Serum microRNAs catalog asthmatic patients by phenotype

Brief running title: Serum miRNAs phenotype asthmatics

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Abstract

Background: Asthma is a chronic inflammatory condition of the airways with a complex pathophysiology. Stratification of asthma subtypes into phenotypes and endotypes should move the field forward, making treatment more effective and personalized. Eosinophils are the key inflammatory cells involved in severe eosinophilic asthma. Due to the health threat posed by eosinophilic asthma, there is a need for reliable biomarkers to identify patients and treat them properly with novel biologics. A promising tool for diagnosis are microRNAs (miRNAs).

Objective: The aim of this study was to find serum miRNAs that can phenotype asthmatic patients.

Methods: Serum miRNAs of eosinophilic (N=40) and non-eosinophilic (N=36) asthmatic individuals were evaluated by next-generation sequencing (NGS), specifically miRNAs-seq, and selected miRNAs were validated by RT-qPCR. Pathways enrichment analysis of deregulated miRNAs was performed.

Results: NGS analysis revealed 15 differentially expressed miRNAs between eosinophilic and non-eosinophilic asthmatic patients, while did not show differences in the miRNome between atopic and non-atopic asthmatic individuals. Of the 15 differentially expressed miRNAs between eosinophilic and non-eosinophilic asthmatics, hsa-miR-26a-1-3p and hsa-miR-376a-3p were validated by RT-qPCR. Expression levels of these two miRNAs were higher in eosinophilic than in non-eosinophilic asthmatics. Furthermore, expression values of hsa-miR-26a-1-3p inversely correlated with peripheral blood eosinophil count and hsa-miR-376a-3p expression values with FeNO values and exacerbations number. Additionally, *in silico* pathway enrichment analysis revealed that these two miRNAs regulate signaling pathways related with asthma pathogenesis.

Conclusion: Hsa-miR-26a-1-3p and hsa-miR-376a-3p could be used to distinguish eosinophilic and non-eosinophilic asthmatic patients.

Key words: Asthmatic patients, Eosinophilic asthma, MicroRNA-seq; Phenotypes/Endotypes, Serum microRNAs.

doi: 10.18176/jiaci.0753

Resumen

Antecedentes: El asma es una enfermedad inflamatoria crónica de las vías respiratorias con una fisiopatología compleja. La estratificación de los subtipos de asma en fenotipos y en endotipos debería hacer avanzar el campo, haciendo que el tratamiento sea más eficaz y personalizado. Los eosinófilos son las células inflamatorias clave implicadas en el asma eosinofílica grave. Debido a la amenaza para la salud que representa el asma eosinofílica, existe la necesidad de biomarcadores confiables para identificar a los pacientes y tratarlos adecuadamente con nuevos biológicos. Una herramienta prometedora para el diagnóstico son los microARNs (miARNs).

Objetivo: El objetivo de este estudio fue encontrar miRNAs séricos que puedan fenotipar a los pacientes asmáticos.

Métodos: Los miARNs séricos de individuos asmáticos eosinofílicos (N = 40) y no eosinofílicos (N = 36) fueron evaluados mediante secuenciación de próxima generación (NGS), específicamente miARN-seq, y los miARNs seleccionados fueron validados por RT-qPCR. Se realizó un análisis de enriquecimiento de rutas de miARNs desregulados.

Resultados: El análisis NGS reveló 15 miARNs expresados diferencialmente entre pacientes asmáticos eosinofílicos y no eosinofílicos, mientras que no mostró diferencias en el miRNoma entre individuos asmáticos atópicos y no atópicos. De los 15 miARNs expresados diferencialmente entre asmáticos eosinofílicos y no eosinofílicos, hsa-miR-26a-1-3p y hsa-miR-376a-3p fueron validados por RT-qPCR. Los niveles de expresión de estos dos miARNs fueron más altos en los asmáticos eosinofílicos que en los no eosinofílicos. Además, los valores de expresión de hsa-miR-26a-1-3p correlacionaron inversamente con el recuento de eosinófilos en sangre periférica y los valores de expresión de hsa-miR-376a-3p con los valores de FeNO y el número de exacerbaciones. Además, el análisis de enriquecimiento de la vía *in silico* reveló que estos dos miARNs regulan vías de señalización relacionadas con la patogénesis del asma.

Conclusión: Hsa-miR-26a-1-3p y hsa-miR-376a-3p podrían usarse para distinguir pacientes asmáticos eosinofílicos y no eosinofílicos.

Palabras clave: Pacientes asmáticos, Asma eosinofílico, MicroARN-seq, Fenotipos/Endotipos, MicroARNs séricos.

doi: 10.18176/jiaci.0753

Introduction

Asthma is a prevalent chronic inflammatory disease of the airways with major public health consequences[1], in which the clinical and pathological characteristics are highly heterogeneous and diverse, thus complicating attempts to control the disease. As a result, there is substantial room for improvement in the diagnostic and therapeutic tools used[2]. Asthma encompasses numerous disease variants[3], and the–phenotyping and endotyping of asthma can facilitate responsiveness to treatment, pinpoint the pathogenic mechanisms involved, and anticipate risks. Based on the predominant inflammatory cell, asthma phenotypes are as follows: eosinophilic, neutrophilic, mixed granulocytic, and paucigranulocytic. Taking an endotype-based approach, asthma is divided into type 2 (T2) asthma, non-T2 asthma, and mixed complex endotypes[4]. Eosinophils emerged as the hallmark of eosinophilic asthma, which mainly involves Th2, but also T2 innate lymphoid cells[5].

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs that are 21 to 25 nucleotides in length[6] and can control gene expression by targeting specific mRNAs for degradation or translational repression. They are involved in multiple biological processes and simultaneously regulate various pathological processes[7]. Serum miRNAs are very stable and resistant to blood RNases, and some have been described as differentially expressed in a range of diseases[8] and have also been shown to function as noninvasive, sensitive and specific biomarkers[9].

The aim of this study is to determine whether miRNAs could serve as biomarkers to classify asthmatic patients in distinct phenotypes/endotypes using next-generation sequencing (NGS) and facilitate the choice of the appropriate treatment.

Methods

Patients' selection

Asthma-diagnosed subjects were recruited from allergy and pulmonology units of Fundación Jiménez Díaz Hospital (FJDH), Complejo Hospitalario de Navarra (CHN), and La Paz Hospital (LPH). Eighteen patients were selected for microRNAs-sequencing (miRNAs-seq) and distributed into 4 different groups: atopic asthma, non-atopic asthma, eosinophilic asthma, and non-eosinophilic asthma. Validation of miRNAs by semi-quantitative real-time polymerase chain reaction (qPCR) was performed including 67 additional patients, i.e., 36 with eosinophilic

doi: 10.18176/jiaci.0753

asthma, and 31 with non-eosinophilic asthma. Descriptive data for demographic, inflammatory, functional, and clinical characteristics of study subjects were compiled.

All patients (N=85) took part in the MEGA project, which uses a cohort of asthma patients of varying grades of severity[10]. The inclusion criteria were the following: (i) acceptance to participate, with signed informed consent; (ii) asthma diagnosis following the 2019 GINA criteria[11]; (iii) age between 18 and 75 years. The definition of atopy and non-atopy was established based on a positive or negative prick test and/or specific IgE to at least one allergen. The definition of eosinophilic (≥500 cells/μL) and non-eosinophilic (<150 cells/μL) asthma was made according to the peripheral blood eosinophil count.

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committees of the above-mentioned hospitals.

Sample collection

Serum was obtained by blood clotting in anticoagulant-free tubes and centrifuged at 3,000 rpm for 10 minutes at 4ºC and stored at -80ºC until use.

MiRNA isolation

RNA (including miRNAs) was obtained from 200 μL of serum using miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Three synthetic miRNA spike-ins (SP) (2, 4, and 5) were added to evaluate optimal RNA extraction (miRCURY LNA RNA Spike-in kit, Qiagen). The RNA enriched in miRNAs was eluted with 20 μL of RNase-free water.

MiRNA-seq: library preparation and sequencing

MiRNA-enriched RNA isolated from serum samples of 18 asthmatic subjects was employed for miRNA-seq.

Small RNA samples were converted to Illumina sequencing libraries using the NEXTFLEX® Small RNA-Seq Kit v3 (Bioo Scientific Corporation, Austin, TX, USA), strictly adhering to the manufacturer user guide. The size profile of the individual libraries was quantified using D1000 DNA High Sensitivity Screen Tape on a 4200 TapeStation System (both Agilent, Santa Clara, CA, USA). Quantified libraries were sequenced on an Illumina

doi: 10.18176/jiaci.0753

NextSeq 550 platform (Illumina, San Diego, CA, USA) using a NextSeq 500/550 75-cycle High Output Kit.

MiRNA bioinformatics analysis

Quality control, pre-processing, and statistical analysis of small RNA-seq data was carried out by the Bioinformatics Unit of IIS-Fundación Jiménez Díaz.

Adaptor removal and trimming of raw reads were accomplished using Cutadapt[12] by following NEXTflex small RNA instructions. Adaptor-trimmed reads between 17 and 25 nt were retained and aligned to the reference genome (GRCh38 assembly) using Bowtie2 as an aligner[13]. Mapping of reads to known miRNAs was performed with HTSeq-count2[14] using mature miRNA annotation retrieved from miRBase database (miRBase v22). Raw miRNA counts across samples were normalized by sequencing depth and RNA composition using TMM function from the NOISeq Bioconductor R package[15]. Subsequent Principal Component Analysis (PCA) on normalized and scaled values was applied using prcomp R function from stats R package[16]. Comparison of normalized expression levels across groups was performed following two alternative methods for testing differential expression in sequencing data: NOISeq[15] and DESeq2[17]. Fold change and adjusted p-values by FDR were calculated and used to identify significant differentially expressed miRNAs.

MiRNAs were considered biologically relevant if they were differentially expressed (adjusted $p<0.05$) and presented a Log_2 fold change$\geq$1.5 between groups.

MiRNA-seq validation of differentially expressed miRNAs by RT-qPCR

For the validation of results obtained by NGS, serum miRNAs from 36 subjects with eosinophilic and 31 non-eosinophilic asthmatic patients were retrotranscribed to cDNA using the miRCURY LNA RT Kit (Qiagen), as described by the manufacturer. Briefly, 4 μL of total RNA was mixed with reverse transcription enzyme and with synthetic miRNAs: SP6 and cel-miR-39-3p, which were used for control of a correct retrotranscription to cDNA. Final volume was 10 μL. The reaction was performed in a Veriti 96 well Thermal Cycler (Applied Biosystems, Warrington, UK), for 60 minutes at 42ºC, then 5 minutes at 95ºC, and indefinitely at 4ºC; cDNA was stored at -20°C until use.

Subsequently, miRNA expression was evaluated by qPCR using miRCURY LNA SYBR Green PCR Kit (Qiagen), following the manufacturer protocol. Based on miRNA-seq results, the

doi: 10.18176/jiaci.0753

following probes were used: hsa-miR-206, hsa-miR-32-5p, hsa-miR-6511a-3p, hsa-miR-202-5p, hsa-miR-26a-1-3p, hsa-miR-200a-3p, hsa-miR-941, hsa-miR-195-5p, hsa-miR-376a-3p, hsa-miR-210-3p, and hsa-miR-450a-5p (Qiagen). Additionally, hsa-miR-103a-3p, hsa-miR-191-5p, SP6 and cel-miR-39-3p were selected as endogenous controls and, hsa-miR-23a-3p and hsa-miR-451a were used as hemolysis controls (Qiagen). All samples were run in triplicate, and reactions were performed in a Light Cycler® 96 thermocycler (Roche, Basel, Switzerland). Cycle threshold (Ct) values were analyzed with LightCycler® 96 SW 1.1 (Roche) software.

The values of RT-qPCR analysis were normalized to the endogenous miRNAs controls using the $2^{-\Delta Ct}$ method,[18] where $\Delta Ct = Ct_{miRNA} - \Delta Ct$ ($Ct_{hsa\text{-}miR\text{-}103a\text{-}5p} + Ct_{hsa\text{-}miR\text{-}191\text{-}5p}$). Hsa-miR-103a-3p and hsa-miR-191-5p, which are known to be stably expressed in whole serum (normal and asthmatic patients), were used as internal controls, as no significant differences in expression were observed between the two groups, that is, eosinophilic and non-eosinophilic asthmatic patients. Relative quantification of differences in expression ($RQ = 2^{-\Delta\Delta Ct}$; where $\Delta\Delta Ct = \Delta Ct_{eosinophilics} - \Delta Ct_{non\text{-}eosinophilics}$) was carried out by the ΔΔCt method[18].

In silico pathway enrichment analysis

Pathway enrichment analysis of dysregulated miRNAs was performed using the DIANA-miRPath v3.0 bioinformatic tool[19].

Statistical analysis

For statistical evaluation GraphPad Prism® v6.0 (GraphPad Software Inc., San Diego, CA) was used.

Results are expressed as median and interquartile range (IQR). Normality was analyzed using the Shapiro-Wilk test. For continuous variables, parametric data comparison between non-paired groups was performed via an unpaired *t* test (compared groups have equal SD) and *t* test with Welch's correction (assumption that the population may have different SD), and non-parametric and non-paired groups were compared by Mann-Whitney test.

Furthermore, Spearman's (for non-parametric data) or Pearson's (for parametric data) correlation was applied for comparisons between miRNA expression levels (ΔCt) and some clinical parameters (quantitative variables); Fisher's exact test on a 2x2 contingency table was performed to test the null hypothesis of independence of two groups and some clinical characteristics (qualitative variables).

doi: 10.18176/jiaci.0753

The expression profile (ΔCt) of each differentially expressed miRNA was used to create receiver operator characteristic (ROC) curves. Logistic regression models were developed for diagnosis.

For all statistical analyses, differences showing $p<0.05$ were considered significant.

Results

Clinical parameters of the study subjects

Descriptive data for demographic, inflammatory, functional and clinical characteristics of 85 study subjects are summarized in Supplementary Table 1S. The patients were distributed into 4 different groups: 40 with eosinophilic asthma, 36 with non-eosinophilic asthma, 5 with atopic asthma, and 4 with non-atopic asthma.

The groups were quite homogeneous in terms of demographic characteristics and did not present any significant differences i.e., age, sex, BMI, and smoking habit. In all studied groups there were more women than men (67.5%, 66.7%, 80%, and 75% female, respectively). Patients from all the groups presented overweight (BMI>25) except the atopic group (BMI=22.1). Smoking habit was similar across all the groups ($p>0.05$). As expected, eosinophilic and non-eosinophilic subjects differed significantly in the peripheral blood eosinophils count (700 *versus* 100 cells/μL, $p<0.0001$). Regarding other inflammatory parameters such as sputum eosinophils, atopy, and IgE levels, we did not observe significant differences, though sputum eosinophils (2.3% and 1%) and IgE levels (209 and 124 IU) were higher in eosinophilic than in non-eosinophilic asthmatics, in contrast to atopy (60% and 77.8%), values for which were slightly higher in non-eosinophilic individuals.

In addition, patients with eosinophilic asthma had extremely significant higher FeNO values than non-eosinophilic asthmatics (50 *versus* 19 ppb, $p<0.0001$). Moreover, although it did not reach statistical significance, the group with eosinophilic asthma showed a greater number of subjects with exacerbations in the past year (22 *versus* 14) and with severe or moderate asthma (22 *versus* 11 and 9 *versus* 5, respectively), as shown in Supplementary Table 1S. On the other hand, neither the ACT scores nor other clinical traits such as treatments with ICS and LABA did not show significant differences (Supplementary Table 1S).

Finally, comparing the groups of atopic and non-atopic asthma, significant difference was only observed in atopy ($p<0.01$).

doi: 10.18176/jiaci.0753

MiRNA-seq data results: differential expression of miRNAs

Analysis of data obtained by NGS (miRNA-seq) showed 15 differentially expressed miRNAs between subjects with eosinophilic and non-eosinophilic asthma (adjusted $p<0.05$). Of these 15 miRNAs, 14 were upregulated and 1 was downregulated in eosinophilic asthmatic individuals. Moreover, when the PCA was performed using miRNA expression values, both groups were clearly differentiated (Supplementary Figure 1S).

In a different population selected based on atopy, the analysis of data obtained by miRNA-seq did not show differentially expressed miRNAs between atopic and non-atopic patients (data not shown).

Validation by RT-qPCR of differentially expressed miRNAs identifyied by miRNA-seq between eosinophilic and non-eosinophilic asthmatics

After miRNA-seq, we performed validation of miRNAs by RT-qPCR. Of the 15 miRNAs differentially expressed by miRNA-seq analysis between eosinophilic and non-eosinophilic asthmatic subjects, 14 were checked (1 was not available). We found a significant increase of hsa-miR-26a-1-3p and hsa-miR-376a-3p expression levels in patients with eosinophilia (Figure 1a and 1b).

Hsa-miR-6513, hsa-miR-1185-1, and hsa-miR-6503 were not detected and the rest of the miRNAs evaluated did not show any significant difference (Figure 1a).

These results confirm that serum hsa-miR-26a-1-3p and hsa-miR-376a-3p are differentially expressed between eosinophilic and non-eosinophilic asthmatic patients, suggesting that these could serve as diagnostic biomarkers.

Correlation of miRNAs with clinical characteristics

In order to establish some relation between the expression levels of these miRNAs (ΔCt) and different clinical parameters, we performed a correlation analysis depending on whether they were quantitative or qualitative variables. The laboratory data and clinical parameters set were as follows: severe asthma, number of peripheral blood eosinophils, percentage sputum eosinophils, atopy, total IgE, percentage of FEV_1/FVC, FeNO, exacerbations over the last year, number of exacerbations last year, ICS and LABA and ACT.

doi: 10.18176/jiaci.0753

Given that higher ΔCt values imply lower miRNA expression level, we emphasize that ΔCt values of hsa-miR-26a-1-3p were inversely correlated with the number of eosinophils in peripheral blood (Spearman r=-0.5736; p<0.001) in all subjects (eosinophilics plus non-eosinophilics; Figure 2a), which means that the count of eosinophils in peripheral blood augmented when the hsa-miR-26a-1-3p expression level was increased. Also, ΔCt values of hsa-miR-376a-3p showed a negative correlation with FeNO levels (Spearman r=-0,2594; p<0.05) in both groups together (Figure 2b) and with the number of exacerbations in the last year (Spearman r=-0,3391; p<0.05) among eosinophilic asthmatics (Figure 2c) and in the total population (Spearman r=-0.2592; p<0.05).

ROC curves and logistic regression models of differentially expressed miRNAs

ROC curves were generated and areas under the curve (AUC) were also calculated. Hsa-miR-26a-1-3p and hsa-miR-376a-3p, showed AUC values of 0.76 and 0.68, respectively and thus only hsa-miR-26a-1-3p was acceptable as univariate predictor (Figure 3).

For a better discrimination of eosinophilic and non-eosinophilic asthma status, multivariate logistic regression models were created using, on the one hand, the values in their original form (continuous predictors) and, on the other hand, the cutoff values of hsa-miR-26a-1-3p and hsa-miR-376a-3p expression defined by the Youden's index as explanatory variables (categorical predictors) (Figure 3). Furthermore, the model combining hsa-miR-26a-3p and hsa-miR-376a-3p, created using the original values, showed an AUC of 0.76, with a sensitivity of 0.79, and a specificity of 0.45 (Figure 3). On the other, the model originated from categorical predictors combining both miRNAs, created using the the cutoff values of miRNAs expression, showed an AUC of 0.79, with a sensitivity of 0.84, and a specificity of 0.91 (data not shown). However, despite the good values of AUC, sensivity and specificity of this last model, it would not be acceptable due to a value <0.1 in the Hosmer-Lemeshow test, used to assess the calibration of the model.

According to these results, hsa-miR-26a-1-3p univariate predictor model seems the best model for discrimination of eosinophilic and non-eosinophilic asthmatic patients, being similar than the hsa-miR-26a-1-3p and hsa-miR-376a-3p multivariate regression model.

Signaling pathways in which dysregulated miRNAs are involved

An *in silico* analysis was carried out with the 2 differently expressed miRNAs to determine their involvement in the biological processes.

doi: 10.18176/jiaci.0753

As Supplementary Figure 2S shows, hsa-miR-26a-1-3p and hsa-miR-376a-3p are involved in the regulation of several crucial pathways: extracellular matrix (ECM)-receptor interaction, non-small cell lung cancer and p53 signaling pathway. Hsa-miR-26a-1-3p modulates two of the pathways, non-small cell lung cancer and p53 signaling pathway, and hsa-miR-376a-3p modulates ECM-receptor interaction. While the p53 and non-small cell lung cancer pathways are more involved in the inflammatory response, ECM-receptor interaction plays a major role in airway remodeling. So, we can infer that the signaling pathways regulated by these miRNAs may be related to the pathogenesis of asthma.

Discussion

This is the first report showing altered expression of serum hsa-miR-26a-1-3p and hsa-miR-376a-3p between eosinophilic and non-eosinophilic asthmatic patients. This profile could be used as a phenotypic biomarker to classify asthmatic individuals in these two groups in order to choose the adequate treatment for each individual.

Asthma is a heterogeneous disease with varying severity[20], therefore, the identification of biomarkers to recognize endotypes and guide therapy has recently become a priority in asthma[21]. These biomarkers include sputum eosinophil percentage, peripheral blood eosinophil count, FeNO, and serum IgE levels[22], being eosinophil count in induced sputum the most sensitive to phenotype patients with severe eosinophilic asthma[23]. Blood eosinophil counts are a potential surrogate biomarker of eosinophilic inflammation in asthma[24] but this relationship may differ per population and per study, in fact, a systematic review and meta-analysis revealed that FeNO, blood eosinophils and serum IgE have moderate diagnostic accuracy, suggesting the need for new techniques to improve this accuracy[25]. Furthermore, there is no consensus regarding eosinophil cutoff levels, the cutoff used in clinical trials to define high blood eosinophil counts ranges between 150 and 300 cells/mL[26] and a blood eosinophil count of $0.22\text{-}0.27\times10^9$/L differentiates eosinophilic and non-eosinophilic asthma with 78%-86% sensitivity[27]. In our study, to ensure membership in the group of eosinophilic patients, the cutoff points were 0.5×10^9 eosinophils/L or higher.

However, more useful biomarkers must be found to catalogue asthmatic patients in different phenotypes/endotypes. In this sense, NGS is a powerful technique being applied to analyze the whole genome profile, including mRNA and small RNA expression[28]. So far, this high-throughput profiling technology has discovered critical miRNAs in certain diseases such as

doi: 10.18176/jiaci.0753

asthma and, due to their advantage of allowing sequencing and detecting low-frequency variants feasible [29,30]. Furtheremore, in this work we obtained higher AUC values than those shown by Hastie et al; where blood eosinophils lack sufficient accuracy for predicting sputum eosinophils in asthma[31].

In the present study, for first time, we found a different profile of 2 miRNAs which could serve for eosinophilic and non-eosinophilic asthma differentiation. Dysregulation of hsa-miR-26a-1-3p and hsa-miR-376a-3p has been previously associated with different aspects of asthma pathogenesis and other respiratory diseases[32–34]. Hsa-miR-26a-1-3p and hsa-miR-376a-3p could be relevant miRNAs with phenotypic implication in the identification of the eosinophilic/non-eosinophilic asthmatic patients, since they were significantly correlated with some of clinical parameters. Thus, hsa-miR-26a-1-3p was related with the eosinophil counts and hsa-miR-376a-3p seems to be linked to the FeNO values and the number of exacerbations in last year. When hsa-miR-376a-3p expression levels are increased, the exacerbations number augmented in the eosinophilic asthmatic patients. It has been shown that blood eosinophil counts, among other clinical variables, may be used to predict frequent asthma exacerbations and that an increased blood eosinophil count (>400 eosinophils/μL) means increased the likelihood of having two or more exacerbations per year[35].

Regarding the downstream effects, we found that hsa-miR-26a-1-3p modulates *in silico* two pathways with statistical significance mainly implicated in inflammation and hsa-miR-376a-3p significantly altered the ECM-receptor interaction signaling pathway. It is very interesting that the pathways regulated by these miRNAs play a key role in the development of airway inflammation and remodeling of asthma[36]. The pathophysiology of airway inflammation associated with asthma has been previously explained by eosinophilic inflammation, favored by airway epithelial barrier involvement, induced by the excessive release of Th2 cytokines[37]. The deregulation of some miRNAs in non-eosinophilic asthmatic individuals could indicate less involvement of the pathways they regulate and a more correct expression of the target genes enabling better asthma control. However, the specific role of these miRNAs in distinguishing these phenotypes of asthmatic patients needs to be further studied.

Finally, in accordance with our findings, we found no differences in the expression of miRNAs between atopic and non-atopic asthma patients, unlike other research teams[38]; this discrepancy in the results can be due to different patient selection because in the Belanger study, patients are selected from a family cohort and most importantly the miRNAs were obtaines

doi: 10.18176/jiaci.0753

from purified eosinophils. Since no differentially expressed miRNAs between atopic and non-atopic subjects were observed, we evaluated the possible statistical differences between the presence or absence of atopy in the population of eosinophilic and non-eosinophilic asthmatics, and the analysis did not show any statistically significant differences (Supplementary Table 1S). Moreover, the ΔCt values of the differentially expressed miRNAs obtained by validation did not correlate with the presence of atopic and non-atopic asthma. Thus, in addition to the lack of differentially expressed miRNAs between atopic and non-atopic patients, it could be said that the presence or absence of atopic asthma is not related to the presence or absence of eosinophilic asthma.

In summary, we describe significant differences in the expression of two miRNAs, hsa-miR-26a-1-3p and hsa-miR-376a-3p, which could be used as biomarkers to phenotype eosinophilic and non-eosinophilic asthmatic patients in order to facilitate the choice of their appropriate treatment.

doi: 10.18176/jiaci.0753

Funding

This study was supported by Fondo de Investigación Sanitaria – FIS and FEDER (Fondo Europeo de Desarrollo Regional) [PI18/00044]; CIBER de Enfermedades Respiratorias (CIBERES), a Carlos III Institute of Health initiative. M.G.-M. was supported by a PFIS contract (FI19/00067) from the Fondo de Investigación Sanitaria (Ministerio de Sanidad y Consumo, Spain). P.M. has a Miguel Servet contract funded by the ISCIII (CP16/00116), L.dF. was supported by the ISCIII contract CA18/00017.

Conflicts of interest

J.S. reports having served as a consultant to Thermofisher, MEDA, Novartis, Sanofi, Leti, Faes Farma, Mundipharma, and GSK; having been paid lecture fees by Novartis, GSK, Stallergenes, Leti, and Faes Farma; as well as having received grant support for research from Thermofisher, Sanofi, and ALK. S.Q. reports personal fees from AstraZeneca, personal fees from Novartis, personal fees from Sanofi, personal fees from Boehringer Ingelheim, personal fees from Teva, personal fees from ALK, personal fees from Mundipharma, personal fees from GSK, personal fees from Chiesi, personal fees from Leti, outside the submitted work. J.M.O. reports grants from Sanofi during the conduct of the study; personal fees from AstraZeneca, personal fees from Mundipharma, outside the submitted work. V.d.P. has received honoraria (advisory board, speaker) and/or institutional grant/research support from Astra-Zeneca and GSK. The rest of authors declare no conflict of interest.

Acknowledgments

The authors recognize Oliver Shaw, English editor of IIS-FJD, for his revision and editing in English.

doi: 10.18176/jiaci.0753

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doi: 10.18176/jiaci.0753

FIGURES

Figure 1 . Serum miRNA deregulation in eosinophilic and non-eosinophilic asthmatic patients (a). Subjects with eosinophilic asthma showed higher expression levels of hsa-miR-26a-1-3p and hsa-miR-376a-3p than non-eosinophilic asthmatics (b). ****, p<0.0001; *, p<0.05.

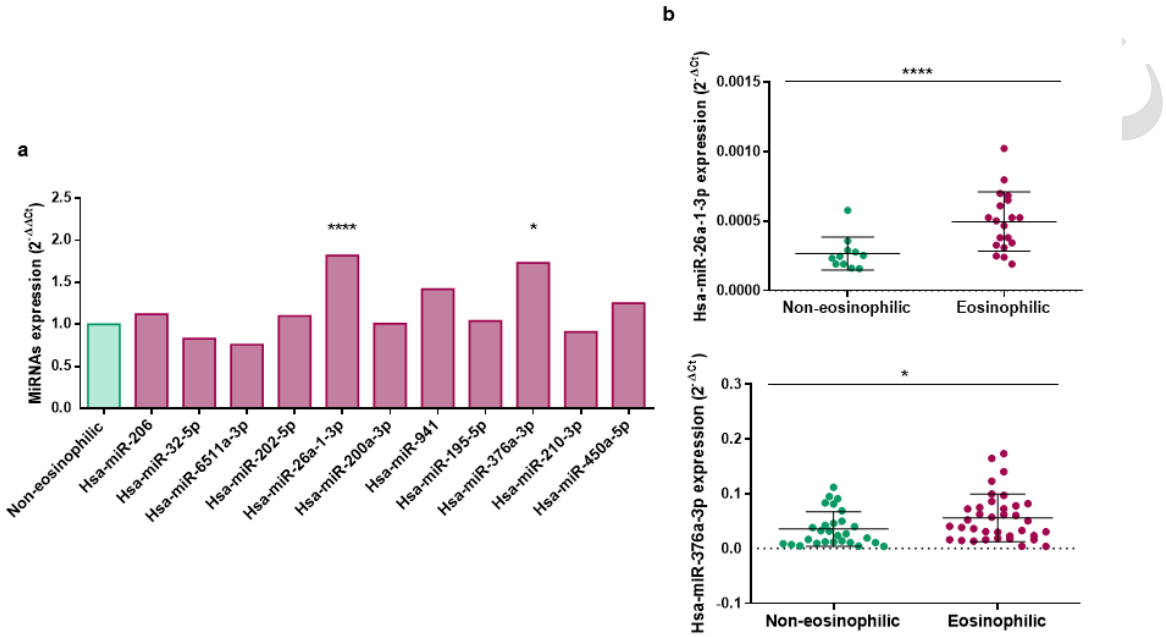

Figure 2. Hsa-miR-26a-1-3p and hsa-miR-376a-3p correlate with clinical parameters. A negative correlation was observed between ΔCt values of hsa-miR-26a-1-3p with peripheral blood eosinophils number in all subjects (a); ΔCt values of hsa-miR-376a-3p also were inversely correlated with FeNO values in both groups together (b) and exacerbations count in eosinophilic asthmatics (c).

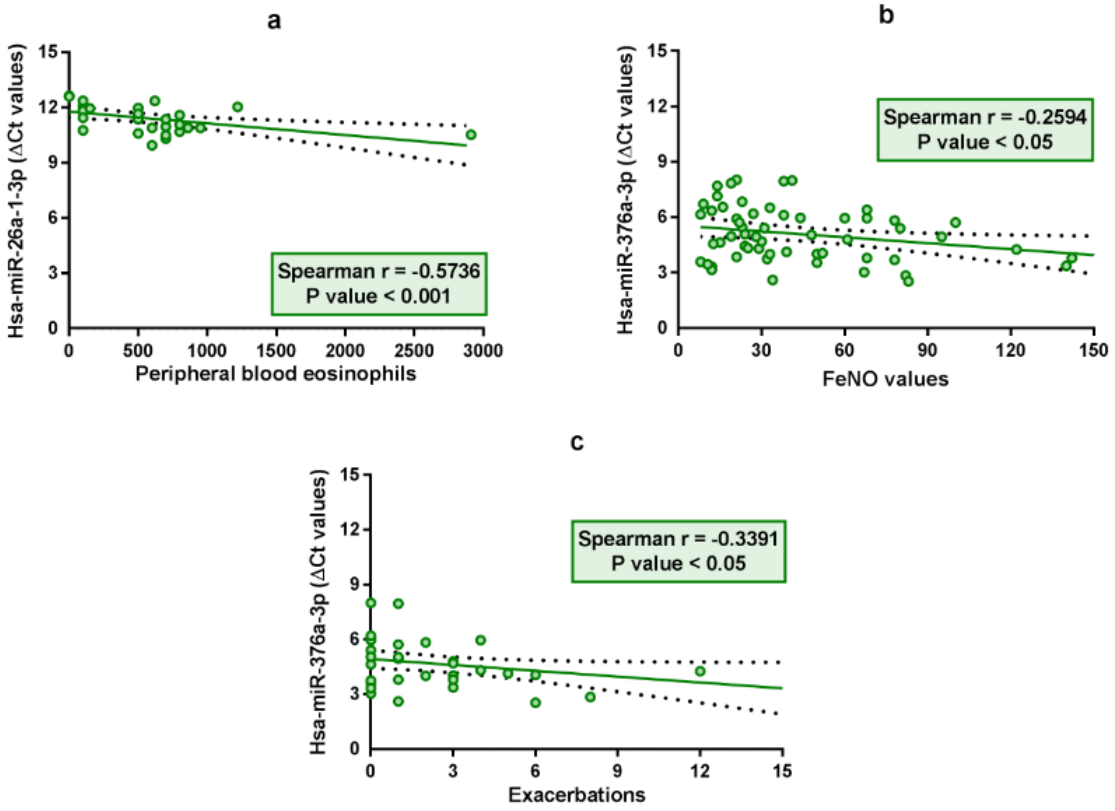

Figure 3. Receiver operator characteristic (ROC) curves of differentially expressed miRNAs and multivariate logistic regression models created.