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Serum microRNA-223 as a potential biomarker for allergic rhinitis and its correlation to eosinophil-derived neurotoxin

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Abstract

Allergic rhinitis (AR) is a global health problem. It is an inflammatory condition defined by a malfunction of the immune system's regulatory mechanism. MicroRNA-223 (miRNA-223) has been linked to the modulation of AR in the last few years. The goal of this study was to determine whether miR-223 can be utilized as a potential biomarker for diagnosis of AR, and whether it correlates with the total nasal symptom score (TNSS) along with serum interleukin-17 (IL-17), interleukin-4 levels (IL-4) and eosinophil-derived neurotoxin (EDN). This study included 76 adult participants, consisted of 38 AR patients and 38 apparently healthy controls. Serum levels of miR-223 were assayed using real-time PCR. The levels of EDN, IL-17 and IL-4 in the serum were determined using an enzyme-linked immunosorbent assay. The optimal cutoff value for the analyzed factors to diagnose AR was determined using a receiver operating characteristic curve analysis (ROC). The demographic features (age and gender) of the two study groups were matched. Patients with pollen-induced AR had significantly higher levels of miR-223 in their serum compared to the controls (median = 3.82; median = 1.03, respectively, $p < 0.001$). In AR cases, a significant positive association was observed between miR-223 expression level and TNSS (r = 0.492, $p = 0.002$), EDN serum level (r = 0.427, $p = 0.008$), IL-4 serum level (r = 0.341, $p = 0.036$) and IL-17 serum level (r = 0.324, $p = 0.047$). MiR-223, at a cutoff value of 1.18, had a sensitivity and specificity of 94.9 % and 92.5%, respectively. In conclusion, miR-223 expression is significantly greater in blood of AR patients. There is a significant association between miR-223 and clinical severity of AR, each of IL-17 and IL-4 as well as EDN. Therefore, miR-223 may be employed as an effective biomarker for AR diagnosis.

Keywords: allergic rhinitis, MicroRNA-223, Biomarker, IL-4, IL-17, EDN

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Introduction

The most prevalent noninfectious chronic rhinitis in the world is allergic rhinitis (AR). It is a major health problem affecting 40% of the global population.[1] Rhinorrhea, congestion, nasal itching, and sneezing are symptoms of AR, which are caused by inflammation of the nasal epithelium induced by allergens as mites, pollens, insects, cigarette smoke, and aspirin or other non-steroidal anti-inflammatory medicines.[2] Sleep disturbances, depression, exhaustion, and poor focus are all symptoms of AR, and they can affect one's quality of life and work productivity.[3]

Mastocytes, eosinophils, macrophages, T lymphocytes, and B lymphocytes are among the inflammatory cells involved in the pathophysiology of AR. Following exposure to an inciting allergen, they invade the mucosa of the nose and throat. In allergic people, the cascade begins with T helper 2 (Th2) cells invasion into the nasal mucosa, followed by the release of a distinct group of cytokines, such as interleukin 3 (IL-3), IL-4, IL-5, and IL-13. This prompts plasma cells to produce immunoglobulin E (IgE), followed by histamine and leukotriene production (early-phase allergic reaction).[4,5] On the other hand, late-phase allergic reactions can cause recurrent AR symptoms that can last for a longer period.[2]

Currently, symptomatic patients with AR are treated with steroids and antihistamines. Although AR pathogenesis is primarily immunological, immunotherapy with specific targets is still restricted.[1,6] Since the morbidity of AR has increased in the recent decades, resulting in a greater economic burden around the world, the discovery of disease-related biomarkers is critical for enhancing AR diagnosis and management.[7]

MicroRNAs (miRNAs) are non-coding, single-stranded RNAs that are 18 to 24 nucleotides long. They regulate the expression of genes by binding to the target messenger RNA's 3'-untranslated region (mRNA) and causing RNA degeneration or protein synthesis inhibition.[8] By switching between Th1 and Th2 polarization and driving persistent epithelial inflammation by innate immune cell activation, miRNAs have been shown to have a role in several inflammatory conditions, including allergy diseases.[9] Circulating miRNAs can be utilized as potential biomarkers due to their involvement in the dysregulation seen in allergic conditions, and they offer more stability than mRNAs.[10]

In the past, inflammation-related miRNAs were reported by several researchers who have also investigated their function in human and animal cells and tissues. MiR-223 is one of the many miRNAs that has been found to have numerous regulatory functions during inflammation.[11]

According to the accumulated data, miR-223 may be implicated during allergic inflammation, which is a major indicator of AR development. Zhou et al., 2021, stressed that miR-223-3p agomir induces infiltration of eosinophils into nasal mucosa of mice infected with AR, whereas miR-223-3p antagomir prevents these pathological alterations.[12]

Eosinophils are known to be crucial in the development of chronic allergy disorders.[13] In individuals with allergic rhinitis, it has been demonstrated that the quantity of eosinophils in the nasal smear is strongly linked with the nasal airflow resistance and the spirometric parameters.[14] After exposure to allergens, significantly increased sums of activated and degranulated eosinophils were found in patients with AR.[15] By encouraging T helper type 2 (Th2) cells recruitment and interacting with dendritic cells, eosinophils can exacerbate allergic inflammation in mice models with allergic lung inflammation.[16] Additionally, eosinophils have the capacity to release Th2 cytokines that have already been produced, such as interleukin (IL) 4 and IL-13, to support Th2 response.[17]

One of the main secretory proteins found in the distinct granules of human eosinophils is called eosinophil-derived neurotoxin (EDN). Cytokines and other pro-inflammatory mediators cause its release, which has the ability to draw in additional immune cells.[18] It has been suggested that eosinophil activity in allergy disorders may be determined by measuring EDN.[19]

The objectives of study were to assess miR-223 as a potential biomarker for diagnosis of AR and to determine the miR-223 inflammatory

role in AR through correlating its level with TNSS, serum IL-4, IL-17, eosinophils count and EDN.

Subjects and Methods

Study Participants

This case-control study included 38 patients with AR recruited from the Allergy and Immunology Clinic at the Faculty of Medicine, Zagazig University during the period from December 2021 to September 2022. In addition, 38 subjects of similar age and who had no family history of atopy volunteered as controls. They didn't have history of AR.

Ethical considerations

A general and local examination was conducted for all the participants, and a thorough medical history was obtained from everyone. All AR patients were mono-sensitized to birch pollen allergen confirmed by a specific IgE assay. Patients who have chronic diseases, or patients who had bronchial asthma or upper or lower respiratory tract infection within the last two months were excluded from the study.

Total nasal symptom score (TNSS)

Nasal congestion, sneezing, itching, and rhinorrhea were assessed by the TNSS. The sum of scores for each nasal symptom at a particular time point was used to calculate the TNSS. TNSS is a four-point scale (0–3). Score (0) indicates the nonexistence of symptoms, score (1) specifies mild tolerable symptoms, score (2) indicates bothersome nevertheless tolerable symptoms, and score (3) specifies severe symptoms that interrupt the daily activity. The score for every single nasal symptom was added to a total out of 12 to calculate the TNSS.[20]

Serum samples

Approximately 10 ml of peripheral blood were collected from every single participant via venipuncture. A small blood aliquot was sent to the university hospital for performing blood cell counts, according to their standard method. Each of the remaining blood sample was centrifuged for 10 min at 1000 xg to split up the serum. A serum fraction was preserved at -20 ºC for subsequent use in specific IgE, IL-4, IL-17 and EDN assays. The remaining serum fraction was further centrifuged for 10 minutes at 14,000 x g and preserved at –80 °C till used for RNA extraction.

Quantitation of specific IgE against common aeroallergens

We measured serum specific anti-IgE levels for common aeroallergens (20 for the food panel and 20 for the inhalant panel) using commercial enzyme-linked immunosorbent assay kits (RIDA Allergy Screen ELISA, R-Biopharm, Germany), according to the manufacturer's instructions. The cutoff value of the ELISA kit was 0.35 kUA/L.

Quantitative measurement of human serum IL-4, IL-17, and EDN

Quantitative determination of serum IL-4 level was accomplished by ELISA kits (Sunlong Biotech, Hangzhou, China). Serum IL-17 was measured using ELISA kits (Abcam, UK). Serum EDN was measured using ELISA Kits (Human EDN, MBL, Nagoya, Japan). All the previously mentioned ELISA kits were performed according to the manufacturer's instructions.

Quantitation of microRNA-223

MicroRNA was extracted by commercial miRNeasy Serum/Plasma Kits (Qiagen, Germany), according to the manufacturer's instructions. After extraction, microRNA was reversibly transcribed by reverse transcription kits (miScript II RT Kit, Qiagen, Germany) according to the manufacturer's instructions. Then, it was incubated in dry bath block (Rocker Sahara 320 dry bath heat block, Rocker Scientific, Taiwan), initially at 37°C for 60 min, followed by a second incubation at 95°C for 5 min to inactivate miScript RT. After that, the reaction mixture was stored at -80°C

Quantitative determination of mature miR-223 was fulfilled by target-specific miScript Primer Assays using commercial kits (miScript SYBR Green real-time PCR Kit, Qiagen, Germany), according to the manufacturer's instructions. Human RNU6B (RNU6-2) was used as a house keeping gene. After the initial activation step of HotStar Taq DNA Polymerase for 15 min at 95°C, the real-time PCR reactions were run for 40 cycles of each of a denaturation

for 15 s at 95 °C, annealing for 30 s at 55°C, and extension for 30 s at 70 °C. The Stratagene Mx3005P platform software (Agilent Technologies, USA) was used to figure out the threshold cycle (Ct) value. The delta-delta Ct method formula was utilized to estimate the fold changes of miR-223 expression.[21]

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) software, Version 20.0 (Armonk, NY: IBM Corp.) was used to analyze the data. The Shapiro-Wilk test was conducted to verify the normality of distribution. Quantitative data are presented through the mean, standard deviation, and range. Qualitative variables are presented as frequency and percentages. To compare the independent groups, independent sample t-test and Mann-Whitney tests were conducted. The Pearson chi-square test was also used to determine the relationship between clinical variables. Pearson correlation was used to obtain the correlation coefficient r. The receiver operating characteristic (ROC) curve analysis was used to establish the optimal cutoff value for the parameters that are under investigation, and to determine the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Statistical significance was defined at *p*-value < 0.05.

Results

Demographic characteristics

The current study involved 38 AR patients and 38 age-matched apparently healthy controls. Their demographic characteristics are presented in Table 1. In the AR group, the percentage of females and males was 44.7% and 55.3%, respectively, and females and males represented 47.4% and 52.6%, respectively in the control group.

Table 1. Demographic data of the study cases and controls.

	Cases (n = 38)	Controls (n = 38)	*p* value
Age			
Mean ± SD.	32.79 ± 10.68	30.63 ± 8.98	NS^{t}
Median (Min. – Max.)	30 (18 – 55)	28.50 (19 – 50)	
Sex			
Male	21(55.3%)	20(52.6%)	$NS^{\chi 2,FE}$
Female	17 (44.7%)	18(47.4%)	
BMI			
Mean ± SD.	26.32 ± 3.46	27.54 ± 2.89	NS^{t}
Median (Min. – Max.)	26.90(18.10 – 32.60)	28.60 (20.60 – 31.80)	

SD: Standard deviation, χ^2: Chi square test, FE: Fisher Exact, t: Student t-test, $p > 0.05$ is not significant (NS).

Measurement of TNSS, specific IgE, eosinophils count

The mean TNSS of AR patients was 8.34 ± 2.60, while the mean specific IgE titer was 17.37 ± 5.16. The mean white blood cells (WBCs) count of AR patients was 8494.3 ± 1891.8. Eosinophils count showed significant higher level in AR patients than controls (median = 245.0, range (145 - 1254), and median = 65.50, range (10 - 128), respectively, $p<0.001$), as presented in Table 2.

Measurement of serum miRNA-223, EDN, IL-4, and IL-17

The miRNA-223 was remarkably expressed in AR group when compared to the control group (median = 3.82, range (1.16 - 5.60); versus median = 1.03, range (0.85 – 3.90), respectively $p<0.001$). Serum EDN showed significantly higher levels in AR patients than in controls (median = 48.21, range (32.76 - 58.45), versus median = 29.38, range (23.45 - 33.91), respectively $p<0.001$). Serum IL-4 showed significantly higher levels in AR patients than in controls (median = 135.0, range (23.90 - 187.7),

and median = 28.44, range (18.75 - 45.98), respectively $p<0.001$). The levels of IL-17 were also significantly higher in AR patients (median=169.7, range (11.95 – 422.9) compared to controls (median=23.45, range (11.34 – 59.20), $p<0.001$, as presented in Table 2.

The values of miR-223, EDN, IL-4 and IL-17 levels were assessed as biomarkers for AR using the ROC curve analysis (Figure 1). The miR-223 optimal cutoff for AR diagnosis was ≥ 1.18 with an area under curve (AUC) of 0.992 (95% CI [0.981 – 1.00]), sensitivity of 94.9 %, specificity of 92.5%, PPV of 92.5%, and NPV of 94.8%. The EDN optimal cut-off in AR diagnosis was 33.68 with an AUC of 0.971 (95% CI [0.922 - 1.00]), sensitivity of 97.37%, specificity of 97.37%, PPV of 97.4% and NPV of 97.4 %. The IL-4 optimal cut-off in AR diagnosis was 45.98 with an AUC of 0.920 (95% CI [0.859 - 0.981]), sensitivity of 81.6%, specificity of 100%, PPV of 100% and NPV of 84.4 %. The IL-17 best cut-off value in AR diagnosis was > 59.2 with an AUC of 0.920 (95% CI [0.854 - 0.986]), sensitivity of 84.2%, specificity of 100%, PPV of 100% and NPV of 86.4 %.

Table 2. Comparison of the measured parameters between the two studied groups.

	Cases (n = 38)	Controls (n = 38)	*p* value
MiRNA-223			
Mean ± SD.	3.56 ± 1.08	1.09 ± 0.47	<0.001[t]
Median (Min. – Max.)	3.82 (1.16 – 5.60)	1.03 (0.85 – 3.90)	
TNSS			
Mean ± SD.	8.34 ± 2.60	-	-
Median (Min. – Max.)	9 (3 – 12)	-	
Specific IgE			
Mean ± SD.	17.37 ± 5.16	-	-
Median (Min. –Max.)	15.50 (8.0 – 26.0)	-	
IL17			
Mean ± SD.	169.36 ± 99.95	27.49 ± 11.80	<0.001[U]
Median (Min. – Max.)	169.7 (11.95 – 422.9)	23.45 (11.34 – 59.20)	
IL4			
Mean ± SD.	118.50 ± 57.80	30.70 ± 9.11	<0.001[U]
Median (Min. – Max.)	135.0 (23.90 –187.7)	28.44 (18.75 – 45.98)	
EDN			
Mean ± SD.	48.03 ± 4.86	29.32 ± 2.88	<0.001[t]
Median (Min. – Max.)	48.21 (32.76 – 58.45)	29.38 (23.45 – 33.91)	
Eosinophils			
Mean ± SD.	335.0 ± 247.8	65.24 ± 27.62	<0.001[t]
Median (Min. – Max.)	245.0 (145 – 1254)	65.50 (10– 128)	
WBCs			
Mean ± SD.	8494.3 ± 1891.8	7664.4 ± 2133.8	NS[t]
Median (Min. – Max.)	8970.0(4565 – 11978)	7052.5 (4982– 12370)	

EDN: Eosinophil-derived neurotoxin, TNSS: total nasal symptom score, SD: Standard deviation, t: Student t-test, U: Mann Whitney test; $p > 0.05$ is not significant (NS).

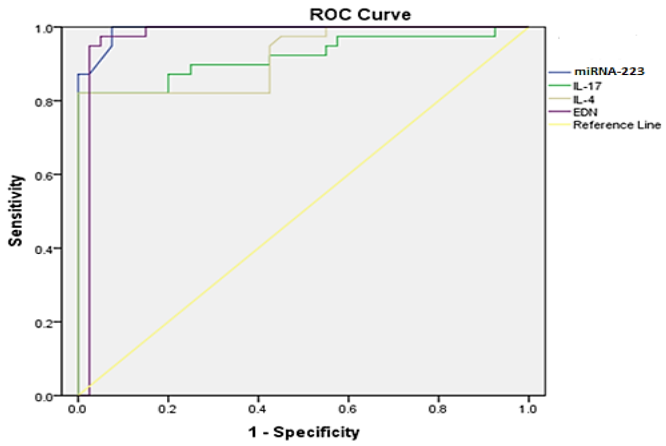

Figure 1. Receiver operating characteristic (ROC) curve analysis showing the performance of miRNA-223, Eosinophil-derived neurotoxin, IL-17, and IL-4 in diagnosis of allergic rhinitis

Correlation of miRNA-223 expression level with laboratory variables

The expression level of miR-223 was significantly associated with EDN (r = 0.427, *p*=0.008, Figure 2A), IL-17 (r = 0.324, *p* = 0.047, Figure 2B), IL4 (r = 0.341, *p* = 0.036, Figure 2C), and eosinophils count (r = 0.795, *p* < 0.001, Figure 2D). However, there was no observed association between miR-223 and specific IgE levels (r = 0.244, *p* = 0.140).

Correlation of miRNA-223 expression level and EDN serum level with TNSS

There was a significant association between miR-223 and TNSS (r = 0.492, *p* = 0.002, Figure 3A), and a significant association between EDN and TNSS (r = 0. 0.405, *p* = 0.012, Figure 3B).

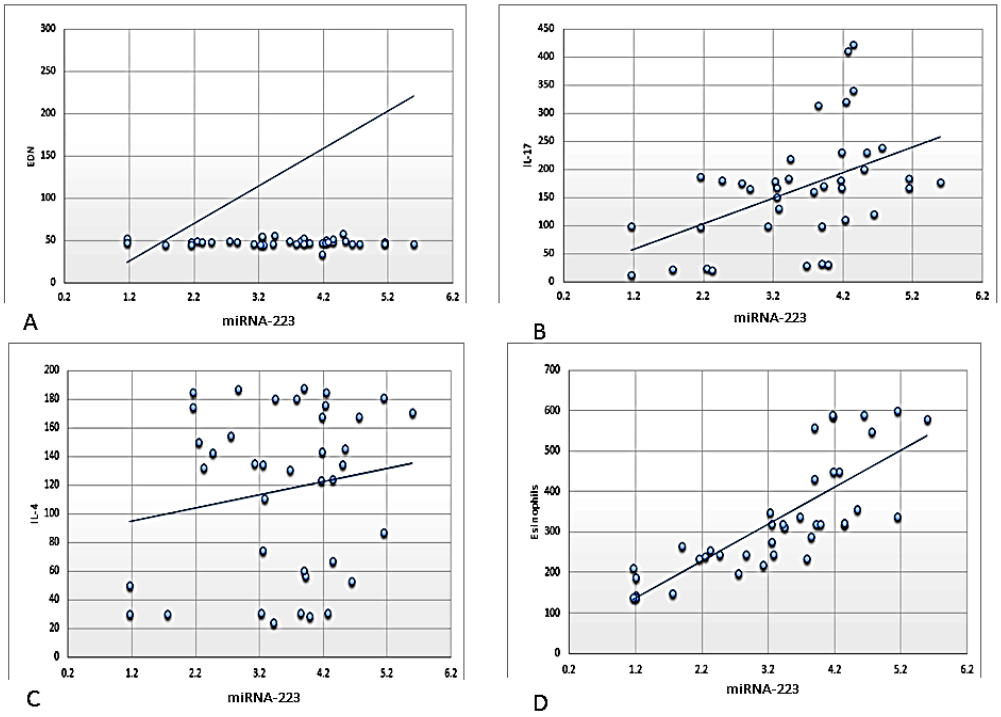

Figure 2. Correlation between miR-223 and Eosinophil-derived neurotoxin (EDN) (A), IL-17 (B), IL-4 (C) and eosinophils count (D) among the 38 AR patients. Pearson's correlation coefficient was applied to analyze the correlation.

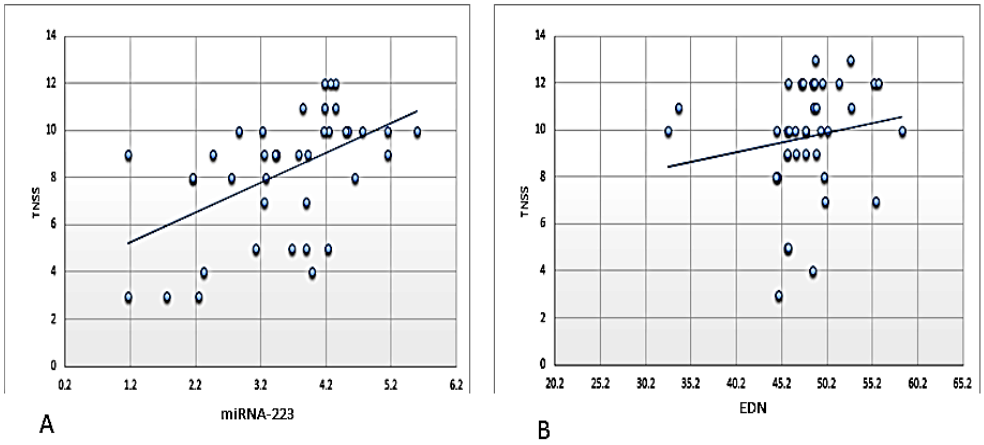

Figure 3. Correlation between miR-223 and total nasal symptom score (TNSS) among the 38 AR patients is shown in A. Correlation between Eosinophil-derived neurotoxin (EDN) and TNSS among the 38 AR patients is shown in B. Pearson's correlation coefficient was applied to analyze the correlation.

Discussion

Several studies have shown that miRNAs have a role in AR pathogenesis, including the regulation of mastocytes, eosinophils, and the Th1/Th2 disproportion.[22,23] The objectives of our study were to assess miR-223 as a potential biomarker for diagnosis of AR and to determine the miR-223 inflammatory role in AR through correlating its level with TNSS, serum IL-4, IL-17, eosinophils count and EDN.

The current study showed that sera of the included AR patients had significantly higher levels of miR-223 than controls. This is in line with what was reported by Zhou et al., 2021.[12] They observed that miR-223-3p expression in serum of AR patients was much higher than in healthy control people, and that miR-223-3p expression in AR mice was also significantly greater than in the control group, and even raised somewhat in miR-223-3p agomir treated AR mice.[12] Another example is a study by Ruan et al., 2020.[24] They reported that the median serum level of miR-223 was considerably greater in AR group than the control group.[24]

Furthermore, in the case of other allergic disorders, when 16 asthmatics who are steroid-free were compared to 12 healthy controls, miRNA profiling revealed that asthma patients had higher expression of miR-223 than healthy controls.[25] Similarly, Maes et al., 2016 [26] found that miR-223 was more copious in severe asthma patients' produced sputum supernatant than in healthy controls. Also, according to Gomez et al., 2020, miR-223 expression was shown to be greater in asthmatic sputum and linked to a neutrophilic asthma phenotype. [27] In addition, miR-223 expression levels were linked to a variety of severe asthma characteristics, bronchodilator responsiveness and nitric oxide levels. On the other hand, miR-223 expression was decreased in blood-derived T cells from mild to moderate asthma patients when compared to healthy people.[28]

A different study reported that smoking during pregnancy raises the likelihood of allergic sensitization, that was linked to reduced numbers of regulatory T cells and elevated expression of miR-223 in cord blood.[29] Regarding atopic dermatitis, the study by Yasuike et al., 2021,[30] stated that plasma levels of miR-223 were higher in severe atopic dermatitis patients.

According to our findings, there was a strong positive correlation between TNSS and serum miR-223 levels. This result agreed with that reported by Ruan et al., 2020,[24] who found that the relative amount of miR-223 in plasma was significantly associated with TNSS. According to the study by Zhou et al., 2021,[12] TNSS increased dramatically in AR mice when compared to control mice and was even greater in AR animals treated with miR-223-3p agomir, although it appeared to decline in miR-223-3p antagomir treated mice.

The current study revealed that IL-4 levels were much greater in AR patients, and significantly associated with miR-223. These findings are consistent with those reported in a study by Ruan et al., 2020.[24] which showed that IL-4 levels in AR patients were higher than in healthy controls, implying that IL4 plays a significant role in immune response regulation. Furthermore, Zhou et al., 2021[12] found that miR-223-3p antagomir reversed the elevated levels of cytokines (IL-4, IL-5, and IFN) in AR mice after miR-223-3p agomir intranasal injection.

The current study observed that IL-17 levels were greater in AR patients, and there was a good association between miR-223 and IL-17 as well. These findings corroborate those reported by Ruan et al., 2020, who observed that IL-17 levels were greater in AR patients than in healthy controls and that miR-223 levels were positively linked with IL-17.[24]

The increase of eosinophils accelerates the development of AR by aggravating airway dysfunction and encouraging tissue remodeling. MiR-223 was shown to work by controlling eosinophil increase in allergic inflammation. [31] In our study, we found a significant correlation between miR-223 and eosinophil count. Our results are in accordance with those of a study by Ruan et al., 2020[24] who found a significant positive correlation between serum levels of miR-223 and eosinophils counts as well as eosinophil cationic protein levels in children with AR. According to a study by Weidner et al., 2020[32] eosinophils and eosinophil cationic protein showed a strong significant correlation with miR-223, suggesting the potential contribution of this miRNA to the growth of eosinophils in asthmatic individuals. [32] Additionally, it was noted that individuals with eosinophilic esophagitis have elevated miR-223, and that the expression level of this gene has the strongest link with esophageal eosinophil counts.[33] Moreover, a study indicated that miR-223 was linked to the development of eosinophil progenitors.[34]

The secretory activity of eosinophils, which is determined by their concentration and propensity to release degranulation products, was proposed as a potential important indicator of allergic diseases activity, and more precisely assessed by eosinophil degranulation products like EDN.[35] Instead of just counting eosinophils, EDN is a more reliable indicator of eosinophil activity. Furthermore, compared to eosinophil cationic protein, EDN is a more readily recoverable degranulation product.[36]

In the current study, there was a significant increase in EDN serum levels in AR patients in comparison to the control subjects. These results are matched with those reported by studies of Morioka et al., 2004 [19] and Kim et al., 2022.[37]

To the best of our knowledge, we are the first to study the correlation between miR-223 and EDN. Our findings indicated a significant positive correlation between EDN and miR-223 serum levels, and this may suggest that miR-223 may act as a new eosinophil regulator in AR. Moreover, we found a significant positive association between EDN and TNSS, suggesting a possible role of EDN as biomarker for eosinophilic inflammation.

According to the ROC analysis, the optimal cutoff for miR-223 in AR diagnosis is 1.18, which has a sensitivity of 94.4 % and a specificity of 92.5 %. Our findings matched with those of Zhou et al., 2021,[12] who determined that miR-223 has a good diagnostic power in discriminating AR patients from healthy people, with an AUC of 0.845. These data suggested that miR-223 could be a potential clinical biomarker for AR diagnosis and a possible positive regulator of pro-allergic variables.

The limitations of this study include its small sample size. In addition, we only looked at miRNA expression in cell-free serum and did not include miR-223 expression in human eosinophils in order to determine its link to these cells.

In conclusion, miRNA-223 was significantly expressed in the sera of AR patients and there was a strong correlation between miRNA-223 and EDN serum levels. According to the findings of this study, miRNA-223 could be suggested as a possible sensitive noninvasive biomarker for AR diagnosis.

Author Contributions

FN; Study design and Sample processing. MAA; Manuscript preparation and Clinical examination. SFS; Research hypothesis and Sample processing. HKAM; Sample collection and Sample processing. YAF; Study design and Manuscript preparation. All authors reviewed the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Institutional Review Board of Faculty of Medicine, Zagazig University (IRB#9126/28-11-2021).

Informed consent

A written informed consent form was obtained from each study participant before included in the study.

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