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Fractional exhaled nitric oxide in childhood is associated with 17q11.2-q12 and 17q12-q21 variants

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Abstract

Background—The fractional concentration of nitric oxide in exhaled air (FeNO) is a biomarker of eosinophilic airway inflammation and associated with childhood asthma. Identification of common genetic variants associated with childhood FeNO may help to define biological mechanisms related to specific asthma phenotypes.

Objective—To identify genetic variants associated with childhood FeNO, and their relation with asthma.

Methods—FeNO was measured in children aged 5 to 15 years. In 14 genome-wide association (GWA) studies (N = 8,858), we examined the associations of ~2.5 million single nucleotide polymorphisms (SNPs) with FeNO. Subsequently, we assessed whether significant SNPs were expression quantitative trait loci (eQTLs) in genome-wide expression datasets of lymphoblastoid cell lines (N = 1,830), and were related with asthma in a previously published GWA dataset (cases: n=10,365; controls: n=16,110).

Results—We identified 3 SNPs associated with FeNO: rs3751972 in *LYR motif containing 9* (*LYRM9*) ($P = 1.97\times10^{-10}$) and rs944722 in *inducible nitric oxide synthase 2* (*NOS2*) ($P = 1.28\times10^{-9}$) both located at 17q11.2-q12, and rs8069176 near *gasdermin B* (*GSDMB*) ($P = 1.88\times10^{-8}$) at 17q12-q21. We found a *cis* eQTL for the transcript *soluble galactoside-binding lectin 9* (*LGALS9*) that is in linkage disequilibrium with rs944722. Rs8069176 was associated with *GSDMB* and *ORM1-like 3* (*ORMDL3*) expression. Rs8069176 at 17q12-q21, and not rs3751972 and rs944722 at 17q11.2-q12, were associated with physician-diagnosed asthma.

Conclusion—This study identified 3 variants associated with FeNO, explaining 0.95% of the variance. Identification of functional SNPs and haplotypes in these regions might provide novel insight in the regulation of FeNO. This study highlights that both shared and distinct genetic factors affect FeNO and childhood asthma.

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Author's contributions
All authors participated substantially in the design of the study, data acquisition or analysis, and writing or revising the manuscript. The manuscript has been reviewed and approved by all authors.

None of the authors have conflict of interest.

Keywords

airway inflammation; asthma phenotypes; biomarker; genetics; genome-wide association study

INTRODUCTION

Asthma is a complex disease with different phenotypes, influenced by many genetic and environmental factors[1]. Why children develop specific asthma phenotypes is still poorly understood[2, 3]. Genetic association studies may help to identify biological pathways underlying the clinical expression of asthma. Recent genome-wide association (GWA) studies provided evidence that different common genetic variants are associated with specific asthma-related outcomes such as childhood onset asthma[4-6], adult asthma[5-7], impaired lung function[8-11], and atopy[12-14].

The fractional concentration of nitric oxide in exhaled air (FeNO) is a noninvasive biomarker of eosinophilic airway inflammation[15-17]. Higher FeNO is associated with childhood asthma symptoms[18], exacerbations[19], physician-diagnosed asthma[15-17] and atopy[20]. Nitric oxide is a reactive free-radical gas generated in the airway epithelium when L-arginine is oxidized to L-citrulline[17]. This reaction is catalyzed by nitric oxide synthases (NOS), that are upregulated in the presence of pro-inflammatory cytokines and inflammatory mediators[17]. Nitric oxide regulates airway and blood vessel tone and high concentrations have antimicrobial effects[17]. Although 60% of the variance in FeNO in adults can be explained by heritability[21], the genetic loci that influence FeNO are largely unknown. Identification of common genetic variants associated with childhood FeNO may help to define biological mechanisms related to specific asthma phenotypes[2, 3, 22, 23].

To identify common genetic variants associated with childhood FeNO, we examined the association of ~2.5 million directly genotyped and imputed single nucleotide polymorphisms (SNPs) with FeNO in 14 independent pediatric discovery GWA studies (N = 8,858).

METHODS

FeNO was measured online in children aged 5 to 15 years according to European Respiratory Society (ERS) and American Thoracic Society (ATS) guidelines[16]. FeNO was natural-log transformed to obtain a normal distribution. We applied linear regression between allele dosages obtained from imputations and natural-log FeNO adjusted for sex and age at time of measurement. Details on the SNP discovery analysis and additional analyses, including the analysis to determine independent SNP effects, explained variance analyses and stratified analysis for current asthma, are presented in the Online Repository Materials methods section, and an overview of our study design is outlined in Figure I. Details on individual study characteristics, SNP genotyping platforms and study association analyses are provided in Repository Table E1.

We assessed whether significant SNPs or SNPs in linkage disequilibrium (LD, a measure of correlation between SNPs) with our lead SNPs were functional annotated SNPs using HaploReg[24] and SIFT (http://sift.jcvi.org/), and were situated in genomic loci that are

involved in the regulation of messenger RNA expression (the so-called 'expression quantitative trait loci' or eQTLs). For the second purpose we used available genome-wide expression datasets of human lymphoblastoid cell lines (N = 1,830)[25, 26].

We tested the relation of significant SNPs with asthma using a previously published GWA dataset of physician-diagnosed asthma (cases: n=10,365; controls: n=16,110)[5]. We explored whether the SNPs identified in the present GWA study were related with FeNO in adults in the Epidemiological study on the Genetics and Environment of Asthma (EGEA) and in Hutterites (N = 1,211).

Finally, we explored whether common genetic variants known to be associated with physician-diagnosed asthma[5] were related with childhood FeNO.

The institutional review boards for human studies approved the protocols and written consent was obtained from the participating subjects or their caregivers if required by the institutional review board.

RESULTS

We identified genome-wide significant ($P < 5\times10^{-8}$) association of childhood FeNO and SNPs at 3 genetic loci. Two SNPs were located at chromosome 17q11.2-q12: the SNP rs3751972 in the *LYR motif containing 9* (*LYRM9*) gene and rs944722 in the *NOS2* gene (Table I). Each C allele of rs3751972 was associated with higher ln(FeNO) (*ß* = 0.09 ppb; S.E. = 0.014; $P = 1.97\times10^{-10}$; explained variance = 0.23%), and each C allele of rs944722 was associated with lower ln(FeNO) (*ß* = -0.07 ppb; S.E. = 0.012; $P = 1.28\times10^{-9}$; explained variance = 0.30%). Rs3751972 and rs944722 are in neighboring loci with low LD, indicating that the two SNPs might not represent the same genetic variation (HapMap pairwise LD, phase II release 22 CEU; D' = 0.237, $r^2 = 0.014$). A third SNP, rs8069176 near the *gasdermin B* (*GSDMB*) gene at 17q12-q21 was also associated with childhood FeNO. Each A allele of rs8069176 was associated with lower ln(FeNO) (*ß* = -0.07 ppb; S.E. = 0.012; $P = 1.88\times10^{-8}$; explained variance = 0.41%). Figure II-IV show the QQ-, Manhattan-, regional association- and forest plots of the 3 signals.

We used the genome-wide complex trait analysis (GCTA) tool to determine if SNP effects were independent. We conditioned on all SNPs of the meta-analysis[27], and showed that rs3751972 and rs944722 were indeed independent signals and did not represent the same genetic variation (Repository Table E2). After conditioning on all SNPs of the meta-analysis, rs3751972 and rs2274894 showed the strongest association in the *LYRM9* gene ($P = 2.06\times10^{-9}$) and in the *NOS2* gene ($P = 1.50\times10^{-8}$, rs2274894 not rs944722 is the strongest signal using GCTA) respectively. Using the same approach, rs8069176 showed the strongest association at 17q12-q21 ($P = 2.14\times10^{-8}$).

The 3 genome-wide significant SNPs showed low heterogeneity between studies (all $P \geq 0.075$, $I^2 = 0 – 37.8\%$). The 3 SNPs together explained 0.95% of the variance in FeNO. Other suggestive loci that were associated with FeNO, but did not reach genome-wide significance ($P < 1\times10^{-5}$), are given in Repository Tables E3 and E4. The associations of genetic variants in the *nitric oxide synthases* or *arginase* genes might be different among

asthmatic versus non-asthmatic children[28]. Therefore, we performed a sensitivity analysis adjusting for current asthma and this produced comparable results for the SNPs in *LYRM9* and *NOS2* and a slightly lower effect for the SNP in the 17q12-q21 locus (Repository Table E5). In addition, we showed that the 3 SNPs were also associated with FeNO in non-asthmatic children (Repository Table E6).

We assessed whether there were common non-synonymous variants with deleterious functional implications in LD ($r^2 > 0.80$) with our 3 genome-wide significant SNPs using HaploReg[24], a data base for functional annotation of SNPs. We found 3 variants, rs11557467, rs2305480 and rs2305479 that were in high LD with rs8069176 at 17q12-q21. Rs11557467 is located in the *zona pellucida binding protein 2* (*ZPBP2*) gene, holding a high risk deleterious effect consisting of a missense variation resulting in a non-conservative amino acid change. Rs2305480 and rs2305479 in the GSDMB gene are both variations with a high risk of deleterious effect resulting from a missense change leading to abolishment of a protein domain. We did not find functional implications for rs3751972 and rs944722 at 17q11.2-q12. The nature of the amino-acid changes, and predicted functional significances using SIFT (http://sift.jcvi.org/), as well as the frequencies, LD with the index SNP at 17q12-q21 and *P* values for FeNO association are depicted in Repository Table E7.

Subsequently, we assessed whether the identified 3 loci were eQTLs in genome-wide expression datasets of lymphoblastoid cell lines (N = 1,830)[25, 26]. We found a *cis* eQTL for the transcript *soluble galactoside-binding lectin 9* (*LGALS9*) in LD with rs944722 in two independent datasets (Repository Tables E8 and E9). *LGALS9* is downstream of the *NOS2* gene. Rs8069176 was associated with both *GSDMB*- and *ORM1-like 3* (*ORMDL3*) gene expression. We did not find eQTLs for rs3751972.

We tested the associations of the 3 FeNO-associated SNPs with physician-diagnosed asthma in a previously published GWA dataset (cases: n=10,365; controls: n=16,110)[5]. The SNP rs8069176 was not available and we used rs2305480 as a proxy. The rs2305480[A] minor allele at the 17q12-q21 locus was associated with a decreased risk of asthma (odds ratio (OR) 0.85; 95% CI 0.81 - 0.88; $P = 7.93\times10^{-17}$; Table II). This is in line with the association with lower FeNO that we found for rs8069176[A]. The SNPs rs3751972 and rs944722 were not associated with an asthma diagnosis ($P \geq 0.3$). The 3 childhood FeNO-associated SNPs were not associated with adult FeNO (N = 1,211, Table II).

Finally, we explored whether common genetic variants known to be associated with physician-diagnosed asthma[5] were related with childhood FeNO. We found that known asthma SNPs rs2305480 at 17q12 (*GSDMB*), rs3894194 at 17q21.1 (*GSDMA*), rs744910 at 15q22.33 (*SMAD3*) and rs1295686 at 5q31 (*IL13*) were indeed associated with childhood FeNO (all $P \leq 0.005$, after Bonferroni correction; Table III). The directions of the SNP effects were as expected. The asthma SNPs together explained 0.32% of the variance in FeNO.

DISCUSSION

We identified associations between FeNO and genetic variants at 3 loci. The common variants in and near the *LYRM9* and *NOS2* genes were located at 17q11.2-q12, the third signal was at 17q12-q21, harboring the *ZPBP2*, *GSDMB*, and *ORMDL3* genes. The three independently associated genetic variants at the 3 loci explained 0.95% of the total variance in FeNO.

The function of the *LYRM9* gene is unknown; variants in the *nitric oxide synthases* and *arginase* genes jointly contributed to differences in FeNO in previous studies[28-31], and variation in *arginase* genes to asthma severity[32]. We did not find associations between the *NOS2* and *LYRM9* SNPs and asthma. It has been shown previously that the inducible NOS2 protein is higher in adults with severe asthma[33]. Unfortunately, we do not have data of the two SNPs and severe asthma cases. Inducible NOS2 is expressed in airway epithelium and is synthesized in response to pro-inflammatory cytokines and mediators. Expression of inducible NOS2 may be beneficial in host defense and in modulating the immune response[17, 34]. In our study genetic variants in *inducible NOS2*, but not in *neuronal NOS1* and *constitutive NOS3*, were robustly associated with childhood FeNO. A previous study suggested that DNA methylation in promotor regions of *arginase* genes were associated with FeNO in children with asthma[29]. Thus, DNA methylation could also play an important role in epigenetic regulation of other genes for NO production.

We found a *cis* eQTL for the transcript *LGALS9* in LD with rs944722, downstream of *NOS2*, and this suggests that the protein Gal-9 may be involved in the regulation of FeNO. Gal-9 plays a crucial role in immune responses, including allergic inflammation. Gal-9 was shown to inhibit allergic airway inflammation, and airway hyperresponsiveness by modulating CD44-dependent leukocyte recognition of the extracellular matrix in mice[35]. Results in guinea pigs showed that Gal-9 might be involved in prolonged eosinophil accumulation in the lung[36]. A recent study suggested a novel function of Gal-9 in mast cells and suggested that Gal-9 might be an interesting new target for the treatment of allergic disorders including asthma[37].

The 17q12-q21 asthma locus, harboring the *ZPBP2*, *GSDMB*, and *ORMDL3* ‘asthma genes’, is a complex region with high LD[4, 5, 38, 39]. *GSDMB* may be involved in the regulation of the growth and differentiation of epithelial cells[40, 41]. The function of the upstream *ORMDL3* gene in humans is not clear. The *ORMDL* family genes encode for transmembrane proteins located in the endoplasmic reticulum membrane. In mice, double knockout of the *ORMDL* genes leads to slower growth and higher sensitivity to toxic compounds in mice[42]. The function of the downstream *ZPBP2* gene is not known. Hence, the mechanisms by which 17q12-q21 variants may regulate FeNO remains to be elucidated.

The three genetic variants identified in the present study explained only a small proportion of the total variance in FeNO, while earlier work on twins indicated that most of FeNO variation is genetically determined. One explanation could be that the heritability of FeNO was overestimated. Lund *et al* estimated the heritability but did not adjust for body height, a determinant of adult FeNO[31]. Furthermore, atopic adults were excluded from their

analysis[21]. In the present study we did not exclude atopic children. Most GWA studies are underpowered to detect a large fraction of the variance conferred by polygenic traits. Big consortia showed consistent genetic architecture of > 1000 alleles for the average polygenic trait[43, 44]. We determined the genetic variance explained at the whole genome SNP level using a GCTA analysis[27], which was 21.3% ($P = 0.100$) in the largest cohort (Generation R Study, Caucasians only, n = 1,332). The missing heritability in our study is most likely explained by other genetic mechanisms, including missing information on causal (rare) variants, interaction between genes, between environmental factors and genes, and by epigenetic mechanisms[45]. It has also been suggested that the association between asthma and FeNO may be entirely explained by atopy[46]. We found an association between the 17q12-q21 childhood asthma locus and FeNO. This suggests that FeNO is related with asthma independent of allergy, as variants at the 17q12-q21 locus are not associated with specific atopic outcomes. The signals in *NOS2* and *LYRM9* were not associated with asthma, which conflicts with a possible causal effect of FeNO on asthma. One explanation could be that FeNO and asthma are not directly related but may have mechanisms in common. Unfortunately, we were not able to assess haplotypes or other types of genetic variation in the *NOS2* and *LYRM9* regions that could play a role in the development of asthma in our *in silico* database of patients with childhood- and adult-onset asthma.

In summary, we identified 3 independent signals that were associated with childhood FeNO in the *LYRM9* and *NOS2* genes, which are both located at 17q11.2-q12, and near the *GSDMB* gene at 17q12-q21. The 3 SNPs together explained 0.95% of the variance in FeNO. Identification of functional SNPs and haplotypes in these regions might provide novel insight in the regulation of FeNO. This study highlights that both shared and distinct genetic factors affect FeNO and childhood asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATS	American Thoracic Society
EGEA	Epidemiological study on the Genetics and Environment of Asthma
eQTLs	expression quantitative trait loci
ERS	European Respiratory Society
FeNO	fractional concentration of nitric oxide in exhaled air
GCTA	genome-wide complex trait analysis
GSDMB	gasdermin B
GWA	genome-wide association
LD	linkage disequilibrium
LGALS9	soluble galactoside-binding lectin 9
LYRM9	LYR motif containing 9
NOS	nitric oxide synthases
ORMDL3	ORM1-like 3
SNPs	single nucleotide polymorphisms
ZPBP2	zona pellucida binding protein 2

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Key messages

■ We identified 3 independent genetic variants associated with childhood FeNO, one of the variants was also associated with physician-diagnosed asthma.

■ Future studies are needed to unravel the mechanisms by which the variants regulate childhood FeNO and asthma.

Capsule summary

This study highlights that both shared and distinct genetic factors affect childhood FeNO and asthma.

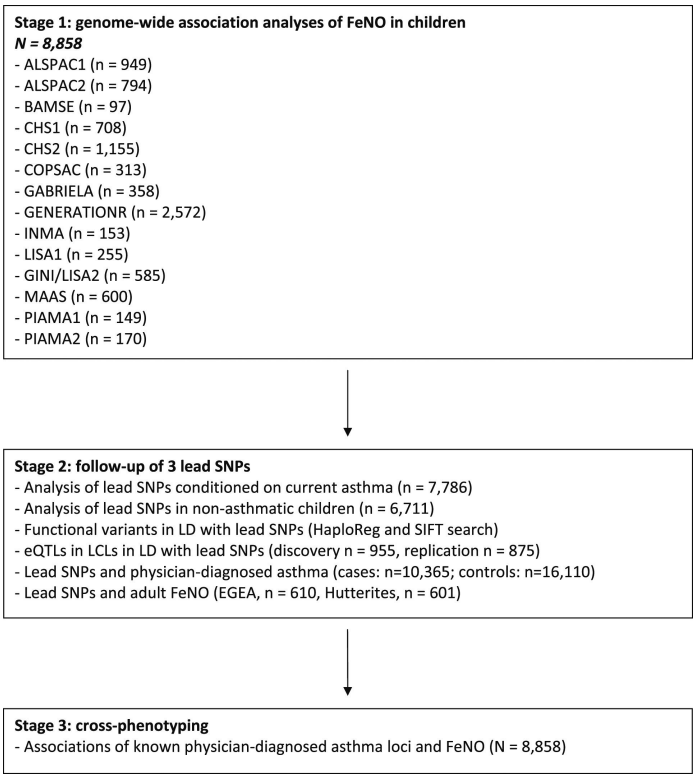

Figure I. Study design

SNPs, single nucleotide polymorphisms; LD, linkage disequilibrium; eQTLs, expression quantitative trait loci; LCLs, lymphoblastoid cell lines.

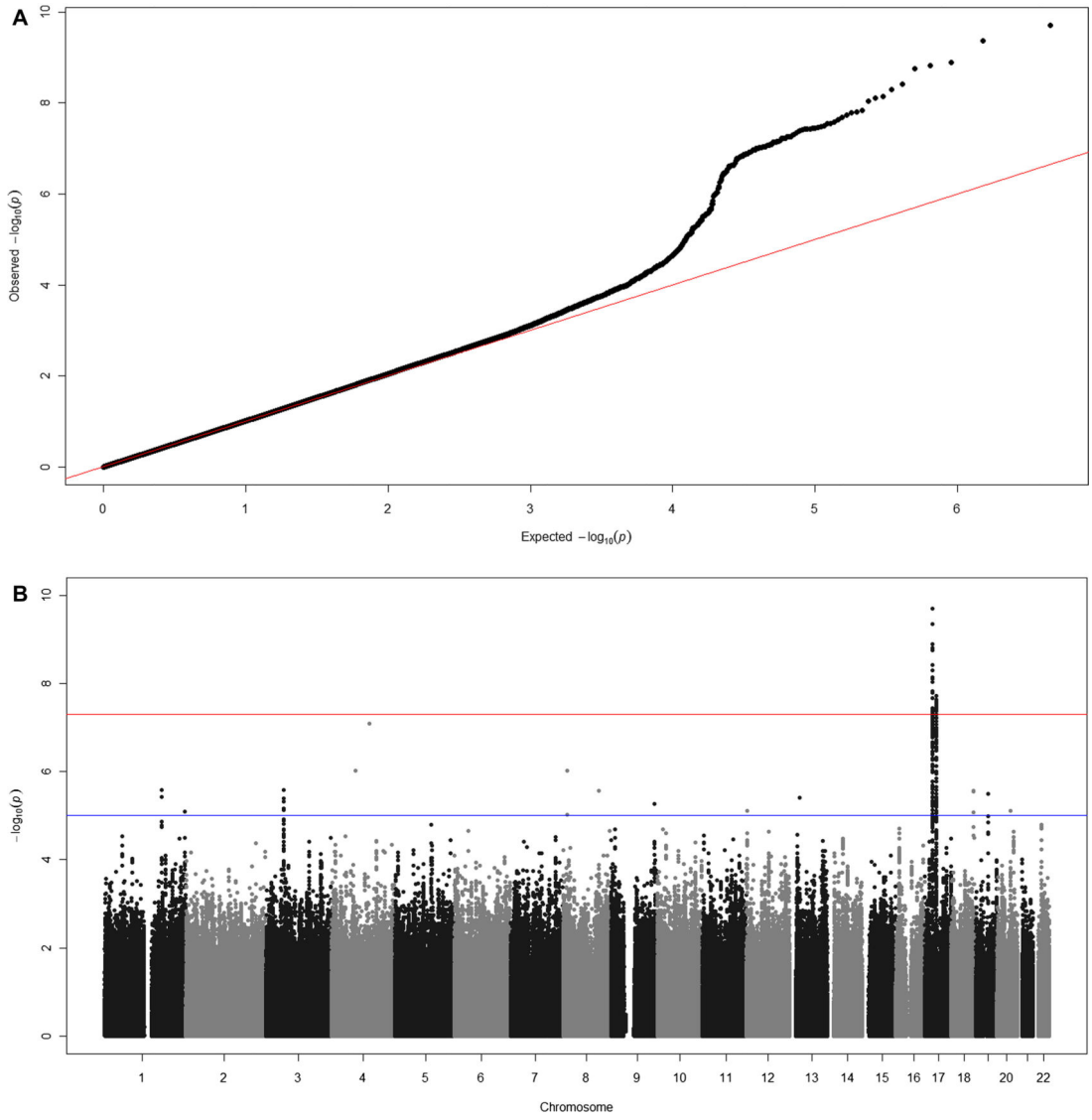

Figure II. QQ and Manhattan plots of 2,253,077 SNPs of 14 GWA studies (N = 8,858)

QQ plot of 2,253,077 SNPs of 14 GWA studies. The black dots represent observed *P* values and the red line represents the expected *P* values under the null distribution. Manhattan plot showing the association *P* values of FeNO of the 14 studies. The $-\log_{10}$ of the *P* value for each of 2,253,077 SNPs (y-axis) is plotted against the genomic position (x-axis).

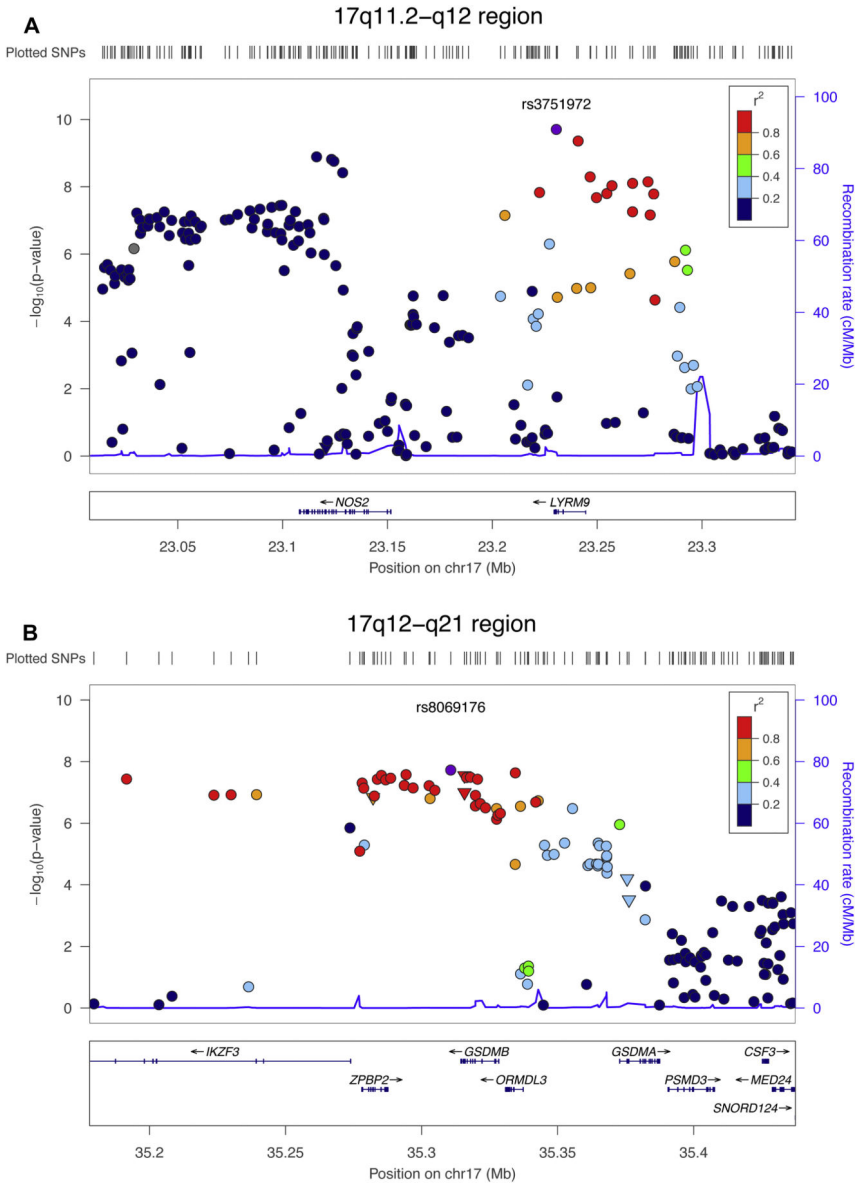

Figure III. Association plots of the 17q11.2-q12 and 17q12-q21 regions

For both the 17q11.2-q12 and 17q12-q21 regions, SNPs are plotted with their *P* values (as $-\log_{10}$ values; left y-axis) as a function of genomic position (x-axis). Estimated recombination rates (right y-axis) taken from HapMap are plotted to reflect the local LD structure around the top associated SNP (purple circle) and their correlated proxies (according to a blue to red scale from $r^2 = 0$ to 1). Triangles represent nonsynonymous SNPs.

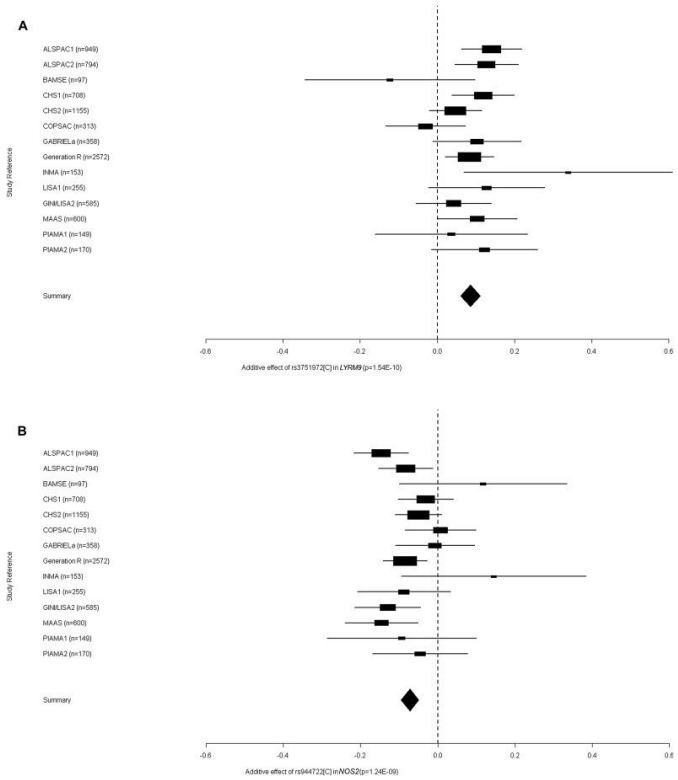

Figure IV. Forest plots of the associations between FeNO and the 3 SNPs associated with FeNO at $P < 5 \times 10^{-8}$

Forest plots of the associations between FeNO and the SNPs in *LYRM9* (a), *NOS2* (b) and near *ZPBP2-GSDMB* (c) at $P < 5 \times 10^{-8}$. In each plot, the triangle indicates the effect size and the confidence interval in the 14 studies. The *P* values in the plots are without genomic control correction.

Table I

Summary statistics of the 3 SNPs at $P < 5\times10^{-8}$.

Marker	MAF	β	S.E.	*P*	I^2	*HetP*
rs3751972[C] at 17q11.2 (*LYRM9*)	0.25	0.086	0.014	1.97×10^{-10}	27.4	0.161
rs944722[C] at 17q11.2-q12 (*NOS2*)	0.38	−0.073	0.012	1.28×10^{-09}	37.8	0.075
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.43	−0.066	0.012	1.88×10^{-08}	0.0	0.668

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Independent SNPs with a genome-wide significant effect on FeNO levels in children are shown ($P < 5\times10^{-8}$). The total sample includes data of 14 independent GWA datasets (N = 8,858). MAF, minor allele frequency; S.E., standard error. β reflects differences in natural log-transformed FeNO per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for sex and age at time of measurement (fixed-effect additive genetic model). Derived inconsistency statistic I^2 and *HetP* values reflect heterogeneity across studies with the use of Cochran's Q tests.

Table II

Association of the 3 SNPs related to childhood FeNO with physician-diagnosed asthma and adult FeNO.

Physician-diagnosed asthma (cases = 10,365 : controls = 16,110)[5]		
Marker	**OR (95% CI)**	***P***
Proxy for rs3751972: rs4796222[A] (r^2=1.000; D′=1.000) at 17q11.2 (*LYRM9*)	0.98 (0.93-1.02)	0.303
Proxy for rs944722: rs2274894[T] (r^2=0.967; D′=1.000) at 17q11.2-q12 (*NOS2*)	1.00 (0.96-1.04)	0.983
Proxy for rs8069176: rs2305480[A] (r^2=1.000; D′=1.000) at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.85 (0.81-0.88)	7.93×10^{-17}

Adult FeNO			
Marker (EGEA, n = 610)	***β***	**S.E.**	***P***
rs3751972[C] at 17q11.2 (*LYRM9*)	0.125	0.065	0.057
rs944722[C] at 17q11.2-q12 (NOS2)	−0.015	0.061	0.802
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	−0.113	0.062	0.067

Marker (Hutterites, n = 601)	**Z score**	***P***
Proxy for rs3751972: rs4796228[G] (r^2=0.659; D′=1.000) at 17q11.2 (*LYRM9*)	−1.536	0.125
Proxy for rs944722: rs2314809[T] (r^2=0.967; D′=1.000) at 17q11.2-q12 (*NOS2*)	−2.322	0.020
Proxy for rs8069176: rs11078927[T] (r^2=1.000; D′=1.000) at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.505	0.613

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). Independent SNPs with a genome-wide significant effect on FeNO levels in children are shown ($P < 5\times10^{-8}$) in relation to physician-diagnosed asthma[5] and adult FeNO. S.E., standard error. Odds ratios (OR) with 95% confidence interval (CI) for physician-diagnosed asthma[5]. *β* reflects differences in natural log-transformed FeNO per minor allele for adult FeNO in EGEA. Z-score reflects the strength of the association between SNP and natural log-transformed FeNO and the direction of the effect of the minor allele in Hutterites.

Table III

Association of known physician-diagnosed asthma loci, from a previous GWA study[5] with childhood FeNO.

Physician-diagnosed asthma[5]						
Marker	**MAF**	***β***	**S.E.**	***P***	***I²***	***HetP***
rs2305480[A] decreasing risk-allele at 17q12 (*GSDMB*)	0.42	−0.065	0.012	2.83×10^{-08}	0.0	0.731
rs3894194[A] increasing risk-allele at 17q21.1 (*GSDMA*)	0.47	0.048	0.012	6.35×10^{-05}	9.5	0.349
rs744910[A] decreasing risk-allele at 15q22.33 (*SMAD3*)	0.49	−0.039	0.012	8.41×10^{-04}	0.0	0.491
rs1295686[T] increasing risk-allele at 5q31 (*IL13*)	0.27	0.044	0.014	1.25×10^{-03}	4.6	0.401
rs1342326[C] increasing risk-allele at 9p24.1 (IL33)	0.17	0.025	0.016	0.119	0.0	0.515
rs9273349[T] decreasing risk-allele at 6p21.3 (*HLA-DQ*)	0.37	−0.022	0.022	0.310	0.0	0.802
rs11071559[T] decreasing risk-allele at 15q22.2 (*RORA*)	0.14	−0.014	0.017	0.415	0.0	0.651
rs3771166[A] decreasing risk-allele at 2q12 (*IL18R1*)	0.35	−0.009	0.012	0.463	7.4	0.371
rs2284033[A] decreasing risk-allele at 22q13.1 (*IL2RB*)	0.42	0.005	0.012	0.705	0.0	0.633
rs2073643[T] increasing risk-allele at 5q23.3 (*SLC22A5*)	0.47	0.000	0.012	0.993	0.0	0.590

Single nucleotide polymorphisms (SNPs) markers are identified according to their standard rs numbers (NCBI build 36). We explored whether common genetic variants known to be related with physician-diagnosed asthma[5] were associated with childhood FeNO. The total sample includes data of 14 independent GWA datasets (N = 8,858). MAF, minor allele frequency; S.E., standard error. β reflects differences in natural log-transformed FeNO per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FeNO adjusted for sex and age at time of measurement (fixed-effect additive genetic model). Derived inconsistency statistic I^2 and *HetP* values reflect heterogeneity across studies with the use of Cochran's Q tests.