FENO and polymorphisms in the NOS genes

SNP- and haplotype-based association analyses

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Gothenburg 2013

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ISBN 978-91-637-3025-2
Printed in Gothenburg, Sweden 2013
Ale Tryckteam AB, Bohus

ABSTRACT

The fraction of exhaled nitric oxide (FENO) is a biomarker reflecting inflammation in the airways. Inter-individual variability of FENO is quite large in the general population. Physiological, biological, environmental and genetic factors contribute to the variability of FENO levels.

The aim of this thesis was to utilize bioinformatics methods to comprehensively characterize genetic variation in the three nitric oxide synthase (*NOS; NOS1, NOS2* and *NOS3*) genes using a tagging single nucleotide polymorphism (SNP) approach, and evaluate the genetic contribution to variation in levels of FENO in a general adult population.

In paper I, a single-SNP association analysis between 49 SNPs in the three *NOS* genes and FENO was performed in 1733 adult subjects. Based on the associations, a list of top-ranked SNPs was selected and used in a forwarded stepwise analysis to identify a reduced set of strongest independently associated SNPs. Two SNPs (rs9901734 and rs3729508) in *NOS2* and one SNP (rs7830) in *NOS3* showed independent associations with levels of FENO. For *NOS2* SNP rs9901734, subjects had 5.3% (95% CI 1.0% to 9.7%) higher levels of FENO per G allele, and for rs3729508, subjects with CC or CT genotypes had 9.4% (95% CI 3.1% to 15.2%) higher levels compared with TT. Subjects with GT or TT in the *NOS3* SNP rs7830 had 5.6% (95% CI 0.4% to 11.1%) higher levels of FENO as compared with those with GG. The effect of this SNP was stronger in subjects with asthma (21.9%, 95% CI 4.6% to 42.0%). In paper II the association between haplotypes in the *NOS2* gene and FENO was investigated in 5912 adult subjects. Ten SNPs across the *NOS2* gene were selected based on previously reported association to FENO. A stepwise linear regression analysis was performed in a forward approach to find a best subset of SNPs with the most significant ($p \leq 0.005$) association to FENO. These SNPs were then used to infer haplotypes. A generalized linear model was used for estimating the effects of all common haplotypes (haplotype frequency ≥ 5%) on FENO using the most common haplotype as the reference group. Seven common haplotypes were inferred representing 84% of all haplotypes. One haplotype ('ACCTT') was significantly associated with lower levels of FENO and three haplotypes ('ACCTC', 'GGCTC' and 'GGCTT') were significantly associated with higher levels of FENO compared with the baseline haplotype (ACTCT), global p-value 3.8×10^{-28} for the haplotype distribution. The association of the haplotype 'ACCTT' with FENO varied by asthma status.

Taken together, our findings suggest that *NOS2* is the major NOS gene determining variability in levels of FENO in the healthy adult population, and also plays a role in subjects with asthma. In addition, a SNP in *NOS3,* and a particular haplotype in *NOS2*, appeared to contribute more strongly to the variation in FENO in subjects with asthma. This study also emphasizes the potential of combining SNP- and haplotype-based approaches in identifying and characterizing the contribution of *NOS* genes to variation in FENO.

Keywords: *FENO, NOS genes, bioinformatics, tagSNPs, haplotype*

ISBN: 978-91-637-3025-2

SAMMANFATTNING PÅ SVENSKA

Koncentrationen av kväveoxid i utandningsluft (FENO) är en biomarkör för inflammation i luftvägarna. Variabiliteten mellan individer i FENO är betydande i den allmänna populationen och faktorer som bidrar till variabiliteten är bl.a. fysiologiska, genetiska samt miljöfaktorer.

Syftet med studierna i denna avhandling var att tillämpa metoder inom bioinformatik för att karaktärisera variationen hos de tre kväeoxidsyntasgenerna *NOS1; NOS2 och NOS3* genom att använda en metod baserad på s.k. tag-SNPar (markör-varianter med förändring i en DNA bas) samt utvärdera det genetiska bidraget till variation i FENO-nivåer hos vuxna.

I delarbete 1 utfördes först analys av sambandet mellan 49 SNPar i de tre *NOS* generna och FENO, en SNP i taget, i en studie-population av 1733 individer. Baserat på graden av association skapades en lista av rangordnade SNPar som sedan användes i en framåt stegvis regressionsanalys för att identifiera ett mindre antal SNPar med starkast oberoende association. Två SNPar (rs9901734 and rs3729508) i *NOS2* och en SNP (rs7830) i *NOS3* visade oberoende association med FENO-nivå. Individer med *NOS2* SNP rs9901734 hade 5.3% (95% CI, 1.0% – 9.7%) högre FENO-nivåer för varje G allel, och individer med genotyperna CC eller CT *NOS2* för SNP rs3729508 hade 9.4% (95% CI 3.1% – 15.2%) högre FENO-nivåer jämfört med TT genotyp. Individer med GT eller TT genotyp i *NOS3* SNP rs7830 hade 5.6% (95%CI, 0.4% – 11.1%) högre FENO-nivåer jämfört med GG genotypen. Effekten av denna SNP var starkare hos individer med astma: 21.9%, 95% CI 4.6% – 42.0%.

I delarbete 2 undersöktes sambandet mellan haplotyper i *NOS2* genen och FENO hos 5912 individer. Tio SNPar utspridda över hela *NOS2* genen valdes ut baserat på tidigare kunskap om association med FENO. Sju haplotyper kunde urskiljas, och dessa representerade 84% av alla haplotyper. En haplotyp ('ACCTT)' var signifikant associerad med lägre FENO-nivåer och tre haplotyper ('ACCTC', 'GGCTC' and 'GGCTT') var signifikant associerade med högre FENO-nivåer jämfört med referens-haplotypen ('ACTCT'), med

ett globalt p-värde på 3.8×10^{-28} för haplotyp-distributionen. Associationen mellan haplotyp 'ACCTT' och FENO varierade med astma-status.

Sammanfattningvis tyder resultaten på att *NOS2* är den av *NOS*-generna som har störst inverkan på variabiliteten av FENO hos den friska, vuxna befolkningen, och även spelar en roll hos individer med astma. Dessutom indikerande resultaten att en SNP i *NOS3* (rs7830) samt en specifik haplotyp ('ACCTT') i *NOS2* bidrog mer till variationen av FENO hos individer med astma. Analysen med haplotyper i *NOS2* genen kunde påvisa det NOS2-relaterade bidraget till variationen i FENO starkare än analysen med individuella SNPar. Denna studie understryker också potentialen med att kombinera SNP- och haplotyp-baserade metoder för att identifiera och karaktärisera *NOS*-genernas bidrag till variation i FENO.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. Dahgam S, Nyberg F, Modig L, Naluai AT, Olin AC. Single nucleotide polymorphisms in the *NOS2* and *NOS3* genes are associated with exhaled nitric oxide. *J Med Genet* 2012;49(3):200-5

II. Dahgam S, Modig L, Naluai AT, Olin AC, Nyberg F. Haplotypes of the inducible nitric oxide synthase (*NOS2*) gene are strongly associated with exhaled nitric oxide levels in adults. (*Manuscript).*

Papers not included in the thesis

- Ehret, G.B., P.B. Munroe, K.M. Rice…….. **Dahgam, S,** ……. C. Newton-Cheh, D. Levy, M.J. Caulfield and T. Johnson. *Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk.*
  Nature, 2011. 478(7367):103-9.

- Soler Artigas M, M., D.W. Loth, L.V. Wain, ……. **Dahgam S**, ……. S.J. London, I.P. Hall, V. Gudnason and M.D. Tobin. *Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function.*
  Nat Genet, 2011. 43(11):1082-90.

- Soler Artigas M, Wain LV, Repapi E, Obeidat M, Sayers I, Burton PR, Johnson T, Zhao JH, Albrecht E, Dominiczak AF, Kerr SM, Smith BH, Cadby G, Hui J, Palmer LJ, Hingorani AD, Wannamethee SG, Whincup PH, Ebrahim S, Smith GD, Barroso I, Loos RJ, Wareham NJ, Cooper C, Dennison E, Shaheen SO, Liu JZ, Marchini J; Medical Research Council National Survey of Health and Development (NSHD) Respiratory Study Team, **Dahgam S**, Naluai AT, Olin AC, Karrasch S, Heinrich J, Schulz H, McKeever TM, Pavord ID, Heliövaara M, Ripatti S, Surakka I, Blakey JD, Kähönen M, Britton JR, Nyberg F, Holloway JW, Lawlor DA, Morris RW, James AL, Jackson CM, Hall IP, Tobin MD; SpiroMeta Consortium.
  Effect of five genetic variants associated with lung function on the risk of chronic obstructive lung disease, and their joint effects on lung function.
  Am J Respir Crit Care Med 2011;184(7):786-95.

ABBREVIATIONS

ADONIX: Adult Onset Asthma and Exhaled Nitric Oxide Cohort

ATS: American Thoracic Society

DNA: Deoxyribonucleic acid

E-M: Expectation-Maximisation

ERS: European Respiratory Society

FENO: Fraction of exhaled nitric oxide

FEV_1: Forced expiratory volume in 1 second

FVC: Forced vital capacity

HWE: Hardy-Weinberg Equilibrium

IgE: Immunoglobulin E

LD: Linkage disequilibrium

MAF: Minor allele frequency

NO: Nitric oxide

NOS: Nitric oxide synthase

eNOS: Endothelial NOS

iNOS: Inducible NOS

nNOS: Neuronal NOS

ppb: Parts per billion

SNP: Single nucleotide polymorphism

CONTENT

BACKGROUND

Human genetics

The human genome is made up of deoxyribonucleic acid (DNA), which is built up by the four nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T). The DNA carries the genetic information which is passed on to the next generation. The four nucleotide bases pair up with each other so that 'A' always pairs with 'T', and 'G' always pairs with 'C'. Total length of the human genome is approximately 3.3 billion base pairs, which is distributed on 23 pairs of chromosomes. Segments of DNA along a region in the genome are called genes. Alternative forms of genes in certain positions, or loci, are called alleles. At each locus there are two alleles in an individual's pair of chromosomes, one from the father and one from the mother. The combination of the two alleles at each locus is called the genotype.

Single nucleotide polymorphisms (SNPs)

As much as 99.9% of the human DNA sequence is identical across populations, but there are also different arrangements in 0.1% of the DNA which make two individuals unique [1, 2]. Of the 0.1%, most of the arrangements (>90%) are attributable to single nucleotide polymorphisms (SNPs). This type of polymorphism occurs when a single nucleotide (A, T, C, or G) in a specific position in the genome sequence is altered (Figure 1). SNPs occur with a minor allele frequency of at least 1% in the population [3], although the frequency can vary across populations and today also rare single nucleotide variants are often called SNPs. According to the current release of the SNP database (dbSNP) build 137 (June 2012) there are 53,558,214 SNPs of frequency ≥1% in the human genome (http://www.ncbi.nlm.nih.gov/projects/SNP). On average one SNP in every 300 base pairs occurs throughout the genome [2, 4]. The position of a SNP is important in determining the nature of effect of the SNP [5, 6]. For example, SNPs situated in the coding region of a gene may change an amino acid in the resulting protein, which in turn could directly change the protein structure or function. SNPs that occur in non-coding regions (introns or promoter) do not directly

involve amino acid change but they may alter gene expression or function of the protein, or be in linkage disequilibrium with (i.e. correlated with - see further below) a causative SNP or SNPs. Therefore, SNPs in a non-coding region are also important markers for assessing association with a trait or disease in genetic association studies.

Figure 1. Illustration of a single nucleotide polymorphism (SNP), where a DNA sequence differs by a single nucleotide.

Tag SNPs

Rather than selecting all the SNPs in a gene, it is possible to select a small subset of SNPs that will provide good information on the total genetic variation in the gene/region. These SNPs are referred to as tag SNPs, and act as proxies for the rest of the SNPs, which can reduce genotyping cost considerably and simplify statistical analysis.

Haplotypes

A haplotype is a set of alleles situated at adjacent loci on the same chromosome. Often, the adjacent nucleotides within the same gene tend to be inherited together more often than expected by chance, a phenomenon called linkage disequilibrium (LD) [7]. There are several measures of LD, including the correlation between two loci represented as r^2 or degree of association between alleles (D′) [8]. The value of r^2 varies between 0 and 1, $r^2 = 1$ means that the SNPs are completely correlated. D′ also varies between 0 and 1, D′ = 1 means complete allelic association.

Association studies of candidate genes

Association studies are often performed to identify if one or more SNPs in a candidate gene are associated with a trait of interest. The trait of interest could be continuous (for example blood pressure) or binary (like many diseases; present or absent) [9-11]. Candidate gene association studies are hypothesis-driven and use data from e.g. a cohort or case-control set of unrelated individuals and can be cross-sectional or longitudinal in nature. In case-control studies, essentially allele or genotype frequencies are compared among the cases and controls. In cohort studies, a linear association between a SNP and a continuous outcome can also be investigated. This thesis emphasizes the cross-sectional cohort study design. In order to estimate the effects of a SNP on a trait, different genetic models can be applied in the statistical analysis, as illustrated in Figure 2. Effect estimates are generally quantified based on number of the copies of minor allele (less common allele) [12]. When the combined effect of two minor alleles is equal to the sum of their individual effects, this is said to be an additive effect. When one or two copies of the minor allele have the same effect on a trait, the effect is said to be dominant. When two copies of the minor allele are required for effect, the effect is said to be recessive.

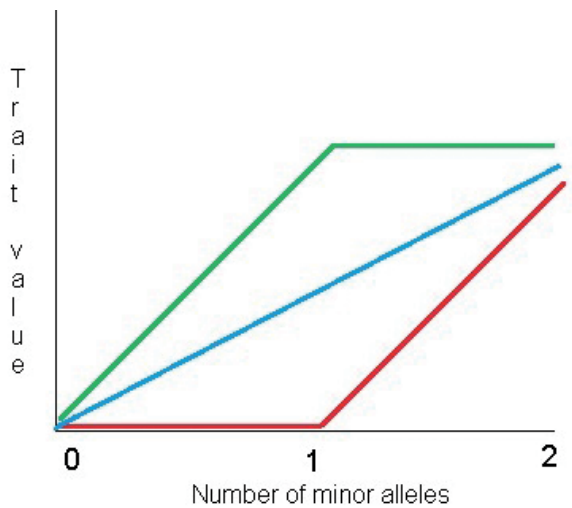

Figure 2. Illustration of the most common genetic models. Blue line indicates a linear (additive effect) relationship between number of minor alleles and trait value. Red and green lines indicate non-linear relationship (recessive and dominant effects, respectively) between number of minor alleles and trait value.

It is likely that a single SNP is often neither necessary nor sufficient for influencing a trait, but instead a combination of SNPs (haplotype) may be responsible for the observed variation in a trait of interest [13]. However, resolving phase of haplotype (i.e. which chromosome each allele resides on) from observed SNP data is complicated in unrelated individuals, because most current genotyping technologies do not provide the phase of maternal or paternal chromosomes [14, 15]. This ambiguity is referred to as haplotype uncertainty and there has been much effort to identify the most likely haplotypes for individuals based on unphased data, with different statistical approaches implemented in several bioinformatics tools [16-20]. Examples include the Bayesian approach [16], the parsimonious approach (the Clark algorithm) [18] and the maximum likelihood approach (Expectation-Maximisation (E-M) algorithm). The E-M algorithm is the most extensively used haplotyping algorithm and it is implemented in several programs for example Haplo.stats [21] , Hapassoc [22] and SNPHAP [23] . In brief, the E-M algorithm is a two-step iterative procedure (the expectation or E step and the maximisation or M step) of calculating the maximum likelihood estimates of the unknown parameters from observed data [24]. In the E step, posterior probabilities for each haplotype are estimated using the genotype information. In the M step, the estimates are iteratively updated until frequencies of haplotypes do not change. Then the inferred haplotypes can be incorporated into regression models to perform association analysis between haplotypes and a trait of interest.

Nitric oxide

Nitric oxide (NO) is an endogenous molecule present throughout the body. In the respiratory tract, NO plays multiple roles, both beneficial and harmful [25]. One of the beneficial effects is that it relaxes respiratory smooth muscles and acts as a bronchodilator. On the other hand, it is involved in various cytotoxic and pro-inflammatory activities such as increased production of reactive oxygen species (ROS) [26], increased bronchial mucus secretion, eosinophilic inflammation [27] and increased airway hyperresposiveness [28].

NOS genes

NO is synthesized from the amino acid L-arginine by specific NO synthase (NOS) enzymes [29]. There are three enzyme isoforms: neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2) and an endothelial form (eNOS; NOS3). The three NOS isoforms are encoded by three distinct genes (*NOS1*, *NOS2* and *NOS3*) located on different chromosomes (12, 17 and 7, respectively), and differentially expressed in different cells [25, 30]. All the *NOS* genes are expressed in airway epithelial cells [31]. *NOS1* and *NOS3* are largely constitutively expressed, resulting in a low basal synthesis of NO; show limited response to physiological stimuli; and are important for physiological functions in the airways [32]. *NOS2*, also called inducible *NOS,* is typically not constitutively expressed to any great extent, but its expression is strongly stimulated by various proinflammatory cytokines [33], resulting in a profoundly greater NO production in the induced state as compared with *NOS1* and *NOS3* [34]. It has also been shown that *NOS2* can be constitutively expressed depending on conditions or factors present in the airways [35]. Continuous exposure to irritants has also been reported to lead to rapid loss of *NOS2* expression in airway epithelial cells in healthy subjects [35].

Exhaled NO

NO produced in the lung diffuses into the respiratory tract and is detectable in exhaled air. NO in exhaled air was first reported by Gustafsson and colleagues in human breath samples [36]. A couple of years later two independent studies reported that asthmatic patients have increased exhaled NO concentrations as compared to healthy individuals [37, 38]. Since then a great interest has been shown for exhaled NO in respiratory research and a substantial number of scientific articles have been published.

Measurements of FENO

In the clinical setting, a single-breath exhalation maneuver is the preferred procedure to measure NO in exhaled air. In the single-breath exhalation procedure, individuals are asked to sit comfortably and inhale NO-free air via a mouthpiece to total lung capacity, then

exhale instantly against an oral pressure into the apparatus (Figure 3). A computer screen attached to the apparatus displays exhalation flow rates together with pre-adjusted oral pressure so that subjects can maintain the pressure to achieve the desirable exhalation flow. This procedure is available for the NIOX® system (Aerocrine AB, Solna, Sweden) (Figure 3). This type of method was initially described by Kharitonov et al 1997 [39] and validated by the American Thoracic Society (ATS) and European Respiratory Society (ERS) [40, 41]. According to ATS and ERS, the NO concentration is recommended to be measured at an exhalation rate of 50 ml/second. However one can measure exhaled NO at different exhalation flow rates. In this thesis the term FENO (fraction of exhaled nitric oxide) is used for describing exhaled NO at the recommended flow rate of 50 ml/s. FENO is relatively easy to measure and highly reproducible in both healthy and non-healthy subjects of different ages [42, 43].

Figure 3. Measuring exhaled NO using the NIOX® system (Aerocrine AB, Solna, Sweden).

Factors influencing levels of FENO

Increased levels of FENO have been reported in many inflammatory lung conditions including asthma, atopy, wheeze, and COPD, [37, 44-46]. FENO levels in patients with asthma decrease after corticosteroid therapy [47-49] and correlate with sputum eosinophils [50] and skin prick test [51]. Decreased levels of FENO have also been reported in other inflammatory lung conditions like chronic fibrosis [52] and respiratory viral infections [53]. A number of other factors such as age, gender, height, atopy, smoking, respiratory tract infections and recent intake of nitrate-rich food are also important in determining levels of FENO [54-57]. In Swedish non-smoking adults the upper normal limit (corresponding to the 95th percentile) for FENO varies between 24 and 54 parts per billion (ppb) (geometric mean of FENO is 16.6 ppb) [54] depending on age, height and atopy. In an earlier population-based analysis in our study population, age and height accounted for 11% of the variability in FENO [54]. Cigarette smoking leads to reduction of FENO levels [58, 59] and the value of FENO for assessing airway inflammation in smokers is not clear; however some studies have documented higher levels of FENO in smokers with asthma compared to healthy smokers [60, 61].

In addition to the factors described above, genetic factors also influence variation in levels of FENO. In a Norwegian twin study, genetic factors explained 60% of the variability in FENO [62]. Several genome-wide linkage studies have demonstrated linkage to chromosomes 7, 12 and 17 for asthma- and atopy-related phenotypes [63-68]. The *NOS1*, *NOS2* and *NOS3* genes are located in clusters of genes on chromosome 12, 17 and 7 respectively. Few studies have examined associations between polymorphisms in the *NOS* genes and FENO levels, and the results of these studies have been inconsistent [69-74]. In adults with asthma an association between AAT repeats in intron 20 in *NOS1* and higher FENO levels was reported [69]. The same AAT repeat was associated with asthma or atopy but not with FENO in children [70, 73]. A recent population-based cohort study of American children has reported significant associations for FENO with genetic variants of *NOS2* but not with *NOS1* or *NOS3* [75]. This result for *NOS2* has also been replicated in adults [74] , but in contrast to the American children study, the investigators also reported an association between SNPs belonging to

NOS3 and FENO levels. A recent gene expression study suggested that expression of *NOS2* in humans is more strongly correlated with FENO than is *NOS3* [76]. For *NOS3*, an association of the T allele of the missense variant G894T (rs1799983) to lower FENO levels was reported in adult asthmatics [72] but not in Chinese children with asthma [73].

AIMS

The overall aim of the thesis was to comprehensively characterize genetic variation in the three nitric oxide synthase (NOS; *NOS1*, *NOS2* and *NOS3*) genes using a tagging SNP approach, and evaluate the genetic contribution to variation in levels of FENO in a general adult population.

Specific aims in paper I and II

Paper I

- To understand which of the three *NOS* genes are most important for determining variation in levels of FENO.
- To investigate if such genetic effects on FENO were different in healthy individuals as compared to asthma or atopy.
- To investigate whether any of the *NOS* SNPs related to FENO were also associated with asthma, atopy or lung function.

Paper II

- To investigate if the association between haplotypes in the *NOS2* gene and FENO is stronger and more distinct than single SNP associations.
- To investigate possible effect modifications by asthma status at the haplotype level.

MATERIALS AND METHODS

Study population

This project is based on the population-based ADONIX (Adult Onset Asthma and Exhaled Nitric Oxide) cohort of randomly selected men and women aged 25-75 years at the time of sampling, and living in the city of Gothenburg and surrounding municipalities in Sweden during the 2001 - 2008. During the period from April 2001 to December 2004 the ADONIX study was established as a sub-project linked to the population-based cohort INTERGENE [77]. There was a break during 2004 because of data preparation. Thereafter, recruitment into the ADONIX study cohort was continued from 2005 to 2008.

In paper I, only 2001-2003 data was used, whereas for paper II the full 2001-2008 cohort was used.

The ADONIX study was approved by the local Ethics Committee at Gothenburg University, Sweden.

Data collection

A postal questionnaire and invitation to a clinical examination was sent to 14554 randomly selected subjects. The overall participation rate of the invited cohort was 46%, 2487 participated during 2001-2003 and 4192 participated during 2005 - 2008. The postal questionnaire included questions on respiratory symptoms, smoking habits and medical history. The clinical examination included anthropometric measurements (height and weight), FENO measurements, lung function measurements, and blood samples.

Measurement of FENO

In this project, FENO was measured by the NIOX® Nitric oxide monitoring system (Aerocrine AB; Stockholm, Sweden) (Figure 3) at exhalation flow rates of 50 mL/s (±10%) against an oral pressure of 5 cm H_2O in accordance with the ATS and ERS recommendations [40,

41]. Individuals were asked not to eat or drink 1 hour before FENO measurements. The FENO measurements were obtained before spirometry because repeated spirometric maneuvers may affect FENO values [78].

During the period from June 2001 to January 2003 measurements were performed in triplicate, and in duplicate from February 2003 to 2008, within 10% deviation, according to the later published revised ATS/ERS recommendations [40, 41]. The mean of these measurements was used as a stable measure of FENO.

Asthma

In the present study, asthma was defined based on a positive answer to at least one of the questionnaire items: 'Have you ever had asthma?'; 'Have you ever had asthma diagnosed by a doctor?'; 'Have you had an attack of asthma during the last 12 months?'; 'Have you had asthma during the past month?'

Atopy

In this study, atopy was defined as the presence of specific serum Immunoglobulin E (IgE) antibodies ≥0.35 kU/L to one or more of eight common inhaled allergens (dog, cat, horse, timothy grass, birch, mugwort, house dust mite, and cladiosporum) [79]. IgE antibodies against these common inhaled allergens were determined by the Phadiatop test (Pharmacia Diagnostics; Uppsala, Sweden) according to the manufacturer's instructions.

Lung function

The lung function parameters forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC)) were determined by a dry wedge spirometer (Vitalograph; Buckingham, UK). The predicted values for spirometric variables (FEV_1 and FVC) were calculated based on age, sex and height [79].

Table 1. Baseline characteristics of the ADONIX population, as well as the geometric FENO levels overall and in subgroups, in paper I and II.

	Paper I		Paper II	
	Subjects (n=1737)	Mean(±SD)	Subjects (n=5633)	Mean(±SD)
Age, years	1737	49 (13.6)	5633	52 (11.7)
Height, cm	1737	172 (9.1)	5633	173 (9.2)
FEV_1/FVC ratio	1312	0.8 (0.1)	5520	0.9 (0.1)
FVC (% predicted)	1312	97.1 (12.6)	5520	110 (15.5)
FEV_1 (% predicted)	1312	93.8 (13.6)	5520	103 (15.3)
FENO levels, ppb				
All	1737	15.9 (1.8)	5633	16.4 (1.8)
Men	853	17.1 (1.7)	2686	18.2 (1.8)
Women	884	14.7 (1.7)	2947	14.9 (1.8)
Smokers	325	11.2(1.8)	995	11.4 (1.8)
Non-smokers	1412	17.5(1.7)	4674	17.6 (1.7)
Asthma	298	17.3 (1.9)	726	17.7 (2.1)
Atopy	434	18.4 (1.8)	1340	18.9 (1.9)

FENO: fraction of exhaled nitric oxide; ppb: parts per billion.
FVC: Forced vital capacity; FEV_1: Forced expiratory volume in 1 second;

Genetic analysis

DNA extraction

Genomic DNA was extracted from blood using a magnetic separation of nucleic acid method (mag DNA Isolation Kit: AGOWA GmbH, Berlin, Germany). All the samples were stored in -80 ℃. The samples were diluted with water to a concentration of 5ng/µl before genotyping.

Genotyping methods

DNA samples were genotyped using the Sequenom MassARRAY method (Sequenom, San Diego, California USA) or a competitive allele specific PCR system, KASPar (KBioscience, Hoddesdon, Hertfordshire, UK).

Genotyped subjects

The number of SNPs and individuals genotyped in papers I and II varies. In paper I, 54 SNPs were genotyped in 2125 individuals. Of these, 2084 (98%) were of European origin and included in paper I. In total 1737 subjects had FENO, genotype, and covariate information and were included in the final analysis set.

In paper II, 10 SNPs were genotyped in 6340 individuals. Of these, 5963 (94%) were of European origin and included in paper II. Among these, 5633 participants had FENO values and constituted the final analysis set.

SNP selection

In Paper I, 54 SNPs in the three *NOS* genes (25 *NOS1*, 17 *NOS2* and 12 *NOS3*) were selected based on the reported association with respiratory disease phenotypes or as tag SNPs (Tables 2, 3 and 4). Six SNPs were selected based on previously reported association with FENO or asthma or other respiratory diseases. The remaining 48 tagSNPs were selected using the HapMap phase III European ancestry data (www.hapmap.org) with pair-wise r^2 for SNPs ≥0.8 at minor allele frequency ≥5%, across the genes including 100 kb upstream and 50 kb downstream of each gene.

In paper II, 10 SNPs in the *NOS2* gene were selected based on previously reported association with levels of FENO (Table 3), and genotyped in the extended ADONIX 2001-2008 cohort dataset [75, 80]. Six SNPs (rs9901734, rs2297514, rs2248814, rs12944039, rs3729508 and rs2779248) resulted from the main findings from our previous analysis [80]. The remaining four SNPs (rs4796017, rs2297520, rs9895453 and rs10459953) were selected from the Salam *et al* Southern California Children's Health Study [75].

Table 2. Genotyped SNPs in the *NOS1* gene in the population.

rs number	Alleles (Major/Minor)	MAF (%)	HWE p-value	Call rate	Previous publications	Reported association
rs2682826	G/A	27.6	0.67	96.1	Leung et al 2005	Increased IgE
rs816347	G/A	8.7	0.17	97.4		
rs2293054	G/A	27.9	0.47	97.1		
rs2293055	G/A	10.0	0.89	98.2		
rs9658350	A/G	19.2	0.52	92.7		
rs6490121	A/G	32.1	0.47	97.2		
rs2293050	C/T	40.4	0.48	98.0		
rs7977109	A/G	48.4	0.10	93.4		
rs7314935	G/A	12.4	0.58	97.6		
rs9658354	A/T	40.1	0.65	98.7		
rs532967	G/A	18.9	0.87	98.0		
rs7310618	C/G	10.9	0.26	98.0		
rs553715	G/T	40.1	0.27	98.3		
rs2077171	C/T	30.1	0.30	97.1		
rs545654	T/C	47.5	0.96	98.3		
rs12578547	T/C	23.9	0.03	95.1		
rs12424669	C/T	12.3	0.26	98.5		
rs1552227	C/T	28.4	0.68	98.4		
rs499262	C/T	18.5	0.28	90.9		
rs693534	G/A	39.6	0.96	97.6		
rs3782218	C/T	16.1	0.31	92.2		
rs1123425	A/G	42.7	0.55	97.9		
rs17509231	C/T	13.6	0.68	97.3		
rs9658253	C/T	19.1	0.53	98.2		
rs41279104	C/T	13.4	0.34	96.9		

MAF: Minor allele frequency; HWE: Hardy-Weinberg Equilibrium; IgE: Immunoglobulin E.

Table 3. Genotyped SNPs in the *NOS2* gene in the population.

rs number	Alleles (Major/Minor)	MAF (%)	HWE p-value	Call rate	Previous publications	Reported association
rs4795051	C/G	42.7	0.37	98.8	Salam et al 2011	FENO
rs9901734*	C/G	23.2	1	98.6	Dahgam et al 2012	FENO
rs2255929	T/A	43.1	0.52	98.2	Hancock et al 2006	PD
rs2297514†	T/C	39.4	0.22	97.9	Dahgam et al 2012	FENO
rs2297515	A/C	14.0	0.48	97.4		
rs2248814†	G/A	41.0	0.73	98.0	Dahgam et al 2012	FENO
rs2314810	G/C	5.1	1	98.5		
rs12944039†	G/A	20.2	1	98.0	Dahgam et al 2012	FENO
rs2297520	C/T	40.2	1	98.6	Salam et al 2011	FENO
rs4795067	A/G	37.0	0.6	98.2		
rs3729508*	C/T	40.5	0.58	98.3	Dahgam et al 2012	FENO
rs9895453	T/C	47.7	0.55	98.6	Salam et al 2011	FENO
rs944725	C/T	41.8	0.55	96.4		
rs8072199	C/T	48.2	0.11	96.2		
rs2072324	C/A	18.9	0.34	96.1		
rs3730013	G/A	31.6	1	98.0		
rs10459953	G/C	35.6	0.14	97.8	Salam et al 2011	FENO
rs2779248†	T/C	38.6	1	97.7	Dahgam et al 2012	FENO
rs2301369	C/G	38.2	0.88	96.5		

MAF: Minor allele frequency; HWE: Hardy-Weinberg Equilibrium; * Top SNPs associated with FENO in multi-SNP analysis and † additional strongest p-values for association with FENO in single-SNP analysis in our previous work [80], and included in paper II. PD: Parkinson's disease.

Table 4. Genotyped SNPs in the *NOS*3 gene in the population.

rs number	Alleles (Major/Minor)	MAF (%)	HWE p-value	Call rate	Previous Publications	Reported association
rs10277237	G/A	21.7	0.62	98.0		
rs1800779	A/G	35.3	0.12	97.6		
rs2070744	T/C	36.1	0.12	98.2		
rs3918226	C/T	7.6	0.3	98.4	Holla et al 2006	Asthma
rs3918169	A/G	16.8	1	97.3	Holla et al 2006	Asthma
rs3793342	G/A	16.0	0.59	98.0		
rs1549758	C/T	28.5	0.44	98.2	Holla et al 2008	Asthma
rs1799983	G/T	29.2	0.35	98.2		
rs3918227	C/A	9.4	0.89	98.0		
rs3918188	C/A	36.0	0.34	97.4		
rs1808593	T/G	19.9	0.88	96.1		
rs7830	G/T	38.0	0.17	98.0		

MAF: Minor allele frequency; HWE: Hardy-Weinberg Equilibrium.

Genotype coding and genetic models

Consider a SNP with two alleles 'A' and 'a'; 'A' is major allele and 'a' is minor allele. Possible genotypes of the SNP are 'AA', 'Aa' and 'aa'. Under the additive genetic model, each SNP was coded to 0 (AA), 1 (Aa) and 2 (aa), where each number indicates the number of copies of the minor allele. Under the dominant model the SNPs were coded as 0 (AA) and 1 (Aa+aa); under the recessive model each SNP was coded as 0 (AA+Aa) and 1 (aa). The other suggested models which were also investigated in paper I, over-dominant (heterozygote risk; 0 (AA+aa) and 1 (Aa)) and co-dominant or genotype-specific, which allows for unconstrained patterns of risk for Aa and aa, coded with two indicator variables with AA as common baseline: 1 (Aa) vs. 0 (AA+aa), and 1 (aa) vs. 0 (AA+Aa).

Statistical analysis

Call rates, MAF and Hardy–Weinberg disequilibrium (HWE) test p values were calculated using the R statistical package 'SNPassoc' [81].

Since FENO had a skewed distribution, the values were log-transformed prior to analyses. All the analyses were adjusted for age, sex, height, atopy and smoking habits. Results are presented as percentage change in the geometric mean of FENO across groups of subjects. Single-SNP association analysis was performed with the R statistical package 'SNPassoc' [81]. Regression analyses were done using SAS version 9.3 (SAS Institute; Cary, NC, USA).

SNP association analysis

In paper I, association analyses between each SNP and FENO were performed using the additive, dominant, recessive, over-dominant and co-dominant genetic models.

First, single-SNP association analysis was performed to identify and rank the associations. Next, we performed an analysis in two stages using forward stepwise multiple linear regression analysis to identify a reduced set of the strongest independently associated SNPs and the most appropriate genetic model for each final SNP. The stepwise regression model approach was also used in paper II.

Analyses stratified by asthma or atopic status, respectively, were also performed for the main effect models. The relationship between the genotypes that had effect on FENO and lung function, asthma or atopy was also investigated using a linear or logistic regression model.

Haplotype association analysis

The 'haplo.stats' package which implements the E-M algorithm was used for inferring common haplotypes (haplotype frequencies ≥5%) in the *NOS2* gene [17]. A generalized linear model using the 'haplo.glm' function implemented in the 'haplo.stats' package was used for estimating the effect of all common haplotypes on levels of FENO,

using the most common haplotype as the reference group, assuming an additive genetic model. The effect of haplotype on FENO by asthma status was investigated, by including product terms between the haplotypes and asthma in the main effect model.

RESULTS

Paper I

Of the 54 genotyped SNPs, 4 SNPs had a genotype call rate below 95% and one showed departure from HWE (p=0.03) (Tables 2-4) and these SNPs were excluded from analysis. Genotype distribution for 49 SNPs in the three *NOS* genes (20 *NOS1*, 17 *NOS2*, and 12 *NOS3*) were in HWE (Tables 2-4). Figure 4 shows the results of single-SNP association between 49 SNPs in the three *NOS* genes and FENO levels.

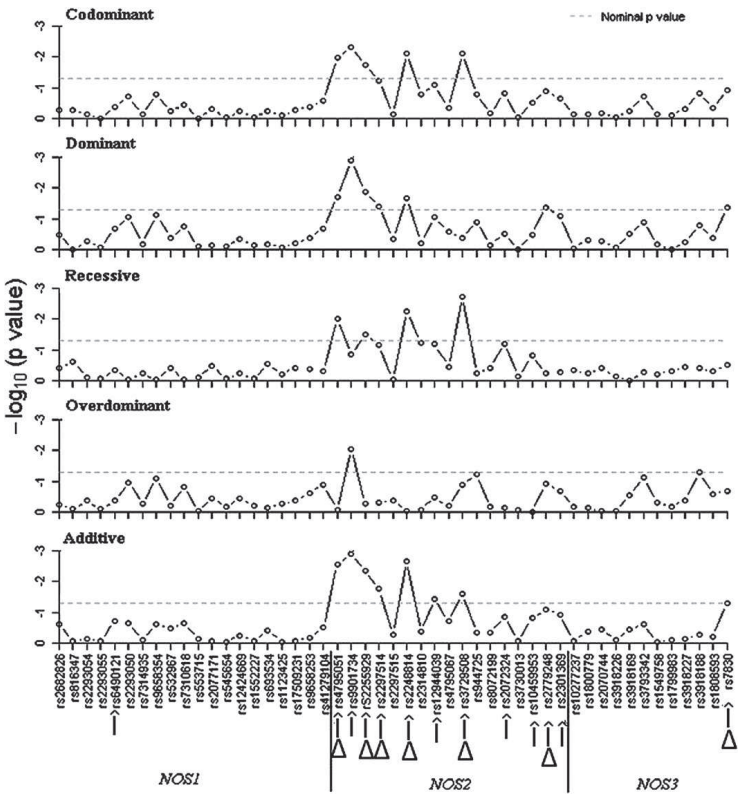

Figure 4. Single-SNP association between FENO and 49 SNPs in the *NOS1*, *NOS2* and *NOS3* genes, by five different genetic models. Each circle represents the minus $\log_{10}$ of the p value for one single *NOS* SNP for each genetic model. The horizontal dotted line shows a statistical significance level at p value 0.05. ↑=SNPs with p≤0.2 from additive model. Δ=SNP with p≤0.05 from at least one non-additive model.

In the multi-SNP model, two SNPs in *NOS2* (rs9901734 and rs3729508) and one in *NOS3* (rs7830) showed independent associations with levels of FENO (Table 5). No independent significant association was seen for any of the SNPs in the *NOS1* gene on FENO.

Table 5. Association of SNPs in the *NOS* genes with FENO among adults. Multi-SNP model.

Gene/SNP	Genetic model	Genotypes	Prevalence n = 1737	Difference in FENO (%), 95% CI	p-value
NOS2					
rs9901734	Additive	CC (ref)	59%		0.016
		CG	36%	5.3 (1.0-9.7)	
		GG	5%	10.7(1.9-19.5)	
rs3729508	Dominant	TT(ref)	17%		0.004
		CC+CT	83%	9.4 (3.1-15.2)	
NOS3					
rs7830	Dominant	GG (ref)	40%		0.034
		GT+TT	60%	5.6 (0.4-11.1)	

FENO: Fraction of exhaled nitric oxide; NOS: Nitric oxide synthase; SNP: Single nucleotide polymorphism.

When we stratified by asthma or atopy, the effect estimate for *NOS2* rs9901734 in 'healthy' subjects (ie, no asthma, wheeze or atopy) was marginally strengthened and remained significant. The *NOS2* rs3729508 estimate was slightly attenuated, while for *NOS3* rs7830 little effect was seen (Figure 5). In subjects with asthma or atopy, the effect estimates were slightly weaker for rs9901734 and slightly stronger for rs3729508, although the difference was not significant. For NOS3, the rs7830 effect estimate was considerably stronger in subjects with asthma (21.9%, 95% CI 4.6% to 42.0%) and significantly different from healthy subjects (p for interaction= 0.01), but less so in atopic subjects.

No significant association was found between the FENO-associated *NOS2* and *NOS3* SNPs and asthma, atopy or lung function.

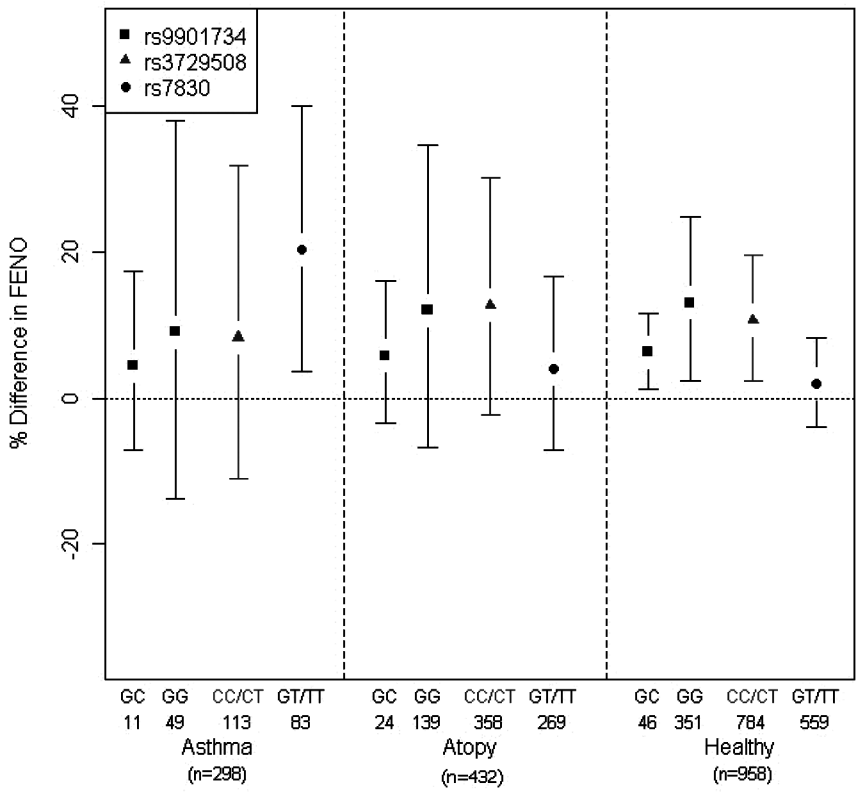

Figure 5. Association between FENO and SNPs in *NOS2* and *NOS3* in subjects with asthma, atopy and in healthy individuals.

Paper II

Genotype distribution for the 10 SNPs in the *NOS2* gene were in HWE. Call rate was ≥95 for all the SNPs (Table 3).

The 5 most significant SNPs in the *NOS2* gene resulting from the stepwise analysis were selected for haplotype analysis (Figure 6).

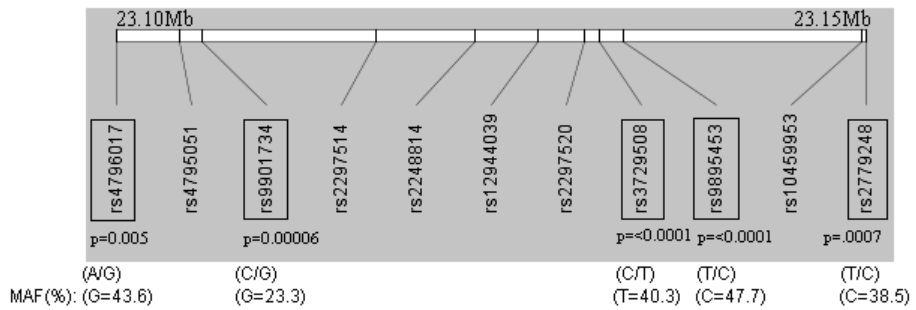

Figure 6. Location of the studied SNPs on chromosome 17. Boxed rs numbers indicate the SNPs most strongly associated with FENO resulting from the stepwise analysis.

A total of 7 common haplotypes were identified, representing 84% of all haplotypes. The most common haplotype alleles were present in the following frequencies: haplotype H1 ('ACTCT ') in 30%, haplotype H2 ('ACCTC') in 14%, haplotype H3 ('ACCTT') in 9%, haplotype H4 ('GCCTC') in 10%, haplotype H5 ('GGCTC') in 5%, haplotype H6 ('GGCTT') in 10% and haplotype H7 ('GGTCT') in 6%.

Haplotype H3 was associated with lower FENO (p=0.006), and haplotypes H2, H5 and H6 were associated with higher FENO (p=0.02, p=0.0002 and $p=7.8\times10^{-13}$) respectively, compared with the baseline haplotype (H1) (Figure 7). In addition to the main findings, there was a statistically significant difference in the association between H3 and FENO in subjects with asthma as compared to in subjects without asthma (p-value for interaction=0.004), with a more strongly negative effect in subjects with asthma than in subjects without asthma [-21.6, 95% CI -33.5, -5.9 vs -4.2, 95% CI -8.2, 0.2).

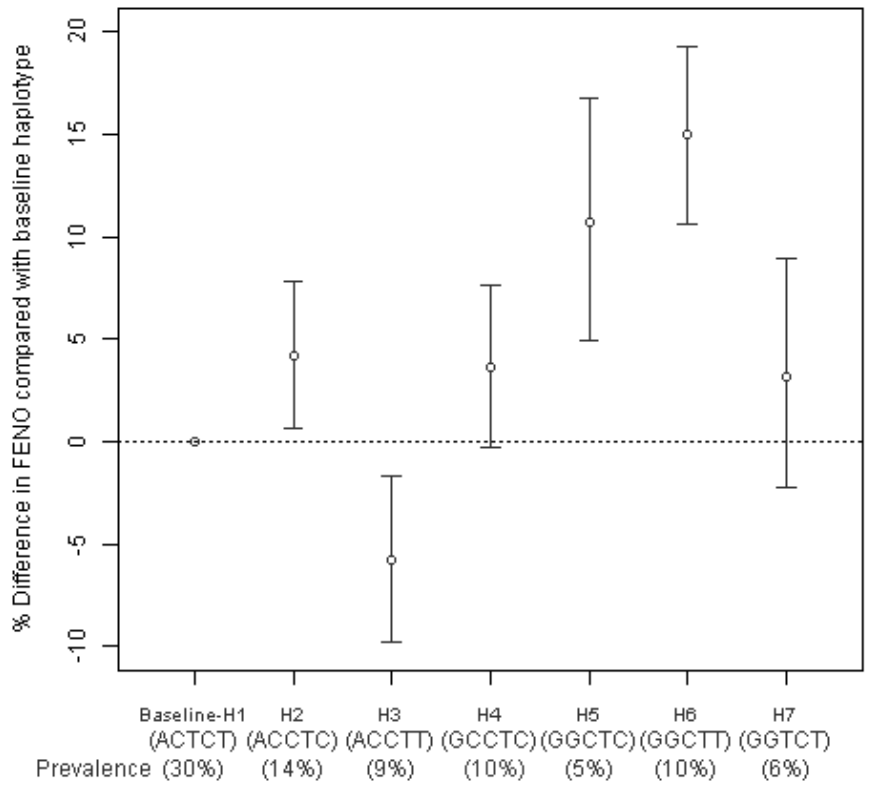

Figure 7. Relative effects of common haplotypes in the *NOS2* gene (with 95% CI) on FENO (n=5633).

DISCUSSION

Our findings that both *NOS2* and *NOS3* are involved in regulating FENO levels are novel, and build a coherent picture with other emerging evidence. The *NOS2* gene is the major *NOS* gene determining variability in FENO in healthy subjects, and also plays a role in subjects with asthma. This is consistent with a priori expectations, since experimental evidence has suggested that *NOS2* encodes for the major enzyme producing NO in exhaled breath [76]. In a population-based study in children, conducted in California also provided evidence of association for SNPs in *NOS2* with FENO levels, supporting the idea that polymorphisms in *NOS2* are the most important *NOS* gene determinants of FENO [75]. On the other hand, the present study found that the genetic contribution of *NOS3* was more prominent in subjects with asthma. *NOS3* has previously been suggested to contribute to the variation FENO levels in asthma [72, 73]. These results have now also been confirmed by a recently published study conducted on adults with and without asthma, which suggested that *NOS2* is the major *NOS* gene determining variability in FENO in healthy subjects and *NOS3* in asthma patients [74]. In the present study no association was observed between SNPs in *NOS1* and FENO. This is well in line with gene expression data, where *NOS2* is highly expressed in the human lung, while *NOS3* is expressed to lesser extent and *NOS1* is undetectable [76].

We also used haplotypes to describe the total common genetic variation in the *NOS2* gene. Seven common haplotypes were identified, constituting 84% of all haplotypes based on our defining SNP set. The common haplotypes in *NOS2* provided much stronger association with FENO than single-SNP associations (global p-value 3.8×10^{-28} for the haplotype distribution). The association of FENO with one haplotype in *NOS2* clearly differed between subjects with and without asthma. Our findings generally support the findings in children with and without asthma by Salam *et al* who also reported that several haplotypes in *NOS2* were associated with higher levels of FENO [75]. However, Salam *et al* used a population-based sample of children that were partly of European and non-European ancestry (non-Hispanic white and Hispanic white), and another set of SNPs to infer all possible haplotypes that occurred to describe variation in

levels of FENO. So neither the two study populations nor the haplotype analysis methods are entirely comparable.

In single-SNP analyses, individuals with all of the studied FENO-associated *NOS2* and *NOS3* SNPs (21% of population) had on average 35% higher FENO than individuals with none of the FENO-associated *NOS2* and *NOS3* SNPs (4% of the population). Likewise in the haplotype analyses, individuals with the haplotype that was associated with higher FENO (H6; frequency 10%) had on average 21% FENO than individuals with the haplotype that was associated with the lowest FENO levels (H3; frequency 9%). In the same population as in this study, current smokers had 30% higher FENO than non-smokers, and subjects with asthma had 16% higher FENO than healthy subjects [54]. When trying to use a single FENO measurement for guiding diagnosis, an important obstacle is the large variation in 'normal FENO value', and our results suggests that this is partly due to genetic differences. We have previously endeavoured to establish normal values for FENO based on FENO measurement from the same population as in this study [54]. Since the variability in FENO related to genetic variation is of a similar magnitude to that related to smoking or asthma, it should potentially be possible to improve the usefulness of FENO for assessing inflammation if the genetic background of subjects could be known.

Most of the SNPs we used in this study are not located in coding or promoter regions. Their function remains unknown. They may influence gene expression, directly affecting variation in FENO. Alternatively, they may be in LD with other coding or functional SNPs that actually affect variation in FENO. Nevertheless, the comprehensive SNP selection and detailed analysis in this study has contributed to a better understanding of which regions within these genes that may be of relevance for the effects seen.

The data used in this thesis come from a population-based cohort of adults (the ADONIX study), where potential participants were randomly selected from the Swedish national population register. The participation rate was 46%, which is less than one would ideally expect. The limited participation rate raises questions regarding the representativity of the study sample to the underlying population. A

non-attendance analysis within the INTERGENE-study, the same study from which the ADONIX-participants originate, was performed based on comparisons with registry data [82]. This study showed that non-participants were more likely to be young, men, less educated, have lower income and to be of non-Nordic origin, which is in line with similar more recent studies [83]. However, the potential response bias is not likely to be related to the genetic background of participants, and hence of less relevance for the present study and its generalizability.

Misclassification of outcome is an important issue to consider. In this thesis we used a stationary chemiluminescence analyzer (the NIOX® system) which is widely used for measurement of FENO. The method is easy to use and reproducible in healthy individuals as well as in subjects with asthma [54, 84]. According to the instrument manufacturer (Aerocrine AB, Solna, Sweden) the variability in FENO measurements is around 3 ppb. This variability would induce some level of misclassification, but this is likely to be unrelated to genetic variation (non-differential misclassification), and would therefore on average tend to slightly bias our results towards the null.

A relatively broad definition of asthma was used, based on a questionnaire response with at least one positive answer to one of several related questions regarding asthma diagnosis and current asthma symptoms. This type of approach clearly carries a potential risk of misclassification. In general, however, such misclassification of asthma is likely to be non-differential with regard to genotypes, and thus would not account for the genetic associations we observed, but may have contributed to decreased statistical power in analyses involving the asthma and for detecting a true effect modification in the analyses stratified by asthma status.

In this thesis, we used a combination of two different approaches for selecting SNPs. The first approach is based on literature looking at the *NOS* genes as candidate genes, and second is a tag SNP approach to complement the first and achieve good genetic coverage. In the first approach, we chose SNPs that previously have been reported to be associated with airway inflammation, asthma and other respiratory diseases; because they might be causative or involved in FENO variation. In the second, tag SNP approach, we attempted to

select an optimal set of SNPs to capture variation in unmeasured SNPs and also to reduce genotyping cost. It is not necessary to genotype all SNPs in a gene since the high correlations between many SNPs (LD) can give rise to redundant information, and it becomes more costly to genotype all SNPs [85].

Population stratification is a common confounding issue in genetic association studies. It implies confounding by ethnicity, in which allele frequencies which vary between ethnically different populations within the study sample may lead to spurious associations, resulting from differences in genetic ancestry which also affect the studied phenotype [86]. In this study we attempted to limit potential confounding by population stratification by including only subjects that were homogeneous with respect to ethnic background (i.e. subjects reporting European country of birth). Therefore, our results are not necessarily generalizable to a population of non-European ancestry. Genotype error, especially genotyping large samples of data in different laboratories can increase rates of misclassification [11, 87]. In this study the genomic DNA samples were genotyped in two different laboratories using two different methods. To increase the validity of the genetic data used, we employed several quality control methods, for example adequate call rate and assessment of HWE (i.e. stable distribution of frequencies of the genotypes in a population). SNPs with call rate ≤95% were excluded from the study, as were SNPs that were clearly and significantly out of HWE.

In our analysis strategy, we used a stepwise regression analysis with a forward approach performed in two stages. We focused on forward selection rather than backward selection due to the high number of variables (8 SNPs, each with five different genetic models), since this approach makes the modeling more practical and easily interpretable than starting by elimination from a very large set of SNPs [88]. In the context of genetic association studies, stepwise regression analysis has been proposed as a useful method to select the most relevant typed variants, and thereby potentially also capture effects of untyped causal functional polymorphisms within the gene or, where sufficient LD structure is present, also in adjacent genes [89]. We further extended this approach by adding a second stepwise regression step to aid in identifying most appropriate genetic models

for each SNP that we observed to be associated. Given the correlation structure that often exists in SNP data the forward selection may fail to identify some important associations, because some SNPs never get to enter the model and in the process may become redundant because of the relationship between SNPs added in and earlier step and SNPs added later. However, the "missed" SNPs with strong results in the single-SNP analysis but not included in our main findings were further considered in haplotype analyses in paper II.

Haplotype analysis, which may provide additional information beyond individual SNP analysis about the genetic basis of complex traits and can be helpful in understanding the unit of biological function, has become of more widespread interest in finding causal connections in candidate genes studies [90]. Haplotype frequencies were estimated using the E-M algorithm, an established method for inferring haplotypes that estimates haplotypes assuming HWE. In our data all the SNPs that were used in the analyses were in HWE, or at least not significantly out of HWE. The inferred frequencies of the haplotypes are therefore likely to be reasonably unbiased. In addition, haplotypes were inferred from a set of SNPs with previously reported association with FENO rather than by just selecting random tag SNPs. Constructing haplotypes from a subset of informative SNPs reduces the haplotype dimensionality and increases power for detecting associations as compared to separate analysis of individual SNPs [13, 91, 92]. Although significant associations were found also for individual SNPs in this study, the haplotype analysis revealed very strong associations, and the haplotype model provided a much stronger global p value (3.8×10^{-28}) for association than individual SNPs.

CONCLUSIONS

This thesis provides strong evidence that the *NOS2* and *NOS3* genes contribute to variation in FENO and identifies relevant SNPs and haplotypes, and it extends this knowledge for the first time to an adult population.

The findings further suggest that the two genes may play different roles in healthy subjects and in subjects with asthma. The *NOS2* gene is the major *NOS* gene determining variability in FENO in healthy subjects and also plays a role in subjects with asthma. *NOS3* contributes to variability in FENO largely in subjects with asthma.

The analysis of haplotypes in the *NOS2* gene described the *NOS2*-related contribution to variation in FENO more strongly than the analysis of individual SNPs, and haplotype-defining SNPs were located relatively far apart in the gene, suggesting that there are likely to be several genetic regions within *NOS2* that are responsible for variation in FENO. Furthermore, one haplotype in the *NOS2* gene contributed to the variation in FENO much more strongly in subjects with asthma than in healthy subjects, whereas the other haplotypes showed less contribution.

An independent effect was also found for one *NOS3* SNP and this effect was stronger in subjects with asthma, indicating that *NOS3* may play a more prominent role for FENO variation in subjects with asthma, and suggesting involvement of different pathways of *NOS2* and *NOS3* for contribution to difference in FENO in subjects with asthma and without asthma.

This study also emphasizes the potential of combining SNP- and haplotype based approaches for identifying and characterizing the contribution of *NOS* genes to variation in FENO.

Potential clinical implications are interesting to consider, given that, the variability in FENO related to the *NOS* genes is approximately similar to that of non-genetic factors. These findings may help provide improvements in predicting normal FENO levels in the general population. However, at present genotyping is not a routine

test, and it is not possible to evaluate measured FENO levels in the scope of presence/absence of *NOS* polymorphisms in a clinical setting. With further development the genotyping techniques this may, however, become feasible, and would enhance the interpretation of an individual FENO value.

ACKNOWLEDGEMENTS

I would like to thank all who were involved in this thesis in different ways.

My first thanks goes to my main supervisor **Fredrik Nyberg**, who is a great advisor. You always supported with your outstanding scientific knowledge and gave excellent guidance all the time and all the way. Even though you are incredibly busy you have never denied me help when asked. I am grateful for your generosity with your time in helping me with English. On top of this work you gave me a wonderful chance to be involved in several international consortia. Thank you for being a great supervisor.

Thanks to **Anna-Carin Olin**, my co-supervisor for recruiting me as a graduate student, for your great support and for your guidance in respiratory research. Your encouragement of learning Swedish made me improve my Swedish language skills.

Thanks to **Åsa Torinssson Nalaui**, my co-supervisor, for your genetic lab expertise. I always saw you as a friend when I asking help and advice.

Thanks to the miljö-och hälsa research school for financial support. My sincere gratitude to the directors of the research school **Dan Strömberg** and **Lotta Dellve**.

Thanks to **Kristina Wass** for your help with the ADONIX data.

Thanks to **Lars Modig**, for encouragement, co-authorship and lunch companion.

Thanks to **Cina Holmer** and **Ann-Sofie Liljenskog Hill** for your support and administrative assistance during these years.

Thanks to **Fredrik Petersson** for helping with computer related issues.

My heartful thanks to **Jaana Gustavsson**, my colleague and friend, for helping me with her English writing skills.

Martin Tondel, my colleague and friend, I wish to thank you for your invaluable support and friendship. All the weekends with beer have given much joy!

All the AMM friends and colleagues, it has been great working with you. Thank you very much for all those chats in 'fika' room.

I am forever grateful to my parents, **Prabhakar Rao** and **Vimala Devi** for their blessings, and my brother **Naresh** and my sister **Neelima** for their endless love that has made me always happy and strong.

Thanks to my in-laws, **Bengt** and **Ingegerd** for all the good care for my children.

Finally, my very special thanks go to my beautiful wife **Anna-Karin** and children **Axel** and **Siri**, for their lovable support in difficult situations.

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I

ORIGINAL ARTICLE

Single nucleotide polymorphisms in the *NOS2* and *NOS3* genes are associated with exhaled nitric oxide

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Received 21 October 2011
Revised 27 January 2012
Accepted 29 January 2012

ABSTRACT

Background Polymorphisms in nitric oxide synthase genes (*NOS1*, *NOS2*, and *NOS3*) have been suggested to have a major impact on fraction of exhaled nitric oxide (FENO), a biomarker of airway inflammation. However, the genetic contribution of *NOS* polymorphisms to FENO is not fully understood. The aim of this study was to investigate comprehensively the association between single nucleotide polymorphisms (SNPs) in all three *NOS* genes and FENO in an adult population, and to assess whether such associations are modified by asthma or atopy.

Method In 1737 adults from a Swedish general population sample, FENO was measured and genetic variation in the *NOS* genes was assessed using 49 SNPs. The genetic effect of *NOS* polymorphisms on FENO, asthma, and atopy was estimated using multiple regression methods.

Results In a multi-SNP model based on stepwise regression analysis, two SNPs in *NOS2* and one in *NOS3* showed independent associations with levels of FENO. For *NOS2* SNP rs9901734, subjects had 5.3% (95% CI 1.0% to 9.7%) higher levels of FENO per G allele, and for rs3729508, subjects with CC or CT genotypes had 9.4% (95% CI 3.1% to 15.2%) higher levels compared with TT. For *NOS3* SNP rs7830, subjects with GT or TT had 5.6% (95% CI 0.4% to 11.1%) higher levels than GG; the genetic effect of this SNP was stronger in asthmatics (21.9%, 95% CI 4.6% to 42.0%).

Conclusion These results suggest that *NOS2* is the major *NOS* gene determining variability in exhaled nitric oxide in the healthy adult population, while *NOS3* may play a more important role in asthmatic adults.

INTRODUCTION

The fraction of exhaled nitric oxide (FENO) is a non-invasive biomarker of airway inflammation and a useful clinical tool for diagnosing and monitoring asthma.[1 2] It is easy to measure and results can be obtained in real-time (online), making it an attractive method in the clinical management of asthma. We have recently reported that increased FENO levels predict new onset of respiratory symptoms in a follow-up cohort.[3] One obstacle, however, to more widespread utility of FENO is that the inter-individual variation in healthy subjects is relatively large, and there is limited knowledge about factors explaining this variation. FENO levels are increased in subjects with asthma[4] and atopy[5] and appear to be related to eosinophilic airway inflammation, such that 26% of the variation in FENO could be explained by the concentration of eosinophils in induced sputum in a large study of asthmatic adults.[6] In healthy adult subjects, the main predictors of FENO are atopy, height, and age, which together explain 11% of the variability of FENO.[7]

In addition, genetic factors are suggested to have a major impact on FENO variability. In a Norwegian twin study, genetic factors explained 60% of the variability in FENO.[8] This study also observed that FENO and airway hyperresponsiveness share a common genetic basis.

Nitric oxide (NO) is synthesised by specific NO synthase (NOS) enzymes, with three distinct isoforms: neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2), and endothelial NOS (eNOS; NOS3).[9 10] All three NOS isoforms are expressed in the lung.[11] The *NOS1* and *NOS3* genes are constitutively expressed resulting in a low basal synthesis of NO,[12 13] whereas expression of *NOS2* seems to be more strongly regulated by gene transcription factors such as nuclear factor κ B ($NF_K\beta$), resulting in an increased NO production.[14] A recent gene expression study suggested that expression of *NOS2* in humans is significantly more detectable and highly correlated with FENO than *NOS3*.[15]

Few studies have examined associations between polymorphisms in the *NOS* genes and FENO levels and, the results of these studies have been inconsistent.[16–20] A reported association between AAT repeats in intron 20 in *NOS1* and higher FENO levels in asthmatic adults[16] was not replicated in asthmatic children.[17 18 20] A recent population based study found tag single nucleotide polymorphisms (SNPs) in *NOS2* to be significantly associated FENO and the association was stronger in asthmatic children.[21] For *NOS3*, an association of the missense variant, G894T (rs1799983), to lower FENO levels was reported in adult asthmatics but not in Chinese children with asthma.[19 20]

The aims of this study were to examine comprehensively the role of SNPs in all three *NOS* genes on the levels of FENO in a large adult cohort; and to study whether any risk variants might be associated with asthma, atopy or lung function, and if the genetic effect on FENO is modified by these respiratory phenotypes.

METHODS

Study population and design

The present study is a part of the ADONIX (adult-onset asthma and exhaled nitric oxide) study cohort of 2200 randomly selected men and women

aged 25–75 years from the city of Gothenburg and surrounding municipalities in Sweden, recruited between June 2001 and December 2003.[7 22] All participants received a postal questionnaire and an invitation to a clinical examination, which included FENO measurements, lung function measurements (forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC)) as well as blood samples. Participants were classified as never, former, and current smokers, based on questionnaire information. In the present study, asthma was defined as a positive response to at least one of the questionnaire items: (1) self reported asthma; (2) doctor diagnosed asthma; (3) asthma attack during the last 12 months; (4) current asthma symptoms during the month preceding clinical examination. Atopy was defined as the presence of specific serum IgE antibodies (≥0.35 kU/1) to any of eight common inhaled allergens (dog, cat, horse, timothy grass, birch, mugwort, house dust mite, and cladiosporum) as determined by the Phadiatop test (Pharmacia Diagnostics; Uppsala, Sweden).[23 24] Spirometry was performed with a dry wedge spirometer (Vitalograph; Buckingham, UK) according to American Thoracic Society (ATS) guidelines, and FEV_1/FVC ratio and percentages of predicted FEV_1 and FVC were calculated based on age, sex, and height.[25] Genomic DNA was extracted from blood using a magnetic separation of nucleic acid method (mag DNA Isolation Kit: AGOWA GmbH, Berlin, Germany). Participants of non-European origin (5%) were excluded from the study to avoid genetic heterogeneity. Of those reporting European country of origin and included in the study, the vast majority (96%) were of Swedish origin.

Exhaled nitric oxide measurements

FENO was measured before spirometry at an expiratory flow rate of 50 ml/s using a chemiluminescence method (NIOX-system; Aerocrine AB, Stockholm, Sweden) according to ATS/European Respiratory Society (ERS) recommendations. Three exhalations were registered during the study period June 2001 to January 2003, and two exhalations during February 2003 to December 2003, according to revised ATS/ERS recommendations,[26 27] and the mean concentration of these was used.

SNP selection and genotyping

Fifty-four SNPs in the *NOS1*, *NOS2*, and *NOS3* genes were selected. Six were selected based on the literature (ie, previously reported association with FENO, asthma, and other respiratory phenotypes) rs2682826,[20] rs41279104,[28] rs2255929,[29] rs3918226,[30] rs3918169,[30] rs1549758[31] and were complemented with 48 tagging SNPs to capture genetic variation across each gene (supplementary table 1). Tag SNP selection was done using the HapMap phase III European ancestry data (http://www.hapmap.org) with a pairwise approach ($r^2 \geq 0.8$) for SNPs with minor allele frequency ≥0.05, extending to 100 kb upstream and 50 kb downstream of each gene. SNPs were genotyped using Sequenom MassARRAY (Sequenom, San Diego, California, USA) or a competitive allele specific PCR system, KASPar (KBioscience, Hoddesdon, Hertfordshire, UK). SNPs with poor genotype call rate (≤95%) and deviation from Hardy–Weinberg Equilibrium (HWE) ($p \leq 0.05$) were excluded.

Statistical analysis

In total, 1737 of 2084 European subjects had available FENO, genotype, and covariate information and were included in the analysis. Association between *NOS* polymorphisms and FENO was analysed using five different genetic models: additive, recessive, dominant, co-dominant (genotype specific risk) and over-dominant (heterozygote risk).[32]

First, we performed a linear regression analysis for each SNP, adjusting for age, sex, height, atopy, and smoking, where the associated SNPs were identified and ranked according to statistical significance. Next we performed an analysis in two stages using forward stepwise multiple linear regression analysis to identify a reduced set of the strongest independently associated SNPs and the most appropriate genetic model for each final SNP.

In the first stepwise analysis stage, SNPs with $p \leq 0.2$ from the additive genetic model in the single SNP association were entered into the stepwise regression analysis, which aimed to determine the most strongly associated SNP or SNPs in a model allowing for independent effects of several SNPs.

In the second stage, significant SNPs ($p \leq 0.05$) from the first stage stepwise regression model, as well as any SNPs from the initial single SNP association analyses with $p \leq 0.05$ from any non-additive (dominant, recessive, over-dominant and co-dominant) genetic models, were included. This selection aimed to ensure that genetic effects not following an additive model would be adequately captured in this final stage. All of the SNPs thus selected and coded into all five genetic models were used as input for a forward stepwise regression analysis in order to identify the most predictive SNPs, each with the best-fitting genetic model, allowing for independent effects of several SNPs.

In both stepwise stages, the p value for an SNP covariate to enter and remain in the model was set at $p=0.10$ and $p=0.20$, respectively. The covariates age, sex, height, atopy, and smoking were forced into the model. In the final multi-SNP model, risk genotype was defined as the genotype that was associated with higher levels of FENO.

Stratified analyses by asthma or atopy status, respectively, were also performed for the final SNP model. The genetic effect was estimated separately for each stratum in a common interaction model, and interaction was tested by including appropriate interaction terms of *NOS* SNPs with asthma or atopy and comparing models with and without interaction terms using a likelihood ratio test. Models were adjusted for age, sex, height, smoking, and asthma medication.

We also performed logistic regression analysis to examine the association between identified risk genotypes and asthma and atopy, respectively, under a dominant genetic model to optimise statistical power. Finally, we analysed the relationship between the risk genotypes and lung function (FEV_1/FVC and per cent predicted FVC and FEV_1) using linear regression.

Single SNP association analyses were performed with the R statistical package 'SNPassoc'. Further regression analyses were done using SAS V.9.2 (SAS Institute). FENO values were log transformed before model fitting. Estimated effects on FENO and lung function are expressed as per cent difference relative to a baseline category, and effects on asthma and atopy as ORs. Values of $p \leq 0.05$ were considered statistically significant and 95% CIs were calculated for all effect estimates.

RESULTS

Baseline characteristics for the study population are presented in table 1. Four SNPs had a genotype call rate ≤95% and one showed departure from HWE ($p=0.03$) and these were excluded from analysis, leaving 49 SNPs (20 *NOS1*, 17 *NOS2*, and 12 *NOS3*). Minor allele frequencies, HWE p values and call rates are shown in supplementary table 1. Linkage disequilibrium (LD) plots for all three *NOS* genes are shown in supplementary figures 1–3.

Thirteen SNPs with $p \leq 0.2$ from the additive model in the single SNP analysis (figure 1, supplementary table 2) were entered into the first stage stepwise regression analysis. This

Table 1 Basic characteristics of 1737 genotyped adults from the Adonix study population, FENO levels overall and in subgroups

Characteristic	Mean (SD)
Age, years	49.2 (13.6)
Height, cm	172.3 (9.1)
FEV_1/FVC ratio*	0.8 (0.1)
FVC (% predicted)*	97.1 (12.6)
FEV_1 (% predicted)*	93.8 (13.6)
FENO levels, ppb	
All (n=1737)	15.9 (1.8)
Men (n=853)	17.2 (1.7)
Women (n=884)	14.7 (1.7)
Asthma (n=298)†	17.3 (1.9)
Atopy (n=432)	18.4 (1.9)
Never smokers (n=878)	17.3 (1.7)
Former smokers (n=534)	16.9 (1.7)
Current smokers (n=325)	11.2 (1.8)

*Lung function data missing for 425 subjects.
†Asthma status missing for four subjects.
FENO, fraction of exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; ppb, parts per billion.

resulted in only one SNP, *NOS2* rs9901734, significantly (p=0.01) associated with FENO.

The SNP rs9901734 and seven SNPs with p≤0.05 from non-additive genetic models in the single SNP analysis (figure 1) were entered into the second stage stepwise regression analysis. Two SNPs in the *NOS2* gene (rs9901734 and rs3729508) and one SNP in the *NOS3* gene (rs7830) were all significantly associated with FENO, each with different genetic models (table 2). For rs9901734, subjects had 5.3% (95% CI 1.0% to 9.7%) higher levels of FENO per each copy of the G allele (additive model). For rs3729508, the optimal fit was provided by a negative recessive genetic effect model for the minor T allele, implying that subjects with CC or CT genotype had 9.4% (95% CI 3.1% to 15.2%) higher FENO levels compared with TT genotype (ie, dominant effect for the major C allele); and for rs7830, subjects with GT or TT had 5.6% (95% CI 0.4% to 11.1%) higher levels of FENO than GG genotype (dominant model).

Haplotype association analysis was performed for the two *NOS2* SNPs rs9910734 (C/G) and rs3729508 (C/T) using an additive model. One haplotype (GC) was significantly associated with 9.1% higher levels of FENO (95% CI 2.8 to 15.8, p=0.004) per haplotype, compared to the baseline haplotype CC, with CT and GT showing no effect versus CC.

When we stratified by asthma or atopy, the effect estimate for *NOS2* rs9901734 in 'healthy' subjects (ie, no asthma, wheeze or atopy) was marginally strengthened and remained significant. The *NOS2* rs3729508 estimate was slightly attenuated, while for *NOS3* rs7830 little effect was seen (table 3). In subjects with asthma or atopy, the effect estimates were slightly weaker for *NOS2* rs9901734 and slightly stronger for *NOS2* rs3729508, although the difference was not significant. For *NOS3*, the rs7830 effect estimate was considerably stronger in asthmatics (21.9%, 95% CI 4.6% to 42.0%) and significantly different from healthy subjects (p for interaction=0.01), but less so in atopic subjects.

No significant association was found between the FENO associated risk genotypes of *NOS2* and *NOS3* SNPs and asthma, atopy or lung function parameters in our cohort (supplementary tables 3 and 4). For future reference, the full results of our single SNP association analyses for all 49 SNPs with FENO are shown in supplementary table 5.

DISCUSSION

In this large Swedish study of an adult general population, we have investigated genetic variation in the *NOS* genes, using SNPs previously reported in the literature as well as tag SNPs. Of the 49 *NOS* SNPs examined, two SNPs in *NOS2* (rs9901734 and rs3729508) and one SNP in *NOS3* (rs7830) were independently associated with variation in FENO levels. The association between rs7830 and FENO varied by asthma status.

The *NOS2* gene, also known as inducible *NOS*, is a priori the most obvious candidate for affecting levels of FENO associated with airway inflammation. A recent population based study, conducted in California, USA by Salam *et al*, provided strong evidence in Hispanic and non-Hispanic white children supporting the proposal that polymorphisms in *NOS2* are important determinants of exhaled NO.[21] They found four SNPs in particular to be most strongly associated with levels of FENO. Our dataset included two SNPs (rs2248814 and rs4795051) in high LD (based on 1000 Genomes Project) with three of their identified SNPs (rs2297512, rs2274894, and rs8081248), and both had the same direction of effect and were significant in our single SNP association analyses (rs2248814: FENO −5.23%, 95% CI −8.6% to −1.9%, p=0.002; rs4795051: FENO −5.1%, 95% CI −8.3% to −1.8%, p=0.003; supplementary table 6). On the other hand, our top *NOS2* SNPs from the stepwise analysis (rs9901734 and rs3729508) both showed low LD (r^2≤0.50) to the four top SNPs in the study by Salam *et al*.[21] Conversely, they had two other SNPs that tagged one of our top SNPs (rs3729508) perfectly, with highly concordant results (supplementary table 6). Overall, these concordant results provide strong evidence that the *NOS2* gene is likely to harbour variants affecting FENO that are common to the studied populations and age groups, and are also consistent with gene expression data showing that the expression of *NOS2* in human airway epithelium cells is an

Table 2 Association of SNPs in *NOS* genes with FENO among adults

Gene/SNP	Genetic model	Genotypes	Prevalence, n=1737	*Difference in FENO (%), 95% CI	p Value
NOS2					
rs9901734	Additive	CC (ref)	59%		0.016
		CG	36%	5.3 (1.0 to 9.7)	
		GG	5%	10.7 (1.9 to 19.5)	
rs3729508	Dominant	TT (ref)	17%		0.004
		CC+CT	83%	9.4 (3.1 to 15.2)	
NOS3					
rs7830	Dominant	GG (ref)	40%		0.034
		GT+TT	60%	5.6 (0.4 to 11.1)	

*FENO values were expressed as percentage change, adjusted for age, sex, height, atopy, and smoking habits.
FENO, fraction of exhaled nitric oxide; NOS, nitric oxide synthase; SNP, single nucleotide polymorphism.

Table 3 Per cent change in the levels of FENO associated with *NOS* gene variants for healthy, asthmatic and atopic subjects

Subgroup	n=1737	*NOS2* rs9901734 (CG/GG vs CC)	*NOS2* rs3729508 (CC/CT vs TT)	*NOS3* rs7830 (GT/TT vs GG)	p Value for interaction, overall model*
Healthy†	n=958	5.8 (0.6 to 11.1)	8.9 (0.6 to 17.8)	1.5 (−4.4 to 7.7)	
Asthmatic	n=298	2.4 (−9.1 to 15.4)	11.3 (−9.1 to 36.4)	21.9‡ (4.6 to 42.0)	0.1
Atopic	n=432	3.8 (−5.5 to 14.0)	13.6 (−2.0 to 31.8)	4.9 (−6.5 to 17.7)	0.94

*Likelihood ratio test p value, comparing a model with to a model without interaction terms for all three SNPs simultaneously (3 degrees of freedom).
†Healthy = no asthma, wheeze and atopy.
‡Significant interaction for *NOS3* SNP rs7830 separately (P for interaction - rs7830*asthma=0.01).
NOS, nitric oxide synthase; SNP, single nucleotide polymorphism.

important determinant of NO in exhaled breath.[15] The results also illustrate that although both we and Salam *et al*[21] used a tag SNP approach, important SNPs from both studies were not well tagged in the other.

The constitutive form of NOS, particularly the *NOS3* gene, has previously been suggested to contribute to the variation in FENO levels in asthmatics,[19 20 33] including a missense polymorphism (G849T) which was reported to explain 16.3% of the variation in levels of FENO in adult subjects with asthma.[19] An interaction between passive smoking and *NOS3* (G849T) on FENO has also been observed in children with asthma.[33] Our finding that both *NOS2* and *NOS3* are involved in regulating FENO levels is novel. Recent experimental evidence has emphasised an important interplay between *NOS3* and *NOS2*. Under inflammatory conditions, *NOS3* knockout mice had an impaired expression of *NOS2* protein in airway epithelium following an aerosol exposure.[34] The rs7830 SNP that was associated with higher levels of FENO in our study was located in the *NOS3* gene. Interestingly, our stratified results suggest that asthma modifies the association between rs7830 and FENO. The effect estimate for rs7830 was substantially higher in asthmatics than healthy subjects (21.9% vs 1.5% higher FENO for GT/TT vs GG). These results thus support the idea that *NOS3* plays a more prominent role in contributing to genetic variation in FENO levels in asthmatic than non-asthmatic subjects.

There was no clear association identified between any *NOS1* SNP and FENO in our data. This is well in line with gene expression data, where *NOS2* is highly expressed in the human lung, while *NOS3* is expressed to a lesser extent and *NOS1* is undetectable.[15]

None of the SNPs selected from previous literature that we examined was clearly associated with FENO in our study. Such inconsistent results are, however, not entirely surprising given differences across studies in factors such as genetic background and environment. Furthermore, earlier studies generally only investigated one or a few candidate SNPs, whereas we used a relatively comprehensive set of tag SNPs to cover genetic variation in the three *NOS* genes.

SNP rs9901734 is located in the intergenic region, close to *NOS2* (3 kb downstream), rs3729508 is located in the intronic region of *NOS2*, and rs7830 is located in the 3-prime untranslated region of *NOS3*. The function of these SNPs remains unknown. They may influence levels of gene expression, directly affecting variation in FENO. Alternatively, they may be in LD with another coding or functional SNP or SNPs that actually affect variation in FENO. Further studies of these *NOS* SNPs can provide more information to better understand their role in airway inflammation and respiratory health.

Our haplotype result is consistent with and provides complementary information to our stepwise results. When the two 'risk' variants (rs9901734 G allele and rs3729508 C allele) are located on the same haplotype (GC), they appear to both mark the same effect (ie, increase FENO levels). Carriers of this haplotype are relatively common in the population (approximately 30%), with individuals carrying one haplotype having increased FENO and individuals carrying two copies (homozygotes) having even higher FENO levels compared to the remaining population.

Although FENO has been used as a tool for the diagnosis and management of asthma for more than a decade, it has not yet fully found its place in the clinical setting. When trying to use a single FENO measurement for guiding diagnosis, an important obstacle is the large variation in 'normal FENO value'. We have previously endeavoured to establish normal values for FENO based on FENO measurement from the same population as in this study.[35] Upper normal values (95th centiles) ranged from 22 parts per billion (ppb) to 57 ppb in different subgroups, depending on age, height, and presence of atopy. FENO is most probably also associated with sex,[36] but this association was rather weak and did not reach statistical significance in this dataset. To date, the new normal values have not been applied in any diagnostic study, so whether they will improve the utility of FENO in the diagnosis of asthma is still unclear. In the present study, individuals with all of the studied risk genotypes in *NOS2* and *NOS3* (21% of population) had on average 35% higher FENO than individuals with none of the risk genotypes (4% of the population). At present it is not possible to evaluate the FENO value in the scope of the presence/absence of *NOS* polymorphisms in a clinical setting, and the practical implications of the results are hence unclear. With further development this may, however, become feasible, which would enhance the interpretation of an individual FENO value.

This study has several strengths. First, it was conducted on a relatively large homogenous Swedish population sample and was restricted to subjects of European origin, to limit population stratification. Second, we comprehensively evaluated common genetic variation within all of the important human *NOS* genes (*NOS1*, *NOS2*, and *NOS3*), by first selecting SNPs with previous evidence of relevance to airway inflammation, asthma, and other respiratory diseases, and then using tag SNPs to complement and achieve good coverage. Third, we applied a structured approach to characterise details of the hypothesised association between NOS genes and FENO, performing a forward stepwise multiple linear regression analysis in two stages rather than multiple testing, since our analysis was based on a clear prior hypothesis. In the context of genetic association studies, stepwise regression analysis has been proposed as a useful method to select the most relevant typed variants, and thereby potentially also capture effects of untyped causal functional polymorphisms

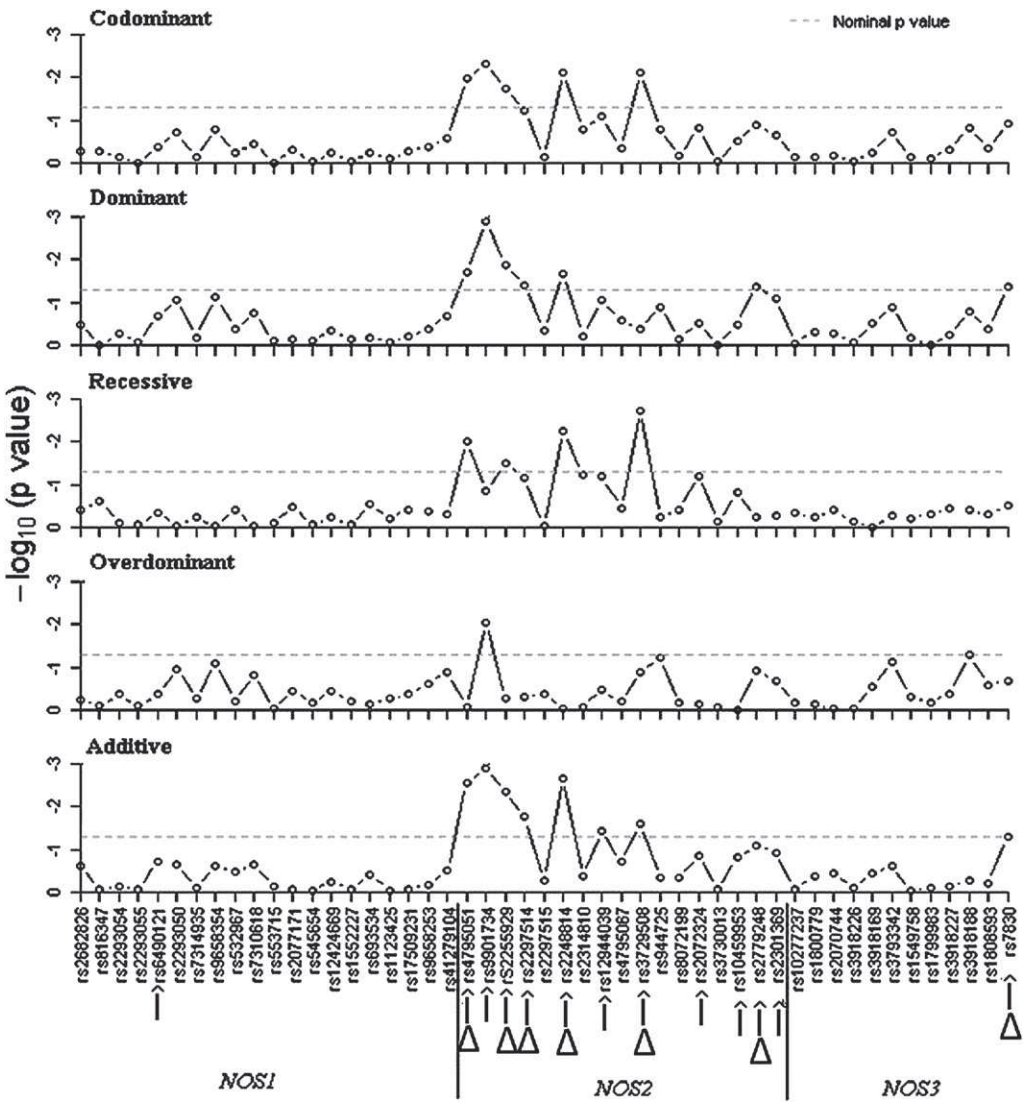

Figure 1 Single-SNP association between fraction of exhaled nitric oxide (FENO) and 49 single nucleotide polymorphisms (SNPs) in the three nitric oxide synthase (*NOS*) genes (*NOS1, NOS2, NOS3*), by five different genetic models. Each circle represents the minus log10 of the p value for one single NOS SNP for each genetic model. The horizontal dotted line shows a statistical significance level at p value 0.05. ↑=SNPs with p≤0.2 from additive model. Δ= SNP with p≤0.05 from at least one non-additive model.

within the gene or, where sufficient LD structure is present, also in adjacent genes.[37] We further extended this approach by adding a second stepwise regression step to aid in identifying most appropriate genetic models for each SNP that we observed to be associated.

Some potential weaknesses of the present study should be noted. According to the instrument manufacturer (Aerocrine AB) the variability in FENO measurements is around 3 ppb. This variability would induce some level of independent misclassification unrelated to genetic variation, and will on average tend to slightly bias our results towards the null. A broad definition of asthma was used, based on a questionnaire response with at least one positive answer to one of several related questions regarding asthma diagnosis and current active asthma, which carries a potential risk of misclassification. However, such misclassification of asthma is likely to be non-differential with

regard to genotypes, and would not account for the genetic associations we observed, but may have contributed to decreased statistical power in analyses involving the asthma phenotype.

Overall, our findings suggest that the observed genetic variation in exhaled NO levels between individuals in this study were predominantly driven by the *NOS2* ('inducible *NOS*', *iNOS*) gene, indicating that *NOS2* is the major *NOS* gene determining variability in exhaled NO in healthy subjects. In asthmatics, *NOS3* ('endothelial *NOS*', *eNOS*) seemed to play a more prominent role for FENO variation.

Contributors SD: statistical analyses, the interpretation of the results, and drafted the article; FN and ACO: development of the research questions, designed and coordinated the study; ATN: involved in genotyping and the interpretation of the results; LM: development of the research questions and participated in the manuscript preparation; all authors read and approved the final version of manuscript.

Funding This study was funded by the Swedish Research Council for Worklife and Social research (FAS), grants 2001-0263, 2003-0139, Swedish Heart and Lung Foundation grant 20050561 and a collaborative research grant from AstraZeneca.

Competing interests None.

Patient consent Obtained.

Ethics approval All subjects gave their written consent, and the protocol was approved by the ethics committee of Gothenburg University.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement The investigators have no current plans to share raw data.

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Supplementary Tables and Figure

Supplementary Table 1 Allele frequencies, Hardy-Weinberg equilibrium (HWE) p-values and call rates for 54 polymorphisms in the 3 *NOS* genes in the Adonix study population (n=1737).

dbSNP id	Alleles (Major/Minor)	Minor allele Frequency	HWE p-value	Call rate	Previous Publications
***NOS1* SNPs**					
rs2682826*	G/A	0.28	0.67	96.14	Leung et al., 2005
rs816347	G/A	0.09	0.17	97.41	
rs2293054	G/A	0.28	0.47	97.12	
rs2293055	G/A	0.1	0.89	98.21	
rs9658350	A/G	0.19	0.52	92.74	
rs6490121	A/G	0.32	0.47	97.24	
rs2293050	C/T	0.4	0.48	98.04	
rs7977109	A/G	0.48	0.1	93.38	
rs7314935	G/A	0.12	0.58	97.64	
rs9658354	A/T	0.4	0.65	98.73	
rs532967	G/A	0.19	0.87	98.04	
rs7310618	C/G	0.11	0.26	98.04	
rs553715	G/T	0.4	0.27	98.27	
rs2077171	C/T	0.3	0.3	97.12	
rs545654	T/C	0.48	0.96	98.27	
rs12578547	T/C	0.24	0.03	95.1	
rs12424669	C/T	0.12	0.26	98.5	
rs1552227	C/T	0.28	0.68	98.39	
rs499262	C/T	0.19	0.28	90.9	
rs693534	G/A	0.4	0.96	97.64	
rs3782218	C/T	0.16	0.31	92.17	
rs1123425	A/G	0.43	0.55	97.98	
rs17509231	C/T	0.14	0.68	97.35	
rs9658253	C/T	0.19	0.53	98.16	
rs41279104*	C/T	0.13	0.34	96.89	Djidjik et al., 2008
***NOS2* SNPs**					
rs4795051	C/G	0.43	0.37	98.79	
rs9901734	C/G	0.23	1	98.56	
rs2255929*	T/A	0.43	0.52	98.16	Hancock et al., 2006
rs2297514	T/C	0.39	0.22	97.93	
rs2297515	A/C	0.14	0.48	97.41	
rs2248814	G/A	0.41	0.73	98.04	
rs2314810	G/C	0.05	1	98.5	
rs12944039	G/A	0.2	1	97.98	
rs4795067	A/G	0.37	0.6	98.16	
rs3729508	C/T	0.41	0.58	98.27	

rs944725	C/T	0.42	0.55	96.43	
rs8072199	C/T	0.48	0.11	96.2	
rs2072324	C/A	0.19	0.34	96.08	
rs3730013	G/A	0.32	1	98.04	
rs10459953	G/C	0.36	0.14	97.81	
rs2779248	T/C	0.39	1	97.7	
rs2301369	C/G	0.38	0.88	96.49	
***NOS3* SNPs**					
rs10277237	G/A	0.22	0.62	98.04	
rs1800779	A/G	0.35	0.12	97.64	
rs2070744	T/C	0.36	0.12	98.16	
rs3918226*	C/T	0.08	0.3	98.39	Holla et al., 2006
rs3918169*	A/G	0.17	1	97.29	Holla et al., 2006
rs3793342	G/A	0.16	0.59	97.98	
rs1549758*	C/T	0.29	0.44	98.21	Holla et al., 2008
rs1799983	G/T	0.29	0.35	98.16	
rs3918227	C/A	0.09	0.89	97.98	
rs3918188	C/A	0.36	0.34	97.41	
rs1808593	T/G	0.2	0.88	96.14	
rs7830	G/T	0.38	0.17	98.04	

* SNPs previously reported to be associated with respiratory disease phenotypes.

Supplementary Table 2 Strongest P-values for the association of *NOS* gene SNPs with FENO for five different genetic models, selected to enter into stages I and stage II of stepwise regression in the statistical analysis. Selection criteria for additive genetic model results into stage I modelling was p≤0.2, and for non-additive genetic model results into stage II modelling p≤0.05 in any of the non-additive models.

Gene	dbSNP ID	Additive	Co-dominant	Dominant	Recessive	Over-dominant
NOS2	rs10459953	0.165				
NOS2	rs12944039	0.034				
NOS2	rs2072324	0.138				
NOS2	rs2248814	0.002	0.008	0.024	0.006	
NOS2	rs2255929	0.005	0.018	0.014	0.031	
NOS2	rs2297514	0.018		0.041		
NOS2	rs2301369	0.114				
NOS2	rs2779248	0.080		0.044		
NOS2	rs3729508	0.028	0.008		0.002	
NOS2	rs4795051	0.003	0.011	0.021	0.010	
NOS1	rs6490121	0.186				
NOS3	rs7830	0.053		0.044		
NOS2	rs9901734	0.001	0.005	0.001		0.009

Supplementary Table 3 Association of the identified risk genotypes of *NOS* SNPs with asthma and atopy

			Asthma		Atopy	
Gene	SNP	Risk genotypes	OR*	95% CI	OR*	95% CI
NOS2	rs9901734	GG or CG	1.16	(0.82-1.66)	0.83	(0.66-1.04)
NOS2	rs3729508	CC or CT	1.07	(0.66-1.74)	0.93	(0.69-1.25)
NOS3	rs7830	GT or TT	1.05	(0.73-1.51)	1.16	(0.92-1.47)

* Adjusted for age, sex, height and smoking habits

OR: odds ratio; CI: confidence interval.

Supplementary Table 4 Association between the identified risk genotypes of *NOS* SNPs and lung function parameters.

			FEV1/FVC		FVC		FEV1	
Gene	SNP	Risk genotypes	(%)*	p	(%)*	P	(%)*	P
NOS2	rs9901734	GG or CG	−0.5	0.9	– 0.5	0.5	– 0.8	0.3
NOS2	rs3729508	CC or CT	0.1	0.8	– 0.3	0.7	– 0.3	0.8
NOS3	rs7830	GT or TT	0.4	0.3	0.2	0.8	0.7	0.4

*% difference in FEV1/FVC and percent predicted FVC and FEV1, adjusted for age and smoking habits. FEV1: Forced Expiratory Volume in 1 sec; FVC: Forced Vital Capacity;

Supplementary Table 5 Results of single-SNP analysis of the 49 SNPs in three NOS genes. Percentage difference with 95% confidence interval of FENO with 5 different genetic models, adjusted for age, sex, height, atopy and smoking habits among 1737 adult subjects in the Adonix study

dbSNP id	Alleles (Major/Minor)	Additive	Dominant	Recessive	Overdominant	Codominant (Heterozygote)	Codominant (Homozygote)
NOS1 SNPs							
rs2682826	G/A	-2.35(-6.16-1.61)	-2.58(-7.33-2.42)	2.17(-12.85-5.48)	0.1(-6.38-3.65)	-2.12(-7.12-3.14)	-5.02(-13.91-4.77)
rs816347	G/A	-0.51(-6.59-5.97)	0.15(-6.24-6.96)	-19.04(-43.57-16.15)	0.87(-5.63-7.82)	0.75(-5.74-7.69)	-18.94(-43.52-16.32)
rs2293054	G/A	-0.7(-4.47-3.21)	-1.58(-6.33-3.41)	1.46(-7.32-11.07)	-2.09(-6.91-2.98)	-2.01(-6.98-3.22)	0.57(-8.38-10.4)
rs2293055	G/A	-0.43(-6.04-5.51)	-0.63(-6.64-5.77)	2.17(-20.75-31.72)	-0.79(-6.91-5.73)	-0.77(-6.9-5.77)	2.02(-20.89-31.58)
rs6490121	A/G	-2.48(-6.04-1.22)	-3.21(-7.91-1.72)	-3.1(-10.56-4.98)	-2.06(-6.84-2.97)	-2.9(-7.87-2.33)	-4.46(-12.16-3.91)
rs2293050	C/T	2.21(-1.33-5.87)	4.56(-0.67-10.06)	0.31(-6.1-7.16)	4.02(-0.98-9.28)	5.05(-0.51-10.93)	3.16(-4.09-10.96)
rs7314935	G/A	0.8(-4.43-6.31)	1.41(-4.32-7.47)	-5.87(-23.89-16.42)	1.95(-3.92-8.18)	1.85(-4.03-8.09)	-5.48(-23.61-16.95)
rs9658354	A/T	2.13(-1.41-5.8)	4.67(-0.55-10.16)	-0.22(-6.65-6.66)	4.44(-0.58-9.71)	5.33(-0.24-11.2)	2.77(-4.5-10.59)
rs532967	G/A	-2.29(-6.53-2.14)	-2.26(-7.21-2.95)	-5.52(-17.15-7.75)	-1.48(-6.61-3.93)	-1.8(-6.95-3.64)	-6.06(-17.72-7.25)
rs7310618	C/G	3.42(-2.11-9.25)	4.36(-1.81-10.92)	-1.35(-19.56-20.97)	4.75(-1.61-11.53)	4.74(-1.64-11.53)	-0.46(-18.86-22.11)
rs553715	G/T	0.64(-2.81-4.22)	0.85(-4.16-6.13)	0.87(-5.55-7.73)	0.3(-4.52-5.37)	0.7(-4.62-6.32)	1.27(-5.81-8.87)
rs2077171	C/T	-0.41(-4.08-3.41)	0.76(-4.1-5.86)	-4.1(-11.81-4.27)	2.31(-2.7-7.57)	1.74(-3.43-7.19)	-3.35(-11.41-5.44)
rs545654	T/C	-0.23(-3.65-3.3)	-0.82(-6.14-4.79)	0.28(-5.45-6.36)	-0.85(-5.62-4.15)	-1.02(-6.64-4.93)	-0.38(-7.1-6.82)
rs12424669	C/T	1.7(-3.63-7.33)	2.39(-3.39-8.52)	-6.02(-25.22-18.12)	2.91(-3-9.18)	2.82(-3.09-9.1)	-5.43(-24.78-18.91)
rs1552227	C/T	0.39(-3.44-4.36)	0.84(-4-5.93)	-0.77(-9.5-8.8)	1.1(-3.83-6.3)	1.06(-4.02-6.41)	-0.31(-9.33-9.61)
rs693534	G/A	-1.54(-4.98-2.02)	-1.1(-6.03-4.09)	-3.63(-9.94-3.13)	0.94(-3.93-6.05)	-0.21(-5.47-5.35)	-3.74(-10.64-3.69)
rs1123425	A/G	0.19(-3.3-3.81)	-0.65(-5.74-4.72)	1.62(-4.71-8.36)	-1.5(-6.24-3.47)	-1.2(-6.55-4.46)	0.88(-6.18-8.47)
rs17509231	C/T	-0.44(-5.38-4.75)	-1.36(-6.82-4.43)	8.36(-9.43-29.64)	-2.27(-7.83-3.62)	-2.08(-7.67-3.84)	7.82(-9.93-29.06)

dbSNP id	Alleles (Major/Minor)	Additive	Dominant	Recessive	Overdominant	Codominant (Heterozygote)	Codominant (Homozygote)
rs9658253	C/T	0.9(-3.44-5.44)	2.11(-3.05-7.54)	-4.95(-16.34-7.98)	3.16(-2.21-8.83)	2.92(-2.48-8.63)	-4.08(-15.67-9.09)
rs41279104	C/T	-2.88(-7.79-2.3)	-3.8(-9.13-1.84)	4.56(-14.46-27.81)	-4.33(-9.75-1.41)	-4.27(-9.7-1.49)	3.51(-15.35-26.57)

NOS2 SNPS dbSNP id	Alleles (Major/Minor)	Additive	Dominant	Recessive	Overdominant	Codominant (Heterozygote)	Codominant (Homozygote)
rs4795051	C/G	-5.08(-8.31--1.75)	-5.93(-10.69--0.92)	-7.89(-13.49--1.94)	-0.42(-5.2-4.59)	-4.2(-9.34-1.24)	-10.18(-16.3--3.62)
rs9901734	C/G	6.99(2.68-11.48)	8.56(3.28-14.12)	8.54(-2.67-21.04)	7.08(1.73-12.71)	8.09(2.61-13.86)	11.79(0.09-24.86)
rs2255929	T/A	5.13(1.55-8.85)	6.74(1.31-12.46)	7.11(0.61-14.03)	1.54(-3.33-6.66)	5.31(-0.37-11.32)	10.47(2.92-18.57)
rs2297514	T/C	4.31(0.71-8.05)	5.43(0.21-10.94)	6.32(-0.57-13.69)	1.67(-3.23-6.81)	4.29(-1.2-10.08)	8.83(1.13-17.11)
rs2297515	A/C	1.51(-3.42-6.68)	2(-3.58-7.9)	-0.55(-15.95-17.67)	2.18(-3.56-8.27)	2.18(-3.59-8.3)	-0.02(-15.55-18.38)
rs2248814	G/A	-5.28(-8.55--1.89)	-5.76(-10.48--0.78)	-8.77(-14.55--2.61)	-0.23(-5.02-4.8)	-3.89(-8.99-1.49)	-10.84(-17.08--4.13)
rs2314810	G/C	3.37(-4.53-11.91)	2.3(-5.79-11.07)	63.13(-1.83-171.07)	1.01(-7.05-9.78)	1.15(-6.92-9.92)	63.32(-1.74-171.45)
rs12944039	G/A	4.72(0.28-9.36)	4.54(-0.67-10.03)	12.51(-0.68-27.46)	2.64(-2.63-8.2)	3.43(-1.93-9.08)	13.77(0.31-29.05)
rs4795067	A/G	2.28(-1.32-6.02)	2.91(-2.13-8.21)	3.15(-3.91-10.74)	1.27(-3.6-6.38)	2.44(-2.86-8.03)	4.51(-3.2-12.82)
rs3729508	C/T	-3.87(-7.19--0.43)	-2.06(-6.96-3.1)	-9.88(-15.62--3.76)	3.97(-1.02-9.22)	0.71(-4.62-6.33)	-9.52(-15.87--2.69)
rs944725	C/T	1.34(-2.21-5.03)	4.14(-1.18-9.74)	-1.85(-8.09-4.82)	4.78(-0.27-10.09)	5.19(-0.47-11.17)	1.15(-6.01-8.87)
rs8072199	C/T	-1.32(-4.67-2.15)	-1.11(-6.46-4.54)	-2.5(-8-3.32)	0.97(-3.93-6.13)	-0.3(-6.05-5.79)	-2.69(-9.2-4.28)
rs2072324	C/A	3.5(-1.09-8.32)	2.75(-2.48-8.26)	14.45(-0.66-31.86)	0.93(-4.33-6.49)	1.58(-3.75-7.21)	15.03(-0.27-32.67)
rs3730013	G/A	0.26(-3.43-4.09)	-0.06(-4.87-4.99)	1.43(-6.59-10.13)	-0.58(-5.4-4.48)	-0.36(-5.41-4.95)	1.25(-7.09-10.35)
rs10459953	G/C	-2.51(-5.94-1.05)	-2.41(-7.16-2.59)	-5.1(-11.72-2.01)	0.06(-4.79-5.17)	-1.36(-6.46-4.02)	-5.76(-12.77-1.8)
rs2779248	T/C	3.24(-0.38-6.99)	5.36(0.14-10.85)	2.28(-4.54-9.59)	3.84(-1.16-9.08)	5.38(-0.14-11.19)	5.31(-2.32-13.54)
rs2301369	C/G	2.95(-0.7-6.72)	4.63(-0.59-10.11)	2.5(-4.43-9.94)	3.08(-1.92-8.34)	4.5(-1-10.31)	5.03(-2.67-13.33)

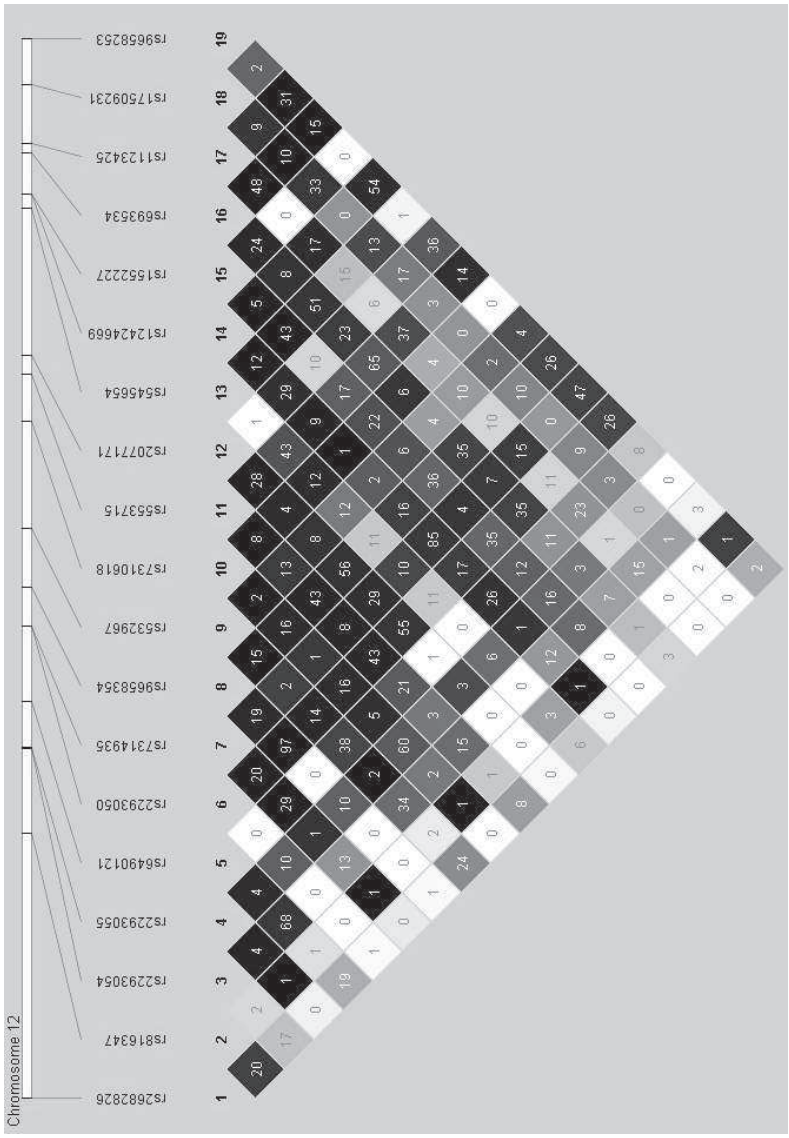
NOS3 SNPs							
rs10277237	G/A	0.29(-3.84-4.59)	-0.42(-5.33-4.75)	4.31(-6.89-16.84)	-1.32(-6.32-3.95)	-1.03(-6.12-4.33)	3.91(-7.39-16.59)
rs1800779	A/G	1.51(-2.2-5.35)	1.82(-3.15-7.06)	2.17(-5.4-10.33)	0.87(-3.98-5.97)	1.54(-3.66-7.02)	3.01(-5.1-11.81)
rs2070744	T/C	1.71(-1.98-5.53)	1.67(-3.31-6.91)	3.35(-4.14-11.42)	0.18(-4.62-5.23)	1.1(-4.09-6.57)	3.97(-4.08-12.7)
rs3918226	C/T	0.88(-5.43-7.62)	0.7(-6.09-7.98)	5.54(-20.47-40.07)	0.39(-6.54-7.82)	0.43(-6.5-7.88)	5.6(-20.45-40.18)
rs3918169	A/G	2.19(-2.45-7.06)	2.89(-2.45-8.52)	-0.02(-13.84-16.03)	3.06(-2.43-8.87)	3.1(-2.43-8.94)	0.86(-13.16-17.15)
rs3793342	G/A	2.97(-1.85-8.03)	4.34(-1.14-10.13)	-4.83(-19.18-12.07)	5.16(-0.5-11.14)	5.04(-0.64-11.04)	-3.5(-18.11-13.7)
rs1549758	C/T	-0.29(-4.09-3.65)	-1.03(-5.78-3.96)	1.98(-6.94-11.75)	-1.64(-6.42-3.39)	-1.46(-6.41-3.74)	1.3(-7.83-11.34)
rs1799983	G/T	0.44(-3.38-4.4)	-0.12(-4.92-4.93)	2.8(-6.07-12.49)	-0.96(-5.77-4.11)	-0.61(-5.61-4.65)	2.51(-6.62-12.53)
rs3918227	C/A	-1.24(-6.97-4.84)	-2.13(-8.19-4.34)	13.99(-13.23-49.75)	-2.93(-9.05-3.6)	-2.81(-8.95-3.73)	13.43(-13.68-49.05)
rs3918188	C/A	-1.08(-4.67-2.64)	-3.45(-8.20-1.53)	3.36(-4.12-11.43)	-4.72(-9.33-0.12)	-4.53(-9.47-0.65)	0.81(-6.98-9.24)
rs1808593	T/G	1.01(-3.33-5.55)	2.16(-3-7.59)	-4.31(-15.69-8.62)	3.09(-2.26-8.74)	2.88(-2.51-8.58)	-3.4(-15-9.78)
rs7830	G/T	3.55(-0.05-7.28)	5.32(0.14-10.76)	3.64(-3.25-11.02)	3.17(-1.8-8.4)	4.97(-0.51-10.75)	6.38(-1.26-14.62)

NOS: Nitric oxide synthase; FENO: Fraction of exhaled nitric oxide

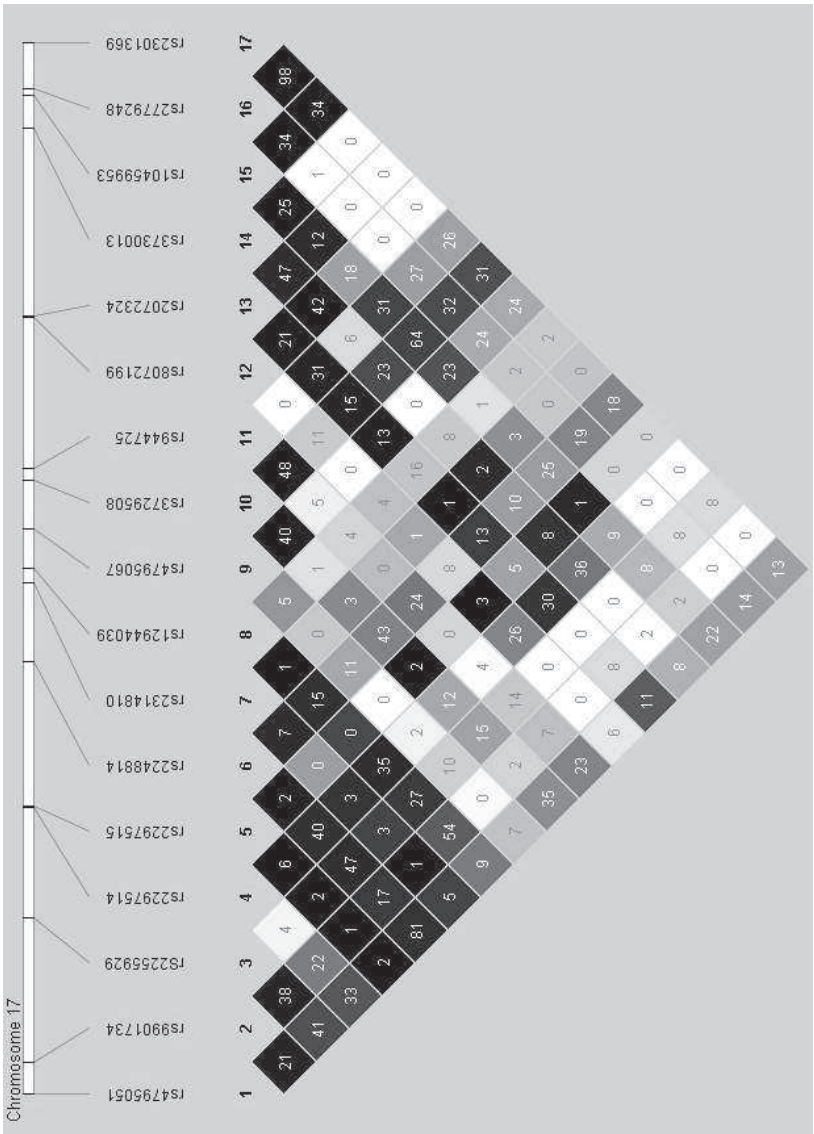
Supplementary Table 6 Comparison between SNPs in the *NOS2* gene reported to be related to FENO reported in the present study in adults to be related to FENO

Primary SNP and association reported					Tag SNPs correlated in the other study and association reported						
Reported NOS2A SNP	Rare allele	Allele frequency	Percent difference in FeNO (95%CI)	p	SNP in LD††	r2	D'	Rare allele	Allele frequency	Percent difference in FeNO (95%CI)	p
Salam et al					Present study						
rs2297512*	NA	0.38/0.38§	-6.0 (-8.9, -3.1)	<.0000001	rs2248814	0.97	1	A	0.41**	-5.3(-8.6,-1.9)†	0.002
					rs4795051	0.60	0.81	G	0.43**	-5.1(-8.3,-1.6)†	0.003
rs2274894*	NA	0.39/0.38§	-5.8 (-8.7, -2.9)	<.0000001	rs2248814	0.97	1	A	0.41**	-5.3(-8.6,-1.9)†	0.002
					rs4795051	0.60	0.84	G	0.43**	-5.1(-8.3,-1.6)†	0.003
rs8081248*	NA	0.42/0.40§	-6.2 (-9.0, -3.2)	<.0000001	rs2248814	0.75	0.92	A	0.41**	-5.3(-8.6,-1.9)†	0.002
					rs4795051	0.87	0.93	G	0.43**	-5.1(-8.3,-1.6) †	0.003
rs4796017*	NA	0.43/0.47§	5.7 (2.6, 9.0)	<.0000001	none						
Present study					Salam et al						
rs9901734‡	G	0.23**	7.0 (2.7,11.5)†	0.001	none						
rs3729508‡	T	0.41**	-3.9 (-7.2,-0.4)†	0.028	rs2297520	0.97	1	NA	0.40/0.33§	-3.3 (-6.3, -0.2)	0.04
					rs9895453	0.82	1	NA	0.49/0.44§	-0.5 (-3.5, 2.6)	0.76
					rs10459953	0.68	0.85	NA	0.35/0.30§	-3.2 (-6.3, 0.0)	0.05

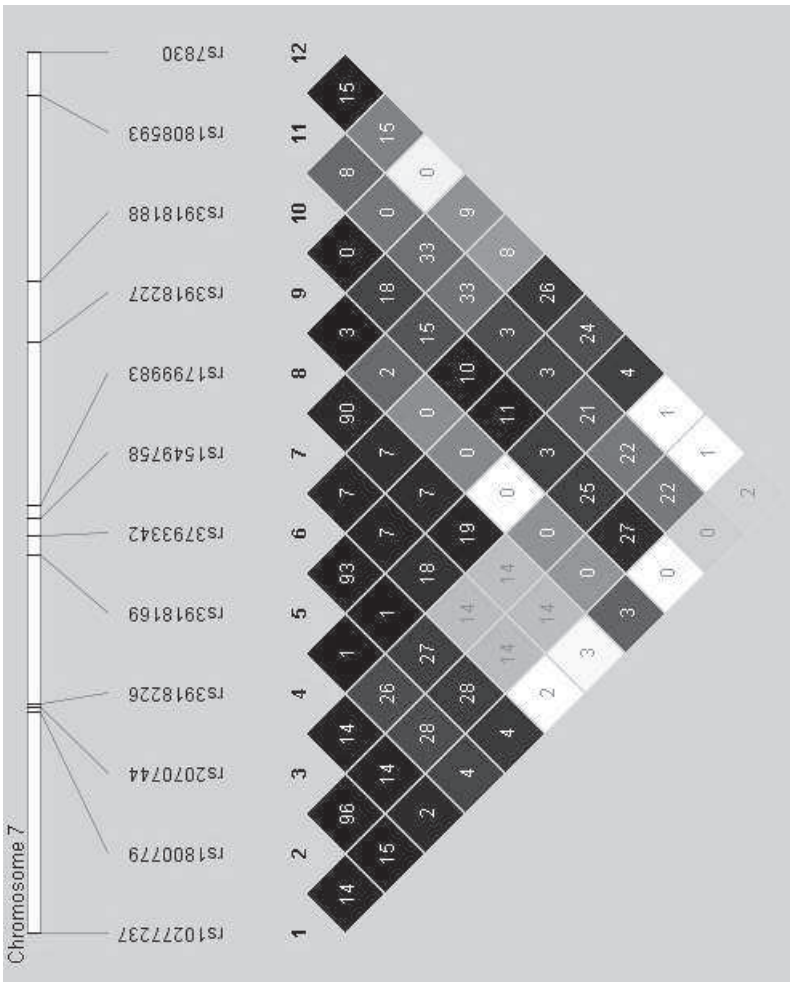
FENO: Fraction of exhaled nitric oxide.
*: rs2297512, rs2274894, rs8081248 and rs4796017: Most significant findings reported in Salam et al 2010.
†: Effect estimates from an additive genetic model from our single-SNP analysis. , Adjusted for age, sex, height, atopy and smoking habits.
‡: rs9901734 and rs3729508: Most significant independent findings in the present study.
§: Rare allele frequency in non-Hispanic white/Hispanic white children
**: Rare allele frequency in western Sweden adults
††: SNPs with a correlation ≥ 0.6; D' (D' = 1indicates complete LD, D' = 0 indicates the absence of LD)



Supplementary Figure 1 Linkage disequilibrium (LD) map of 19 *NOS1* SNPs on chromosome 12. The number in each box corresponds to the pairwise correlation coefficient r2 (a measure of LD) between any two SNPs and shading indicates D' (a measure of LD; white shade indicates low D' and black shade indicates high D'). Of the 20 *NOS1* SNPs, 19 SNPs are shown in the map. Since rs41279104 was located quite far away outside the gene, it is not included in the map.



Supplementary Figure 2 Linkage disequilibrium (LD) map of 17 *NOS2* SNPs on chromosome 17. The number in each box corresponds to the pairwise correlation coefficient r2 (a measure of LD) between any two SNPs and shading indicates D' (a measure of LD; white shade indicates low D' and black shade indicates high D').



Supplementary Figure 3 Linkage disequilibrium (LD) map of 12 *NOS3* SNPs on chromosome 7. The number in each box corresponds to the pairwise correlation coefficient r2 (a measure of LD) between any two SNPs and shading indicates D' (a measure of LD; white shade indicates low D' and black shade indicates high D').

II

Haplotypes of the inducible nitric oxide synthase gene are strongly associated with exhaled nitric oxide levels in adults: a population-based study

Manuscript

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ABSTRACT

Background: Previous genetic association studies have reported evidence for association of SNPs in the inducible nitric oxide synthase (iNOS), encoded by the *NOS2* gene to variation in levels of fractional exhaled nitric oxide (FENO) in children and adults. In this study we evaluated the region spanning from SNP rs4796017 (23.10 Mb) to rs2779248 (23.15 Mb) to further understand the contribution of *NOS2* to variation in levels of FENO.

Methods: In a cohort of 5912 adults 25-75 years of age, we investigated the relationship between *NOS2* haplotypes and FENO, and effect modifications by asthma.

Results: Seven common haplotypes (H1-H7) were inferred from all possible haplotype combinations. One haplotype H3 was significantly associated with lower levels of FENO; -5.8% (95%CI -9.8 to -1.7; p=0.006) compared with the most common baseline haplotype H1. Three haplotypes (H2, H5 and H6) were significantly associated with higher levels of FENO, +4.2% (95%CI 0.6 to 7.8; p=0.02), +10.7% (95%CI 5.0 to 16.7; p=0.0002) and +14.9% (95%CI 10.6 to 19.3; $p=7.8\times10^{-13}$) respectively. The effect of haplotype (H3) was stronger in subjects with asthma -21.6% (95%CI, -33.5 to -5.9) than in subjects without asthma -4.2 (95%CI -8.4 to 0.2). P-value for interaction between H3 and asthma status was 0.004.

Conclusion: Our findings suggest several common haplotypes in the *NOS2* gene are contributes to variation in FENO in adults. We also saw some evidence of effect modification by asthma status on haplotype (H3).

INTRODUCTION

The fractional concentration of nitric oxide in exhaled air (FENO; fraction of exhaled NO) is suggested as a useful biomarker of airway inflammation [1]. Studies have shown increased levels of FENO in individuals with asthma [2, 3] and FENO correlates with eosinophilic airway inflammation while inhaled corticosteroids reduce FENO [1]. Increased levels of FENO can also be observed in adults and children without respiratory symptoms or asthma, and there are indications that increased FENO may be a marker of future risk of new onset of respiratory symptoms or asthma [4, 5].

Nitric oxide (NO) is formed by conversion of L-arginine to L- citrulline in the presence of one of three distinct isoforms of NO synthase (NOS) known as neuronal NOS (nNOS;NOS1), inducible NOS (iNOS;NOS2) and endothelial NOS (eNOS;NOS3), coded by the three genes *NOS1, NOS2* and *NOS3*, respectively [6]. nNOS and eNOS each generate small amounts NO in the lung. iNOS on the other hand generates substantial amounts of NO primarily in response to inflammatory stimuli such as cytokines, oxidants and infections [7]. The expression of *NOS2* in human airway epithelium cells is regulated by various inflammatory (e.g. nuclear factor kappa B (NFκB)) and non-inflammatory (e.g. Kruppel-like factor) transcriptional factors suggesting that several diverse pathways are involved [7, 8]. A gene expression study has demonstrated that enhanced expression of the *NOS2* gene in the epithelial cells of the airways is related to increased FENO levels in healthy individuals [9]. Genetic association studies in both children and adults have reported that polymorphisms in the *NOS2* gene influence levels of FENO [10-12]. In a previous study we reported two tagging SNPs in *NOS2*, rs9901734 and rs3729508, that were associated with FENO levels in adults without asthma or respiratory symptoms or atopy [12]. A recent study by Bouzigon et al also provided evidence of a relationship between SNPs in the *NOS2* gene and FENO in non-asthmatic adults [11]. In an earlier by Salam et al. have identified comprehensive sets of

haplotypes in the promoter, coding and downstream regions in *NOS2* and found associations of these haplotypes with FENO in children with and without asthma. However, important details about the contribution of the *NOS2* gene to variation in FENO levels are still lacking. In this study, we fine mapped the previous findings by others and us to identify sequence specific combinations of alleles (haplotypes) that influence FENO levels.

MATERIALS AND METHODS

Study population: We used the population-based Adult Onset Asthma and Nitric Oxide (ADONIX) cohort of men and women, aged 25-75 years at the time of sampling and recruited between 2001 and 2008 in Gothenburg, Sweden. The ADONIX cohort includes in total 6679 participants. Part of the study population (1737 subjects) recruited during 2001-2003 were also included in our previous work on the *NOS* genes and FENO[12].

The original study design and protocol of the ADONIX study has been described in detail elsewhere [13, 14]. In brief, all participants received a questionnaire related to current respiratory health status, medical history and smoking habits. Participants who responded to the questionnaire were also invited for clinical examination, which included blood sampling and measurements of FENO. In the present study, asthma was defined based on a positive answer to at least one of the questionnaire items: 'Have you ever had asthma?'; 'Have you ever had asthma diagnosed by a doctor?'; 'Have you had an attack of asthma during the last 12 months?'; 'Do you having asthma during the past month?' Atopy was defined as the presence of specific serum IgE antibodies (≥0.35 kU/1) to any of eight common inhaled allergens (dog, cat, horse, timothy grass, birch, mugwort, house dust mite, and cladiosporum) as determined by the Phadiatop test (PharmaciaDiagnostics; Uppsala, Sweden) [15]. Participants were classified as smokers or non-smokers based on their reported smoking habits.

Measurements of FENO: FENO was measured with an online NO monitoring system (NIOX® ; Aerocrine AB, Stockholm, Sweden), at an exhalation flow of 50 ml/s, according to the 2005 ATS/ERS recommendations [16], after at least 4 hours of fasting. Exhalations were registered for each subject within 10% deviation, and in triplicate between June 2001 and January 2003, and in duplicate from February 2003 through 2008 according to the revised ATS/ERS recommendations [16]. The mean concentration was used for analyses.

SNP selection and genotyping: Ten SNPs in the region spanning from 23.10 Mb to 23.15 Mb of the *NOS2* gene (the NCBI build 36) were selected for analysis based on previously published data regarding their association with FENO (table 2) [10, 12]. Six SNPs (rs9901734, rs2297514, rs2248814, rs12944039, rs3729508 and rs2779248) were main findings from our previous analysis [12]. The remaining four SNPs (rs4796017, rs2297520, rs9895453 and rs10459953) were SNPs from the Salam et al Southern California Children's Health Study that were not in close LD with our earlier findings [10]. SNPs were genotyped using a Sequenom MassARRAY platform (Sequenom San Diego, CA, USA) or a competitive allele specific PCR system, KASPar (KBioscience, Hoddesdon Herts, Great Britain). Genotyping call rate for all SNPs was ≥98% and all the SNPs were in Hardy-Weinberg Equilibrium (HWE) ($p \geq 0.001$). Samples with genotyping success rate ≤80% across the 10 SNPs were excluded from the study. Only subjects reporting European country of birth were included in the present study; 96% were of Swedish origin.

Statistical analysis: Stepwise regression in a forward approach was performed in SAS (Version 9.2, SAS Institute) assuming five different genetic models as previously described [12], to find a subset of SNPs in the *NOS2* gene that were strongly associated with FENO. In the stepwise regression analysis, p-values were set to 0.10 and 0.20 for a SNP to enter and remain in the model, respectively. Subsequently, we considered SNPs with a p-value of ≤0.005 from the stepwise regression for haplotype association analysis. We obtained all

possible haplotype pairs along with their respective likelihoods for each individual, and resulting population frequencies using an E-M (expectation-maximization) algorithm as implemented in the 'haplo.stats' package in the R statistical program [17]. The E-M algorithm estimates haplotype frequencies and posterior probabilities of each pair of haplotypes in each individual given observed genotypic data using a likelihood approach. A generalized linear model using the haplo.glm function implemented in the 'haplo.stats' package was used for estimating the effect of all common haplotypes (haplotype frequencies ≥5%) on levels of FENO, using the most common haplotype as the reference group, assuming an additive genetic model. We evaluated the haplotype effects on FENO by asthma status, by including product term between the haplotypes and asthma in the main effect model.

Since the distribution of FENO was skewed, values were log-transformed prior to analyses. We adjusted all the analyses for age, sex, height, atopy and smoking habits. Results are presented as a percentage change in the geometric mean of FENO across group of subjects, comparing each SNP or haplotype to the respective reference.

RESULTS

In the total cohort (n=6679), DNA was available for 6340 participants. Of these, 5963 (94%) were of European origin and 377 (6%) of non-European origin. We excluded fifty-one individuals due to poor genotyping quality, leaving 5912 European participants. Among these, 5633 participants had FENO values and constituted the final analysis set. Basic characteristics of the study population and FENO levels are presented in Table 1. Geometric mean (±SD) of FENO level was 16.4 (±1.8) parts per billion (ppb) for all subjects. Descriptive statistics and publication source of the ten SNPs are shown in Table 2. The LD pattern of the ten SNPs in the *NOS2* gene, with corresponding r^2 values are shown in supplementary Figure 1.

The stepwise analysis identified a subset of five SNPs with independent associations with FENO (Table 3). The minor allele of one SNP, rs3729508(C/T), showed a negative association, and the other four SNPs (rs4796017(C/G), rs9901734(C/G), rs9895453 (T/C) and rs2779248 (T/C)) showed positive association for the minor allele, with different genetic models.

Seven common haplotypes, each with a frequency of ≥5% in the population, accounted for 84% of all possible haplotype combinations, and rare haplotypes for approximately 16%. The haplotypes and their frequencies defined by the subset of 5 SNPs are presented in Table 4. The most common haplotype 'ACTCT ' was chosen as the baseline haplotype (H1). We found significant associations between four haplotypes (H2, H3, H5 and H6) and FENO (Table 4). Haplotypes H2 (ACCTC), H5 (GGCTC) and H6 (GGCTT) were significantly associated with higher levels of FENO, +4.2% (95%CI 0.6 to 7.8; p=0.02), +10.7% (95%CI 5.0 to 16.7; p=0.0002) and +14.9% (95%CI 10.6 to 19.3; $p=7.8 \times 10^{-13}$), respectively, compared with the baseline haplotype. Haplotype H3 (ACCTT) was significantly associated with lower levels of FENO, -5.8% (95%CI -9.8 to -1.7, p=0.006).

Among subjects with asthma, H2, H4, H5 and H7 were associated with lower FENO values while an opposite effect was observed among subjects without asthma. For H6 the effect was positive in both groups, but stronger in subjects with asthma. However, these differences were statistically not significant. There was a statistically significant difference in the association between H3 and FENO in subjects with asthma as compared to in subjects without asthma (p-value for interaction=0.004), with a more strongly negative effect in subjects with asthma than in subjects without asthma [-21.6, 95%CI -33.5, -5.9 vs -4.2, 95%CI -8.2, 0.2) Table 5.

DISCUSSION

In this study of Swedish adults, we identified four haplotypes in the *NOS2* gene that were strongly and significantly associated with levels of FENO in adults. One haplotype (H3) was associated with lower FENO and the three other (H2, H5 and H6) with higher FENO. In addition to the main effects, we also observed an effect modification by asthma status for association with haplotype H3.

The human *NOS2* gene is one of several genes encoding a NOS enzyme isoform, and it has been demonstrated that expression of this isoform was associated with exhaled NO levels in children [9]. So far, only our previous study and two others have attempted to study association between polymorphisms in the *NOS2* gene and FENO levels in either adults or children [10-12]. In children, various SNPs including rs2297512, rs2774894, rs8081248 and rs4796017 (Salam et al. [10]) and in adults rs9901734, rs2297514 (Dahgam et al. [12]), and most recently rs12601458, rs6505510 (Bouzigon et al. [11]) have been reported to be associated with FENO among subjects without asthma. In our analysis, of the 10 investigated SNPs selected from our study and Salam et al. five showed significant, and independent, evidence of association with FENO levels. This result is in line with the most recent report from the French Epidemiological study on the Genetics and Environment of Asthma (EGEA) assessing effects of genetic variants of the *NOS* genes on exhaled NO among non-asthmatic adults [11]. However, direct experimental evidence to support a functional role of the studied *NOS2* polymorphisms is currently lacking.

Haplotype analysis, which may provide additional information beyond individual SNP analysis about the genetic basis of complex traits and can be helpful in understanding the unit of biological function, has become of more widespread interest in finding casual connections in candidate genes studies. [18-20]. Furthermore, constructing haplotypes from a subset of

informative SNPs reduces the haplotype dimensionality and increases power for detecting associations as compared to separate analysis of individual SNPs [20-22]. Using this approach in our study five-SNP haplotype analysis revealed four haplotypes with strong and significant association with FENO. Although significant associations were found also for individual SNPs, the haplotype analysis revealed very strong associations, and the haplotype model provided a much stronger global p value (3.8×10^{-28}) for association than individual SNPs. Our results generally support the findings in children with and without asthma by Salam et al. who also report several haplotypes of *NOS2* that were associated with increased FENO levels. However, Salam et al [10] used a population-based sample of children that were partly of European and non-European ancestry (non-Hispanic white and Hispanic white), and another set of SNPs to infer all possible haplotypes that occurred to describe variation in levels of FENO. So neither the two study populations nor the haplotype analysis methods are entire comparable.

This study extends our previous work on FENO association. Strengths of this study include a large study sample from a homogenous adult population and a strong biological *a priori* hypothesis. In addition, haplotypes were inferred from a set of SNPs with previously reported association with FENO rather than by just selecting random tag SNPs. Finally, non-European subjects were excluded from the analysis to avoid potential confounding effects of population stratification.

Our findings thus suggest that the haplotype structure across the *NOS2* gene contributing to variation in FENO in adults. Furthermore, we demonstrated that the effect of some of these haplotypes differ by asthma status. The SNPs we used to infer the haplotypes are not located in coding or promoter regions. Potential biological reasons for our results could be that the haplotypes are in strong LD with unmeasured causal genetic alterations or that they are

involved in more complex regulation of gene expression [20]. Further studies can be build on these findings to search for causal genetic variants with respect to the studied gene region.

Tables

Table 1. Baseline characteristics of the study population (n=5633) and FENO levels overall and in subgroups.

Variable	Mean (±SD)
Age, years	52 (±11.7)
Height, cm	173 (±9.2)
$FENO_{50}$ levels, ppb	
All (n=5633)	16.4 (±1.8)
Men (n=2686)	18.2 (±1.8)
Women (n=2947)	14.9 (±1.8)
Smokers (n=959)	11.4 (±1.8)
Non-smokers (n=4674)	17.6 (±1.7)
Asthma (n=726)	17.7 (±2.1)
Atopy (n=1340)	18.9 (±1.9)

FENO: fractional of exhaled nitric oxide; ppb: parts per billion.

Table 2. Summary of genotyped single nucleotide polymorphisms (SNPs) in the *NOS2* gene.

rs-number (dbSNP)	Major/minor allele	SNP position‡	SNP location	MAF(%)	HWE p-value	Previous publications
rs4796017	A/G	23099118	Intergenic	43.6	0.13004	Salam et al 2011
rs9901734*	C/G	23105156	Intergenic	23.3	0.381615	Dahgam et al 2012
rs2297514†	T/C	23117442	Intron	40	0.22077	Dahgam et al 2012
rs2248814†	G/A	23124448	Intron	40.6	1	Dahgam et al 2012
rs12944039†	G/A	23128891	Intron	21.2	0.784704	Dahgam et al 2012
rs2297520	C/T	23132167	Intron	40.2	0.54992	Salam et al 2011
rs3729508*	C/T	23133157	Intron	40.3	0.786645	Dahgam et al 2012
rs9895453	T/C	23134884	Intron	47.7	0.834329	Salam et al 2011
rs10459953	G/C	23151645	UTR-5′	35.7	0.55049	Salam et al 2011
rs2779248†	T/C	23151959	Near-gene-5‖	38.5	0.868869	Dahgam et al 2012

NOS2: inducible Nitric oxide synthase; MAF : Minor allele frequency; HWE: Hardy-Weinberg Equilibrium; * Top SNPs associated with FENO in multi-SNP analysis and † strongest p-values for association with FENO in single-SNP analysis in our previous work [12]. All SNPs are oriented to the forward strand of the NCBI build 36. ‖ Intergenic, but within 2000 bases of a transcribed region.

Table 3. Effect of gene variants of NOS2 on levels of FENO. Results from the stepwise regression with different genetic models.

dbSNP	Genotype	Prevalance (%)	Genetic model	% Change in FENO, (95%CI)	P-value
rs4796017	AA‡	32.5			
	AG	48.5			
	GG	19.2	Additive	4 (1.1,6.7)*	0.00542
rs9901734	CC‡	59.2			
	CG+GG	40.8	Dominant	7.8 (3.9,11.8)	0.00006
rs3729508	CC‡	35.5			
	TC	48.3			
	TT	16.2	Additive	-12.7(-12.7,-17.3)*	<0.0001
rs9895453	TT‡	27			
	TC	56.4			
	CC	22	Additive	10.5(6.1,15.2)*	<0.0001
rs2779248	TT‡	37.8			
	TC+CC	62.2	Dominant	6.3 (2.6,10.1)	0.00074

iNOS:NOS2: Inducible Nitric oxide synthase. FENO: Fraction of exhaled nitric oxide.‡ Reference genotype. *Effect per minor allele.

Table 4. Association between haplotypes of the *NOS2* gene and levels of FENO (n=5633): Main effect model.

Haplotypes	rs4796017	rs9901734	rs3729508	rs9895453	rs2779248	Frequency (%)	% Change in FENO, (95%CI)	p-value
H1(Baseline)	A	C	T	C	T	30	Reference	
H2	A	C	C	T	C	14	4.2 (0.6,7.8)	0.02
H3	A	C	C	T	T	9	-5.8 (-9.8,-1.7)	0.006
H4	G	C	C	T	C	10	3.6 (-0.3,7.7)	0.07
H5	G	G	C	T	C	5	10.7 (5.1,16.7)	0.0002
H6	G	G	C	T	T	10	14.9 (10.6,19.3)	7.8×10^{-13}
H7	G	G	T	C	T	6	3.2 (-2.3,8.9)	0.26
Rare	*	*	*	*	*	16	16 (12.3,19.8)	<0.001

FENO: Fraction of exhaled nitric oxide; NOS2:iNOS: Inducible Nitric oxide synthase; CI confidence interval. Effects of haplotype on FENO was expressed as percentage difference in levels of FENO compared with most common haplotype .The model was adjusted for age, sex, height, atopy and smoking habits, assuming additive genetic model. P-value for global test for association: 3.88×10^{-28}.

Table 5. Association between haplotypes of the *NOS2* gene and levels of FENO, by asthma status.

	Asthma status		
	Yes(n=726)	No(n=4907)	
Haplotype	% Change in FENO, (95%CI)	% Change in FENO, (95%CI)	P-value for interaction(haplotype*asthma)
(H2)ACCTC	-2.8(-16.5,12.2)	5.3(1.5,9.2)	0.138
(H3)ACCTT	-21.6(-33.5,-5.9)	-4.2(-8.4,0.2)	0.004
(H4)GCCTC	-5.6(-20.2,10.5)	4.9(1.0,9.4)	0.075
(H5)GGCTC	-1.6(-22.8,21.4)	12.1(6.0,18.5)	0.092
(H6)GGCTT	24.3(7.6,47.1)	13.8(9.4,18.5)	0.091
(H7)GGTCT	-6.4(-26.1,17.4)	3.8(-2.0,9.9)	0.224

FENO: Fraction of exhaled nitric oxide; NOS2:iNOS: Inducible Nitric oxide synthase; CI confidence interval .The model was adjusted for age, sex, height, atopy and smoking habits, assuming additive genetic model. Effect modifications were calculated as follows: when asthma = Yes, the effect of haplotype is β coefficient of haplotype+ β coefficient of interaction term, and when asthma = NO, the effect of haplotype is β coefficient of haplotype. SNPs order in haplotypes: rs4796017, rs9901734, rs3729508, rs9895453 and rs2779248.

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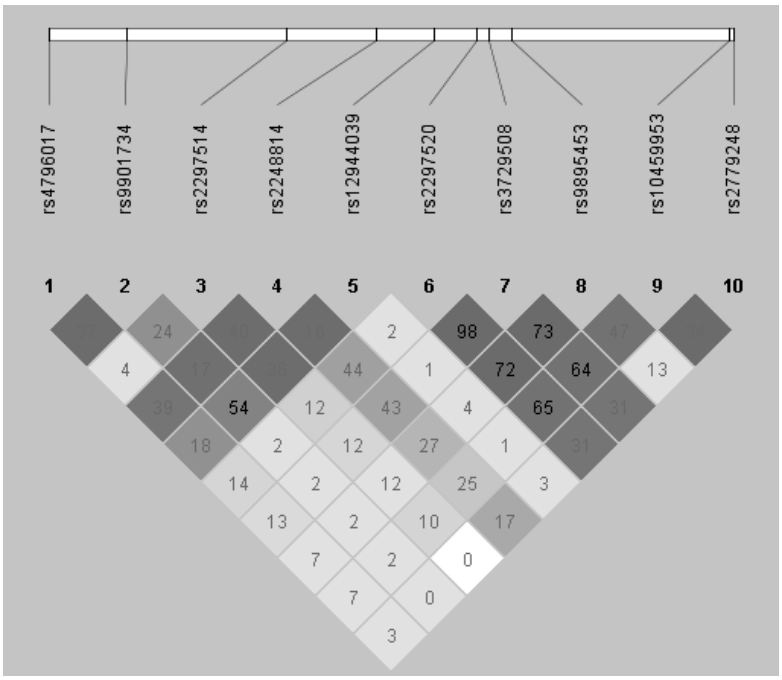
Supplementary figure



Supplementary figure 1. Linkage disequilibrium (LD) map of 10 SNPs in *NOS2* gene on chromosome 17. The number in each box corresponds to the pairwise correlation coefficient r^2 (a measure of LD) between any two SNPs and shading indicates D' (a measure of LD; red shade indicates high D' and light red shade indicates low D').