

## ORIGINAL ARTICLE

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# In Vitro Analysis of Nine MicroRNAs in CD8+ T Cells of Asthmatic Patients and the Effects of Two FDA-approved Drugs

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## ABSTRACT

In this study, we first tried to determine whether the expression level of 9 miRNAs in the peripheral blood CD8+ T cells of asthmatic patients varies from that of controls, and secondly, we investigated the effects of fluticasone furoate and vilanterol on the expression level of these miRNAs.

Fifteen subjects including 8 healthy individuals and 7 asthmatic patients were included in this study. CD8+T cells were isolated from participants' peripheral blood by a negative selection method using magnetic-activated cell sorting (MACS). The expression of 9 miRNAs was examined between the healthy individuals and asthmatic patients. Then the expression level of 9 miRNAs before and after treatment with the drugs was examined by quantitative real-time PCR.

No significant changes in the expression level of 9 miRNAs were observed in asthmatic patients compared to the healthy controls. Fluticasone and vilanterol, in combination, had the greatest effect on miRNA expression. MiR-150 and miR-106a were the most and the least miRNAs, respectively, present in CD8+ T cells of patients and controls. MiR-106a and miR-126 had a positive correlation in CD8+ cells of asthmatic patients.

Although no significant difference in the expression level of studies miRNAs was observed, the correlations among miRNAs were significant. Therefore, we suggest that the correlation between miRNAs would be a very important factor in physiological and pathological conditions in healthy individuals and asthmatic patients. Such a miRNA-miRNA correlation network can be even more critical than any changes in the variation of their expression in the CD8+ T cells.

**Keywords:** CD8+ T cells; Fluticasone furoate; MicroRNAs; Vilanterol

## INTRODUCTION

Asthma is a chronic lung disease characterized by hyper-responsiveness, high secretion of mucus and inflammation of the airways. Asthma patients

experience wheeze, shortness of breath, chest tightness and cough. The disease affects 300 million people across the world.<sup>1</sup> The vast majority of research has addressed the role of the most important lymphocytes, i.e. CD4+ T cells, involved in asthma pathogenesis; however, another type of lymphocyte called cytotoxic CD8+ T cells have been also shown to play roles in the disease.<sup>2</sup> Although there are few studies on CD8+ T cells in asthma,<sup>3</sup> it is well established that CD8+ T cells are present in airways of patients with asthma.

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Moreover, CD8+ T cells have been isolated from bronchial biopsies of asthmatic patients.<sup>4</sup> There are controversial aspects regarding the role of CD8+ T cells in asthma including the triggering or suppressing the inflammation conditions.<sup>5</sup> Hence, CD8+ T cells are expected to possibly play the same important roles as CD4+ T cells in the pathogenesis of asthma. Peripheral blood CD8+ T cells have been shown to produce T helper type 2 cytokines in patients with asthma in comparison to healthy subjects in both adult<sup>6</sup> and children population.<sup>7</sup>

MicroRNAs (miRNAs) are small non-coding RNAs that regulate the expression of protein-coding genes as post-transcriptionally and act on their target mRNAs.<sup>8</sup> These are believed to adjust the expression of more than a half of all human genes.<sup>9</sup> Several miRNAs have been recognized to be differently expressed in different diseases including asthma.<sup>10</sup> In this study, nine miRNAs involved in asthma pathogenesis were selected for investigation in peripheral blood CD8+ T cells, which include: miR-21, miR-106a, miR-126, miR-146a, miR-150, miR-155, miR-181a, miR-221, and miR-223. The miRNAs selection was based on some publications indicating the role of these miRNAs in asthma.<sup>11-15</sup> These studies had evaluated the mentioned miRNAs in different cells including lower airway cells, epithelial cell specimens, peripheral CD4+, and CD8+ T cells..

Dudda et al showed that miR-155 has been increased proportionally to the strength of signaling via CD8+ T cell receptors (TCR).<sup>16</sup> miR-155 regulates the functions of CD8+ T cells via TCR signaling, increasing responses against viruses. In two other studies, the down-regulation of miR-21 and miR-155 resulted in down-regulation of inhibitors of TCR signaling including SOCS1, DUSP10, and BCL6.<sup>17,18</sup> Two miRNAs miR-150 and miR-223 have been shown to increase the proliferation and the activation of CD8+T cells. In contrast, miR-146a has a negative effect on CD8+ T cell activation.<sup>19</sup> The expression of miR-181a is decreased as CD8+ T cells become mature and is associated with decreased TCR sensitivity to antigens.<sup>20</sup> The role of some selected miRNAs such as miR-106a, miR-126 and miR-221 in CD8+ T cells have not been identified; however, different studies reported their increased expression in asthma.<sup>14,21,22</sup>

The expression levels of miRNAs in a population can differ from other populations.<sup>23</sup> Therefore the

expression of miRNAs in CD8+ T cells from asthmatic patients can also be different in Iran. The present study aimed to: 1) to determine whether the selected miRNAs show any changes in CD8+ T cells taken from asthmatic patients compared to those of healthy controls, 2) to examine the effects of two drugs used for treatment of asthma, including fluticasone furoate and vilanterol, on the expression level of these miRNAs in CD8+ T cells from asthmatic patients and healthy controls and 3) to evaluate the correlation of miRNAs in asthma and healthy conditions.

## MATERIALS AND METHODS

### Participants

A total of 15 participants (10 males and 5 females) entered this study. Seven of them were patients with moderate asthma and 8 were non-asthmatic healthy controls. The most important manifestations of the disease were as follows: cough, chest tightness, wheezing and shortness of breath. As shown in Table 1, asthmatic patients took corticosteroid (budesonide, prednisone or beclomethasone) in combination with a short- (salbutamol) or long-acting (salmeterol or formoterol) beta 2 agonist continually, but not the drugs, i.e. fluticasone furoate and vilanterol studied in this research. The inclusion criterion was the diagnosis of asthma by a physician, based on spirometry and questionnaire. Our exclusion criterion was chronic obstructive pulmonary disease (COPD) and other respiratory diseases in patients. This study was approved by Immunology, Asthma and Allergy Research Institute's Ethics Committee. Informed written consent was obtained from all patients and controls. Type of asthma was determined according to guidelines of Global Initiative for Asthma (GINA) along with physician's confirmation.

### Isolation of CD8+ T Cells by MACS

30 mL of heparinized peripheral blood samples were obtained from patients and non-asthmatic healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Paque gradient (Cedarlane, Canada) and centrifuged. The resulting pellet was used to isolate CD8+ T cells using magnetic-activated cell sorting (MACS) by negative selection. CD8+ T cell isolation was done according to the manufacturer's instructions. Briefly, two

antibody sets (Miltenyi Biotec, Germany) were added to the pellet. The first set was a cocktail of biotin-conjugated antibodies to bind CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCR $\gamma/\delta$  and glycophorin A positive cells. Then the second microbead-conjugated antibody was added to the cells in order to bind both biotin-conjugated antibodies and the LS column used for MACS (Miltenyi Biotec, Germany). Finally, isolated cells were counted using a hemocytometer. The viability and the purity of the cells were >99% and >95% respectively.

#### **Treating the CD3/CD28-Stimulated TCD8+ cells with Fluticasone Furoate (FF) /Vilanterol (VI)**

An equal number of CD8+ T cells (approximately  $2 \times 10^6$  cells) were poured into 4 wells of a 24-well plate coated by anti-CD3. These 4 wells were named as "without treatment (W/O.T)", "treatment with fluticasone furoate (FF)", "treatment with vilanterol (VI)" and "treatment with both (FFV)". This procedure was performed for CD8+ T cells isolated from both asthmatic patients and healthy controls. In order to uptake drugs, the cells must be stimulated and activated. Therefore, TCD8+ cells were stimulated with coated anti-CD3 and soluble anti-CD28 (eBioscience, USA). The plate was placed in an incubator (5% CO<sub>2</sub>, 37°C) for 18 hours. After this period, the concentration of  $10^{-7}$   $\mu\text{g}/\text{mL}$  for fluticasone furoate and vilanterol were prepared in phosphate-buffered saline (PBS) and added to respective wells. The plate was incubated in 5% CO<sub>2</sub> at 37°C for an additional 4 hours.

#### **3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay**

To evaluate the toxicity of fluticasone furoate and vilanterol on CD8+ T cells, MTT test was performed. The CD8+ cells were cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) and 10  $\mu\text{L}$  anti-CD28 in 96 well culture plates coated by anti-CD3 at 10000 cells per well and incubated in 5% CO<sub>2</sub> at 37°C. After 18 hours, the concentration of  $10^{-7}$   $\mu\text{g}/\text{mL}$  fluticasone furoate and vilanterol dissolved in PBS were added to the cultured CD8+ T cells for 4 hours. Then 10  $\mu\text{L}$  MTT was added to the cells. 4 hours later, the plate was centrifuged and then the supernatant was removed. In this step, formazan crystals had been formed. In order to solubilize the crystals, 100  $\mu\text{L}$  of dimethyl sulfoxide (DMSO) was poured in all wells. Finally, using a plate reader, the absorbance was

measured at 570 nm. The absorbance values of the untreated sample were considered as 100% and other absorbance values were expressed according to those values. No cytotoxic effects of fluticasone furoate and vilanterol on CD8+ T cells were observed (data not shown).

#### **RNA Extraction and cDNA Synthesis**

After incubation, the cells were harvested for RNA extraction. According to miRCURY RNA Isolation Kit protocol (Exiqon, Germany), total RNA was extracted from all samples. A nanodrop spectrophotometer (Thermo Scientific, USA) was used to determine the concentrations of extracted RNAs. For cDNA synthesis, 2  $\mu\text{g}$  RNA samples were used. Two steps, polyadenylation and reverse transcription of RNAs into cDNA were performed using BONmiR miRNA 1st-Strand cDNA Synthesis Kit (Stem Cell Technology, Iran). RNA polyadenylation was done in 20 reactions by poly (A) polymerase enzyme at 37°C for 30 min. One-half of polyadenylation reaction products were used to synthesize cDNA. Polyadenylated RNAs were reverse transcribed in 20- $\mu\text{L}$  reactions using oligo dT as primer according to the manufacturer's protocol.

#### **Evaluation of miRNAs by Quantitative PCR**

Forward primers of 9 miRNAs and general reverse primer were purchased (Stem Cell Technology, Iran). The qPCR reaction comprised of 5  $\mu\text{L}$  SYBR green PCR 2X master mix (Ampliqon, Denmark), 200 ng template cDNA, 2.5  $\mu\text{M}$  forward primer, and 2.5  $\mu\text{M}$  reverse primer that reached a final volume of 10  $\mu\text{L}$  with water. The expression of miRNAs was normalized to RNU44 (a small nucleolar RNA/a housekeeping gene). The PCR reaction was done with the following program: a cycle at 95°C for 15 min, followed by 45 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 15 s. To verify whether nonspecific products are present at the end of reaction, the melting curve analysis was performed with a cycle at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Analysis of real time data was performed using the comparative CT method ( $\Delta\Delta\text{CT}$  method).

#### **Statistical Analysis**

Data management and analysis were performed using IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA). Due to the non-

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normalized distribution of data, nonparametric tests were used to compare different groups. The comparison of the expression level of miRNAs between the normal and patients groups was done using the Mann-Whitney U test. The correlation between all miRNAs was analyzed using the Spearman test. The different expression levels of miRNAs among untreated samples and treated ones (before and after the treatments) were compared using the Wilcoxon signed-rank test. The graphs were plotted with GraphPad Prism 6 software (California, USA). The significant level was set at a *p*-value <0.05.

### RESULTS

#### Patients' Characteristics

Characteristics of all the participants in the study are shown in Table 1. All patients except one had unrelated parents. Four patients with asthma had a history of smoking but they had given up smoking prior to the current study. Healthy controls did not have any history of shortness of breath or wheezing. They did not take any medications. They also were not an active or passive smoker.

**Table 1. Characteristics of patients with moderate asthma and non-asthmatic controls in a study on nine MicroRNAs in CD8+ T cells**

|                                   | Non-asthmatic healthy controls | Patients with Moderate Asthma  |
|-----------------------------------|--------------------------------|--|
| No.                               | 8                              | 7  |
| Age (year), mean±SD               | 39.38±12.25                    | 45.29±19.96  |
| Sex, (Male/Female)                | 4/4                            | 6/1  |
| BMI (kg/m <sup>2</sup> ), mean±SD | 23.91±4.17                     | 23.75±4.28   |
| FEV1 (%), mean±SD                 | ND                             | 69.85±11.56*   |
| FVC (%), mean±SD                  | ND                             | 87.28±16.93*   |
| FEV1/FVC ratio (%), mean±SD       | ND                             | 85.00±14.45*   |
| Smoker/Non-smoker                 | 0/8                            | 4/3  |
| Medications                       | ND                             | Salbutamol, Prednisone (P1), Salbutamol, Beclomethasone, Mometasone furoate (P2), Salbutamol, Beclomethasone (P3, P5), Salmeterol, Formoterol, Budesonide (P4), Formoterol, Budesonide (P6), Budesonide, Formoterol (P7) |

ND=Not Done, BMI=Body Mass Index, FEV1= Forced expiratory volume in 1 second, FVC=Forced vital capacity, SD=Standard Deviation, \*=>80% predicted is considered normal, P1 to P7=Patients P1 to P7.

#### The Expression of 9 miRNAs Was not Altered in CD8+ T Cells of Asthmatic Patients Compared to Healthy Controls

The expression of 9 miRNAs was analyzed by qPCR in CD8+ T cells taken from each participant. miR-146a was the only miRNA with decreased expression in the asthmatic patient group, but insignificantly (*p*=0.093). Eight other miRNAs were not differentially expressed in CD8+ T cells from patients with asthma compared with non-asthmatic healthy controls. The results can be seen in Figure 1.

#### The Highest and the Lowest Expression of miRNAs in Asthmatic Patients and Healthy Controls

Figure 2 indicates that miR-150 was a miRNA with high expression in circulatory CD8+ T cells in both patients and controls. In contrast, miR-106a was expressed at a very low level. Three miRNAs, miR-155, miR-21 and miR-223, were expressed at intermediate levels. They were expressed less than miR-150 but more than the expression of 5 other miRNAs.

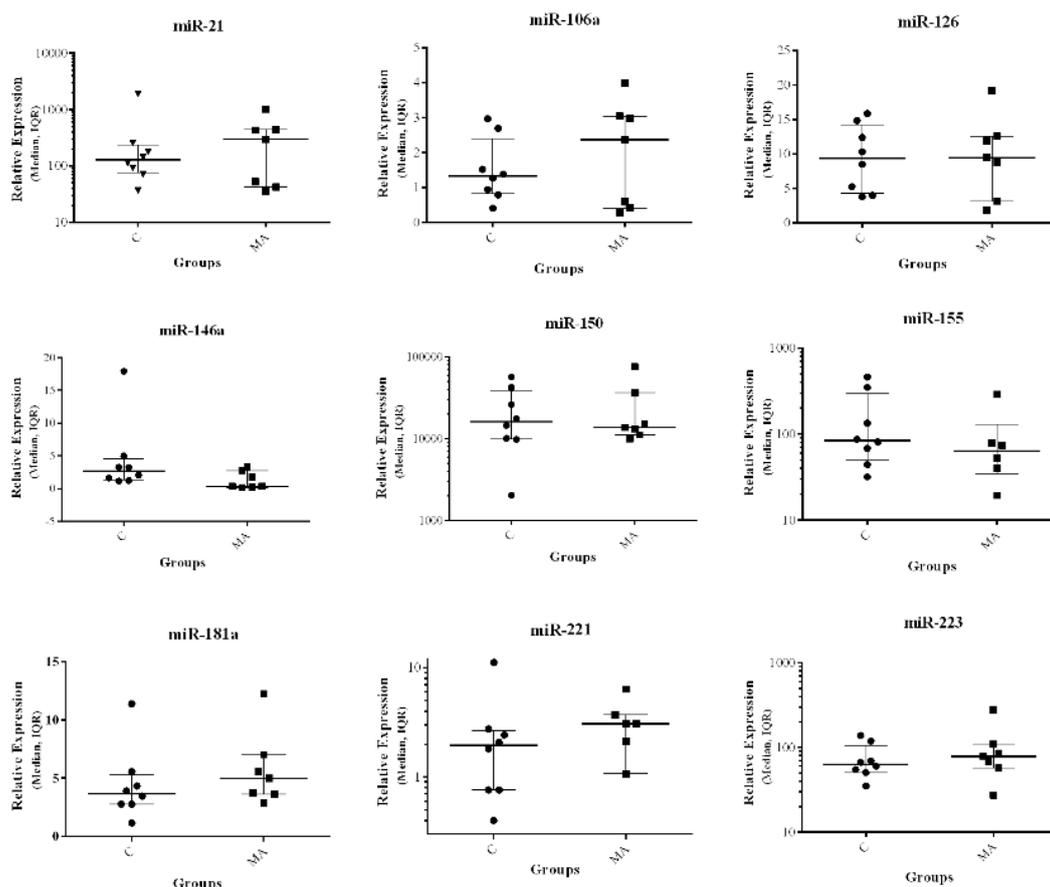


Figure 1. miRNA expression in CD8+ T cells of asthmatic patients compared to that of healthy controls. No significant changes was found in the expression of 9 miRNAs in patients compared to controls. C=control, MA=moderate asthma, IQR=interquartile range.

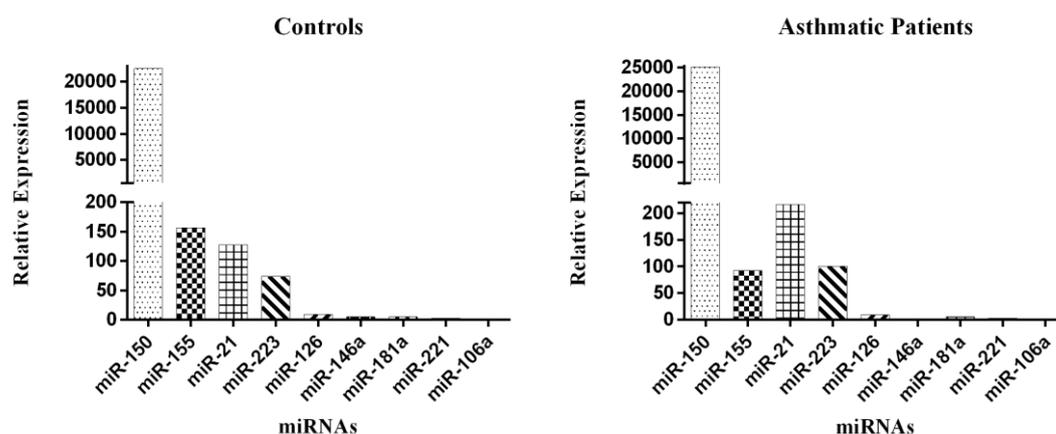


Figure 2. The relative expression of 9 miRNAs in circulatory CD8+ T cells taken from asthmatic patients and controls. miR-150 was highly expressed in both groups. The expression of miR-106a and miR-221 was very low. miRNAs miR-155, miR-21, and miR-223 were expressed at an intermediate level in proportion to the expression of other miRNAs.

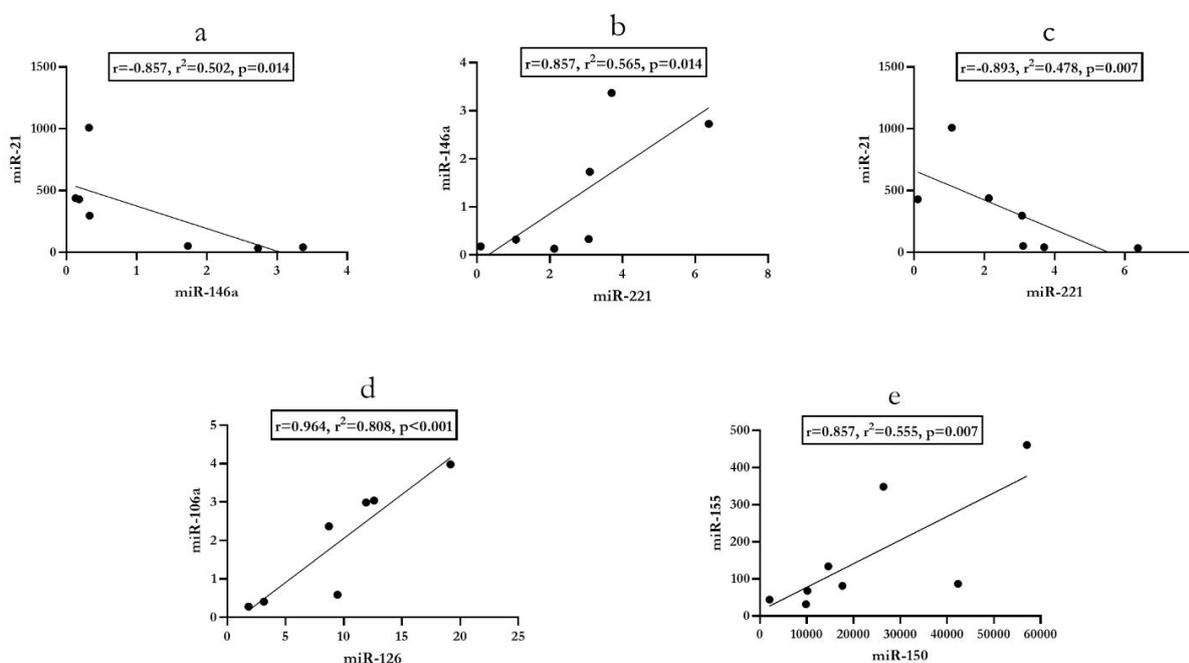
**Correlations between miRNAs in Patients and Controls**

Correlations between miRNAs are shown in Figure 3. In the control group, a positive correlation ( $p < 0.01$ ) between miR-150 and miR-155 was observed. In asthmatic patients, 4 types of correlations were found: a negative correlation ( $p < 0.05$ ) between miR-21 and miR-146a, a negative correlation ( $p < 0.01$ ) between miR-21 and miR-221, a positive correlation ( $p < 0.001$ ) between miR-106a and miR-126, a positive correlation ( $p < 0.05$ ) between miR-146a with miR-221.

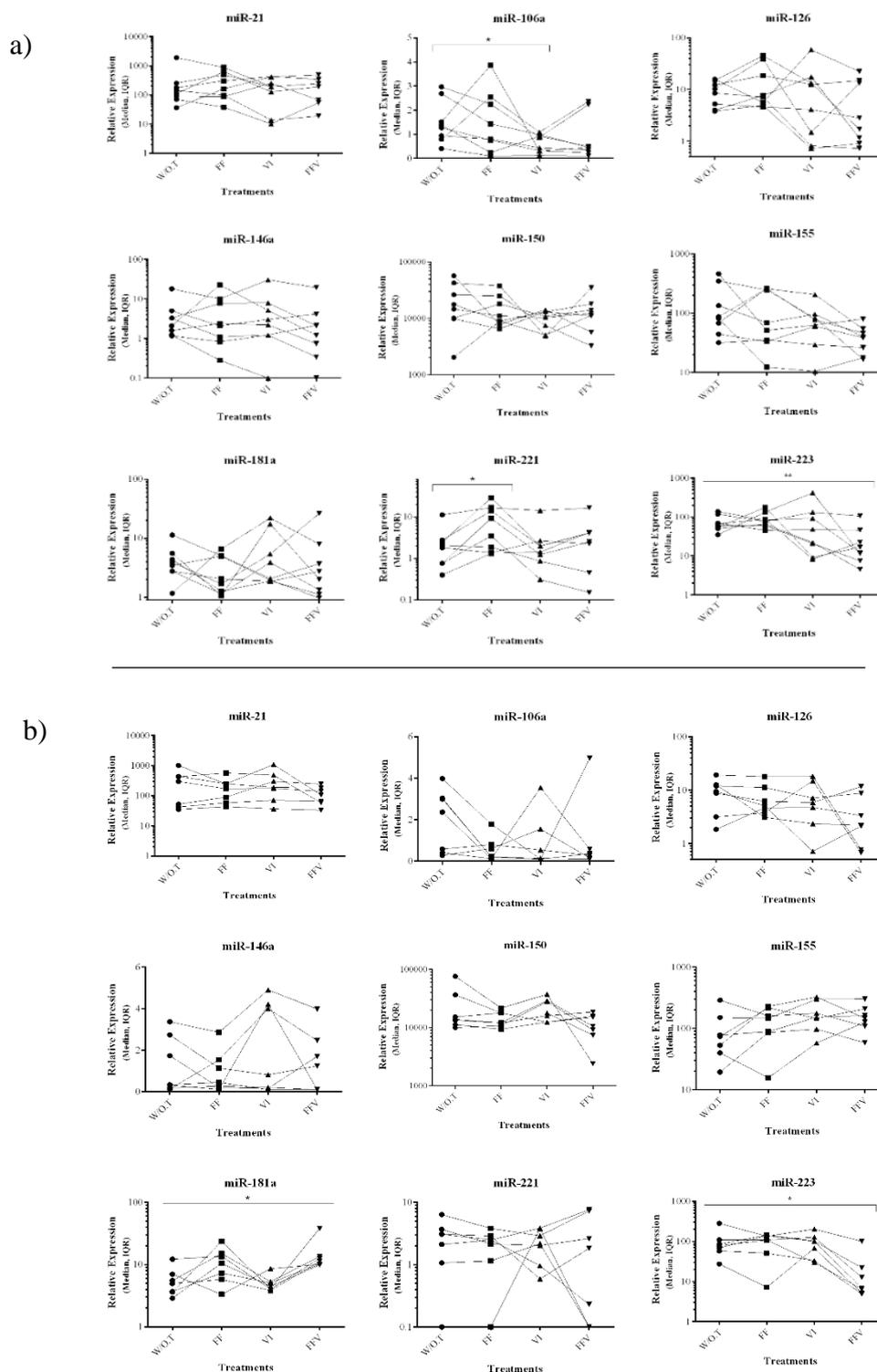
**Down-regulation of miR-223 following Exposure to Fluticasone Furoate and Vilanterol in Asthmatic Patients and Healthy Controls**

Figure 4 shows the effects of fluticasone furoate

and vilanterol on the expression of miRNAs in asthma and control groups. In the control group (Figure 4a) vilanterol decreased miR-106a expression; however, it had no impact on the expression levels of the 8 other miRNAs. Fluticasone furoate led to increased expression of miR-221 compared to that of untreated cells. The combination of two drugs resulted in the down-regulation of miR-223. In the asthma group (Figure 4b), it seems fluticasone furoate and vilanterol, alone, did not affect the expression of 9 miRNAs of CD8+ cells collected from asthma patients (as shown in Table 2). In this group, miR-181a expression was decreased by fluticasone furoate and vilanterol when they were used in combination. Therefore, the two drugs in combination down-regulated the expression of miR-223 both in the asthma and control groups.



**Figure 3.** the correlation between miRNAs in Asthma (a-d) and Control Group (e) in a study on nine MicroRNAs in CD8+ T cells. Rx=Spearman rank correlation coefficient, p=p value



**Figure 4. The effects of fluticasone furoate and vilanterol on miRNA expression in Control group (a) and Asthma group (b). W/O.T= without treatment, FF=fluticasone furoate, VI=vilanterol, FFV= fluticasone furoate and vilanterol simultaneously, IQR=interquartile range, \* $p < 0.05$ , \*\* $p < 0.01$ . Up-regulation of miR-221 by FF, down-regulation of miR-106a by VI and down-regulation of miR-223 by FFV was observed in the control group. Up-regulation of miR-181a and down-regulation of miR-223 by FFV was observed in the asthma group.**

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**Table 2. Expression of 9 miRNAs in CD8+ cells collected from asthma and control group; receiving treatment with fluticasone furoate ( FF)", "treatment with vilanterol ( VI)", "treatment with both ( FFV) fluticasone furoate and vilanterol “ and those without treatment**

| Moderate Asthma Group | Control Group | Treatments         | miRNAs   |
|-----------------------|---------------|--------------------|----------|
|                       |               | No treatment       | miR-21   |
| 0.6875                | 0.6406        | Treatment with FF  |          |
| 0.6875                | 0.9453        | Treatment with VI  |          |
| 0.2188                | 0.9453        | Treatment with FFV |          |
|                       |               | No treatment       | miR-106a |
| 0.1094                | 0.9959        | Treatment with FF  |          |
| 0.3750                | 0.0234        | Treatment with VI  |          |
| 0.2188                | 0.1484        | Treatment with FFV |          |
|                       |               | No treatment       | miR-126  |
| 0.2188                | 0.3125        | Treatment with FF  |          |
| 0.5781                | 0.8438        | Treatment with VI  |          |
| 0.1094                | 0.4609        | Treatment with FFV |          |
|                       |               | No treatment       | miR-146a |
| 0.3750                | 0.8438        | Treatment with FF  |          |
| 0.6875                | 0.4609        | Treatment with VI  |          |
| 0.8125                | 0.1953        | Treatment with FFV |          |
|                       |               | No treatment       | miR-150  |
| 0.2188                | 0.3828        | Treatment with FF  |          |
| 0.8125                | 0.1484        | Treatment with VI  |          |
| 0.4688                | 0.3125        | Treatment with FFV |          |
|                       |               | No treatment       | miR-155  |
| 0.4688                | 0.6406        | Treatment with FF  |          |
| 0.1563                | 0.3828        | Treatment with VI  |          |
| 0.2969                | 0.0078        | Treatment with FFV |          |
|                       |               | No treatment       | miR-181a |
| 0.1094                | 0.2500        | Treatment with FF  |          |
| 0.6875                | 0.7578        | Treatment with VI  |          |
| 0.0156                | 0.6406        | Treatment with FFV |          |
|                       |               | No treatment       | miR-221  |
| 0.2188                | 0.0391        | Treatment with FF  |          |
| 0.6875                | 0.6406        | Treatment with VI  |          |
| 0.5625                | 0.1094        | Treatment with FFV |          |
|                       |               | No treatment       | miR-223  |
| 0.9375                | 0.7422        | Treatment with FF  |          |
| 0.9375                | 0.2500        | Treatment with VI  |          |
| 0.0156                | 0.0078        | Treatment with FFV |          |

## DISCUSSION

In this study, we did not observe any changes in the expression of 9 miRNAs in patients with moderate asthma compared to control group. Few studies have addressed the expression profile of miRNAs in CD8+ T cells in asthma. However, the role of numerous miRNAs has been explored in the immune system.<sup>24,25</sup> In a study by Tsitsiou et al down-regulation of two miRNAs, miR-28-5p and miR-146a, was observed in CD8+ T cells taken from asthmatic patients compared to those of control subjects.<sup>15</sup> We showed the down-regulation of miR-146a in CD8+ T cells of patients with asthma compared to that of controls, but the decrease was not statistically significant.

In addition, there is some evidence for the different role of miR-146a in CD8+ differentiation while its expression differs among T cell subsets.<sup>13</sup> Tsitsiou et al did not report any changes in the expression of miR-21, miR-126, miR-155 and miR-181a in CD8+ T cells from patients with asthma.<sup>15</sup> These findings are in accordance with ours. miR-155 has a key role in regulating immune responses in T cells. Upon CD8+ T cell activation the expression of miR-155 is increased which results in their expansion. This increase in the levels of miR-155 leads to different functions of CD8+ T cells at different stages of differentiation.<sup>12</sup>

In a study by Wu et al the expression of miR-21 has been shown to be higher in effector CD8+ T cells than naïve ones, suggesting its important role in the effector phase.<sup>26</sup> In agreement, we found higher expression of miR-21 in both patients and controls. A large number of miRNAs have been reported to express in all subsets of T cells, but some are highly expressed, including 7 miRNAs let-7f, miR-15b, -16, -21, -142-3p, -142-5p, and miR-150.<sup>26</sup> These differences in the expression of miRNAs have previously been reported in a different population.<sup>23</sup> Although in the current study no significant change has been seen in the expression of 9 miRNAs in CD8+ T cells of asthmatic patients compared to normal subjects, we showed that miR-150 was the most expressed miRNA in CD8+ T cells. The expression of miR-150 alone accounted for ~50 fold higher than other 8 miRNAs. This finding was not in accordance with that of Wu et al because in our study miR-155 had higher expression levels than that of miR-21. miR-106a and miR-221 were miRNAs with the least expression in both control and patient groups. The expression change in miRNAs miR-106a, -126, -221 and -223 in CD8+ T cells from asthmatic patients

compared to healthy controls has not been reported in previous studies.<sup>15</sup> These results were confirmed in our study. In the current study, we could not find any difference of miR-221 between asthmatic patients and healthy controls. A study by Liu et al reported an increase in miR-221 and -485-3p in peripheral blood lymphocytes in pediatric asthma patients compared to those in controls.<sup>14</sup> Another study by Qin et al in the lung biopsy of an ovalbumin-induced murine asthma model reported similar results.<sup>11</sup> However, this finding has yet to be investigated in CD8+ T cells of animal models.

The second part of the present study aimed to examine the effects of two drugs, fluticasone furoate and vilanterol, on the expression level of 9 miRNAs in both asthma patients and healthy controls. To our knowledge, no study has yet to address the effects of glucocorticoids on miRNA expression in CD8+ T cells. Vilanterol, a long-acting beta2 agonist, in combination with fluticasone furoate, an inhaled corticosteroid (ICS), was approved by FDA at the dose of 100 and 25 µg, respectively, for the treatment of COPD and asthma, though both drugs individually seem to have efficacy in controlling asthma.<sup>27</sup> In the present study, these two drugs, individually or in combination, did not have any effects on the expression of 5 miRNAs miR-21, -126, -146a, -150, and -155. Fluticasone furoate gave rise to an increase in miR-221 expression in the healthy group, but not in asthmatic patients. This increase in miR-221 in asthma group can be due to the resistance of CD8+ T cells from asthmatic patients rather than those in healthy controls. We did not expect to observe that because down-regulation of miRNA-221 has been reported to decrease airway inflammation in murine asthma models.<sup>11</sup> Vilanterol resulted in a decrease in the expression of miR-126 in controls, but it did not affect patients with asthma. In a study by Wu et al up-regulation of miR-126 was associated with asthma; therefore, down-regulation of this miRNA can result in improvement of asthma symptoms.<sup>28</sup> In our study, the combination of two drugs (FFV) led to a decrease in the expression of miR-223 in both groups and up-regulation of miR-181a in asthmatic patients. miR-223 up-regulation has been shown in different studies on asthma<sup>29,30</sup> and this down-regulating can be associated with decrease of inflammation and asthma. However, we suggest that these drugs play role in controlling the expression of miRNAs, probably via those other miRNAs not studied here. Given that some

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of the results, i.e. increase or decrease of miRNA expression, have been observed only in one of our study groups, the effects of the drugs on the level of other miRNAs in CD8+ cells needs to be further explored.

Correlations between the expressions of 9 miRNAs in CD8+ T cells from controls differed from those from asthmatic patients, which means that the phenotype of CD8+ T cells might be different in controls compared to asthma patients. On the other hand, these results determine which miRNAs function together in similar pathways and which have different pathways. MiR-21 expression was inversely correlated with miR-146a and miR-221 expression levels in CD8+ T cells. The negative correlation between miR-21 and miR-146a seems logical, since miR-21 targets IL12p35 mRNA which inhibits the differentiation of Th2, and miR-146a has been reported to be down-regulated in CD8+ T cells in asthma.<sup>15</sup> The expression level of miR-106a was strongly correlated to that of miR-126, suggesting that they might have similar functions. Suppressing miR-106a or miR-126 has been shown to alleviate the hallmark features of asthma including airway hyper-responsiveness and inflammation.<sup>31</sup> Considering that miRNA can regulate a large number of target mRNAs,<sup>32</sup> a disease's conditions may change the default pathway of a miRNA in special cells or tissues. However, these correlations need to be further studied.

We had two main limitations in the current study one of which was selecting 9 miRNAs instead of studying miRNA profiling involved in asthma. In order to collect more details about the role of circulatory CD8+ T cells in the pathogenesis of asthma, it is necessary to study more miRNAs with known roles in asthma. The other limitation was that we did not conduct the study of miRNA expression after patients' taking two drugs fluticasone furoate and vilanterol.

Our study suggests that there is no difference in the expression of the 9 selected miRNAs in peripheral CD8+ T cells of patients with asthma and control group. However, these miRNAs can form a miRNA network that cooperates together to affect the pathogenesis of asthma. CD8+ T cells from Iranian patients and healthy controls had a distinct pattern of miRNA expression with high expression of 4 miRNAs miR-150, miR-155, miR-21, and miR-223. This finding suggests that every population may have a distinct miRNA expression pattern. On the other hand, the drugs used by asthmatic patients can modulate the

expression of miRNAs by their down- or up-regulation. This will help characterize the exact mechanisms of action of the drugs prescribed for the treatment of asthma on the expression of miRNAs.

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