Published in final edited form as:

Clin Exp Allergy. 2015 April ; 45(4): 797–806. doi:10.1111/cea.12461.

A Common variant in *RAB27A* gene is associated with fractional exhaled nitric oxide levels in adults

Emmanuelle Bouzigon^{1,2}, Rachel Nadif^{3,4,*}, Emma E. Thompson^{5,*}, Maria Pina Concas⁶, Susan Kuldane⁵, Gaixin Du⁵, Myriam Brossard^{1,2}, Nolwenn Lavielle^{1,2}, Chloé Sarnowski^{1,2}, Amaury Vaysse^{1,2}, Philippe Dessen⁷, Ralf JP van der Valk^{8,9,10}, Liesbeth Duijts^{9,10,11}, A John Henderson¹¹, Vincent WV Jaddoe^{8,9,10}, Johan C de Jongste⁹, GABRIEL consortium, EARly Genetics & Lifecourse Epidemiology (EAGLE) Consortium, Stefania Casula^{6,12}, Marie-Hélène Dizier^{1,2}, Isabelle Pin^{13,14,15}, Régis Matran^{16,17}, Mark Lathrop¹⁸, Mario Pirastu^{6,19}, Florence Demenais^{1,2}, and Carole Ober⁵

¹Inserm, UMR-946, F-75010, Paris, France ²Univ Paris Diderot, Sorbonne Paris Cité, Institut Universitaire d'Hématologie, F-75007, Paris, France ³Inserm, U1018, Centre for research in Epidemiology and Population Health (CESP), Respiratory and Environmental Epidemiology Team, F-94807, Villejuif, France ⁴Univ Paris-Sud, UMRS 1018, F-94807, Villejuif, France ⁵Department of Human Genetics, University of Chicago, Chicago, IL 60637, USA ⁶Institute of Population Genetics, National Council of Research, Sassari, Italy ⁷Inserm, UMRS 985, Institut Gustave Roussy, F-94805, Villejuif, France ⁸The Generation R Study Group, Erasmus Medical Center, Rotterdam, The Netherlands ⁹Department of Pediatrics, Erasmus Medical Center, Rotterdam, The Netherlands ¹⁰Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands ¹¹School of Social and Community Medicine, University of Bristol, UK ¹²Institute of Molecular Genetics, National Council of Research, Pavia, Italy ¹³INSERM, IAB, Team of Environmental Epidemiology applied to Reproduction and Respiratory Health, F-38000 Grenoble, France ¹⁴Univ. Grenoble Alpes, IAB, Team of Environmental Epidemiology applied to Reproduction and Respiratory Health, F-38000 Grenoble, France ¹⁵Pédiatrie, CHU de Grenoble, F-38043, Grenoble, France ¹⁶Univ Lille Nord de France, F-59000, Lille, France ¹⁷CHU, F-59000, Lille, France ¹⁸McGill University and Génome Québec Innovation Centre, Montréal, Canada ¹⁹Ogliastria Genetic Park, Perdasdefogu, Sardinia, Italy

Abstract

Background—Exhaled nitric oxide (FeNO) is a biomarker for eosinophilic inflammation in the airways and for responsiveness to corticosteroids in asthmatics.

Correspondence: Emmanuelle Bouzigon, MD, PhD, UMR946, INSERM / Université Paris-Diderot, Institut de Génétique Moléculaire, 27 rue Juliette Dodu, 75010 Paris, France, Tel: +33 1 53 72 50 23 / Fax: +33 1 53 72 50 49, emmanuelle.bouzigon@inserm.fr.

*These authors are joint second authors.

Conflict of interest

Dr Liesbeth Duijts is the recipient of a European Respiratory Society/Marie Curie Joint Research Fellowship and from the Lung Foundation Netherlands (no 3.2.12.089; 2012). The other authors declare no conflict of interest.

Objective—We sought to identify in adults the genetic determinants of fractional exhaled nitric oxide (FeNO) levels and to assess whether environmental and disease-related factors influence these associations.

Methods—We performed a genome-wide association study of FeNO through meta-analysis of two independent discovery samples of European ancestry: the outbred EGEA study (French Epidemiological study on the Genetics and Environment of Asthma, N=610 adults) and the Hutterites (N=601 adults), a founder population living on communal farms. Replication of main findings was assessed in adults from an isolated village in Sardinia (Talana study, N=450). We then investigated the influence of asthma, atopy and tobacco smoke exposure on these genetic associations and whether they were also associated with FeNO values in children of the EAGLE (EARly Genetics & Lifecourse Epidemiology, N=8,858) consortium.

Results—We detected a common variant in *RAB27A* (rs2444043) associated with FeNO that reached the genome-wide significant level ($P=1.6 \times 10^{-7}$) in the combined discovery and replication adult datasets. This SNP belongs to member of RAS oncogene family (*RAB27A*) and was associated with an expression quantitative trait locus for *RAB27A* in lymphoblastoid cell lines from asthmatics. A second suggestive locus (rs2194437, $P=8.9 \times 10^{-7}$) located nearby the sodium/calcium exchanger 1 (*SLC8A1*) was mainly detected in atopic subjects and influenced by inhaled corticosteroid use. These two loci were not associated with childhood FeNO values.

Conclusions and Clinical Relevance—This study identified a common variant located in *RAB27A* gene influencing FeNO levels specifically in adults and with a biological relevance to the regulation of FeNO levels. This study provides new insight into the biological mechanisms underlying FeNO levels in adults.

Keywords

Exhaled nitric oxide; genetics; airway inflammation; genome-wide association study; asthma; atopy

INTRODUCTION

Nitric oxide (NO) and related compounds are produced by a wide variety of inflammatory cells in the respiratory system [1]. The fractional exhaled nitric oxide (FeNO) is a biomarker for eosinophilic airway inflammation and for responsiveness to corticosteroids in asthmatics [2]. FeNO levels are elevated in both children and adults with asthma, generally lower in children than in adults and are decreased in current smokers [2]. FeNO levels of atopic asthmatics are higher than those of non-atopic asthmatics, whereas no differences in FeNO levels were observed between non-allergic asthmatics and non-allergic non-asthmatic adults [3–5].

Nitric oxide is endogenously produced by three NO synthase isoforms (NOSs, *EC 1.14.13.39*) from L-arginine: a neuronal isoform (nNOS encoded by *NOS1*), an inducible isoform (iNOS, encoded by *NOS2*) and a vascular endothelial isoform (eNOS encoded by *NOS3*) [6]. The human airway epithelial cells express all NOS isoforms [7] and produce FeNO in response to pro-inflammatory cytokines and inflammatory mediators [8].

The genetic contribution to FeNO levels is substantial with genetic effects accounting for around 67% of the variation in FeNO in adults [9]. Previous studies of FeNO focused mainly on candidate genes and identified associations of FeNO with genetic variants at the *NOS2* promoter region in both non-asthmatic children and adults, and with variants in *NOS3* and *ARG2* (arginase 2) in adults and children respectively [10–12]. Recently, a genome-wide association study (GWAS) of FeNO in children identified three associated loci: *LYR motif containing 9* (*LYRM9*), *NOS2* and near *gasdermin B* (*GSDMB*) at the 17q21 asthma locus [13].

The goal of the current study was to identify genetic loci associated with FeNO levels through meta-analysis of GWAS conducted in adults of European ancestry from two discovery cohorts: the French Epidemiological study on the Genetics and Environment of Asthma (EGEA) and the Hutterites, an isolated population of European descent now living in South Dakota, with replication in adults from an isolated village in Sardinia (Talana study). We then investigated whether the detected genetic associations were modified by environmental and disease-related factors influencing FeNO levels: asthma, atopy and tobacco smoke exposure and whether they were also associated with FeNO values in children of the EAGLE (EARly Genetics & Lifecourse Epidemiology) consortium.

METHODS

Discovery cohorts

Two independent populations of European descent were included in the discovery phase: the EGEA cohort and the Hutterites that have been described in detail previously [14, 15]. Briefly, the EGEA study combines a case-control and a family-based study of asthma with a 12-year longitudinal survey (EGEA2, 2003–2007). The whole study population included 388 asthmatic probands recruited in chest clinics and their 1,317 family members (probands' parents and/or siblings) plus 415 population-based controls. The present analysis uses all adult participants with FeNO data measured at EGEA2 (N=610).

The Hutterites of North America are a founder population of European descent. The 601 adult participants of the study belong to a single 13-generation (3,671 persons) pedigree and live on communal farms in South Dakota [15]. The communal lifestyle of the Hutterites results in low environmental heterogeneity. Of particular to this study is that smoking is prohibited.

Ethical approval was obtained for all cohorts from the appropriate institutional ethics committee and written consent was obtained from all participants.

Phenotypes

In EGEA, FeNO measurements at the 50mL/s flow rate were performed according to ATS/ERS recommendations (ATS Workshop 2005) [16] before any other pulmonary function test as previously described [17]. In the Hutterites, FeNO was measured at the 50mL/s flow rate using a Sievers Nitric Oxide Analyzer (NOA) 280i according to ATS guidelines during visits to Hutterite farms between 2006–2009 [16].

Genotyping

The EGEA sample was genotyped using the Illumina 610 Quad array (Illumina, San Diego, CA) at the Centre National de Génotypage (CNG, Evry, France), as part of the European Gabriel consortium [18]. The Hutterites were genotyped using the Affymetrix GeneChip® 500k or 6.0 Mapping Array. Stringent quality criteria were applied to select both individuals and SNPs and have been previously detailed [15, 19]. After the quality control (QC) process, a total of 513,460 and 266,727 SNPs were available in EGEA and Hutterites respectively. To generate a common set of SNPs between EGEA and Hutterites for meta-analysis, imputations were conducted in EGEA using MACH software (<http://www.sph.umich.edu/csg/abecasis/MACH/>), with reference haplotype panels from HapMap Phase 2 CEU subjects. Imputed allele dosage values of SNPs with imputation quality score (Rs_q) greater than or equal to 0.5 and minor allele frequency $\geq 3\%$ were kept for analysis allowing investigation of both common and low frequency alleles [20]. A total of 247,463 SNPs available in both EGEA and Hutterites were examined.

Statistical analysis and strategy of analysis

Discovery study—After the study-specific QC, a total of 1,211 adult individuals from the two discovery studies were included in the present study. We examined log-transformed FeNO levels adjusted for age at time of measurement, sex, height, smoking status (current, ex- and non-smokers) and examination centre in EGEA, and adjusted for age at time of measurement and sex in Hutterites. Association between log-transformed FeNO and each SNP was investigated under an additive genetic model by linear regression based methods (see online supplementary). Genome-wide association studies of FeNO were conducted in EGEA and Hutterites separately, and combined through meta-analysis in order to increase power and to obtain more robust findings. The meta-analysis was performed using weighted Z-score method [21]. The SNPs showing suggestive evidence of association ($P \leq 5 \times 10^{-5}$) with FeNO levels in this discovery dataset meta-analysis were selected for replication in adults from the Talana study.

Replication study and sensitivity analyses—The Talana study comes from a cross-sectional population based study performed in a secluded area of Sardinia (Ogliastra), as previously described [22]. Data from 450 adult subjects with genotypes, FeNO measurements and asthma evaluations were included in the replication study (see online supplementary). FeNO measurements at the 50mL/s flow rate were carried out with stationary chemiluminescence analyzer CLD 88 sp (Eco Physics AG) according to ATS guidelines [16]. Association studies were performed on log-transformed FeNO adjusted for age, sex, height and smoking status (current, ex- and non-smokers), using linear regression based method (see online supplementary). Association results between discovery and replication datasets were combined through meta-analysis of weighted Z-scores [21]. In order to control for multiple tests, a critical P-value threshold equal to 2.02×10^{-7} was considered genome-wide significant based on a standard Bonferroni correction (0.05 error / 247,463 SNPs) for meta-analysis of both discovery and replication studies and a P-value of less than 0.0056 (0.05/9 independent SNPs taken forward for replication) for the analyses of the replication sample.

For SNPs showing increased evidence for association in the meta-analysis of discovery and replication studies, a sensitivity analysis was then performed by adding inhaled corticosteroid (ICS) use in the regression model. Moreover, to investigate the effect of asthma, atopy (defined by a positive skin prick test response to at least one aeroallergen) and tobacco smoke exposure on these main genetic associations, analyses were conducted in each dataset stratified by each of these factors. The FeNO values were unadjusted for smoking in the stratified analyses on that factor. The association results across studies were summarized within strata through meta-analysis of weighted Z-scores [21]. Then, tests for heterogeneity of SNP effect on FeNO levels between strata were performed. Finally, we explored whether SNPs identified in the adult discovery GWAS were related to FeNO levels in children of the EAGLE (N=8,858) consortium (see online supplementary) [13].

Pairwise linkage disequilibrium (LD) measures (D' and r^2) between genetic polymorphisms were estimated using the SNAP program [23].

eQTL analysis and functional annotation

We assessed whether our two most significant SNPs (or their proxies) were expression quantitative trait loci (eQTLs). We used eQTL browser (<http://www.hsph.harvard.edu/liming-liang/software/eqtl/>) to search for eQTLs in genome-wide expression data of lymphoblastoid cell lines from British asthmatic subjects [24]. Moreover, functional annotations of these two SNPs (or their proxies) were obtained using HaploReg [25].

Pathway analysis

The single SNP analysis was complemented by pathway analysis. Gene Set Enrichment Analysis (GSEA) implemented in GenGen program [26], which searches for gene ontology (GO) classes enriched with association signals, was performed from the meta-analysis results of the discovery datasets. A total of 173,882 SNPs were within 20kb of 29,418 genes or pseudo-genes. These genes were assigned to 327 level-4 GO classes that met our pathway size criteria (at least 20 genes and at most 200 genes). Significance of GO categories was assessed by false discovery rate (FDR) based on 10,000 SNP permutations. To explore biological connections and potentially identify new pathways, we reported GO categories with P-value ≤ 0.05 and FDR ≤ 0.25 as recommended by the authors (<http://www.broadinstitute.org/gsea/>).

RESULTS

The characteristics of the participants included in the discovery phase (EGEA and Hutterites, N=1,211) as well as in the adult replication population (Talana, N=450) are shown in Table 1. Due to the mode of ascertainment, asthma was more prevalent in EGEA (43.3%) than in Hutterites (17.1%) whereas the proportion of atopic subjects (sensitization to any of the allergens tested) did not differ between the two studies (56.2% *versus* 52.2%). However, among sensitized subjects, Hutterite individuals were more often poly-sensitized (positive skin prick test responses ≥ 3 allergens) than EGEA samples (56.4% *versus* 44.9%, $P=6 \times 10^{-5}$). The FeNO values were higher in the Hutterites than in EGEA (23.2 with

interquartile range [17.0;32.3] *versus* 16.0 with IQR [10.6;22.5] ppb). GWAS were then conducted separately in EGEA and Hutterites samples.

The Manhattan plot for the genome-wide meta-analysis of FeNO levels is shown in Figure 1, and the quantile-quantile plot is shown in online supplementary figure E1.

Genetic variants associated with FeNO levels

We detected 14 SNPs, corresponding to nine independent markers ($r^2 < 0.8$) that were associated with FeNO levels at $P = 5 \times 10^{-5}$ in the discovery meta-analysis (Figure 1 and Table 2). The most significant signal was in the *plexin C1* (*PLXNC1*) gene on chromosome 12 with four SNPs in strong LD ($r^2 > 0.8$) having P-values ranging from 2.5×10^{-6} to 9.5×10^{-6} . Among the nine independent SNPs taken forward for replication, two showed association with FeNO in the Talana samples: rs2444043 in *RAB27A* on chromosome 15 ($P = 0.0007$) that showed significant association when corrected for multiple testing and rs2194437 in an intergenic region between *SLC8A1* (*solute carrier family 8 (sodium/calcium exchanger) member 1*) and *HNRNPA1P57* (*heterogeneous nuclear ribonucleoprotein A1 pseudogene 57*) on chromosome 2 ($P = 0.02$). These two loci showed increase evidence for association in the combined discovery and replication meta-analysis (Table 2). The strongest association was detected for rs2444043 located in the *RAB27A* gene (15q21) that reached genome-wide significance after the meta-analysis of discovery and replication samples ($P = 1.6 \times 10^{-7}$, explained FeNO variance = 1.65%). Each additional T allele of rs2444043 was associated with higher FeNO levels. Suggestive evidence of association was found for rs2194437 in 2p23-p22 region ($P = 8.9 \times 10^{-7}$, explained FeNO variance = 1.46%) with each C allele associated with lower values of FeNO. Interestingly, accounting for inhaled corticosteroid use in the regression model showed improvement in the evidence for association of rs2194437 ($P = 1.3 \times 10^{-7}$) whereas the result for rs2444043 was unchanged. The regional association plots for these two replicated loci are shown in online supplementary figure E2.

We next investigated the influence of smoking (using the FeNO values unadjusted for smoking), asthma and atopic status on the two main association signals in the three adult datasets (Table 3). The association between FeNO and rs2444043 was mainly detected in non-atopics ($P = 1.8 \times 10^{-4}$ *vs* $P = 0.009$) and the association with rs2194437 was mainly present in atopics ($P = 3.8 \times 10^{-5}$ *vs* $P = 0.03$) or in never smokers ($P = 9.2 \times 10^{-7}$ *versus* $P = 0.01$).

Finally, we tested whether the nine loci detected at $P = 5 \times 10^{-5}$ in the adult discovery meta-analysis were associated to FeNO values in childhood using the 14 pediatric studies of the EAGLE consortium. None of these SNPs showed evidence for association ($P > 0.14$), suggesting that our signals were specific to adult FeNO levels (Table 4).

eQTL and functional annotations

Using the eQTL browser, we found that two SNPs (rs8042411 and rs11855490) in LD with rs2444043 ($D' = 1$, $r^2 = 0.1$) were associated with *RAB27A* gene expression ($P = 2.8 \times 10^{-8}$) in lymphoblastoid cell lines from asthmatics [24]. Interestingly, haplotype reconstruction showed that the rs2444043 T allele that is associated with increased FeNO levels in our

study, was always associated with rs11855490 G allele for which positive correlation with *RAB27A* expression is observed. We did not find eQTL for rs2194437.

Using the functional annotation HaploReg database, we found that the two loci with associated SNPs (2p23-p22 and 15q21) contained marks of active regulatory elements notably in lung fibroblasts and small airway epithelial cells: enhancer histone marks, DNase hypersensitivity sites and binding protein sites (online supplementary table E1).

Pathway analysis

We applied gene set enrichment analysis to the discovery GWAS meta-analysis results to explore biological connections and identify pathways associated with FeNO levels. We identified enrichment of 18 partially overlapping GO categories that achieved a category-specific P-value 0.05 and FDR 0.25 among which two had P-values equal to 0.005: ‘Oxidoreductase activity’ function and ‘Coenzyme metabolic process’ (online supplementary table E2 and figure E3). Thirteen GO categories were connected in a large cluster including four GO categories that were closely linked two by two. Among these GO categories, two were related to GTPase activity: ‘Ras guanyl-nucleotide exchange factor activity’ and ‘Regulation of small GTPase mediated signal transduction’; but did not include our top signals.

DISCUSSION

In this meta-analysis, we found a new locus (*RAB27A* gene on chromosome 15q21) associated with FeNO levels in adults. Our most significantly associated SNP was rs2444043 that was formally replicated in an independent study and reached a genome-wide significant level after the meta-analysis of discovery and replication adult samples. This SNP is located in intron 1 of *RAB27A* within a DNaseI hypersensitive site. The protein encoded by *RAB27A* belongs to the small GTPase superfamily. Interestingly, two of the main GO categories detected by the GSEA analysis (‘Regulation of small GTPase mediated signal transduction’ and ‘Ras guanyl-nucleotide exchange factor activity’) are related to small GTPase activity. These GOs regulate two other GOs that include *RAB27A*: ‘GTPase activity’ function and ‘small GTPase mediated signal transduction’ process, but that were not part of the pathway analysis since these GOs did not meet our pathway size criteria. This pathway analysis supports and strengthens the evidence for a role of GTPase in FeNO levels. The Rab family functions as regulators of specific intracellular traffic pathway. Rab27a protein is located in the cytoplasm and is membrane-bound; it is expressed in the lungs of both mice and humans [27]. Pulmonary epithelium atrophy has been described in lungs of double knockout mice (Rab27a and Rab27b), and interestingly these morphologic changes were observed in aged mice only (over 12–18 months) [27]. Moreover, a reduced expression of *RAB27A* was found in old mouse lung tissue (25–31 months), as well as in human senescent lung fibroblasts and lung epithelial cells [28]. These observations are in agreement with the absence of association signal between FeNO levels and SNPs at the *RAB27A* locus in the EAGLE children cohorts. Rab27A is also expressed in human blood eosinophils, and recently, a direct role of Rab27a in eosinophil degranulation has been

shown in Rab27a-deficient mouse strain [29]. In humans, blood eosinophils produce NO and participate in the regulation of the NO pool in pulmonary tissues [30].

The second most significant signal detected at a suggestive level was with rs2194437 located in an intergenic region between *SLC8A1* and *HNRNPA1P57* (2p23-p22), and was mainly found in atopic samples and with increase evidence for association when accounting for ICS use. This SNP is in LD ($D'=1$, $r^2=0.3$) with SNP in intron 2 of *SLC8A1*, also called *NCX1* (sodium/calcium exchanger 1). NCX1 is ubiquitously expressed in several tissues, including lung [31]. The expression of NCX1 is enhanced by pro-inflammatory cytokines Tumor Necrosis Factor alpha (TNF α) and IL-13 in cultured human airway smooth muscle, thereby increasing the intracellular Ca²⁺ concentration, and thus enhancing airway contractility [32]. Interestingly, the two constitutive NO-synthases (NOS1 and NOS3) are calcium dependent and activated by small rises in intracellular calcium [33].

We previously showed significant associations of FeNO with *NOS3* and promoter *NOS2* SNPs in EGEA non-asthmatics and with a *NOS3* SNP in EGEA asthmatics [10]. In the present study, only weak associations with FeNO levels were detected for SNPs in *NOS2*, *NOS3* and *ARG2* candidate genes ($0.02 < P < 0.05$, results not shown). These modest associations may be due to insufficient SNP coverage of those regions in our meta-analysis as the previously reported SNPs were not part of the common set of markers available for the meta-analysis.

FeNO values are generally lower in children than in adults whether they have asthma or not [2]. We identified two genetic determinants of FeNO levels that have an effect in adults but not in children. We investigated environmental and disease-related factors influencing FeNO values that might differ between adults and children under study: tobacco smoke exposure, asthma and atopy. None of these factors could explain the difference for *RAB27A* locus effects between children and adults because the genetic association signal was mainly observed in non-smoker, non-asthmatic or non-atopic individuals. Instead, the specific effect of the *RAB27A* polymorphism on FeNO levels in adults might reflect a strong gene by age interaction, which would be consistent with several lines of evidence implicating *RAB27A* in age-related changes of bronchial airways [27, 28]. In that light, it is possible that the adult-specific association could be related to ageing process or other factors throughout life that might influence FeNO levels. In contrast, the effect of the 2p23-p22 locus on FeNO levels may be related to the allergy or inflammatory process, as it was mainly detected in atopic subjects and influenced by ICS use. Indeed, the proportion of atopic subjects was very high in both adult studies (52% and 56% in Hutterites and EGEA respectively).

Neither of the two most significant signals detected in the present study was associated with asthma in the largest asthma GWAS conducted to date ($P = 0.23$ for ever asthma, childhood onset asthma and adult onset asthma) [18]. Furthermore, using the NCBI dbGaP database, we identified that genetic polymorphisms located in the surrounding region of our two most significant associations have been associated with an inflammation/oxidative stress biomarker [34], and neutrophil counts [35] for the 15q21 region, and with blood lipid phenotypes [36] for the 2p23-p22 region, but not with asthma *per se*.

Although the current study is the largest effort so far to determine genetic determinants of FeNO levels in adults, we are still limited by the number of subjects included to generate enough statistical power to detect all variants with modest effects. As in most GWAS, the genome-wide significant level was reached when combining several datasets. The power to detect a variant with genetic effect and characteristics similar to rs2444043 was equal to 31% in the discovery sample (EGEA and Hutterites) and reached 53% in the combined discovery and replication samples [37]. Larger or more functionally focused studies are needed to characterize the many loci with modest effects that remain to be identified for FeNO in adults.

In summary, we have identified a common variant located in *RAB27A* gene associated with FeNO levels specifically in adults of both inbred and outbred populations living on different environments and that explained 1.65% of the variance in FeNO levels. Several lines of evidence suggest that this gene is a strong candidate for FeNO levels. In addition, suggestive association was detected with SNPs nearby *SLC8A1*. Further genetic investigation of the relationship between these loci and other airway inflammatory phenotypes is warranted. These results provide new insight into the biological mechanisms that underlie the regulation of FeNO levels in adults.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank all those who participated to the setting of the study and on the various aspects of the examinations involved: interviewers, technicians for lung function testing and skin prick tests, blood sampling, IgE determinations, coders, those involved in quality control, data and sample management and all those who supervised the study in all in all EGEA centers and during all Hutterite field trips. The authors are grateful to the three CIC-Inserm of Necker, Grenoble and Marseille who supported the EGEA study and in which subjects were examined. They are also grateful to the three biobanks in Lille (CIC-Inserm), Evry (Centre National de Génotypage) and at Annemasse (Etablissement français du sang) where EGEA biological samples are stored. Further acknowledgements are given in the Online Supplement.

Fundings

EGEA: Research was funded by French National Agency for Research (ANR-CES-2009, ANR-11-BSV1-027-GWIS-AM), Region Nord Pas-de-Calais and GABRIEL, a multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community (contract 018996 from the European Commission).

Hutterites: Studies in the Hutterites were supported by NIH grant R01 HL085197 to C.O.

Talana - Ogliastro Genetic Park study: Project ICT SIAI 101 "Creating a center of excellence in bio-computing technologies applied to personalized medicine" Sardegna Ricerche, Sardinia Region, Ministry of Economy and Finance.

EAGLE consortium: The UK Medical Research Council, the Wellcome Trust (Grant Ref. 092731) and the University of Bristol provide core support for ALSPAC.

References

1. Ricciardolo FL, Di Stefano A, Sabatini F, Folkerts G. Reactive nitrogen species in the respiratory tract. *Eur J Pharmacol.* 2006; 533:240–52. [PubMed: 16464450]

2. Szeftler SJ, Wenzel S, Brown R, et al. Asthma outcomes: biomarkers. *J Allergy Clin Immunol*. 2012; 129:S9–23. [PubMed: 22386512]
3. Dweik RA, Boggs PB, Erzurum SC, et al. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. *Am J Respir Crit Care Med*. 2011; 184:602–15. [PubMed: 21885636]
4. Jang WN, Park IS, Choi CH, et al. Relationships between exhaled nitric oxide and atopy profiles in children with asthma. *Allergy Asthma Immunol Res*. 2013; 5:155–61. [PubMed: 23638314]
5. Scott M, Raza A, Karmaus W, et al. Influence of atopy and asthma on exhaled nitric oxide in an unselected birth cohort study. *Thorax*. 2010; 65:258–62. [PubMed: 20335297]
6. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J*. 2001; 357:593–615. [PubMed: 11463332]
7. Asano K, Chee CB, Gaston B, et al. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc Natl Acad Sci U S A*. 1994; 91:10089–93. [PubMed: 7524082]
8. Robbins RA, Barnes PJ, Springall DR, et al. Expression of inducible nitric oxide in human lung epithelial cells. *Biochem Biophys Res Commun*. 1994; 203:209–18. [PubMed: 7521161]
9. Thomsen SF, Ferreira MA, Kyvik KO, Fenger M, Backer V. A quantitative genetic analysis of intermediate asthma phenotypes. *Allergy*. 2009; 64:427–30. [PubMed: 19175595]
10. Bouzigon E, Monier F, Boussaha M, et al. Associations between nitric oxide synthase genes and exhaled NO-related phenotypes according to asthma status. *PLoS One*. 2012; 7:e36672. [PubMed: 22590587]
11. 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; 49:200–5. [PubMed: 22368302]
12. Salam MT, Bastain TM, Rappaport EB, et al. Genetic variations in nitric oxide synthase and arginase influence exhaled nitric oxide levels in children. *Allergy*. 2011; 66:412–9. [PubMed: 21039601]
13. van der Valk RJ, Duijts L, Timpson NJ, et al. Fraction of exhaled nitric oxide values in childhood are associated with 17q11. 2-q12 and 17q12-q21 variants. *J Allergy Clin Immunol*. 2014; 134:46–55. [PubMed: 24315451]
14. Kauffmann F, Dizier MH, Pin I, et al. Epidemiological study of the genetics and environment of asthma, bronchial hyperresponsiveness, and atopy: phenotype issues. *Am J Respir Crit Care Med*. 1997; 156:S123–9. [PubMed: 9351592]
15. Yao TC, Du G, Han L, et al. Genome-wide association study of lung function phenotypes in a founder population. *J Allergy Clin Immunol*. 2014; 133:248–55. e1–10. [PubMed: 23932459]
16. ATS Workshop Proceedings: Exhaled nitric oxide and nitric oxide oxidative metabolism in exhaled breath condensate: Executive summary. *Am J Respir Crit Care Med*. 2006; 173:811–3. [PubMed: 16556701]
17. Nadif R, Matran R, Maccario J, et al. Passive and active smoking and exhaled nitric oxide levels according to asthma and atopy in adults. *Ann Allergy Asthma Immunol*. 2010; 104:385–93. [PubMed: 20486328]
18. Moffatt MF, Gut IG, Demenais F, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med*. 2010; 363:1211–21. [PubMed: 20860503]
19. Imboden M, Bouzigon E, Curjuric I, et al. Genome-wide association study of lung function decline in adults with and without asthma. *J Allergy Clin Immunol*. 2012; 129:1218–28. [PubMed: 22424883]
20. Frazer KA, Murray SS, Schork NJ, Topol EJ. Human genetic variation and its contribution to complex traits. *Nat Rev Genet*. 2009; 10:241–51. [PubMed: 19293820]
21. Rosenthal R. Meta-analytic procedures for social research. Newbury Park, CA: Sage; 1984.
22. Biino G, Balduino CL, Casula L, et al. Analysis of 12,517 inhabitants of a Sardinian geographic isolate reveals that predispositions to thrombocytopenia and thrombocytosis are inherited traits. *Haematologica*. 2011; 96:96–101. [PubMed: 20823129]

23. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI. SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. *Bioinformatics*. 2008; 24:2938–9. [PubMed: 18974171]
24. Liang L, Morar N, Dixon AL, et al. A cross-platform catalogue of 14,177 expression quantitative trait loci derived from lymphoblastoid cell lines. *Genome Res*. 2013
25. Ward LD, Kellis M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. *Nucleic Acids Res*. 2012; 40:D930–4. [PubMed: 22064851]
26. Wang K, Li M, Bucan M. Pathway-based approaches for analysis of genomewide association studies. *Am J Hum Genet*. 2007; 81:1278–83. [PubMed: 17966091]
27. Bolasco G, Tracey-White DC, Tolmachova T, et al. Loss of Rab27 function results in abnormal lung epithelium structure in mice. *Am J Physiol Cell Physiol*. 2011; 300:C466–76. [PubMed: 21160031]
28. Rolewska P, Simm A, Silber RE, Bartling B. Reduced expression level of the cyclic adenosine monophosphate response element-binding protein contributes to lung aging. *Am J Respir Cell Mol Biol*. 2014; 50:201–11. [PubMed: 23991634]
29. Kim JD, Willetts L, Ochkur S, et al. An essential role for Rab27a GTPase in eosinophil exocytosis. *J Leukoc Biol*. 2013; 94:1265–74. [PubMed: 23986549]
30. Iijima H, Duguet A, Eum SY, Hamid Q, Eidelman DH. Nitric oxide and protein nitration are eosinophil dependent in allergen-challenged mice. *Am J Respir Crit Care Med*. 2001; 163:1233–40. [PubMed: 11316664]
31. Khananshvil D. The SLC8 gene family of sodium-calcium exchangers (NCX) - structure, function, and regulation in health and disease. *Mol Aspects Med*. 2013; 34:220–35. [PubMed: 23506867]
32. Sathish V, Delmotte PF, Thompson MA, Pabelick CM, Sieck GC, Prakash YS. Sodium-calcium exchange in intracellular calcium handling of human airway smooth muscle. *PLoS One*. 2011; 6:e23662. [PubMed: 21858195]
33. Dweik RA. Nitric oxide, hypoxia, and superoxide: the good, the bad, and the ugly! *Thorax*. 2005; 60:265–7. [PubMed: 15790976]
34. Benjamin EJ, Dupuis J, Larson MG, et al. Genome-wide association with select biomarker traits in the Framingham Heart Study. *BMC Med Genet*. 2007; 8 (Suppl 1):S11. [PubMed: 17903293]
35. Ramsuran V, Kulkarni H, He W, et al. Duffy-null-associated low neutrophil counts influence HIV-1 susceptibility in high-risk South African black women. *Clin Infect Dis*. 2011; 52:1248–56. [PubMed: 21507922]
36. Kathiresan S, Manning AK, Demissie S, et al. A genome-wide association study for blood lipid phenotypes in the Framingham Heart Study. *BMC Med Genet*. 2007; 8 (Suppl 1):S17. [PubMed: 17903299]
37. Gauderman, WJ.; Morrison, JM. QUANTO 1.1: A computer program for power and sample size calculations for genetic-epidemiology studies. 2006. Available: <http://biostats.usc.edu/Quanto.html>

Figure 1.

Manhattan plot of meta-analysis GWAS results of FeNO levels for all EGEA and Hutterites samples. X-axis shows chromosome position and Y-axis shows $-\log_{10}(P\text{-value})$. The horizontal lines are drawn at $P=2.02 \times 10^{-7}$ and $P=5 \times 10^{-5}$ for genome-wide and suggestive significant thresholds respectively. Gene symbol and full name: *TAF1B* (TATA box binding protein (TBP)-associated factor, RNA polymerase I, B), *SLC8A1* (solute carrier family 8 (sodium/calcium exchanger), member 1), *SLC6A11* (solute carrier family 6 (neurotransmitter transporter), member 11), *PLEKHG4B* (pleckstrin homology domain containing, family G (with RhoGef domain) member 4B), *FSTL4* (follistatin-like 4), *PLXNC1* (plexin C1), *RAB27A* (RAB27A, member RAS oncogene family), *ZNF40* (human immunodeficiency virus type I enhancer binding protein 1) and *ZADH2* (zinc binding alcohol dehydrogenase domain containing 2).

Table 1

Characteristics of adult participants of the discovery phase (EGEA and Hutterites) and of the replication phase (Talana) at the time of FeNO measurement

	Discovery samples		Replication
	EGEA (n=610)	Hutterites (n=601)	Talana (n=450)
Age, year, mean (SD)	40.5 (16.8)	38.8 (15.3)	48.5 (17.9)
Sex, men, n, %	299 (49.0)	272 (45.3)	172 (38.2)
Smoking habits, n, % ^a			
Never smokers	308 (50.5)	601 (100)	276 (61.3)
Ex-smokers	157 (25.7)	0	99 (22.0)
Current smokers	145 (23.8)	0	75 (16.7)
Asthma			
Ever asthma, n, %	265 (43.4)	103 (17.1)	67 (14.8)
Current asthma, n, %	251 (41.1)	-	47 (10.4)
Age of asthma onset, mean (SD)	12.7 (14.3)	-	31.4 (13.4)
Allergic rhinitis, n, % ^b	233 (38.2)	-	62 (13.8)
Atopic dermatitis, n, %	234 (38.4)	61 (10.1)	43 (9.6)
FEV ₁ % predicted, mean (SD)	103.7 (17.5)	98.5 (13.5)	108.3 (16.3)
Inhaled corticosteroids, last 3 months, n, %	40 (6.6)	6 (1.0) ^c	2 (0.04)
SPT+ ^d , n, %	343 (56.2)	314 (52.2)	62 (32.6)
SPTQ ^e , n, %			
1	110 (32.1)	53 (16.9)	26 (42.0)
2	79 (23.0)	84 (26.7)	11 (17.7)
3	154 (44.9)	177 (56.4)	25 (40.3)
FeNO (ppb), median [Q1–Q3]	16.0 [10.6–22.5]	23.2 [17.0–32.3]	10.7 [6.8–17.8]
IgE (IU/mL), median [Q1–Q3]	79.0 [26.5–212.0]	26 [10–68]	39.6 [15.1–90.5]
Eosinophils, median [Q1–Q3] ^f	178 [100–280]	124 [76–180]	3.3 [2.1–4.0]

^aSmoking is prohibited in Hutterites

^bNo Rhinitis data is available for Hutterites.

^cIndicates use of inhaled corticosteroids the week prior to testing.

^dSkin Prick Test positivity (SPT+) was defined by a positive skin-prick test response to at least one airborne allergen. In Talana study, SPT was only measured in 190 subjects.

^eSPTQ: number of positive skin prick tests

^fEosinophils are expressed as number of cells/mm³ except in Talana study (%)

Main association results with FeNO (P 5×10^{-5}) in adult discovery studies (EGEA & Hutterites) and replication in Talana study

Table 2

Discovery phase: EGEA & Hutterites (N=1211)																Replication phase: Talana (N=450)				Meta-analysis Adults only			
Baseline allele frequency																Meta-analysis				EGEA & Hutterites & Talanas			
Chr	Region	Locus	SNP	Position ^a	Base/Effect Allele	Hutt.	EGEA	Z-score	P-value	Hutt. Z-score	EGEA Z-score	Base. allele frequency	Z-score	P-value	Z-score	P-value							
2	2p25	TAF1B	rs17364189	10064791	G/A	0.63	0.59	-4.09	4.3×10 ⁻⁵	-3.41	-2.36	0.62	-0.18	0.86	-3.59	3.4×10 ⁻⁴							
2	2p23-p22	SLC8A1/HNRNPA1P57	rs2194437	41343221	G/C	0.94	0.96	-4.35	1.3×10 ⁻⁵	-3.24	-2.90	0.96	-2.30	0.02	-4.92	8.9×10 ⁻⁷							
3	3p25.3	SLC6A11	rs2697159	10974427	G/A	0.39	0.51	-4.14	3.5×10 ⁻⁵	-2.74	-3.10	0.57	1.52	0.13	-2.74	6.1×10 ⁻³							
5	5p15	PLEKHG4B	rs7717734	181463	T/C	0.31	0.29	4.29	1.7×10 ⁻⁵	2.72	3.33	0.42	0.96	0.34	4.17	3.1×10 ⁻⁵							
5	5q31.1	FSTL4	rs256257	132916408	T/C	0.38	0.41	4.12	3.7×10 ⁻⁵	2.24	3.57	0.36	-1.50	0.13	2.74	6.3×10 ⁻³							
11	11p14.3	LOC100288844/LUZP2	rs12791623	24277924	T/A	0.93	0.89	-4.06	5.0×10 ⁻⁵	-2.03	-3.69	0.82	0.13	0.89	-3.39	6.9×10 ⁻⁴							
12	12q23.3	PLXNC1	rs3847800	94546413	A/G	0.34	0.16	4.70	2.6×10 ⁻⁶	3.85	2.78	0.11	-1.44	0.15	3.26	1.1×10 ⁻³							
15	15q21.1	RAB27A	rs2444043	55534342	C/T	0.47	0.50	4.07	4.6×10 ⁻⁵	4.01	1.73	0.51	3.39	0.0007	5.24	1.6×10 ⁻⁷							
18	18q22.3	ZNF407/ZADH2	rs10163698 ^b	72907278	C/T	0.80	0.95	-4.39	1.1×10 ⁻⁵	-1.87	-4.32	0.33	-1.01	0.31	-4.28	1.9×10 ⁻⁵							

^aPosition in base pairs (bp) – build 37.3 NCBI

^bIn Talana study, rs1047521 SNP's was used as proxy of rs10163698 (D' = 1, r² = 0.03)

Table 3

Heterogeneity test of SNP effect between A) asthmatic and non-asthmatic adult groups, B) atopic and non-atopic adult groups and C) according to tobacco smoke exposure for the two SNPs for which increased evidence of association was observed in the combined meta-analysis of adult discovery and replication sets^a

Part A		Meta-analysis Asthmatic subjects (N=417)					Meta-analysis Non-asthmatics (N=994)		Test for heterogeneity between groups			
Chr	Gene	Previous Gene	Next Gene	SNP	Position ^b	Base/Alt Allele	Z-score	P-value	Z-score	P-value	Chi ²	P-value
2	SLC8A1	HNRNPA1P57		rs2194437	41343221	G/C	-3.01	0.003	-3.28	0.001	0.23	0.63
15	RAB27A			rs2444043	55534342	C/T	1.62	0.11	4.86	1.2×10 ⁻⁶	1.57	0.21
Part B		Atopic subjects (N=657)					Non-atopic subjects (N=533)					
2	SLC8A1	HNRNPA1P57		rs2194437	41343221	G/C	-4.12	3.8×10 ⁻⁵	-2.24	0.03	3.07	0.08
15	RAB27A			rs2444043	55534342	C/T	2.59	0.009	3.75	1.8×10 ⁻⁴	2.94	0.08
Part C		Current & Ex-smokers (N=475)					Never smokers (N=1,185)					
2	SLC8A1	HNRNPA1P57		rs2194437	41343221	G/C	-2.56	0.01	-4.91	9.2×10 ⁻⁷	3.32	0.06
15	RAB27A			rs2444043	55534342	C/T	3.00	0.003	4.08	4.4×10 ⁻⁵	2.38	0.12
		Current smokers (N=220)					Ex- & never smokers (N=1,440)					
2	SLC8A1	HNRNPA1P57		rs2194437	41343221	G/C	-2.79	0.005	-4.26	2.0×10 ⁻⁵	1.33	0.25
15	RAB27A			rs2444043	55534342	C/T	1.77	0.08	4.70	2.6×10 ⁻⁶	1.96	0.16

^aTalana data were not used in the atopy stratified analysis since skin prick tests were only measured in 190 subjects having an Allergy & Asthma questionnaire score 5.

^bPosition in base pairs (bp) – build 37.3 NCBI

Association between SNPs related to adult FeNO levels at $P < 5 \times 10^{-5}$ in the discovery sets and childhood FeNO levels (N=8,858) in pediatric studies of the EAGLE consortium

Table 4

Chr	Locus	SNP	Baseline/Effect allele	Baseline allele frequency	Regression coefficient	Standard Error	P-value	Cochran P-value	I ²
2	TAF1B	rs17364189	G/A	0.65	-0.0001	0.012	0.99	0.61	0
2	SLC8A1/HNRNP57	rs2194437	G/C	0.92	-0.002	0.027	0.94	0.68	0
3	SLC6A11	rs2697159	G/A	0.46	-0.011	0.012	0.33	0.22	21.6
5	PLEKHG4B	rs7717734	T/C	0.30	-0.001	0.013	0.46	0.36	8.7
5	FSTL4	rs256257	T/C	0.42	-0.003	0.012	0.78	0.46	0
11	LOC100288844/LUZF2	rs12791623	T/A	0.90	-0.029	0.020	0.14	0.52	0
12	PLXNC1	rs3847800	A/G	0.16	0.008	0.016	0.61	0.51	0
15	RAB27A	rs2444043	C/T	0.50	-0.002	0.012	0.85	0.12	31.7
18	ZNF407/ZADH2	rs10163698	C/T	0.95	-0.048	0.034	0.16	0.10	35.9