Stability of asthma control implies no changes in microRNAs expression

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Asthma is a chronic disease that affects 4.3% of 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 clinical phenotypes responsible to specific treatments. 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, it has been reported that microRNAs (miRNAs) present in body fluids meet several of these criteria and are used as diagnostic markers in many areas [2-4]. Eosinophils, a central cell in asthma pathogenesis, have the ability to secrete exosomes. These structures contain miRNAs. MiRNAs are single-stranded RNA sequences (around 19-22 nucleotides) that do not code for proteins with crucial functions in the development and continuation of the pathogenic mechanisms of asthma, but can instead be implicated in asthma physiopathology by regulating the translation of proteins related to asthma processes [5]. MiRNAs can be encapsuled in exosomes or bound to proteins in biofluids and in both cases they present a high resistance to degradation by RNases [6,7]**.**

Released exosomes and miRNAs inside them have been found in serum; however, the precise role played in asthma has not been fully defined as well as its stability in the same patient over time [3-5].

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In order to establish if miRNAs expression remain steady over time in asthmatic patients, when their disease remains clinically stable, we selected 20 asthmatic patients from a national cohort (MEGA project) [8]. These subjects were recruited in the allergy department of the Fundación Jiménez Díaz University Hospital, (Madrid) in a randomized manner. Clinical and epidemiological characteristics are in Table 1 in supplementary material. Subjects were properly informed and signed informed consent. The study was conducted following the principles of the Declaration of Helsinki. The study was approved by Fundación Jiménez Díaz Ethics Committee. All selected patients presented a confirmed diagnosis of asthma with greater than 12% improvement in FEV1 15 minutes following administration of inhaled Salbutamol (400µg) or had methacholine airway hyperresponsiveness (PC20 methacholine <16 mg/mL) [9]. All of these patients had moderate persistent asthma and were treated with a combination of IC/LABA at medium doses (400 micrograms of budesonide and 12 micrograms of formoterol fumarate dihydrate daily or equivalent). No change was made in the treatment received for asthma during the period of study, from baseline to the follow-up visits. Serum was obtained by blood centrifugation, and stored at -80ºC before analysis for a period of time fewer than two years.

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

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The miRNAs that have been analyzed are the following: miR-320-a, miR-144-5p, miR-1246, miR-21-5p, and miR-185-5p. These miRNAs have been selected because in a previous published article, we found that these miRNAs profile in eosinophils can be used as asthma diagnosis biomarker in serum [10]. MiR-191-5p was measured as the endogenous control, and Sp2, Sp4, Sp5 and Sp6 were measured for evaluation of correct extraction and reverse transcription.

The statistical analysis was carried out with the GraphPad Instat® program, T-test for parametric samples (meet Gaussian distribution) and Mann Whitney for non-parametric samples. Paired tests were performed for baseline-follow-up comparisons.

As can be observed in Table I (supplementary files), asthma was stable over time, in terms of asthma control test (ACT) 21.1±3.7 *vs* 20.8±3.1; and lung function (FEV1%) 97.7±12.9 *vs* 97.5±13.9. Besides the stability in clinical parameters for these subjects no statistically significant differences were found between the results obtained in asthmatics at baseline and follow-up visits in any of the analyzed miRNAs. For miR-1246 baseline expression ($2^{-\Delta Ct}$) was 0.72±0.33 and follow-up expression was 1.21±0.89 ($P = 0.34$); for miR-144-5p baseline expression was 0.18±0.11 *vs.* 0.22±0.22 ($P = 0.70$); for miR-320a baseline expression was 1.89±0.75 *vs.* 3.14±2.30 ($P = 0.22$); baseline expression for miR-185-5p was 4.50±1.95 *vs.* 5.70±2.53 ($P = 0.34$), and finally miR-21-5p baseline expression was 11.53±2.59 vs. 8.22±5.32 at the follow-up visit ($P = 0.19$) as shown in Figure 1.

The lack of significant differences between baseline and follow-up visits in asthmatic patients (for whom no therapeutic changes were made) could mean that the miRNAs remain stable over time in the same patient, when no changes on therapy were made and clinical parameters are continuous. Our hypothesis is that potential changes in expression of miRNAs in the same asthmatic subject over time could be due to

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modifications in the health status of the individual spontaneously or after new therapeutic interventions.

As expression of these miRNAs does not change in clinically stable asthma patients we can deduce that the expression of this miRNAs may be used for diagnosis when asthma circumstances are unchanged.

To our knowledge, this is the first study showing the stability of miRNAs over time in asthmatic subjects to whom no changes were made in their treatment and no clinical changes were observed. MiRNAs stable expression implies that they may be used for asthma diagnosis at different time points of their controlled disease.

Conflict of interest:

JS reports having served as a consultant to Thermofisher, Novartis, Sanofi, Leti, FAES FARMA, Mundipharma, and GSK; having been paid lecture fees by Novartis, GSK, Stallergenes, LETI, and FAES FARMA; and received grant support for research from Thermofisher and ALK.

VDP reports having served as a speaker/consultant to Astra-Zeneca.

MJR, JMRM and BS declare no conflicts of interest.

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Figure 1. Expression of miRNAs by qPCR at baseline and follow-up visits. Relative expression ($2^{-\Delta Ct}$) is shown as the mean±SD.