

# NOS1 polymorphism is associated with atopy but not exhaled nitric oxide levels in healthy children

Ali M, Khoo S-K, Turner S, Stick S, Le Souëf P, Franklin P. NOS1 polymorphism is associated with atopy but not exhaled nitric oxide levels in healthy children.

Pediatr Allergy Immunol 2003; 14:261–265. ©2003 Blackwell Munksgaard

Exhaled nitric oxide ( $FE_{NO}$ ) is raised in atopy. The mechanism for this is unclear. The aim of this study was to investigate whether the number of AAT repeats in intron 20 of the *NOS1* gene, recently associated with variations in  $FE_{NO}$  in adults with asthma and cystic fibrosis, was associated with the raised  $FE_{NO}$  in healthy atopic children. Eighty-seven healthy children (44 girls, 42 atopic, age range 6–18 years) underwent measurements of  $FE_{NO}$ , spirometry, airway responsiveness and skin prick testing. Genotyping was carried out to determine the number of AAT repeats. There was no association between the number of AAT repeats and  $FE_{NO}$  in either the whole sample of healthy children ( $n = 87$ ) or in the subsample of healthy atopics ( $n = 42$ ). However, a greater number of atopic children had two high repeat alleles compared with non-atopic children (33.3% vs. 13.6%, respectively,  $p = 0.03$ ). This suggests that variations in the *NOS1* gene may contribute to atopy without this relationship being reflected by  $FE_{NO}$ .

**May Ali<sup>1</sup>, Siew-Kim Khoo<sup>1</sup>, Stephen Turner<sup>1</sup>, Stephen Stick<sup>2</sup>, Peter Le Souëf<sup>1</sup> and Peter Franklin<sup>1</sup>**

<sup>1</sup>Department of Paediatrics, University of Western Australia and <sup>2</sup>Department of Respiratory Medicine, Princess Margaret Hospital for Children, Perth, Australia

Key words: exhaled nitric oxide; nitric oxide synthase; atopy; children

May Ali, Department of Paediatrics, University of Western Australia, Perth, Australia 6009

Tel.: +61 8 94897874

Fax: +61 8 93408983

E-mail: niluwana@cylle.uwa.edu.au

Accepted 15 February 2003

Nitric oxide (NO) is a free radical which is formed when L-arginine is oxidized by the enzyme NO synthase (NOS) (1). Three distinct isoforms of NOS have been identified, two of which are constitutive (NOS1 and 3) and one that is inducible (NOS2). Nitric oxide measured in the exhaled air (fractional concentration of NO in exhaled breath –  $FE_{NO}$ ) is elevated in both asthmatic children (2) and adults (3) compared with non-asthmatic subjects. Increased  $FE_{NO}$  is thought to be due to an up-regulation of NOS2, a hypothesis supported by its increased expression in airway epithelial cells of asthmatic compared with non-asthmatic subjects (4). However, there is now evidence to suggest that NOS1 may have an important role in  $FE_{NO}$  in asthma (5).

Within the asthmatic population, there is a large variation in  $FE_{NO}$  that may not be directly related to the degree of asthma severity (6). This variation may, in part, be attributed to atopy. Exhaled NO is higher in atopic compared with non-atopic asthmatics (7). An association between raised  $FE_{NO}$  and atopy has also been

demonstrated in healthy adults (8) and children (9). Furthermore, there is evidence to suggest that increased  $FE_{NO}$  is associated with the degree of atopy (9). The mechanisms for this association are unclear.

Recently, an association was reported between  $FE_{NO}$  and a polymorphism in the *NOS1* gene in adults with mild asthma (5). The study reported an inverse relationship between the number of AAT repeats at a locus in intron 20 of the *NOS1* gene and  $FE_{NO}$ . Subjects having two alleles with 12 or more AAT repeats at this locus had lower  $FE_{NO}$  than patients with at least one allele < 12 repeats. Similar associations between  $FE_{NO}$  and this polymorphism have been observed in cystic fibrosis patients (10) and children with sickle cell disease (11).

We postulated that the low AAT repeat genotype may explain some of the increase in  $FE_{NO}$  associated with atopy. The aim of this study was to investigate if there was an association between this polymorphism and  $FE_{NO}$  in healthy atopic and non-atopic children.

## Methods

### Subjects and procedure

Subjects were participants in an ongoing prospective birth cohort study (12). Eighty-seven, non-smoking, non-asthmatic children (44 girls), aged between 6 and 18 years, were assessed as part of this study. None of the children had a history of allergic respiratory disease (asthma or rhinitis) and all were well at the time of testing. The children underwent an assessment that included FE<sub>NO</sub> measurements, spirometry, inhaled histamine challenge and skin prick testing and blood was collected for genetic analysis. The study was approved by the Medical Ethics Committee of the Princess Margaret Hospital for Children.

### Phenotypic assessments

Exhaled NO was measured with a fast response chemiluminescence NO analyzer (NOA 280, Sievers Instruments Inc., Boulder, CO, USA) using a single breath technique as previously described (9). Children inhaled to total lung capacity and immediately exhaled while maintaining an expiratory flow of 35 ml/s and a mouth pressure of 15 cm H<sub>2</sub>O, in accordance with recent recommendations (13). Measurements were collected prior to spirometry and histamine challenge and at least 1 hour after exercise. Pulmonary function testing was performed using a hand-held spirometer (Pneumocheck Spirometer 6100; Welch-Allyn, Skaneateles Falls, NY, USA) in accordance with published guidelines (14). Airway responsiveness (AR) to histamine was determined using the rapid dosimeter technique (15). Responsiveness was defined as a provocative dose of < 7.8 µmol that provoked at least a 20% fall in FEV<sub>1</sub> (PD<sub>20</sub>). Further, a dose-response slope (DRS) was calculated using the method of O'Connor (16). Skin prick reactivity to 10 common allergens was assessed using the method described by Pepys (17). The allergens tested were: cow's milk, egg white, rye grass, mixed grass, *Dermatophagoides farinae*, *D. pteronyssinus*, cat dander, dog dander, *Alternaria alternans* and *Aspergillus fumigatus* (Hollister-Stier, Elkhart, IN, USA). Atopy was defined as the presence of at least one positive skin prick test (SPT).

### Genetic analysis

Total genomic DNA was isolated from peripheral blood cells using the phenol/chloroform method (18).

The region spanning the AAT repeats in intron 20 of the *NOS1* gene was amplified from genomic

DNA by the polymerase chain reaction (PCR). The 25-µl PCR mix contained 100 ng of genomic DNA, 1× buffer (Invitrogen, Melbourne, Australia), 200 µM deoxynucleotide triphosphate, 25 ng of each of the forward and the reverse primers (forward primer: 5'-ATG GTG TGT TGG TGA AC-3', reverse primer: 5'-GTC ATA GTG GGA ATG TTT AC-3'), 0.5 U of Platinum *Taq* polymerase (Invitrogen, Melbourne, Australia), and sterile water. PCR conditions were 10 min at 94°C followed by 12 touchdown cycles of 94°C for 1 min, annealing temperature for 30 s (touchdown programme was used where the annealing temperature was lowered by 0.5°C per cycle from 64°C to 58°C) and 72°C for 20 s. This was then followed by 35 cycles under the same conditions but with a fixed annealing temperature of 58°C and a final elongation step at 72°C for 10 min upon completion of the final cycle. These conditions generated an amplicon of 116 base pairs for an allele that contained 10 AAT repeats. A denaturing PAGE system was used for amplicon analysis. Five microliters of each PCR was then mixed with an equal volume of loading dye containing formamide (90% v/v), bromophenol blue and xylene cyanol. The DNA was then denatured by incubating in boiling water for 5 min and subsequently snap chilled on ice. The denatured DNA mixture was then electrophoresed for 48 min at 20 W in 6% polyacrylamide denaturing gels containing 7 M urea and 0.6× TBE. A 0.6× TBE solution was used as the gel running buffer. The gels were run at room temperature but were preheated prior to loading of the samples by 15-min pre-runs at 30 W. Upon completion of electrophoresis, gels were immediately fixed, silver stained and developed using the Bio-Rad Silver Stain kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

### Statistical analyses

Exhaled NO levels were skewed to the right so all values were transformed to their natural logarithm (ln) in order to achieve a normal distribution. Student's *t*-test and one-way analysis of variance (ANOVA) were used to compare FE<sub>NO</sub> between children grouped according to atopic status, presence of increased AR and the number of AAT repeats at the *NOS1* gene (homozygous for ≥12 AAT repeats or at least one allele < 12 AAT repeats). Factors that may affect FE<sub>NO</sub>, including age, atopy, AR and the *NOS1* polymorphism (as a dichotomous variable), were investigated in a multiple linear regression model. The relationship between the sum of AAT

repeats in both alleles and FE<sub>NO</sub> was investigated using Pearson's correlation. Differences in allele distribution between atopic and non-atopic children were determined using chi-square analysis. Exhaled NO levels are reported as geometric mean (GM) with 95% confidence intervals (CI).

#### Power of the study

We calculated the sample size required for finding a difference in FE<sub>NO</sub> between children with two high repeat (i.e. ≥12) alleles and those with at least one allele with <12 repeats with 80% power and 5% significance level (19). Using the relative difference in FE<sub>NO</sub> concentrations reported by Wechsler et al. (5) (62%) and the standard deviation of our transformed data, 13 children were needed in each group.

#### Results

Of the 87 children involved in the study, 42 were atopic. There were no differences in the mean age, gender distribution and spirometric measures (*FVC* and *FEV*<sub>1</sub>) between the atopic and non-atopic groups (Table 1). Atopic children had higher FE<sub>NO</sub> levels than non-atopics (GM 16.1 p.p.b. vs. 7.3 p.p.b., respectively,  $p < 0.0001$ ) (Table 1), and there was a positive correlation between FE<sub>NO</sub> and the number of positive SPT recorded ( $r = 0.67$ ,  $p < 0.001$ ). There was a difference in FE<sub>NO</sub> between children with and without increased AR ( $p = 0.02$ ). In a multiple linear regression model both atopy ( $p < 0.001$ ) and increased AR ( $p = 0.004$ ) remained predictors of FE<sub>NO</sub>.

#### Allele distribution

The relative frequency of the various alleles of the (AAT)<sub>n</sub> repeat in intron 20 of the *NOS1* gene

was bimodally distributed. The distribution of the alleles and the number of healthy children harboring two high repeat alleles was very similar to that reported by Wechsler et al. (5) for healthy adults (Table 2).

#### FE<sub>NO</sub> and NOS1 polymorphism

There was no difference in FE<sub>NO</sub> concentrations between children who were homozygous for ≥12 repeats and those with at least one low repeat allele [GM (95% CI) 12.7 p.p.b. (8.9–18.0 p.p.b) vs. 10.3 p.p.b. (8.8–12.1 p.p.b), respectively,  $p = 0.32$ ]. Similarly within the atopic group ( $n = 42$ ) there was no difference in FE<sub>NO</sub> levels between children with different genotypes. Geometric mean FE<sub>NO</sub> concentrations were 16.2 p.p.b. (11.5–22.7 p.p.b) for children with two high repeat alleles and 16.1 p.p.b. (12.6–20.7 p.p.b) for children with at least one allele with <12 AAT repeats (Fig. 1). The atopic group was split into those with 1–3 positive SPT ( $n = 22$ ) and 4 or more positive SPT ( $n = 20$ ) to investigate if there was a relationship between FE<sub>NO</sub> and the NOS1 polymorphism that was dependent on the degree of atopy. There was no difference in FE<sub>NO</sub> for children with different genotypes in these two groups. Furthermore, FE<sub>NO</sub> concentrations did not correlate with the sum of AAT repeats in intron 20 for either the whole group ( $p = 0.36$ ) or just the atopic group ( $p = 0.66$ ).

#### Atopy and NOS1 polymorphism

There was a higher number of atopic than non-atopic children harboring two high repeat alleles (33.3% vs. 13.6%, respectively,  $\chi^2$   $p = 0.03$ )

Table 1. Characteristics of atopic and non-atopic healthy children

|                            | Non-atopic<br>n = 45 | Atopic<br>n = 42  |
|----------------------------|----------------------|-------------------|
| Sex (m/f)                  | 22/23                | 21/21             |
| Age (years)*               | 12.0 ± 2.7           | 11.8 ± 2.9        |
| FVC (L)*                   | 2.5 ± 0.7            | 2.6 ± 1.0         |
| FEV (L)*                   | 2.3 ± 0.6            | 2.4 ± 0.9         |
| PD <sub>20</sub> < 7.8‡    | 8 (18.2)             | 13 (31.7)         |
| FE <sub>NO</sub> (p.p.b.)† | 7.3 (6.1–8.8)        | 16.1 (13.2–19.6)§ |
| Two high repeat alleles‡   | 6 (13.6)             | 14 (33.3)§        |

\*Mean ± SD.

†GM (95% CI).

‡n (%).

§ $p < 0.05$ .

Table 2. Relative frequency of each NOS1 allele in healthy children and adults

| No. repeats                                              | Healthy children<br>(present study) n = 87<br>(alleles n = 174) | Healthy adults<br>[Wechsler et al. (5)]<br>n = 305 (alleles n = 610) |
|----------------------------------------------------------|-----------------------------------------------------------------|----------------------------------------------------------------------|
| 17                                                       | 0.000                                                           | 0.003                                                                |
| 16                                                       | 0.006                                                           | 0.000                                                                |
| 15                                                       | 0.017                                                           | 0.052                                                                |
| 14                                                       | 0.191                                                           | 0.161                                                                |
| 13                                                       | 0.264                                                           | 0.267                                                                |
| 12                                                       | 0.017                                                           | 0.010                                                                |
| 11                                                       | 0.006                                                           | 0.010                                                                |
| 10                                                       | 0.455                                                           | 0.438                                                                |
| 9                                                        | 0.045                                                           | 0.052                                                                |
| 8                                                        | 0.000                                                           | 0.003                                                                |
| 6                                                        | 0.000                                                           | 0.003                                                                |
| No. of subjects<br>with both alleles<br>with ≥12 repeats | 20/89 (22.5%)                                                   | 70/305 (23.0%)                                                       |

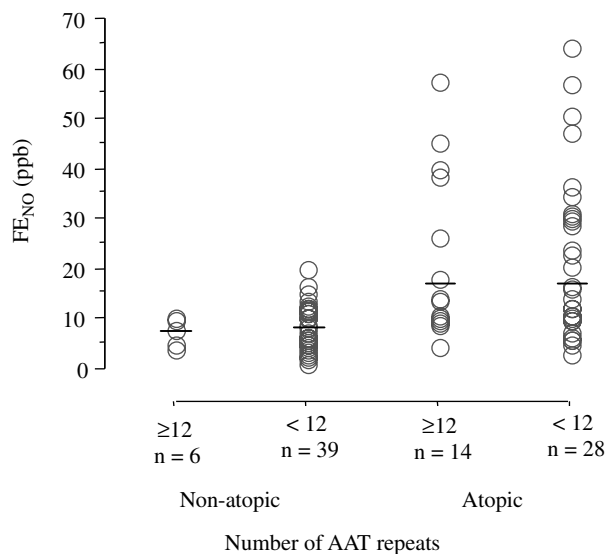


Fig. 1. Exhaled nitric oxide levels for atopic and non-atopic children with either two high repeat alleles ( $\geq 12$ ) or at least one low repeat allele ( $< 12$ ). — indicates the geometric mean for each of the four groups.

(Table 1). There was no association between AR (PD20 or DRS) and the *NOS1* gene polymorphism.

## Discussion

Exhaled NO is elevated in atopic compared with non-atopic subjects with (7) and without (8,9) respiratory disease. The mechanism for this is unclear but may involve changes in NO synthesis or regulation due to mutations in the NOS genes. A polymorphism in the *NOS1* gene has been identified and was found to be associated with variations in FE<sub>NO</sub> in adults with asthma (5) or cystic fibrosis (10), and children with sickle cell disease (11). The role of atopy in this association was not considered in these studies. We investigated the role of atopy in the relationship between the *NOS1* polymorphism and FE<sub>NO</sub> in healthy children. We did not find an association between the number of AAT repeats and FE<sub>NO</sub> in this study. However, there was an association between the *NOS1* polymorphism and atopy, suggesting that this polymorphism may influence the production of NO in a way that affects the allergic response in children but does not contribute significantly to FE<sub>NO</sub> in this age group.

The allele distribution in our subjects was similar to that reported for healthy adults by Wechsler and colleagues (5). Therefore, the different findings between the two studies are unlikely to be due to genetic differences in the studied populations. There are important

differences between the two study groups, pertaining to age and disease status, which may account for the conflicting results. However, as an association between the *NOS1* polymorphism and FE<sub>NO</sub> has been reported in children (11), it is likely that disease status is the main contributing factor to these differences. Nitric oxide synthase expression and regulation is highly complex and may vary depending on different airway chemistry between disease states (20). We have studied a healthy population while other studies observed an association between the *NOS1* polymorphism and FE<sub>NO</sub> in different disease groups.

An interesting finding in the present study was a higher prevalence of atopic children harboring two high repeat alleles compared with non-atopic children. This finding was surprising as the atopic children in this study had significantly elevated FE<sub>NO</sub> while the high AAT repeat *NOS1* polymorphism has been associated with relatively lower FE<sub>NO</sub> (5,10,11). Our data suggest that an association independent of FE<sub>NO</sub> can exist between the *NOS1* polymorphism and atopy in healthy children. It is unclear how this may work. One possibility is that this polymorphism may manifest itself phenotypically by way of linkage with other loci that are relevant to atopy. It has been demonstrated that *NOS1* is able to regulate *NOS2* expression (21). Therefore, it is possible that the *NOS1* polymorphism may play a role in the immune system as NO formed by *NOS2* may modulate the Th1/Th2 balance by favoring Th2 responses (22).

Alternatively, the association between high AAT repeats and atopy could be a direct consequence of the polymorphism itself influencing the production of NO in a way that affects the allergic response as a physiological messenger, but not contribute significantly to FE<sub>NO</sub>. There is increasing evidence pointing to the likelihood of a functional relevance of introns whereby their RNA products are part of a complex communication network that is capable of influencing phenotypic outcomes through various transcriptional, translational and post-translational regulation mechanisms (23).

The regulation and role of NO in airway patho-physiology through the various NOS isoforms is very complex and is still far from being fully understood. Further investigation of these findings is necessary for developing a better understanding of the role of *NOS1* in atopy.

## References

1. BARNES PJ, KHARITONOV SA. Exhaled nitric oxide: a new lung function test. *Thorax* 1996; 51: 233–7.

2. ARTLICH A, HAGENAH JU, JONAS S, AHRENS P, GORTNER L. Exhaled nitric oxide in childhood asthma. *Eur J Pediatr* 1996; 155: 698–701.
3. ALVING K, WEITZBERG E, LUNDBERG JM. Increased amount of nitric oxide in exhaled air of asthmatics. *Eur Respir J* 1993; 6: 1368–70.
4. HAMID Q, SPRINGALL DR, RIVEROS-MORENO V, et al. Induction of nitric oxide synthase in asthma. *Lancet* 1993; 342: 1510–3.
5. WECHSLER ME, GASEMANN 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.
6. KHARITONOV SA, BARNES PJ. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 2001; 163: 1693–722.
7. HO LP, WOOD FT, ROBSON A, INNES JA, GREENING AP. Atopy influences exhaled nitric oxide levels in adult asthmatics. *Chest* 2000; 118: 1327–31.
8. MOODY A, FERGUSON W, WELLS A, BARTLEY J, KOLBE J. Increased nitric oxide production in the respiratory tract in asymptomatic pacific islanders: an association with skin prick reactivity to house dust mite. *J Allergy Clin Immunol* 2000; 105: 895–9.
9. FRANKLIN PJ, TAPLIN R, STICK SM. A community study of exhaled nitric oxide in healthy children. *Am J Respir Crit Care Med* 1999; 159: 69–73.
10. GASEMANN H, KNAUER N, BUSCHER R, HUBNER K, DRAZEN JM, RATJEN F. Airway nitric oxide levels in cystic fibrosis patients are related to a polymorphism in the neuronal nitric oxide synthase gene. *Am J Respir Crit Care Med* 2000; 162: 2172–6.
11. SULLIVAN KJ, KISSOON N, DUCKWORTH LJ, et al. Low exhaled nitric oxide and a polymorphism in the NOS 1 gene is associated with acute chest syndrome. *Am J Respir Crit Care Med* 2001; 164: 2186–90.
12. YOUNG S, LE SOUËF PN, GEELHOED GC, STICK SM, TURNER KJ, LANDAU LI. The influence of a family history of asthma and parental smoking on airway responsiveness in early infancy. *New Eng J Med* 1991; 324: 1168–73.
13. KISSOON N, DUCKWORTH LJ, BLAKE KV, MURPHY SP, TAYLOR CL, SILKOFF PE. FE (NO): relationship to exhalation rates and online versus bag collection in healthy adolescents. *Am J Respir Crit Care Med* 2000; 162: 539–45.
14. ANONYMOUS. Standardization of spirometry – 1987 update. Statement of the American Thoracic Society. *Am Rev Respir Dis* 1987; 136: 1285–98.
15. YAN K, SALOME C, WOOLCOCK AJ. Rapid method for measurement of bronchial responsiveness. *Thorax* 1983; 38: 760–5.
16. O'CONNOR G, SPARROW D, TAYLOR D, SEGAL M, WEISS S. Analysis of dose-response curves to methacholine. An approach suitable for population studies. *Am Rev Respir Dis* 1987; 136: 1412–7.
17. PEPYS J. Skin tests for immediate, type I, allergic reactions. *Proc R Soc Med* 1972; 65: 271–2.
18. SAMBROOK J, FRITSCH EF, MANIATIS T. *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
19. FLOREY CD. Sample size for beginners. *BMJ* 1993; 306: 1181–4.
20. KONNO S, HIZAWA N, YAMAGUCHI E, JINUSHI E, NISHIMURA M. (CCTTT)<sub>n</sub> repeat polymorphism in the NOS2 gene promoter is associated with atopy. *J Allergy Clin Immunol* 2001; 108: 810–4.
21. TOGASHI H, SASAKI M, FROHMAN E, et al. Neuronal (type I) nitric oxide synthase regulates nuclear factor kappaB activity and immunologic (type II) nitric oxide synthase expression. *Proc Natl Acad Sci USA* 1997; 94: 2676–80.
22. KOLB H, KOLB-BACHOFEN V. Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator? *Immunol Today* 1998; 19: 556–61.
23. MATTICK JS. Non-coding RNAs: the architects of eukaryotic complexity. *EMBO* 2001; 2: 986–91.