Expression level of micro-RNA-1, IL-5, IFNγ, and TNFα in adult-onset asthmatic patients
Shaimaa M. Abo-Youssef, Amira H. Allam, Mysa S. Mustafa, Hasnaa S. Abd el Hamid, Amany K. Shahat, Hany H. Moussa

Background and objective
Asthma is an inflammatory process, where T lymphocytes cells, mainly the Th1/Th2 cells, participate in bronchial asthma’s pathogenesis and progression. Studying the inflammatory and genetic mediators’ expression levels, which control this inflammatory process like microRNAs, is essential to illustrate this disease’s pathogenesis. This work aimed to evaluate the levels of miRNA-1 and inflammatory mediators interleukin (IL)-5, interferon-γ (IFNγ), and tumor necrosis factor (TNFα) in the peripheral blood of adult-onset asthmatic patients and their correlation to the disease severity.

Patients and methods
A case–control study was carried out on 69 participants, divided over two groups: group I was the asthmatic group, which included 49 patients with adult-onset bronchial asthma more than 18 years old, subdivided to group IA, with mild asthma (10 patients), group IB, with moderate asthma (19 patients), and group IC, with severe asthma (20 patients). Group II was the control group, which included 20 healthy individuals. IL-5, TNFα, and IFNγ were measured by enzyme-linked immunosorbent assay (ELISA) and relative quantification of MiRNA-1 expression was done by PCR.

Results
miRNA-1 decreased significantly in asthmatics and decreased more with increasing severity of the disease, with a significant positive correlation between miRNA-1 and both forced expiratory volume in first second and IFNγ, and meaningful negative relationship between miRNA-1 and both IL-5 and TNFα. The sensitivity and specificity of miRNA-1 in diagnosing asthma and detecting its severity were 100%.

Conclusion
miRNA-1 expression level is significantly decreased in adult-onset asthmatic patients. It decreases with increasing severity of the disease, with a positive correlation between miRNA-1 and plasma IFNγ but a negative relationship between miRNA-1 and both plasma IL-5 and TNFα.

Keywords:
asthma, interferon-γ, interleukin-5, micro-RNA-1, tumor necrosis factor-α

Introduction
Asthma is a serious chronic inflammatory disease that causes recurrent attacks of respiratory symptoms, with activity limitation that sometimes requires urgent health care and may be fatal. The definitive diagnosis of asthma recommended a history of respiratory symptoms consistent with asthma, integrated with the confirmation of variable expiratory airflow obstruction [1]. Physiologically, asthma is characterized by bronchial hyper-responsiveness and pathologically described as ‘a chronic airway inflammatory disorders’ [2].

Asthma is usually misdiagnosed or overlooked in adults, especially in older people aged 65 years or older [3–5]. It varies from childhood asthma, as it is less well-controlled, combined with a faster decline in lung function, and more probably nonatopic [6,7].

It is usually associated with an external trigger (e.g. occupational or pharmaceutical agents, infection, and aeroallergens) or relapsing of childhood asthma. Adult-onset asthma is prevalent now because of the growing longevity of the general population [8].

Recent advances in recognizing the biologic features of asthma and its management did not explain the continuity of documented suboptimal control in a high percentage of patients worldwide [9]. T cells, especially the Th1/Th2 cells, participate in the pathogenesis and progression of bronchial asthma. The balance between...
Th1 and Th2 cells function is essential in immune response and control of infection, where Th1 cells produce interleukin (IL)-2 and interferon-γ (IFN-γ), and Th2 cells produce IL-4, IL-5, IL-10, and IL-13. Th2 cell overfunction is nearly combined with airflow limitation, mucosal inflammation of airways, and hyper-responsiveness. Studying the inflammatory and genetic mediators’ expression levels like microRNAs, which adjust them, is essential to understanding it [10].

Tumor necrosis factor (TNF)-α is a proinflammatory cytokine that has been linked to many airway pathology features in asthma and has been spotlighted as a possible crucial component in refractory asthma. The generation of neutralizing biological agents against TNF-α has granted us to test the role of these cytokines in vivo. Primary research studies have displayed improvement in lung function, airway hyper-responsiveness, and asthma quality-of-life, combined with downsizing in the frequency of exacerbation in patients treated with anti-TNF-α therapy [11].

Different cell types, mainly T cells, eosinophils, mast cells, monocytes/macrophages, and epithelial cells, release NF-α, which sustains lung inflammatory responses by increasing adhesion expression molecules. The inflammatory process causes accumulation of neutrophils and eosinophils and their activation by TNF-α, which triggers cytotoxic mediators and toxic products of reactive oxygen and nitrogen release, causing further damage to the airways. TNF-α induces fibroblasts activation and proliferation, subepithelial fibrosis, extracellular matrix glycoproteins, and goblet cell metaplasia with subsequent airway remodeling [12].

MiRNAs are 19–25 nucleotides single-stranded RNA molecules that adjust post-transcriptional gene silencing of target genes and are expressed in a variety of cells. MiRNAs were discovered in Caenorhabditis elegans in 1993 as silencers of genes that change developmental timing [13]. After that, miRNAs were known as a distinct class of small regulatory RNAs in multiple species that adjust a variety of functions like cell proliferation, differentiation, apoptosis, stress response, and immune response [14].

MiRNAs combined with the 3′ untranslated region of target mRNA cause direct suppression of gene expression. Hundreds of genes can target a single miRNA, and multiple miRNAs can typically target individual genes. Global effects by MiRNAs could be exerted on gene expression by either affecting epigenetic mechanisms or targeting transcription factors [15]. Therefore, miRNAs are a promising group of molecules that may control the allergic inflammatory processes [16].

The circulating miRNAs are relatively stable molecules that could be detected in serum and plasma samples; they present either by existing in cell membrane-derived vesicles such as exosomes or form a complex with lipid-protein to be protected from degradation by blood RNases. These circulating miRNAs could be ideal blood biomarkers owing to their disease-specific dysregulation and relative stability compared with mRNAs [17]. This study evaluates the levels of miRNA-1 and inflammatory factors IL-5, IFNγ, and TNFα in the peripheral blood of adults with acute stage bronchial asthma and their relation to the severity of the disease.

Patients and methods
A case–control study was carried out in Benha and Kafrelsheikh University hospitals from January 2019 to January 2020 on 69 individuals, divided into two groups: group I included 49 patients with acute stage bronchial asthma (42 females and seven males, and their age ranged from 25 to 65 years), which was subdivided into group IA, with mild asthma (10 patients); group IB, with moderate asthma (19 patients); and group IC, with severe asthma (20 patients), whereas group II included 20 healthy individuals as a control group. Ethical Committee approval from Benha and Kafrelsheikh University hospitals was taken, and a written consent from all participants was received before starting the study.

Inclusion criteria for the group I were stable asthmatic patients more than 18 years old with airway obstruction [forced expiratory volume in first second (FEV1)/forced vital capacity (FVC)<70%] and with FEV1 of greater than 12% or 200 mL reversibility from the prebronchodilator value [18]. Corticosteroids should be stopped for 12 h before laboratory tests for severe persistent asthmatic patients.

Exclusion criteria
The following were the exclusion criteria:

1. Patients with high-dose systemic corticosteroid (prednisone 1 mg/kg/day).
2. Patients with systemic inflammatory diseases.
3. Active smokers or ex-smokers.
Methods

All participants were exposed to the following: first, thorough history and clinical examination; second, plain chest radiography (P-A view); third, pulmonary function tests (spirometry prebronchodilator and postbronchodilator, using P.K. Morgan Limited; Chatham, Kent, UK) [13]; and fourth, plasma levels of IL-5, TNFα, and IFNγ [human IL-5, TNFα, and IFNγ were measured in serum using a commercially available ELISA kit (Human IL-5 PicoKine ELISA Kit, catalog # EK0407; Boster Biological Technology, Pleasanton, California, USA; Human TNFα, PicoKine ELISA Kit, catalog # EK0407; Boster Biological Technology; and Human IFNγ PicoKine ELISA Kit, catalog # EK0407; Boster Biological Technology, respectively), according to the manufacturer's protocol]. In principle, an antigen detection immunoassay utilizes the quantitative technique of a sandwich enzyme-linked immunosorbent assay in which wells of kit's microplates are already coated with antibodies (primary antibodies) specific for particular epitopes on human IL-5. Diluted serum samples were briefly incubated in the wells, which were then repeatedly washed to remove unbound proteins. Biotinylated conjugated antihuman IL-5 antibody (secondary antibody) specific for the target epitopes was added into the microwells, incubated, and cleaned. Streptavidin horseradish peroxidase enzyme conjugated to the constant heavy chain of the secondary antibody was inserted into the microwells, incubated, and the wells were rewashed. The bound conjugate was developed as a colored product (corresponding to the antigen concentration) by adding a substrate. After the color is entirely developed, the enzymatic reaction was terminated by adding an acid solution. The optical density of each microwell was measured at 450 nm. Obtained optical density values were recorded as pg/ml by the Bio-Rad ELISA data analysis software, USA. The calculated cut-off value equals the mean absorbance value of the negative controls plus three standard deviations. The same steps were done for TNFα and IFNγ.

Relative quantification of MiRNA-1 expression

Blood samples were collected from fasting adults in both groups in the early morning. These samples were handled using the following protocols: plasma was separated and kept at −80°C for more processing. Total RNA was drawn out by the use of the miRNeasy Mini Kit (Qiagen, Düsseldorf, Germany). cDNA was synthesized from miRNA by using the miScript II RT Kit (Qiagen) according to the manufacturer's instructions. Quantitative real-time PCR was done following the SYBR Green PCR protocol by the use of QuantiTect SYBR Green PCR Kit (Qiagen) with a One-Step real-time PCR. Each reaction mix contained 2× QuantiTect SYBR Green PCR Master Mix, 10× miScript Primer Assay specific for miR-1, template cDNA, and RNase-free water in a total volume of 25 μL. The real-time PCR instrument Rotor-Gene Q (Qiagen) was used with the following cycling conditions: enzyme activation at 95°C for 15 min, annealing at 55°C for the 30 s followed by 45 cycles of denaturation at 94°C for 15 s, and extension at 70°C for 30 s. The primer sequences of miR-1 and QAPDH used as an internal reference are shown in Table 1. Relative quantification was reported according to the $\Delta\Delta$Ct method, and results were recorded in the linear form by using the formula $2^{-\Delta\Delta C_t}$ [13] and expressed as relative units (RU) using Step One software (Applied Biosystems, Waltham, Massachusetts, USA) [19].

Statistical analysis

The analysis was calculated by SPSS version 21 (Released 2009-PASW Statistics for Windows Version 21.0; SPSS Inc., Chicago, Illinois USA). The number of frequency and percentage described the qualitative parameters, whereas the quantitative variables were described by mean, SD, and range. Moreover, the comparison of independent quantitative variables in four groups was calculated by the analysis of variance test and a Tukey test for post hoc. However, a comparison between qualitative variables was made by the $\chi^2$ test, Monte Carlo, and Fisher's exact test. The Pearson test calculated the correlation between two quantitative variables. Furthermore, receiver operating characteristic curve and calculation of area under the curve were used to evaluate the test accuracy.

Results

This study shows no significant difference among studied groups regarding age and sex. There is a considerable difference in plasma levels of miRNA, IFNγ, TNFα, and IL-5 ($P<0.001$) between asthmatics and nonasthmatics and among different stages of asthma in patients; however, there was no significant difference in FEV1 between mild stage and control; in FEV1/FVC between mild vs moderate stages, between

<table>
<thead>
<tr>
<th>Table 1 Primers used in PCR steps</th>
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<tr>
<td>Primer name</td>
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<tr>
<td>MIR-1 Forward: 5'-GGGGTGGAATGTAAGAA-3'</td>
</tr>
<tr>
<td>GAPDH Forward: 5'-GAATCTCCATACCCATCTCAGG-3'</td>
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mild vs moderate, and between moderate vs severe; in micro-RNA between mild vs moderate; in IL-5 and TNF-alpha between moderate vs severe; and in INF-gamma between moderate vs severe stages (Table 2). There was a significant positive correlation between miRNA-1 and FEV₁, FEV₁/FVC, and IFNγ and a meaningful negative relationship between miRNA-1 and IL-5 and TNFα, as shown in Table 3. Receiver operating characteristic curve analysis confirms the sensitivity, specificity, and accuracy of different markers in diagnosing asthma and detecting its severity (Fig. 1). AUC was highest in miRNA-1 and FEV₁ followed by TNFα, IFNγ, and then IL-5, with a cutoff point of 1.57, 61.89, 682.27, 56.9, 33.87, and 66.12%, respectively; their sensitivity was 100, 100, 75, 100, 70, and 65%, respectively, and specificities were 100, 100, 66, 62, 56, and 59%, respectively, as shown in Table 4 and Fig. 2a–b.

**Discussion**

This study reported significantly lower levels of MiRNA-1 and IFNγ in the serum of adults with bronchial asthma compared with healthy participants. In contrast, the expression levels of IL-5 and TNF-α were considerably higher compared with healthy participants. These significant differences denote Th1/Th2 dysfunction or imbalance in asthmatic patients.

MiRNA-1 levels have been shown to have significant differences among different stages of bronchial asthma and between asthma and control groups. MiRNA levels decreased gradually with increasing severity of the disease, with a significant positive correlation between miRNA-1 and FEV₁, FEV₁/FVC ratio, and IFNγ, and a meaningful negative relationship between miRNA-1 and IL-5 and TNFα, suggesting that the downregulation of miRNA-1 expression and Th1/Th2 dysfunction may play essential roles in the progression of asthma. Therefore, downregulation of miRNA-1 may directly affect Th2 cell overfunction and Th1 cell dysfunction.

The expression levels of multiple miRNAs are reported to be altered in childhood asthma [20].

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**Table 2** Sex, age, FEV₁, FEV₁/FVC, MiRNA-1, IL-5, TNFα, and IFNγ levels in studied groups

<table>
<thead>
<tr>
<th>Sex [n (%)]</th>
<th>N</th>
<th>Mean±SD (range)</th>
<th>P value</th>
<th>Post hoc</th>
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<tbody>
<tr>
<td>Mild asthma</td>
<td>10</td>
<td>50±15</td>
<td>&lt;0.001</td>
<td>P₁=0.006, P₂=0.007, P₃=0.003, P₄=0.001</td>
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<tr>
<td>Moderate</td>
<td>15</td>
<td>60±20</td>
<td>&lt;0.001</td>
<td>P₁=0.006, P₂=0.007, P₃=0.003, P₄=0.001</td>
</tr>
<tr>
<td>Severe</td>
<td>20</td>
<td>70±25</td>
<td>&lt;0.001</td>
<td>P₁=0.006, P₂=0.007, P₃=0.003, P₄=0.001</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>80±30</td>
<td>&lt;0.001</td>
<td>P₁=0.006, P₂=0.007, P₃=0.003, P₄=0.001</td>
</tr>
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FEV₁, forced expiratory volume in first second; FVC, forced vital capacity; IL, interleukin; IFNγ, interferon-γ; P₁ comparison between mild asthma and moderate; P₂ comparison between mild asthma and severe; P₃ comparison between mild asthma and control; P₄ comparison between moderate and severe; P₅ comparison between moderate and control; P₆ comparison between severe and control; TNFα, tumor necrosis factor-α. Table 2 shows no significant difference among studied groups regarding age and sex. There is a considerable difference in plasma levels of miRNA, IFNγ, TNFα, and IL-5 (P<0.001) between asthmatics and nonasthmatics and among different stages of asthma in patients; however, there was no significant difference in FEV₁ between mild stage and control, in FEV₁/FVC between mild vs moderate stages, between mild vs severe and between moderate vs severe, in micro-RNA between mild vs moderate, in IL-5 and TNFα between moderate vs severe, in IFNγ between moderate vs severe stages.
Midyat et al. [21] isolate total RNA from peripheral blood of 124 patients, age-matched and sex-matched asthmatic and nonasthmatic children, to analyze their altered expression levels. They identified 122 different expressed miRNAs and reported three downregulated expressed genes in all three asthma models, measured from 1.82-fold to 3.57-fold, and miR-21 was the most up-regulated miRNA through an IL-13Rα-independent pathway.

It is motivating to guess that the miR-1 down-regulation contributes to the smooth muscle’s hypertrophy and the remodeling seen in bronchial asthma [27]. The miR-21 upregulation appears to stimulate Th2 and reduces Th1 responses by targeting IL-12 expression [28]. Several other miRNAs regulate T-cell differentiation and function, such as MiR-10a, miR-17-92, miR-181a, miR-182, and miR-29a/b; however, the role of miRNA-1 in inducing immune system imbalance and Th1/Th2 dysfunction is still unclear, and future studies are needed to clarify its specific tasks in asthma [29,30].

Atopic background of asthma represents the majority of asthmatic patients. Th2 subtype cells are responsible for the augmentation of the inflammatory response in bronchial asthma by expression of multiple cytokines, such as IL-4 and IL-5, which plays a crucial role in the pathogenesis of asthma. At the same time, Th1 cells cross-regulate Th2 cells in some systems through secretion of IFN-γ; however, the role of miRNA-1 in inducing immune system imbalance and Th1/Th2 dysfunction is still unclear, and future studies are needed to clarify its specific tasks in asthma [31].

Like this study, Silvestri et al. [32] concluded that TNF-alpha and sIL-8 are biomarkers of systemic
inflammation in severe asthmatics, combined with augmented circulating neutrophils. This suggests the participation of neutrophil-derived cytokine in severe asthma. Matera et al. [33] reported increased levels of TNF-α in the tissues of the asthmatic patients’ airways and upregulated expression of TNF-α in alveolar macrophages, mast cells, and bronchial epithelial cells. They concluded that TNF-α exerts a variety of proinflammatory actions in the tissues of the airways, including cytokines, chemokines, adhesion molecules, and mucins.
Conclusion
Plasma miRNA-1 level is significantly decreased in asthmatic patients. Plasma miRNA-1 substantially decreases with increasing severity of the disease and positively correlates with FEV1/FVC and plasma IFNγ. However, a negative correlation was found with plasma IL-5 and TNFα. The role of plasma miRNA-1 in inducing immune system imbalance and Th1/Th2 dysfunction is unclear, and further studies are required to clarify its role in asthma pathogenesis.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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