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Research Article

Circulating Serum Levels of Fox P3, GATA-3 and IL-17 A as Potential Biomarkers in Patients with Symptomatic Asthma

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I N F O

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A B S T R A C T

Background: The lack of information on the association of certain factors in the pathophysiology of the chronic inflammatory disorder, Asthma limits its early detection and therapy. Keeping these facts in view, an endeavour was made to investigate the roles, if any, such as IL-17A, FoxP3 and GATA-3 in the occurrence and intensity of asthma in north Indian population.

Objective: The study was conducted to evaluate the level of FoxP3, GATA-3 and IL-17A in symptomatic asthma patients as compared to healthy controls.

Methods: In the study we had taken 125 cases and 125 controls. Levels of total interleukin 17A, transcription factors FoxP3 and GATA-3 were measured by ELISA in serum of asthmatic patients. Student's T-test and ANOVA test was applied for quantitative variables. The qualitative variables analyzed using Chi-Square test /Fisher's exact test.

Results: Clinical symptoms like breathlessness, cough, headache, disturbed sleep, congestion, and wheezing were found significantly elevated in asthmatics as compared to the controls. Except haemoglobin; in the cases the levels of blood eosinophils, Absolute Eosinophil Count (AEC), Total Leukocyte Count (TLC) and serum Immunoglobulin E (IgE) were observed higher as compared to the controls and also the higher serum levels of IL-17A and GATA-3 were found in the cases. Contrary to IL-17A and GATA-3; the levels of FoxP3 was increased in the control, and was lower in the patient serum. A positive correlation was found between total IgE and GATA-3 (Pearson Correlation=0.283, P=0.042). We have shown that GATA-3 is increased in the asthmatic patients (0.541±0.140) than in the controls (0.312±0.076). The GATA-3 was found over expressed in patients with severe asthma.

Conclusion: Increased level of GATA-3 and IL-17A might be useful as diagnostic marker for patients with symptomatic asthma there is need for further larger scale studies to establish the role of these biomarkers in asthmatic patients.

Keywords: Asthma, GATA-3, FOXP3, IL-17A, Indian Population

Introduction

Among many persistent inflammatory respiratory diseases, asthma is the most common. It has been characterized by bronchial hyper-responsiveness, short breaths, and frequent events of wheezing, chest tightness, coughing and increase in total immunoglobin (IgE) levels.[1] Being one of the most prevalent chronic diseases of airways, asthma has been reported to adversely affect >300 million people worldwide for the most part to the children and adults.[2] In United States, asthma has affected >23 million adults. As compared to men, women are more vulnerable to asthma with greater morbidity.[3] In India, currently about 30 million people are reported to be afflicted with this disease. Looking at the data of the last one decade, the prevalence of asthma in children has been observed to be constantly increasing in the Indian subcontinent.

In previous decade, a major investigation has dealt with the role of T-helper cells (Th1, Th2 and Th17) and their mediators/ cytokines that have been associated in the onset of airway inflammatory response in asthma.[4] It has been reported that the cytokines released by Th2 cells are essential for Immunoglobulin E (IgE) synthesis, chemokine production, and mucous production in the airways.[5] The chronic respiratory inflammation and hyper-responsiveness have been indicated to be an outcome of a coordinated interaction between T and B-cells, as well as the mast cells and eosinophils.[6] We have demonstrated that Th17 cells may be responsible for pathogenesis of inflammation due to allergy in asthma. They have further indicated that allergic asthma may enhance the number of Th17 cells and hence the production of Th17 cytokines in the patients.[7] This conclusion suggests that there may be involvement of Th17 immunity in the systemic immune responses of allergic asthma.[8] The results of certain clinical experiments have suggested that Th17 mediated inflammation plays significant role in the patients suffering from severe asthma and neutrophilic inflammation, as well as steroid-resistance.[9] The interleukin 17 (IL17) is the main cytokine of Th17 cells which plays a key role in imparting protection against infection. IL17 has also been implicated to induce and maintain chronic inflammatory disorders.[10] The IL-17A and IL-17F are the two essential members belonging to the IL-17 family of cytokines.[11]

Recent data have indicated that the expression of IL-17A in sub mucosa of bronchia is increased,[12] which reflects the possible involvement of IL-17A in the pathogenesis of asthma.[13] Also, Treg has been shown to play a part in prevention of the allergic diseases via suppression of the production and activities of Th2 cytokines.[14,15] Like Treg, some other factors which include CD4, CD25, and FoxP3 (fork head box transcription factor) have been shown to be involved in the development as well as in the functioning of Th2.[16-19] The role of FoxP3 has been assigned to regulate differentiation of native T-cells into Treg phenotype.[18-20] These cells suppress the functions of T cells which are auto-reactive in nature and may control Th2 cells implicated in pathogenesis and also the Th1 cells which are expected to be involved into initiation of asthma.[19] Th2 cells and GATA-3 gene have important role in IgE production, allergic inflammation and asthma.[21] The low expression of GATA-3 gene in asthmatics found under basal conditions and even after *in vitro* stimulation which was expected to be higher alike younger asthmatic groups.[22,23] GATA-3 expression in both groups was analyzed in basal as well as in activated states. The increased FoxP3 transcription factor modulates the immune response.[20,24] The aim of study was to analyse the concentrations of IL-17A, FoxP3, GATA-3 and IgE in the blood serum of asthmatic and healthy control groups.

Materials and Methods

Period of Study

The study was conducted for 12 month duration in 2016. Prior approval was obtained from institutional ethics committee constituted by King George's Medical University (KGMU), Lucknow comprising of external experts as members.

Study Population

The study population included cases and control with 125 cases and 125 controls were enrolled on the bases of inclusion and exclusion criteria from the OPD of the Department of Pulmonary and Critical Care Medicine, and King George's Medical University (KGMU), Lucknow. Spirometry test for both cases and controls were assessed in the study for lung function by Spirometry method and predicted values of FEV_1 were calculated. Spirometry was defined by a percentage of FEV_1 within 3 hours after included in OPD of the Department of Pulmonary and Critical Care Medicine, King George's Medical University (KGMU), were considered as one of the parameters.

Collection of Demographic and Epidemiological Information

A pre-designed Performa was employed to collect demographic and epidemiological information on age, sex, occupation, family history, clinical symptoms with more than five days based on records available with the patient and history of illness in the last 12 months suggestive of infectious nature viz, chest infections, chest tightness, Breathlessness, Cough Headache, Disturbed sleep, Chest tightness, Wheezing for which treatment was sought from local practitioners.

Clinical Specimens

Blood samples were collected from patient's visiting the OPD. The patients were classified as cases and controls and

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blood were collected in the plain vial for the separation of serum.

Inclusion and Exclusion Criteria

All the samples from controls and cases were categorized under following inclusion and exclusion criteria:

The Inclusion Criteria for Cases

- Age: 18 years to 65 years
- Cases were taken irrespective of the stage of asthma
- Patient who has symptoms of persistent cough, sputum production, or dyspnea, and/or a history of exposure to risk factors for the disease. The diagnosis is confirmed by spirometry (as per GINA criteria)
- Asthma: >12% reversibility of FEV1 with inhaled bronchodilators

Exclusion Criteria for Cases

- Age<18 years
- Other associated diseases (TB, Pneumonia and other)
- Pregnancy
- COPD Patients have <80% predicted and FEV1/FVC<70%

Inclusion Criteria of Control

- Age sex matched
- Not to be primary relative of the patients

Exclusion Criteria for Control

- Age <18 years
- Other associated disease (COPD)
- Pregnancy
- Patients have <80% predicted and FEV1/FVC<70%

The clinical specimens, comprising of blood and serum samples, were received by the Department of Pulmonary and Critical Care Medicine of KGMU Hospital under standard transport conditions. The specimens were processed for identification as per standard protocol techniques and the levels of haemoglobin, eosinophils, Absolute Eosinophil Count (AEC), Total Leukocyte Count (TLC) and serum Immunoglobulin E (IgE) were estimated. Total serum protein levels was determined for IL-17F, GATA-3 and FoxP3 using standard ELISA kits (M/ s Sunred). 17F levels was determined using ELISA Kits (Sunred M/ s, ELISA Kit Catalogue no. 201-12-0047), FOXP3 ELISA Kit Catalogue no. DZE201120693), GATA3 ELISA Kit Catalogue no. DZE201123855). The tests were performed on 125 cases and 125 controls, as per the manufacturer's protocol.

The ELISA was performed as per the standard procedure. The wells of the ELISA plates were coated with 40 µl of test sample to which 10 µl of biotin labelled IL 17F antibody were added. Further, 50 µl of Streptavidin HRP was added and the plates were sealed and gently mixed. The plates were incubated at 37°C for 60 minutes. The membrane was carefully removed and the liquid was decanted and the wells were briefly washed. 50 µl of chromagen solution A and solution B each were added in the order and were gently mixed followed by incubation in dark at 37°C for 10 minutes. The reaction was then stopped by adding 50 of the stop solution.

The blank wells were coated with buffer solution in place of the samples. Rest of the procedure was same. Similarly, standard solution was added in place of the samples in the wells labelled as standard.

The plates were read at 450nm wave length soon after adding the stop solution. The OD readings were normalized using blank reading taken as zero.

Data Evaluation and Statistical Analysis

Quantitative variables were compared using Unpaired Student's T-test between two groups and ANOVA test was applied among three groups. Chi-Square test /Fisher's exact test was applied to Qualitative variables and p value found <0.05 considered statistically significant. The Statistical Package for Social Sciences (SPSS) version 16.0 was used to analyze the data.

Result

Characteristics of the Study Population

The demographic profile of patients suffering from cases and the controls have been summarised in Table 1. Our study included 125 cases consisting 46 males and 79 females. Four samples with 18 years, seventy seven cases between 18 to 35 years, forty three cases between 36 and 50 years and one case above 60 years of age were considered for the study. The control group included 45 males and 80 females of age 18 years, seventy seven controls between 18 to 35 years, forty two were between 36 and 50 years and above 60 years. In the present study, we observed tobacco smoking habits were similar in the cases and control (Table 2).

Clinical Symptoms in Study Population

The occurrence of breathlessness, cough, headache, disturbed sleep, chest tightness, and wheezing were recorded to be 89.6%, 87.2%, 84.0%, 76.8%, 76.8%, 91.2% and 81.6%, respectively, in the patients. The FEV1 (%) changes (18.3520± 5.160) were also observed. The values of the clinical indices for above parameters in the cases were much higher than that in the controls (Table 3 and Figure 1).

Laboratory Parameters in Study Population

The values of parameters of laboratory investigations of the cases and controls are depicted in Table 4. The levels of IgE, Eosinophil, AEC, TLC and Hb (Mean±SD) were observed to be 399.6±11.27 IU/ml, 7.330±0.4110 % , 541.2±15.67 cell/cum and 6861±236.4 cell/cum 10.95±0.1247 (gm/

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dl), respectively, in the cases. Except Hb, the values of other clinical parameters were significantly higher than the controls. The level of Hb, however, was lower 0.95 ± 0.1247 (gm/dl) in the cases when compared to the control (13.01 ± 0.1567 gm/dl).

The levels of IL-17A, FOXP3 and GATA-3 in serum by ELISA of cases and controls were estimated and the sensitivity and specificity was determined as described in the materials and methods section. The results thus obtained are been summarised in Table 5. The IL-17A levels were found to be 0.439±0.110 ng/l in the cases and 0.318±0.073 ng/l in the controls. The GATA-3 and FoxP3 levels in cases and in controls as shown in Table 5 were estimated to be 0.541±0.140 ng/l in cases and 0.312±0.076 ng/l in controls. The levels of FOXP3 were 0.397± 0.101 ng/l in the asthmatics and 0.583±0.143 ng/l in the controls. It was observed in cases contained higher levels of IL-17A and GATA-3 but there were decreased level of FOXP3 in cases (Figure 2).

Table 1.Demographic profile of asthmatics and control: descriptive statistics

			Groups		Total
			Cases	Controls	
Age intervals	18 years	Count	4	5	9
		% within Groups	3.2%	4.0%	3.6%
	18 to 35 years	Count	77	77	154
		% within Groups	61.6%	61.6%	61.6%
	36 to 50 years	Count	43	42	85
		% within Groups	34.4%	33.6%	34.0%
	Above 60 years	Count	1	1	2
		% within Groups	.8%	.8%	.8%
Total		Count	125	125	250
		% within Groups	100.0%	100.0%	100.0%

Table 2.Status of smoking

			Groups		Total
			Cases	Controls	
Status of smoking	Absent	Count	64	69	133
		% within Groups	51.2%	55.2%	53.2%
	Present	Count	61	56	117
		% within Groups	48.8%	44.8%	46.8%
Total		Count	125	125	250
		% within Groups	100.0%	100.0%	100.0%

Table 3.Clinical symptoms

Symptoms	Cases (N=125)		Controls (N=125)		P-value
	N	%	N	%	
Breathlessness	112	89.6	56	44.8	<0.001*
Cough	109	87.2	69	55.2	<0.001*
Headache	105	84.0	76	60.8	<0.001*
Disturbed sleep	96	76.8	76	60.8	0.006*
Chest tightness	114	91.2	86	68.8	<0.001*
Wheezing	102	81.6	80	64.0	0.002*

Chi-square test Applied. *Significance.

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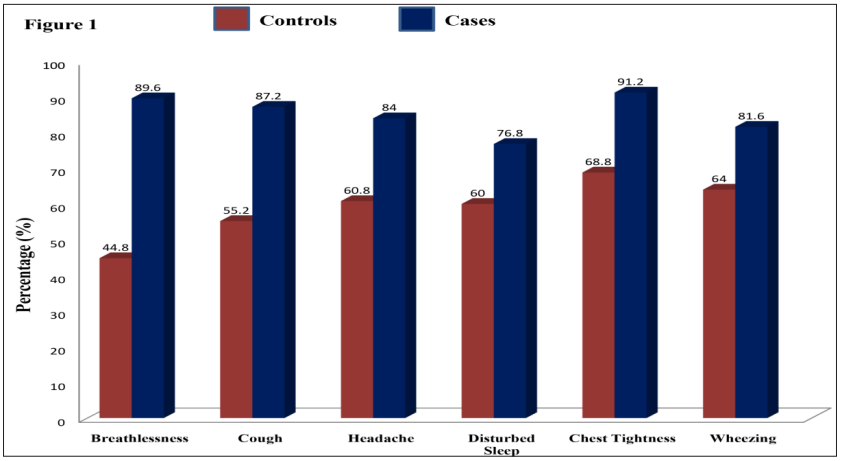

Figure 1.The symptomatic categories of cases in asthmatic patients. The values of these clinical indices as recorded for the above parameters. In the cases the indices were much higher than that in the controls

Table 4.Clinical indices in cases and controls

	Groups	Number	Mean	Std. Deviation	p-value
Hb (gm/dl)	Cases	125	10.8480	1.15742	<0.001*
	Controls	125	12.9712	1.58612	
Eosinophil (%)	Cases	125	7.1280	3.81207	<0.001*
	Controls	125	3.9920	2.14587	
TLC (cell/cum)	Cases	125	6.4667E3	2434.10330	<0.001*
	Controls	125	4.7287E3	1254.31956	
IGE (IU/mL)	Cases	125	4.0147E2	112.58293	<0.001*
	Controls	125	1.9281E2	78.27698	
AEC (cell/cum)	Cases	125	5.3013E2	146.65842	<0.001*
	Controls	125	4.2441E2	84.45824	
FEV1 (%)	Cases	125	18.3520	5.16073	<0.001*
	Controls	125	8.8080	1.67859	

Table 5.The levels of IL-17 A, FOXP3, GATA3 in serum

Protein	Groups	Number	Mean	Std. Deviation	p-value
IL-17 A (ng/l)	Cases	125	0.439	0.110	<0.001*
	Controls	125	0.318	0.073	
FOXP3 (ng/l)	Cases	125	0.429	0.124	<0.001*
	Controls	125	0.583	0.143	
GATA3 (ng/l)	Cases	125	0.541	0.140	<0.001*
	Controls	125	0.312	0.076	

Correlation among Interleukin and Transcription Factors in Different Categories of Cases

The correlations among the proteins IL-17F, GATA-3, FoxP3 and IgE have been shown in Table 6. A positive correlation established between total IgE and GATA-3 levels (Pearson Correlation=0.283, P=0.042) (Figure 3A). But there no correlation was found for the other transcription factor (FoxP3) and IL-17A, with the IgE values (Figure 3B and C).Statistical differences were observed between these analytes of asthmatics and controls for the IL-17A/ GATA-3 (Figure 2). The balance between Treg/ Th effector cells may also regulate the level of inflammation in asthmatic (Figure 3).

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Table 6.Correlations among the Proteins

		IGE (IU/mL)	IL-17 A (ng/l)	GATA3 (ng/l)	FOXP3 (ng/l)
IGE (IU/mL)	Pearson correlation	1.0	0.097	0.182*	-0.103
	Sig. (2-tailed)		0.283	0.042	0.255
	N	125.0	125.0	125.0	125.0
IL-17 A (ng/l)	Pearson correlation	0.097	1.0	0.230**	0.063
	Sig. (2-tailed)	0.283		0.010	0.488
	N	125.0	125.0	125.0	125.0
GATA3 (ng/l)	Pearson correlation	0.182*	0.230**	1.0	0.026
	Sig. (2-tailed)	0.042	0.010		0.775
	N	125.0	125.0	125.0	125.0
FOXP3 (ng/l)	Pearson correlation	-0.103	0.063	0.026	1.0
	Sig. (2-tailed)	0.255	0.488	0.775	
	N	125.0	125.0	125.0	125.0
*. Correlation is significant at the 0.05 level (2-tailed).					
**. Correlation is significant at the 0.01 level (2-tailed).					

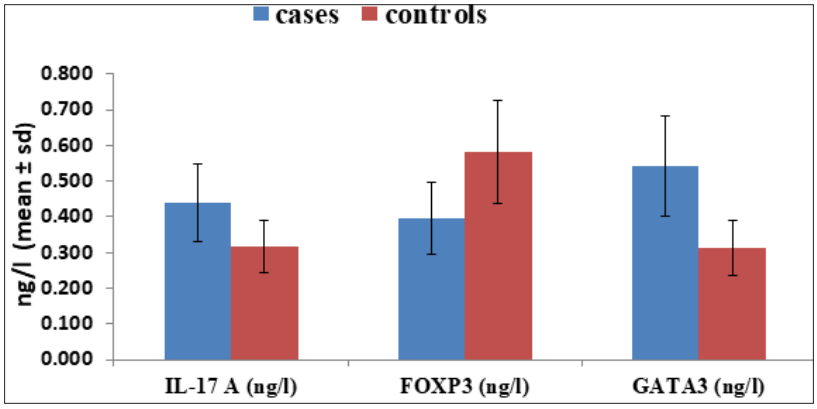

Figure 2.IL-17A, FoxP3 and GATA3 value for asthma patients (n=25) and healthy control (n=25) groups. Results are expressed as ng/l. The results are statistically significant p-value= <0.001*. IL-17A, FoxP3 and GATA3 Mean+Standard Deviation, Asthma vs control. 0.439±0.110 vs 0.318±0.101., 0.397±0.101 vs 0.583± 0.143 and 0.541±0.140 vs 0.312±0.076

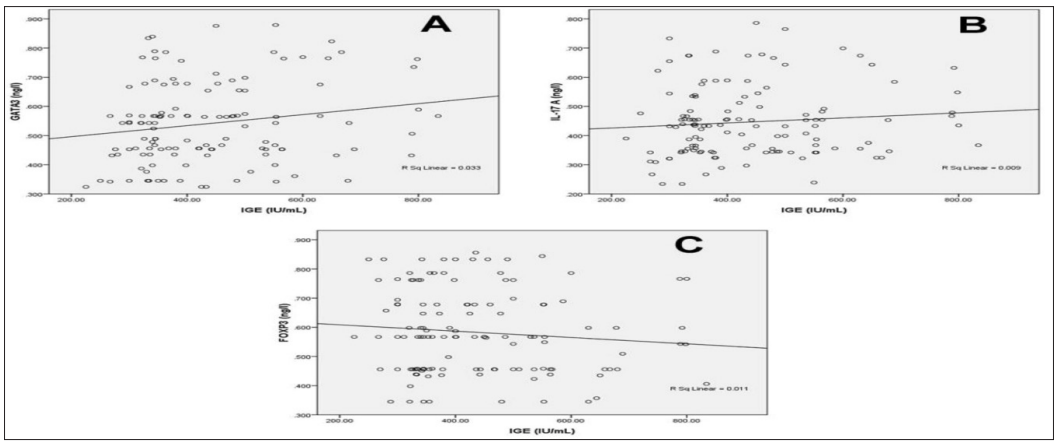

Figure 3. Correlations of IgE with all the three antigenic proteins (IL-17A, GATA-3 and FoxP3) as determined by ELISA. GATA-3 and IgE show a positive correlation

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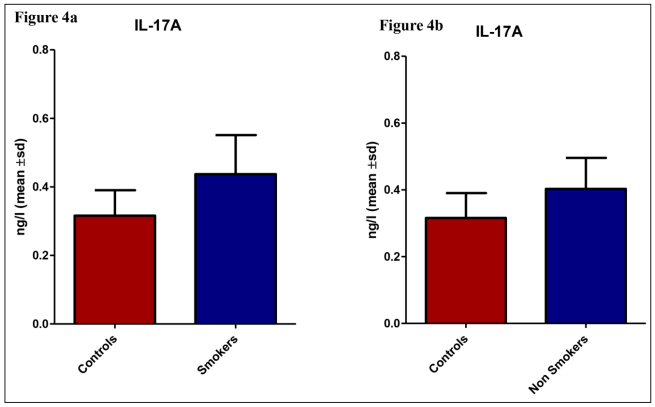

Figure 4.The comparison of antigenic response between smokers (a) and non-smokers (b) using IL-17A. The ELISA was repeated three times and mean±SD was determined

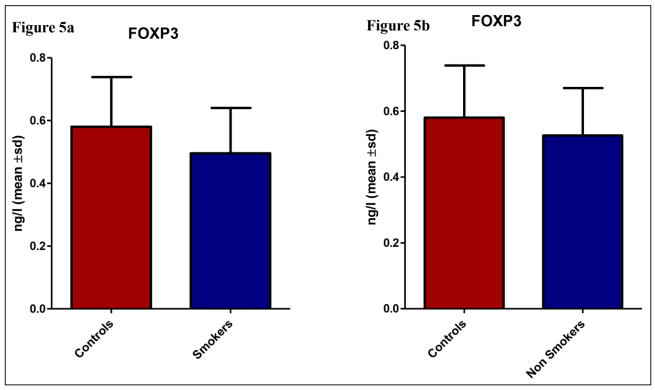

Figure 5.The comparison of antigenic response between smokers (a) and non-smokers (b) using FoxP3. The ELISA was repeated three times and mean±SD was determined

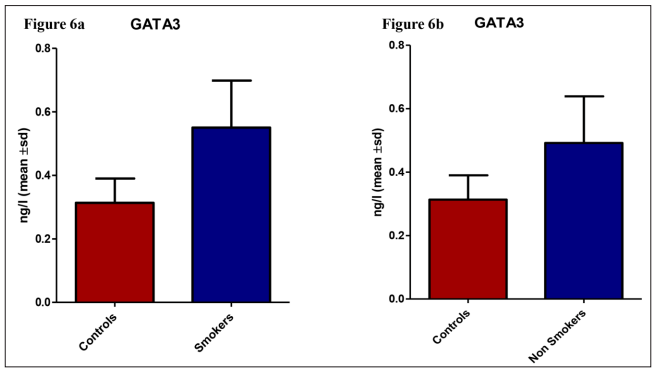

Figure 6.The comparison of antigenic response between smokers (a) and non-smokers (b) using GATA-3. The ELISA was repeated three times and mean±SD was determined

Comparative of Smoking and Non-smoking Asthmatics

In order to look for the correlation between the expression of different factors and the effect of tobacco inhalation in asthmatic patients, we have categorically determined the levels of IL-17A, FoxP3 and GATA-3 in smokers and in non-smokers. Surprisingly, the levels of IL-17A (Figure 4a and 4 b), FoxP3 (Figure 5a and 5b) and GATA-3 (Figure 6a and 4b) were not able to discriminate the patients between smokers and non-smoker groups.

Discussion

Asthma, a chronic disease affecting kids, young and adults at greater intensity, is often characterized by different levels of chronic inflammation as well as structural alterations in airway causing morbidity and mortality. Alyasin et al. have demonstrated that both the inflammatory as well as structural changes (airway remodeling) may be cause of inflammation in asthma.[25,26] The structural changes include sub-epithelial thickening, epithelial detachment, airway smooth muscle hypertrophy, hyperplasia of goblet cell, enlargement of bronchial gland, angiogenesis and changes in the components of extracellular matrix, which are influenced by a complex cytokines network.[27] Thus, a thorough knowledge about the cytokine network's modulation during asthma may significantly contribute to understanding of pathogenesis of disease and also towards developing new therapeutic strategies to cure.

The data obtained from this study suggested that though the age and height were almost the same in asthmatics and in the controls; the cases displayed loss of weight in comparison to that of controls. Also among the total people recruited for this study, the smokers in the cases were higher with longer smoking duration than controls. However, the family history of asthma, breathlessness, cough, headache, disturbed sleep, chest tightness and wheezing were significantly higher in the asthmatics in comparison to the controls. Our results corroborate with the findings earlier reported while studying the expression of mRNA IL-17A in atopic asthmatics.[28]

In the onset of asthma attacks, the cytokine IL-17A plays a crucial role but in varied ways. It has been shown by various groups that IL-17 is involved in influencing the number of respiratory tract neutrophil, generates hyper-reactivity in respiratory tract, induces hyper-secretion of mucus, causes remodelling of airway, and also the steroid resistance.[29-32] possibly, these cascades of events are the causes of pathogenesis of inflammation of airway due to allergic reactions. The variations in different genes associated with the occurrence and severity of asthma have also been recently demonstrated by Piva SR et al.[33]

The results from the present investigation reflected enhanced Immunoglobulin E (IgE) levels, eosinophils, AEC and TLC in the cases. However, the Hb content was lower in the cases in comparison to the controls. The elevated total IgE level is considered as one of the chief characteristics in the atopic asthmatics or allergic asthmatics.[28] Mowahed

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M et al. have also reported the significantly higher concentration of serum IgE in the asthmatics than the normal individuals.[34] The results of present investigation showing increased levels of eosinophils, AEC and TLC in the patients corroborate with the findings earlier presented, who have indicated that there could be some asthmatics with both eosinophilic and neutrophilic inflammations.[35]

The recent studies have indicated that T helper 17 (Th17) cells may be involved in the onset/ severity of asthma via inducing inflammation in airways through secreting interleukin IL-17A, a cytokine of Th17. Mowahed et al. have indicated that the serum IL-17A concentration got significantly elevated in the asthmatics in comparison to the controls.[34] In our study, this pattern was recorded to be equivalent to their finding i.e. the serum IL-17A level was higher in the cases than the controls. The increased level of serum IL17-A seems to be associated to the pathophysiology of allergic asthma.

Mowahed et al. have further expressed that IL-17A, like the IgE, rises in sera of asthmatics as the different indicators i.e. IgE increased in the serum consistently with the disease severity whereas IL-17A increased in serum with increase in severity of the disease.[34] Thus they demonstrated almost no correlation between these two clinical indices. The results of our investigation reflected increase in the levels of these two parameters with the onset/ increase in the severity of the disease there by indicating a positive correlation between them.

In another study, it has been demonstrated that asthmatics exhibited a significantly higher level of serum IL-17A in comparison to controls (healthy) subjects thereby suggesting that IL-17A can be exploited as a potential clinical biomarker for prospective diagnosis and therapeutic management of asthma.[36] In our study, the FoxP3 concentration was found significantly higher in asthmatic in comparison to healthy controls (p=0.000). The FoxP3 levels were also significantly higher in asthmatic group on corticosteroids. According to the Hori S et al. FoxP3 expression was also higher in CD4+CD25+ Treg cells in asthmatics as anti-inflammatory.[37,38] In contrast; Karengiannidis et al. found no significant difference in FoxP3 level in both healthy and asthmatics.[39] There is an increased level of suppressive cytokines and transcription factor FoxP3 expression found on corticosteroid treatment.[39-42] No data were yet found in the previous studies concerning the effect of clinical variables and other asthma medications on Treg FoxP3 expression. Our study have shown that FoxP3 levels were not affected by the age, exposure to environmental tobacco smoke, disease control, asthma severity, lymphocyte percentage, eosinophil percentage or FEV1%. FoxP3 levels are increased in healthy control as compared to asthmatic patients. FoxP3 levels are usually low in smoker and non-smoker in asthmatic patients group compare to the control group (Figure 5a and b). These findings propose that FoxP3 is important to predict the long-lasting asthma in the elderly, and can be implied for future therapeutic approach and asthma therapy. The zinc finger transcription factor GATA-3 has been found to control the expression and production of Th2 specific interleukins in isolated cell systems and invertebrates, which is found to be crucial transcription factor for immune activation.[43-45] However, there has been demonstrated the expression of GATA-3 in mast cells and also found to be expressed in airway epithelial cells, associated with the allergic reaction.[46-48] In our findings (Table 5) we have shown that GATA-3 is increased in the asthmatic patients (0.541 ± 0.140) than in the controls (0.312 ± 0.076) indicating over expression of GATA-3 in patients with severe asthma as reported by other groups.[49]

The biomarkers were found to be responsive to the asthma in humans. The blood samples of the patients with other complications such as, cardiac disease that are symptomatically similar to asthma were excluded in the study. The response of these biomarkers needs to be studied in those complications as well before being proposed as diagnostic biomarkers for confirming asthma. These biomarkers are thus, candidate for further studies.

Conclusion

The increase in the extent of serum IL-17A, GATA-3, and IgE in the asthmatic patients may have a key role in disease pathogenesis. FoxP3 is generally increased in the control and is lower in patient serum. FoxP3 protein secreted by Treg may be a natural anti inflammatory in hyperimmune states like bronchial asthma. The FoxP3 production may be the cause of anti inflammation by anti-inflammatory agent (which is cortisol). Thus, the serum IL-17A, GATA-3 and FoxP3 as well as IgE, may be exploited as a potential clinical biomarker for the suitable diagnosis and timely therapy of the disease.

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Conflict of Interests: None

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