Fraction of exhaled nitric oxide values in childhood are associated with 17q11.2-q12 and 17q12-q21 variants

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Background: The fraction of exhaled nitric oxide (FENO) value is a biomarker of eosinophilic airway inflammation and is associated with childhood asthma. Identification of common genetic variants associated with childhood FENO values might help to define biological mechanisms related to specific asthma phenotypes.

Objective: We sought to identify the genetic variants associated with childhood FENO values and their relation with asthma.

Methods: FENO values were measured in children age 5 to 15 years. In 14 genome-wide association studies (N = 8,858), we examined the associations of approximately 2.5 million single nucleotide polymorphisms (SNPs) with FENO values. Subsequently, we assessed whether significant SNPs were expression quantitative trait loci in genome-wide expression data sets of lymphoblastoid cell lines (n = 1,830) and were related to asthma in a previously published genome-wide association data set (cases, n = 10,365; control subjects: n = 16,110).

Results: We identified 3 SNPs associated with FENO values: rs3751972 in LYR motif containing 9 (*LYRM9*; $P = 1.97 \times 10^{-10}$) and rs944722 in inducible nitric oxide synthase 2 (*NOS2*; $P = 1.28 \times 10^{-9}$), both of which are located at 17q11.2-q12, and rs8069176 near gasdermin B (*GSDMB*; $P = 1.88 \times 10^{-8}$) at 17q12-q21. We found a *cis* expression quantitative trait locus 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, but not rs3751972 and rs944722 at 17q11.2-q12, were associated with physician-diagnosed asthma.

Conclusion: This study identified 3 variants associated with FENO values, explaining 0.95% of the variance. Identification of functional SNPs and haplotypes in these regions might provide novel insight into the regulation of FENO values. This study highlights that both shared and distinct genetic factors affect FENO values and childhood asthma. (J Allergy Clin Immunol 2014;134:46-55.)

Key words: *Airway inflammation, asthma phenotypes, biomarker, genetics, genome-wide association study*

Abbreviations used

eQTL:	Expression quantitative trait locus
FENO:	Fraction of exhaled nitric oxide
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 synthase
ORMDL3:	ORM1-like 3
SNP:	Single nucleotide polymorphism
ZPBP2:	Zona pellucida binding protein 2

Asthma is a complex disease with different phenotypes that is influenced by many genetic and environmental factors.[1] Why children have specific asthma phenotypes is still poorly understood.[2,3] Genetic association studies might 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 fraction of exhaled nitric oxide (FENO) value is a noninvasive biomarker of eosinophilic airway inflammation.[15-17] Higher FENO values are 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 (NOSs), which are upregulated in the presence of proinflammatory 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 values in adults can be explained by heritability,[21] the genetic loci that influence FENO values are largely unknown. Identification of common genetic variants associated with childhood FENO values might help to define biological mechanisms related to specific asthma phenotypes.[2,3,22,23]

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

METHODS

FENO values were measured online in children age 5 to 15 years according to European Respiratory Society and American Thoracic Society guidelines.[16] FENO values were natural log transformed to obtain a normal distribution. We applied linear regression between allele dosages obtained from imputations,

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and natural log FENO values were adjusted for sex and age at the time of measurement. Details on 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 Methods section in this article's Online Repository at www.jacionline.org, and an overview of our study design is outlined in Fig 1. Details on individual study characteristics, SNP genotyping platforms, and study association analyses are provided in Table E1 in this article's Online Repository at www.jacionline.org.

We assessed whether significant SNPs or SNPs in linkage disequilibrium (LD; a measure of correlation between SNPs) with our lead SNPs were functionally annotated SNPs by using HaploReg[24] and SIFT (http://sift.jcvi.org/) and were situated in genomic loci that are involved in the regulation of mRNA expression (the so-called expression quantitative trait loci [eQTLs]). For the second purpose, we used available genome-wide expression data sets of human lymphoblastoid cell lines (n = 1,830).[25,26]

We tested the relation of significant SNPs with asthma by using a previously published GWA data set of physician-diagnosed asthma (cases, n = 10,365; control subjects, n = 16,110).[5] We explored whether the SNPs identified in the present GWA study were related to FENO values in adults in the Epidemiological Study on the Genetics and Environment of Asthma and in Hutterites (n = 1,211).

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

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 ($P < 5 \times 10^{-8}$) association of childhood FENO values and SNPs at 3 genetic loci. Two SNPs were located at chromosome 17q11.2-q12: the SNPs 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) values (β = 0.09 ppb; SE = 0.014; $P = 1.97 \times 10^{-10}$; explained variance = 0.23%), and each C allele of rs944722 was associated with lower ln(FENO) values (β = −0.07 ppb; SE = 0.012; $P = 1.28 \times 10^{-9}$; explained variance = 0.30%). rs3751972 and rs944722 are in neighboring loci with low LD, indicating that the 2 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, which is located near the gasdermin B *(GSDMB)* gene at 17q12-q21, was also associated with childhood FENO values. Each A allele of rs8069176 was associated with lower ln(FENO) values (β = −0.07 ppb; SE = 0.012; $P = 1.88 \times 10^{-8}$; explained variance = 0.41%). Figs 2 to 4 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 whether 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 (see Table E2 in this article's Online Repository at www.jacionline.org). After conditioning on all SNPs of the meta-analysis, rs3751972 and rs2274894 showed the strongest association in the *LYRM9* gene ($P = 2.06 \times 10^{-9}$) and in the *NOS2* gene ($P = 1.50 \times 10^{-8}$, rs2274894 and not rs944722 is the strongest signal by using GCTA), respectively. By using the same approach, rs8069176 showed the strongest association at 17q12-q21 ($P = 2.14 \times 10^{-8}$).

The 3 genome-wide significant SNPs showed low heterogeneity between studies (all $P \geq .075$, $I^2 = 0\%$ to 37.8%). The 3 SNPs together explained 0.95% of the variance in FENO values. Other suggestive loci that were associated with FENO values but did not reach genome-wide significance ($P < 1 \times 10^{-5}$) are shown in Tables E3 and E4 in this article's Online Repository at www.jacionline.org. The associations of genetic variants in the *NOS*s or arginase genes might be different among asthmatic versus nonasthmatic 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 (see Table E5 in this article's Online Repository at www.jacionline.org). In addition, we showed that the 3 SNPs were also associated with FENO values in nonasthmatic children (see Table E6 in this article's Online Repository at www.jacionline.org).

We assessed whether there were common nonsynonymous variants with deleterious functional implications in LD ($r^2 > 0.80$) with our 3 genome-wide significant SNPs by using HaploReg,[24] a database 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 nonconservative 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 determined by 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 Table E7 in this article's Online Repository at www.jacionline.org.

Subsequently, we assessed whether the identified 3 loci were eQTLs in genome-wide expression data sets 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 2 independent data sets (see Tables E8 and E9 in this article's Online Repository at www.jacionline.org). *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 data set (cases, n = 10,365; control subjects, 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, 0.85; 95% CI, 0.81-0.88; $P = 7.93 \times 10^{-17}$; Table II). This is in line with the association with lower FENO values that we found for rs8069176[A]. The SNPs rs3751972 and rs944722 were not associated with an asthma diagnosis ($P \geq .3$). The 3 childhood FENO-associated SNPs were not associated with adult FENO values (n = 1,211, Table II).

Finally, we explored whether common genetic variants known to be associated with physician-diagnosed asthma[5] were related to childhood FENO values. We found that the known asthma

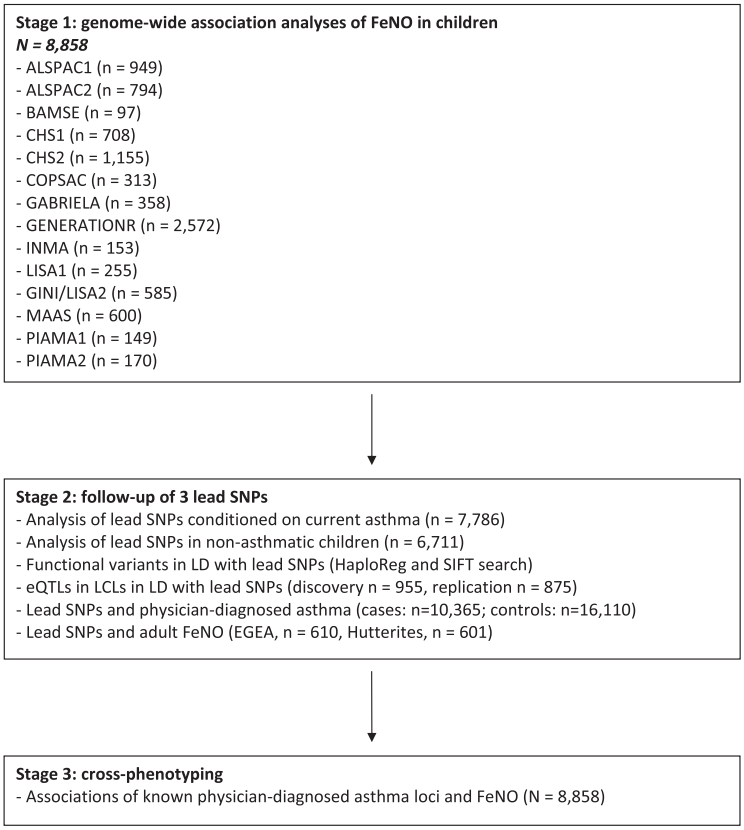

FIG 1. Study design. *LCL*, Lymphoblastoid cell line.

TABLE I. Summary statistics of the 3 SNPs at a *P* value of less than 5×10^{-8}

Marker	MAF	β	SE	*P* value	I^2	*HetP*
rs3751972[C] at 17q11.2 *(LYRM9)*	0.25	0.086	0.014	1.97×10^{-10}	27.4	.161
rs944722[C] at 17q11.2-q12 *(NOS2)*	0.38	−0.073	0.012	1.28×10^{-9}	37.8	.075
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	0.43	−0.066	0.012	1.88×10^{-8}	0.0	.668

SNP markers are identified according to their standard rs numbers (National Center for Biotechnology Information build 36). Independent SNPs with a genome-wide significant effect on FENO values in children are shown ($P < 5 \times 10^{-8}$). The total sample includes data of 14 independent GWA data sets (N = 8,858). β reflects differences in natural log-transformed FENO values per minor allele. *P* values are obtained from linear regression of each SNP against natural log-transformed FENO values adjusted for sex and age at the time of measurement (fixed-effect additive genetic model). The derived inconsistency statistic I^2 and *HetP* values reflect heterogeneity across studies with the use of Cochran Q tests.
MAF, Minor allele frequency.

SNPs rs2305480 at 17q12 *(GSDMB)*, rs3894194 at 17q21.1 *(GSDMA)*, rs744910 at 15q22.33 *(SMAD3)*, and rs1295686 at 5q31 *(IL13)* were associated with childhood FENO values (all $P \leq .005$ 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 values.

DISCUSSION

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

The function of the *LYRM9* gene is unknown; variants in the *NOS*s and arginase genes jointly contributed to differences in FENO values in previous studies,[28-31] and variation in arginase genes contributed to asthma severity.[32] We did not find associations between the *NOS2* and *LYRM9* SNPs and asthma. It has been shown previously that levels of the inducible NOS2 protein are higher in adults with severe asthma.[33] Unfortunately, we do not have data for the 2 SNPs and patients with severe asthma. Inducible NOS2 is expressed in airway epithelium and is synthesized in response to proinflammatory cytokines and mediators. Expression of inducible NOS2 might 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 values. A previous study suggested that DNA methylation in promotor regions of arginase genes were associated with

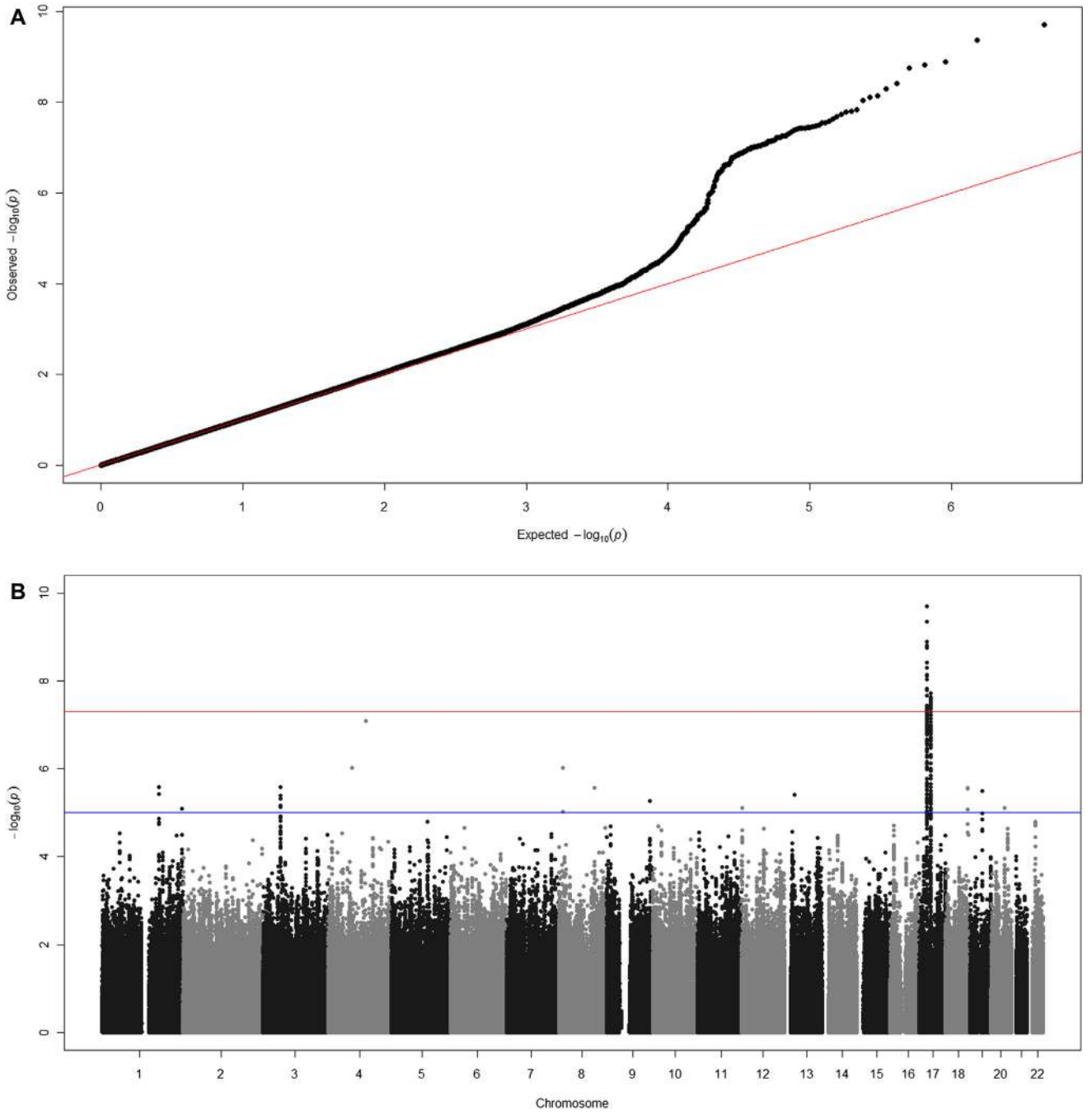

FIG 2. A, QQ plot of 2,253,077 SNPs of 14 GWA studies (N = 8,858). The *black dots* represent observed *P* values, and the *red line* represents the expected *P* values under the null distribution. **B,** Manhattan plot showing the association *P* values of FENO values of the 14 studies. The $-\log_{10}$ of the *P* value for each of 2,253,077 SNPs *(y-axis)* is plotted against the genomic position *(x-axis)*.

FENO values 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, which is downstream of *NOS2*, and this suggests that the protein Gal-9 might be involved in the regulation of FENO values. 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

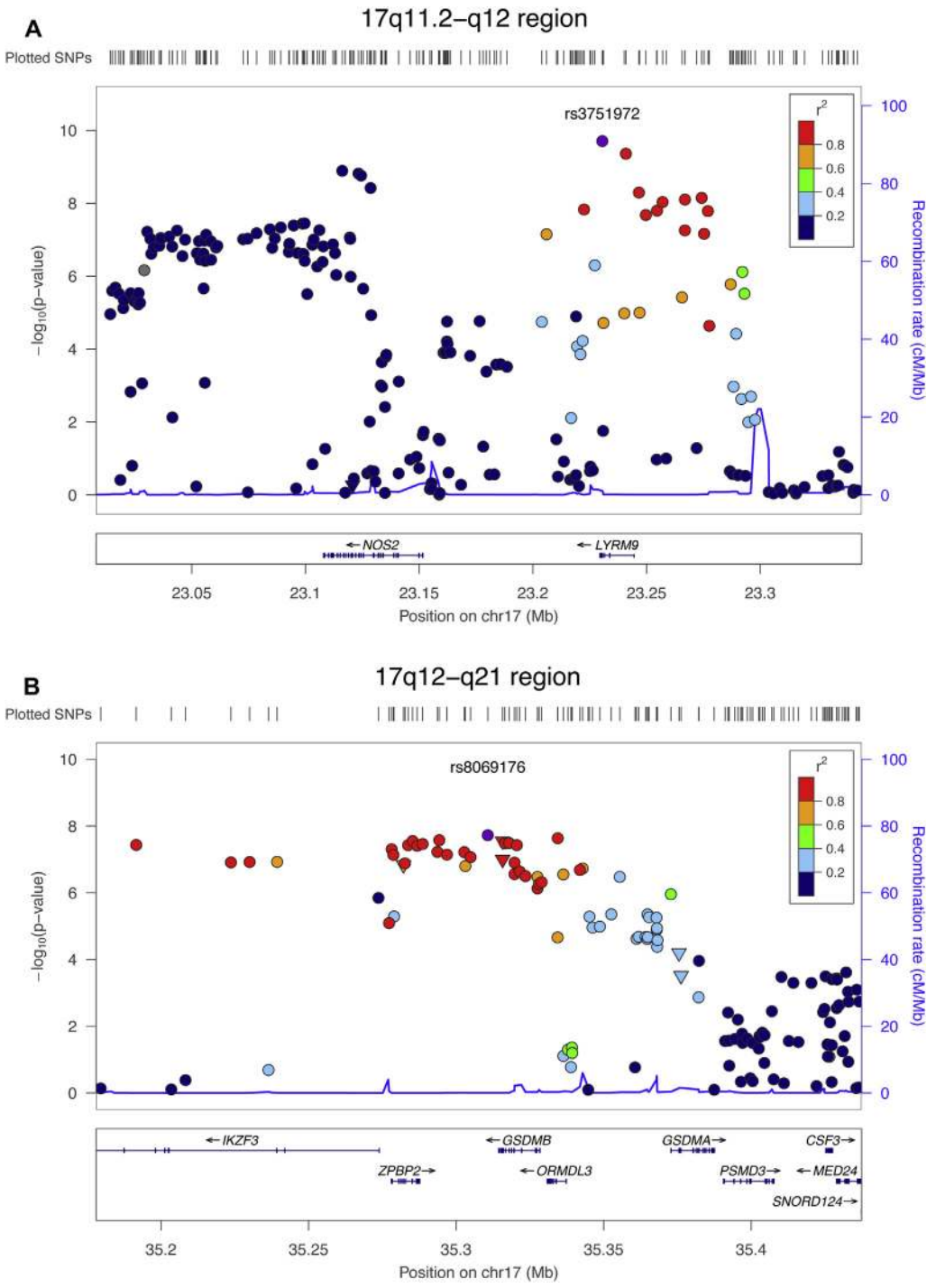

FIG 3. Association plots of the 17q11.2-q12 **(A)** and 17q12-q21 **(B)** regions. For both the 17q11.2-q12 and 17q12-q21 regions, SNPs are plotted with their *P* values (as $-\log_{10}$ values, *left y-axis*) as a function of genomic position *(x-axis)*. Estimated recombination rates *(right y-axis)* taken from HapMap are plotted to reflect the local LD structure around the top associated SNP *(purple circle)* and correlated proxies (according to a blue to red scale from $r^2 = 0$ to 1). *Triangles* represent nonsynonymous SNPs.

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* might be involved in the regulation of the growth and differentiation of epithelial cells.[40,41] The function of the upstream *ORMDL3* gene in human subjects 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

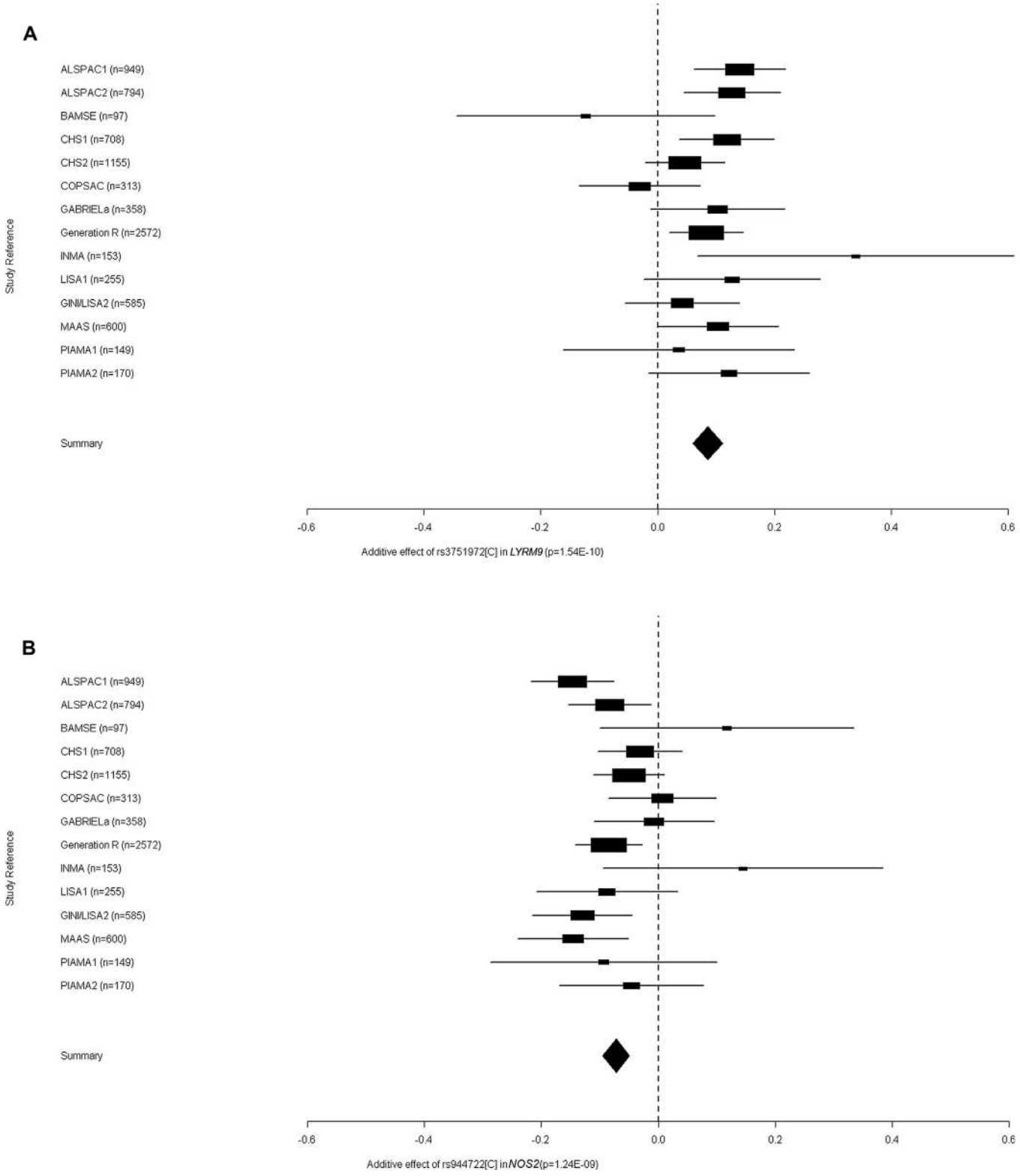

FIG 4. Forest plots of the associations between FENO values and the 3 SNPs associated with FENO values at a *P* value of less than 5×10^{-8}. The SNPs in *LYRM9* **(A)**, *NOS2* **(B)**, and near *ZPBP2-GSDMB* **(C)** are shown. In each plot the *triangle* indicates the effect size and the CI in the 14 studies. The *P* values in the plots are without genomic control correction.

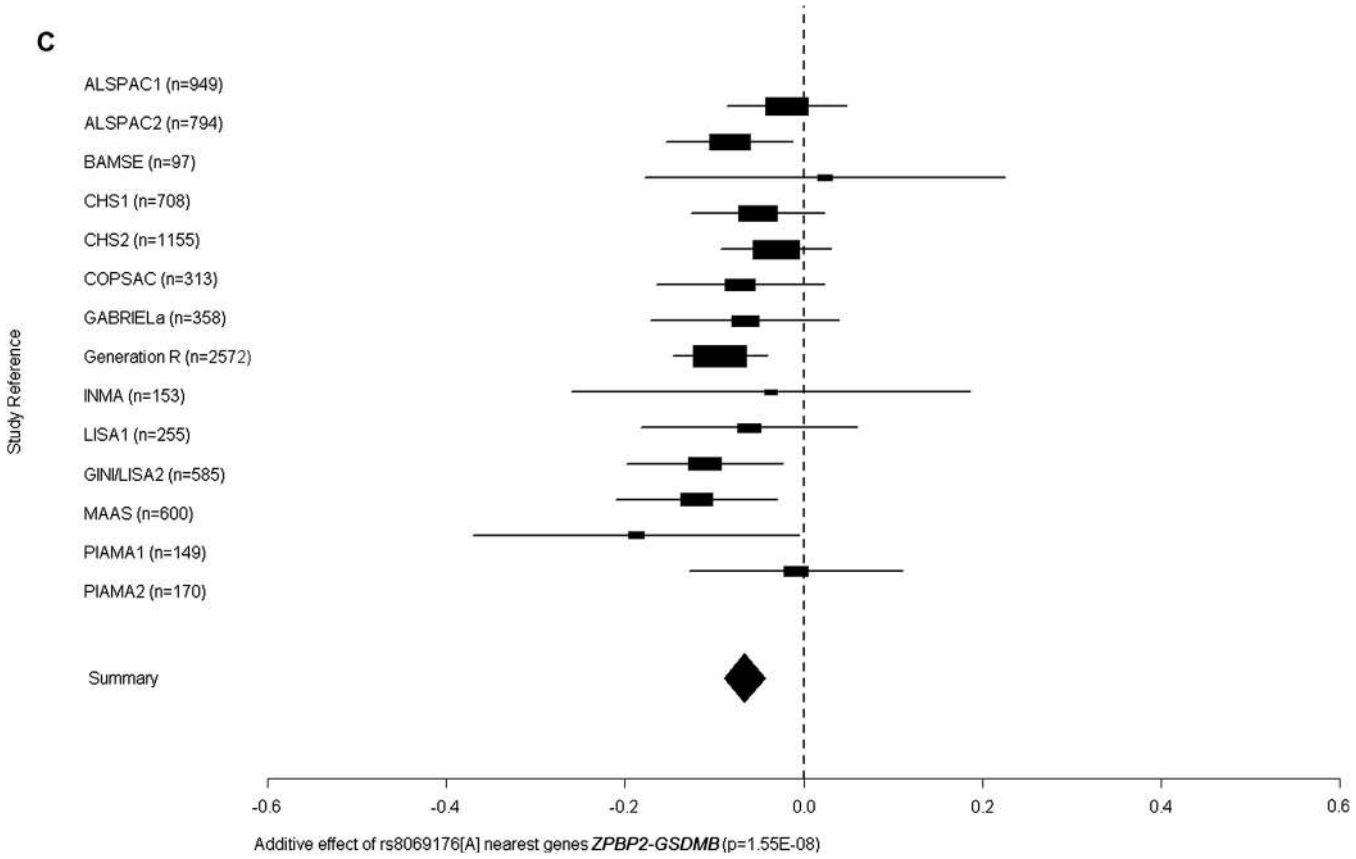

FIG 4. *(Continued)*

by which 17q12-q21 variants might regulate FENO values remains to be elucidated.

The 3 genetic variants identified in the present study explained only a small proportion of the total variance in FENO values, whereas earlier work on twins indicated that most of the FENO value variation is genetically determined. One explanation could be that the heritability of FENO values was overestimated. Lund et al[21] estimated the heritability but did not adjust for body height, a determinant of adult FENO values.[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 more than 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% ($P = .100$) in the largest cohort (Generation R Study; white subjects 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 values might be entirely explained by atopy.[46] We found an association between the 17q12-q21 childhood asthma locus and FENO values. This suggests that FENO values are related to asthma independent of allergy because 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 values on asthma. One explanation could be that FENO values and asthma are not directly related but might 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 values 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 values. Identification of functional SNPs and haplotypes in these regions might provide novel insight into the regulation of FENO values. This study highlights that both shared and distinct genetic factors affect FENO values and childhood asthma.

Acknowledgements per study can be found in this article's Online Repository at www.jacionline.org.

TABLE II. Association of the 3 SNPs related to childhood FENO values with physician-diagnosed asthma and adult FENO values

Physician-diagnosed asthma (cases, n = 10,365; control subjects, n = 16,110)[5]			
Marker		**OR (95% CI)**	***P* value**
Proxy for rs3751972: rs4796222[A] (r^2 = 1.000, D′ = 1.000) at 17q11.2 *(LYRM9)*		0.98 (0.93-1.02)	.303
Proxy for rs944722: rs2274894[T] (r^2 = 0.967, D′ = 1.000) at 17q11.2-q12 *(NOS2)*		1.00 (0.96-1.04)	.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 value			
Marker (EGEA, n = 610)	**β**	**SE**	***P* value**
rs3751972[C] at 17q11.2 *(LYRM9)*	0.125	0.065	.057
rs944722[C] at 17q11.2-q12 *(NOS2)*	−0.015	0.061	.802
rs8069176[A] at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)	−0.113	0.062	.067
Marker (Hutterites, n = 601)		***Z* score**	***P* value**
Proxy for rs3751972: rs4796228[G] (r^2 = 0.659, D′ = 1.000) at 17q11.2 *(LYRM9)*		−1.536	.125
Proxy for rs944722: rs2314809[T] (r^2 = 0.967, D′ = 1.000) at 17q11.2-q12 *(NOS2)*		−2.322	.020
Proxy for rs8069176: rs11078927[T] (r^2 = 1.000, D′ = 1.000) at 17q12-q21 (nearest genes *ZPBP2-GSDMB*)		0.505	.613

SNP markers are identified according to their standard rs numbers (National Center for Biotechnology Information build 36). Independent SNPs with a genome-wide significant effect on FENO values in children are shown ($P < 5 \times 10^{-8}$) in relation to physician-diagnosed asthma[5] and adult FENO values. Odds ratios *(OR)* with 95% CIs are shown for physician-diagnosed asthma.[5] β reflects differences in natural log-transformed FENO values per minor allele for adult FENO values in EGEA. The *z* score reflects the strength of the association between SNPs and natural log-transformed FENO values and the direction of the effect of the minor allele in Hutterites.
EGEA, Epidemiological Study on the Genetics and Environment of Asthma.

TABLE III. Association of known physician-diagnosed asthma loci from a previous GWA study[5] with childhood FENO values

Physician-diagnosed asthma[5]						
Marker	**MAF**	**β**	**SE**	***P* value**	I^2	***HetP***
rs2305480[A] decreasing risk allele at 17q12 *(GSDMB)*	0.42	−0.065	0.012	2.83×10^{-8}	0.0	.731
rs3894194[A] increasing risk allele at 17q21.1 *(GSDMA)*	0.47	0.048	0.012	6.35×10^{-5}	9.5	.349
rs744910[A] decreasing risk allele at 15q22.33 *(SMAD3)*	0.49	−0.039	0.012	8.41×10^{-4}	0.0	.491
rs1295686[T] increasing risk allele at 5q31 *(IL13)*	0.27	0.044	0.014	1.25×10^{-3}	4.6	.401
rs1342326[C] increasing risk allele at 9p24.1 *(IL33)*	0.17	0.025	0.016	.119	0.0	.515
rs9273349[T] decreasing risk allele at 6p21.3 *(HLA-DQ)*	0.37	−0.022	0.022	.310	0.0	.802
rs11071559[T] decreasing risk allele at 15q22.2 *(RORA)*	0.14	−0.014	0.017	.415	0.0	.651
rs3771166[A] decreasing risk allele at 2q12 *(IL18R1)*	0.35	−0.009	0.012	.463	7.4	.371
rs2284033[A] decreasing risk allele at 22q13.1 *(IL2RB)*	0.42	0.005	0.012	.705	0.0	.633
rs2073643[T] increasing risk allele at 5q23.3 *(SLC22A5)*	0.47	0.000	0.012	.993	0.0	.590

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

Key messages

- **We identified 3 independent genetic variants associated with childhood FENO values. 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 values and asthma.**

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