Stability of Asthma Control Implies No Changes in microRNAs Expression

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Asthma is a chronic disease that affects 4.3% of the population worldwide [1]. Pulmonary function tests and bronchial provocation tests are still the gold standard in diagnosing and assessing the severity of respiratory diseases; however, they are not able to differentiate between the clinical phenotypes responsible for specific manifestations. An ideal biomarker must be measurable with minimal invasiveness, be specific and sensitive, and be able to be detected quickly and accurately. In this context, microRNAs (miRNAs) present in body fluids have been reported to meet several of these criteria and are used as diagnostic markers in many areas [2-4]. Eosinophils, which play a key role in the pathogenesis of asthma, have the ability to secrete exosomes. These structures contain miRNAs, which are single-stranded RNA sequences (around 19-22 nucleotides) that do not code for proteins with crucial functions in the development and maintenance of the pathogenic mechanisms of asthma, but may instead be implicated in the pathophysiology of asthma by regulating the translation of proteins related to asthma processes [5]. miRNAs can be encapsulated in exosomes or bound to proteins in biofluids; in both cases, they are highly resistant to degradation by RNases [6,7].

Released exosomes and the miRNAs inside them have been found in serum. However, neither the precise role of miRNAs in asthma nor their stability in the same patient over time has been fully defined [3-5].

In order to establish whether expression of miRNA in clinically stable asthma patients remains steady over time, we selected 20 asthmatic patients from a national cohort (MEGA project) [8]. These patients were recruited randomly in the Allergy Department of Fundación Jiménez Díaz University Hospital, Madrid, Spain. Clinical and epidemiological characteristics are shown in the Supplementary Material. Patients received all necessary information and gave their written informed consent to participate. The study was conducted following the principles of the Declaration of Helsinki and approved by Fundación Jiménez Díaz Ethics Committee. All selected patients had a confirmed diagnosis of asthma with >12% improvement in FEV_1 15 minutes after inhaling salbutamol (400 µg) or methacholine airway hyperresponsiveness (PC_{20} methacholine <16 mg/mL) [9]. They also had moderate persistent asthma and were being treated with a combination of inhaled corticosteroids/long-acting ß-agonists at medium doses (400 µg of budesonide and 12 µg of formoterol fumarate dihydrate daily or equivalent). No change was made in the treatment received for asthma during the study period, ie, from baseline to the follow-up visits. Serum was obtained by centrifugation and stored at –80ºC before analysis for no more than 2 years.

Serum miRNAs were extracted using the miRCURY RNA Isolation Kit-Biofluids (Qiagen) and retrotranscribed to cDNA using the Universal cDNA Synthesis kit II (Qiagen) following the manufacturer's instructions. Synthetic spike-ins were added during the RNA extraction (Sp2, Sp4, and Sp5) and reverse transcription (Sp6) processes to ensure appropriate extraction and cDNA synthesis. miRNA expression was evaluated using quantitative polymerase chain reaction as previously described [10] at baseline and 6-12 months later at follow-up visits.

The miRNAs analyzed were miR-320-a, miR-144-5p, miR-1246, miR-21-5p, and miR-185-5p. These miRNAs were selected because we previously found that their profile in eosinophils can be used as a serum biomarker of asthma [10]. MiR-191-5p was measured as the endogenous control, and Sp2, Sp4, Sp5, and Sp6 were measured to ensure correct extraction and reverse transcription.

The statistical analysis was carried out using the GraphPad Instat program. The *t* test was performed for normally distributed samples (those meeting a Gaussian distribution) and the Mann-Whitney test for non-normally distributed samples. Paired tests were performed to compare baseline data with follow-up data.

Asthma was stable over time in terms of the mean (SD) Asthma Control Test score (21.1 [3.7] vs 20.8 [3.1]) and lung function (FEV_1%, 97.7 [12.9] vs 97.5 [13.9]). In addition to the stable clinical parameters, no statistically significant differences were found between the results obtained in asthmatics at baseline and follow-up visits for any of the

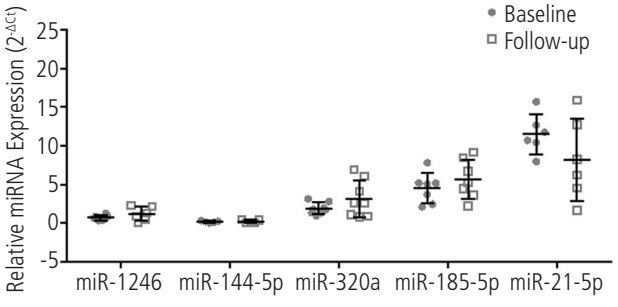

Figure. Expression of miRNAs by qPCR at baseline and at follow-up visits. Relative expression ($2^{-\Delta Ct}$) is shown as mean (SD).

miRNAs analyzed. The baseline expression values ($2^{-\Delta Ct}$) compared with the follow-up values were as follows: miR-1246, 0.72 (0.33) vs 1.21 (0.89) (*P*=.34); miR-144-5p, 0.18 (0.11) vs 0.22 (0.22) (*P*=.70); miR-320a, 1.89 (0.75) vs 3.14 (2.30) (*P*=.22); miR-185-5p, 4.50 (1.95) vs 5.70 (2.53) (*P*=.34); and miR-21-5p, 11.53 (2.59) vs 8.22 (5.32) (*P*=.19) (Figure).

The lack of significant differences between baseline and follow-up visits in asthmatic patients (whose therapy remained unchanged) could mean that the miRNAs remain stable over time in the same patient, with no change in therapy or clinical parameters. Our hypothesis is that changes in the expression of miRNAs in the same asthmatic patient over time could be due to spontaneous modifications in health status or new therapeutic interventions.

As expression of these miRNAs does not change in clinically stable asthma patients, we can deduce that their expression may prove useful for diagnosis when the circumstances of asthma are unchanged.

To our knowledge, this is the first study to show the stability of miRNAs over time in asthmatic patients in whom no changes were made to treatment and no clinical changes were observed. Stable miRNA expression implies that these biomarkers may be used for diagnosis of asthma at different time points during the disease course.

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Conflicts of Interest

JS reports having served as a consultant to Thermo Fisher, Novartis, Sanofi, Leti, FAES FARMA, Mundipharma, and GSK and having received lecture fees from Novartis, GSK, Stallergenes, LETI, and FAES FARMA. He has also received grant support for research from Thermo Fisher and ALK.

VDP reports having served as a speaker/consultant to AstraZeneca.

MJR, JMRM, and BS declare that they have no conflicts of interest.

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