OVA (grade VI; Sigma Aldrich Chemie)/Alum (2.5 mg; Pierce Chemical Co, Rockford, IL, USA) in phosphate buffered saline (PBS), or PBS alone for controls, on Days 0, 7, 14, 28, 42 and 56. Sensitisation to OVA was verified by high levels of OVA-specific plasma IgE and IgG1. On days 70 and 71 mice were challenged for 20 min with 1%-LPS-depleted OVA in PBS or with PBS alone delivered by Pari-Boy nebulizer (Pari GmbH, Starnberg, Germany). Moderate allergic airway inflammation was confirmed by goblet cell metaplasia in the lungs, elevated numbers of inflammatory cells and increased CCL17 titres in BALF (2).

For house dust mite (HDM) induced asthma, mice were challenged intranasally (i.n.), 3 times per week over 5 weeks with 20 µg whole body extract of *Dermatophagoides pteronyssinus* (Nr. 218234, GREER®, Lenoir, USA) in 30 µl sterile PBS, or PBS alone for controls as previously described (2). HDM-induced airway inflammation was confirmed by lung inflammatory cell infiltration, BAL cell counts, differential cell counts and airway hyperreactivity in response to metacholine.

Statistical analyses of murine experiments

For the miRNA plasma panels, expression levels and fold changes were determined by global mean normalization (GMN) as described by D'haene *et al.* (3) and Hellemans *et al.* (4). The arithmetic mean of replicate raw Ct values (mean Ct) was converted to relative quantities (RQ) using the formula $RQ = 2^{-(\text{mean Ct})}$. Normalised relative quantities (NRQs) were then calculated by the quotient of RQ to the geometric mean of all valid RQ values from a given sample. The Kolmogorov-Smirnov test was used to determine whether data were normally distributed. Student's t test was applied for Ct' values, which were calculated by the formula $Ct' = \log_2(NRQ)$ (5). To account for multiple testing, the Benjamini-Hochberg critical value was calculated for each sample and compared to the p-value obtained by t-test.

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As GMN is not possible when looking at fewer miRs, normalization was achieved by calculating miR-ratios all analyses presented from Figure 2 onwards. miRNA ratios were calculated as pairwise differences between Ct values ($\Delta Ct = Ct_1 - Ct_2$). Student's t-test was used to identify miRNA ratios that were significantly regulated in both the OVA and the HDM model.

Statistical analysis of the human studies

For normalisation, pairwise differences between Ct values were calculated to obtain miRNA ratios, using the same methodology as in the mice studies. In the training cohort the disease status (asthma vs control) was modelled with a regularised logistic regression using all miRNA ratios as covariates. Model selection was performed with a generalised linear model via penalised maximum likelihood with lasso regularisation (6). The optimal lambda value was calculated using leave-one-out cross validation. The model accuracy was tested on the test cohort and assessed via the area under the receiver operator characteristics curve (ROC AUC).

For the five ratios that were selected in the final model, correlation with clinical characteristics was analysed using the data from both cohorts combined (training + test cohort) to overcome limitations in numbers of patients per subgroup. Analyses of variables available in patients with asthma and controls, i.e. age, sex, smoking status, sensitisation and allergic rhinitis were performed using data from both groups; the other variables were tested among patients with asthma only. A complete list of categorical and interval scale variables tested is displayed in supplemental table S1.

Categorical variables were tested for difference in the ratios using a one-way ANOVA. For interval scale variables, significance was determined using Pearson's product moment

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correlation coefficient. Only associations with a controlled false discovery rate of less than 10% are shown. All p-values were adjusted using the Benjamini Hochberg procedure.

SUPPLEMENTARY FIGURES & LEGENDS

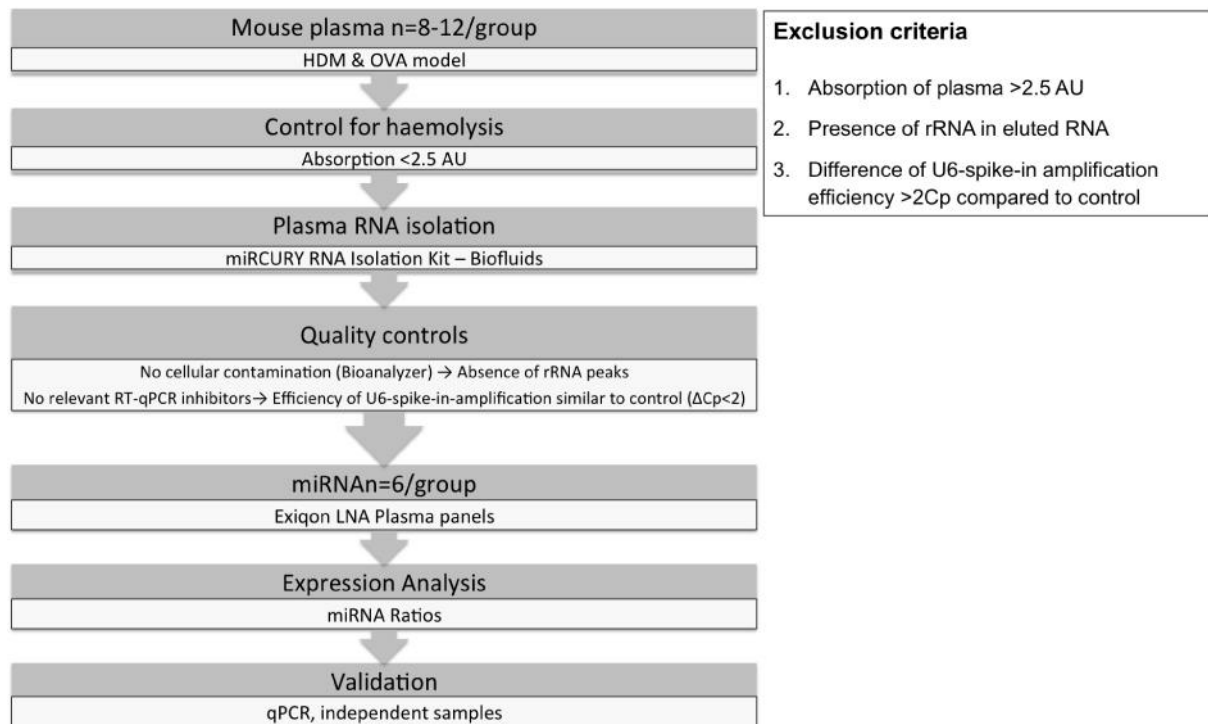


Figure S1 Overview of workflow for miRNA profiling from plasma. Abbreviations: HDM = house dust mite, OVA = ovalbumin.

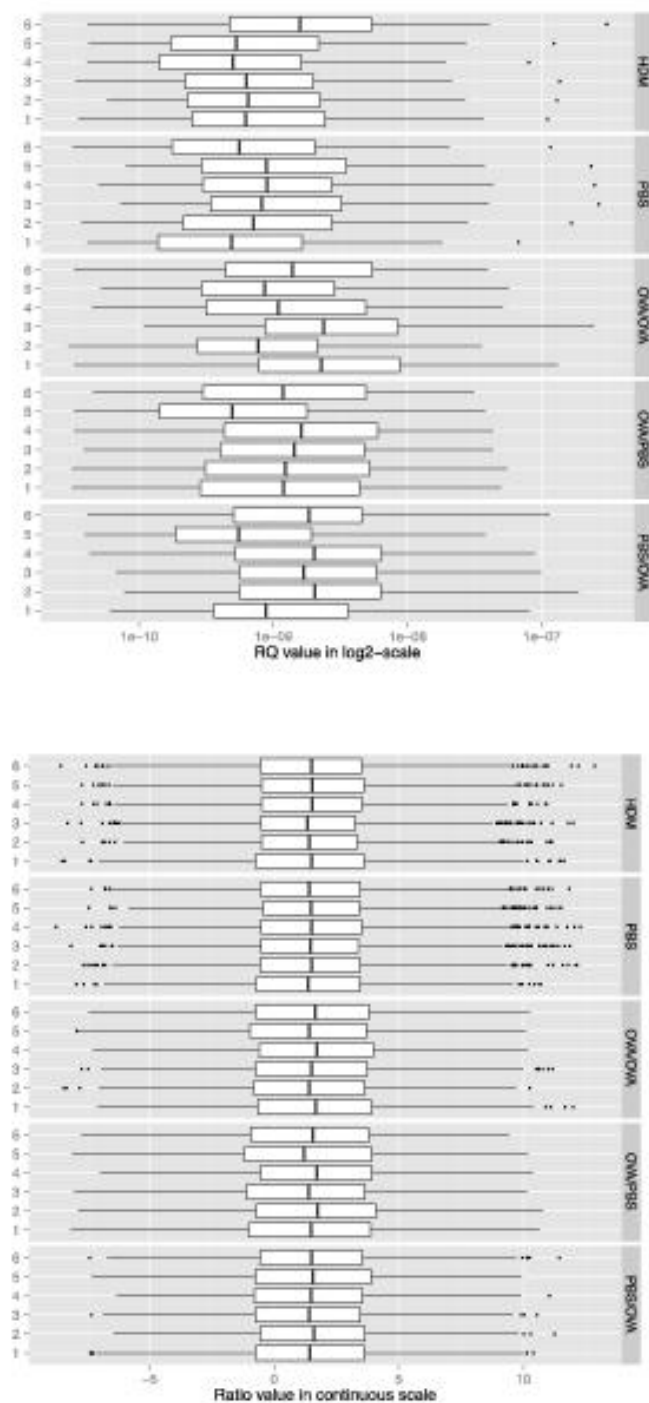
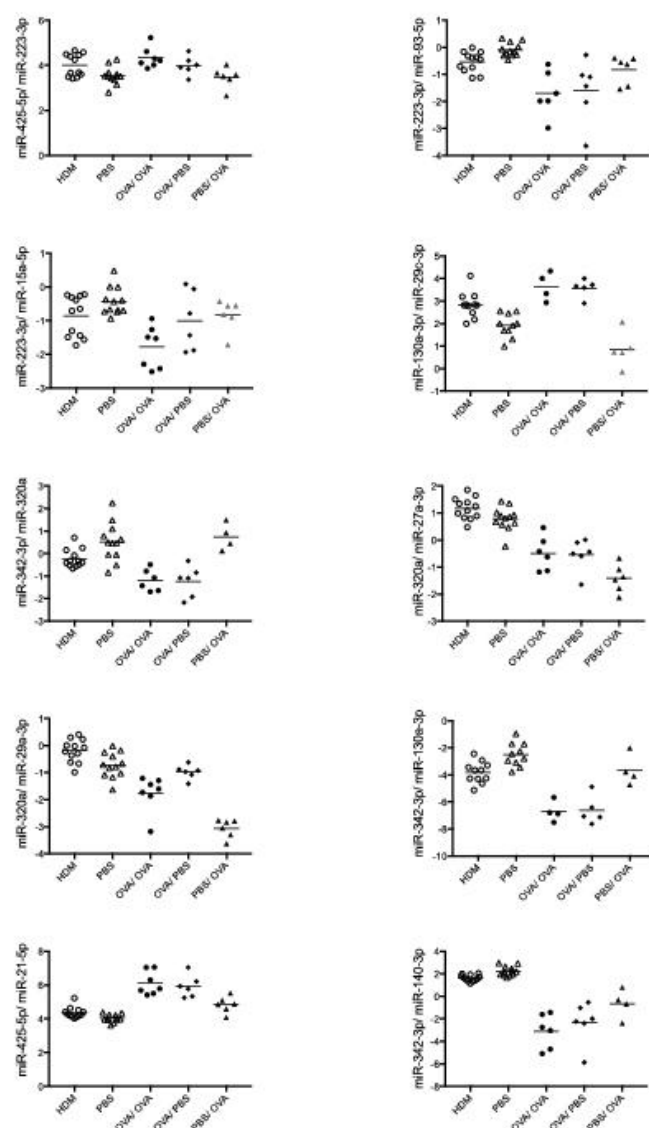


Figure S2 Selection of a normalization strategy for miRNA analysis

The relative quantities (RQ) of all miRNAs are shown in the upper panel, the miRNA ratios in the lower panel. Each boxplot represents all miRNA expression data from one animal.

Figure S3

**B**

miR Ratios validation	p_{HDM}	p_{OVA}
miR-425-5p / miR-223-3p	0.0290	0.0032
miR-223-3p / miR-93-5p	0.0355	0.0026
miR-223-3p / miR-15a-5p	0.0478	0.0107
miR-130a-3p / miR-29c-3p	0.0019	<0.0001
miR-342-3p / miR-320a	0.0157	<0.0001
miR-320a / miR-27a-3p	0.0324	0.0049
miR-320a / miR-29a-3p	0.0130	<0.0001
miR-342-3p / miR-130a-3p	0.0050	<0.0001
miR-425-5p / miR-21-5p	0.0082	<0.0001
miR-342-3p / miR-140-3p	0.0003	0.0154

Figure S3: Validated miR-ratios from independent samples of animal models (data underlying Table 2B). Ratios were significantly regulated ($p < 0.05$ by t-test) between asthmatic and control in both models (HDM vs control and OVA/ OVA vs PBS/ OVA), p-values are depicted in B. Each dot represents the ratio of one animal. Assayed sample numbers were $n(HDM)=12$, $n(PBS)=12$, $n(OVA/ OVA)=7$, $n(OVA/PBS)=6$, $n(PBS/ OVA)=6$ for all miR-ratios. Please note that the number of dots is lower for certain ratios as some miRs were expressed below the detection limit ($C_p > 35$).

SUPPLEMENTARY TABLES

Supplementary Table S1 Variables used for correlations with clinical data

Categorical variables	Interval scale variables
Sex	age
Smoking status (current/ former / never)	blood leukocyte count
Sensitisation (polyvalent, pollen, HDM, animal, moulds)	blood eosinophil count
asthma control (controlled/ partially controlled/ uncontrolled)	FeNO (ppm)
asthma severity (mild/ moderate/ severe)	asthma control score
current asthma medications (inhaled corticosteroids (ICS)	forced expiratory volume in 1 second (FEV ₁)
ICS dosage (low/ medium/ high)	% predicted FEV ₁
inhaled β_2 -agonist	forced vital capacity (FVC)
inhaled anticholinergic	
oral corticosteroid	
antileukotriene	
anti-IgE	
allergic rhinitis	

Supplementary Table S2 Correlation of miRNA ratios in plasma with categorical clinical data.

Clinical data		miRNA ratios in plasma (adjusted p-values)				
		miR-21-5p/ miR-15a-5p	miR-27a-3p/ miR-15a-5p	miR-29c-3p/ miR-15a-5p	miR-223-3p/ miR-425-5p	miR-15a/ miR-342-3p
Sex		0.23	0.55	0.23	0.05	0.23
Smoking status		0.20	0.48	0.61	0.61	0.67
Sensitisation	Pollen	0.40	0.40	0.44	0.18	0.44
	HDM	0.35	0.35	0.35	0.81	0.50
	Animal dander	0.39	0.39	0.39	0.35	0.39
	Moulds	0.93	0.93	0.93	0.93	0.93
	Polyvalent	0.52	0.52	0.36	0.14	0.39
Asthma control status		0.25	0.25	0.25	0.25	0.25
Asthma severity		0.13	0.10	0.10	0.58	0.20
Current medication	ICS	0.93	0.93	0.39	0.93	0.72
	ICS dose	0.76	0.88	0.76	0.76	0.76
	Inhaled beta2-agonist	0.90	0.90	0.90	0.90	0.90
	Inhaled anticholinergic	0.24	0.38	0.13	0.99	0.33
	Oral steroids	0.01	0.01	0.01	1.00	0.01
	Omalizumab	0.96	0.83	0.96	0.62	0.96
	Antileukotriene	0.97	0.10	0.91	0.05	0.97
Allergic rhinitis		0.59	0.59	0.59	0.59	0.59

ICS inhaled corticosteroid.

Supplementary Table S3 Correlation of miRNA ratios in plasma with numerical clinical data.

Clinical data		miRNA ratios in plasma (adjusted p-values)				
		miR-21-5p/ miR-15a- 5p	miR-27a- 3p/ miR- 15a-5p	miR-29c- 3p/ miR- 15a-5p	miR-223- 3p/ miR- 425-5p	miR-15a/ miR-342-3p
Age		0.83	0.59	0.59	0.83	0.83
Asthma Control Test		0.55	0.55	0.55	0.55	0.55
FeNO		0.48	0.94	0.94	0.94	0.94
	IgE	0.27	0.50	0.27	0.50	0.27
	Total Leukocytes	0.79	0.79	0.79	0.79	0.79
Laboratory Parameters	Eosinophils (%)	0.35	0.35	0.58	0.58	0.58
	Total Eosinophils	0.53	0.53	0.64	0.53	0.53
Lung Function	FVC	0.71	0.78	0.71	0.71	0.78
	FVC % pred	0.27	0.27	0.27	0.42	0.03
	FEV ₁	0.95	0.95	0.95	0.95	0.95
	FEV ₁ % pred	0.93	0.43	0.76	0.76	0.16
	FEV ₁ /FVC	0.55	0.87	0.87	0.87	0.87

FeNO Fractional exhaled nitric oxide; FVC Forced vital capacity; FEV₁ Forced expiratory volume in 1 second.

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