

Exhaled Nitric Oxide in Patients with Asthma

Association with *NOS1* Genotype

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An increased concentration of nitric oxide (NO) in exhaled air (FeNO) is now recognized as a critical component of the asthmatic phenotype. When we identified patients with asthma on the basis of a standard case definition alone, we found that they were remarkably heterogeneous with respect to their FeNO. However, when we included genotype at a prominent asthma candidate gene (i.e., *NOS1*) in the case definition, and determined the number of AAT repeats in intron 20, we identified a remarkably homogenous cohort of patients with respect to FeNO. Both mean FeNO ($p = 0.00008$) and variability around the mean ($p = 0.000002$) were significantly lower in asthmatic individuals with a high number (≥ 12) of AAT repeats at this locus than in those with fewer repeats. These data provide a biologically tenable link between genotype at a candidate gene in a region of linkage, *NOS1*, and an important component of the asthmatic phenotype, FeNO. We show that addition of *NOS1* genotype to the case definition of asthma allows the identification of a uniform cohort of patients, with respect to FeNO, that would have been indistinguishable by other physiologic criteria. Our isolation of this homogenous cohort of patients ties together the well-established associations among asthma, increased concentrations of NO in the exhaled air of asthmatic individuals, and variations of trinucleotide repeat sequences as identified in several neurologic conditions.

Multiple genetic studies in families have established linkage between the diagnosis of asthma and the distal region of human chromosome 12 (1–3), containing the highly polymorphic gene encoding for the neural form of nitric oxide synthase (*NOS1*) (4). *NOS1* has been implicated as an attractive asthma candidate gene (5) for a number of reasons. First, the fraction of nitric oxide (NO) in exhaled air (FeNO) is, on average, increased in patients with asthma (6–8), but there is wide dispersion about the mean (9, 10). Second, numerous experimental data from studies of both animals and humans indicate that neurogenic factors are important in asthma (11, 12). Third, neuronal nitric oxide synthase (NOS) is important in models of asthmatic airway hyperresponsiveness: mice with targeted deletions of *nos1* have diminished FeNO values and fail to manifest greater airway responsiveness than wild-type mice after allergen sensitization and challenge (13, 14).

Patients with asthma are clinically quite diverse, have variable levels of FeNO, and are often difficult to distinguish phenotypically from one another. Given the well established association between allelic variations of trinucleotide repeat sequences

and several human neurologic conditions (15, 16), we reasoned that there may be an association between various alleles at a trinucleotide repeat locus in the *NOS1* gene and FeNO in asthma. Furthermore, we postulated that we could perhaps distinguish otherwise phenotypically similar asthmatics individuals by *NOS1* genotype. We tested this hypothesis in a cohort of patients with asthma by relating FeNO to the number of AAT repeats at a locus in intron 20 of the *NOS1* gene (4). Since this analysis established a strong association between low FeNO levels (i.e., the normal, nonasthmatic phenotype) and genotype at this *NOS1* locus, we performed an additional study in which we sought a genetic association between the same *NOS1* polymorphism and the diagnosis of asthma.

METHODS

Patient Recruitment and Eligibility Criteria

Ninety-seven asthmatic subjects in two cohorts (a hypothesis-generating and a hypothesis-testing cohort) were recruited from the database of the Partners Asthma Research Center at Brigham and Women's Hospital. The subjects, whose ages ranged from 19 to 54 yr (mean 33 yr), included 38 men and 59 women, of whom 74 were white, 16 black, four Hispanic, one Native American, and two Asian. The subjects' mean FEV₁ was 82% predicted, and was similar in all groups; the mean for each racial group ranged from 77% to 85% predicted. The mean FeNO was 15.2 ppb (15.8 ppb in white versus 13.9 ppb nonwhite subjects) (Table 1).

Subjects were considered asthmatic if they had had a clinical history of asthma (as defined by the American Thoracic Society [17]) for at least 1 yr and had a history within the year preceding the study of at least one of the following: 12% reversibility of FEV₁ in response to a bronchodilator; 25% decrease in FEV₁ in response to cold-air challenge; or a methacholine (MCh) challenge with a provocative concentration that decreased FEV₁ by 20% (PC₂₀) of < 8 mg/ml. All phenotypic assessments were made without knowledge of the patient's genotype. Subjects were excluded if they had used inhaled or systemic corticosteroids within 30 d before the study, if they: had had an upper respiratory tract infection within the preceding 30 d; had smoked tobacco products within the preceding 6 mo or had a > 10 pack-year smoking history; were pregnant; had a history of other documented pulmonary disease (such as chronic obstructive pulmonary disease, cystic fibrosis, or bronchiectasis); or had a history of other major medical problems. Upon presentation to the Asthma Research Center, each subject gave written informed consent for spirometry and genetic screening (approved by the institutional review board at Brigham and Women's Hospital) and completed a patient information sheet. The 97 subjects were studied in two cohorts, consisting respectively of 51 subjects who constituted the initial hypothesis-generating cohort and an additional 46 subjects who were recruited to form the hypothesis-testing cohort. In the second study, which compared 495 white asthmatic subjects with 305 white controls, similar criteria were used for defining asthmatic subjects. Subjects were considered asthmatic if they had a clinical history of asthma and an FEV₁ between 40% and 80% predicted, and if their FEV₁ improved by > 12% and 200 ml after treatment with albuterol. Neither MCh responsiveness nor response to cold-air challenge was assessed. Subjects were excluded if they received any long-term controller asthma therapy including corticosteroids. Medical history and smoking criteria in the second study were identical to those used in the FeNO study. The control popula-

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tion had no history of asthma or atopy by self-report, and had normal IgE values, as recently described (18).

NO Collection

While seated and wearing noseclips, qualified subjects performed normal tidal breathing for 30 s from a source of air containing a low concentration of NO gas. Gas exhaled by each subject was collected in three separate Mylar bags at 2-min intervals under conditions of controlled flow and airway opening pressure (375 ml/s, 10 mm Hg) (19–21). NO concentrations in exhaled gas were determined by chemiluminescence, and the median NO values were recorded. Baseline spirometry was followed by a bronchodilator, cold-air, or MCh challenge.

Genetic Analysis

DNA was extracted from each subject's blood by standard techniques (QIAamp Blood Kit; Qiagen Inc., Valencia, CA). Alleles at the AAT repeat in intron 20 of *NOS1* were amplified from genomic DNA by the polymerase chain reaction (PCR). Each forward and reverse primer (20 pmol) was radiolabeled with [γ - 32 P]deoxyadenosine triphosphate, via the use of polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany). The PCR mix contained 100 ng of genomic DNA, PCR buffer (Boehringer Mannheim), 200 μ M of each deoxynucleotide triphosphate, 1 μ l of radiolabeled primers, and 1.5 U of Taq polymerase in a 25- μ l reaction mixture. PCR conditions were 6 min at 94° C, followed by 35 cycles at 94° C for 1 min, 59° C for 1 min, and 72° C for 1 min. Chain elongation was continued for 5 min after the last cycle. Primers used included 5'-CTG GGG GCA ATG GTG TGT-3' as the forward primer and 5'-GAG TAA AAT TAA GGG TCA GC-3' as the reverse primer (22). Simple sequence-length polymorphism was used for DNA analysis (23) as modified by our group (18). Gels were run at room temperature at 60 W, dried, and exposed to X-ray film as required. To determine the number of AAT repeats representing different alleles, we cloned PCR products into TA vectors (Invitrogen, Carlsbad, CA). DNA minipreps (QIAprep Miniprep; Qiagen, Inc.) were prepared and sequenced in an automated sequencer (Applied Biosystems, Foster City, CA), as recently reported (24).

Statistical Analysis

Statistical differences in expired NO among three groups of subjects (homozygous for ≥ 12 AAT repeats, heterozygous for ≥ 12 AAT repeats, or homozygous for < 12 AAT repeats) were determined with the Kruskal–Wallis test and differences between the two groups having alleles with ≥ 12 AAT repeats and all other genotypes were analyzed with Welch's modified *t* test. Differences in variance were calculated with the F test. Differences in overall allele distribution and race were determined by chi-square testing in $2 \times n$ contingency tables (Sigmastat, San Rafael, CA). The Hardy–Weinberg equilibrium was examined through the Markov chain method, with a program for population genetics data analysis. A value of $p < 0.05$ was considered statistically significant.

RESULTS

FeNO Phenotype and *NOS1* Genotype

We first studied two cohorts of subjects with mild asthma who had used neither inhaled nor systemic corticosteroids within

the preceding 30 d. The first cohort consisted of 51 patients from whom the association noted in the following discussion was first identified; a second, independently recruited cohort consisted of 46 patients in whom the findings in the first cohort were replicated. The two cohorts were thus considered together in the final analysis. In these patients, FEV₁ was $82 \pm 8\%$ (mean \pm SD) predicted, and the mean FeNO was 15.3 ± 6.2 ppb. Among these individuals, we identified nine distinct alleles of the intronic (AAT)_n repeat in intron 20 of *NOS1*, and numbered them according to the number of repeats (range: 8 to 17 repeats). The relative frequency of the various alleles was broadly, and—more important—bimodally distributed (Figure 1). Differences in FeNO across genotypes defined by AAT repeat sizes of 12 or more or of less than 12 repeats were assessed with the Kruskal–Wallis test. Because significant differences were found ($p = 0.016$), pairwise *t* tests were performed. On the basis of these results, we segregated the patients into two groups: one consisting of individuals in the lower portion of the distribution (i.e., individuals harboring at least one *NOS1* intronic trinucleotide allele with fewer than 12 repeats [$n = 75$; 61.3% female; mean FEV₁: 82.2% predicted; mean age: 33.1 yr]), and the other consisting of individuals in the higher portion of the distribution (asthmatic subjects harboring two *NOS1* alleles with at least 12 repeats ($n = 22$; 63.6% female; mean FEV₁: 82.3% predicted, mean age: 32.9 yr)).

The mean FeNO was significantly lower in subjects harboring two high-AAT-repeat alleles than in subjects with at least one low-repeat allele (10.8 ± 3.6 ppb versus 16.6 ± 10.1 ppb; *t* test $p = 0.00008$). In addition, the variance about the mean was significantly smaller in this group (13.2 ppb versus 101.9 ppb; F-test $p = 2 \times 10^{-6}$) (Figure 2). A single allele with fewer than 12 repeats appeared to convey the high-FeNO (i.e., asthmatic) phenotype, since patients with only one such allele had increased (on average) and highly variable FeNO levels. Results were similar when the low-repeat group included only the 23 subjects who harbored two alleles with fewer than 12 AAT repeats: FeNO remained higher, with borderline significance (14.3 ± 7.2 ppb versus 10.8 ± 3.6 ppb; $p = 0.06$), but variance about the mean differed significantly (62.3 ppb versus 13.2 ppb; $p = 0.0005$). Boerwinkle-measured genotype analysis indicated that 7.2% of total variance, exhibited by the group of patients harboring two *NOS1* alleles containing more than 12 repeats, is explained by this locus (25, 26); this subset of the population is therefore characterized by a very narrow distribution of FeNO, with a lower mean. Thus, within a group of otherwise phenotypically indistinguishable asthmatic individuals (the groups of asthmatic individuals harboring two high-AAT-repeat alleles and the group harboring at least one low-repeat allele were similar with respect to FEV₁; $p = 0.23$), allelic differences in *NOS1* were strongly associated with varying levels of exhaled NO, and patients harboring at least one allele with fewer than 12 AAT repeats were responsible for the majority (92.8%) of the variability in this outcome indicator.

TABLE 1
CHARACTERISTICS OF ETHNIC SUBGROUPS

Ethnic Subgroup	Male/Female	Mean FEV ₁	Mean FeNO (ppb)
White	30/44	83%	15.8
Black	6/10	85%	12.6
Hispanic	0/4	77%	14.3
Other	2/1	80%	20.3

Definition of abbreviation: FeNO = fraction of nitric oxide in exhaled air.

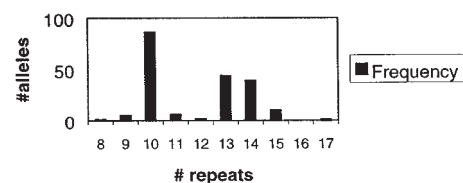


Figure 1. Distribution of alleles in 97 asthmatic subjects ($n = 194$ alleles). The bimodal distribution of alleles led to the division of subjects into those with < 12 or ≥ 12 intronic AAT repeats.

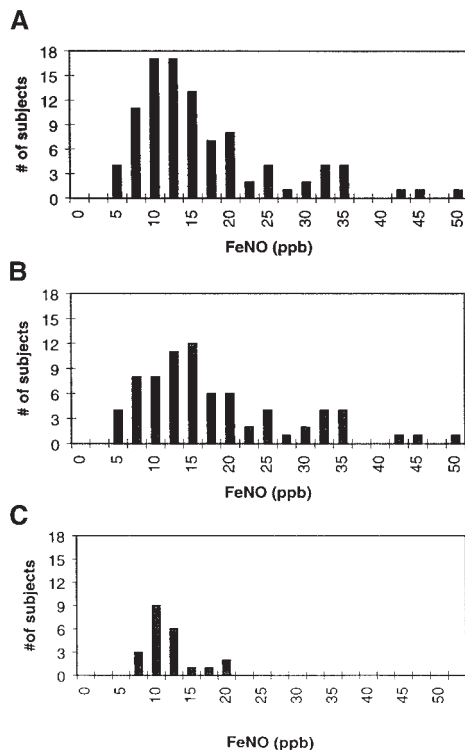


Figure 2. Distribution of NO in entire study population (A) and in two subgroups based on number of allele repeats at a locus in intron 20 of the *NOS1* gene: (B) at least one allele with fewer than 12 repeats ($n = 75$) and (C) both alleles with greater than or equal to 12 repeats (of $n = 22$). Subjects with 12 or more intronic AAT repeats had significantly lower mean $F_{E}NO$ values (10.8 ppb versus 16.6 ppb; $p = 0.00008$) and variance (13.2 ppb versus 101.9 ppb; $p = 0.000002$) than did subjects with fewer than 12 repeats.

NOS1 Allele Frequencies in Asthmatic versus Control Subjects

Because increased $F_{E}NO$ is a phenotypic characteristic of asthma (27–29), we postulated an association between the alleles associated with low $F_{E}NO$ (i.e., the nonasthmatic phenotype) in patients with asthma and the lack of a diagnosis of asthma in a different study population. Therefore, we studied allele frequencies of the *NOS1* (AAT)_n repeat in 495 white asthmatic subjects and 305 white controls, none of whom were included in the first study. The control population was in Hardy–Weinberg equilibrium at this locus, as recently described (18). Although no significant association was found between the presence of asthma and any single allele of the intronic trinucleotide repeat, genotypes that had only high repeat numbers (which were associated with lower $F_{E}NO$ among asthmatic individuals in the first study) were more frequent in controls (70 of 305) than in asthmatic individuals (77 of 495) ($p = 0.0087$; odds ratio: 0.62; 95% confidence interval: 0.43 to 0.89) (Table 2).

Interestingly, 22 of 97 (22.7%) asthmatic individuals in the $F_{E}NO$ phenotypic study harbored two alleles with ≥ 12 repeats; this constituted a higher percentage than the 15.6% (77 of 495) of asthmatic individuals with a similar genotype in the population study. Since the asthmatic individuals in the population study were solely whites whose mean FEV_1 was lower (mean FEV_1 : 56.4% predicted in the population study [56.8% predicted in those with 12 or more repeats, and 56.2% predicted in those with < 12 repeats; $p = 0.64$] versus 82% predicted in the $F_{E}NO$ cohort), we speculated that the different proportions of asthmatic individuals with two high-repeat alleles in the studies

TABLE 2
FREQUENCY OF EACH *NOS1* ALLELE GENOTYPE IN NORMAL CONTROLS AND ASTHMATIC SUBJECTS

No. Repeats	Normal Controls n = 305; (alleles n = 610)	Asthmatic Subjects n = 495 (alleles n = 990)
17	0.003 (2)	0.001 (1)
15	0.052 (32)	0.048 (48)
14	0.161 (98)	0.139 (138)
13	0.267 (163)	0.234 (232)
12	0.010 (6)	0.012 (12)
11	0.010 (6)	0.005 (5)
10	0.438 (267)	0.496 (491)
9	0.052 (32)	0.064 (63)
8	0.003 (2)	0 (0)
6	0.003 (2)	0 (0)
No. of patients with both alleles with ≥ 12 repeats	70/305 (23.0%)	77/495 (15.6%)*

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

* $p = 0.0087$; OR = 0.62; 95% CI: 0.43 to 0.89.

might in part have reflected differences in race and asthma severity. We therefore calculated the genotype frequency in a subset of the $F_{E}NO$ cohort that included only white subjects whose FEV_1 was less than 80% predicted (range: 47% to 79% predicted; mean: = 69.8% predicted). In this subset of patients, five of 32 (15.6%) patients harbored two alleles with ≥ 12 repeats. Thus, when one considers white asthmatic individuals with an arbitrary FEV_1 cutpoint, one finds them somewhat similar in genotypic constitution at the *NOS1* locus.

DISCUSSION

A major problem in the study of complex medical disorders is phenotypic heterogeneity. Several common conditions, including hypertension, obesity, schizophrenia, and asthma are defined by their phenotypic characteristics, but because they probably result from multiple environmental and genetic causes, even the best case definition usually results in the identification of a phenotypically heterogeneous group. In addition to using standard physiological criteria, one way to identify a phenotypically homogenous group of patients is to use genotypic criteria for case definition. In the present study we tested this idea by using genotype at a locus in the *NOS1* gene to identify a group of patients with asthma with a very limited range of exhaled NO within a group of patients with a wide range of exhaled NO levels. Our isolation of this homogenous cohort of patients, based on *NOS1* genotype, ties together many well-established associations among the diagnosis of asthma, the increased concentrations of NO in exhaled air of patients with asthma, and variations of trinucleotide repeat sequences as identified in several human neurologic conditions.

A number of investigative groups have detected genetic linkage between the diagnosis of asthma and the distal region of human chromosome 12q (1, 2, 30). Among the asthma candidate genes in this region of the human genome is that for the neural form of nitric oxide synthase, or *NOS1* (5). Although it is telomeric from the point of maximum score for the logarithm of the odds favoring genetic linkage, *NOS1* is a particularly interesting candidate gene for asthma because of the recently recognized role of NO in asthma (31). On average, patients with asthma have higher levels of $F_{E}NO$ than do normal subjects (6–8, 27). In the absence of asthma-provoking events or asthma treatments, $F_{E}NO$ remains relatively fixed over time in individual patients; however, among members of a particular population, $F_{E}NO$ is highly variable, and many

asthmatic individuals may have the low levels of FENO that are characteristic of normal subjects (9, 10). Although the molecular source of the NO in exhaled air remains unknown, data from mouse models indicate that *nos1* contributes substantially to FENO (14). Our data extend the body of information on the linkage at the human chromosome 12 locus in two important ways. First, we have established a strong association between classes of *NOS1* alleles and FENO among patients with asthma. Second, we have shown an association between the class of *NOS1* alleles in asthma patients that are associated with low levels of FENO and the lack of a diagnosis of asthma. These data provide a biologically plausible connection between the linkage established on chromosome 12 and a mechanism of occurrence of asthma.

The relationship we have established is between a polymorphism within an intron of a gene encoding the neural protein NOS1 and a particular component of the asthma phenotype (i.e., FENO), as well as between this polymorphism and the diagnosis of asthma. Our data are consistent with the considerable evidence that neural mechanisms in general and NO in particular contribute to the pathophysiology and symptomatology of asthma. For example, there is known to be complex interaction of inflammation and neural control of airways, with inflammatory mediators affecting neurotransmission and neurotransmitters in turn modulating the inflammatory response in the airways (11). In addition to the classic cholinergic and adrenergic neural pathways, nonadrenergic noncholinergic (NANC) pathways, in which NO has recently been recognized as the main neurotransmitter, are present in the airways and modulate bronchomotor tone (11). Thus, one component of the neurogenic regulation of asthma may be neurally derived NO, a product of NOS1, which, along with the NO derived from other NOS enzymes, may be variably involved in airway inflammation. Since the approximately 25% of asthma patients in our study who harbored two high-repeat *NOS1* alleles had low levels of exhaled NO, we speculate that this cohort of asthmatic individuals is missing part of the neural mechanism responsible for the asthma phenotype of other patients with asthma.

Our previously reported findings in mice with a targeted deletion of *nos1* support a role for neurally derived NO in the physiologic manifestations of asthma. Mice with a targeted deletion of *nos1* have lower levels of FENO than do wild-type mice (13) and fail to develop airway hyperresponsiveness after allergen sensitization and challenge (14). Moreover, these mice develop phenotypic changes compatible with hypertrophic infantile pyloric stenosis, a disorder that has also been associated with DNA sequence variants in *NOS1* (32, 33). These data from murine models provide a biologic basis for interpretation of our findings in human subjects with asthma, in that they relate disruption of a neural pathway to components of the asthma phenotype. If differences in allele size at the *NOS1* locus examined in our study reflect differences in the neurogenic component of asthma, we speculate that patients with two alleles, each containing high numbers of AAT repeats, could have either dysfunctional NOS1 or diminished amounts of this enzyme. In such subjects, we propose that only two isoforms of NOS (i.e., NOS2 and NOS3), rather than all three NOS isoforms, contribute to the lower values and decreased variance of FENO. Extension of this reasoning leads to the conclusion that NOS1 is the enzyme isoform responsible for the wide variability of FENO observed in most asthmatic subjects.

If there is a suppression of neural amplification of asthmatic bronchoconstriction, it could be explained by the observed important role of trinucleotide repeats in the pathogenesis of a number of neurodegenerative diseases, such as myotonic dys-

trophy, Huntington's disease, spinocerebellar ataxia, and fragile X syndrome (15, 16). For example, in spinocerebellar ataxia, a difference of only one trinucleotide repeat in intronic DNA can serve as a dividing factor between neural dysfunction and its absence in the context of total numbers of trinucleotide repeats on the order of 20 (34). Thus, it is possible that patients who harbor two alleles with high AAT repeat numbers at this *NOS1* locus have dysfunction of nitridergic nerves, and that this dysfunction accounts for their lower mean FENO values and smaller variance about this mean. Our data did not allow us to determine whether the locus we identified is the causative locus of a decreased FENO itself or whether it is in linkage disequilibrium with a distinct causative locus. Indeed, given the diversity of *NOS1* (35), it is quite possible that the locus we identified is a marker for the functional polymorphism rather than being the polymorphism itself. One possibility is that the microsatellites in intron 20 of *NOS1* and the last exon preceding intron 20 are in linkage disequilibrium with more functionally relevant sequence variants. Alternatively, variations in airway levels of NO derived from NOS1 may be locally involved in the regulation of an inflammatory process that could secondarily influence FENO via a regulatory effect of *NOS1* on *NOS2* expression (36). Most previous genetic association studies have focused on effects on phenotype mean rather than phenotype variance. The significant difference in variance of FENO among subjects stratified by their number of *NOS1* trinucleotide repeats could be caused by linkage disequilibrium, gene-gene interaction, gene-environment interaction or, less likely, by genotype selection of environments (37). Our findings open this field to appropriate follow-up research.

Our additional finding in separate populations of normal and asthmatic individuals of an association ($p = 0.0087$) between classes of *NOS1* alleles (based on length of the intronic trinucleotide repeat) and the diagnosis of asthma lends further credence to our findings within the asthmatic population. Asthma is recognized as a complex phenotype of which increased FENO is but a single component. For instance, in our populations, FEV₁ was similar between groups, yet subjects differed significantly with respect to FENO and *NOS1* genotype. Nevertheless, although clinical diagnosis of asthma united these individuals, the diagnosis is likely to be most evident in a patient manifesting all facets of the disease. Therefore, an asthma patient with low FENO values could be considered closer to normal than an asthmatic subject with increased levels of FENO. Indeed, in our case-control association analysis, we found that the genotype associated with low (nonasthmatic) FENO values was also the genotype more prevalent in normal than in asthmatic individuals. If asthma represents a complex phenotype resulting from multiple distinct defects, it stands to reason that the genotype associated with low values of FENO would be the one occurring less commonly in patients with asthma.

To clarify the role of NO in the airway, and to ascertain the meaning of low levels of FENO in a subset of asthmatic individuals, further studies are required. For instance, we propose that studying FENO in a population of patients with more severe asthma, to see whether there is a similar NO distribution, would demonstrate a potentially pivotal role of NO in the pathogenesis of asthma, or might suggest that NO plays a role only in patients with asthma of a particular degree of severity. Establishing this may be difficult, since patients with more severe asthma tend to be treated with corticosteroids, and this is associated with lower NO levels. Other potentially useful studies may include assessing other phenotypic characteristics in asthmatic individuals with both low and high trinucleotide repeats. For instance, the degree of airway responsiveness to inhaled brady-

kinin, hypertonic saline, or various allergens has been associated with levels of exhaled NO, and in each of these challenges there is a heterogeneous response among asthmatic individuals (38, 39). Because some asthmatic individuals are more sensitive to some stimuli than are other asthmatic individuals, some of these differences may be explained by *NOS1* genotype.

Our approach to analysis within the population of patients with asthma was distinct from that used by other investigators. We examined FeNO according to stratification by genotype at the *NOS1* locus rather than by assessing whether a given genotype at this locus was likely to be associated with a given level of FeNO. Although this distinction may appear subtle, it is of substantial clinical importance. Stratification by genotype at the *NOS1* locus offers an unambiguous method for isolating subgroups among what otherwise appear to be clinically similar patients with asthma. Since genotypes with a high trinucleotide identify a cohort of patients who have lower and less variable levels of exhaled NO, we speculate that in an asthmatic cohort harboring two alleles with ≥ 12 AAT repeats, the contribution of *NOS1* to the total FeNO level may be diminished. These patients represent a common and easily identifiable subset of patients in whom the complexity of the asthmatic phenotype is reduced.

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