Serum Anti-TPO and TPO Gene Polymorphism as a Predictive Factor for Hidden Autoimmune Thyroiditis in Patient with Bronchial Asthma and Allergic Rhinitis

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Thyroid peroxidase (TPO) is the key enzyme in the biosynthesis of thyroid hormones T3 and T4. Autoimmune thyroiditis is a common disorder affecting 10% of population worldwide. A key feature of autoimmune thyroiditis is the presence of anti TPO antibodies, and some mutation of the TPO gene. Association between autoimmune thyroiditis and other autoimmune disorders has been reported but little is known about association with allergic diseases. In this study, we aimed to evaluate frequency of hidden autoimmune thyroiditis among allergic patient and examine possible relationship between anti-TPO levels and polymorphism at the TPO gene A2173/C exon 12 and different types of allergens. The study included 50 adult Egyptian patients with allergic rhinitis and /or bronchial asthma and 50 controls. For each subject, thyroid stimulating hormone (TSH), thyroxin 4 (T4) and Triiodothyronine (T3) hormones were measured. Anti-thyroid peroxidase (anti-TPO) level was detected by ELISA; and TPO gene polymorphism 2173A>C exon 12 was analyzed using restriction fragment length polymorphism (RFLP). Skin prick test was done to assess allergic response in patients. Serum levels of T3, T4 and TSH did not show any statistical significant difference between patients and groups. However, mean serum anti-TPO level was statistically higher in patients than controls, and correlated positively with body mass index, age, diastolic blood pressure, suggesting higher prevalence of hidden autoimmune thyroiditis in allergic patients than in control group. 2173A>C Genotyping revealed that the frequency of C allele is increased in the patient group. C allele represents a risk factor with odds ratio of 2.37 (1.035-5.44) and a significant *P* value <0.05. It is concluded that TPO 2173A>C polymorphism may be considered as a risk factor for developing autoimmune thyroiditis in patients with allergic rhinitis and asthma and that these patients should regularly be checked for hidden thyroiditis.

Allergic rhinitis and bronchial asthma are among the most common allergic diseases. Bronchial asthma is a syndrome of bronchial obstruction due to inflammation in the airways precipitated by different types of allergens. It is characterized by reduction of air flow, dyspnea, wheezing, and coughing (Barnes, 2008). Allergic rhinitis is historically known as Hay fever, it is characterized by sneezing, runny nose, nasal obstruction, headache and itchy eyes may be also present (AAAAI, 2003).

The prevalence of autoimmune diseases is about 8% of the population (Whitacre, 2001) Hashimoto's thyroiditis is the most common cause of hypothyroidism in iodine-sufficient areas of the world. Hypothyroidism affects 10 % of the population worldwide (Hollowell *et al.,* 2002) It is characterized clinically by gradual thyroid failure, with or without goiter formation, due to autoimmune-mediated destruction of the thyroid gland involving apoptosis of thyroid epithelial cells. Nearly all patients have high serum concentrations of antibodies against one or more thyroid antigens; diffuse lymphocytic infiltration of the thyroid, which includes predominantly thyroid-specific B and T cells; and follicular destruction, which is the characteristic hallmark of thyroiditis (Tamai *et al.,* 1980).

Co-existence of autoimmune thyroiditis with allergic rhinitis and bronchial asthma has been observed before. Both disorders have been found to affect the severity of each other, as allergic attacks increase the severity of autoimmune disorders, meanwhile, if the

severity of an autoimmune disease increased, the allergic condition would be worse (Lindberg *et al.,* 1998, Takeoka *et al.,* 2003, Abd El-Aziz *et al.,* 2010).

Until now, the development of autoimmune diseases and allergic diseases are not fully understood, although, it is widely accepted that the development of both types of diseases requires the interaction between several genetic and environmental factors. As a result, the pathogenic relationship between thyroid dysfunction and allergic disorders is not clearly understood. It is observed, however, that changes in the thyroid hormones affects prostaglandin, leukotriene, and catecholamine levels in tissue and circulation which may affects immune response (Greer & McCombe, 2012). Thus, through investigation of both genetic and environmental factors are crucial to understand the pathogenesis, optimize the diagnosis and adapt the early detection, prevention and treatment protocols (Wang, 2005). Previous studies demonstrated that A2173C polymorphism at exon 12 can be considered as a potential risk for autoimmune hypothyroidism (Balmiki *et al.,* 2014).

TPO, enzyme plays a key role in thyroid hormone synthesis as it is responsible for both the iodination of Thyroglobulin and the coupling of some of the iodotyrosyl residues to generate the thyroid hormones T3 and T4. (Zaletel & Gaberščck, 2011). It is the major antigen involved in autoimmune thyroiditis (High levels of anti TPO are found in sera of patients with autoimmune thyroiditis (Hadj-Kacem *et al.,* 2009).

The human TPO gene ID: 7173 is located on chromosome 2p25 spans approximately 150 Kb, containing 17 exons and 16 introns. More than 50 TPO gene mutations have been identified including deletion, insertion, or change in DNA building blocks (Cipollini *et al.,* 2013). Effects of different mutation are variable, while some can change the 3 dimensional structure of the enzymes; others can prevent the enzyme from attaching to the cell membrane or even cause mild or severe iodide organification defects. Anti TPO recognize conformational epitopes that are highly dependent on the three-dimensional structure of the TPO molecule. A2173/C mutation are known to change the conformation of TPO epitope and thus stimulate the pathogenesis of autoimmune thyroiditis (Neves *et al.,* 2010). Many studies had also found relations between autoimmune thyroiditis and other autoimmune disorders like Psoriasis, Rheumatoid arthritis and Type 1 diabetes mellitus (Metwalley & El-Saied, 2014)

To date, very few studies have reported the relationship between autoimmune thyroiditis and allergic diseases. To our knowledge, this is the first study trying to detect the role of *TPO* gene mutation at A2173/C in autoimmune thyroiditis with allergic rhinitis and bronchial asthma in Egyptians. Moreover, we aimed to detect the frequency of hidden autoimmune thyroiditis among patients suffering from allergic rhinitis and bronchial asthma and to clarify the possible relationships of polymorphism at the A2173/C, anti-TPO levels and different types of allergens causing allergy.

Material and Methods

This case–control study included 100 unrelated subjects: 23 patients with bronchial asthma, 27 patients with allergic rhinitis and 50 healthy controls and they were matched to cases by age. All patients were recruited from Allergy and Immunology Unit and outpatient clinics of Internal Medicine Department, Faculty of Medicine, Zagazig University, Egypt. The diagnosis of asthma was based on patient's symptoms (chronic coughing, dyspnea, wheezing), clinical examination of the chest, lung function tests and patient's response to bronchodilators. Diagnosis of allergic rhinitis was based on clinical symptoms (sneezing, runny nose, nasal obstruction, nasal itching) and physical examination of nose and pharynx, X-rays and CT as required. All subjects underwent complete

history, and clinical examination. We assessed clinical and anthropometrical variables, including blood pressure and body mass index (BMI) and diagnose the precipitating allergic condition using skin prick testing. Patients who had positive skin testing results were enrolled in the study.

Skin prick test was used to identify different types of allergens causing the allergic conditions; patients were exposed to standardized commercial allergen extracts (Omega laboratories, Canada). The panel of allergen extracts used included: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, Timothy pollen, Ragweed pollen, Grass pollen, Brich, Hay dust, Moulds (Alternaria, *Penicillium notatum*, *Aspergillus niger*), Tobacco, dog epithelium and cockroach. Histamine was used as positive control and a negative control was also used. We followed the guidelines of American Academy of Allergy, Asthma and Immunology (AAAAI) during practicing and interpretation of results. For reading, we considered a wheal positive if it is 3 mm larger than the negative control. Before testing, patients were asked to stop anti-histaminic for 4-7 days according to the type of H2 blocker antihistaminic, one day for H1 blocker, mast cell stabilizer for one day and oral corticosteroid foe one day if the dose excedded30mg/day. Topical corticosteroids at the site of skin testing were stopped for 3 weeks before testing (Heinzerling *et al.,* 2009).

Exclusion criteria included age less than 18 years old, pregnancy, history of diabetes mellitus, hypertension, liver, kidney, or thyroid diseases (The aim of the study was to detect hidden autoimmune thyroiditis). None of the participants was on medication known to affect endocrine parameters, metabolism, or inflammation. Patients who could not stop pharmacological therapy as indicated for measuring the anti TPO or for skin testing were also excluded from the study. A written informed consent was signed by each participant after explaining the study, and benefits as well as risks of the procedure. The ethical committee of Faculty of Medicine, Zagazig University approved this study protocol and the written informed consent.

Blood Sampling: All patients were asked to stop oral steroid therapy for one month before blood collection in order not to interfere with the level of anti-TPO. Management of allergic conditions was obtained by local steroid therapy, anti histaminics and leukotriene inhibitors as required. 5 ml of blood was withdrawn from each patient, 2 ml was collected in heparinized blood tubes, and these tubes were used for DNA extraction, while the other three ml was collected in plain tube to separate sera for ELISA assay (TSH/T3/T4 and anti-TPO), all sera were kept frozen at -20°C until assay time

TSH/T3 and T4 assay: were measured using (Cobas e411, Roche Diagnostics, GmbH, Mannheim, Germany) The assay was done according to the manufacturers guidelines. Test results for TSH were considered positive if TSH level was greater than 4.2 µIU/ml, T3: 0.80 - 2.0 ng/ml and T4: 5.1-14.1µg/dl

Anti- TPO level in serum

It was measured using (Anti-TPO IgG EISA kit, Epitope diagnostic, Inc. USA). The assay was done according to the manufacturers guidelines. Test results for thyroid autoimmunity were interpreted according to the manufacture guidelines, the result was considered positive if anti-TPO antibody level was > 35 U/ml and borderline if between 20 and 35 U/ml and negative when the anti-TPO antibody level was below 20U/ml (Abd El-Aziz *et al.,* 2010).

TPO gene polymorphism To detect A2173/C (rs732609). Thr725Pro polymorphism at exon 12.

1- DNA Extraction & Amplification

Genomic DNA was isolated from the blood using QIAamp Blood Kit (QIAGEN, Hilden, Germany). Amplification was done by polymerase chain reaction (PCR). PCR was performed in a Thermocycler (Biometra, Germany) using following primers. The primer sequences used were F: CGGGTCATCTGTGA CAACAC and R: AGCTCCTGGGGAAGATAAGC. Primers were designed according to Primer 3 tool of primer designing of NCBI (Gutiérrez-Achury *et al.,* 2009).20 µl PCR reaction tube contained 6 µl master mix (iNtRON Biotechnology, Korea), 2 µl of extracted genomic DNA, 1µl of each primer and 6 µl of distilled water. PCR protocol used was as followed: Denaturation at 95°C for 30 seconds, annealing at 55-60°C for 30 seconds, and extension at 72°C for 30 seconds x 44 cycles were performed. Final extension at 75 °C for 10 min to amplify 205pb (Gutiérrez-Achury *et al.,* 2009).

- Quality control measures for PCR

-Positive control: Internal Quality control kit was used (Investigator 24plex QS Kit) (Qiagen, Germany)

-Negative control: Dnase/Rnase free water was added to the master mix in place of target nucleic acid. To ensure that the master mix and final processing reagents are not contaminated

2- Restriction fragment polymorphism (RFLP)

RFLP is reliable and accurate method to detect single nucleotide polymorphism (SNP). For RFLP, The PCR

products were analyzed by RFLP using BseDI fast digest restriction enzymes (Fermantas, UK). The enzymatic reaction was done in 30 µl tube, contained 10 µl of PCR product, 1µl of restriction enzyme, 2 µl of fast digest buffer and then complete with 17 µl Dnase free water for 10 min at 37°C. The enzyme produces several cuts that can differentiate different genotypes as follows: AA genotype produces 5 different segments which are of the following lengths: 168bp, 71bp, 25bp, 9bp, 9bp. AC genotype produce 8 segments of different lengths which are 168bp, 110bp 71bp, 58bp, 44bp, 25bp, 9bp and 9bp. CC genotype produce 6 segments of different lengths which are 110bp, 58bp, 44bp, 25bp, 9bp and 9bp.(NEB Cutter, new England biolabs) Analysis of the results were done using 2.5% agarose gel electrophoresis and 50bp DNA ladder (Qiagen, Germany, GmbH).

- Quality control for restriction mapping

-Positive control: A pre-made restriction map supplied from nebcutter (New England biolabs) was used to verify the activity of the restriction enzyme.

-Negative control- 1: A sample of the amplicon, prepared and processed in the same manner as the positive control, but with no addition of the restriction enzyme was prepared to verify that digestion of the nucleic acids resulted from the added restriction enzyme, and not contaminating enzymes.

-Negative control- 2 Dnase/Rnase free water was added to the master mix in place of nucleic acid. To ensure that the master mix and final processing reagents are not contaminated

Other methods to avoid contamination throughout PCR techniques were also applied these include: laminar air-flow hoods, use of bleach to decontaminate the surfaces, use of filtered tips and perform different steps in different areas

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences for Windows (version 17.0; SPSS, Chicago, IL, USA). Data were expressed using descriptive statistic (mean± standard deviation) and were analyzed using T test. One-way analysis of variance (ANOVA) test was done to compare different parameters between more than two groups. Genotype frequencies in cases and controls were tested for Hardy–Weinberg equilibrium, and any deviation between the observed and expected frequencies was tested for significance.

Results

Among case individuals, 34% were males and 66% were females, and in control individuals, 44% were males and 56% were females. The mean age of case group was 35.56±7.48 years and in controls 35.96±5.75 years. The case and control individuals were thus balanced in terms of age and Gender. Clinical, anthropometric and biochemical characteristics of the studied groups were summarized in (Table 1).

Our results show that T3, T4 and TSH levels were normal among patients and controls. However, in the control group, anti TPO level was found to be negative (below 20 U/ml in 48 patients, while 2 patients had shown to be on the border line group anti TPO of 26.2 and 28.04. Meanwhile, in the patient group, 9 patients had anti TPO of levels below20 U/ml, 29 patients were classified as being in the borderline group (20-35 U/ml) and 12 patients show high levels of anti TPO above 35 U/ml.

Table 1. Clinical, anthropometric and biochemical characteristics of patients with Bronchial Asthma and Allergic Rhinitis (case group) compared with controls.

Parameter	Healthy control group (*n*=50)	*Case group (*n*=50)	§*P* value
Age (years)	35.96±5.75	35.56±7.48	NS
Systole (mmHg)	111.36±9.34	106.5±8.10	<0.001*
Diastole (mmHg)	75.64±4.089	74.22±5.64	NS
BMI (Kg/m^2)	25.38±2.078	32.92±9.13	<0.001*
T3 (pg/ml)	2.52±1.358	1.44±0.478	NS
T4 (ng/dl)	3.33±2.267	6.18±2.500	NS
TSH (µIU/ml)(	2.24±1.086	2.42±.70982	NS
Anti TPO(IU/ml)	16.8± 5.272	30.76±17.918	<0.001*

*case group: patients with Bronchial Asthma and Allergic Rhinitis, BMI: body mass index; TSH: thyroid stimulating hormone; T3, T4, anti-TPO antibodies. §*P* < 0.05 is significant when compared with control group.

Patient had significantly higher values of BMI (32.92±9.13) compared to controls (25.38±2.07). Moreover, anti-TPO was significantly higher in patients (30.76±17.918) compared to controls (16.8± 5.272), (*P*<0.05). On the contrary, systolic blood pressure was significant lower in patients (106.5±8.10) than in the control group (111.36±9.34). On the other hand; there were no significant difference between patients and controls as regard age, diastolic blood pressure; T3, T4 and TSH (*P*> 0.05).

Pearson correlations of anti-TPO levels with anthropometric, biochemical characteristics and tested allergens in case group were presented in Table 2. In patients with allergy, anti-TPO levels were positively correlated with BMI, age, diastolic blood pressure, T3, T4 and TSH. On the contrary, there were none significant correlations between anti-TPO levels, systolic blood pressure, and the tested allergen; Pollen, mould, hay dust, tobacco, cockroach, dog epithelium and mite (*P* > 0.05).

Table 2. Pearson Correlations of Anti TPO levels with anthropometric, biochemical characteristics and allergen in case group

Characteristics	Case group (n=50)	
	r	p
Age (years)	0.519	<0.001*
Systole (mmHg)	0.109	NS
Diastole (mmHg)	0.352*	<0.05
BMI (Kg/m^2)	0.316	<0.05
T3 (pg/ml)	0.467	<0.01
T4 (ng/dl)	0.339	<0.05
TSH (µIU/ml)	0.316	<0.05
Pollen	0.259	NS
Cockroach	107	NS
Mould	-0.007-	NS
Dog epithelium	-0.080-	NS
Hay dust	0.213	NS
Tobacco	-0.149-	NS
Mite	0.131	NS

P>0.05 is significant

Prevalence of different allergens in allergic patients are shown in figure.

According to our results, AA genotype was found in 21(42%) of the cases and 30 (60%) of controls, while, AC genotype was detected in 10 (20%) cases and 12(24%) of controls. CC genotype, however, was found in 19 (38%) of cases and 8(16%) of control. As a result, C allele is more frequent in patients than in controls. When we calculated the risk of having C allele, we found that OR is 2.37 (1.035-5.44) with a statistically significant $P<0.05$ as shown in table 3 and figure 2.

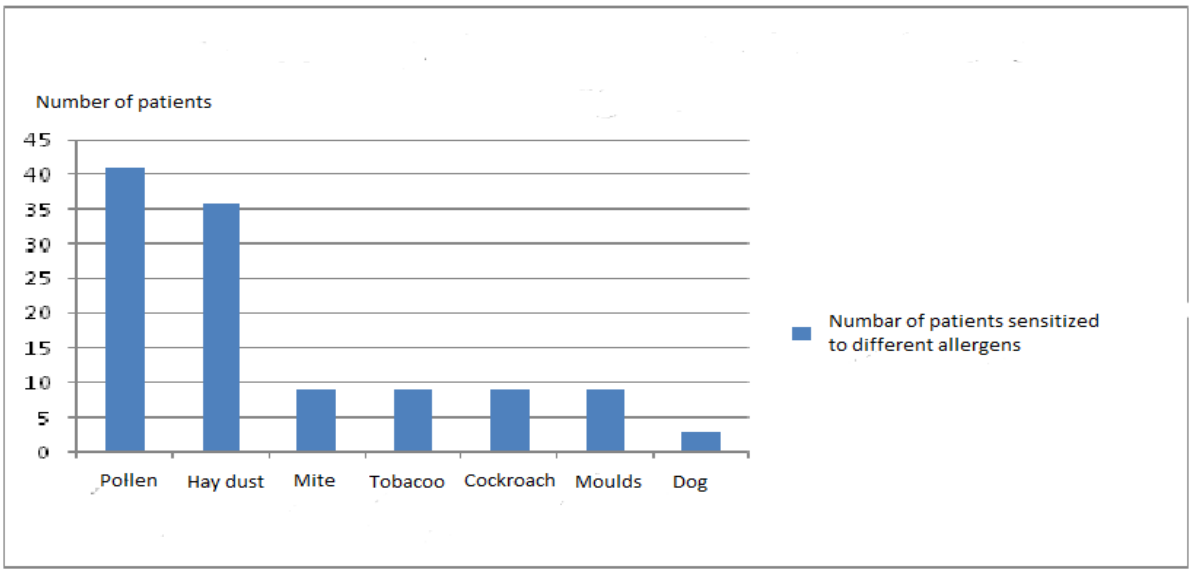

Figure 1. Number of patients sensitized to different types of allergens

Table 3. shows the distribution of several genotypes and alleles in cases and controls.

	Cases	Controls
Allele		
C	24(48%)	14(28%)
A	26(52%)	36(72%)
Genotypes		
AA	21(42%)	30(60%)
AC	10(20%)	12(24%)
CC	19(38%)	8(16%)
OR	2.37 (1.035-5.44)	
P value	0.0412	

P<0.05 is significant

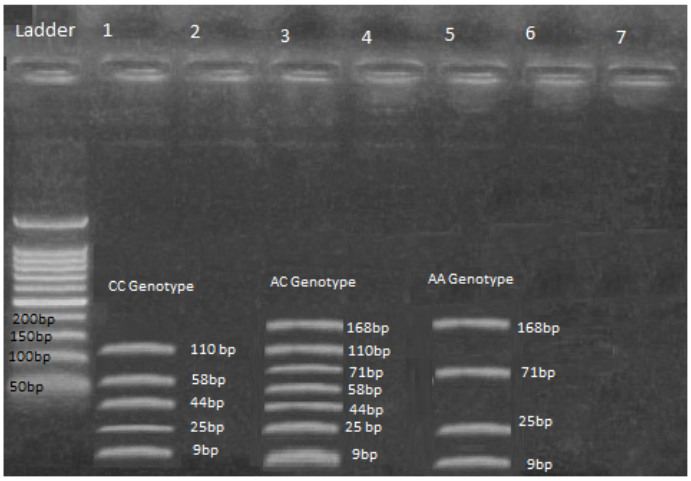

Figure 2. Results of RFLP genotyping. Lane 1 shows: CC genotype produce 6 segments of different lengths which are 110bp, 58bp, 44bp, 25bp, 9bp and 9bp. Lane 3 shows: AC genotype produce 8 segments of different lengths which are 168bp, 110bp 71bp, 58bp, 44bp, 25bp, 9bp and 9bp. Lane 5 shows: AA genotype was shown in lane 5 showing 5 different segments with the following lengths: 168bp, 71bp, 25bp, 9bp, 9bp.

Discussion

Auto immune thyroiditis (AITDs) is the most common cause of hypothyroidism in the United States after 6 years old, affected about 2 to 4 % of women and up to 1% of men. The pathophysiology of AITDs is affected by several genetic and environmental factors, as well as hormonal influences (Helvaci *et al.,* 2006).

As asthmatic patients are more reactive to exogenous antigens, it is probable for them to be hyper-reactive to endogenous antigens as well (Lindberg *et al.,* 1998). Earlier studies have shown that hypothyroidism ameliorates and hyperthyroidism exacerbates bronchial asthma (Ayers & Clark, 1981; Rowe, 1991). The study of Toru Hikta and colleagues on 13 asthmatics showed that aminophyline injection caused catecholamine release, B2 adrenergic receptor stimulation, thyroid releasing hormone (TRH) release from hypothalamus and resultant increase in thyroid stimulating hormone (TSH) and T4. They recommended further assessment of the relationship between thyroid hormones and asthma (Hikta *et al.,* 1998).

Although allergic rhinitis and bronchial asthma are common, to date, very few studies have been reported about association between allergic rhinitis and bronchial asthma and thyroiditis. In the present study, we investigated the possible association of polymorphism at the A2173/C, anti-TPO levels, allergic rhinitis and bronchial asthma to suggest new pathogenesis between thyroiditis from one side and allergic rhinitis and bronchial asthma from the other side. Moreover, we investigated relationships of allergic rhinitis and bronchial asthma with different types of allergens and body mass index with thyroid function tests as well as anti-TPO as markers of autoimmune thyroiditis in Egyptian patients.

The main finding of the present study was the significant high level of anti TPO levels in allergic cases than control group (P<0.05). This indicates that patients with allergic rhinitis and bronchial asthma are more exposed to autoimmune thyroiditis. This

finding was although supported by the significant increase in the BMI in the patient group than in the control group (P<0.05) and the significant lowering in the patient systolic blood pressure value than in the control group (P<0.05). Other previous studies yield the same results (Amino *et al.,* 2003, Abd El-Aziz *et al.,* 2010). They explained that by the fact that allergic responses are usually mediated by Th2 cells. Th2 cells secrete IL-4 and IL-5, theses interleukins are known B-cell stimulators which may explain increased autoantibody production [20]. However, there were no statistical significant difference in the level of T3, T4 or TSH between patients and controls (P<0.05). While, Abdel Aziz *et al.,* got the same result (Abd El-Aziz *et al.,* 2010) others found that there were statistically significant decrease in the level of thyroid hormones in allergic patients than in control group (Rapoport & Mclachlan, 2001). To solve this conflict, studies with larger number of patients may be needed.

In this study, we have chosen a common polymorphism A2173/C at TPO gene exon 12 to study the prevalence of this polymorphism in Egyptian patients suffering from bronchial asthma and allergic rhinitis and autoimmune disease. Alternation of TPO gene at this site can yield different protein as threonine amino acid is changed to proline amino acid (Neves *et al.,* 2010). The two amino acids are different in many aspects, while, threonine has uncharged polar hydrophilic side chain, proline has hydrophobic side chain. Hydrophobicity and charge of molecule are known factors affecting the antigen antibody interaction and this may increase the ability of molecule to stimulate autoantibody formation (Ferrier, 2014). Also this alternation in amino acid sequence affects the function of the thyroperoxidase enzyme and hence results in hypothyroidism (Bakker *et al.,* 2000).

According to our results, among the control group, A genotype represented 72 %, while C genotype represented 28 %. The distribution of this allele in control group is near to that found in other study done on Italian and Spanish population; those populations are of special importance in this study as they belong to the same ethnic Caucasian population as Egyptians (Cipollini *et al.,* 2013). While in the patient group, C genotype represented 48% and A genotype represent 52%. When we tried to compare the distribution of these alleles in the patient group, we found that this work is apparently the first to study this polymorphism in allergic patients. However, we found that, C allele comprises a risk factor for increased anti-TPO production OR 2.37 (1.035-5.44); this difference is statistically significant (P<0.05). This results matches results obtained by other researchers, found that this mutation may play an important role not only in increased anti-TPO production, but also in the development of diabetes type 1, hypothyroidism, thyroid dysgenesis and thyroid cancer (Neves *et al.,* 2010, Balmiki *et al.,* 2014, Guria *et al*., 2014).

Finally, we concluded that although the levels of T3 and T4 were found to be normal in the studied allergic groups, evidence of hidden autoimmune thyroiditis were present, represented in increased level of anti-TPO in the serum, increase body mass index and decrease the systolic blood pressure values. These changes were seemed to be genetically related as we found that the studied allergic groups not only suffered from increased serum anti-TPO levels but also they had increased frequency of C allele polymorphism at the A2173/C 48% of patients compared with only 28% of control. Consequently, we recommend close monitoring for evidence of hypothyroidism in patients with allergic rhinitis and asthma especially in the presence of genetic predisposition. Indeed, further in-depth studies with larger number of patients are needed to detect the biological bases of

interaction between allergic and autoimmune disease and to determine genetic factors.

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