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ORIGINAL RESEARCH

MicroRNA-146a-5p and microRNA-210-3p Correlate with T Regulatory Cells Frequency and Predict Asthma Severity in Egyptian Pediatric Population

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Background: Severe bronchial asthma (BA) affects 5–10% of children, which imposes socioeconomic burden. Therefore, it is crucial to identify biomarkers for risk stratification in children with BA. T regulatory cells (Tregs) play a balancing role in allergic response regulation. We aimed to investigate the relationship between Treg, miR-210-3p, and miR-146a-5p in relation to asthma phenotypes in search of novel biomarkers of disease severity.

Methods: This study included 50 children with BA classified into Group 1 (n = 25) children with mild to moderate asthma and Group 2 (n = 25) children with severe asthma. In addition to 26 control subjects. Flow cytometry was used to detect Tregs. Plasma miR-210-3p and miR-146a levels were determined using quantitative real-time PCR. Patients' FEV1 (Forced Expiratory Volume in the first second) was measured.

Results: miR-210-3p level correlated negatively with Treg frequency ($r = -0.828$, $P < 0.001$) and FEV1 ($r = -0.621$, $P < 0.001$). The level of miR-146a-5p positively correlated positively with Treg% ($r = 0.303$, $P = 0.032$). ROC curve analysis revealed that miR-210-3p was the most sensitive biomarker of severity, with the area under curve (AUC) = 0.923, 96% sensitivity, and 60% specificity. According to multivariate analysis, miR-210-3p is an independent risk factor for BA severity [OR =3.119, P = 0.030], while miR-146a-5p is a protective factor [OR =0.811, P = 0.049].

Conclusion: Treg frequency is linked to FEV1, miR-146a-5p and miR-210-3p in childhood BA. Upregulation of miR-210-3p is a sensitive biomarker and an independent risk factor for BA severity in Egyptian children.

Keywords: T reg, miR-210-3p, miR-146a-5p, childhood asthma, asthma classifications

Introduction

Bronchial asthma (BA) is the most prevalent chronic disease in children and a significant source of distress for children and their families. Young children are especially diverse, with numerous and variable phenotypic manifestations in early life corresponding to several outcomes.[1]

Despite advances in disease understanding and the availability of effective treatments, asthma remains one of the leading causes of emergency department visits and multiple hospitalizations in children.[2]

Numerous classifications of BA based on phenotype and endotype have been proposed. The phenotype comprises the clinical characteristics of asthma, such as onset age, triggers, comorbidities, treatment response, and evolution over time.[3]

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The difficult-to-define endotype includes the disease's underlying inflammatory cells and immunopathological mechanisms.[4]

The phenotypes can change over time in response to alterations in the disease's primary causative factors. A patient with early-onset disease has a poor prognosis for asthma, with a high risk of disease persistence and severity during childhood.[3]

T regulatory cells (Tregs) are immune cell balancers. They inhibit inflammatory responses and maintain immune tolerance in steady state immune systems. Numerous studies have demonstrated that the frequency of regulatory T cells (Treg) is downregulated in BA in favor of the upregulation of T helper 17 (Th17).[5] Foxp3 is a transcriptional regulator that is expressed by Treg. Reduced Foxp3 expression compromises the suppressive function of Treg cells.[6]

MicroRNAs (miRs) are short single-stranded RNA molecules that silence gene expression post-transcriptionally, resulting in transcript degradation or inhibition of translation of the target genes.[7] Observed modifications of miRs reported in BA in easily accessible body fluids (serum and plasma) have suggested miRs as biomarkers for disease diagnosis[8] and identifying patients at risk for severe asthma.[9]

According to reports, miR-210-3p inhibits the protein expression of Foxp3 and modulates the immunosuppressive properties of Tregs which induces immune dysfunction via T regs in Psoriasis vulgaris patients,[10,11] but this link has not been investigated in childhood BA.

In addition, miR-146a-5p, which was found to be expressed in Tregs, is essential for their suppressive function. The absence of miR-146a-5p in Treg cells led to the breakdown of immunological tolerance, which was manifested as IFNγ-dependent immune-mediated lesions. miR-146a-5p is responsible for the optimal range of activation of Treg signal transducer and activator transcription 1 (Stat1), which is essential for Treg-mediated control of Th1 responses and associated autoimmunity.[12] As the origins of childhood BA initiation are frequently beyond the control of the health care system, it is desirable to develop methods for regulating immune responses that influence BA outcomes in children.

In search of new severity biomarkers and therapeutic targets in childhood asthma, we aimed to investigate the possible relationship between the frequency of circulating Treg with miR-210-3p and miR-146a-5p and asthma severity.

Patients and Methods

This cross-sectional study was conducted on 50 BA children classified as belonging to Group 1 (n = 25) with mild to moderate asthma and Group 2 (n = 25) with severe asthma. The asthmatic children were obtained from the Pediatric Allergy and Pulmonology unit of the Children's Hospital at the Cairo University, Egypt. In addition to the healthy children (n = 26) who served as the normal control group.

The current study complies with the Declaration of Helsinki. Research Ethical Committee of the Faculty of Medicine for Girls-Institutional Research Board approved the study (IRB no. 2022081494). Before beginning, informed consents were obtained from the legal guardians of the study participants.

The sample size was determined by Epi Info Version 7 with a confidence level of 95% and an assumption of 8.2% for the percentage of children based on the rate of asthma cases during this period. One random sample out of two was collected systematically.

Inclusion Criteria

1. Asthma was identified in children in accordance with GINA 2019.[13] Asthma that is effectively controlled with only as-needed controller medication or low intensity maintenance controller therapy, such as low dose inhaled corticosteroids (ICS) and leukotrienes receptor antagonists, is referred to as mild asthma. Moderate asthma is asthma that is well controlled on low dose ICS- LABA. Severe asthma is defined as asthma that requires high dose ICS-LABA or low doses of systemic steroids to prevent it from being uncontrolled or asthma that remain uncontrolled despite this treatment.
2. Regarding control group, during their normal exam, healthy children who were age- and gender-matched to the cases were chosen at random as the controls.

Exclusion Criteria

a) Children with systemic immunological disorders, malignancy, or anatomic abnormalities of the respiratory tract. b) Children below 5 years as they showed no compliance in performing pulmonary function tests.

Tools of the Study

Questionnaire for collecting demographic information and disease history.

Children with asthma were evaluated for forced expiratory volume in the first second (FEV1) (% of predicted) using spirometry.

Each child's 4 mL of blood was separated into 2 tubes of disodium ethylenediamine tetra-acetic acid for sample processing (EDTA). The initial 2 mL of blood was drawn for a complete blood count (CELL-DYN Ruby, Hematology analyser, 5-part differential, Abbott Diagnostics, USA), blood film, and flow cytometric analysis of Treg. The second tube was centrifuged to separate 200 µL of plasma, which was immediately frozen at −20°C, pending RNA isolation processing.

Laboratory Research

At Al-Zahraa Hospital, Al-Azhar University, the expression levels of plasma miR-210-3p and miR-146a-5p were analyzed by quantitative real-time polymerase chain reaction (q-PCR) following reverse transcription using a miScript RT kit from QIAGEN according to the manufacturer's instructions.

Plasma miR was extracted using the miRNeasy Mini Kit (catalog number 217004) according to the manufacturer's instructions. The concentration and purity of total RNA were determined using the spectrophotometer NanoDrop (ND-1000, Thermo Fisher Scientific, USA), and 75 ng of the RNA was used in the reverse transcription reaction.

In accordance with the manufacturer's instructions, complementary DNA was synthesized from the extracted RNA during this step. miScript II RT Kit (cat. no. 218161) was utilized.

The q-PCR was performed on a PCR ViiA ^{TM} 7 System (no. 278880908, USA) with human miscript SYBER green Master Mix and primers for miR-210-3p (cat. no. MS0000380, lot. no. 00160602113) and miR-146a-5p (cat. no. MS00003535, Lot 20160510009s). The endogenous control was the housekeeping microRNA SNORD 68 (catalog number MS00033712, lot number 201601113022s) supplied by QIAGEN, Germany. Fluorescence was measured on a cycle-by-cycle basis. After analyzing melting curves, a single, distinct peak was found for each target. MicroRNA data were expressed as the median of relative expression (fold changes). The P values were calculated manually based on replicate 2-ΔΔCT values for each miR in the studied groups [8].

Flow Cytometry Assay

A four-color FACS Calibur flow cytometry assay was conducted at the Clinical Pathology Department, AL-Azhar University (BD, Biosciences, San Jose, USA). For data analysis, CellQuest Pro software (BD Biosciences, San Jose, USA) was utilized. Next, using color calibrate beads, the compensation setting was determined prior to sample acquisition (BD, Biosciences, San Jose, USA, lot. no 5093879). For the detection of non-specific binding, isotype controls (BD, Biosciences, San Jose) were obtained.

Two tubes were used for each participant in the study. Each received 100 µL of the adjusted sample. The first tube contained 5 µL of a monoclonal antibody of CD4 FITC-conjugated Ab (Immunotech, Beckman Coulter, Marsellia, France, Catalog number: AO7750. Lot number.100), PerCP-conjugated anti-human, CD3 (BD Biosciences, USA Cat. no. 95131, lot no.345766), CD25 APC-conjugated Ab (R&D systems, Minneapolis, Minnesota, USA, Cat. no. FAB1020A. lot. no. LXJ0215071) and PE-conjugated FoxP3 (eBioescience, Europe/International, Clone: 236A/E7. Cat. no 12-4777-42. lot. no. E11467-1633) using FOXP3 Intercellular permeabilization concentrate reagent (eBioescience, Europe/International, Cat. no. 00-5123-43. lot. no. 4273423) as manufacture procedures instructions.

The second tube was incubated with a mixture of 5 ug FITC (IgG)/ PE (IgG) surface isotypic control and 5 ug APC (IgG) surface isotypic control (BD, Biosciences, San Jose). Both tubes were incubated for 20 minutes in the dark. The samples were then resuspended in 200–500 ug PBS and analyzed. The number of cells to acquire was set to (100,000).

Gating strategy for Treg frequency detection: Using dot plot forward and side scatter (FS/SS), the mature lymphocyte gate was detected (R1), and another graph was taken for detection of the CD3+CD4+ co-expressing T lymphocyte gate (R2), A quadrant plot was drawn from R1 and R2 representing CD25-APC on Y axes and FoxP3-PE on X axes. Then the gate of co-expression of CD25 surface marker and the intracellular PE-conjugated FoxP3 was considered as the Treg frequency in the sample in the upper right quadrant (area detected by red arrow) (Figure 1)

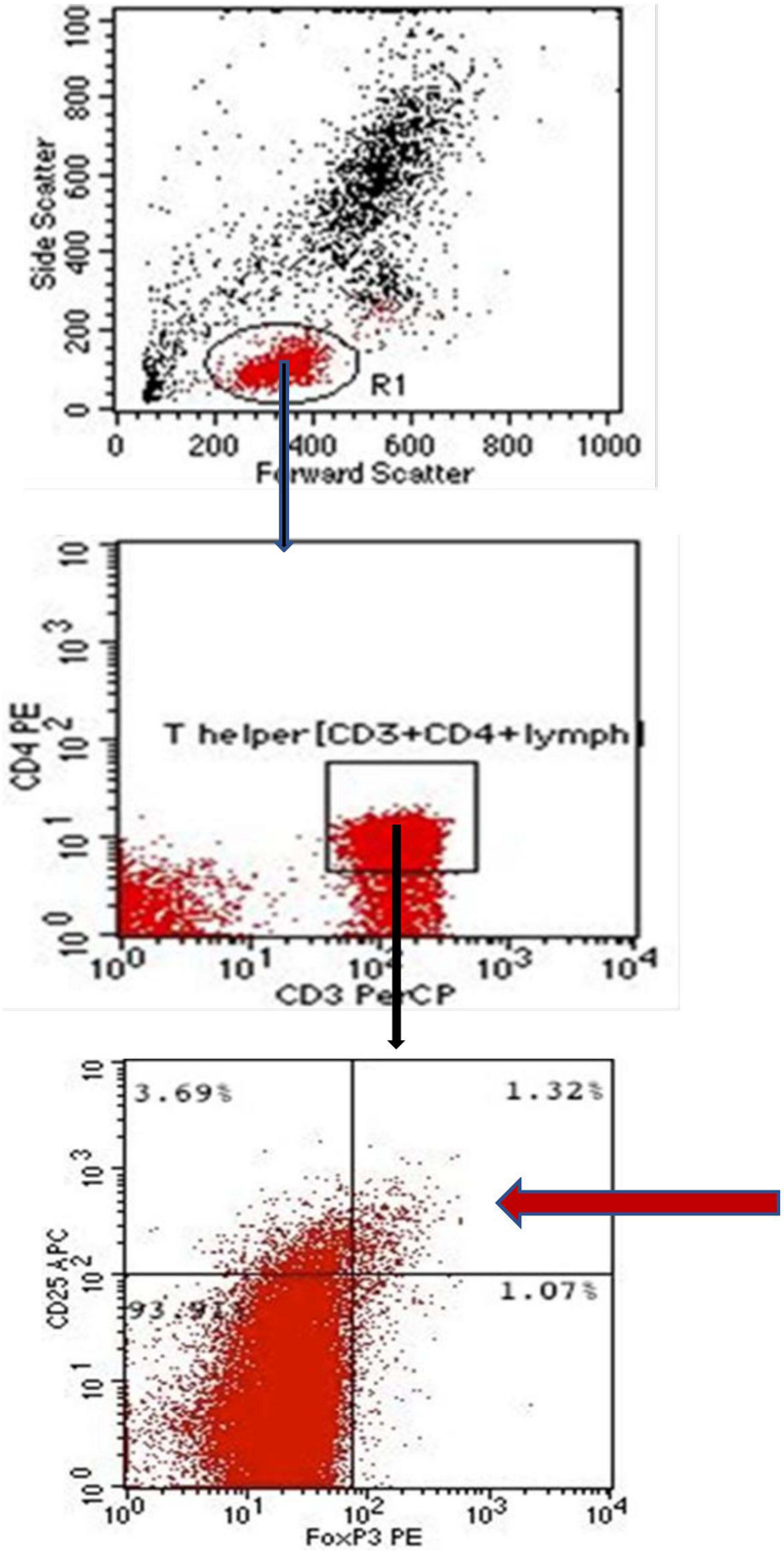

Figure 1 Gating strategy for Treg frequency detection.

Statistical Design

The collected data was reviewed, coded, and statistically analyzed using version 18 of Statistical Package for the Social Sciences (SPSS) (Inc, Chicago, Illinois, USA). The descriptive statistics for the data were mean ± standard deviation (SD),

median, and percentage. The Chi-square test was used to compare qualitative data. The Student's *t*-test was utilized to compare quantitative data. Additionally, a correlation test was conducted to determine the relationship between various biomarkers in asthmatic children. The level of significance was set at $p < 0.05$, and the results were presented in tables and graphs. ROC curve was performed with an Area under curve (AUC) calculated. Logistic regression analysis was performed odds ratio (OR) with 95% confidence intervals were computed to assess the association between each possible parameter and the occurrence of severe BA.

Results

In this study, 50 asthmatic children were divided equally between groups of mild to moderate asthma (n = 25) and severe asthma (n = 25). In addition, 26 healthy children of the same age and sex served as controls, allowing the expression of microRNA to be standardized to the average.

Clinical information on children with BA is documented in (Table 1). Age, pulmonary function test, and laboratory data comparisons between study groups are detailed in (Table 2).

Age and sex were comparable between groups, as revealed by a comparison of the groups. No significant deviation was observed in the mode of delivery.

The percentage of Treg was significantly reduced in the severe group compared to the mild to the moderate group and the control group ($P < 0.001$), whereas there was no significant difference between the mild to the moderate group and the control group (Table 2, Figure 2A).

Table 1 Clinical Data of the Bronchial Asthma Children (n=50)

		Severe Cases		Mild to Moderate Cases		P value
		Count	%	Count	%	
Rhinorrhea	**Y**	12	48.0%	10	40.0%	0.569
	N	13	52.0%	15	60.0%	
Sinusitis	**Y**	9	36.0%	0	0.0%	0.002
	N	16	64.0%	25	100.0%	
Wheeze	**Y**	25	100.0%	25	100.0%	–
	N	0	0.0%	0	0.0%	
Cough	**Y**	25	100.0%	25	100.0%	–
	N	0	0.0%	0	0.0%	
Dyspnea	**Y**	13	52.0%	13	52.0%	1
	N	12	48.0%	12	48.0%	
Sputum	**Y**	14	56.0%	12	48.0%	0.571
	N	11	44.0%	13	52.0%	
Rhinitis	**Y**	8	32.0%	2	8.0%	0.034
	N	17	68.0%	23	92.0%	
Eczema	**Y**	9	36.0%	4	16.0%	0.107
	N	16	64.0%	21	84.0%	
Conjunctivitis	**Y**	1	4.0%	1	4.0%	1
	N	24	96.0%	24	96.0%	

(*Continued*)

Table 1 (Continued).

		Severe Cases		Mild to Moderate Cases		P value
		Count	%	Count	%	
Drug allergy	**Y**	0	0.0%	0	0.0%	–
	N	25	100.0%	25	100.0%	
Inhaled Steroids	**Y**	20	80.0%	20	80.0%	1
	N	5	20.0%	5	20.0%	
Inhaled B2 agonist	**Y**	25	100.0%	25	100.0%	–
	N	0	0.0%	0	0.0%	
Leukotriene antagonist	**Y**	4	16.0%	4	16.0%	1
	N	21	84.0%	21	84.0%	
Systemic steroids	**Y**	8	32.0%	1	4.0%	**0.023***
	N	17	68.0%	24	96.0%	
Family hist.	**Positive**	19	76.0%	11	44.0%	0.021
	Negative	6	24.0%	14	56.0%	

Note: *Statistically significant (P value <0.05) in bold.

Table 2 Comparison of Demographic Data, Pulmonary Function Test, and Flow Cytometry Data Between Study Groups

		Severe Cases N=25		Mild to Moderate Cases N=25		Control Group N=26		P1	P2	P3
		Count	(%)	Count	(%)	Count	(%)			
Sex	F	5	20%	6	24%	6	23.1%	0.733	0.789	0.938
	M	20	80%	19	76%	20	76.9%			
Mode of delivery	NVD	11	44.0%	14	56.0%	8	30.8%	0.396	0.124	0.017
	CS	14	56.0%	11	44.0%	18	69.2%			
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**P1**	**P2**	**P3**
Age/ years		9.16	2.23	7.72	3.09	8.35	2.26	0.065	1.00	1.00
No of attacks /month		7.84	2.94	1.52	0.51	–	–	< 0.001	–	–
FEV1		73.88	5.00	89.20	6.98	98.27	5.42	< 0.001	0.056	<0.001
		Median	**1st-3rd Quartile**	**Median**	**1st-3rd Quartile**	**Median**	**1st-3rd Quartile**	**P1**	**P2**	**P3**
T regulatory %		1.99	0.78–2.2	2.40	2.40–5.10	3.05	2.49–3.77	< 0.001	1	<0.001

Notes: P1 referring to comparison between mild and severe; P2 referring to comparison between mild and control; P3 referring to comparison between severe and control.
Abbreviations: F, female; M, male; P, Probability value; SD, Standard Deviation; FEV1, Forced expiratory volume 1st second; NVD, Normal vaginal delivery; CS, Cesarean section.

miR146a-5p was upregulated in the mild to moderate group compared to the severe group when miRs expression was analyzed in relation to the average expression. The fold changes of expression in the mild to the moderate group were approximately 5.8 times greater than the mean expression (P = 0.001). In contrast, miR-210-3p was significantly

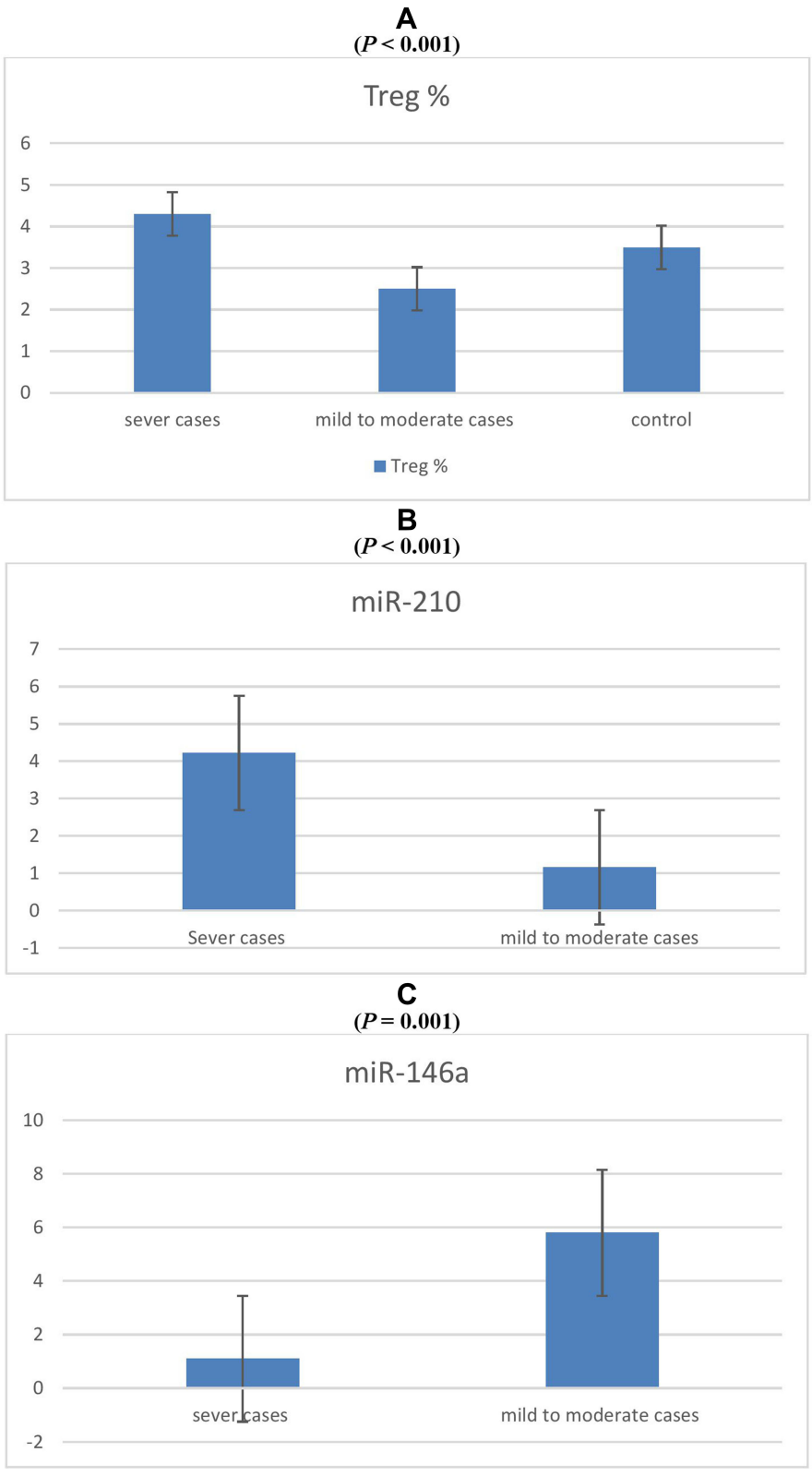

Figure 2 Comparison of frequency of circulation Treg, expression level of miR146a and miR 210 between severe group and mild to moderate group. (**A**) Treg was down regulated in severe group (P < 0.001. (**B**) MiR-210 was significantly up-regulated in severe group (P < 0.001). (**C**) miR146a was significantly up regulated in mild to moderate group (P = 0.001).

Table 3 Comparison of miR-146a, miR-210-Fold Changes and Complete Blood Picture, Between Children with Different Degrees of Asthma

	Severe Cases N=25			Mild to Moderate Cases N=25			P value
	Median	1st Quartiles	3rd Quartiles	Median	1st Quartiles	3rd Quartiles	
microRNA-146a fold change	1.1	0.12	4.34	5.8	3.2	17.74	0.001
microRNA-210-fold change	4.22	2.87	7.58	1.16	0.44	2.03	<0.001
TLC × 10^3/mm^3	6.55	5.60	9.10	7.80	6.90	9.40	0.277
ANC × 10^3/mm^3	2.30	2.00	3.00	2.90	2.10	3.10	0.460
ALC × 10^3/mm^3	3.50	2.90	4.00	4.00	3.10	5.00	0.213
AEC × 10^3/mm^3	0.40	0.30	0.58	0.70	0.60	0.90	< 0.001
PLT × 10^3/mm^3	264	228	298	302	267	349	0.001

Abbreviations: TLC, total leucocytic count; ANC, absolute neutrophil count; ALC, absolute lymphocytic count; AEC, absolute eosinophilic count; PLT, platelet.

upregulated in the severe group, with a (4.22-fold) increase in expression relative to the mean ($P < 0.001$), as shown in Table 3 and Figures 2B and C.

There was a significant increase in AEC in mild to moderate cases when compared to severe cases ($p< 0.001$) as shown in Table 3.

Comparison of Treg frequency in children with asthma subclassified according to presence or absence of another allergic disease shown in Table 4.

Children with BA (n = 50) showed a negative correlation between Treg% and miR-210-3p level ($r = -0.828$, $P < 0.001$). Treg% correlated positively with miR-146a-5p concentration ($r = 0.303$, $P = 0.032$) and FEV1 ($r = 0.507$, $P < 0.001$), as shown in (Table 5, Figure 3).

There was an inverse correlation between miR-210-3p and absolute eosinophilic count while Treg frequency and miR-146a-5p showed no correlation with AEC.as displayed in Table 5.

Treg at cutoff 2.7 with the area under the curve (AUC) of 0.828 yielded 80.0% sensitivity and 60% specificity, miR-210-3p at cutoff 1.65 fold change with AUC = 0.923 yielded 96% sensitivity and 68% specificity, and miR-146a-5p at cut off 4.4 with AUC = 0.771 yielded 76% sensitivity and 60% specificity could distinguish mild to moderate from severe patients. as evident in (Table 6).

Table 4 Comparison of Treg Frequency in Children with Asthma Subclassified According to Presence or Absence of Another Allergic History

		T Regulatory %			P value
		Median	1st Quartile	3rd Quartile	
Rhinorrhea	**Yes** (n=22)	2.20	0.82	2.90	0.245
	No (n=28)	2.58	1.99	4.45	
Sinusitis	**Yes** (n=9)	0.85	0.70	2.00	0.003
	No (n=41)	2.60	1.99	4.60	
Rhinitis	**Yes** (n=10)	1.43	0.70	2.30	0.034
	No (n=40)	2.60	1.25	4.62	
Eczema	**Yes** (n=13)	0.82	0.60	2.20	0.002
	No (n=37)	2.60	2.00	4.60	

Table 5 Correlation of T Regulatory Frequency, miR-146a-5p and miR-210-3p with Other Study Markers in Children with Asthma n=50

All Patients (n=50)		Treg %	miR-146a-5p	miR-210-3p
Age/ years	**Correlation Coefficient**	−0.039	−0.162	0.146
	P value	0.786	0.261	0.313
	N	50	50	50
Age of Onset/ year	**Correlation Coefficient**	0.263	0.177	−0.277
	P value	0.065	0.218	0.051
	N	50	50	50
No of attacks /month	**Correlation Coefficient**	−0.494	−0.498	0.671
	P value	< 0.001	< 0.001	< 0.001
	N	50	50	50
AEC	**Correlation Coefficient**	0.270	0.338	−0.388
	P value	0.058	0.016	**0.005***
	N	50	50	50
miR-210	**Correlation Coefficient**	−0.828	−0.348	–
	P value	< 0.001	0.013	–
	N	50	50	–
miR-146a	**Correlation Coefficient**	0.303	–	−0.348
	P value	0.032	–	0.013
	N	50	–	50
FEV1	**Correlation Coefficient**	0.507	0.437	−0.621
	P value	< 0.001	0.001	< 0.001
	N	50	50	50

Note: *Statistically significant (P value < 0.05)
Abbreviations: FEV1, Forced expiratory volume 1st second; AEC, absolute eosinophilic count.

In order to evaluate altered Treg frequency, miR-146a-5p and miR-210-3p as independent risk factors for asthma severity, a logistic regression analysis was conducted with adjustment of cofounders (age and family history), as shown in Table 7. The results showed that, miR-210-3p could be considered as independent risk factor [OR =3.119 (1.119–8.692), P = 0.030], while miR-146a-5p could be considered as protective factor. [OR =0.811 (0.658–0.999), P = 0.049].

Discussion

Asthma affects approximately 14% of children and adolescents worldwide, making it the most prevalent chronic respiratory disease in childhood. Despite the high prevalence, the outcomes for childhood asthma are poor. Due to numerous diagnostic challenges, overdiagnosis and underdiagnosis of pediatric asthma continue to be a challenge. For effective asthma control, the prevention of asthma triggers must be viewed holistically. Subsequently, using individualized asthma action plans, it is vital to modify the risk factors and steps taken during acute attacks.[14] Understanding the processes that sustain asthmatic inflammation is essential for the development of precise treatments, so this was our objective.

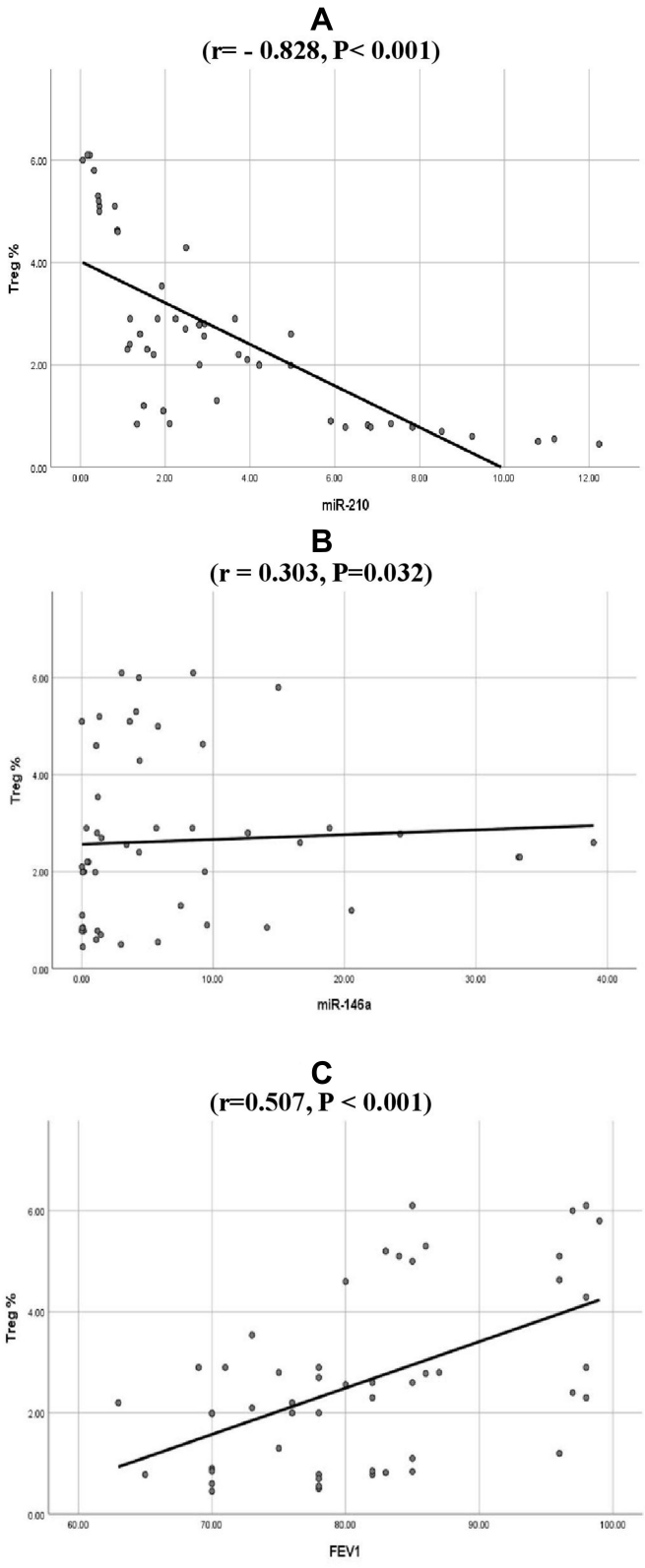

Figure 3 Correlation of Treg with miR-210-3p, miR-146a-5p and FEV1. (**A**) There was a significant negative correlation between Treg % and miR-210-3p level of expression (r= - 0.828, P< 0.001). (**B**) There was a significant positive correlation between Treg % and miR-146a-5p level of expression (r = 0.303, P=0.032). (**C**) There was a significant positive correlation between Treg % and FEV1 (r=0.507, P < 0.001).

Table 6 Output Data of ROC Curve Regarding the Study Markers Discriminative Ability to Differentiate Severe Group from Mild to Moderate Group

	Area Under the Curve	P value	95% Confidence Interval		Cut Off	Sensitivity %	Specificity %
			Lower Bound	Upper Bound			
T regulatory %	0.828	< 0.001	0.716	0.940	2.7	80	60
miR-210 fold change	0.92	< 0.001	0.85	0.994	1.65	96	68
miR-146a fold change	0.771	0.001	0.63	0.91	4.4	76	60

Table 7 Multivariate Analysis Performed in Children with Asthma to Test Study Markers as Predictors of Severity in Bronchial Asthma

		P value	OR	95% C.I.	
				Lower	Upper
Severe asthma	**T regulatory %**	0.336	0.645	0.264	1.577
	miR-146a fold change	0.049	0.811	0.658	0.999
	miR-210 fold change	0.030	3.119	1.119	8.692
	Age/ years	0.252	1.268	0.845	1.904
	Family hist.	0.712	1.509	0.170	13.365

When compared to individuals with mild to moderate asthma, children with severe asthma showed a significantly increase in incidence of associating sinusitis and rhinitis ($p = 0.002$ and $p = 0.034$, respectively). Samitas et al found that rhinitis, sinusitis, and asthma frequently coexist. and that illness mechanisms that manifest in the upper airways may be a reflection of dynamic tissue reorganization events that occur in the lower airways.[15] According to Humbert et al 2017, multi-morbidities, most of which are allergic in nature, share a common underlying inflammatory pathophysiological mechanism, frequently coexist with allergic asthma. Asthma severity, and patients' responses to treatment may be impacted by coexisting multi-morbidities.[16]

T lymphocyte-induced inflammation is believed to be the primary pathophysiological mechanism underlying asthma. An imbalance between Th17 and Treg cells may precipitate the development of asthma in asthmatics.[17] Tregs are essential for suppressing effector cells and maintaining self-tolerance. Ineffective differentiation and Treg dysfunction may contribute to the development of asthma.[18] In Treg cells, a number of transcriptional cofactors interact with the Foxp3 transcription factor to support their suppressive function.[19]

Compared to the mild to moderate group, the percentage of Treg was lower in the severe group ($P < 0.001$). Bakr et al demonstrated that regarding Egyptian asthmatic children, have decreased Treg cells, leading to an increase in effector cells that mediate airway inflammation. However, Bakr et al found that patients with moderate asthma had a lower frequency of Treg cells than those with severe asthma.[20] Moreover, Song et al demonstrated that enhancing Treg responses suppresses allergen-induced airway inflammation.[21]

No significant difference in Treg frequency was observed between the mild to the moderate group and the control group, indicating that only severe cases have clinical relevance for Treg frequency downregulation (Table 3, Figure 2A). This contradicts the findings of Robinson, who found that asthma cases with low Foxp3 expression had inadequate Treg numbers and suppressive function in the airway. The fact that Robinson examined the airway Treg while we examined the circulating Treg explains the controversy.[22]

Endotype molecular biomarkers can reclassify the numerous asthma phenotypes. There are currently two asthma endotypes: eosinophilic T helper-2 (Th2) high and non-eosinophilic Th2 low.[23] Th2-high asthma is associated with

adaptive immunity and allergic asthma.[21] Non-Th2-type asthma is associated with non-allergic asthma, which is triggered by emotion, obesity, and environmental factors such as air pollution, including ozone and cigarette smoke, among others. Non-Th2-type asthma is distinguished by neutrophilic inflammation, M1 macrophage migration to the airways, Il-17 and IFN- production by activated Th1 cells, and an initial hyperresponsive reaction.[24]

The type 2 immune response, is based on the participation of the following cells: Th2 cells, B cells, NK cells, mast cells, eosinophils, basophils, and their cytokines, and is believed to be involved for allergic asthma and rhinitis, according to Berghi et al.[25] Shi et al noted that the overexpression of Th2 was connected to the decrease in Treg cells. Furthermore, it has been demonstrated that the fall of Treg cells in people with moderate to severe asthma plays a part in the development of the disease.[26] According to Palmer et al, type 2 inflammation is aberrant and persistent in allergic diseases is associated with altered Treg frequency.[27]

According to Norlander et al, the loss of Treg restraining function on the innate and adaptive immune systems to maintain homeostasis led to the emergence of severe asthma cases. Tregs exert a negative influence on allergic airway inflammation, which is characterized by a Th2 response caused by a loss of tolerance to harmless environmental antigens.[28] In addition, Harb et al discovered that the severity of the disease and the decrease in Treg-mediated suppression were related to an increase in Notch4 receptors on circulating and lung tissue Tregs in asthmatic patients. Without Notch4, allergens and particle pollution cannot cause airway inflammation. Notch4 amplifies the inflammatory response by transforming Tregs into Th2 and Th17 effector T cells.[19]

Foxp3 is a "master" (multiple pathways) regulator gene that manages the stability and development of Treg cells, according to various definitions. Recent research demonstrates that the epigenetic mechanisms, specifically the miRs responsible for regulating the expression of the Foxp3 gene, are crucial components of the suppressive action of Treg cells.[29]

Furthermore, group 2 innate lymphoid cells (ILC2s) have a powerful potential to create Th2-type cytokines and are crucial in the development of allergy disorders and asthma.[30] They also highlighted that Induced Tregs (iTregs) can regulate immune responses and suppress the production of ILC2-driven, IL-5 and IL-13 which contribute to asthma development.

In miRs analysis, we found that miR-210-3p was significantly upregulated in the severe group compared to the mild to moderate group ($P < 0.001$), with approximately (4.2) folds higher expression than the average expression (Table 2). In contrast, Kyyaly et al found that severe asthmatics had significantly lower serum levels of miR-210-3p than mild asthmatics. His study included only four adult BA patients in each group, making our data statistically more credible.[9]

Two members of the miR-146 family, miR-146a and miR-146b are expressed as negative feedback in response to pro-inflammatory stimuli to prevent excessive inflammation.[31] In this study, the expression of miR146a-5p was significantly upregulated in the mild to moderate group compared to the average expression by 5.8-fold ($P = 0.001$). Tahamtan et al reported that miR-146a has an anti-inflammatory function that negatively regulates the interferon (IFN) response and adaptive immunity by targeting adaptor protein (AP)-1 activity and IL-2 expression, as well as immune cell activation and cytokine production.[32]

According to Liu et al, the targeting of miR-146a results in a negative feedback loop for Nuclear factor kappa B (NF-κB) signaling. Thus, pro-inflammatory cytokines such as tumor necrosis factor-alpha, interleukin-1 (IL-1), interleukin-6 (IL-6), and chemokines are diminished.[33]

There was significant decrease of Treg in children suffering of sinusitis and rhinitis when compared to children without sinusitis and rhinitis ($p = 0.003$ and $p = 0.034$, respectively), as displayed in Table 4 which is in agreement with Palmer et al, who stated that type 2 inflammation characterises rhinitis and chronic rhinosinusitis and is associated with altered Treg frequency.[27] According to Berghi et al. Tregs have the ability to regulate T-effector function and reduce the generation of Th2 inflammatory cytokines in rhinitis and chronic rhinosinusitis.[25]

Platelet count was significantly lower in children with severe asthma compared to those with mild to moderate symptoms (Table 3). This was consistent with the findings of Kowal et al, who discovered a correlation between platelet count and allergen challenge in allergic asthma. This correlation was attributed to platelet intravascular activation resulting from an increase in plasma B-thromboglobulin.[34]

Treg frequency was negatively correlated with the miR-210-3p level in all children with asthma (n = 50) (r = −0.828, P < 0.001). Long et al found that miR-210-3p targets FoxP3 expressed by Treg and inhibits it, which explains why miR-210-3p has an exacerbating role in the development of asthma.[35] In addition, Zhao et al found that miR-210-3p regulates the immunosuppressive activities of Tregs by binding to the 3′-UTR region of Foxp3 and suppressing Foxp3 protein expression.[10]

The current study discovered a correlation between Treg frequency and miR-146a-5p concentration (r = 0.303, P = 0.032). According to Lu et al, miR-146a-5p is a key regulator of Treg cell activity and is required for Treg cell suppression of IFN-mediated pathogenic Th1-associated inflammation. However, Lu et al discovered that the absence of miR-146a causes Treg cells to proliferate but function poorly. Instead of plasma miR-146a-5p, they investigated the intracellular miR that is expressed by Treg cells.[12] Liu et al discovered a significant inverse correlation between the quantity of miR-146a-5p and the number of Treg cells and Foxp3 expression. This controversy can be explained by the differences in disease nature between the current study and that of Liu et al which examined this relationship in rheumatoid arthritis.[36]

As demonstrated by the present study, there was a positive correlation between the Treg proportion and FEV1 (r = 0.507, P < 0.001) (Table 5). In agreement with our findings, Kraszula et al reported that in patients with severe adult asthma, activated Treg cells expressing chemokine receptor 5 (CCR5) reflect high suppressive activity, can home to the lung, and correlate positively with FEV1.[37] According to Zou et al, the ratio of Treg to Th17 cells is correlated with asthma severity and disease management. The ratios of IL-17A/IL-10 and RORt/Foxp3, which are inversely correlated with FEV1, indicate that Treg has a positive effect on FEV1.[38] In addition, Zheng et al reported that AHR is associated with a decrease in Treg and an imbalance in the Th17/Treg ratio in asthmatic children.[39]

Also, there was an inverse correlation between miR-210-3p and AEC which indicated that miR-210-3p is not only linked to the phenotypic classification of asthma but also relates to the endotypic classification of eosinophilic Th2 type[23] and highlights its importance as target therapy.

ROC curve analysis of the examined markers established the superiority of miR-210-3p as a biomarker of severity that can distinguish mild to moderate from severe patients at cutoff = 1.65-fold change with AUC = 0.92, 96% sensitivity, and 68% specificity. Following this, miR-146a at cutoff 4.4 with AUC = 0.771 exhibited 76% sensitivity and 60% specificity, whereas the frequency of circulating Treg at cutoff 2.7 with AUC of 0.828 exhibited 80% sensitivity and 60% specificity (Table 5). When considering the stability of miRs in plasma and the convenience of storage, the significance of miR-210-3p as a biomarker of asthma severity in children is heightened.

Logistic regression analysis denoted that he upregulation of miR-210-3p is an independent risk factor for BA severity while miR-146a-5p is considered as protective factor which could be explained by their opposing relation with Tregs frequency which suppresses Th2.[26,28]

Limitations of the Study

Due to a lack of funding, airway Treg cells and the cytokines produced by Treg were not evaluated in the current study, but they should be in future studies in order to gain a deeper understanding of the relationship between Treg functions and plasma miR-210-3p and miR-146a-5p. Also, it is recommended in future studies to correlate the current study markers with IgE levels and IgE-mediated immunologic pathways.

Conclusion

A decrease in the frequency of Treg cells, downregulation of miR-146a-5p, and overexpression of miR-210-3p exacerbate asthma in Egyptian pediatric population. Treg frequency is linked to, FEV1, miR-146a-5p and miR-210-3p in childhood BA. Upregulation of miR-210-3p is a sensitive biomarker and an independent risk factor for BA severity in Egyptian children.

Data Sharing Statement

Data is available upon request.

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Disclosure

The authors report no conflicts of interest in this work.

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