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Lack of Association of FOXP3 Gene with Risk of Asthma in Children: A Case-Control Study

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ABSTRACT

Background: Loss of Treg cell suppressive activity due to FOXP3 gene disruption is the leading hypothesis for the development of allergy disorders. Thus, asthma susceptibility appears to be determined by host genetic variables affecting FOXP3. A number of research have looked at the role of polymorphisms in the FOXP3 gene in relation to allergy susceptibility.

Objective: We aimed at studying the association between FOXP3 gene single nucleotide polymorphism (SNP) and bronchial asthma, asthma severity as well as atopy in Egyptian children.

Patients and Methods: The study comprised 101 children with asthma and a control group of 101 children without asthma. Subgroup analyses revealed that there were 44 "atopic" a nd 57 "non-atopic" asthmatics in the total asthmatic group. Polymerase chain reaction sequence specific primers (PCR-SSP) were used to investigate the FOXP3 genotypes (rs3761548, rs2232365).

Results: There was no significant difference in the genotypes tested between the asthmatic group and the control group. The AC of rs3761548 and the GG (rs2232365) polymorphisms were the most frequent genotype among the studied children. And only C allele of (rs3761548) was more frequent among asthmatic cases compared to control group. Moreover, no statistically significant difference was found among atopic asthmatic children as regards FOXP3 genotypes (rs3761548, rs2232365) and degree of asthma severity.

Conclusions: No link between FOXP3 gene polymorphism (rs3761548 and rs2232365) and asthma susceptibility was found in Egyptian children with asthma. The severity of atopic asthma in children was not shown to be correlated with FOXP3 polymorphism in the present study.

Keywords: Asthma, FOXP3, Polymorphism, Atopy.

INTRODUCTION

Atopy and non-atopy are the two basic categories used to classify asthma phenotypes [1]. Multiple immunological processes (endotypes) determine the variety of clinical manifestations associated with this disease (phenotypes). It is essential to understand endotypic processes in order to more accurately categorise individuals and develop more effective, individualized therapy strategies [2].

Therefore, phenotypes are the observable traits of an organism that emerge from the interplay between its genotype and its environment [3]. Tolerance against allergens is maintained or acquired by the help of CD4+ CD25+ regulatory T cells (Treg). Therefore, a lack of Tregs or impaired Treg function may play a role in the onset and maintenance of atopic eczema and asthma. Further evidence that FOXP3 may have a role in atopic illness comes from the association between the Xp11.23 FOXP3 chromosomal locus and asthma and atopy [4].

Variations in a single nucleotide's base pair the vast majority of phenotypic diversity among humans may be traced back to single-nucleotide polymorphisms (SNPs). Further, it has been hypothesized that genetic variants in the FOXP3 gene are linked to T cell dysfunction [5, 6]. By generating the necessary cell programme, FOXP3 can promote Treg cell growth and function. Several studies showed that Treg cells could lose their phenotypic features and be transformed into effector T cells during inflammation due to the alterations in FOXP3 expression and stability [7]. Although FOXP3 gene polymorphisms have been connected to a number of allergy diseases, their role in the onset of allergic asthma remains unclear [8].

The purpose of this research was to examine the connection between two significant SNPs in the FOXP3 gene and the development of asthma.

PATIENTS AND METHODS

The research was conducted in the Clinical Pathology Department and the Pulmonology Unit of the Children's Hospital Faculty of Medicine, Zagazig University. The study was conducted through the period from October 2019 to March 2020.

Type of the study: Our study was designed as a case-control study.

Subjects: Subjects included in the study were 2 groups: The asthmatic group included 101 children aged 5 to 15 years (mean age 7.30 ± 2.02 years) diagnosed as bronchial asthma according to The Global Initiative For Asthma (GINA) guidelines, 2019 [9]. They were 50 males and 51 females. Asthmatic group was subdivided into 2 subgroups according to the presence and evidence of atopy into 44 atopic and 57 non-atopic. The control group included 101 children, 52 males and 49 females with a mean age of 7.71 ± 2.31 years, who had no evidence of bronchial asthma, allergy or atopy and also

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had no first degree relatives with bronchial asthma, allergy or atopy.

Exclusion criteria: Patients with Autoimmune disorders, cardiovascular diseases, acquired immunodeficiency and other chronic diseases (including mental disorders), children below 5 years were excluded from the study.

Methodology:

At the time of study enrollment, all patients had a comprehensive clinical evaluation (history and physical examination), with a focus on chest symptoms, additional atopic signs, and disease severity according to The Global Initiative for Asthma Management and Prevention (GINA) recommendations for asthma diagnosis and severity evaluation [9].

FOXP3 gene polymorphism using PCR-SSP technique was performed for all patients and controls. A blood sample for FOXP3 genotyping was collected.

For all asthmatic children: CBC, neutrophils, serum total IgE and eosinophilic count were performed and pulmonary function test was done for them using forced spirometry (D-97024 Hochberg, Germany).

Genotyping:

The blood samplel was taken from a vein and placed in sterile EDTA-containing tubes. The DNA was extracted using the Intron Biotechnology Gspin TM Total DNA Extraction Mini Kit (Korea).

We used PCR-SSP (polymerase chain reaction–sequence specific primers) to genotype two polymorphisms. To amplify rs3761548 and rs2232365 by polymerase chain reaction. In a total volume of 25 ul, we added 50 ng of genomic DNA, 200 M of each dNTP (combination of dATP, dTTP, dCTP, dGTP), 0.2 M of each primer, 1.5 mM of MgCl2, 10 mM of Tris hydrochloride (pH 8.3) and 1 U of TaqDNA polymerase, then the mixture was incubated for 5 min at 95 ◦C (Fermentas, Italy).

The parameters for the cycling were as follows: an initial denaturation phase of 94°C (4 minutes), followed by 35 cycles at 94°C (30 sec), 61°C (30 sec), and 72°C (40 sec), and finally an extension step of 5 minutes at 72°C. Electrophoresis on an agarose gel at 1.5% resolution allowed us to see the PCR products stained with 0.5 g/ml ethidium bromides under an ultraviolet light. Each polymorphism was genotyped in duplicate from 10% of the participants, and the results were 100% consistent.

Interpretation:

Regarding rs3761548, the homozygous CC and AA genotypes appeared as single band in both female and male groups. And the heterozygous. AC genotypes appeared as two bands only in female group (Figure 1). Regarding rs2232365, the homozygous GG and AA genotypes appeared as single band in both female and male groups, and the heterozygous AG genotypes appeared as two bands only in female group (Figure 2).

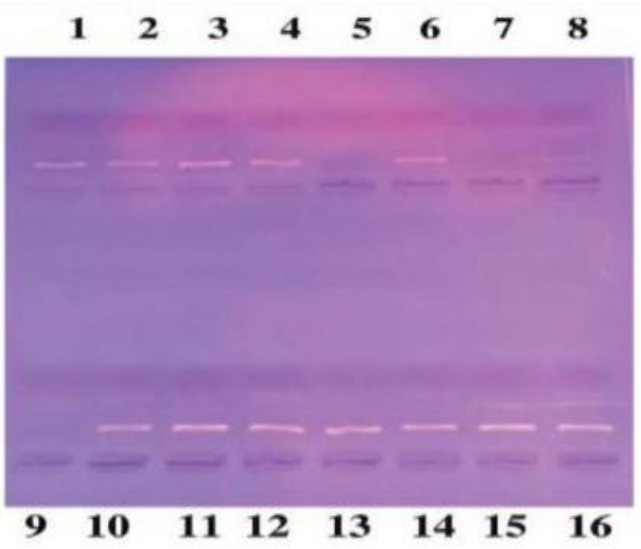

Figure (1): Photographing Gel Electrophoresis results of PCR based SSP analysis of A> C (rs3761548) polymorphism.

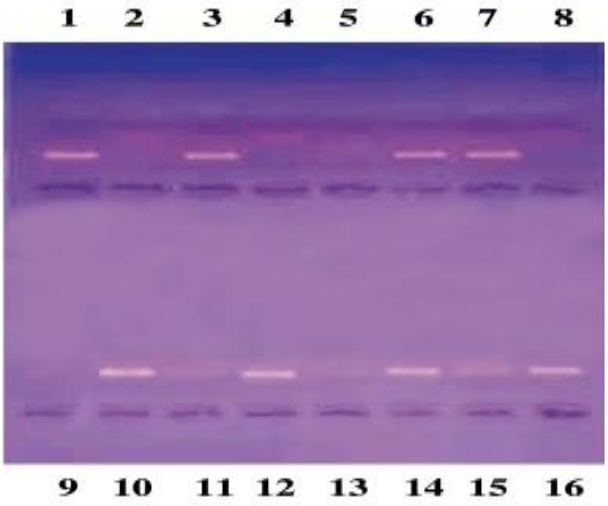

Figure (2): Photographing Gel Electrophoresis results of PCR based SSP analysis of A> G (rs2232365) polymorphism.

Ethical approval:

The research protocol was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Zagazig University (IRB Approval No. (#5627/21-1-2019). All children's parents gave their informed consents before being included in our study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical Analysis

In order to analyze the data acquired, Statistical Package of Social Sciences version 20 was used to execute it on a computer (SPSS). In order to convey the findings, tables and graphs were employed. The quantitative data was presented in the form of the mean, median, standard deviation, and confidence intervals. The information was presented using qualitative

statistics such as frequency and percentage. The student's t test (T) is used to assess the data while dealing with quantitative independent variables. Pearson Chi-Square and Chi-Square for Linear Trend (X^2) were used to assess qualitatively independent data. The significance of a P value of 0.05 or less was determined.

RESULTS

The study included 101 asthmatic children; 50 (49.5%) experienced mild asthma, 49 (48.5%) had moderate asthma and 2 (2%) showed severe persistent asthma. Patient diagnosis and assessment of severity classification were done according to GINA guidelines for asthma, 2019 [9].

Mean patients age was 7.30 ± 2.02 years. Fifty were males and fifty-one were females. We classified our asthmatic patients according to evidence of atopy (by clinical history, Ige level, absolute eosinophilic count and the presence of other allergic symptoms as allergic rhinitis, dermatitis) into two groups atopic and non-atopic groups. Atopic manifestations were found in 44 cases. 101 sex and age matched healthy children with no previous history of chest disease or atopic disorders were included as controls. Table (1) showed the basic data of asthmatic patients.

Table (1): Comparison between the two studied groups according to gender, atopy and asthma severity

Variables	Asthmatic patients (N=101) N (%)
Gender	
Male	50(49.5%)
Female	51(50.5%)
Atopic manifestations	
Present	44(43.6%)
Absent	57(56.4%)
Asthma severity	
Mild persistent	50 (49.5%)
Moderate persistent	49 (48.5%)
Severe persistent	2 (2%)

We also found significant increase in serum levels of IgE and eosinophils in atopic asthmatics compared to non-atopic asthmatics as demonstrated in table (2).

Table (2): Distribution of the studied asthmatic children according to mean serum IgE, eosinophils and their atopic state (n = 101).

Variables	Atopy		U	p
	Not atopic (n= 57)	Atopic (n= 44)		
IgE (IU/ml)				
Mean ± SD.	175.24 ± 41.81	291.55 ± 68.35	669.0*	<0.001*
Eosinophil's (%)				
Mean ± SD.	0.39 ± 0.07	1.07 ± 0.16	601.50*	<0.001*

There were no statistically significant differences between patients and controls in the genotypic distribution or allele frequencies of the FOXP3 gene polymorphism as shown in table (3).

For rs3761548, heterozygous AC genotype represented the most frequent genotype in cases and control (57.4% and 66.3% respectively) followed by homozygous CC (30.7% and 21.8% respectively) and then homozygous AA genotype (11.9% and 11.9% respectively).

For rs2232365, homozygous GG genotype represented the most frequent genotype in cases and control (53.5% and 51.5% respectively) followed by heterozygous AG genotype (25.7% and 27.7% respectively) and then homozygous AA genotype (20.8% and 20.8% respectively) (Table 3).

Table (3): Comparison between the two studied groups according to FOXP3 polymorphisms.

FOXP3 polymorphism	Group A (n = 101)		Group B (n = 101)		χ^2	p
	No.	%	No.	%		
rs3761548						
AA	12	11.9	12	11.9	2.176	0.337
AC	58	57.4	67	66.3		
CC	31	30.7	22	21.8		
rs2232365						
AA	21	20.8	21	20.8	0.112	0.946
AG	26	25.7	28	27.7		
GG	54	53.5	52	51.5		

C allele was more prevalent in cases compared to control (59.4% and 55% respectively), while the G allele had nearly the same frequency in cases and control (66.3% and 65.3% respectively) (Table 4).

Table (4): Comparison between the two studied groups according to the frequency of A, C and G alleles in FOXP3 polymorphisms.

FOXP3 polymorphism	Group A (n = 101)		Group B® (n = 101)		χ^2	p	OR (95% C.I)
	No.	%	No.	%			
rs3761548 allele							
A	82	40.6	91	45.0	0.819	0.366	0.834 (0.562 – 1.237)
C	120	59.4	111	55.0	0.819	0.366	1.200 (0.809 – 1.780)
rs2232365 allele							
A	68	33.7	70	34.7	0.044	0.834	0.957 (0.634 – 1.444)
G	134	66.3	132	65.3	0.044	0.834	1.045 (0.693 – 1.577)

Atopic asthmatic patients were subdivided into 3 groups according to disease severity; mild, moderate and severe persistent asthma. Genotypic distribution did not show a significant difference between mild, moderate and severe persistent cases in rs3761548 and rs2232365 (p-value = 0.625, p-value 0.782 respectively) as shown in tables (5 and 6).

Table (5): Relation between gene polymorphism (rs3761548) and degree of asthma severity in Atopic cases (n= 44).

Degree of asthma severity	FOXP3 polymorphism (rs3761548)n=44						χ^2	^{MC}p
	AA (n = 5)		AC (n = 22)		CC (n = 17)			
	No.	%	No.	%	No.	%		
Mild persistent	2	40.0	10	45.5	6	35.3	2.993	0.625
Moderate persistent	3	60.0	12	54.5	9	52.9		
Severe persistent	0	0.0	0	0.0	2	11.8		

Table (6): Relation between gene polymorphism (rs2232365) and degree of asthma severity in Atopic cases (n=44).

Degree of asthma severity	FOXP3 polymorphism (rs2232365)n=44						χ^2	^{MC}p
	AA (n = 10)		AG (n = 11)		GG (n = 23)			
	No.	%	No.	%	No.	%		
Mild persistent	5	50.0	3	27.3	10	43.5	2.155	0.782
Moderate persistent	5	50.0	7	63.6	12	52.2		
Severe persistent	0	0.0	1	9.1	1	4.3		

DISCUSSION

The role of SNPs in the development of allergy illnesses has been the subject of several Genome-Wide Association Studies, but the X chromosome has been largely excluded from these analyses [10]. There are over a hundred SNPs in the FOXP3 gene, and approximately twenty have been investigated for their possible links to illness [11]. Expression of FOXP3 is lower in people with asthma and allergies compared to healthy control, and this gene has been demonstrated to be responsible for suppressing the Th2 response. Asthma-related genetic variants and epigenetic processes both contribute to impaired FOXP3 function [12].

Primary immunodeficiency due to FOXP3 gene mutations is medically recognised as Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX). The major pathogenic event leading to multiorgan autoimmunity in IPEX is the malfunction of thymus-derived regulatory T (tTreg) cells, which are maintained by the transcription factor FOXP3. IPEX causes severe enteropathy, type 1 diabetes, and eczema as clinical manifestations [13]. To our knowledge, all the studies on FOXP3 were on allergic rhinitis and atopy but asthma was poorly studied. In our study, we focused on exploring the association between FOXP3 gene polymorphism and asthma, atopy and asthma severity in Egyptian population.

Our study was a case-control study, which included 101 asthmatic children who were subdivided into 44 atopic patients and 57 non-atopic patients, and 101 healthy control children. Both asthma group and control group were closely matched regarding their age and gender distribution with no significant gender between both groups. Regarding the degree of asthma severity according to **GINA** [9], 49.5% of asthmatic were mild persistent, 48.5% were moderate persistent and 2% of asthmatic were severe persistent. **Elrifai *et al.*** [14] divided asthmatic cases into 49.3% mild persistent, 22.7% were moderate persistent and 27% had non-atopic manifestations and 73% were atopic. When comparing the asthmatic patients according atopic state and eosinophil count, the mean was 0.39 ± 0.07% in non-atopic cases and 1.07 ± 0.16% in atopic cases.

There was significantly higher eosinophils among atopic group (p value< 0.001). **Inoue *et al.* [15]** found that the asthmatic group had a significantly higher eosinophil count with median 389.7 /µl vs 164.0/µl in children without asthma (p<0.001). When comparing the asthmatic patients according to atopic state and serum IgE, the mean serum IgE level was 175.24 ± 41.81 IU/ml in non-atopic cases and in atopic cases was 291.55 ± 68.35 IU/ml. There was significantly higher IgE among atopic group (p value<0.001). This agrees with **Mouthuy *et al.* [16]** who found that atopic asthmatics present with elevated levels of IgE with mean 290 IU/ml compared to non-atopic asthma with mean 38.5 IU/ml. Those with atopy, such as those with allergic rhinitis, often have an imbalance of T helper cytokines, which may stimulate the synthesis of mucosal immunoglobulins E (IgE). In a study done by **Hassannia *et al.* [17]** where total IgE levels and eosinophil counts were compared between groups, those with allergic rhinitis clearly had higher amounts and more of these cells than the control group (p=0.001).

We investigated the FOXP3 gene SNPs (rs3761548, rs2232365) by using PCR-SSP technique. In contrary to our research hypothesis, our results showed no significant association between different genotypes and bronchial asthma in the studied population. Moreover, we did not find any correlation between allele frequency and the occurrence of bronchial asthma among our children. In this study, the AA, AC and CC genotypes of the FOXP3 gene (rs3761548) polymorphism didn't show any statistically significant difference in distribution between asthmatic patients and control. Generally, AC genotype represented the highest prevalent genotype in both patients and control, while the AA genotype was the least. For rs2232365, homozygous GG genotype represented the most frequent genotype in cases and controls while the AA genotype was the least with no statistically significant difference .C allele was more prevalent in cases as compared to controls while the G allele had nearly the same frequency in cases and controls. Contradictory to our results, **Hassannia *et al.* [17]** reported that AC genotype for this rs3761548 allele was protective for allergic rhinitis in female using polymerase chain reaction with sequence-specific primers PCR-SSP. **Fodor *et al.* [6]** Hungarian researchers found that only women with the AA genotype of the rs3761548 FOXP3gene polymorphism were protected from developing allergic rhinitis. It may be due to minor differences in immunologic mechanisms in males and females. Moreover, **Hori *et al.* [18]** found that FOXP3 was significantly overexpressed in CD4+CD25+ Treg cells from asthmatics and discussed how this may serve as a counter regulatory mechanism by acting as an anti-inflammatory agent. Also we disagree with **Raedler *et al.* [1]** who reported increased FOXP3 expression in asthmatic patients compared to healthy control. Treg cell-suppressing potential by FOXP3 protein was detected in both groups. While **Provoost *et al.* [19]** found that Treg-cell FOXP3 protein expression is considerably lower in asthma patients. Recently, **Shah *et al.* [8]** have discovered that increased blood levels of IL-13 and IL-4, together with the FOXP3 gene SNPS rs3761548, are risk factors for the development of allergic asthma.

These discrepancies may be attributable to variations in study design, to the use of various Treg sub-types, or to the fact that the research was conducted on peripheral blood mononuclear cells, which reflect a systemic response that is susceptible to environmental influences. It's also hard to compare research because of the variations in demographic and sample size **[12].** Possible lack of statistical significance in our findings may be because of the very small size of our sample.

In atopic asthmatic patients, genotypic distribution did not show significant difference between mild, moderate and severe persistent cases in rs3761548 and rs2232365. To date, no studies are available addressing the association between the FOXP3 gene polymorphism in atopic asthmatic patients and asthma severity.

We recommend that large-scale studies of the Egyptian population are needed to assess the role of the two studied FOXP3 gene polymorphisms and other FOXP3 SNPs. Also estimation of FOXP3 protein level in relation to asthma, atopy and asthma severity.

CONCLUSION

In conclusion, comparison of our results with other studies indicates that FOXP3 gene polymorphism is not associated with asthma, or disease severity in atopic asthmatic patients as genetic basis of asthma that may differ between different ethnic groups.

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REFERENCES

1. **Raedler D, Ballenberger N, Klucker E *et al.* (2015):** Identification of novel immune phenotypes for allergic and nonallergic childhood asthma. Journal of Allergy and Clinical Immunology, 135 (1): 81-91.
2. **Khalaf K, Paoletti G, Puggioni F *et al.* (2019):** Asthma from immune pathogenesis to precision medicine. Seminars in Immunology, 46: 1-8
3. **Nissen F, Douglas I, Müllerová H *et al.* (2019):** Clinical profile of predefined asthma phenotypes in a large cohort of UK primary care patients (Clinical Practice Research Datalink). Journal Asthma Allergy, 12: 7-19.
4. **Bottema R, Kerkhof M, Reijmerink N *et al.* (2010):** X-chromosome Forkhead Box P3 polymorphisms associate with atopy in girls in three Dutch birth cohorts. Allergy, 65(7): 865-874.

5. **Torgerson D, Ampleford E, Chiu G *et al.* (2011):** Meta-analysis of genome-wide association studies of asthma in ethnically diverse North American populations. Nature Genetics, 43 (9): 887-892.
6. **Fodor E, Garaczi E, Polyánka H *et al.* (2011):** The rs3761548 polymorphism of FOXP3 is a protective genetic factor against allergic rhinitis in the Hungarian female population. Human Immunology, 72 (10): 926-929.
7. **Colamatteo A, Carbone F, Bruzzaniti S *et al.* (2020):** Molecular Mechanisms Controlling Foxp3 Expression in Health and Autoimmunity: From Epigenetic to Post-translational Regulation. Frontiers in Immunology, 10: 1-20.
8. **Shah Z, Beigh A, Rasool R *et al.* (2020):** Influence of single Gene variants of FOXP3 on Allergic Asthma predisposition. Gene, 763: 1-5.
9. **GINA, Global Initiative for Asthma (2019):** Global Strategy for Asthma Management and Prevention. Pp. 1-201. https://ginasthma.org/wp-content/uploads/2019/06/GINA-2019-main-report-June-2019-wms.pdf
10. **Wise A, Gyi L, Manolio T (2013):** Exclusion: toward integrating the X chromosome in genome-wide association analyses. The American Journal of Human Genetics, 92 (5): 643-647.
11. **Gao L, Li K, Li F *et al.* (2010):** Polymorphisms in the FOXP3 gene in Han Chinese psoriasis patients. Journal of Dermatological Science, 57 (1): 51-56.
12. **Marques C, Costa R, de Oliveira Costa G *et al.* (2015):** Genetic and epigenetic studies of FOXP3 in asthma and allergy. Asthma Research and Practice, 1(1): 1-10.
13. **Bacchetta R, Barzagh F, Roncarolo M (2016):** From IPEX Syndrome to FOXP3 Mutation: A Lesson on Immune Dysregulation. Annals of the New York Academy of Sceinces, 1417 (1): 5-22.
14. **Elrifai N, Al-Wakeel H, Osman H *et al.* (2019):** FOXO3a gene polymorphism and bronchial asthma in Egyptian children. The Egyptian Journal of Pediatric Allergy and Immunology, 17 (1): 31-36.
15. **Inoue T, Akashi K, Watanabe M *et al.* (2016):** Periostin as a biomarker for the diagnosis of pediatric asthma. Pediatric Allergy and Immunology, 27 (5): 521-526.
16. **Mouthuy J, Detry B, Sohy C *et al.* (2011):** Presence in sputum of functional dust mite-specific IgE antibodies in intrinsic asthma. American Journal Respiratory Critial Care Medicine, 184 (2): 206-214.
17. **Hassannia H, Abediankenari S, Ghaffari J (2011):** FOXP3 and TGF-β Gene Polymorphisms in Allergic Rhinitis. The Iranian Journal of Immunology, 8 (4): 218-225.
18. **Hori S, Nomura T, Sakaguchi S (2003):** Control of regulatory T cell development by the transcription factor Foxp3. Science, 299 (5609): 1057-1061.
19. **Provoost S, Maes T, van Durme Y *et al.* (2009):** Decreased FOXP3 protein expression in patients with asthma. Allergy, 64: 1539-1546.