

ORIGINAL ARTICLE

Genetic regulation of gene expression in the lung identifies *CST3* and *CD22* as potential causal genes for airflow obstruction

Maxime Lamontagne,¹ Wim Timens,² Ke Hao,³ Yohan Bossé,^{1,4} Michel Lavolette,¹ Katrina Steiling,⁵ Joshua D Campbell,⁵ Christian Couture,¹ Massimo Conti,¹ Karen Sherwood,⁶ James C Hogg,^{6,7} Corry-Anke Brandsma,² Maarten van den Berge,⁸ Andrew Sandford,^{6,9} Stephen Lam,¹⁰ Marc E Lenburg,⁵ Avrum Spira,⁵ Peter D Paré,^{6,9} David Nickle,¹¹ Don D Sin,^{6,9} Dirkje S Postma⁸

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For numbered affiliations see end of article.

Correspondence to

Professor Dirkje S Postma, University of Groningen, University Medical Center Groningen, Department of Pulmonary Medicine and Tuberculosis, GRIAC Research Institute, AA11, Hanzeplein, PO Box 30001, Groningen 9700 RB, The Netherlands; d.s.postma@umcg.nl

ML, WT, and KH contributed equally.

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ABSTRACT

Background COPD is a complex chronic disease with poorly understood pathogenesis. Integrative genomic approaches have the potential to elucidate the biological networks underlying COPD and lung function. We recently combined genome-wide genotyping and gene expression in 1111 human lung specimens to map expression quantitative trait loci (eQTL).

Objective To determine causal associations between COPD and lung function-associated single nucleotide polymorphisms (SNPs) and lung tissue gene expression changes in our lung eQTL dataset.

Methods We evaluated causality between SNPs and gene expression for three COPD phenotypes: FEV₁% predicted, FEV₁/FVC and COPD as a categorical variable. Different models were assessed in the three cohorts independently and in a meta-analysis. SNPs associated with a COPD phenotype and gene expression were subjected to causal pathway modelling and manual curation. *In silico* analyses evaluated functional enrichment of biological pathways among newly identified causal genes. Biologically relevant causal genes were validated in two separate gene expression datasets of lung tissues and bronchial airway brushings.

Results High reliability causal relations were found in SNP–mRNA–phenotype triplets for FEV₁% predicted (n=169) and FEV₁/FVC (n=80). Several genes of potential biological relevance for COPD were revealed. eQTL-SNPs upregulating cystatin C (*CST3*) and *CD22* were associated with worse lung function. Signalling pathways enriched with causal genes included xenobiotic metabolism, apoptosis, protease–antiprotease and oxidant–antioxidant balance.

Conclusions By using integrative genomics and analysing the relationships of COPD phenotypes with SNPs and gene expression in lung tissue, we identified *CST3* and *CD22* as potential causal genes for airflow obstruction. This study also augmented the understanding of previously described COPD pathways.

INTRODUCTION

Genome-wide association studies (GWAS) have revolutionised our ability to identify common genetic variants that are associated with complex chronic diseases.¹ This approach has been applied

Key messages

What is the key question?

- What are the causal genetic variants changing gene expression in the lung that in turn associate with lower lung function and COPD?

What is the bottom line?

- Lung function-associated genetic variants alter the mRNA expression of nearby genes involved in biological pathways underpinning pulmonary function and COPD pathogenesis.

Why read on?

- New genes of airflow obstruction are identified with a generalised framework for the identification of causal genes from joint examination of genome-wide genotyping and gene expression data in the same patients.

to COPD, a lung disease that is caused predominantly by cigarette smoking in the western world.^{2–4} It is well known that only a subset of heavy smokers (15–20%) develop clinically relevant COPD and there is considerable evidence that there is a substantial genetic component involved in its pathogenesis.⁵ Although GWAS have identified novel loci that harbour susceptibility genes, they do not allow precise identification of the causal variant (or variants). In addition, GWAS do not provide information on how and to what extent the gene (or genes) within the susceptibility loci contribute to the phenotype. Interestingly, the majority of genetic variants which have been associated with disease traits by GWAS do not affect the coding sequence of genes but are located in intergenic regions or introns.⁶ Possible explanations are that the associated alleles are in linkage disequilibrium (LD) with rarer coding alleles with large effect sizes⁷ and/or that the genetic variants control the level of expression of genes involved in pathogenetic pathways. For complex genetic diseases, such as COPD, the effects of susceptibility alleles may primarily act by regulating gene



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expression rather than by altering protein coding as in most Mendelian diseases.⁸

We recently reported the discovery of a large number of lung-specific expression quantitative trait loci (eQTLs)⁹ and identified the most likely causal genes within three GWAS-nominated COPD susceptibility loci.¹⁰ The aim of the present study is to use the power of genome-wide mRNA expression arrays combined with genome-wide interrogation of single nucleotide polymorphisms (SNPs) to pinpoint specific SNPs that are related to lung tissue gene expression and to COPD phenotypes. Identification of susceptibility alleles that function as strong eQTLs increases the likelihood of identifying the true susceptibility gene within loci with large areas of LD.⁸ Moreover, the use of integrative genomics by combining environmental exposure data with susceptibility alleles, RNA expression levels, and different COPD phenotypes can unravel causal genetic relationships.¹¹ The basic principle of the current study is to map the genetic regulation of gene expression to identify DNA variants that induce changes in transcriptional networks that in turn contribute to COPD and airway obstruction pathogenesis.

METHODS

Subject selection

The methods for subject selection and phenotyping, and for interrogation of gene expression and genotype were recently described.⁹ The lung tissue used for discovery of eQTLs was from 1111 human subjects who underwent lung surgery at three academic sites, Laval University, University of British Columbia (UBC) and University of Groningen, henceforth referred to as Laval, UBC and Groningen, respectively. All lung specimens from Laval were obtained from patients undergoing lung cancer surgery and were harvested from a site distant from the tumour. At UBC, the majority of samples were from patients undergoing resection of small peripheral lung lesions. Additional samples were from autopsy and at the time of lung transplantation. At Groningen, the lung specimens were obtained at surgery from patients with various lung diseases, including patients undergoing therapeutic resection for lung tumours, harvested from a site distant from the tumour, and lung transplantation. For the present study, the principal aim was to examine smoking-related airway obstruction. Thus, we excluded subjects whose lung function may have been influenced by lung diseases other than COPD and lung cancer. Exclusion criteria are provided in the online supplement.

COPD phenotypes and GWAS

Genome-wide association was performed using linear or logistic regression models on the three phenotypes: FEV₁% predicted and FEV₁/FVC as continuous variables, and COPD defined dichotomously based on an FEV₁/FVC < 0.7 cut-off (see online supplement). Single-marker association tests were run within each cohort adjusting for age, gender and smoking status. Fixed-effects meta-analysis was then performed combining the three cohorts using inverse SE weighting.

Expression trait processing

Expression traits were adjusted for age, gender and smoking status as described previously.⁹ Gene expression data are available in the Gene Expression Omnibus repository through accession number GSE23546.

Causality models

We evaluated three competing causality models to describe the relationships between lung eQTL-SNPs, RNA expression and

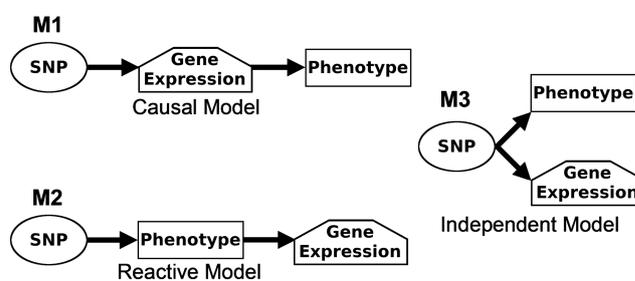


Figure 1 Causality models showing the relationships between the expression of a gene, a phenotype and a single nucleotide polymorphism (SNP). Three models are depicted including a causal model (M1), a reactive model (M2), and an independent model (M3). M1 is the simplest, and states that the genotype at an expression quantitative trait loci SNP acts directly on gene expression pattern to produce the phenotype. M2 states that the gene expression pattern is reactive to the phenotype, and M3 states that the gene expression pattern and phenotype are independent.

COPD phenotypes¹² (figure 1). Model 1 indicates a potential causal relationship where the SNP acts on gene expression to produce the phenotype, which was the main interest of this work. Model 2 indicates that the gene expression pattern is reactive to the phenotype, that is, the phenotype drives gene expression. Model 3 is the independent model where the SNP acts on the phenotype and gene expression independently. We required a p value $< 1 \times 10^{-3}$ for SNP associated with phenotype before examining a particular variable triplet (ie, SNP, gene expression and phenotypes). The causal model (model 1) was selected when p value < 0.05 was found for SNP associated with gene expression adjusting for phenotype and p value > 0.05 was found for SNP associated with phenotype adjusting for gene expression. More details are provided in the online supplement. Analyses were performed in the three cohorts separately and then combined into a meta-analysis. We then conducted sample bootstrapping and repeated the causality test for $N=1000$ realisations. The reliability score of the molecular relationship is the fraction of bootstrap realisations that supports the call observed. Threshold for p values, number of bootstrap reselections and reliability cut-off (> 0.8) were selected based on previous literature.¹²

Manual curation and pathway analyses on reliable causal models

The biology and possible role of genes in the causal model were reviewed by manual curation and bioinformatics tools, including Ingenuity Pathway Analysis (IPA), MetaCore and Partek (see online data supplement).

Replication studies

Biologically relevant causal genes were validated in two datasets. First, a bronchial airway epithelial dataset where genome-wide gene expression levels were obtained from bronchial brushing of 238 individuals and associated with lung function as previously described.¹³ Second, a regional lung tissue dataset where genome-wide gene expression was obtained for eight regions of the same lung and for eight patients. The association between gene expression levels and micro-CT-based regional emphysema severity was assessed.¹⁴ More details are provided in the online data supplement.

RESULTS

Of the 1111 subjects in whom there were data on genotype and gene expression, 848 with sufficient phenotypic information

Table 1 Clinical characteristics of patients

Characteristics	Laval (n=403)	UBC (n=270)	Groningen (n=175)
Age (years)	63.4±9.8	63.9±10.0	59.7±10.0
Male/female (n)	224/179	144/126	83/92
Body mass index (kg/m ²)	26.6±5.2	25.7±5.4	24.6±4.1
FEV ₁ % predicted	82.2±17.6	83.6±22.3	72.4±24.9
FEV ₁ /FVC	0.68±0.10	0.69±0.13	0.64±0.16
COPD (%)	209 (51.9)	114 (42.2)	112 (64.0)
Stage 1: mild	80 (38.2)	43 (37.7)	16 (14.3)
Stage 2: moderate	117 (56.0)	60 (52.6)	30 (26.8)
Stage 3: severe	11 (5.3)	2 (1.8)	11 (9.8)
Stage 4: very severe	1 (0.5)	9 (7.9)	44 (39.3)
Missing data	0	0	11 (9.8)
Non-COPD (%)	194 (48.1)	156 (57.8)	63 (36.0)
Smoking (%)			
Smoker	89 (22.1)	91 (33.7)	12 (6.9)
Ex-smoker	281 (69.7)	150 (55.6)	117 (66.9)
Non-smoker	33 (8.2)	16 (5.9)	43 (24.6)
Missing data	0	13 (4.8)	3 (1.7)
Pack years (years)	48.6±27.4	46.0±28.6	32.1±18.1

Continuous variables are presented as means±SDs.
UBC, University of British Columbia.

were included in the analysis. The demographic and clinical features of the subjects in the three cohorts are described in table 1.

Causal pathways that fit model 1

Model fitting was restricted to SNPs that were significantly associated with at least one of the lung function variables or COPD as a categorical variable at a p value cut-off of 10⁻³. SNPs were considered an eQTL if they had a p value ≤10⁻⁵. Using these

criteria, there were 4465 SNP–mRNA–phenotype triplets. Of these, 249 triplets showed a significant fit to the causal model for at least one of the phenotypes with a reliability score >0.8 in at least one cohort and/or in the meta-analysis. A fit to this model means that the SNP was associated with one of the phenotypes and affected gene expression in a direction that supported a causal relationship.

The list of causal models with a confidence score >0.8 in any one of the cohorts or in the meta-analysis is shown in online supplementary table S1. For FEV₁% predicted, 169 causal models were found, including 122 unique SNPs and 169 probe sets. The 169 models included 168 *cis* eQTLs and 1 *trans* eQTL, respectively. For FEV₁/FVC, 80 causal models were found involving 63 unique SNPs and 80 probe sets. Among these 80 models, 79 were *cis* eQTLs and 1 was a *trans* eQTL. No causal model was found when using COPD as a categorical variable. There was no overlap between causal pathway models for FEV₁% predicted and FEV₁/FVC.

Manual curation of significant and reliable causal models

Significant causal models were inspected manually. We identified a number of models of potential biological relevance for FEV₁% predicted and FEV₁/FVC. The most biologically relevant models are listed in table 2. The p values and direction of effect for each of the SNP associations with the gene expression and the phenotype are also indicated. For example, SNP rs6048956 was significantly associated with cystatin C (*CST3*) transcript. The common allele was associated with higher expression of the transcript (figure 2 and positive eQTL Z score in table 2) and with lower FEV₁% predicted (figure 2 and negative Z score with phenotype in table 2). Taken together, these results suggest that the common allele confers susceptibility to a lower FEV₁% predicted value through upregulation of the *CST3* mRNA expression levels in the lung. This was confirmed in a second causality model that interrogated a different probe

Table 2 Models of biological relevance identified by manual curation

SNPs	Reference allele (freq)*	Gene (probe set)	Lung eQTL p value [†]	eQTL Z score [‡]	p Value phenotype [§]	Z score phenotype [¶]
FEV ₁ % predicted						
rs769178	G (0.91)	<i>NCR3</i> (100125842_TGI_at)	8.03×10 ⁻³⁰	11.3	4.7×10 ⁻⁴	3.5
rs6048956	C (0.77–0.79)	<i>CST3</i> (100307577_TGI_at)	1.54×10 ⁻⁶⁸	17.5	7.8×10 ⁻⁴	-3.4
rs6515375	G (0.79–0.81)	<i>CST3</i> (100125967_TGI_at)	3.65×10 ⁻⁶	4.6	5.8×10 ⁻⁴	-3.4
rs2270859	G (0.83–0.88)	<i>CSTA</i> (100148334_TGI_at)	1.74×10 ⁻⁹	6.0	3.1×10 ⁻⁶	-4.7
rs4550905	G (0.26–0.31)	<i>PPARGC1A</i> (100131093_TGI_at)	1.69×10 ⁻⁵	-4.3	4.4×10 ⁻⁴	-3.5
rs3803761	G (0.71–0.77)	<i>FLCN</i> (100135396_TGI_at)	3.77×10 ⁻²⁹	11.2	3.4×10 ⁻²	-2.1
rs1543438	A (0.76–0.82)	<i>BCL2L1</i> (100158784_TGI_at)	3.89×10 ⁻⁵	-4.1	7.94×10 ⁻⁵	3.9
rs9880397	G (0.62–0.64)	<i>CADM2</i> (100162763_TGI_at)	1.3×10 ⁻⁸	-5.7	2.9×10 ⁻⁴	3.6
rs2466183	T (0.84–0.86)	<i>TNFRSF10B</i> (100153254_TGI_at)	3.76×10 ⁻⁵	-4.1	6.6×10 ⁻⁴	3.4
FEV ₁ /FVC						
rs17754977	A (0.30–0.32)	<i>GSTO2</i> (100132911_TGI_at)	3.96×10 ⁻⁵	4.1	8.2×10 ⁻⁴	3.3
rs9987135	T (0.28–0.31)	<i>DEPDC6</i> (100154484_TGI_at)	6.13×10 ⁻⁵⁹	16.2	1.1×10 ⁻⁴	-3.9
rs10411704	T (0.79–0.82)	<i>CD22</i> (100154732_TGI_at)	1.70×10 ⁻⁴⁰	-13.3	4.6×10 ⁻⁴	3.5
rs12179536	A (0.80–0.84)	<i>MUC22</i> (100304000_TGI_at)	3.47×10 ⁻³³	-12.0	3.0×10 ⁻³	3.0
rs2287765	T (0.91–0.93)	<i>SPINK5</i> (100305138_TGI_at)	2.85×10 ⁻¹⁰	6.3	9.2×10 ⁻⁴	-3.3

p Values and Z scores in this table are from the meta-analysis. As indicated in the text, the significant causal models were selected based on results of both individual cohorts and meta-analysis (SNP associated with phenotype with p value <10⁻³, SNPs associated with gene expression with p value ≤10⁻⁵, and reliability score >0.8 in at least one cohort and/or in the meta-analysis). Known role of genes are provided in online supplementary table S2.

*Frequency of the reference allele in the three cohorts.

†Lung eQTL p-value from the meta-analysis.

‡Z score from the eQTL meta-analysis showing the direction of effect for the SNP on gene expression.

§p Value for association between the SNP and phenotype from the meta-analysis.

¶Effect size (Z score) for association between the SNP and the phenotype from the meta-analysis, showing the direction of effect for the SNP on the phenotype.

eQTL, expression quantitative trait loci; SNP, single nucleotide polymorphism.

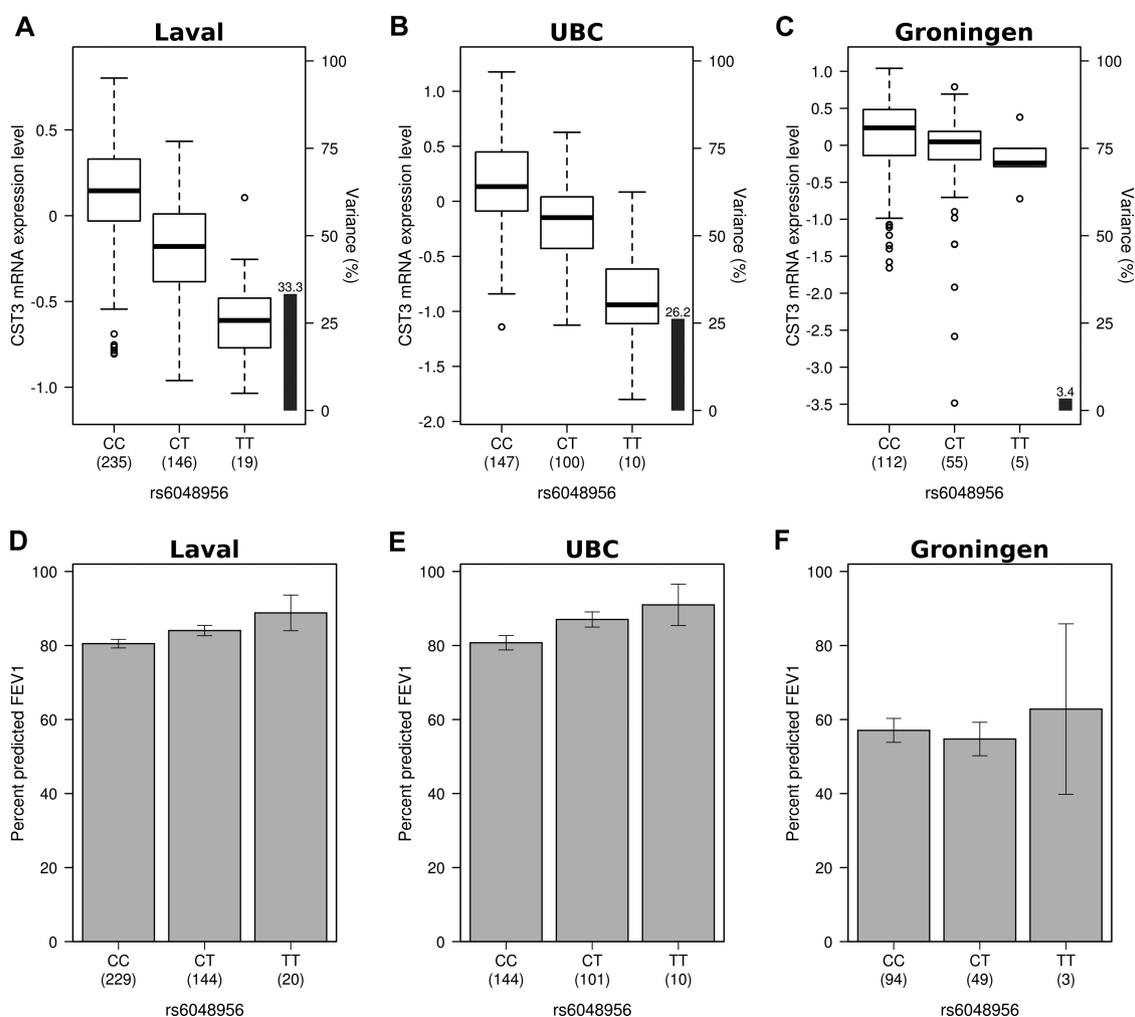


Figure 2 Direction of effects for causality model with rs6048956, mRNA expression of cystatin C (*CST3*) and FEV₁% predicted. The left, centre and right panels show the results for Laval, University of British Columbia (UBC) and Groningen samples, respectively. (A–C) are boxplots of gene expression levels in the lung for *CST3* according to genotype groups for single nucleotide polymorphism (SNP) rs6048956. The left y-axis shows the mRNA expression levels for *CST3*. The x-axis represents the three genotype groups for SNP rs6048956. The right y-axis shows the proportion of the gene expression variance explained by the SNP (black bar). Box boundaries, whiskers and centre mark in boxplots represent the first and third quartiles, the most extreme data point which is no more than 1.5 times the IQR, and median, respectively. (D–F) are barplots of the mean and SE of FEV₁% predicted according to genotype groups for SNP rs6048956.

set for *CST3*, FEV₁% predicted and rs6515375 (table 2 and see online supplementary figure S1). Analogous observations were made for natural cytotoxicity triggering receptor 3 (*NCR3*), cystatin A (*CSTA*), peroxisome proliferation-activated receptor γ coactivator 1 α (*PPARGC1A*), folliculin (*FLCN*), BCL2-like 1 (*BCL2L1*), cell adhesion molecule 2 (*CADM2*), and tumour necrosis factor receptor superfamily member 10b (*TNFRSF10B*) for FEV₁% predicted, and glutathione S-transferase ω 2 (*GSTO2*), DEP domain containing 6 (*DEPDC6*), CD22 molecule (*CD22*), mucin 22 (*MUC22*), and serine peptidase inhibitor Kazal type 5 (*SPINK5*) for FEV₁/FVC (table 2). The direction of effects for all these causality models are illustrated in online supplementary figures S2–13.

Pathway analyses

To find key biological pathways involved in COPD, causality genes were overlaid on canonical pathways available in IPA. One hundred and sixty-nine genes and 80 causality genes for FEV₁ and FEV₁/FVC were considered, respectively (see online supplementary table S1). Table 3 shows all canonical pathways enriched with causality genes ($p < 0.05$). Of interest was the aryl

hydrocarbon receptor signalling pathway, which is involved in xenobiotic clearance. Five causality genes were noted in this canonical pathway, including *ARNT* (aryl hydrocarbon receptor nuclear translocator), *IL6* (interleukin 6), *GSTO2*, *ALDH8A1* (aldehyde dehydrogenase 8 family, member A1) and *TRIP11* (thyroid hormone receptor interactor 11). The aryl hydrocarbon receptor signalling pathway and the location of the causality genes are illustrated in online supplementary figure S14. Another pathway involved in xenobiotic handling, the xenobiotic metabolism signalling pathway, was also enriched for causality genes, many of which overlapped with those in the aryl hydrocarbon receptor signalling pathway (*ARNT*, *IL6*, *GSTO2* and *ALDH8A1*). The former also includes *PPARGC1A* and *PRKCE* (protein kinase C, ϵ). This pathway is illustrated in online supplementary figure S15.

For the Partek analyses 118 and 45 transcripts were identified for FEV₁% predicted and FEV₁/FVC, respectively. These transcripts mapped to a total of 115 genes in Partek GS (based on official gene symbols) and were parsed to Partek Pathway Suite. Causality genes were overlaid onto canonical pathways from the REACTOME and KEGG databases. Table 4 shows the canonical

Table 3 Canonical pathways enriched for causality genes using the Ingenuity Pathway Analysis system

Pathways	p Value
Cdc42 signalling	0.00002
Crosstalk between dendritic cells and natural killer cells	0.00015
Graft-versus-host disease signalling	0.00069
OX40 signalling pathway	0.00151
Antigen presentation pathway	0.00468
Neuroprotective role of THOP1 in Alzheimer's disease	0.00589
<i>Taurine and hypotaurine metabolism</i>	0.00617
Autoimmune thyroid disease signalling	0.00661
Regulation of actin-based motility by Rho	0.00676
Communication between innate and adaptive immune cells	0.00676
<i>Aryl hydrocarbon receptor signalling</i>	0.00759
Integrin signalling	0.00891
Allograft rejection signalling	0.00977
Cytotoxic T-lymphocyte-mediated apoptosis of target cells	0.01023
<i>Cyanoamino acid metabolism</i>	0.01047
<i>Glutathione metabolism</i>	0.01072
Amyotrophic lateral sclerosis signalling	0.01230
Systemic lupus erythematosus signalling	0.01230
PXR/RXR activation	0.01995
Macropinocytosis signalling	0.02455
Selenoamino acid metabolism	0.02692
<i>Xenobiotic metabolism signalling</i>	0.02951
Cellular effects of sildenafil (Viagra)	0.03162
Aminoacyl-tRNA biosynthesis	0.03631
Actin cytoskeleton signalling	0.04365
FAK signalling	0.04467
Apoptosis signalling	0.04677

Pathways discussed in the text are given in italic.

pathways enriched with causality genes ($p < 0.05$). TNF-related apoptosis-inducing ligand (TRAIL) signalling was the most significant pathway, with several other apoptosis-related pathways included in the list of top significant associations. The glutathione metabolism pathway connects to both the cyanoamino acid and taurine and hypotaurine metabolism pathways (see online supplementary figure S16). These three pathways were identified in our enrichment analysis and were also identified using the IPA system. GO functional categories also showed enrichment for causality genes (see online supplementary figure S17) including the γ -glutamyl transferase activity.

Replication of the most biologically relevant causality models

No other large-scale lung eQTL dataset is available in patients with and without COPD. To replicate the most biologically relevant causality models, we relied on two genome-wide expression datasets. Causality genes were first validated in a gene expression study of bronchial airway epithelial cells obtained by bronchoscopy. All 13 genes represented in table 2, except MUC22, were assayed in this airway dataset. Two genes were significantly associated with lung function at a false discovery rate (FDR) of 5% in the bronchial airway epithelial dataset: *CSTA* (FDR=0.002 with FEV₁% predicted) and *TNFRSF10B* (FDR=9.35×10⁻⁵ with FEV₁% predicted). In both cases, the direction of effect was the same as that in the current study (table 5). For example, higher *CSTA* mRNA levels were

Table 4 Canonical pathways enriched for causality genes using Partek

Pathways	p Value	Pathway ID
TRAIL signalling	0.0003	reactome_pathway_506
<i>Cyanoamino acid metabolism</i>	0.001	kegg_pathway_36
Cell adhesion molecules	0.002	kegg_pathway_139
Extrinsic pathway for apoptosis	0.002	reactome_pathway_502
Death receptor signalling	0.002	reactome_pathway_503
<i>Taurine and hypotaurine metabolism</i>	0.002	kegg_pathway_34
<i>Glutathione metabolism</i>	0.003	kegg_pathway_39
<i>Aminoacyl-tRNA biosynthesis</i>	0.004	kegg_pathway_81
<i>Apoptosis</i>	0.006	reactome_pathway_501
Cytosolic tRNA aminoacylation	0.006	reactome_pathway_1127
Glutathione conjugation	0.007	reactome_pathway_256
Natural killer cell mediated cytotoxicity	0.011	kegg_pathway_152
The NLRP1 inflammasome	0.015	reactome_pathway_469
tRNA aminoacylation	0.018	reactome_pathway_1126
<i>Apoptosis</i>	0.023	kegg_pathway_126
BoNT light chain types B, D and F cleave VAMP/synaptobrevin	0.024	reactome_pathway_647
Amyloids	0.026	reactome_pathway_643
Nucleotide-binding domain, leucine rich repeat containing receptor (NLR) signalling pathways	0.026	reactome_pathway_465
BH3-only proteins associate with and inactivate anti-apoptotic BCL-2 members	0.034	reactome_pathway_517
Axonal growth inhibition (RHOA activation)	0.039	reactome_pathway_984
Vitamin C (ascorbate) metabolism	0.039	reactome_pathway_188
Import of palmitoyl-CoA into the mitochondrial matrix	0.039	reactome_pathway_70
Downregulation of ERBB4 signalling	0.039	reactome_pathway_1023
p75NTR regulates axonogenesis	0.043	reactome_pathway_982
Caspase-8 is formed from procaspase-8	0.043	reactome_pathway_507
Activation of procaspase-8	0.043	reactome_pathway_508
Endosomal/vacuolar pathway	0.043	reactome_pathway_413
Phase II conjugation	0.047	reactome_pathway_249
SLBP independent processing of histone pre-mRNAs	0.048	reactome_pathway_1111
Arachidonic acid metabolism	0.050	kegg_pathway_56

Pathways in italic are also found in ingenuity pathway analysis. TRAIL, tumour necrosis factor related apoptosis-inducing ligand.

associated with worse lung function (table 2 and see online supplementary figure S3).

Causality genes identified in this study were also compared with a second lung transcriptomic study that evaluated the impact of regional emphysema severity on gene expression. Interestingly, *CD22* was positively associated with regional emphysema severity within individuals ($p = 5.8 \times 10^{-5}$) and was a part of the 127 gene signature for emphysema identified in that study.¹⁴ This observation is consistent with the current study showing that carriers of the rare allele for rs10411704 had greater mRNA expression of *CD22* and worse FEV₁/FVC (table 2 and see online supplementary figure S11). Associations with regional emphysema severity were also observed for three other genes in table 2 including *NCR3* ($p = 0.058$), *PPARGC1A* ($p = 0.081$) and *BCL2L1* ($p = 0.051$), but these were not statistically significant. However, the direction of effect was consistent only for *PPARGC1A*. The expression of this gene was found to decrease with emphysema severity in the regional lung tissue dataset and, in the current study, carriers of the rare allele for

Table 5 Replication of causality genes in the bronchial airway epithelial and the regional lung tissue datasets

	Replication datasets	
	Bronchial airway epithelium ¹³	Regional lung tissue ¹⁴
FEV ₁ % predicted		
<i>NCR3</i>	True	False
<i>CST3</i>	True	
<i>CSTA</i>	True*	
<i>PPARGC1A</i>	False	True
<i>FLCN</i>	False	
<i>BCL2L1</i>	True	False
<i>CADM2</i>	False	
<i>TNFRSF10B</i>	True*	
FEV ₁ /FVC		
<i>GSTO2</i>	False	
<i>DEPDC6</i>	False	
<i>CD22</i>	True	True*
<i>MUC22</i>		
<i>SPINK5</i>	True	

*Significant in the original studies.^{13 14}

rs4550905 were associated with less mRNA expression of *PPARGC1A* in the lung and lower FEV₁ (table 2 and see online supplementary figure S4).

DISCUSSION

This investigation integrated a genome-wide eQTL study on lung tissue with genome-wide genetic association results for lung function and COPD in the same subjects. For the discovery of eQTLs we used the entire dataset which consisted of 1111 tissue samples from subjects who had lung surgery for a variety of reasons. In order to focus on COPD, we limited the study to 848 subjects with sufficient phenotypic information and without a lung disease (other than COPD and lung cancer) which could cause abnormalities of pulmonary function. We limited the causal pathway analysis to SNPs that were both significantly related to gene expression ($p < 10^{-5}$) and were associated with one of three COPD phenotypes ($p < 10^{-3}$), that is, FEV₁% predicted, FEV₁/FVC and COPD defined as FEV₁/FVC < 0.7. Of the 4465 SNP–mRNA–phenotype triplets which met our inclusion criteria, 249 triplets showed a significant fit to the causality model for at least one of the phenotypes with a reliability score ≥ 0.8 . We thus provide evidence that these SNPs influence disease susceptibility by altering gene expression. Causality pathway genes were enriched in pathways involved in xenobiotic handling, antiprotease and antioxidant activity and apoptosis.

Two of the eQTL-SNPs in *CST3* (rs6515375 and rs6048956) and another for *CSTA* (rs2270859) were in the causal pathway for FEV₁% predicted. The two *CST3* SNPs were in perfect LD and were eQTLs for two different probe sets. *CST3* and *CSTA* are cysteine antiproteases; *CST3* antagonises cysteine cathepsins such as cathepsin L and S, and *CSTA* acts similarly for cathepsins B, H and L.^{15 16} Interestingly the direction of association is such that the alleles that are associated with a higher mRNA level of *CST3* and *CSTA* are associated with lower FEV₁/FVC.

The above findings might seem paradoxical since cystatins are antiproteases and if one simply invoked the protease–antiprotease hypothesis, then one might expect that individuals with higher levels to be protected from COPD. One possibility is that

upregulation of *CST3* mRNA and protein could be the result of a feedback loop stimulated by high protease levels. This would be supported by observations in bronchoalveolar lavage fluid and serum reporting higher cystatin C level in patients with emphysema.^{17 18} Alternatively excessive inhibition of proteases may confer biological effects on a yet to be discovered mechanism which contributes to the pathogenesis of airflow obstruction. Further molecular studies of the involved proteins might shed more light on this. We previously reported SNPs associated with *CST3* mRNA and protein levels in alveolar macrophages.¹⁹ However, in the latter study, higher *CST3* mRNA in alveolar macrophages was associated with higher FEV₁. Together, these studies suggest that the cystatin genes are important in the pathogenesis of COPD, however the relationship between *CST3* mRNA expression levels and lung function remains to be elucidated in relevant tissues and cell types.

With respect to antioxidant activity, eQTLs from *PPARGC1A* and *GSTO2* were found. *PPARGC1A* binds to peroxisome proliferation-activated receptor γ (PPAR γ) by induction of PPAR γ ligands, coactivating PPAR γ target genes, involved in antioxidant activity. Compared with controls, expression levels of PPAR γ , PGC-1 α and γ glutamylcysteine synthetase (γ -GCS) have been reported to be significantly increased in the lungs of patients with mild COPD, and progressively decreased in more severe disease.²⁰ These authors concluded that γ -GCS showed compensatory upregulation in the early stage of COPD, which progressively decompensated with disease progression and that the activation of the PPAR γ /PGC-1 α pathway may protect against COPD progression by upregulating γ -GCS and relieving oxidative stress. Furthermore *PPARGC1A* has been described to be involved in bronchial smooth muscle remodelling and skeletal muscle wasting.^{21 22} For *GSTO2*, a strong association of the Asn142Asp SNP with FEV₁ and FVC was found in the Framingham Heart Study.²³ In a latter study, the Asn142Asp polymorphism in *GSTO2* and the *GSTO1* 140Asp/*GSTO2* 142Asp haplotype were associated with increased risk of COPD but failed to reveal an association between lung function parameters and non-synonymous coding SNPs in the *GSTO* genes.²⁴ Polymorphisms in *GSTO2* were also associated with COPD either with or without lung cancer.²⁵

Pathways involved in apoptosis and in handling of xenobiotic pathways were significantly enriched for causal genes. The results of the present study aid in understanding the underlying genetic dysregulation of apoptosis. In general, increased apoptosis of endothelial cells and fibroblasts has been shown to contribute to the development of emphysema.^{26 27} This is partly due to an imbalance caused by excess oxidant and protease effects related to cigarette smoking and to intrinsic dysregulation of apoptosis induced in susceptible individuals. This may explain why the apoptotic effects are larger in smokers with COPD than smokers without COPD. Dysregulation of apoptosis can also work the other way: decreased apoptosis in cells of the immune system can lead to sustained inflammation, and when occurring in fibroblasts (eg, in the bronchial wall) can induce fibrosis. Emphysema is characterised by alveolar cell apoptosis, which was shown to be mediated by increased levels of apoptotic proteins including TRAIL receptors.²⁸ Interestingly, TRAIL signalling was the most significant pathway enriched with causality genes using Partek. TRAIL signalling has been shown to regulate immune responses in the lung and can lead to a sustained, proinflammatory response that contributes to vascular disease. An opposite effect is related to the death receptor signalling pathway, which, in humans, binds with TRAIL, induces formation of a death-inducing signalling complex, ultimately leading to caspase activation and initiation of apoptosis.²⁹

The aryl hydrocarbon receptor signalling pathway is involved in xenobiotic clearance and therefore of relevance to the known vulnerability of patients with COPD to (cigarette) smoke. Furthermore additional metabolic pathways including the glutathione metabolism pathway and two subpathways involving metabolism of the cyanoamino acids, taurine and hypotaurine were also significantly enriched in the causal analysis. Genetic variants in a number of genes in the glutathione pathway have previously been associated with risk for COPD.^{25 30}

No other large-scale lung eQTL dataset is available in patients with and without COPD. To replicate the most biologically relevant causality models, we relied on two genome-wide expression datasets. Causality genes were first validated in a gene expression study of bronchial airway epithelial cells obtained by bronchoscopy and then in a transcriptomic study of whole lung explanted at surgery. Whole genome genotyping is not available for these datasets. Accordingly, replication of significant triplets (ie, SNP–mRNA–phenotype) can only evaluate the concordance between gene expression and phenotype. Additional studies with phenotype, genotype and gene expression in the lung would be required to provide full validation.

In conclusion, integration of lung-specific eQTL data with GWAS from the same individuals has revealed interesting and potentially causal pathways of airflow obstruction. GWAS have revolutionised our ability to identify gene variants that contribute to susceptibility for common complex genetic diseases but often do not pinpoint the exact genes or mechanisms. Causal pathway analysis involving the joint examination of genetic and genomic data is a vital next step in discovering novel biomarkers and therapeutic targets in airflow obstruction as evidenced in the present study.

Author affiliations

¹Institut universitaire de cardiologie et de pneumologie de Québec, Québec, Canada

²Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Groningen, The Netherlands

³Department of Genetics and Genomics Sciences, Mount Sinai School of Medicine, New York, New York, USA

⁴Department of Molecular Medicine, Laval University, Québec, Canada

⁵Division of Computational Biomedicine, Bioinformatics Program, Boston University, Boston, Massachusetts, USA

⁶University of British Columbia Center for Heart Lung Innovation and Institute for Heart and Lung Health, St Paul's Hospital, Vancouver, British Columbia, Canada

⁷Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada

⁸Department of Pulmonology, University of Groningen, University Medical Center Groningen, GRIAC Research Institute, Groningen, The Netherlands

⁹Respiratory Division, Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

¹⁰British Columbia Cancer Agency, Vancouver, British Columbia, Canada

¹¹Merck & Co Inc, Rahway, New Jersey, USA

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