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Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients

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Abstract

Background—Asthma is characterized by increased airway narrowing in response to nonspecific stimuli. The disorder is influenced by both environmental and genetic factors. Exosomes are nanosized vesicles of endosomal origin released from inflammatory and epithelial cells that have been implicated in asthma. In this study we characterized the microRNA (miRNA) content of exosomes in healthy control subjects and patients with mild intermittent asthma both at unprovoked baseline and in response to environmental challenge.

Objective—To investigate alterations in bronchoalveolar lavage fluid (BALF) exosomal miRNA profiles due to asthma, and following subway air exposure.

Methods—Exosomes were isolated from BALF from healthy control subjects (n = 10) and patients with mild intermittent asthma (n = 10) after subway and control exposures. Exosomal RNA was analyzed by using microarrays containing probes for 894 human miRNAs, and selected findings were validated with quantitative RT-PCR. Results were analyzed by using multivariate modeling.

Results—The presence of miRNAs was confirmed in exosomes from BALF of both asthmatic patients and healthy control subjects. Significant differences in BALF exosomal miRNA was

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detected for 24 miRNAs with a subset of 16 miRNAs, including members of the let-7 and miRNA-200 families, providing robust classification of patients with mild nonsymptomatic asthma from healthy subjects with 72% cross-validated predictive power ($Q^2 = 0.72$). In contrast, subway exposure did not cause any significant alterations in miRNA profiles.

Conclusion—These studies demonstrate substantial differences in exosomal miRNA profiles between healthy subjects and patients with unprovoked, mild, stable asthma. These changes might be important in the inflammatory response leading to bronchial hyperresponsiveness and asthma.

Keywords

microRNA; allergic asthma; IL-13; exosome; lung function

Asthma is a common chronic inflammatory respiratory disorder with increasing morbidity[1] characterized by airway narrowing in response to various stimuli, such as allergens, infections, and air pollutants.[2,3] The pathogenesis is influenced by both genetic factors and environmental triggers.[4] The pathophysiologic changes leading to asthma remain unclear, particularly in terms of nonallergic responses, and currently, there are no fully effective therapies.

Exosomes, nanosized vesicles of endosomal origin released from both immune and structural cells in the lung,[5–9] have recently been shown to play a role in allergy and asthma.[10,11] Although previously thought to be intracellular debris, exosomes are now viewed as important mediators in intercellular communication[12–14] capable of loading their content into recipient cells both proximally and distally.[15,16] We recently showed that exosomes from bronchoalveolar lavage fluid (BALF) of patients with asthma differ in phenotype and function compared with those from healthy subjects.[17] Furthermore, we have demonstrated that exosomes can induce cysteinyl leukotriene and IL-8 release in lung epithelial cells, an effect that was significantly higher for exosomes from asthmatic patients compared with those from healthy control subjects.[17] These findings suggest that exosomes play a role in local regulation of asthma pathology in the lung.

Exosomes are present in most body fluids, and their composition differs depending on their cellular origin.[8,18–23] The presence of RNA has previously been confirmed in exosomes from saliva, plasma, and breast milk.[20] Exosomes can transfer genetic material to nearby cells, thereby affecting the function of the recipient cell.[20,24,25] The presence of microRNA (miRNA) in exosomes from certain bodily fluids, including saliva, has also been confirmed.[20,21,26] miRNAs are small noncoding RNAs that exert posttranscriptional fine tuning of gene expression by suppressing translation or through mRNA degradation. A single miRNA can regulate large numbers of genes, and multiple miRNAs can regulate the same gene with additive or synergistic effects.[27] miRNAs play fundamental roles in several pulmonary diseases, including interstitial lung disease, chronic obstructive pulmonary disease, and asthma.[28–33] Altered miRNA expression has recently been associated with airway smooth muscle hyperresponsiveness[34] and mild asthma in human subjects.[33,35] Exosomes and miRNAs have thus been implicated in asthma separately; however, alterations in exosomal miRNA content caused by asthma have not yet been described. The

primary goal of this study was to characterize differences in exosomal miRNA between patients with mild intermittent asthma and healthy subjects.

Air pollutants are important factors in causing asthma exacerbations.[36–38] Urban air contains many pollutant factors, including particulate matter (PM), ozone, and nitric oxides. In large cities the subway system represents a major form of transportation and is a significant source of PM emission, yet few studies have focused on the health effects of this environment. We have previously described differences in oxylipin profiles in asthmatic patients exposed to subway air, suggesting impaired anti-inflammatory responses to the initial proinflammatory response triggered by subway PM stimuli in asthmatic patients, thus prolonging the inflammation compared with that seen in healthy subjects.[39] As such, a secondary aim of this study was to investigate exosomal miRNA profiles after exposure to Stockholm subway air.

METHODS

Study subjects and sampling

Patients with mild intermittent asthma (n = 10) and healthy subjects (n = 10, Table I)[39] were included. Detailed descriptions of study design, exposure regimen, PM monitoring results, and subjects are given elsewhere.[40,41]

Patients with asthma were hyperresponsive, which was defined as a 20% or greater decrease in FEV_1 after methacholine challenge. β_2-Agonist use on demand was allowed, but other medications, including inhaled corticosteroids, were not permitted. Participants were nonsmokers, were not habitual subway commuters, and refrained from subway use for more than 3 weeks before study initiation. *In vitro* allergy tests were conducted on all subjects (Phadiatop; Pharmacia-Upjohn, Uppsala, Sweden).[39] The study was performed out of the pollen season, and the washout period for respiratory tract infections was 6 weeks. All subjects underwent 2 bronchoscopies 14 hours after a 2-hour exposure regimen in a Stockholm subway station at rush hour (Odenplan, Stockholm, Sweden) and an ambient control air environment, which were performed in random order with a minimum interval of 3 weeks. During exposure, breathing rates were normalized to 20 L/min/m^2 body surface through mild exercise on an exercise bicycle with individually adjusted resistance. Bronchoalveolar lavage (BAL) was performed, as previously described.[42] BAL cells were pelleted by means of centrifugation at 400*g* for 10 minutes at 4°C, and the cell-free supernatant (BALF) was stored at −80°C until exosome preparation. The study was approved by the Stockholm Local Ethical Committee (case nos. 2006/643-31/4 and 2007/748-31/3), and all subjects provided written consent.

Exosomal preparation

Exosomes were isolated from 100 ± 1 mL of BALF per patient, minus 6 mL for fluorescence-activated cell sorting (FACS) analysis, by using serial ultracentrifugation: 3,000*g* for 30 minutes to remove debris and thereafter 10,000*g* for 30 minutes at 4°C (Beckman Coulter Optima L-100 XP Ultracentrifuge with Ti45 rotor; Beckman Coulter, Fullerton, Calif). The supernatant was filtered through a 0.2-μm filter (DISMIC-25cs

cellulose acetate; Advantec, Dublin, Calif), followed by a second ultracentrifugation at 140,000*g* for 2 hours at 4°C to pellet the exosomes. The pellet was resuspended directly in Nucleospin miRNA lysis reagent (Macherey-Nagel, Düren, Germany) and frozen at −80°C until use.

Exosome characterization by FACS analyses

BALF was added directly to Dynabeads (2 mL/μL beads) coated with anti–MHC class II (MHCII) antibodies (clone HKB1; Invitrogen/Dynal, Paisley, United Kingdom), as previously described.[43] Beads were labeled with fluorescein isothiocyanate–labeled anti–MHC class I, anti–MHC class II, CD63, and CD86 and phycoerythrin-labeled anti-CD54 or isotype-matched controls (BioLegend, San Diego, Calif). Samples were analyzed in a FACSCalibur (BD Biosciences, San Jose, Calif) by using forward scatter/side scatter bead gating, and mean fluorescence intensity (MFI) ratios were calculated as the geometric mean of the marker divided by the geometric mean of the isotype control.

RNA extraction and miRNA microarray

RNA was extracted and separated into small RNA (including miRNAs, 18–200 nt) and large RNA (>200 nt) fractions by using Nucleospin miRNA, according to the manufacturer's instructions. RNA quality was assessed by using UV 260/280 and 230/260 absorbance ratios obtained by using Nanodrop (Thermo Scientific, Wilmington, Del), resulting in a mean 260/280 ratio of 1.95. RNA size distribution was examined on RNA Pico LabChips (Agilent Technologies, Palo Alto, Calif) processed on the Agilent 2100 Bioanalyzer small RNA electrophoresis program.

An aliquot of 1 μL was used for validation by means of quantitative RT-PCR, and the rest was concentrated (SpeedVac, Thermo Fisher) to a volume of 4 μL and used for amplification. RNA was labeled with Cy3-CTP by using the miRCURY LNA microRNA power labeling kit (Exiqon, Woburn, Mass), according to the manufacturer's protocol. Labeled RNA was hybridized to 1-color Agilent custom UCSF miRNA v3.5 multi-species 8×15K Ink-jet arrays (Agilent Technologies) containing 894 different miRNAs.

Nanoscale quantitative RT-PCR validation

High-throughput nanoscale quantitative PCR (qPCR) was used to confirm the differential expression of selected miRNAs. An aliquot of 1.0 μL, corresponding to a starting material of 4.6 mL of BALF, was used for multiplex stem-loop reverse transcription and preamplification, as previously described.[44] After purification of multiplex PCR products, uniplex nanoscale qPCR was run on a microfluidics chip (Fluidigm, South San Francisco, Calif). After removing miRNAs that were not detected in any samples, reactions that did not amplify were set to a cycle threshold of 26, corresponding to a value less than the limit of detection. Log_2-transformed relative normalized expression values were generated by subtracting the cycle threshold for each miRNA of interest from the sample-specific mean of 26 miRNAs expressed in all samples.[45]

Statistical analyses

The microarray dataset was normalized by using quantile normalization.[46] No background subtraction was performed, and the median feature pixel intensity was used as the raw signal before normalization. miRNAs with a $\log_2$ expression value of less than 5.5, corresponding to a signal-to-noise ratio of less than 2, were excluded from all statistical analyses. Univariate statistical analyses were performed with a 2-way ANOVA linear model, and *P* value correction to account for a high false-positive rate was performed by using the false discovery rate method according to Hochberg and Benjamini[47] with the R package limma in Bioconductor.[48,49]

Multivariate analyses were performed with SIMCA P version 13.0 (Umetrics AB, Umeå, Sweden) by using principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) analysis. In contrast to the more commonly used PCA modeling, OPLS analysis is a supervised method designed to separate structured noise unrelated (orthogonal) to the predictive variance of interest (eg, asthmatic patients vs healthy subjects), thereby increasing the interpretability of the multivariate model, particularly in deriving the observed group separation back to miRNA variables of interest.[50] Analyses were performed on $\log_2$-transformed, quantile-normalized, mean-centered data scaled to unit variance. Variable selection for model optimization was performed iteratively based on variable importance in the projections (VIP) and scaled loadings of the predictive component of the OPLS models as (p[corr]), applying cutoffs of a VIP of greater than 1 and |p(corr)| of greater than 0.5 (see Fig E1 in this article's Online Repository at www.jacionline.org). Model performance is reported as cumulative correlation coefficients for the model (R^2), predictive performance based on 7-fold cross-validation (Q^2), as well as cross-validated ANOVA (CV-ANOVA) *P* values for OPLS-based group separation. To evaluate differences between the disease states, we subdivided the analyses into comparisons of control exposures separately from the subway exposures. Individual models were compared through shared-and-unique-structures (SUS) analyses, in which the scaled loadings (p[corr]) of the respective models are plotted against each other.[51] Shared variables cluster along the diagonals, whereas variables unique for either model are found along the axes.

Pathway analysis

The cumulative effects on gene expression of the observed alterations in exosomal miRNA expression between healthy subjects and asthmatic patients at baseline was calculated by using miRSystem.[52] The fold changes of the 24 significantly altered miRNAs were used as input, including only experimentally validated target genes in the output. The resulting list of target genes displaying an observed-to-expected ratio of greater than 1.0 (244 genes) was included in downstream pathway analysis by using the DAVID tool, version 6.7,[53,54] with *Kyoto Encyclopedia of Genes and Genomes* (KEGG) Pathways.[55,56]

RESULTS

FACS characterization of exosomes

Each BALF sample was analyzed with Dynabeads to detect surface proteins. Pilot experiments showed very low binding to latex beads coated with anti-CD63 (data not

shown); hence anti-MHCII beads were chosen for further analysis. Exosomes from all samples showed the presence of CD63, supporting an endosomal origin. Furthermore, exosomes displayed a strong signal for MHCII. MHC class I, CD54, and CD86 were not detected (Fig 1). No significant differences were seen between groups. A representative image of the bead gating is displayed in Fig E2 in this article's Online Repository at www.jacionline.org.

Asthmatic patients versus healthy control subjects at baseline

All samples from healthy control subjects and patients with mild asthma breathing ambient air passed quality control and were used for analysis.

Univariate statistical analysis of miRNA microarray expression values revealed that 24 miRNAs were present at significantly different levels in asthmatic patients compared with healthy control subjects (false discovery rate, <.05; Table II). Correlation analyses with clinical data, as well as BAL cell differential data, revealed a strong correlation between the expression profile of the 24 altered miRNAs and FEV_1 within the asthmatic group at baseline ($R^2 = 0.74$, PLS inner relation; Fig 2, right panel) but not within the other groups. No relevant correlations were found between BAL cell differential counts and miRNA expression profiles.

Subsequent multivariate modeling with OPLS revealed a significant separation between asthmatic patients and healthy control subjects at baseline ($P = 1.4 \times 10^{-5}$, CV-ANOVA; Fig 2, left panel). Results of the corresponding supervised hierarchic clustering based on univariate analysis of miRNA expression values are displayed in Fig E3 in this article's Online Repository at www.jacionline.org. The optimal OPLS model achieved after iterative variable selection (Fig E1) identified 16 of the significantly altered miRNAs to be the most important for separating asthmatic patients from healthy control subjects at baseline (Table II and Fig 2), resulting in a predictive power of 73% ($R^2 = 0.77$, $Q^2 = 0.73$) based on 7-fold cross-validation.

Asthmatic patients versus healthy control subjects after subway air exposure

Samples 03S and 34S were removed from final analyses because of failure to pass microarray quality control criteria. In addition, initial PCA analysis identified 20S as a strong outlier, driving the first 2 principal components (Fig E4 in this article's Online Repository at www.jacionline.org). Accordingly, these 3 samples were removed from all statistical analyses.

OPLS modeling of exosomal miRNA profiles after subway exposure resulted in significant separation between groups (Fig 3; $P = .049$, CV-ANOVA; $R^2 = 0.40$; $Q^2 = 0.35$). The optimized model consisted of 11 miRNAs, 3 of which were not prominent in the baseline model: miRNA-125b, miRNA-130a, and miRNA-203. SUS analysis revealed a very high correlation between the variables driving the separation between groups after baseline and subway exposures, respectively ($R^2 = 0.98$, Fig 4). The response to subway air exposure consisted of a slight but non-significant increase in most altered miRNAs in all subjects,

regardless of diagnosis (see Fig E5 in this article's Online Repository at www.jacionline.org).

miRNA profiles in relation to atopy

The changes seen in miRNA profiles at baseline were further analyzed in relation to the degree of atopy, which was defined as the maximal response to Phadiatop allergen testing for each subject.[39] OPLS analysis based on the subset of 16 miRNAs driving the separation between healthy control subjects and asthmatic patients at baseline but with the maximal allergy response as the response variable resulted in a robust significant model with a predictive power of 71% (R^2 = 0.86; Q^2 = 0.71; P = .005, CV-ANOVA). The group separation corresponded well with asthma diagnosis (Fig 5), with the exception of 2 asthmatic patients who clustered together with the healthy control subjects. Further examination of the clinical characteristics revealed that these 2 patients had mild to no atopy (maximal allergy response, 0 or 2), whereas the rest of the asthmatic patients had an allergy response of 4 to 6 (scale of 0-6). Removing these 2 patients from the model improved the predictive power and strengthened the overall model (R^2 = 0.91; Q^2 = 0.81; P = .002, CV-ANOVA). SUS analysis between the OPLS model based on asthma diagnosis and allergy response, respectively, showed a very high similarity between the models, as evident by the clustering of the variables along the diagonal (Fig 5, lower panel).

Validation by nanoscale qPCR

Validation of 18 of the 24 miRNAs significantly altered because of asthma at baseline was performed by using high-throughput nanoscale qPCR (Table II). Reliable primer pairs could not be obtained for the remaining 6 miRNAs. The presence of all 18 miRNAs was verified. Significant alteration in miRNA levels in accordance with those detected by means of microarray was confirmed for 8 miRNAs (let-7a, miRNA-21, miRNA-658, miRNA-24, miRNA-26a, miRNA-99a, miRNA-200c, and miRNA-1268), and 5 additional miRNAs displayed trends of alterations in accordance with the microarray results but did not reach significance (P = .06–.20; let-7b, let-7c, miRNA-200b, miRNA-27a, and miRNA-665). Alterations opposite to those detected by using microarrays were indicated for 2 miRNAs: miRNA-34c-5p and miRNA-34b-5p.

Pathway analysis

Investigation of the cumulative effects on gene expression of the observed alterations between healthy control subjects and asthmatic patients at baseline followed by downstream pathway analysis was performed with a combination of the miRSystem and DAVID tools. The resulting list of 244 target genes was associated with significant alteration of 3 distinct KEGG pathways: the mitogen-activated protein kinase (MAPK) signaling pathway (13 miRNAs targeting 11 genes, P = .012), cytokine–cytokine receptor interaction (11 miRNAs targeting 10 genes, P =.028), and the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling pathway (11 miRNAs targeting 7 genes, P = .048). Four of the target genes (*IL6*, *IL8*, *IL10*, and *IL13*) were also validated in BioCarta, with significant alterations of the pathways "cytokine network" (P = .011) and "cytokines and inflammatory

response" (P =.023). The 3 affected KEGG pathways with targeted genes marked are displayed in Figs E6 to E8 in this article's Online Repository at www.jacionline.org.

DISCUSSION

In spite of the fact that miRNAs have been shown to regulate a wide variety of inflammatory processes, including chronic lung diseases such as asthma and chronic obstructive pulmonary disease,[17,29,33,57,58] no studies have previously investigated the miRNA content of exosomes isolated from BALF. In this study we demonstrated the presence of miRNAs in exosomes from BALF of both healthy subjects and asthmatic patients. We also showed that exosomal miRNA profiles are altered because of asthma. The most striking differences were observed at baseline levels, with 24 miRNAs significantly altered between asthmatic patients compared with healthy subjects. Even though these asthmatic patients had mild intermittent stable disease with normal lung function, the expression profiles of the 24 identified miRNAs were highly correlated with FEV_1 in these subjects ($R^2 = 0.74$). Pathway analysis to evaluate the possible biological effects of these alterations revealed a concerted action of a subset of these miRNAs on cytokines of known importance in asthma, including IL-13, IL-10, IL-6, and IL-8. The MAPK and JAK-STAT signaling pathways were also significantly affected by subsets of these miRNAs. Finally, multivariate models performed to evaluate the predictive capacity of these findings on similar cohorts of patients showed that a subset of 16 miRNAs provided a highly significant separation of the asthmatic and healthy groups ($P = 1.4 \times 10^{-5}$), with a predictive power of 73% (Fig 2).

Members of the let-7 family (a-e), as well as the miRNA-200 family (200b and 141), were among the most prominent in driving the separation between the asthmatic patients and healthy control subjects in the multivariate models. All of these miRNAs were downregulated because of asthma at baseline. The miRNA-200 family has been shown to regulate epithelial mesenchymal transition,[59] which has been implicated in airway remodeling in asthmatic patients.[60] The let-7 family of miRNAs has also been shown to be important in patients with allergic asthma.[61]Kumar et al[61] recently demonstrated that airway inflammation in a murine IL-13 model was associated with a reduction in let-7 family members and that administration through inhalation of a let-7 mimic to the lungs of these mice resulted in decreased IL-13 levels, resolution of airway inflammation and hyperresponsiveness, and attenuation of mucus metaplasia and subepithelial fibrosis. miRNA-21, levels of which are also decreased in asthmatic patients at baseline, has also been implicated in the regulation of IL-13 in mouse models.[62,63] IL-13 plays a central role in patients with allergic asthma, and thus a hampered ability to fine tune IL-13 transcription through miRNA-mediated posttranscriptional regulation might represent an important factor in asthma pathology. A combination of cumulative gene target and pathway analyses confirmed that IL-13 is targeted by a number of the miRNAs with altered exosomal expression in asthmatic patients. Two of these (miRNA-34b-5p and miRNA-34c-5p) were recently shown to be regulated by IL-13 in primary airway epithelial cells.[33] Our data thus suggest that alterations in exosome-mediated miRNA transport between different cell types in the lung might provide an additional layer of complexity in this dysregulation.

The differences in exosomal miRNA content between asthmatic patients and healthy control subjects at baseline could be related to the severity of allergy. Multivariate models based on the miRNAs identified as markers for mild asthma but using degree of atopy (ie, maximal response to Phadiatop allergen challenge) as the response variable resulted in a significant separation between groups ($P = 5.4 \times 10^{-3}$), with a similar predictive power to the model based on asthma diagnosis ($Q^2 = 0.71$). As demonstrated in Fig 5, lower panel, the same variables drove the separation in both the allergy response–based model and the asthma diagnosis–based model, indicating that the observed alterations in exosomal miRNA content were associated with allergic asthma. It is noteworthy that these differences were observed in unprovoked subjects with stable mild disease outside of the allergy season.

miRNAs have also been implicated in response to injury by exposure to environmental pollutants.[27] Air pollution is a considerable health problem causing inflammation in the lungs after inhalation of, for example, PM. These particulates might cause more damage in vulnerable subjects with an underlying inflammatory condition, such as asthma. The composition of subway PM differs significantly from that of the more well-studied exhaust from combustion motor vehicles, primarily consisting of iron oxides as opposed to the predominant polycyclic aromatic hydrocarbons in roadside emissions. Farraj et al[64] reported downregulation of 14 miRNAs in the heart in response to exposure to metal-rich PM matter. However, investigations of the effects on lung function (FEV_1, peak expiratory flow, and fraction of exhaled nitric oxide) in the healthy[40] and asthmatic[41] subjects participating in this study did not reveal any significant effects on lung function of the subway exposure, which was also confirmed by others investigating more chronic effects on subway personnel.[65]

Subway exposure did not result in any significant alterations of exosomal miRNA expression levels within either group by using univariate measures. Overall, the OPLS models comparing asthmatic patients and healthy control subjects after subway exposure were highly correlated to the corresponding baseline models (R^2=0.98), with the exception of 3 miRNAs that became more prominent for group separation after subway air exposure: miRNA-203, miRNA-130, and miRNA-125b, all of which were downregulated in asthmatic patients.

miRNA-203 has been shown to bind GABA-A by using computational predictions.[66] Activation of airway smooth muscle GABA(A) receptors with agonists promotes relaxation of airway smooth muscle[67] and has been suggested as a therapeutic target for airway relaxation.[68] miRNA-125b inhibits the expression of TNF-α, which has been implicated in asthma, and has been shown to be downregulated in LPS-stimulated macrophages.[69] miRNA- 125b has also been demonstrated to be involved in Toll-like receptor signaling and cytokine release in asthmatic patients.[70] Gene target analysis using MicroCosm also revealed that human prostaglandin E synthase 2 is the primary target of miRNA-125b (P-base = 8.7×10^{-3}, P-org = 5.8×10^{-5}, score = 18). We have previously shown higher baseline levels of a number of oxylipins, including prostaglandin E_2, in the healthy compared with the asthmatic group.[39] These oxylipins are thought to be broncho-protective and have anti-inflammatory effects. A reduced anti-inflammatory response in asthmatic patients could be detected after subway exposure, and this was interpreted as a reduced

protective response to noxious stimuli in vulnerable subjects with an already ongoing pulmonary inflammation.

It would be helpful to know the origin of the exosomes to evaluate the importance and function of the altered miRNAs in asthma pathology. Previous studies have shown that both epithelial and immune cells secrete exosomes.[6–8,23,71,72] However, specific markers of cellular origin are not yet available.[73] Known exosomal surface markers, such as CD63, CD81, and CD9, are primarily used for evaluating the exosomal content of the preparation. The presence of CD63 in all samples supports an endosomal origin in the current study, and we have previously demonstrated the purity of exosomes isolated by using the same method from BALF by means of Western blotting and electron microscopy.[22] However, the differences in the abundance of CD63 previously reported between similar cohorts of healthy subjects and patients with mild asthma could not be confirmed in the current study. These discrepancies might be due to the lower sensitivity achieved with the methods of direct coating of BALF onto beads used here as opposed to preconcentration of samples used in the previous study.[17]

Furthermore, the presence of MHCII but not MHC class I might be an indication of a more prominent exosomal secretion from antigen-presenting cells than from structural cells in these samples. However, no discernible differences between groups were observed. Solberg et al[33] recently showed that all but 2 of the 24 miRNAs found to be altered in BALF exosomes in the current study also are altered in the airway epithelium of steroid-naive patients with mild asthma. The trajectory of upregulation or downregulation was the same in the epithelium as in exosomes, albeit to a lower degree, for all miRNAs but the 3 miRNAs primarily altered because of subway exposure. However, given the lack of relevant exosomal surface markers, it is unclear whether these correlations might be due to the exosomes being of epithelial origin or the epithelial cells being recipients of the exosomal content.

In this study we have, for the first time, verified the presence of miRNA in exosomes isolated from BALF. Furthermore, we revealed alterations in the exosomal miRNA profiles from asthmatic patients and healthy control subjects both at baseline and after subway air exposure. These differences were seen in a distinct small group of miRNAs and were related to allergic asthma. The most striking differences were observed in patients with unprovoked asthma at baseline, even though the asthmatic patients had mild stable disease with normal lung function, suggesting an intrinsic dysregulation of exosome production in asthmatic patients. Furthermore, the altered regulation of a subset of miRNAs in the asthmatic group was highly correlated to lung function. The majority of the altered miRNAs were downregulated, and a number of them appear to be implicated in the regulation of IL-13, as confirmed by means of pathway analysis. As such, these results might indicate that exosomal miRNAs exert a moderating regulatory function in allergy-associated pathways, which are downregulated in asthmatic patients at baseline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
CV-ANOVA	Cross-validated ANOVA
FACS	Fluorescence-activated cell sorting
JAK	Janus kinase
KEGG	*Kyoto encyclopedia of genes and genomes*
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHCII	MHC class II
miRNA	MicroRNA
OPLS	Orthogonal projections to latent structures
PCA	Principal component analysis
PM	Particulate matter
qPCR	Quantitative PCR
STAT	Signal transducer and activator of transcription
SUS	Shared and unique structures
VIP	Variable importance in the projections

REFERENCES

1. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy. 2004; 59:469–478. [PubMed: 15080825]
2. Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. Nat Immunol. 2002; 3:715–720. [PubMed: 12145657]
3. von Mutius E. Gene-environment interactions in asthma. J Allergy Clin Immunol. 2009; 123:3–13. [PubMed: 19130922]

4. Renz H, Autenrieth IB, Brandtzaeg P, Cookson WO, Holgate S, von Mutius E, et al. Gene-environment interaction in chronic disease: a European Science Foundation Forward Look. J Allergy Clin Immunol. 2011; 128(suppl):S27–S49. [PubMed: 22118218]
5. Esser J, Gehrmann U, D'Alexandri FL, Hidalgo-Estevez AM, Wheelock CE, Scheynius A, et al. Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration. J Allergy Clin Immunol. 2010; 126:1032–1040. e1–e4. [PubMed: 20728205]
6. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. J Exp Med. 1996; 183:1161–1172. [PubMed: 8642258]
7. Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. J Immunol. 2002; 168:3235–3241. [PubMed: 11907077]
8. van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, et al. Intestinal epithelial cells secrete exosome-like vesicles. Gastroenterology. 2001; 121:337–349. [PubMed: 11487543]
9. Kesimer M, Scull M, Brighton B, DeMaria G, Burns K, O'Neal W, et al. Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense. FASEB J. 2009; 23:1858–1868. [PubMed: 19190083]
10. Admyre C, Telemo E, Almqvist N, Lotvall J, Lahesmaa R, Scheynius A, et al. Exosomes—nanovesicles with possible roles in allergic inflammation. Allergy. 2008; 63:404–408. [PubMed: 18315728]
11. Almqvist N, Lonnqvist A, Hultkrantz S, Rask C, Telemo E. Serum-derived exosomes from antigen-fed mice prevent allergic sensitization in a model of allergic asthma. Immunology. 2008; 125:21–27. [PubMed: 18355242]
12. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia. 2006; 20:847–856. [PubMed: 16453000]
13. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. Leukemia. 2006; 20:1487–1495. [PubMed: 16791265]
14. Mack M, Kleinschmidt A, Bruhl H, Klier C, Nelson PJ, Cihak J, et al. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. Nat Med. 2000; 6:769–775. [PubMed: 10888925]
15. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem. 2010; 285:17442–17452. [PubMed: 20353945]
16. Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int. 2010; 78:838–848. [PubMed: 20703216]
17. Torregrosa Paredes P, Esser J, Admyre C, Nord M, Rahman QK, Lukic A, et al. Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma. Allergy. 2012; 67:911–919. [PubMed: 22620679]
18. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. J Immunol. 2007; 179:1969–1978. [PubMed: 17641064]
19. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. Int Immunol. 2005; 17:879–887. [PubMed: 15908444]
20. Lasser C, Alikhani VS, Ekstrom K, Eldh M, Paredes PT, Bossios A, et al. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. J Transl Med. 2011; 9:9. [PubMed: 21235781]
21. Michael A, Bajracharya SD, Yuen PS, Zhou H, Star RA, Illei GG, et al. Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis. 2010; 16:34–38. [PubMed: 19627513]

22. Qazi KR, Torregrosa Paredes P, Dahlberg B, Grunewald J, Eklund A, Gabrielsson S. Proinflammatory exosomes in bronchoalveolar lavage fluid of patients with sarcoidosis. Thorax. 2010; 65:1016–1024. [PubMed: 20880880]
23. Thery C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, et al. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. J Cell Biol. 1999; 147:599–610. [PubMed: 10545503]
24. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007; 9:654–659. [PubMed: 17486113]
25. Yuan A, Farber EL, Rapoport AL, Tejada D, Deniskin R, Akhmedov NB, et al. Transfer of microRNAs by embryonic stem cell microvesicles. PLoS One. 2009; 4:e4722. [PubMed: 19266099]
26. Zomer A, Vendrig T, Hopmans ES, van Eijndhoven M, Middeldorp JM, Pegtel DM. Exosomes: fit to deliver small RNA. Commun Integr Biol. 2010; 3:447–450. [PubMed: 21057637]
27. Sonkoly E, Pivarcsi A. MicroRNAs in inflammation and response to injuries induced by environmental pollution. Mutat Res. 2011; 717:46–53. [PubMed: 21329708]
28. Ezzie ME, Crawford M, Cho JH, Orellana R, Zhang S, Gelinas R, et al. Gene expression networks in COPD: microRNA and mRNA regulation. Thorax. 2012; 67:122–131. [PubMed: 21940491]
29. Pottelberge GR, Mestdagh P, Bracke KR, Thas O, Durme YM, Joos GF, et al. MicroRNA expression in induced sputum of smokers and patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2011; 183:898–906. [PubMed: 21037022]
30. Cho JH, Gelinas R, Wang K, Etheridge A, Piper MG, Batte K, et al. Systems biology of interstitial lung diseases: integration of mRNA and microRNA expression changes. BMC Med Genomics. 2011; 4:8. [PubMed: 21241464]
31. Garbacki N, Di Valentin E, Huynh-Thu VA, Geurts P, Irrthum A, Crahay C, et al. MicroRNAs profiling in murine models of acute and chronic asthma: a relationship with mRNAs targets. PLoS One. 2011; 6:e16509. [PubMed: 21305051]
32. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, et al. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. J Exp Med. 2010; 207:1589–1597. [PubMed: 20643828]
33. Solberg OD, Ostrin EJ, Love MI, Peng JC, Bhakta NR, Hou L, et al. Airway epithelial miRNA expression is altered in asthma. Am J Respir Crit Care Med. 2012; 186:965–974. [PubMed: 22955319]
34. Chiba Y, Misawa M. MicroRNAs and their therapeutic potential for human diseases: MiR-133a and bronchial smooth muscle hyperresponsiveness in asthma. J Pharmacol Sci. 2010; 114:264–268. [PubMed: 20953121]
35. Jardim MJ, Dailey L, Silbajoris R, Diaz-Sanchez D. Distinct microRNA expression in human airway cells of asthmatic donors identifies a novel asthma-associated gene. Am J Respir Cell Mol Biol. 2012; 47:536–542. [PubMed: 22679274]
36. Gauderman WJ, Avol E, Lurmann F, Kuenzli N, Gilliland F, Peters J, et al. Childhood asthma and exposure to traffic and nitrogen dioxide. Epidemiology. 2005; 16:737–743. [PubMed: 16222162]
37. Halonen JI, Lanki T, Yli-Tuomi T, Kulmala M, Tiittanen P, Pekkanen J. Urban air pollution, and asthma and COPD hospital emergency room visits. Thorax. 2008; 63:635–641. [PubMed: 18267984]
38. Penttinen P, Vallius M, Tiittanen P, Ruuskanen J, Pekkanen J. Source-specific fine particles in urban air and respiratory function among adult asthmatics. Inhal Toxicol. 2006; 18:191–198. [PubMed: 16399661]
39. Lundstrom SL, Levanen B, Nording M, Klepczynska-Nystrom A, Skold M, Haeggstrom JZ, et al. Asthmatics exhibit altered oxylipin profiles compared to healthy individuals after subway air exposure. PLoS One. 2011; 6:e23864. [PubMed: 21897859]
40. Klepczynska Nystrom A, Svartengren M, Grunewald J, Pousette C, Rodin I, Lundin A, et al. Health effects of a subway environment in healthy volunteers. Eur Respir J. 2010; 36:240–248. [PubMed: 20032018]

41. Klepczynska-Nystrom A, Larsson BM, Grunewald J, Pousette C, Lundin A, Eklund A, et al. Health effects of a subway environment in mild asthmatic volunteers. Respir Med. 2012; 106:25–33. [PubMed: 21996054]
42. Eklund A, Blaschke E. Relationship between changed alveolar-capillary permeability and angiotensin converting enzyme activity in serum in sarcoidosis. Thorax. 1986; 41:629–634. [PubMed: 3024350]
43. Johansson SM, Admyre C, Scheynius A, Gabrielsson S. Different types of in vitro generated human monocyte-derived dendritic cells release exosomes with distinct phenotypes. Immunology. 2008; 123:491–499. [PubMed: 17949417]
44. Moltzahn F, Olshen AB, Baehner L, Peek A, Fong L, Stoppler H, et al. Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. Cancer Res. 2011; 71:550–560. [PubMed: 21098088]
45. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, et al. A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol. 2009; 10:R64. [PubMed: 19531210]
46. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19:185–193. [PubMed: 12538238]
47. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. Stat Med. 1990; 9:811–818. [PubMed: 2218183]
48. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bio-conductor: open software development for computational biology and bioinformatics. Genome Biol. 2004; 5:R80. [PubMed: 15461798]
49. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004; 3 Article3.
50. Trygg J, Wold S. Orthogonal projections to latent structures (OPLS). J Chemometrics. 2002; 16:119–128.
51. Wiklund S, Johansson E, Sjostrom L, Mellerowicz EJ, Edlund U, Shockcor JP, et al. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. Anal Chem. 2008; 80:115–122. [PubMed: 18027910]
52. Lu TP, Lee CY, Tsai MH, Chiu YC, Hsiao CK, Lai LC, et al. miRSystem: an integrated system for characterizing enriched functions and pathways of microRNA targets. PLoS One. 2012; 7:e42390. [PubMed: 22870325]
53. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009; 4:44–57. [PubMed: 19131956]
54. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009; 37:1–13. [PubMed: 19033363]
55. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res. 2012; 40:D109–D114. (Database issue). [PubMed: 22080510]
56. Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 1999; 27:29–34. [PubMed: 9847135]
57. Ariel D, Upadhyay D. The role and regulation of microRNAs in asthma. Curr Opin Allergy Clin Immunol. 2012; 12:49–52. [PubMed: 22157155]
58. Oglesby IK, McElvaney NG, Greene CM. MicroRNAs in inflammatory lung disease—master regulators or target practice? Respir Res. 2010; 11:148. [PubMed: 21029443]
59. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008; 10:593–601. [PubMed: 18376396]
60. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. Curr Opin Allergy Clin Immunol. 2012; 12:53–59. [PubMed: 22217512]

61. Kumar M, Ahmad T, Sharma A, Mabalirajan U, Kulshreshtha A, Agrawal A, et al. Let-7 microRNA-mediated regulation of IL-13 and allergic airway inflammation. J Allergy Clin Immunol. 2011; 128:1077–1085. e1–e10. [PubMed: 21616524]
62. Lu TX, Munitz A, Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. J Immunol. 2009; 182:4994–5002. [PubMed: 19342679]
63. Mattes J, Collison A, Plank M, Phipps S, Foster PS. Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease. Proc Natl Acad Sci U S A. 2009; 106:18704–18709. [PubMed: 19843690]
64. Farraj AK, Hazari MS, Haykal-Coates N, Lamb C, Winsett DW, Ge Y, et al. ST depression, arrhythmia, vagal dominance, and reduced cardiac micro-RNA in particulate-exposed rats. Am J Respir Cell Mol Biol. 2011; 44:185–196. [PubMed: 20378750]
65. Bigert C, Alderling M, Svartengren M, Plato N, Gustavsson P. No short-term respiratory effects among particle-exposed employees in the Stockholm subway. Scand J Work Environ Health. 2011; 37:129–135. [PubMed: 20686740]
66. Zhao C, Huang C, Weng T, Xiao X, Ma H, Liu L. Computational prediction of microRNAs targeting GABA receptors and experimental verification of miR-181, miR-216 and miR-203 targets in GABA-A receptor. BMC Res Notes. 2012; 5:91. [PubMed: 22321448]
67. Mizuta K, Xu D, Pan Y, Comas G, Sonett JR, Zhang Y, et al. GABAA receptors are expressed and facilitate relaxation in airway smooth muscle. Am J Physiol Lung Cell Mol Physiol. 2008; 294:L1206–L1216. [PubMed: 18408071]
68. Gallos G, Yim P, Chang S, Zhang Y, Xu D, Cook JM, et al. Targeting the restricted alpha-subunit repertoire of airway smooth muscle GABAA receptors augments airway smooth muscle relaxation. Am J Physiol Lung Cell Mol Physiol. 2012; 302:L248–L256. [PubMed: 21949156]
69. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol. 2007; 179:5082–5089. [PubMed: 17911593]
70. Androulidaki A, Iliopoulos D, Arranz A, Doxaki C, Schworer S, Zacharioudaki V, et al. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity. 2009; 31:220–231. [PubMed: 19699171]
71. Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. Mol Biol Cell. 1997; 8:2631–2645. [PubMed: 9398681]
72. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med. 2001; 7:297–303. [PubMed: 11231627]
73. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J Proteomics. 2010; 73:1907–1920. [PubMed: 20601276]

Key messages

- Exosomal miRNA profiles differ between healthy subjects and patients with mild intermittent asthma with normal lung function.
- Pathway analysis revealed a concerted significant effect on IL-13, IL-10, IL-8, and IL-6, as well as on the MAPK and JAK-STAT signaling pathways.
- The expression profile of the altered miRNAs was highly correlated with lung function (FEV_1) in the asthma group at baseline.

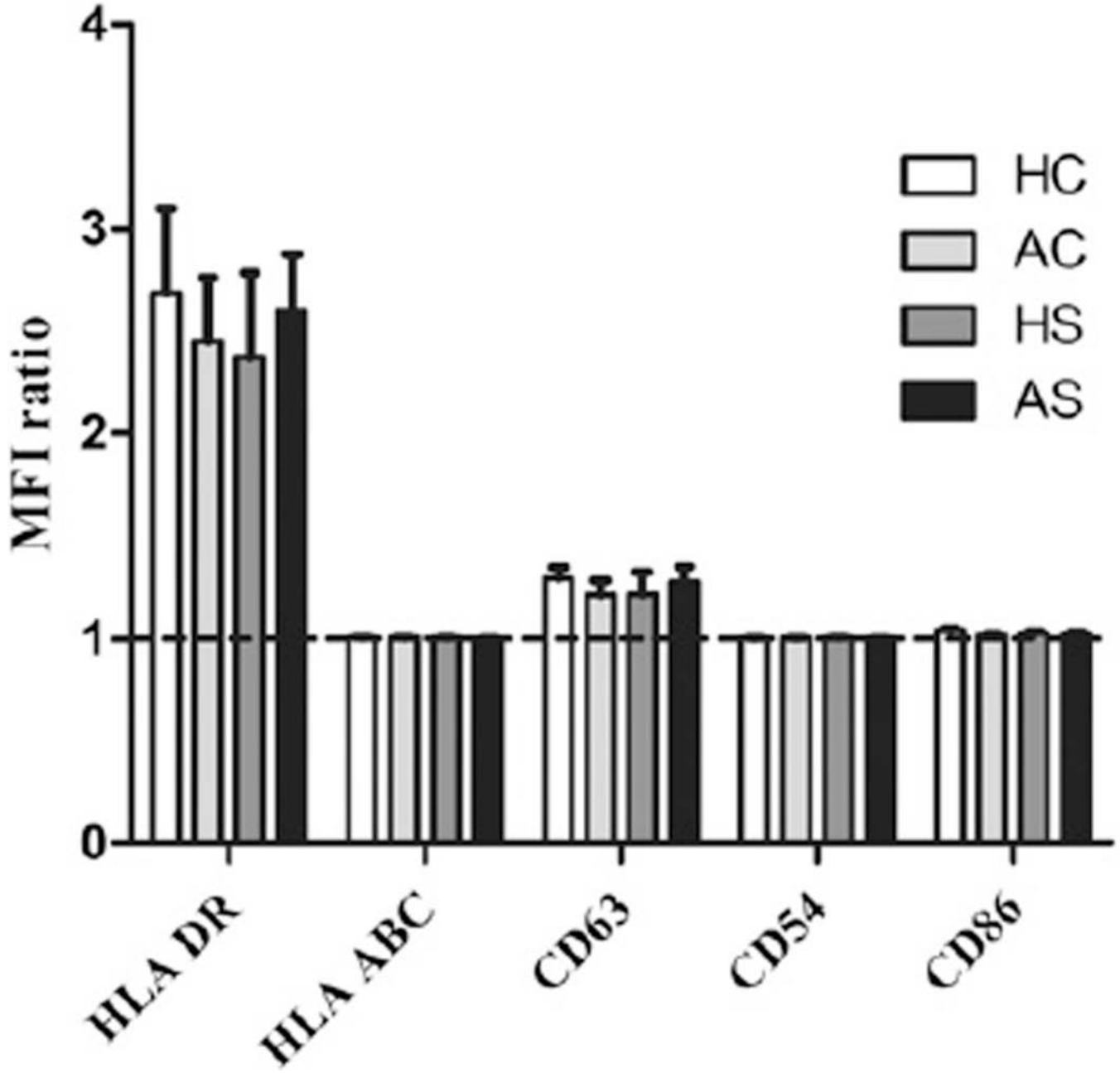

FIG 1.

Flow cytometric analysis of BALF exosomes displayed expression of MHCII and CD63 surface markers, whereas MHC class I, CD54, and CD86 were not detected. Results are shown as the MFI for the detected molecule divided by the MFI for the isotype control. Data represent means + SEMs (n = 10 in each group). *AC*, Asthmatic patients after control exposure; *AS*, asthmatic patients after subway exposure; *HC*, healthy control subjects after control exposure; *HS*, healthy control subjects after subway exposure.

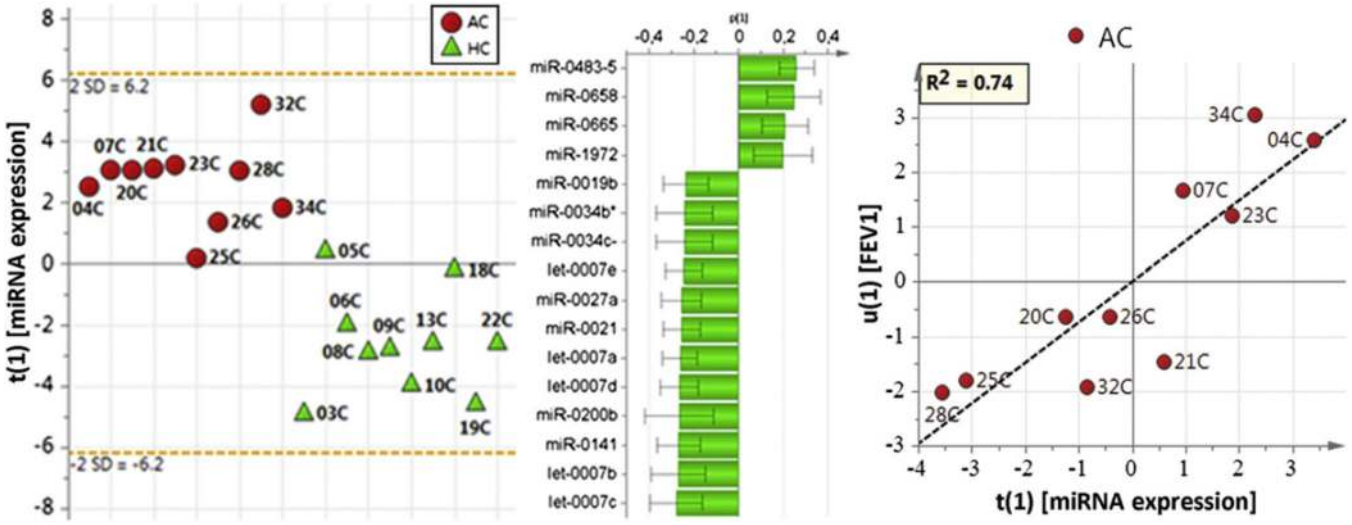

FIG 2.

OPLS analysis of the asthmatic versus healthy groups at baseline resulted in a robust separation between groups (*scores plot*, *left panel*). The optimized model of 16 miRNAs generated a highly significant classification model ($P = 1.4 \times 10^{-5}$, CV-ANOVA), with a predictive power of 73% ($R^2 = 0.77$, $Q^2 = 0.73$). The loadings for the predictive component of the 16 miRNAs are shown in the *middle panel*. Correlation analysis with PLS showed a strong correlation between the expression pattern of the 24 miRNAs significantly altered between the 2 groups ($q < 0.05$) and FEV_1 in asthmatic subjects (PLS inner relation, $R^2 = 0.74$; *right panel*). *AC*, Asthmatic patients after control exposure; *HC*, healthy control subjects after control exposure.

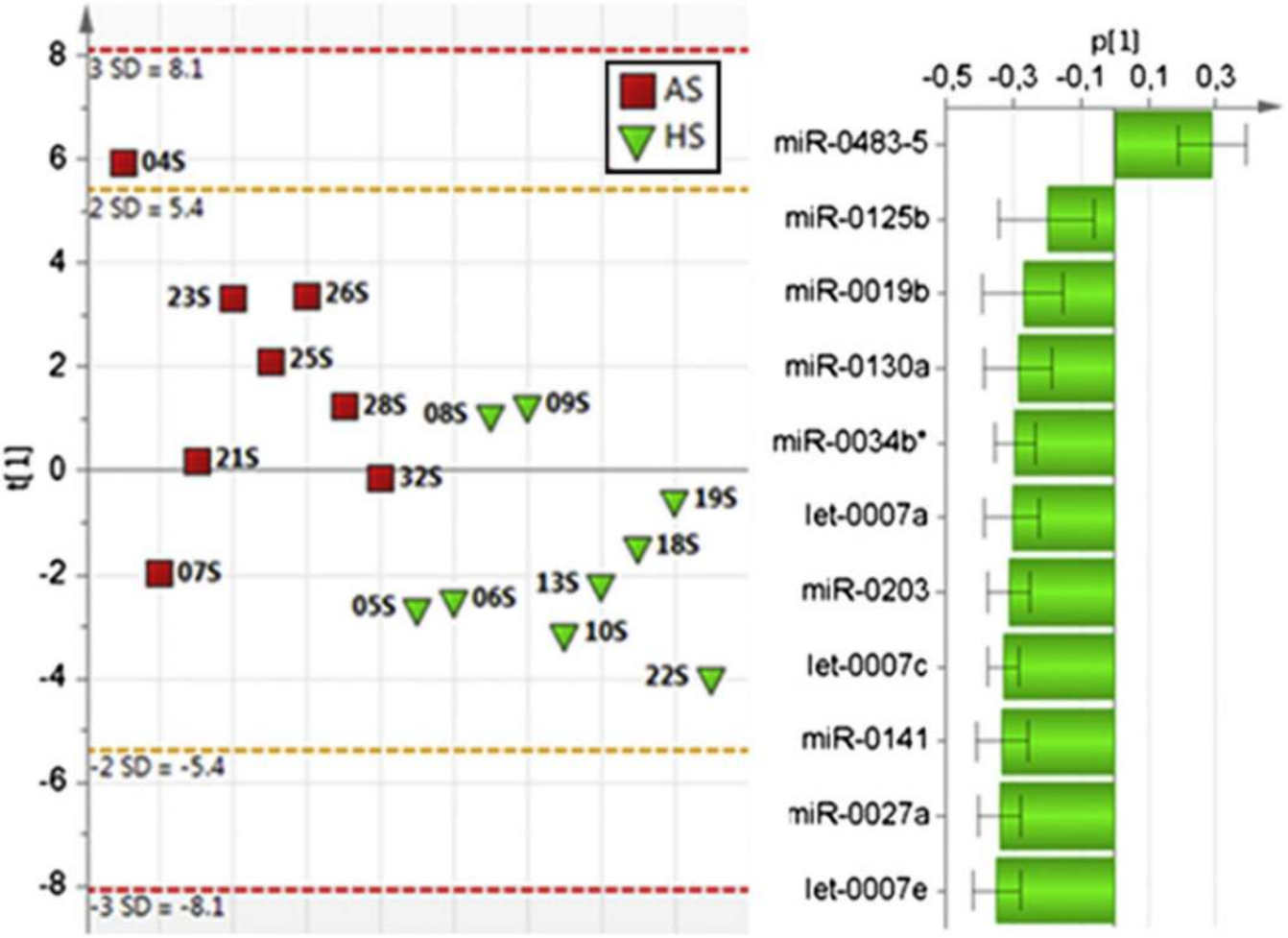

FIG 3.

OPLS analysis of the asthmatic versus healthy groups after subway exposure yielded a significant (P = .049, CV-ANOVA) separation, although with some overlap between the 2 groups (*scores plot*, *left panel*). The optimal model consisting of 11 miRNA variables (loadings, *right panel*) resulted in 35% predictive power (R^2 = 0.40, Q^2 = 0.35). *AS*, Asthmatic patients after subway exposure; *HS*, healthy control subjects after subway exposure.

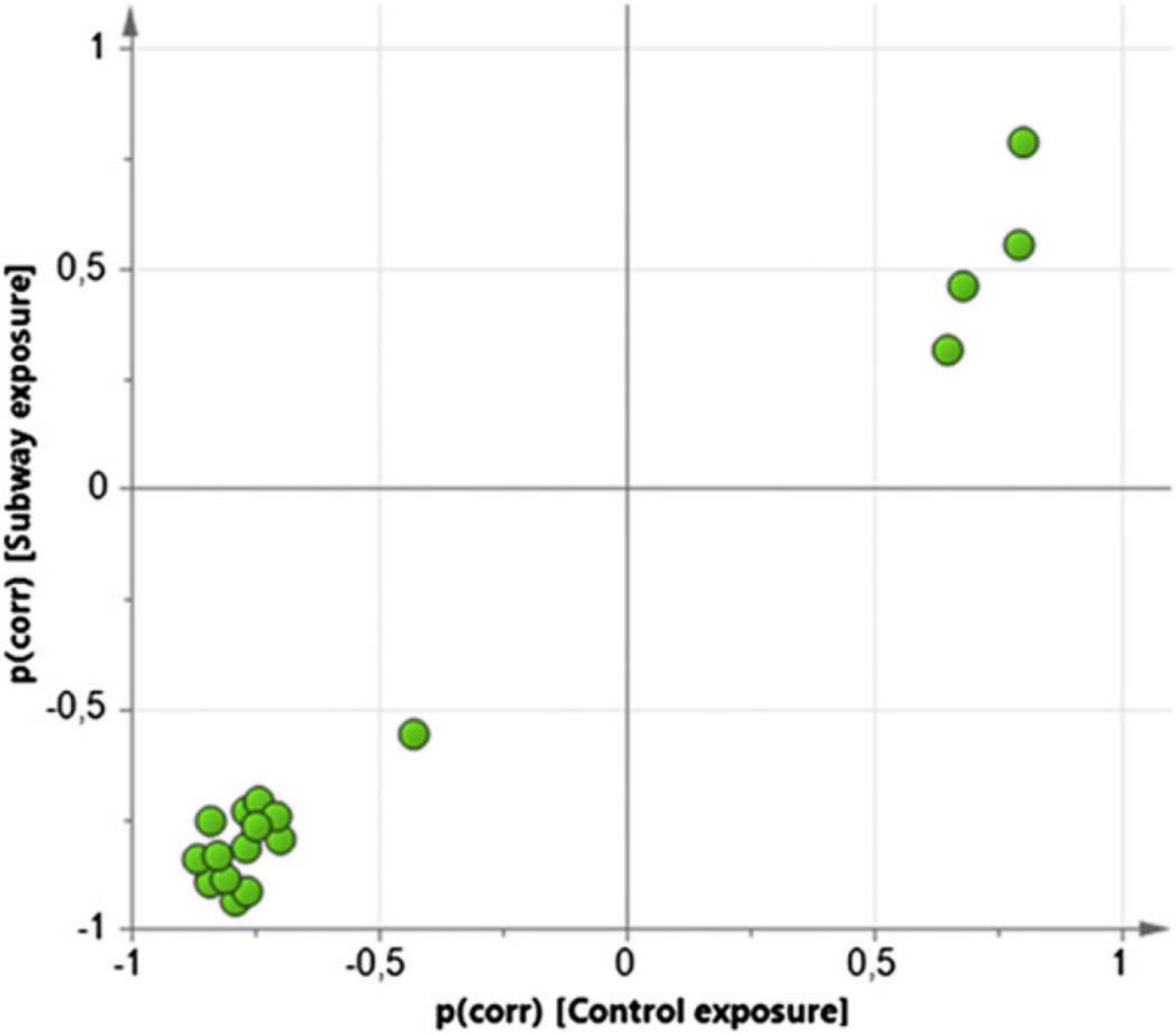

FIG 4.

Comparison of the 2 OPLS models for classification of the asthmatic and healthy groups after control exposure *(x-axis)* and subway exposure *(y-axis)* through an SUS plot revealed high correlation between the models (R^2 = 0.98). Clustering of the variables along the diagonal indicates that the miRNA alterations driving the separation between the 2 groups were the same at baseline and after subway exposure.

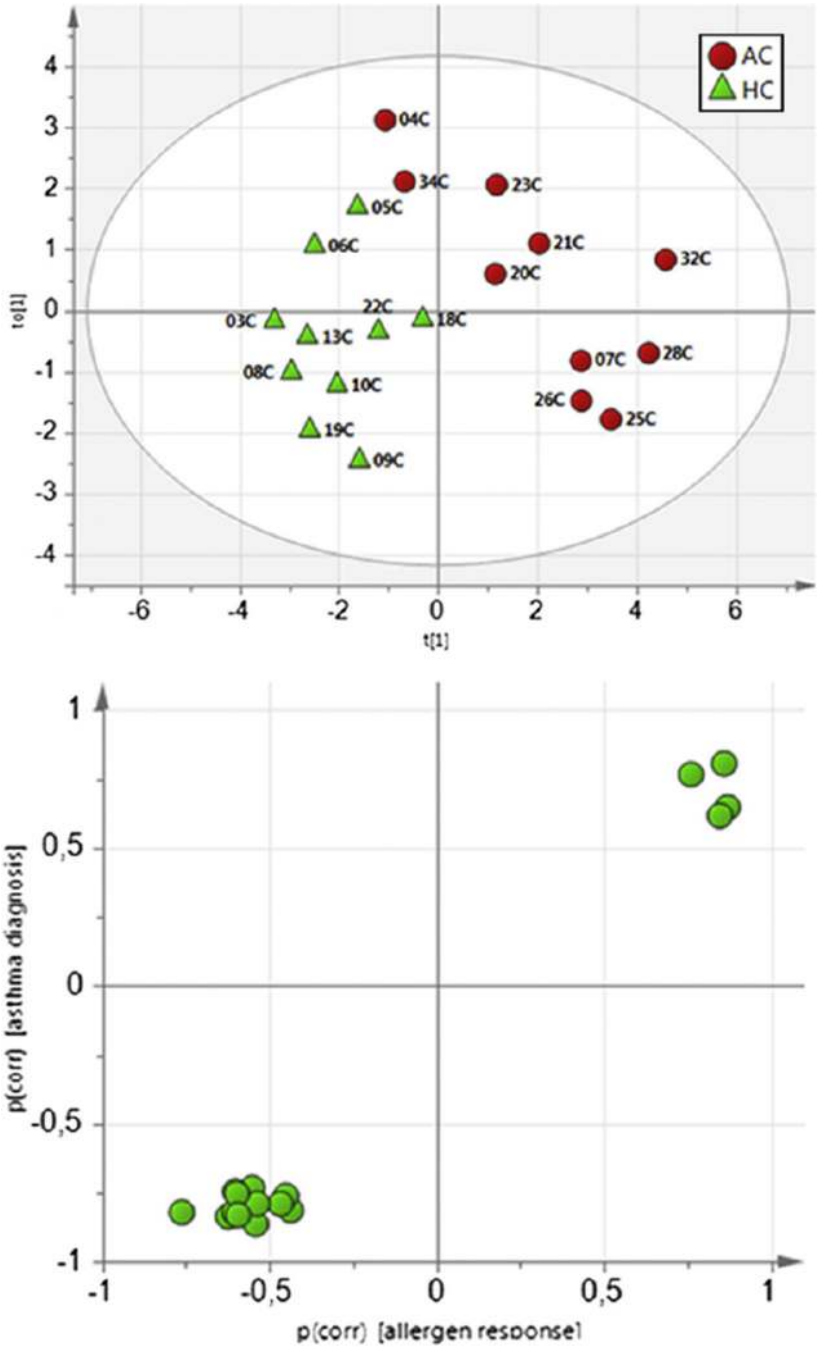

FIG 5.

OPLS analysis based on maximal allergy response using the 16 biomarker miRNAs identified in Fig 2 showed a strong separation according to the expected groups, with 2 outliers from the asthmatic group clustering with the healthy control subjects. These 2 patients had no (subject 4; allergy response = 0) or very mild (subject 34; allergy response = 2) atopic responses, in contrast to the other asthmatic subjects, who all had an allergy response of greater than 4 to at least 1 of the allergens tested. The apparent relation between the alterations in miRNA profile detected at baseline levels to allergic asthma were verified by the high correlation between the allergen response model and the asthma diagnosis model in the SUS plot (R^2 =

0.98, *lower panel*). Notably, these subjects had stable, asymptomatic, mild intermittent asthma, with no allergen challenge for at least 3 weeks.

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TABLE I

Patients' data

Diagnosis	Subject no.*	Sex	Age (y)	Allergy response†	Control exposure		Subway exposure	
					FVC (%)	FEV_1 (%)	FVC (%)	FEV_1 (%)
Asthmatic patients	4	F	40	0	118	122	114	126
	7	F	31	4	113	114	109	121
	20	M	25	4	100	94	102	92
	21	M	25	5	98	87	94	80
	23	M	23	4	113	110	118	110
	25	F	21	5	98	84	102	88
	26	F	21	6	98	94	104	101
	28	F	20	5	101	82	97	80
	32	F	19	5	78	83	76	83
	34	M	18	2	113	126	111	125
Healthy control subjects	3	M	42	0	113	109	113	106
	5	F	36	0	136	122	139	120
	6	F	31	0	94	85	101	87
	8	F	29	0	90	74	108	88
	9	F	28	0	121	101	112	95
	10	F	27	0	107	110	107	111
	13	M	26	0	99	100	102	102
	18	M	24	0	104	105	106	104

Diagnosis	Subject no.*	Sex	Age (y)	Allergy response†	Control exposure		Subway exposure	
					FVC (%)	FEV_1 (%)	FVC (%)	FEV_1 (%)
	19	M	24	0	125	116	119	109
	22	M	22	0	111	86	112	87

F, Female; *FVC*, forced vital capacity; *M*, male.

*Subject ID numbers correspond to IDs used in the study by Lundstrom et al.[39]

†Allergy response test values are classified from 0 to 6 (negative: 0, <0.35 kU/L; positive: 1, 0.35–0.69; 2, 0.7–3.4; 3, 3.5–17.4; 4, 17.5–49; 5, 50–99; and 6, >99 kU/L) (Phadiatop, Pharmacia-Upjohn). Allergy was tested against timothy grass, *Acarus* species, horse, cat, mugwort, birch, dog, olive, lichwort, fish, and *Cladosporium* species. The maximum response level for each subject is indicated.

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TABLE II

Statistical differences and fold changes between asthmatic patients and healthy control subjects in microarray analysis, multivariate modeling, and qPCR validations

	Microarray, AC vs HC			Fluidigm nanoscale qPCR, AC vs HC	
	P(corr)	FDR	Fold	*P* value	Fold
let-7c	−0.86	.0010	−2.2	.14	−1.5
let-7b	−0.84	.0036	−2.0	.20	−1.5
miR-141	−0.83	.00088	−2.4		ND
miR-200b	−0.82	.0038	−1.8	.067	−1.9
let-7d	−0.82	.0038	−1.8		ND
let-7a	−0.81	.0036	−2.4	.017	−9.4
miR-21	−0.79	.0038	−2.3	.001	−17
miR-27a	−0.79	.0036	−2.5	.16	−2.1
let-7e	−0.76	.0036	−1.7		ND
miR-34c-5p	−0.75	.0038	−2.4	.11	3.6
miR-34b-5p	−0.75	.0038	−2.3	.12	2.4
miR-19b	−0.73	.0093	−1.8		ND
miR-1972	0.62	.025	1.6		ND
miR-665	0.65	.014	1.7	.10	3.1
miR-658	0.77	.014	1.7	.039	3.8
miR-483-5p	0.81	.0038	2.2	.45	1.8
miR-0022	NA	.0036	−2.2	.94	1.0

	Microarray, AC vs HC			Fluidigm nanoscale qPCR, AC vs HC	
	P(corr)	FDR	Fold	*P* value	Fold
miR-0024	NA	.0064	−1.9	.050	−1.6
miR-0026a	NA	.0054	−2.2	.024	−1.8
miR-0099a	NA	.027	−1.6	.045	−1.9
miR-0200c	NA	.0038	−2.0	.018	−1.6
miR-1268	NA	.050	1.9	.0010	3.8
miR-0203	NA	.012	1.5		ND
miR-0130a	NA	.0038	1.8	.30	−1.5

FDR, False discovery rate; *NA*, not applicable; *ND*, not determined because of a lack of specific primer pairs.