Original article

Endothelial nitric oxide synthase gene polymorphisms are associated with sensitization to seasonal aeroallergens in asthmatic children

Maria Iordanidou, Emmanouil Paraskakis, Anna Tavridou, Athanasios Chatzimichael, Vangelis G. Manolopoulos

Alexandroupolis, Greece

Background: **Childhood asthma phenotype is the consequence of interaction between environment and genetic factors. Nitric oxide (NO) formation is affected by polymorphisms in nitric oxide synthase (NOS) enzymes, which play a significant role as inflammatory factors in the airways. This study was undertaken to estimate the correlation of -786C>T and 894G>T polymorphisms of the *eNOS* gene with the sensitization of asthmatic children to common aeroallergens.**

Methods: **A total of 193 asthmatic children and 96 healthy controls, who were of Mediterranean origin, living in the same geographical area, were enrolled in the study. 894G>T and -786T/C polymorphisms of the *eNOS* gene were analyzed using a PCR-RFLP method.**

Results: **The 894GG genotype was more frequent (68.6%) in children with asthma sensitized to Oleaeuropaea than in those with asthma non-sensitized (43.0%) (*P*=0.004). Likewise, -786TT genotype frequency was higher in children with asthma sensitized to Oleaeuropaea (51.0%) than in those with asthma non-sensitized (31.7%) to this allergen (*P*=0.035). For the aeroallergens Parietariajudaica and mixed grass, the frequency of -786C allele carriage was associated with protection from sensitization to Parietariajudaica and mixed grass in asthmatic children (*P*=0.021 and *P*=0.017, respectively). In the healthy control group, the genotype frequencies for these polymorphisms were similar to genotype frequencies of children with asthma non-sensitized to these three specific aeroallergens.**

Conclusion: **In children with asthma, 894G>T and -786T/C polymorphisms of the *eNOS* gene were correlated with sensitization to common seasonal aeroallergens.**

World J Pediatr 2017;13(1):34-40

Key words: **asthma;**
atopy;
genetics;
nitric oxide

Introduction

Asthma, a multifactorial chronic inflammatory lung disease, is the result of interactions between environmental and genetic factors including the exposure to common allergens.[1] Atopy characterized by increased specific IgE levels against common environmental allergens is considered one of the most significant risk factors for childhood asthma.

Previous studies[2,3] have shown associations between various polymorphisms and atopic asthma. The studied loci included those encoding nitric oxide synthase enzymes (NOS), since nitric oxide (NO) seems to play an important role in asthma pathophysiology.[4] NO is generated from L-arginine by a family of NOS, neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3) and inducible NOS (iNOS, NOS2), all of which are expressed in the epithelium of the human airways.[5,6]

NO regulates the airway function significantly and is associated with the pathophysiology of inflammatory airway diseases.[5] It is well established that the inflammation of the airways is of paramount importance in the pathophysiology of asthma. Although one of the beneficial effects of NO is its bronchodilatory action, it has been shown that exhaled NO (eNO) is increased in asthmatic patients compared with

Author Affiliations: Respiratory Unit, Department of Pediatrics, University Hospital of Alexandroupolis, Alexandroupolis, Thrace, Greece (Iordanidou M, Paraskakis E, Chatzimichael A); Laboratory of Pharmacology, Medical School, Democritus University of Thrace, Alexandroupolis, Greece (Tavridou A, Manolopoulos VG)

Corresponding Author: Emmanouil Paraskakis, MD, PhD, Respiratory Unit, Department of Pediatrics, Academic General Hospital of Alexandroupolis, 68100 Dragana Alexandroupolis, Thrace, Greece (Tel: +302551074415; Fax: +302551074433; Email: eparaska@med.duth.gr)

doi: 10.1007/s12519-016-0043-9
Online First, June 2016

control subjects and in allergen-induced late asthmatic reactions, and reduced when corticosteroids are used in asthma exacerbations.[7-9] Changes in exhaled NO levels in asthmatic patients appear to be associated with quantitative markers of airway inflammation.[10] Additionally, atopy is strongly correlated with elevated eNO levels in asthmatic subjects.[11]

Recent studies[5,12-14] have shown that except the induction of iNOS, which seems to act as the main source of increased eNO in asthma, *eNOS* might as well play a role in asthma pathophysiology. NO production is regulated by factors that affect the expression or activity of *eNOS* enzyme itself or through alterations in the availability of activating cofactors or endogenous activator molecules.[5,12] Data from animal models showed that the non-inducible NOS systems play a key role in the regulation of airway reactivity and inflammation.[13] A previous study showed that *eNOS* contributes to NO-related physiology and pathophysiology in the airways and may be associated with the pathogenesis of asthma.[14]

The human *eNOS* gene consists of 26 exons spanning 21 kb and producing 1203-1205 residues.[15] The most widely studied variants in the *eNOS* gene are two single nucleotide polymorphisms, the 894G>T variant in exon 7 that leads to an amino acid change from Glu to Asp (Glu298Asp) and a T to C change at position -786 in the promoter region (5' flanking region).[16] Both of these Single Nucleotide Polymorphisms (SNPs) are functional and result in lower NO production.[17,18]

Until now, genetic studies examining the correlation of polymorphisms in the *eNOS* gene with asthma and allergic manifestations have focused on adult populations with the exception of two, carried out in Chinese and American children, respectively.[19,20] Asthma may have different phenotypes and it is well established that childhood asthma is different from the adulthood asthma.[21] Thus, we investigated in children of Mediterranean origin the possible association of the polymorphisms of -786T/C and 894G>T of the *eNOS* gene in childhood asthma.

Methods

Population

The asthmatic study population included 193 children of Mediterranean origin with asthma (134 males and 59 females) and 96 healthy controls. All children were patients of a Pediatric Clinic of University General Hospital and were enrolled in the study from March 2008 to September 2010. The selection of the study population was consecutive. All parents of children included in this study gave written informed consent. The protocol of the study was approved by the Scientific Council and the Ethics Committee of the Academic General Hospital.

Information about demographic data, anthropometric measurements, risk factors and possible confounders as well as family history of atopy or asthma was also collected in the questionnaire. Data about the medical history, including bronchiolitis, were retrieved by the personal medical card of each patient, filled in by their pediatrician annually, retrospectively. Children with asthma were included in the study according to physical examination, clinical asthma symptoms, medical history, and spirometry. Asthma definition was based on the following criteria: a) at least 1 symptom of asthma including cough, wheezing chest tightness and breathlessness and b) an increase of at least 15% in baseline forced expiratory volume in 1 second (FEV_1) after bronchodilator use. Atopy was defined as a total serum IgE level higher than 100 IU/mL measured by chemiluminescence or a positive prick test for at least 1 of 9 common aeroallergens. In children under 5 years old asthma diagnosis was based on GINA guidelines and the presence of atopy, since it is usually not possible to determine FEV_1 in these patients.

Spirometry was carried out according to the American Thoracic Society recommendations, using a volume-displacement hand-held spirometer (MIR Spirolab, Roma, Italy). Bronchodilator reversibility was estimated 20 minutes after administration of 200 mg of albuterol by a metered-dose inhaler through a spacer. A total of 110 asthmatic children (57%) were diagnosed as having allergic rhinitis according to clinical history and physical examination. Skin prick testing for 9 common aeroallergens (dermatophagoides pteronyssinus, dermatophagoides farinae, alternaria alternata, canis familiaris, felis domesticus, parietaria judaica, mixed grass, olea europaea, and cupressus sempervirens) was carried out in all children using standardized extracts (Prick Test Diagnostic, ALK Abello, Madrid, Spain). Also, appropriate positive (histamine) and negative control solutions were used. A positive skin prick test was defined as (diameter of weal reaction) a wheal size ≥3 mm larger than the negative control as recorded 20 minutes after the subtraction. The healthy control group comprised children without familiar or personal history of asthma or atopy. All children in the healthy control group were not sensitized to the above-mentioned aeroallergens using skin prick testing.

Exclusion criteria for patient enrollment were previous or current history of significant medical illness, parasite infection, allergen hyposensitization, or respiratory infection four weeks prior to the study.

Genotyping

Genomic DNA extraction was carried out from white

blood cells in peripheral venous blood by Puregene DNA Purification System (Gentra, Minnesota, MI, USA) according to the manufacturer's instructions. A PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) was applied for the identification of the -786T/C and 894G>T polymorphisms in the *eNOS* gene according to the protocol described by Ragia et al.[22] The resulting PCR products were digested, at 37°C overnight, using 5U of HapII restriction enzyme and MboI restriction enzyme for the -786T/C and 894G>T polymorphism, respectively. The products of restriction were separated on a 2.5% agarose gel and visualized by ethidium bromide staining. Genotyping for all the study participants were carried out in duplicate with identical results for all subjects genotyped.

Statistical analysis

All continuous variables are recorded as mean ± standard error of mean (SEM). Genotype distributions differences from that expected for Hardy-Weinberg equilibrium (HWE) were tested using the Chi-square test. Relative frequencies of genotypes, carriage of the variant allele or alleles were estimated for each group and a Chi-square analysis was used to compare the distribution of genotypes, carriage of the variant allele and alleles between the studied groups. Multiple testing was adjusted using Bonferroni's correction. A *P* value of 0.025 was considered statistically significant ($P<0.05/n$, $P<0.05/2=0.025$). To calculate the risk of sensitization to allergens associated with -786T/C and 894G>T polymorphisms of the *eNOS* gene, logistic regression analysis was performed with a stepwise forward selection procedure with sensitization as a dependent variable. The carriage of the variant allele as an independent variable before and after adjustment for age, sex and total IgE was used to calculate the odds ratios (OR) with 95% confidence intervals (95% CI). A *P* value of <0.05 was considered statistically significant. Analyses were made using the SPSS software package (15.0 for Windows).

Results

Study population

A total of 193 asthmatic children (134 males, 59 females) and 96 healthy non atopic controls (45 males, 51 females) were recruited in the study. The demographic, clinical and laboratory characteristics of the study participants are shown in Table 1. In our study population, 60 of the 193 asthmatic children were not sensitized to any of the studied aeroallergens but they had the clinical diagnosis of allergic rhinitis according to ARIA criteria and/or had IgE ≥100 IU/mL.

The 894G>T and -786T/C polymorphisms

Hardy-Weinberg equilibrium (HWE) ($P>0.05$) was confirmed for the 894G>T and 786T/C polymorphisms of the *eNOS* gene in both groups. No association was found between genotype frequencies of 894 G>T and -786T/C polymorphisms and susceptibility to childhood asthma. The frequencies of GG, GT and TT genotypes of the 894G>T polymorphism were 49.7%, 40.5% and 9.8%, respectively in the asthmatic group, and the frequencies of GG, GT and TT genotypes of the *eNOS* gene were 47.9%, 42.7% and 9.4% in the healthy control group, respectively ($P>0.05$). In the asthmatic group, 36.8% of the subjects were genotyped as TT, 48.7% as TC and 14.5% as CC for the -786T/C polymorphism of the *eNOS* gene. The frequencies of TT, TC and CC genotypes in the healthy control group were 34.4%, 47.9% and 17.7%, respectively ($P>0.05$).

Asthmatic patients with positive skin prick tests for olea europaea were more often homozygous for the common genotype (894GG) (68.6%) than those with negative skin prick test for olea europaea (43.0% 894GG) ($P=0.004$) (Table 2).

Additionally, the frequency of 894T allele carriers was significantly lower in children with asthma and positive olea europaea prick test (31.4%) in contrast with those with asthma and negative olea europaea prick test (55.0%) ($P=0.002$) (Table 2). Similarly, the frequency of 894T allele was lower in asthmatic children sensitized to olea europaea (20.6%) than in those non-sensitized to olea europaea (33.5%) ($P=0.015$). After Bonferroni's correction, 894G>T polymorphism of the *eNOS* gene was still significantly correlated with the lower risk of sensitization to olea europaea where a *P* value of 0.025 (0.05/2) was considered statistically significant. For the other studied aeroallergens, there were no significant differences when we compared the genotype distribution of 894G>T polymorphism between sensitized and non-sensitized children with asthma.

Table 1. Demographic characteristics of the participants

Variables	Asthmatic children *n*=193	Healthy control *n*=96	*P* value
Sex (males/females)	134/59	45/51	<0.001
Age (y)	8.5±0.2	9.5±0.3	0.017
Weight (kg)	37.8±1.2	38.9±1.7	NS
BMI (kg/m^2)	19.8±0.3	19.2±0.4	NS
Breast feeding, *n* (%)	154 (79.8)	84 (87.5)	NS
Allergic rhinitis, *n* (%)	110 (57.0)	-	
Bronchiolitis, *n* (%)	81 (42.0)	12 (12.5)	<0.001
Smoking, *n* (%)	100 (51.8)	56 (58.3)	NS
Total IgE	291.2±37.3	73.4±14.7	<0.001
FVC	94.4±1.2		
FEV_1%	98.6±1.3		
FEV_1/FVC	1.04±0.0		

BMI: body mass index; NS: not significant; FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 second.

For -786T/C polymorphism, significant differences in genotype distribution were recorded between sensitized and non-sensitized children with asthma to olea europaea, parietaria judaica and mixed grass (Table 2). Particularly, the frequency of -786TT genotype was higher in children with asthma sensitized to olea europaea (51.0%) than in those with asthma non-sensitized to this allergen (31.7%) (P=0.035). Similarly, this correlation was verified when the results were analyzed according to the carriage of the variant -786T allele or the -786T/C allele frequencies since the frequencies of -786C allele carriage (TC and CC genotypes) and -786C allele were lower in children with asthma sensitized to olea europaea than in those with asthma non-sensitized to olea europaea (49.0% *vs.* 68.3%, P=0.014 and 57.4% *vs.* 70.6%, P=0.019) (Table 2).

The wild-type -786TT genotype was significantly more frequent in children with asthma sensitized to parietaria judaica (52.5%) than in those with asthma non-sensitized to parietaria judaica (32.7%) (P=0.067) (Table 3). Although this association was marginally significant, the -786C allele carriage (TC and CC genotypes) was more frequent in asthmatic children non-sensitized to parietaria judaica (67.3%) than in those sensitized to this allergen (47.5%) (P=0.021) (Table 3). This association was verified when the results were analyzed for allele frequencies as the frequency of -786C allele was significantly higher in children with asthma non-sensitized to parietaria judaica (41.8%) than in those with asthma sensitized to this allergen (28.7%) (P=0.033) (Table 3).

Moreover, the distribution of genotypes of -786T/C polymorphism was different between children with asthma sensitized to mixed grass (51.0% TT, 32.7% TC, 16.3% CC) and those with asthma non-sensitized to mixed grass (31.9%TT, 54.2% TC,

Table 2. Results of the analysis of 894G>T and -786T/C polymorphisms of the *eNOS* gene in relation to sensitization to olea europaea

Variables	Olea europaea+IgE, n=51		Olea europaea–IgE, n=142		P value
	n (%)	95% CI	n (%)	95% CI	
894G>T					
GG	35 (68.6)	55.14-80.07	61 (43.0)	35.03-51.17	0.004
GT	11 (21.6)	12.04-34.22	67 (47.1)	39.10-55.38	
TT	5 (9.8)	3.84-20.16	14 (9.9)	5.76-15.57	
T allele carriage					
GG	35 (68.6)	55.14-80.07	61 (43.0)	35.03-51.17	0.002
GT+TT	16 (31.4)	19.93-44.86	81 (57.0)	48.83-64.97	
Alleles					
G	81/102 (79.4)	70.81-86.37	189/284 (66.5)	60.92-71.85	0.015
T	21/102 (20.6)	13.63-29.19	95/284 (33.5)	28.15-39.08	
-786T/C					
TT	26 (51.0)	37.52-64.33	45 (31.7)	24.47-39.65	0.035
TC	21 (41.2)	28.46-54.86	73 (51.4)	43.23-59.53	
CC	4 (7.8)	2.71-17.58	24 (16.9)	11.43-23.70	
C allele carriage					
TT	26 (51.0)	37.52-64.33	45 (31.7)	24.47-39.65	0.014
TC+CC	25 (49.0)	35.67-62.48	97 (68.3)	60.35-75.53	
Alleles					
T	72/102 (70.6)	61.26-78.76	163/284 (57.4)	51.59-63.05	0.019
C	30/102 (29.4)	21.24-38.74	121/284 (42.6)	36.95-48.41	

eNOS: endothelial nitric oxide synthase; CI: condifence interval.

Table 3. Results of the analysis of -786T/C polymorphism of the *eNOS* gene in relation to sensitization to parietaria judaica and mixed grass

Variables	Parietariajudaica+IgE n=40		Parietariajudaica–IgE n=153		P value	Mixed grass+IgE n=49		Mixed grass–IgE n=144		P value
Genotypes	n (%)	95% CI	n (%)	95% CI		n (%)	95% CI	n (%)	95% CI	
TT	21 (52.5)	37.30 -67.35	50 (32.7)	25.63-40.38	0.067	25 (51.0)	37.30-64.62	46 (31.9)	24.75-39.86	0.027
TC	15 (37.5)	23.79-52.95	79 (51.6)	43.75-59.46		16 (32.7)	20.81-46.48	78 (54.2)	46.01-62.15	
CC	4 (10.0)	3.47-22.04	24 (15.7)	10.59-22.07		8 (16.3)	8.03 -28.46	20 (13.9)	8.98-20.23	
C allele carriage										
TT	21 (52.5)	37.30-67.35	50 (32.7)	25.63-40.38	0.021	25 (51.0)	37.30-64.62	46 (31.9)	24.75-39.86	0.017
TC + CC	19 (47.5)	32.65 -62.70	103 (67.3)	59.62 -74.37		24 (49.0)	35.38 -62.70	98 (68.1)	60.14-75.25	
Alleles										
T	57/80 (71.3)	60.71 -80.28	178/306 (58.2)	52.59-63.60	0.033	66/98 (67.3)	57.66-76.02	169/288 (58.7)	52.93-64.26	0.129
C	23/80 (28.7)	19.72-39.29	128/306 (41.8)	36.40-47.41		32/98 (32.7)	23.98-42.34	119/288 (41.3)	35.74-47.07	

eNOS: endothelial nitric oxide synthase; CI: confidence interval.

13.9%CC) (*P*=0.027) (Table 3). This difference was significant when the results were analyzed according to the carriage of the variant -786C allele (TC and CC genotypes). Particularly, the frequency of the -786C allele carriage was significantly higher in asthmatic children non-sensitized to mixed grass (68.1%) than in those sensitized to this allergen (49.0%) (*P*=0.017) (Table 3). After Bonferroni's correction, the -786T/C polymorphism of the *eNOS* gene was still significantly correlated with the lower risk of sensitization to olea europaea, parietaria judaica or mixed grass according to the carriage of the -786C allele, where a *P* value equal to 0.025 (0.05/2) was considered statistically significant. Finally, no significant differences were found when we compared the genotype distribution of-786T/C polymorphism between children with asthma sensitized and non-sensitized to the other allergens.

Using logistic regression analysis with sensitization to olea europaea as a dependent variable and 894T allele carriage as an independent variable, we found that the 894T allele carriage was associated with the lower risk of sensitization to this allergen before (OR=0.34, 95% CI=0.17-0.67, *P*=0.002) and after adjustment for age, sex and total IgE (OR=0.28, 95% CI=0.13-0.62, *P*=0.002). Also, using logistic regression analysis with sensitization to olea europaea, parietaria judaica and mixed grass as dependent variables and -786C allele carriage as an independent variable, a significantly lower risk of sensitization to these aeroallergens was correlated with the presence of the -786C allele before (OR=0.44, 95% CI=0.23-0.85, *P*=0.015; OR=0.43, 95% CI=0.21-0.89, *P*=0.022 and OR=0.45, 95% CI=0.23-0.87, *P*=0.056, respectively) and after adjustment for age, sex and total IgE (OR=0.41, 95% CI=-0.20-0.86, *P*=0.019; OR=0.41, 95% CI=0.19-0.89, *P*=0.025 and OR=0.46, 95% CI=0.22-0.96, *P*=0.040, respectively).

Asthma related phenotypes and *eNOS* polymorphisms

Neither 894G>T nor -786T/C polymorphisms of *eNOS* was correlated with total serum IgE levels (data not shown). Also, no correlation was found between these polymorphisms of the *eNOS* gene and peripheral blood eosinophils and pulmonary function tests in the asthmatic group (data not shown).

Discussion

The present study is the first to examine the role of -786T/C and 894G>T polymorphisms of the *eNOS* gene in asthmatic children of Mediterranean origin. Our results have shown the existence of significant differences in the frequency of these genotypes and alleles between sensitized and non-sensitized children with asthma to common seasonal aeroallergens, such as olea europaea, parietaria judaica, and mixed grass.

eNOS 894T allele was shown to be more susceptible to proteolytic cleavage than *eNOS* 894G allele and the cleaved fragments are expected to lack NO synthase activity and the steady-state level of eNOS enzyme in carriers of the 894T variant allele might therefore be lower, with a resultant reduction in capacity for NO production.[18] The 786T/C SNP reduces *eNOS* gene promoter activity by approximately 50%[17] and similarly eNOS expression and serum nitrite/nitrate levels[23] since the -786C variant allele markedly reduces the transcription rate of the *eNOS* gene, and consequently NO production, likely because the -786C allele creates a binding site for a replication protein A1 that suppresses *eNOS* transcription.[17]

Taken together, these findings indicate that the polymorphisms may have a functional role in pediatric asthma, as they regulate eNOS protein function. The higher prevalence of the wild type genotypes for both polymorphisms in asthmatic children with positive skin prick test reactivity to the above allergens in comparison with those with negative tests could reflect a possible effect of these polymorphisms on skin test reactivity and asthma, or alternatively, the *eNOS* variant may be in linkage disequilibrium with another susceptibility gene in the same region.

Our study has shown that sensitization to parietaria judaica, mixed grass and olea europaea, the three most frequent seasonal aeroallergens in our population as reported in previous studies,[24,25] is associated with *eNOS* polymorphisms in children with asthma. In our population the most frequent allergens are *D pteronissinus* and *D farinae*. However, no correlation was shown between *eNOS* polymorphisms and *D pteronyssinus* and *D farinae* in our study. The latter may be due to their perennial character. Until now, it is not elucidated whether NO regulation is different from each allergen. A possible explanation for the correlation of *eNOS* -786T/C and *eNOS* G894T polymorphisms only with seasonal but not with perennial aeroallergens could be the different pathophysiology of allergic sensitization to seasonal or perennial allergens. As shown in a recent study, different memory CD4(+) T cell responses are elicited in the context of perennial versus seasonal stimulation with the allergens.[26] This means that the different allergic pattern of each allergen and the different characteristics of this geographic area could be a possible explanation for these results. Furthermore, the genotype frequencies of 894G>T and -786T/C polymorphisms in the healthy control group are in accordance with the genotype frequencies of children with asthma non-sensitized to these aeroallergens. The results of our study have shown that

the genotype frequencies are almost the same between asthmatic children and healthy controls.

Studies on the role of the *eNOS* gene with asthma have been carried out only in a limited number of patients including mainly adult populations. In children, only two studies examining the role of *eNOS* gene polymorphisms in asthma were carried out. The first was conducted in 295 Chinese children indicating no significant correlation of 894G>T variant of the *eNOS* gene with asthma, atopy (specific-D pteronissinus IgE or specific-cat IgE) or FeNO (fraction of eNO).[19] The second study by Salam et al investigated another polymorphism of the *eNOS* gene (27 bp repeat) and found no correlation between this polymorphism and FeNO in asthmatic children.[20] One possible explanation for this diversity in replication results could be the heterogeneity between study populations, such as ethnicity and different environmental exposures.

Other studies about *eNOS* gene polymorphisms and their correlation with asthma or atopy phenotypes that were carried out in adult populations showed contradictory results. Although no association of 894G>T and -786T/C polymorphisms of the *eNOS* gene with asthma or atopy was found in many studies,[16,27-30] -786T/C polymorphism of the *eNOS* gene was correlated with asthma only in men or women separately[30,31] and the common haplotype -786T/691C/27-bp 5 repeat variant/774C/894G/11T was associated with a lower risk of asthma.[28] However, other polymorphisms of the *eNOS* gene were correlated with asthma, atopy phenotypes or skin prick test positivity in adult populations.[14,16,27,30] These contradictory results could be explained by the heterogeneity among study populations included in each study, such as age, ethnicity, sex, and environmental exposures as well as the multiple roles of NO in the airways. While this study demonstrated significant effects of *eNOS* SNPs on sensitivity to common aeroallergens in asthmatic children, there were some study limitations. One of the limitations is the small number of participants, which may decrease the statistical power of this study. Furthermore, we did not have a replication population for this study, which could have provided additional confirmation of our findings. Indeed, our results need a replication in a large number of subjects.

In conclusion, this study provides evidence that *eNOS* polymorphisms may contribute to genetic susceptibility to asthma in children. Our findings in *eNOS* polymorphisms support the idea of a possible involvement of the *eNOS* gene in asthma. The two studied polymorphisms seem to be associated with sensitization to common seasonal aeroallergens in children with asthma of Mediterranean origin. These genes should be added in a list of susceptibility genes for atopy, and our findings expand our understanding of biological pathways that are dysregulated in these conditions and are candidate genes for the treatment of atopy and asthma. However, additional studies are required to show precisely whether the *eNOS* gene influences childhood asthma and atopy and whether its regulation may provide a novel target in the prevention and treatment of chronic inflammatory respiratory diseases.

Funding: The study was not funded.
Ethical approval: The study was approved by the Scientific Council and the Ethics Committee of the Academic General Hospital.
Competing interest: All authors declare no competing interests.
Contributors: Iordanidou M contributed to paper writing patient enrolment and laboratory assessments; Paraskakis E contributed to the design of the study, paper writing and editing; Tavridou A contributed to paper writing and laboratory assessments; Chatzimichael A and Manolopoulos V contributed to the design of the study and paper editing.

References

1 Sengler C, Lau S, Wahn U, Nickel R. Interactions between genes and environmental factors in asthma and atopy: new developments. Respir Res 2002;3:7.
2 Batra J, Das S, Chatterjee R, Chandra S, Ghosh B. Monocyte chemotactic protein (MCP3) promoter polymorphism is associated with atopic asthma in the Indian population. J Allergy Clin Immunol 2011;128:239-242. e3.
3 Murk W, Walsh K, Hsu LI, Zhao L, Bracken MB, Dewan AT. Attempted replication of 50 reported asthma risk genes identifies a SNP in RAD50 as associated with childhood atopic asthma. Hum Hered 2011;71:97-105.
4 Bove P F, van der Vliet A. Nitric oxide and reactive nitrogen species in airway epithelial signaling and inflammation. Free Radic Biol Med 2006;41:515-527.
5 Baraldi E, deJongste JC, European Respiratory Society/ American Thoracic Society (ERS/ATS) Task Force. Measurement of exhaled nitric oxide in children, 2001. Eur Respir J 2002;20:223-237.
6 Barnes PJ. NO or no NO in asthma? Thorax 1996;51:218-220.
7 Sippe JM, Holden WE, Tilles SA, O'Hollaren M, Cook J, Thukkani N, et al. Exhaled nitric oxide levels correlate with measures of disease control in asthma. J Allergy Clin Immunol 2000;106:645-650.
8 Lehtimaki L, Kankaanranta H, Saarelainen S, Hahtola P, Jarvenpaa R, Koivula T, et al. Extended exhaled NO measurement differentiates between alveolar and bronchial inflammation. Am J Respir Crit Care Med 2001;163:1557-1561.
9 Paraskakis E, Brindicci C, Fleming L, Krol R, Kharitonov SA, Wilson NM, et al. Measurement of bronchial and alveolar nitric oxide production in normal children and children with asthma. Am J Respir Crit Care Med 2006;174:260-267.
10 Berlyne GS, Parameswaran K, Kamada D, Efthimiadis A, Hargreave FE. A comparison of exhaled nitric oxide and induced sputum as markers of airway inflammation. J Allergy Clin Immunol 2000;106:638-644.
11 Gratziou C, Lignos M, Dassiou M, Roussos C. Influence of

atopy on exhaled nitric oxide in patients with stable asthma and rhinitis. Eur Respir J 1999;14:897-901.
12 Charles IG, Scorer CA, Moro MA, Fernandez C, Chubb A, Dawson J, et al. Expression of human nitric oxide synthase isozymes. Methods Enzy mol 1996;268:449-460.
13 Tulic MK, Wale JL, Holt PG, Sly PD. Differential effects of nitric oxide synthase inhibitors in an in vivo allergic rat model. Eur Respir J 2000;15:870-877.
14 Lee YC, Cheon KT, Lee HB, Kim W, Rhee YK, Kim DS. Gene polymorphisms of endothelial nitric oxide synthase and angiotensin-converting enzyme in patients with asthma. Allergy 2000;55:959-963.
15 Marsden PA, Heng HH, Scherer SW, Stewart RJ, Hall AV, Shi XM, et al. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. J Biol Chem 1993;268:17 478-17 488.
16 Holla LI, Buckova D, Kuhrova V, Stejskalova A, Francova H, Znojil V, et al. Prevalence of endothelial nitric oxide synthase gene polymorphisms in patients with atopic asthma. Clin Exp Allergy 2002;32:1193-1198.
17 Miyamoto Y, Saito Y, Nakayama M, Shimasaki Y, Yoshimura T, Yoshimura M, et al. Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a -786T-->C mutation associated with coronary spastic angina. Hum Mol Genet 2000;9:2629-2637.
18 Tesauro M, Thompson WC, Rogliani P, Qi L, Chaudhary PP, Moss J. Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: cleavage of proteins with aspartate vs. glutamate at position 298. Proc Natl Acad Sci U S A 2000;97:2832-2835.
19 Leung TF, Liu EK, Tang NL, Ko FW, Li CY, Lam CW, et al. Nitric oxide synthase polymorphisms and asthma phenotypes in Chinese children. Clin Exp Allergy 2005;35:1288-1294.
20 Salam MT, Bastain TM, Rappaport EB, Islam T, Berhane K, Gauderman WJ, et al. Genetic variations in nitric oxide synthase and arginase influence exhaled nitric oxide levels in children. Allergy 2011;66:412-419.
21 Gelfand E W. Pediatric asthma: a different disease. Proc Am Thorac Soc 2009;6:278-282.
22 Ragia G, Nikolaidis E, Tavridou A, Arvanitidis KI, Kanoni S, Dedoussis GV, et al. Endothelial nitric oxide synthase gene polymorphisms -786T > C and 894G > T in coronary artery bypass graft surgery patients. Hum Genomics 2010;4:375-383.
23 Ohtoshi K, Yamasaki Y, Gorogawa S, Hayaishi-Okano R, Node K, Matsuhisa M, et al. Association of (-)786T-C mutation of endothelial nitric oxide synthase gene with insulin resistance. Diabetologia 2002;45:1594-1601.
24 Arshad SH, Karmaus W, Matthews S, Mealy B, Dean T, Frischer T, et al. Association of allergy-related symptoms with sensitisation to common allergens in an adult European population. J Investig Allergol Clin Immunol 2001;11:94-102.
25 Kaleyias J, Papaioannou D, Manoussakis M, Syrigou E, Tapratzi P, Saxoni-Papageorgiou P. Skin-prick test findings in atopic asthmatic children: a follow-up study from childhood to puberty. Pediatr Allergy Immunol 2002;13:368-374.
26 Wambre E, Bonvalet M, Bodo VB, Maillère B, Leclert G, Moussu H, et al. Distinct characteristics of seasonal (Bet v 1) vs. perennial (Der p 1/Der p 2) allergen-specific CD4(+) T cell responses. Clin Exp Allergy 2011;41:192-203.
27 Holla LI, Jurajda M, Pohunek P, Znojil V. Haplotype analysis of the endothelial nitric oxide synthase gene in asthma. Hum Immunol 2008;69:306-313.
28 Storm van's Gravesande K, Wechsler ME, Grasemann H, Silverman ES, Le L, Palmer LJ, et al. Association of a missense mutation in the NOS3 gene with exhaled nitric oxide levels. Am J Respir Crit Care Med 2003;168:228-231.
29 Gao PS, Kawada H, Kasamatsu T, Mao XQ, Roberts MH, Miyamoto Y, et al. Variants of *NOS1, NOS2*, and *NOS3* genes in asthmatics. Biochem Biophys Res Commun 2000;267:761-763.
30. Djidjik R, Ghaffor M, Brun M, Gharnaout M, Salah SS, Boukouaci W, et al. Constitutive nitric oxide synthase gene polymorphisms and house dust mite respiratory allergy in an Algerian patient group. Tissue Antigens 2008;71:160-164.
31 Holla LI, Stejskalova A, Znojil V, Vasku A. Association study of promoter polymorphisms within the *NOS3* gene and allergic diseases. Int Arch Allergy Immunol 2006;141:103-109.

Received October 21, 2014
Accepted after revision February 16, 2015