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To cite this article: Simone Accordini *et al* 2024 *J. Breath Res.* **18** 016001

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Journal of Breath Research

OPEN ACCESS

RECEIVED
5 April 2023

REVISED
14 September 2023

ACCEPTED FOR PUBLICATION
21 September 2023

PUBLISHED
6 October 2023

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PAPER

SNPs in *FAM13A* and *IL2RB* genes are associated with FeNO in adult subjects with asthma

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Keywords: asthma, *FAM13A*, FeNO, *IL2RB*, machine learning, polymorphism, SNP

Supplementary material for this article is available online

Abstract

Nitric oxide has different roles in asthma as both an endogenous modulator of airway function and a pro-inflammatory mediator. Fractional exhaled nitric oxide (FeNO) is a reliable, quantitative, non-invasive, simple, and safe biomarker for assessing airways inflammation in asthma. Previous genome-wide and genetic association studies have shown that different genes and single nucleotide polymorphisms (SNPs) are linked to FeNO. We aimed at identifying SNPs in candidate genes or gene regions that are associated with FeNO in asthma. We evaluated 264 asthma cases (median age 42.8 years, female 47.7%) who had been identified in the general adult population within the Gene Environment Interactions in Respiratory Diseases survey in Verona (Italy; 2008–2010). Two hundred and twenty-one tag-SNPs, which are representative of 50 candidate genes, were genotyped by a custom GoldenGate Genotyping Assay. A two-step association analysis was performed without assuming an *a priori* genetic model: step (1) a machine learning technique [gradient boosting machine (GBM)] was used to select the 15 SNPs with the highest variable importance measure; step (2) the GBM-selected SNPs were jointly tested in a linear regression model with natural log-transformed FeNO as the normally distributed outcome and with age, sex, and the SNPs as covariates. We replicated our results within an independent sample of 296 patients from the European Community Respiratory Health Survey III. We found that SNP rs987314 in family with sequence similarity 13 member A (*FAM13A*) and SNP rs3218258 in interleukin 2 receptor subunit beta (*IL2RB*) gene regions are significantly associated with FeNO in adult subjects with asthma. These genes are involved in different mechanisms that affect smooth muscle constriction and endothelial barrier function responses (*FAM13A*), or in immune response processes (*IL2RB*). Our findings contribute to the current knowledge on FeNO in asthma by identifying two novel SNPs associated with this biomarker of airways inflammation.

1. Introduction

Asthma is a complex chronic disorder of the conducting airways, which is related to an immunological reaction, inflammation of bronchial walls, and increased mucus secretion [1]. Asthma involves the interaction among multiple genetic, environmental, and lifestyle factors [2], and it presents with different phenotypes. The most prevalent phenotype is type 2 inflammation (T_H2)-associated asthma, which is strongly linked to atopy, allergy, and the response to corticosteroids [1, 3]. In T_H2-associated asthma, the immune process initiates with the development of T_H2 cells, which produce interleukin (IL) 4, IL5, and IL13 cytokines. These cytokines are responsible for both the stimulation of the allergic and eosinophilic inflammation, and the epithelial and smooth-muscle changes that contribute to asthma pathobiology

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[3]. Pro-inflammatory cytokines [interferon gamma (IFNγ), IL1β, IL13, and tumour necrosis factor alpha (TNFα)] induce the production of nitric oxide (NO) in the airway epithelial cells by promoting the expression of the enzyme T_H2-regulated inducible NO synthase (iNOS) [4]. NO has different roles in asthma as both an endogenous modulator of airway function and a pro-inflammatory mediator [4].

NO levels can be measured in human breath. Fractional exhaled NO (FeNO) is a reliable, quantitative, non-invasive, simple, and safe biomarker for assessing airways inflammation in subjects with asthma [5]. FeNO is higher in males and increases with increasing age and height [6], but it is negatively associated with tobacco smoking and obesity [7]. Furthermore, previous genome-wide and genetic association studies have shown that different genes [8] and single nucleotide polymorphisms (SNPs) [9–17] are linked to FeNO.

Typically, genetic association studies consist of single-SNP-based tests under the assumptions that genetic variants independently contribute to a given phenotype and that the underlying genetic model of inheritance is additive. The main limitation of this approach is that single SNPs can only explain a small proportion in the genetic variation of complex traits, which results in the missing heritability problem [18]. Furthermore, true associations can be missed when assuming an additive genetic model in case the correct model is recessive or dominant, which also leads to a reduction in statistical power [19]. These shortcomings have encouraged the application of statistical learning methods that allow to jointly analyse a large number of SNPs in a high dimensional setting without an *a priori* specification of the underlying genetic model, such as gradient boosting machine (GBM) [19].

The present study is a candidate gene association analysis aimed at identifying SNPs that are associated with FeNO in adult subjects with asthma. To fulfil this purpose, we jointly analysed data from the Gene Environment Interactions in Respiratory Diseases (GEIRD) survey [20]. In the original survey (2008–2010), an overall panel of 384 SNPs tagging 53 candidate genes or gene regions (see table S1) was assessed. The selection of the 53 genes or gene regions was based on their association with asthma, chronic obstructive pulmonary disease (COPD), or allergic rhinitis, as observed in previous studies identified from literature [21–23] (the full list of references is reported in table S2), or on their involvement in possible related biological pathways (such as inflammation, innate immunity and immunoregulation, oxidative stress and xenobiotic metabolism, regulation of protease-antiprotease equilibrium, and tissue remodelling) [24]. Then, we replicated our findings within the European Community Respiratory Health Survey (ECRHS) III (*www.ecrhs.org*) [25–27].

2. Materials and methods

2.1. GEIRD study

GEIRD is an Italian, multi-centre, (multi)case-control study on the role of genetic and modifiable factors in asthma, COPD, chronic bronchitis, and allergic rhinitis (the protocol is fully described elsewhere [20]). Briefly, the cases and the controls were identified in pre-existing cohorts [25–28] and in new random samples of the general adult population through a two-stage process, which consists of a mailed screening questionnaire (stage 1) and a clinical examination for accurate phenotyping (stage 2) (figure 1). The participants in GEIRD stage 2 also provided blood samples for genetic data collection.

Asthma cases were the individuals who had reported at least one of the following two conditions:

(a) ever asthma;
(b) asthma-like symptoms [asthma attacks, wheezing, chest tightness, shortness of breath (SoB) at rest, SoB at night time, SoB following strenuous activities] or the utilization of anti-asthmatic drugs in the previous 12 months, having fulfilled at least one of the following clinical characteristics:
    1. positive methacholine challenge test [provocative dose (PD_{20}) <1 mg causing a 20% fall in forced expiratory volume in one second (FEV_1)];
    2. pre-bronchodilator (BD) airflow obstruction (AO) [FEV_1/forced vital capacity (FVC) <lower limit of normal (LLN) [29] or <70%] and a positive reversibility test (increase in post-BD FEV_1 >12% and >200 ml with respect to pre-BD FEV_1 after 400 mcg of salbutamol);
    3. pre- but not post-BD FEV_1/FVC <LLN or <70%, and post-BD FEV_1 ⩾80% predicted.

The subjects with current asthma were those who (i) had reported asthma-like symptoms or the utilization of anti-asthmatic drugs in the previous 12 months, or (ii) had pre-BD AO or (iii) had a positive methacholine challenge test. The criteria used to identify the cases of COPD, chronic bronchitis, or allergic rhinitis are described elsewhere [20]. The controls were the subjects without asthma, COPD, chronic bronchitis, and allergic rhinitis who had pre-BD FEV_1 >70% predicted and pre-BD FEV_1/FVC ⩾LLN and ⩾70%. The subjects not fulfilling the criteria for cases and controls were included in a residual group.

2.2. FeNO and genetic protocols

In GEIRD, FeNO (exhalation flow rate of 50 ml s^{-1}) was measured according to international guidelines

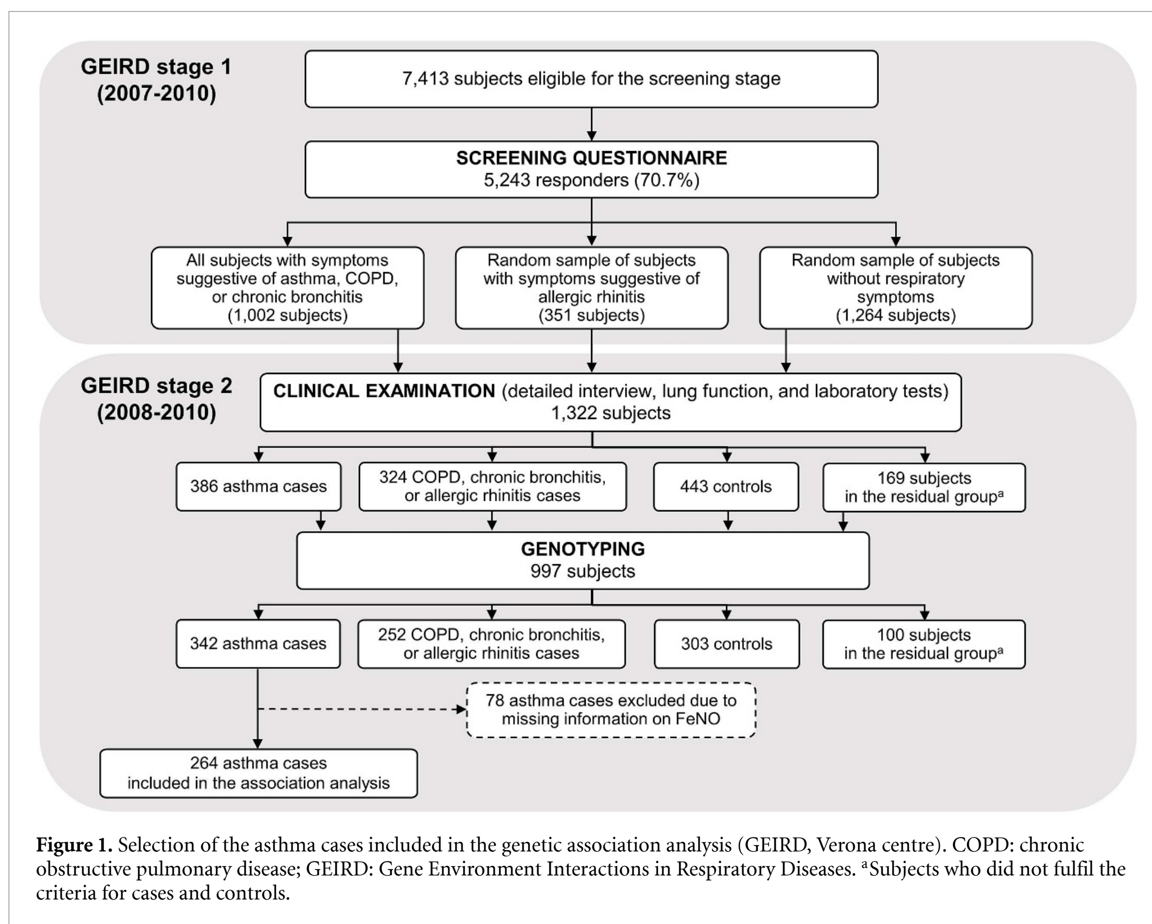

Figure 1. Selection of the asthma cases included in the genetic association analysis (GEIRD, Verona centre). COPD: chronic obstructive pulmonary disease; GEIRD: Gene Environment Interactions in Respiratory Diseases. [a]Subjects who did not fulfil the criteria for cases and controls.

[30] by using a chemiluminescence analyser (CLD88, Ecomedics, Switzerland). FeNO measurements were expressed as 'part per billion' (ppb) absolute values. Blood samples were collected and stored for genomic DNA extraction according to standardized international protocols [20]. The selection of the 384 SNPs in the 53 genes or gene regions [31] was based on including SNPs tagging most of haplotype variability in the CEU population (HapMAp phase II) and SNPs from literature (*www.ncbi.nlm.nih.gov/snp*). These SNPs were chosen by STAMPA application (GEVALT software; *acgt.cs.tau.ac.il/gevalt/#ver2*) and constitute the optimal set of tag-SNPs that are representative of a given genomic region with high linkage disequilibrium (LD) and maximum prediction accuracy. Candidate gene SNPs were analysed using a high degree genotyping method (GoldenGate Genotyping assay, Illumina).

2.3. Study subjects

In the present analyses, only the participants in GEIRD stage 2 from the Verona centre were included due to the availability of their genetic data. In this centre, 1322 cases and controls were identified at the clinical stage and 997 of these individuals were genotyped (figure 1). Genetic data from all the genotyped subjects were used for additional SNP quality checks. Of all the cases of asthma who had provided genetic data in the original study in Verona (342 subjects), 264 patients with a FeNO measurement were included in this genetic association analysis.

The appropriate ethics committee ('*Comitato Etico per la Sperimentazione dell'Azienda Ospedaliera Istituti Ospitalieri di Verona*') approved the GEIRD survey in Verona and all the aspects of the research project were fully explained to the participants, who gave their written informed consent.

2.4. Genetic association analysis

Of the 384 SNPs assessed in the original survey (GEIRD), 221 SNPs tagging 50 genes or gene regions (see table S1) met the following criteria and were included in the present analysis:

1. genotype failure rate ⩽5% in the 997 genotyped subjects;
2. genotype failure rate ⩽5% in the 342 asthma cases;
3. minimum genotype frequency ⩾5% in the 342 asthma cases;
4. allele frequencies needed to respect Hardy–Weinberg equilibrium (HWE) in the 303 controls (the SNPs not available for the controls were excluded from the analysis) [32]. *P*-values for testing deviation from HWE were corrected for the false discovery rate by using the Benjamini–Yekutieli procedure [33, 34].

In order to identify the SNPs that are associated with FeNO, a two-step approach was adopted: GBM [19, 35] was used to select the SNPs (step 1) that were simultaneously included as covariates in a multivariable linear regression model for significance testing (step 2). In both steps, natural log-transformed FeNO (log-FeNO) was the normally distributed outcome and the covariates were age, sex, and the SNPs (the genotype data for all SNPs were coded without assuming an *a priori* genetic model: 0 = reference = homozygous with higher allele frequency, 1 = heterozygous, 2 = homozygous with lower allele frequency). Other covariates (such as cigarette smoking, obesity, or allergic rhinitis) were not analysed because these variables are not confounders of the relationship between SNPs and FeNO.

At step 1, GBM was used to rank-order the 221 SNPs according to their variable importance measure (VIM), which quantifies the total contribution of each SNP to the prediction of log-FeNO. Because VIM in GBM is biased for SNPs in LD, the 221 SNPs were divided into ten overlapping subsets of low correlated SNPs (within-subset correlation <0.1) [36], and GBM was applied to each subset in parallel ('gbm' package in R software; *cran.r-project.org/web/packages/gbm*) [37]. The tuning of GBM hyper-parameter was performed by ten-fold cross-validations, which suggested a shrinkage rate of 0.01, at least five observations per node of each tree, a bagging fraction of 0.8, and a training fraction of one. Five hundred trees were used to build the first deep learning model and the interaction depth was set equal to one. An aggregate VIM was obtained as the median of the VIMs computed in the overlapping subsets. The 15 SNPs that had the highest aggregate VIM were selected for step 2 because of the relatively small sample size.

At step 2, the GBM-selected SNPs were included as covariates in a multivariable linear regression model for significance testing. The strength of the association between each SNP and log-FeNO was measured through the beta regression coefficient, which represents the difference in the expected log-FeNO (Δlog-FeNO) between the heterozygous genotype (or the homozygous genotype with lower allele frequency) and the reference for a given SNP, with the genotype of the other SNPs held constant. A sensitivity analysis was performed to evaluate the generalizability of the results to the European population, repeating the genetic association analysis (step 2) by including only the asthma cases with both parents born in Europe.

2.5. Replication analysis

The GBM-selected SNPs in GEIRD were tested for association with log-FeNO in a replication sample of 296 asthma cases who had participated in ECRHS III (from 15 centres located in Estonia, France, Germany, Norway, Spain, Sweden, and United Kingdom) [27]. ECRHS is a population-based, cohort study that recruited subjects aged 20–44 at baseline (ECRHS I; 1991–1993) [25]. The study subjects answered a mailed screening questionnaire (stage 1) and a 20% 'random sample' of the responders underwent a detailed clinical examination (stage 2). The follow-up of the participants in ECRHS I stage 2 took place in 1998–2002 (ECRHS II) [26] and 2010–2013 (ECRHS III) [27]. A standardized clinical interview, lung function, and laboratory tests were performed on all occasions. Blood samples for genotyping were collected in ECRHS II, and FeNO (exhalation flow rate of 50 ml s^{-1}) was measured in ECRHS III. Additional information regarding the ECRHS III survey, including ethics approvals, was included as supplementary material.

The definition of asthma in ECRHS III was comparable with that used in GEIRD. Asthma cases were the subject who had fulfilled at least one of the following criteria:

1. ever asthma OR (asthma attacks/asthma-like symptoms/anti-asthmatic drugs in the previous 12 months AND PD_{20} <1 mg) OR (asthma attacks/asthma-like symptoms/anti-asthmatic drugs in the previous 12 months AND pre-BD FEV_1/FVC <LLN [29] or <70%) at ECRHS I;
2. ever asthma OR (asthma attacks/asthma-like symptoms/anti-asthmatic drugs in the previous 12 months AND PD_{20} <1 mg) OR (asthma attacks/asthma-like symptoms/anti-asthmatic drugs in the previous 12 months AND pre-BD FEV_1/FVC <LLN or <70%) at ECRHS II;
3. ever asthma OR (asthma attacks/asthma-like symptoms/anti-asthmatic drugs in the previous 12 months AND pre-BD FEV_1/FVC <LLN or <70% AND post-BD FEV_1 >12% and >200 ml with respect to pre-BD FEV_1 after 400 mcg of salbutamol) OR (asthma attacks/asthma-like symptoms/anti-asthmatic drugs in the past 12 months AND pre- but not post-BD FEV_1/FVC <LLN or <70% AND post-BD FEV_1 >80% predicted) at ECRHS III.

The subjects with current asthma at ECRHS III were those who (i) had reported asthma attacks, asthma-like symptoms, or anti-asthmatic drugs in the previous 12 months or (ii) had pre-BD FEV_1/FVC <LLN or <70%.

A 2-level (subject: level 1 unit; centre: level 2 unit) random-intercept linear regression model, with age, sex, and all the GBM-selected SNPs as fixed-effect covariates, was used to account for the ECRHS hierarchical data structure. One-sided *p*-values were computed for the beta regression coefficients that were statistically significant in GEIRD and were in the same direction in GEIRD and ECRHS III.

All statistical analyses were performed by using R software (version 3.6.2; The R Foundation for Statistical Computing, Vienna, Austria) and STATA software (release 16; StataCorp, College Station, Texas, USA).

3. Results

3.1. Main characteristics of the asthma cases

The 264 asthma cases identified in GEIRD and included in the genetic association analysis (step 1) had a median age of 42.8 years (female 47.7%) and a median BMI of 24.0 (table 1). The vast majority (96.6%) of these patients had both parents born in Europe. Past, current light, and current heavy smokers were 29.2%, 11.0%, and 12.5%, respectively. Of the subjects who reported asthma in their life, about 43% developed the disease before the age of 10. Sixty-two per cent of the asthma cases had current asthma, those with allergic rhinitis were 54.6% and those with chronic cough or phlegm were 12.1%. The median pre-BD FEV_1, FVC, and FEV_1/FVC % predicted was 96.8, 100.5, and 93.9, respectively, and the median FeNO was 20.1 ppb. The distribution of the demographic and clinical variables, and smoking habits was not significantly different between the 264 study subjects and the 122 eligible patients in GEIRD who were excluded from the analysis due to missing information on genetic data and/or FeNO (see table S3).

Of the 264 asthma cases included in the genetic association analysis at step 1 (GBM), 19 patients with a missing value in at least one of the GBM-selected SNPs were excluded from the analysis at step 2. Compared to the 245 GEIRD patients included in the genetic association analysis (step 2), the 296 asthma cases identified in ECRHS III and assessed in the replication analysis had a higher percentage of females (58.8% vs 46.9%, $p = 0.006$), a higher age (median: 54.0 vs 42.4 years, $p < 0.0001$), a higher BMI (median: 26.7 vs 23.9, $p < 0.0001$), an older age of asthma onset ($p < 0.001$), a higher percentage of current asthmatics (69.3% vs 60.8%, $p = 0.040$), a higher percentage of subjects with coexisting cough or phlegm (24.0% vs 11.8%, $p = 0.001$), a lower pre-BD FEV_1% predicted (median: 85.6 vs 97.2, $p < 0.0001$), a lower FVC % predicted (median: 96.3 vs 100.4, $p < 0.001$), a lower FEV_1/FVC % predicted (median: 86.2 vs 93.8, $p < 0.0001$), and a lower FeNO (median: 17.0 vs 20.0, $p = 0.004$) (table 1).

3.2. Genetic association and replication analyses

At step 1, the following 15 SNPs were selected by GBM (table 2): rs2735014 (*HLA-G*; VIM = 44.54), rs2523793 (*HLA-G*; VIM = 27.93), rs13022785 (*TNS1*; VIM = 19.48), rs1419779 (*NPSR1*; VIM = 17.35), rs987314 (*FAM13A*; VIM = 13.97), rs2069812 (*IL5*; VIM = 13.50), rs1063320 (*HLA-G*; VIM = 13.21), rs2869546 (*CHRNA3*; VIM = 12.76), rs174579 (*FADS2*; VIM = 8.56), rs11639224 (*IREB2*; VIM = 8.15), rs944725 (*NOS2*; VIM = 7.33), rs647041 (*CHRNA5*; VIM = 5.83), rs953569 (*HAVCR1*; VIM = 5.66), rs1610696 (*HLA-G*; VIM = 4.97), and rs3218258 (*IL2RB*; VIM = 4.84).

At step 2, SNP rs2523793 in *HLA-G* was not evaluated because of multi-collinearity (variance importance factor >10). Six out of the remaining 14 GBM-selected SNPs were associated with log-FeNO (table 2): rs13022785 (*TNS1*; TC vs TT, $p = 0.012$), rs1419779 (*NPSR1*; GG vs AA, $p = 0.002$), rs987314 (*FAM13A*; TC vs CC, $p = 0.011$), rs174579 (*FADS2*; TT vs CC, $p = 0.020$), rs953569 (*HAVCR1*; AA vs CC, $p = 0.006$), and rs3218258 (*IL2RB*; TC vs CC, $p = 0.048$). The observed associations did not change after including only the 237 asthma cases with both parents born in Europe (table S4). Of these polymorphisms, only rs987314 (*FAM13A*; AG vs GG, $p = 0.038$) and rs3218258 (*IL2RB*; AG vs GG, $p = 0.030$) were significantly associated with log-FeNO in the replication analysis using the ECRHS III data (table 2).

4. Discussion

Out of six polymorphisms in *TNS1, NPSR1, FAM13A, FADS2, HAVCR1*, and *IL2RB* genes that were significantly linked to FeNO in Italian adult subjects with asthma, SNPs rs987314 in *FAM13A* and rs3218258 in *IL2RB* were replicated in an independent sample of patients from other European countries. Compared to previous studies from literature, our findings contribute to the current knowledge on FeNO by identifying two novel SNPs in candidate genes that are associated with this biomarker of airways inflammation in asthma, supporting accordingly a possible causal role for FAM13A and IL2RB genes in this phenotype.

4.1. FAM13A

FAM13A gene is involved in different mechanisms that could affect smooth muscle constriction and endothelial barrier function responses, leading to increased FeNO levels and asthma risk. In more detail, *FAM13A* gene encodes a Ras homologous GTPase-activating protein (Rho-GAP) domain-containing protein, which is expressed in mucosal cells, epithelial cells, alveolar cells, and alveolar macrophages [38–40]. Proteins containing this domain are involved in GTP phosphatase (Rho-GTPases) activity modulation in lung diseases and they are key regulators in cytoskeletal and cellular processes. These proteins regulate Ras homolog family member A (RhoA) activity, actin cytoskeleton dynamics (induction of F-actin stress fibres), and epithelial-mesenchymal transition by marker modulation (E-cadherin, α-smooth muscle actin and vimentin) [38, 39, 41]. Moreover, Rho-GTPases are involved in the pulmonary endothelial barrier function, which is dysregulated in several lung

Table 1. Main characteristics of the asthma cases included in the genetic association analysis (GEIRD dataset) and in the replication analysis (ECRHS III dataset).

		Genetic association analysis			
		Step 1	Step 2	Replication analysis	*P*-value[b]
Sample, *n*		264	245[a]	296	—
Females, %		47.7	46.9	58.8	0.006
Age (years), median (IQR)		42.8 (35.7, 49.5)	42.4 (35.2, 49.4)	54.0 (48.0, 59.0)	<0.0001
European-born parents[c], %	Both	96.6	96.7	—	—
	Only one	1.5	1.2	—	
	None	0.8	0.8	—	
	Unknown	1.1	1.2	—	
BMI, median (IQR)		24.0 (21.9, 26.7)	23.9 (21.7, 26.6)	26.7 (23.5, 30.3)	<0.0001
Tobacco smoking, %	Never	47.4	46.5	42.4	0.197
	Past	29.2	29.8	35.6	
	Current light	11.0	11.4	7.5	
	Current heavy	12.5	12.2	14.6	
Age of asthma onset (years)[d], %	0–9	42.9	42.0	21.2	<0.001
	10–19	17.9	19.1	18.7	
	⩾20	37.1	36.6	52.5	
	Unknown	2.1	2.3	7.6	
Current asthma, %		61.7	60.8	69.3	0.040
Allergic rhinitis[e], %	Absent	44.7	44.9	51.4	0.092
	Present	54.6	54.3	48.7	
	Unknown	0.8	0.8	0.0	
Chronic cough or phlegm[f], %	Absent	87.1	87.4	75.0	0.001
	Present	12.1	11.8	24.0	
	Unknown	0.8	0.8	1.0	
Pre-BD FEV_1% predicted, median (IQR)		96.8 (87.6, 109.0)	97.2 (88.1, 108.9)	85.6 (75.9, 99.5)	<0.0001
Pre-BD FVC % predicted, median (IQR)		100.5 (91.9, 110.5)	100.4 (92.4, 110.7)	96.3 (86.8, 106.3)	<0.001
Pre-BD FEV_1/FVC % predicted, median (IQR)		93.9 (88.7, 101.2)	93.8 (89.1, 101.1)	86.2 (82.7, 96.2)	<0.0001
FeNO (ppb), median (IQR)		20.1 (12.2, 40.2)	20.0 (12.3, 39.7)	17.0 (12.0, 25.5)	0.004

Gene Environment Interactions in Respiratory Diseases; ECRHS: European Community Respiratory Health Survey; IQR: interquartile range; BMI: body mass index; pre-BD: pre-bronchodilator; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; FeNO: fractional exhaled nitric oxide; ppb: part per billion.

[a] Of the 264 asthma cases included in the genetic association analysis at step 1 (gradient boosting machine, GBM), 19 patients with a missing value in at least one of the GBM-selected SNPs were excluded from the analysis at step 2.

[b] Pearson chi-squared test, Fisher's exact test, or Wilcoxon rank-sum test were used when needed to compare the distribution of the main characteristics between the 245 GEIRD patients included in the genetic association analysis at step 2 and the 296 ECRHS III patients included in the replication analysis.

[c] Not measured in ECRHS III.

[d] Information obtained from the patients who reported asthma in their life.

[e] Having reported any nasal allergies, including hay fever.

[f] Having reported cough and/or phlegm from the chest, usually in winter and on most days for as long as three months each year.

diseases, such as asthma, COPD, and cystic fibrosis [38, 39, 42, 43]. In addition, *FAM13A* plays a role in asthma by regulating β-catenin stability and increasing Wingless/Integrase-1 (WNT) signalling pathways. Finally, alterations in these signalling pathways have been linked to airway remodelling pathogenesis [40, 44].

Different SNPs in *FAM13A* gene, which are in LD with rs987314, may affect Rho-GTPase activity and cellular pathways associated with *FAM13A*, such as

Table 2. SNPs identified in the genetic association analysis (GEIRD dataset) and tested in the replication analysis (ECRHS III dataset). The statistically significant associations are reported in bold.

		Genetic association analysis						Replication analysis				
		Step 1			Step 2							
Gene or gene region	SNP	Genotype	Sample ($n = 264$)	VIM	Sample[a] ($n = 245$)	Δlog-FeNO[b] [95% CI]	Two-sided p-value	Genotype[c]	Sample ($n = 296$)	Δlog-FeNO[d] [95% CI]	Two-sided p-value	One-sided p-value[e]
HLA-G	rs2735014	GG	134	44.54	122	0.00	—	CC	163	0.00	—	—
		TG	107		101	−0.14 [−0.36, 0.08]	0.225	AC	120	−0.02 [−0.19, 0.14]	0.773	—
		TT	22		22	0.16 [−0.27, 0.58]	0.468	AA	13	−0.14 [−0.56, 0.28]	0.508	—
HLA-G	rs2523793	GG	134	27.93	—	—	—	—	—	—	—	—
		AG	103		—	—	—	—	—	—	—	—
		AA	22		—	—	—	—	—	—	—	—
TNS1	rs13022785	TT	123	19.48	115	0.00	—	TT	116	0.00	—	—
		TC	109		101	**0.27 [0.06, 0.48]**	**0.012**	TC	138	0.04 [−0.12, 0.20]	0.612	0.306
		CC	31		29	0.06 [−0.26, 0.38]	0.712	CC	42	0.16 [−0.07, 0.40]	0.165	—
NPSR1	rs1419779	AA	112	17.35	104	0.00	—	AA	121	0.00	—	—
		AG	131		124	−0.02 [−0.22, 0.18]	0.853	AG	139	−0.05 [−0.21, 0.11]	0.508	—
		GG	21		17	**−0.63 [−1.03, −0.24]**	**0.002**	GG	36	−0.09 [−0.34, 0.16]	0.469	0.235
FAM13A	rs987314	CC	83	13.97	75	0.00	—	GG	114	0.00	—	—
		TC	123		115	**0.29 [0.07, 0.51]**	**0.011**	AG	135	0.14 [−0.02, 0.30]	0.076	**0.038**
		TT	57		55	0.23 [−0.04, 0.49]	0.098	AA	47	0.20 [−0.02, 0.43]	0.073	—
IL5	rs2069812	CC	124	13.50	115	0.00	—	GG	131	0.00	—	—
		TC	106		99	−0.14 [−0.34, 0.06]	0.165	AG	135	−0.15 [−0.30, 0.004]	0.056	—
		TT	33		31	−0.29 [−0.60, 0.01]	0.058	AA	30	−0.02 [−0.27, 0.23]	0.874	—
HLA-G	rs1063320	CC	73	13.21	70	0.00	—	CC	76	0.00	—	—
		GC	123		115	−0.08 [−0.38, 0.21]	0.585	GC	145	−0.04 [−0.27, 0.18]	0.716	—
		GG	68		60	0.11 [−0.30, 0.52]	0.596	GG	75	0.01 [−0.30, 0.32]	0.948	—
CHRNA3	rs2869546	TT	109	12.76	102	0.00	—	TT	129	0.00	—	—
		TC	130		121	0.01 [−0.47, 0.49]	0.971	TC	135	0.10 [−0.26, 0.46]	0.572	—
		CC	25		22	0.24 [−0.47, 0.94]	0.509	CC	32	−0.11 [−0.67, 0.44]	0.687	—
FADS2	rs174579	CC	168	8.56	160	0.00	—	CC	192	0.00	—	—
		TC	82		75	0.16 [−0.04, 0.37]	0.124	TC	94	−0.10 [−0.26, 0.05]	0.197	—
		TT	13		10	**−0.57 [−1.06, −0.09]**	**0.020**	TT	10	−0.10 [−0.51, 0.32]	0.650	0.325
IREB2	rs11639224	AA	99	8.15	92	0.00	—	AA	114	0.00	—	—
		AG	125		119	0.16 [−0.10, 0.42]	0.228	AG	137	−0.07 [−0.27, 0.13]	0.490	—
		GG	37		34	0.18 [−0.27, 0.64]	0.433	GG	45	−0.39 [−0.72, −0.06]	0.020	—

(Continued.)

Table 2. (Continued.)

		Genetic association analysis						Replication analysis				
		Step 1			Step 2							
Gene or gene region	SNP	Genotype	Sample ($n = 264$)	VIM	Sample[a] ($n = 245$)	Δlog-FeNO[b] [95% CI]	Two-sided p-value	Genotype[c]	Sample ($n = 296$)	Δlog-FeNO[d] [95% CI]	Two-sided p-value	One-sided p-value[e]
NOS2	rs944725	CC	109	7.33	98	0.00	—	CC	109	0.00	—	—
		TC	107		102	0.09 [−0.12, 0.31]	0.395	TC	139	0.05 [−0.11, 0.22]	0.540	—
		TT	47		45	0.27 [−0.01, 0.54]	0.056	TT	48	−0.15 [−0.36, 0.07]	0.185	—
CHRNA5	rs647041	CC	100	5.83	94	0.00	—	CC	118	0.00	—	—
		TC	130		121	−0.11 [−0.62, 0.41]	0.682	TC	134	−0.03 [−0.41, 0.34]	0.858	—
		TT	33		30	−0.09 [−0.87, 0.69]	0.820	TT	44	0.30 [−0.27, 0.86]	0.305	—
HAVCR1	rs953569	CC	66	5.66	62	0.00	—	GG	83	0.00	—	—
		AC	139		129	−0.20 [−0.43, 0.02]	0.080	TG	128	0.06 [−0.12, 0.24]	0.508	—
		AA	59		54	**−0.39 [−0.66, −0.11]**	**0.006**	TT	85	0.13 [−0.07, 0.32]	0.203	—
HLA-G	rs1610696	GG	137	4.97	131	0.00	—	CC	150	0.00	—	—
		GC	86		81	0.14 [−0.11, 0.40]	0.269	CG	127	0.004 [−0.18, 0.19]	0.970	—
		CC	33		33	0.36 [−0.02, 0.75]	0.065	GG	19	0.14 [−0.23, 0.51]	0.463	—
IL2RB	rs3218258	CC	139	4.84	131	0.00	—	GG	150	0.00	—	—
		TC	101		96	**0.21 [0.002, 0.41]**	**0.048**	AG	127	0.15 [−0.01, 0.30]	0.059	**0.030**
		TT	21		18	0.36 [−0.02, 0.74]	0.067	AA	19	−0.07 [−0.38, 0.23]	0.716	—

SNP: single nucleotide polymorphism; GEIRD: Gene Environment Interactions in Respiratory Diseases; ECRHS: European Community Respiratory Health Survey; VIM: variable importance measure; FeNO: fractional exhaled nitric oxide; 95% CI: 95% confidence interval; *HLA-G*: human leukocyte antigen G; *TNS1*: tensin 1; *NPSR1*: neuropeptide S receptor 1; *FAM13A*: family with sequence similarity 13 member A; *IL5*: interleukin 5; *CHRNA3*: cholinergic receptor nicotinic alpha 3 subunit; *FADS2*: fatty acid desaturase 2; *IREB2*: iron responsive element binding protein 2; *NOS2*: nitric oxide synthase 2; *CHRNA5*: CHRNA 5 subunit; *HAVCR1*: hepatitis A virus cellular receptor 1; *IL2RB*: IL 2 receptor subunit beta.

[a] Of the 264 cases of asthma included in the genetic association analysis at step 1 (gradient boosting machine, GBM), 19 patients with a missing value in at least one of the GBM-selected SNPs were excluded from the analysis at step 2.

[b] Difference in the expected log-FeNO between the heterozygous genotype (or the homozygous genotype with lower allele frequency) and the reference for a given SNP, with the genotype of the other SNPs held constant, obtained by a linear regression model with sex, age, and the 14 GBM-selected SNPs (coded without imposing an *a priori* genetic model) as covariates.

[c] Identified by alleles reported in the forward orientation; differences between GEIRD and ECRHS III genotypes are due to the strand (forward or reverse) that was sequenced in the two surveys.

[d] Obtained by a 2-level (subject: level 1 unit; centre: level 2 unit) linear regression model with sex, age, and the 14 GBM-selected SNPs (coded without imposing an *a priori* genetic model) as fixed-effect covariates.

[e] Computed for the associations that were statistically significant in GEIRD and were in the same direction in GEIRD and ECRHS III.

endothelial barrier function, contributing to inefficient gas exchanges that are common in lung diseases. Furthermore, these SNPs may influence *FAM13A* gene expression, as most of them have been identified in non-coding regions downstream the Rho-GAP domain [38, 40, 45, 46]. In particular, SNP rs987314 is reported to be negatively associated with *FAM13A* gene expression levels in the whole blood [47]. In addition, the percentage of asthma cases with non-normal FeNO (⩾25 ppb) in GEIRD was significantly higher among the patients with TT or TC genotypes than among those with CC genotype in this SNP (45.3% vs 30.7%, $p = 0.032$). Finally, the observed association between SNP rs987314 and FeNO should not depend on the confounding effect of the main characteristics of the asthma cases in GEIRD, as the distribution of these variables was not significantly different among the SNP genotypes (see table S5).

4.2. IL2RB

IL2RB gene encodes IL2Rβ, which is a subunit of IL2 receptor that is involved in receptor-mediated endocytosis and in transduction of the mitogenic signals of IL2. It belongs to the type I cytokine receptor family, it is devoid of intrinsic kinase activity, and its presence influences receptor affinity (intermediate-high affinity forms) [48, 49]. IL2, which is a pro-inflammatory cytokine secreted by activated T cells after antigen stimulation, is expressed in the hematopoietic system (including lymphoid linages T, B, and NK cells, as well as myeloid cells, such as macrophages, monocytes, and neutrophils) and plays a critical role in controlling immune system homeostasis [50, 51]. IL2 is a T cell growth and survival factor that promotes survival and proliferation of regulatory T cells (primary and memory immune responses) by binding its receptor and by activating its downstream signalling, which leads to the transcription of IL2-dependent genes [48, 50].

SNPs in *IL2RB* gene, which are not in LD with rs3218258 ($r^2 < 0.025$), are associated with asthma severity, inflammation, and dysregulation of the immune system [49, 52]. However, SNP rs3218258 is negatively associated with *IL2RB* gene expression levels in the whole blood [53] and, in GEIRD, non-normal FeNO was largely more prevalent among the asthma cases with TT genotype than among the patients with TC or CC genotypes for this SNP (72.2% vs 38.3%, $p = 0.005$). As observed for SNP rs987314, the association between rs3218258 and FeNO should not depend on the potential confounding effect of the main characteristics of the GEIRD patients (see table S6).

4.3. TNS1, NPSR1, FADS2, and HAVCR1

SNPs rs13022785 in *TNS1*, rs1419779 in *NPSR1*, rs174579 in *FADS2*, and rs953569 in *HAVCR1* genes were not significantly associated with FeNO in the ECRHS III dataset. Despite lack of replication in this independent sample of patients, these genes might be interesting targets for further investigations into their role in asthma (see table S7).

4.4. Strengths and limitations of the study

A major strength of the current report is the replication analysis that was carried out within an independent sample of asthma cases from different European countries. Secondly, an accurate phenotyping with similar protocols was carried out in GEIRD [20] and ECRHS III [27]. Thirdly, the study designs of GEIRD and ECRHS should guarantee that representative samples of asthma cases, presenting a wide range of asthma phenotypes, were identified from the general adult population in Europe. Finally, we used a statistical approach (GBM) that does not require an *a priori* specification of the genetic model and that permits inclusion of a large number of predictors. Therefore, GBM can be used for jointly exploring multiple SNPs (step 1) and for reducing the number of SNPs for statistical testing within a parametric framework (step 2).

A few limitations of our study should be mentioned. Both the sample size and the number of genotyped SNPs are relatively small. In particular, the limited sample size did not allow the analysis to be stratified by different asthma phenotypes, as asthma SNPs may be associated with one or more disease-related traits (e.g. eosinophilia, airway obstruction, atopy) in affected subjects [54], and to create a validation set for GBM. In the latter case, however, we used a cross-validation technique (k-fold) that permits an accurate performance estimation [55]. In addition, the asthma cases identified in ECRHS III had a different range of age (40–66 years), as compared to the patients in GEIRD (20–66 years). This difference between the datasets could reduce the replicability and generalizability of our results. Finally, we tested an Italian cohort of European ancestry and replicated our results in an independent cohort from other European countries, which limits the generalizability of our findings to other populations because ethnicity has also been shown to influence FeNO [56].

5. Conclusions

Two novel SNPs in *FAM13A* and *IL2RB* genes are associated with FeNO in adult subjects with asthma who were identified from random samples of the general European population. This positive association supports a possible causative role for FAM13A and IL2RB genes in airway inflammation in asthma, therefore establishing the role of these genes deserves further investigations. Moreover, these genes represent an interesting target because of their involvement in different mechanisms that affect smooth muscle constriction and endothelial barrier function responses (*FAM13A*), or in immune response processes (*IL2RB*).

Data availability statement

The data cannot be made publicly available upon publication because they contain sensitive personal information.

Funding

GEIRD in Verona was funded by Cariverona Foundation (Bando 2006), the Italian Ministry of Health (Ricerca finalizzata 2009, RF-2009-1471235), Chiesi Farmaceutici S.p.A., and the Italian Medicines Agency—AIFA. The coordination of ECRHS III was supported by the Medical Research Council. Local funding agencies, PIs and team members of ECRHS III are reported in the supplemental information.

Author contributions

S A, L C, and M O conceived and designed the work. S A, C B, G M, C M, J P, and M O collected the data. V L, L C, and S A performed the statistical analyses. S A, V L, and L C drafted the article. All authors interpreted results, revised the article critically for important intellectual content, and gave final approval of the version to be submitted.

Conflict of interest

The authors declare no competing interests.

Ethical statement

The appropriate ethics committee ('Comitato Etico per la Sperimentazione dell'Azienda Ospedaliera Istituti Ospitalieri di Verona') approved the GEIRD survey in Verona and all the aspects of the research project were fully explained to the participants, who gave their written informed consent. Additional information regarding the ECRHS III survey, including ethics approvals, was included as supplementary material.

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