

Prevalence of endothelial nitric oxide synthase gene polymorphisms in patients with atopic asthma

L. I. Hollá*, D. Bučková*, V. Kuhrová†, A. Stejskalová*, H. Francová†, V. Znojil‡ and J. Vácha*

*Institute of Pathological Physiology, †Research Institute of Child Health and ‡Centre for Mathematical Modelling, Masaryk University Brno, Czech Republic

Summary

Background Asthma is a common multifactorial disease, the aetiology of which is attributable to both environmental and genetic factors. The endothelial nitric oxide synthase (NOS3) gene has been implicated in asthma pathogenesis.

Objective This study investigated associations of 27 base-pair tandem repeat polymorphism in intron 4 and the Glu298Asp (G894T) variant of the NOS3 gene with atopic asthma in a Czech population.

Methods Polymerase chain reaction was used to determine the NOS3 genotypes in subjects with atopic asthma ($n = 163$) and random controls ($n = 209$).

Results The NOS3 allele or genotype distributions did not differ significantly between the control and asthma groups. However, the common genotype (bb) of the NOS3 polymorphism in intron 4 was found to be significantly associated with total IgE levels ($P = 0.006$), specific IgE levels for feathers ($P = 0.0002$) and a positive skin prick test for hay ($P = 0.004$). In one atopic patient, we identified an additional 27-bp repeat in the NOS3 gene (NOS3c), which occurred in heterozygous combination with the NOS3b allele (NOS3b/c genotype). In addition, we describe a new polymorphism (A5495G) in the NOS3 gene, which was in almost complete linkage disequilibrium with the NOS3 repeat polymorphism in intron 4. The Glu298Asp variant was not associated with asthma and/or related atopic phenotypes in our study.

Conclusion Neither the NOS3 'b' allele nor the NOS3 'b/b' genotype showed any general association with atopic asthma, but they were associated with atopy-related phenotypes. We conclude that the NOS3 gene polymorphisms may act as disease modifiers in atopic asthma phenotype in our population.

Keywords asthma, atopy, gene, IgE, nitric oxide synthase, NOS, polymorphism

Submitted 31 July 2001; revised 5 April 2002; accepted 15 April 2002

Introduction

Atopic asthma is a common multifactorial disease resulting from an interaction between a genetic predisposition and environmental factors, such as pollen, house-dust mite and smoke. Both candidate and whole human genome screenings have identified a number of possible gene linkages to asthma [1, 2], one of which was the endothelial nitric oxide synthase gene (NOS3) [1, 3].

There is increasing evidence that nitric oxide (NO), produced by the enzymes NOSs, may play an important role in regulating airway function and in the pathophysiology of inflammatory airway diseases [4]. NO plays a key role as a vasodilator, neurotransmitter and inflammatory mediator in the airways, being produced in increased amounts in asthma and allergic rhinitis [5, 6]. One of the potentially beneficial effects of NO derives

from its bronchodilatory action. Moreover, it may seem paradoxical that asthmatics have elevated levels of exhaled NO in the context of a disease characterized by bronchial hyper-reactivity [7]. On the other hand, NO may have deleterious effects on the airways as a vasodilator by increasing plasma exudation, and may also amplify the asthmatic inflammatory response [8]. NO in exhaled air correlates with asthma severity, sputum eosinophils, methacholine reactivity and peak flow variability, probably related to airway inflammation [9].

NO is generated from L-arginine by the enzyme NOS, of which there are three known isoforms. Inducible NO synthase (NOS2), constitutive neuronal NO synthase (NOS1) and constitutive endothelial NO synthase (NOS3) are encoded by a gene family located on different chromosomes and expressed in different cells [10]. Some types of gene polymorphisms of the NOS1 gene (chromosome 12q24) have in previous studies been associated with the pathogenesis of asthma [11–13]. Two previous studies of the role of the NOS3 gene in the pathogenesis of atopic asthma produced conflicting results [3, 14]. One reported an association of 27 base-pair tandem repeat polymorphism in intron 4 of the NOS3 gene (chromosome

Correspondence: Lydie Izakovičová Hollá, Department of Pathological Physiology, Medical Faculty, Masaryk University, Kom. nám.2, CZ 662 43 Brno, Czech Republic. E-mail: holla@med.muni.cz

7q35–36) [15] with bronchial asthma in Orientals [3], whereas the Glu298Asp variant in exon 7 was not associated with asthma and/or related atopic phenotypes in a study by Gao et al. [14].

The aim of our present study was to test whether atopic asthma or related atopic phenotypes are associated with selected mutations (polymorphisms) in the NOS3 gene in a highly homogeneous Czech population. Association analysis was used in the study, as it is known to be a more powerful tool than linkage analysis in families when searching for genes with weak to moderate effects underlying complex phenotypes [16].

Methods

Subjects

Caucasian subjects of exclusively Czech ethnicity ($n = 372$) were included in the study. All subjects were selected on a detailed questionnaire modified from the American Thoracic Society respiratory questionnaire [17] relating to lifetime symptoms suggestive of asthma and rhinitis, extended with additional questions on symptoms and therapy as well as on other diseases. Phenotype status was assigned without previous knowledge of genotypes independently by two investigators.

A total of 163 patients with clinically manifested atopic asthma (93 men and 70 women), aged 28 ± 14 years (mean $\pm$ SD) were studied. A total of 209 reference subjects (113 men and 96 women), aged 36 ± 15 years, who were free of lung, skin and cardiovascular disease and had no clinical evidence of personal or familial atopy, asthma, hay fever or eczema (detailed personal and family history questions were asked at the time of enrolment), were recruited as controls.

Subjects with bronchial asthma were defined according to the criteria of the American Thoracic Society [17]. The diagnosis of bronchial asthma was confirmed by a clear clinical history with clinical symptoms (episodic wheezing, chest tightness and dyspnoea) and a 15% or greater reversibility of forced expiratory volume (FEV1) spontaneously or after nebulized β_2 -agonist. At presentation, the total number of asthmatic patients was distributed relatively evenly between two categories of severity, 55% being in the mild group and 45% in the moderate group. Some subjects also had allergic rhinitis with symptoms of either hay fever or year-round rhinitis characterized by watery rhinorrhoea, itching of the nasal mucosa and episodes of sneezing paroxysms. Some asthma patients had concomitant atopic dermatitis. The atopy diagnosis was based on several criteria, including clinical signs of atopic disease and the following criteria, as described previously [18]:

Positive skin prick tests (greater than or equal to 3 mm above a negative control) to one or more common aeroallergens.

These were house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinum*), common grass and tree pollen mixtures (see Appendix 1), and common moulds (*Alternaria*, *Cladosporium* and *Aspergillus*). Histamine was used as a positive and saline as a negative control. We used standardized test procedures and a set of commercially available allergens routinely used in practice in our country (Sevapharm, Prague, Czech Republic).

Raised specific serum IgE levels (> 0.35 kU/L, by Ala-stat, DPC-Biermann, Bad-Nauheim, Germany) produced in

response to one or more of the common allergens including *Dermatophagoides farinae*, grass pollens, animal danders and mold.

Raised total serum IgE levels above normal values (greater than 150 IU/mL in non-smoking adults, by a nephelometric test (Dade-Behring, Mannheim, Germany).

All the subjects gave written informed consent to participation in the study which was approved by the Committee for the Ethics of Medical Experiment on Human Subjects, Medical Faculty, Masaryk University, Brno, Czech Republic.

Genotyping

Leucocyte DNA was extracted from peripheral blood leucocytes by standard methods using proteinase K digestion.

To determine the NOS3 genotype, a genomic DNA fragment of intron 4 of the NOS3 gene was amplified by polymerase chain reaction (PCR) as described previously [3]. PCR products in 2% agarose gel show a 602-bp fragment in the b allele and a 575-bp fragment in the a allele (genotypes described as bb, 602 bp; ba, 602 + 575 bp; aa, 575 bp).

The NOS3894G/T genotypes were determined by PCR as described by Novoradovsky et al. [19]. Digestion with *Ban*II revealed fragments of 92 and 62 bp lengths for the G allele, and 154 bp for the T allele.

Sequence analysis

The PCR was performed with NOS primers as described above. The generated PCR products were separated on a 2% agarose gel and atypical fragments were used as sequencing templates. Sequence analysis was performed by the cycle-sequencing method, using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham, Aylesbury, UK) and Redivue ^{33} P labelled terminator pack (Amersham). The sequencing reaction was performed according to the manufacturer's protocol. Electrophoresis was run on Bio-Rad (Hercules, CA, USA) sequencing apparatus in 6% polyacrylamide, 8 mol/L urea at constant 22 mA for 2 and 4 h.

A5495G polymorphism

Applying a direct sequencing technique revealed the existence of a novel variant in intron 4 of the NOS3 gene leading to a G→A substitution at position 5495. To confirm the mutation, a rapid detection method was developed where a 251-bp DNA fragment was amplified (primers 5'-CCCAGTTCC-CCTGCTTCG-3' and 5'-GGCAAGTGTCAGATAGGATT-3') and its product digested with *Dde*I restriction endonuclease. The PCR was performed in a final volume of 15 μ L, containing 50 mM KCl, 10 mM Tris-HCl buffer, pH 8.4, 0.9 mM MgCl₂, 6 μ M of each primer, 200 μ M dNTP and 0.1 μ g of genomic DNA in the presence of 0.1 U of Taq polymerase (MBI Fermentas, Vilnius, Lithuania) to provide 251-bp products. After the initial denaturation step (95 °C for 2 min), each cycle (of further 30 cycles) consisted of a 95 °C denaturation for 30 s, a 56 °C annealing for 30 s, a 72 °C extension for 45 s, with the final extension lasting 5 min at 72 °C. Equal amounts of 10 μ L of the PCR product were digested with *Dde*I for 3 h at 37 °C and separated on 2% agarose gel. In the absence of the mutation (allele A present), the 251-bp fragment was cleaved into three segments of 156-, 64- and 31-bp. In the presence of the mutation (G allele), we found two fragments of 156- and 95-bp.

Statistical analysis

Allele frequencies were calculated from the observed numbers of genotypes. χ^2 analysis was used to test for a deviation of genotype distribution from Hardy–Weinberg equilibrium. The significance of differences in the allele frequencies between each two groups was determined by Fisher's exact test. Except for allelic frequencies, χ^2 test was used for all other statistical analyses. A non-parametric Mann–Whitney test was used to assess phenotypic differences (in total IgE levels) between the carriers of different genotypes of the eNOS [the bb and ba genotypes were compared; the aa genotype was excluded for a small number of patients (only 4)]. Contingency table analysis, odds ratio (OR), 95% confidence intervals and significance values were estimated with the use of the programme package Statistica v 3.0 (Statsoft Inc., Tulsa, OK, USA). Corrections for multiple comparisons (Holm's method) were performed and only the values of P_{corr} (P corrected) less than 0.05 were considered significant.

Results

PCR amplification results and the sequencing of some PCR products

The PCR of the NOS3 gene resulted in a 602-bp fragment (NOS3b) from a human genomic DNA template provided that five 27-bp repeats were present in intron 4, and a 575-bp product (NOS3a) if only four repeats were present [3, 20]. DNA from individuals heterozygous for the NOS3 polymorphism produced both the 602-bp and 575-bp fragments (NOS3b/a). Gel separation of the PCR product of one patient showed an abnormal banding pattern (629-bp = NOS3c allele, see Fig. 1). Sequencing and the sequence comparison revealed an additional 27-bp repeat in the NOS3 gene, which occurred in a heterozygous combination with the NOS3b allele (NOS3b/c genotype) as described previously by Sigusch *et al.* [20].

NOS3 polymorphism (27-bp repeat in intron 4) in the control and asthmatic population

Genotype distributions for the NOS3 variant in intron 4 for both groups did not differ significantly from those expected under conditions of Hardy–Weinberg equilibrium (data not shown).

In the control group ($n = 209$), the frequencies of the bb, ba and aa genotypes of the NOS3 gene were 145 (69.4%), 60 (28.7%) and 4 (1.9%), respectively. In the population of patients with atopic asthma ($n = 163$), the distributions of the bb, ba, aa (and bc) genotypes were 113 (69.3%), 44 (27.0%), 5 (3.1%) and 1 (0.6%), respectively. There were no significant differences in the distribution of these genotypes or allele frequencies in patients with atopic asthma as compared to control subjects. When the atopic asthma group was subdivided into subjects with mild and those with moderate asthma, there were no significant differences between the two groups ($P > 0.05$, data not shown).

Skin test reactivity and specific IgE levels

When asthmatic patients were subdivided in subjects with positive and those with negative skin prick tests (or specific IgE levels), the NOS3 polymorphism was significantly associated with a positive reactivity to hay and feathers (Table 1). Patients with atopic asthma and positive skin prick tests (or specific IgE levels) for these antigens (hay and feathers) were more often homozygous for the common genotype (bb) of the NOS3 gene than homozygous for the (aa) genotype and heterozygous (ab) [for hay: $P = 0.004$, $P_{\text{corr}} < 0.05$, for feathers: $P = 0.0002$, $P_{\text{corr}} < 0.05$, respectively]. No associations were found for house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinum*, data not shown).

Association of 27-bp repeat polymorphism with total serum IgE levels

Among our atopic asthmatic patients, those with the (bb) genotype had higher total serum IgE levels than the (ab) heterozygotes [aa homozygotes were excluded due to a small number of patients ($n = 4$) in this group]. The mean value of total serum IgE levels was 407.4 ± 63.5 IU/mL (mean $\pm$ SE) in the (bb) group, which was significantly higher than that of the (ab) heterozygotes (124.4 ± 33.9 IU/mL, $P = 0.006$).

G894T polymorphism (Glu298Asp)

The genotype distributions were in Hardy–Weinberg equilibrium, both within each group and in the population as a whole. In agreement with our hypothesis, there were no differences in genotype or allele frequencies between the atopic asthmatic patients [$n = 157$, allele G/T = 0.672/0.328, genotype: GG = 70 (44.6%), GT = 71 (45.2%) and TT = 16 (10.2%)] and the control

Fig. 1. Polymorphism of the 27-bp repeat in intron 4 of the endothelial NO synthase (NOS3) gene. Polymerase chain reaction (PCR) products were separated by agarose gel electrophoresis. Lane 1 = NOS3b/c, lane 2 = NOS3b/a, lane 3 = NOS3b/b, lane 4 = NOS3a/a, MW indicates molecular weight marker, the band of 602-bp indicates five repeats, the band of 575-bp indicates four repeats, and one very rare genotype of 629-bp indicates six 27-bp repeats.

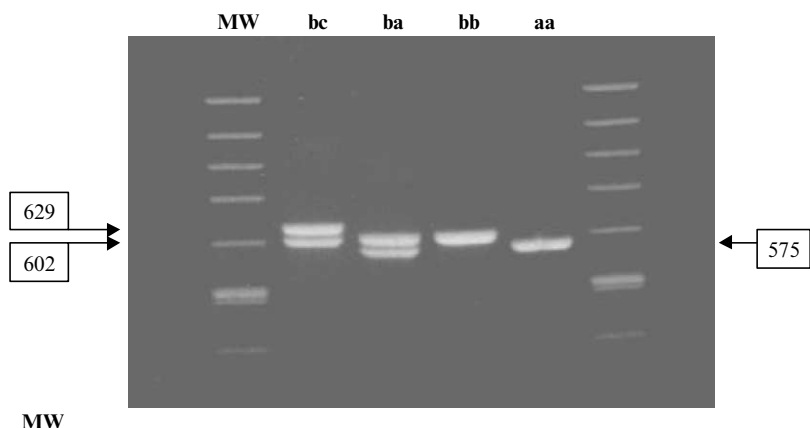


Table 1. Association of the 27-bp repeat polymorphism in intron 4, the A5495G and G894T variants of the NOS3 gene with skin prick tests (or specific IgE levels) in the Czech asthmatic patients

NOS3 genotypes	Hay (prick test)		Feathers (specific IgE levels)	
	Negative	Positive	Negative sIgE	Positive sIgE
Intron 4	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
aa	3 (12.0)	0 (0)	3 (16.7)	0 (0)
ab	6 (24.0)	21 (25.9)	2 (11.1)	23 (28.4)
bb	15 (60.0)	60 (74.1)	12 (66.7)	58 (71.6)
bc	1 (4.0)	0 (0)	1 (5.6)	0 (0)
	$P = 0.004, P_{\text{corr}} < 0.05$		$P = 0.0002, P_{\text{corr}} < 0.05$	
A5495G	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
AA	12 (60.0)	54 (74.0)	8 (61.5)	54 (72.0)
AG	5 (25.0)	19 (26.0)	2 (15.4)	21 (28.0)
GG	3 (15.0)	0 (0)	3 (23.1)	0 (0)
	$P = 0.003, P_{\text{corr}} < 0.05$		$P = 0.0001, P_{\text{corr}} < 0.05$	
G894T	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
GG	8 (33.3)	35 (44.3)	9 (52.9)	33 (41.8)
GT	14 (58.3)	36 (45.6)	8 (47.1)	37 (46.8)
TT	2 (8.3)	8 (10.1)	0 (0)	9 (11.4)
	n.s		n.s	

P-value: the statistical significance for the comparison of genotype distributions (χ^2 test). P_{corr} -value: the statistical significance after a correction (Holm's test).

subjects [$n = 200$, allele G/T = 0.670/0.330, genotype: GG = 89 (44.5%), GT = 90 (45.0%) and TT = 21 (10.5%)]. In addition, there were no significant associations between this polymorphism and any of the patient subgroups classified by the severity of asthma (data not shown) or serum IgE concentration and/or other atopy-related phenotypes (Table 1).

A5495G polymorphism

Applying a direct sequencing technique revealed the existence of a novel variant in intron 4 of the NOS3 gene leading to a G→A substitution at position 5495. To confirm the mutation, a rapid detection method was developed where a 251-bp DNA fragment was amplified as described in method section.

In the subgroups of the subjects, we showed the frequency of the A allele to be 82.9% in the asthmatics and 85.9% in the control sample ($n = 126$, $n = 152$, respectively). In asthmatics, 87 (69.0%) patients were homozygous for the common A allele, 35 (27.8%) were heterozygous (AG) and four (3.2%) were homozygous for the rare G allele. One hundred and eleven (73.0%) subjects homozygous for the A allele, 39 (25.7%) heterozygous and two (1.3%) subjects homozygous for the G allele were detected in control group. No significant differences were detected between both groups. This polymorphism was in almost complete linkage disequilibrium with the NOS3 27-bp repeat polymorphism in intron 4 (position 5257–5283 del 27-bp). The mean value of total serum IgE levels was 469.1 ± 75.7 IU/mL (mean $\pm$ SE) in the (AA) group, which was significantly higher than that of the (AG) heterozygotes (150.6 ± 41.9 , $P = 0.003$) (aa homozygotes were excluded due to a small number of patients ($n = 4$) in this group). Patients with atopic asthma and positive skin prick tests (or specific IgE levels) for some allergens (hay and feathers) were more often homozygous for the common genotype (AA) of the NOS3 gene than homozygous for the (GG) genotype and heterozygous (AG) [for hay: $P = 0.003$, $P_{\text{corr}} < 0.05$, for feathers: $P = 0.0001$,

$P_{\text{corr}} < 0.05$, respectively, Table 1]. No associations were found for house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinum*, data not shown).

Discussion

Asthma predisposition is regarded as a multifactorial condition whose onset and severity are influenced by both genetic and environmental factors. Our study represents an investigation of the role of the NOS3 gene in atopic asthma in a Czech population (the ethnic Czech population is a highly homogenous group of Caucasians living in the central Europe).

Firstly, we analysed a possible association of the NOS3 gene. The results of previous studies suggest that the 894G/T mutation in exon 7 (Glu298Asp) in this gene may have a functional consequence as it affects the function of the eNOS protein [19, 21, 22]. There exists only one study, by Gao et al. [14], examining this polymorphism with respect to the pathophysiology of atopic asthma. The authors found no significant association between the 894G/T variant of the NOS3 gene and atopic asthma in a British population. This study in a Czech population also shows no association between this polymorphism and atopic asthma either, which is consistent with the findings of the previous study [14].

Secondly, we examined the potential role of the 27-bp repeat polymorphism in intron 4 in the pathogenesis of asthma. This NOS3 gene locus may be responsible for variations in the genetic control of plasma NO. The mean plasma NO level of subjects who were homozygous for the a allele was nearly 20% lower than in subjects with the b allele [23]. Lee et al. [3] recently reported an association between the 27-bp variant in intron 4 of the NOS3 gene and asthma in Orientals living in South Korea. In our study we were unable to detect any association of the NOS3 genotype with atopic asthma. These data are at odds with

those reported by Lee et al. [3], who found an association between the (bb) genotype of NOS3 gene and asthma. Our negative results may reflect some involvement of variable combinations of risk alleles in different populations. Although there were no differences in the genotype or allele frequencies between asthmatics and controls, there was a higher prevalence of the (bb) genotype in the subgroup of asthmatic subjects with a positive skin prick test reactivity (or positive specific IgE levels) to hay (and/or feathers) compared to those with negative tests for these allergens. These statistical associations between the polymorphism of the NOS3 gene (7q35–36) could reflect a direct effect of the marker allele on skin test reactivity and possibly atopic asthma, or alternatively, the NOS3 variant may be in a tight linkage disequilibrium with a putative susceptibility gene in this region. Linkages between TCR- β and nearby markers (D7S684) with both IgE and asthmatic phenotypes were found in a previous study [24]. Skin reactivity to other common environmental allergens, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinum* (house-dust mite), showed no significant association with genotype NOS3. However, a significant genetic association between the 27-bp repeat variant (bb = five repeats) in intron 4 of the NOS3 gene and the increased serum IgE levels in atopic asthmatic patients was found in our population.

Thirdly, we identified a novel mutation in intron 4 (A5495G) of the NOS3 gene, which was in a nearly complete linkage disequilibrium with the NOS3 27-bp repeat polymorphism in this intron. The functional significance of the nucleotide exchange is unclear at present.

NO is an important endogenous regulatory molecule synthesized in the lung and, in asthma (especially atopic asthma), the airway NO levels are increased [25]. The levels of exhaled NO were significantly higher in patients with asthma who were both sensitized and exposed to a relevant allergen than in those who were sensitized but not exposed [26]. Recently, it has been shown that exhaled NO levels in asthmatics correlate more closely with atopy (with total IgE and a number of positive skin prick tests) than with bronchial hyper-reactivity and lung functions [27], which might correspond with our results and indicate a certain participation of the gene for NOS3 in the pathophysiology of atopic asthma.

In conclusion, this study provides evidence for a role of the NOS3 polymorphisms in genetic susceptibility to atopic asthma in the Czech population. Moreover, we showed the potential importance of complex interactions between the genetic background and the expression of the atopic asthma phenotype. However, because differences in genetic background influence the allele frequencies of disease-associated genes, further studies might provide more information on the involvement of the NOS3 gene in asthma.

Acknowledgements

We would like to thank H. Melicharova, A. Pazdirkova, L. Siskova, B. Novotna, V. Izakovic, A. Spacilova, I. Nemcova and J. Teturova for their collection and phenotyping of subjects. The study was supported by project CEZ: 307/98: 141100002 provided by the Ministry of Education, Youth and Physical Training of the Czech Republic.

References

- Holgate ST. The cellular and mediator basis of asthma in relation to natural history. *Lancet* 1997; 350:5–9.
- Dizier MH, Besse-Schmittler C, Guillaud-Bataille M et al. Genome screen for asthma and related phenotypes in the French EGEA study. *Am J Respir Crit Care Med* 2000; 162:1812–8.
- 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; 55:959–63.
- Barnes PJ. Nitric oxide and airway disease. *Ann Med* 1995; 27:389–93.
- Sippel JM, Holden WE, Tilles SA et al. Exhaled nitric oxide levels correlate with measures of disease control in asthma. *J Allergy Clin Immunol* 2000; 106:645–50.
- Djupesland PG, Chatkin JM, Qian W, Haight JS. Nitric oxide in the nasal airway. A new dimension in otorhinolaryngology. *Am J Otolaryngol* 2001; 22:19–32.
- al-Ali MK, Eames C, Howarth PH. Exhaled nitric oxide; relationship to clinicophysiological markers of asthma severity. *Respir Med* 1998; 92:908–13.
- Barnes PJ. NO or no NO in asthma? *Thorax* 1996; 51:218–20.
- Lim S, Jatakanon A, John M et al. Effect of inhaled budesonide on lung function and airway inflammation. Assessment by various inflammatory markers in mild asthma. *Am J Respir Crit Care Med* 1999; 159:22–30.
- Nathan C, Xie QW. Nitric oxide synthases. roles, tolls, and controls. *Cell* 1994; 78:915–8.
- Grasemann H, Yandava CN, Drazen JM. Neuronal NO synthase (NOS1) is a major candidate gene for asthma. *Clin Exp Allergy* 1999; 29:39–41.
- Wechsler ME, Grasemann H, Deykin A et al. Exhaled nitric oxide in patients with asthma: association with NOS1 genotype. *Am J Respir Crit Care Med* 2000; 162:2043–7.
- Grasemann H, Yandava CN, Storm van's Gravesande K et al. A neuronal NO synthase (NOS1) gene polymorphism is associated with asthma. *Biochem Biophys Res Commun* 2000; 272:391–4.
- Gao PS, Kawada H, Kasamatsu T et al. Variants of NOS1, NOS2, and NOS3 Genes in Asthmatics. *Biochem Biophys Res Commun* 2000; 267:761–3.
- Marsden PA, Heng HH, Scherer SW et al. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem* 1993; 268:17478–88.
- Scott WK, Pericak-Vance MA, Haines JL. Genetic analysis of complex diseases (letter). *Science* 1997; 275:1327, discussion 1329–30.
- American Thoracic Society. Definition and classification of chronic bronchitis, asthma and pulmonary emphysema. *Am Rev Respir Dis* 1988; 136:225–44.
- Izakovićová Hollá L, Vaškó A, Izaković V, Znojil V. The interaction of the polymorphisms in transporter of antigen peptides (TAP) and lymphotoxin α (LT- α) genes and atopic diseases in the Czech population. *Clin Exp Allergy* 2001; 31:1418–23.
- Novoradovsky A, Brantly ML, Wacławski MA et al. Endothelial nitric oxide synthase as a potential susceptibility gene in the pathogenesis of emphysema in α 1-antitrypsin deficiency. *Am J Respir Cell Mol Biol* 1999; 20:441–7.
- Sigusch HH, Surber R, Lehmann MH et al. Lack of association between 27-bp repeat polymorphism in intron 4 of the endothelial nitric oxide synthase gene and the risk of coronary artery disease. *Scand J Clin Lab Invest* 2000; 60:229–36.
- Tesaro 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 USA* 2000; 97:2832–5.

- 22 Yoshimura M, Yasue H, Nakayama M et al. A missense Glu298Asp variant in the endothelial nitric oxide synthase gene is associated with coronary spasm in the Japanese. *Hum Genet* 1998; 103:65–9.
- 23 Tsukada T, Yokoyama K, Arai T et al. Evidence of association of the eNOS gene polymorphism with plasma NO metabolite levels in humans. *Biochem Biophys Res Commun* 1998; 245:190–3.
- 24 Noguchi E, Shibasaki M, Arinami T et al. Evidence for linkage between the development of asthma in childhood and the T-cell receptor beta chain gene in Japanese. *Genomics* 1998; 47:121–4.
- 25 Gustafsson LE. Exhaled nitric oxide as a marker in asthma. *Eur Respir J* 1998; 11:49–52.
- 26 Simpson A, Custovic A, Pipis S, Adisesh A, Faragher B, Woodcock A. Exhaled nitric oxide, sensitization, and exposure to allergens in patients with asthma who are not taking inhaled steroids. *Am J Respir Crit Care Med* 1999; 160:45–9.
- 27 Ho LP, Wood FT, Robson A, Innes JA, Greening APOD. Atopy influences exhaled nitric oxide levels in adult asthmatics. *Chest* 2000; 118:1327–31.

Appendix 1

The grass and tree pollens mixtures were used as follows:

- 1 Pollinare mixtum: *Agrostis alba*, *Alopecurus pratensis*, *Anthoxanthum odouratum*, *Apera spica-venti*, *Arrhenatherum elatius*, *Avenastrum sp.*, *Dactylis glomerata*, *Festuca rubra*, *Holcus lanatus*, *Lolium sp.*, *Phleum pratense*, *Poa pratensis*, *Secale cereale*, *Trisetum flavescens*, *Plantago lanceolata*.
- 2 Mixture of grass I: *Arrhenatherum elatius*, *Dactylis glomerata*, *Festuca rubra*, *Lolium sp.*, *Phleum pratense*, *Secale cereale*.
- 3 Mixture of grass II: *Argopyrum repens*, *Bromus erectus*, *Calamagrostis epigeios*, *Deschampsia flexuosa*.
- 4 Early spring mixture: *Alnus glutinosa*, *Betula pendula*, *Carpinus betulus*, *Corylus avellana*, *Fraxinus excelsior*, *Salix caprea*.
- 5 Late spring mixture: *Taraxacum officinale*, *Sambucus nigra*, *Urtica dioica*.
- 6 Autumn mixture of pollen: *Artemisia absinthium*, *A. vulgaris*, *Solidago canadensis*.
- 7 Artemissia mixtum: *Artemissia absynth.*, *A. vulgaris*.