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ORIGINAL RESEARCH

Differential Expression of Vitamin E and Selenium-Responsive Genes by Disease Severity in Chronic Obstructive Pulmonary Disease

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Abstract

Antioxidant nutritional status is hypothesized to influence chronic obstructive pulmonary disease (COPD) susceptibility and progression. Although past studies relate antioxidants to gene expression, there are no data in patients with COPD. This study investigated the hypothesis that antioxidant status is compromised in patients with COPD, and antioxidant-responsive genes differentially express in a similar pattern. Lung tissue samples from patients with COPD were assayed for vitamin E and gene expression. Selenium and vitamin E were assayed in corresponding plasma samples. Discovery based genome-wide expression analysis compared moderate, severe, and very severe COPD (GOLD II-IV) patients to mild and at-risk/normal (GOLD 0-I). Hypotheses-driven analyses assessed differential gene expression by disease severity for vitamin E-responsive and selenium-responsive genes. GOLD II-IV COPD patients had 30% lower lung tissue vitamin E levels compared to GOLD 0-I participants (p = 0.0082). No statistically significant genome-wide differences in expression by disease severity were identified. Hypothesis-driven analyses of 109 genes found 16 genes differentially expressed ($p_{adjusted}$ < 0.05) by disease severity including 6 selenium-responsive genes (range in fold-change -1.39 to 2.25), 6 vitamin E-responsive genes (fold-change -2.30 to 1.51), and 4 COPD-associated genes. Lung tissue vitamin E in patients with COPD was associated with disease severity and vitamin E-responsive genes were differentially expressed by disease severity. Although nutritional status is hypothesized to contribute to COPD risk, and is of therapeutic interest, evidence to date is mainly observational. The findings reported herein are novel, and support a role of vitamin E in COPD progression.

Keywords: Antioxidants, gene expression, tocopherol, selenium, COPD

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive irreversible airflow limitation (1). Death rates from COPD have been steadily rising over the last few decades and in 2009 COPD was the third leading cause of death in the United States (2–5). The substantial costs of COPD include losses in life expectancy and quality of life as well as mounting costs for medical care, which were estimated at $14.5 billion per year in the United States in 2000 (6, 7).

The primary risk factor for COPD is cigarette smoking; however, evidence supports a role for genetic variation as a contributor to disease susceptibility and progression (1, 8). Investigations of genetic risk for COPD include candidate gene studies, genome-wide association studies (GWAS), and gene

expression studies (8). Gene expression studies, either genome-wide or candidate-gene-based, provide insights into COPD pathogenesis by directly studying the transcriptome in lung tissue (8) to provide clues about causal mechanisms and identify informative biomarkers (9). Prior work on genetic variation, including both sequence and expression variation, provides insights into COPD etiology that may ultimately offer novel preventive and/or therapeutic targets (10–23).

An imbalance between oxidants and antioxidants is hypothesized to play an important role in COPD pathogenesis (24–26). Oxidative stress in the lungs is a hallmark of COPD (27–29), and patients with COPD have lower antioxidant status compared to non-diseased individuals, with further declines in antioxidant status during acute exacerbations of COPD (30, 31). Published studies support a protective role for nutrients with antioxidant properties, which defend the lungs and thereby prevent damage from oxidative stress arising from both endogenous and exogenous oxidants (29,32–35).

A separate line of research has identified genes that are responsive to manipulation of antioxidant nutrients (10,36–48), and, an as yet unanswered question is whether antioxidant-responsive genes are differentially expressed in the antioxidant-depleted environment of the COPD lung. Given the central role of oxidant/antioxidant balance in COPD pathogenesis, we tested the hypothesis that the status of nutrients with antioxidant properties is compromised in patients with COPD, and that genes related to antioxidant function are differentially expressed in COPD.

Methods

Participants and specimens

The Lung Tissue Research Consortium (LTRC), a National Heart, Lung, and Blood Institute-sponsored tissue bank (http://www.ltrcpublic.com), provided clinical and demographic data and plasma, frozen lung tissue, and RNAlater-preserved lung tissue specimens from 24 patients with a major diagnosis of COPD/emphysema and surgical treatment for disease. Dietary supplement usage information was not collected by the LTRC. Disease status was staged using the Global Obstructive Lung Disease (GOLD) 2001 guidelines (49), where stage 0 indicates no obstructive lung disease based on pulmonary function testing, but presence of respiratory symptoms (50). Stages I through IV correspond to levels of severity of COPD based on objective pulmonary function testing (51). Recent revisions to GOLD guidelines omit stage 0. In light of these considerations, stages 0/I (indicating absent to mild disease) were combined and compared with stages II-IV in all analyses (Table 1).

The study was reviewed and granted exempt status by the Institutional Review Board (IRB) of Cornell University, Ithaca, NY. Further details on methods are provided in supplementary materials, including Supplemental Table 1.

Gas chromatography mass spectrometry (GC-MS) determination of α-tocopherol

Quantitative determination of α-tocopherol concentration in plasma, the high density lipoprotein fraction of plasma (HDL), and lung tissue used a method comprising lipid extraction, trimethylsilyl derivitization of tocopherols, and isothermal detection and resolution of tocopherols and cholesterol by GC-MS (compared to δ_9-α-tocopherol internal standard). Plasma samples were assayed in duplicate for total α-tocopherol with average coefficient of variation (CV) of 4.2%; the CV was 2.4% after adjusting for cholesterol (as measured within the GC-MS method). Sample quantity limitations necessitated single measurements of HDL (cholesterol adjusted) and lung tissue α-tocopherol concentrations. Lung tissue α-tocopherol concentrations were not adjusted for cholesterol due to heterogeneity of lung tissue samples.

Inductively coupled plasma mass spectrometry (ICP-MS) determination of selenium

Quantitative determination of selenium in plasma used a method comprising sample dilution and acidification and detection by ICP-MS (Agilent 7500 cs/ce quadrupole ICP-MS in *Spectrum* mode, ion intensity m/z 78 and ^{89}Y internal standard). Two reference standard solutions, 1 ppb and 5 ppb, were run at regular intervals with standard deviations of 0.023 and 0.073, respectively. The selenium concentration of four reference samples was determined at regular intervals, and all values were within the range of acceptable results throughout the assay.

Chemiluminescence determination of plasma cholesterol

Total cholesterol was determined using chemiluminescence under standard methods on a Siemens Dimension

Table 1. Distribution of study participants by Global Obstructive Lung Disease (GOLD) classification (51)

GOLD classification	0[a]	I: mild	II: moderate	III: severe	IV: very severe
FEV_1/FVC	> 0.70	< 0.70	< 0.70	< 0.70	< 0.70
%predicted FEV_1	>80%	>80%	>50% and <80%	>30% and <50%	<30%b
Number of participants	5	3	4	5	5

[a]Original GOLD guidelines, stage 0 defined as "at-risk of COPD," and identified as normal spirometry with > 1 respiratory symptom (breathlessness, cough, and/or sputum production); 2005 revised guidelines omitted this stage.
[b]Or <50% plus the presence of chronic respiratory failure.

Xpand, a Center for Disease Control and Prevention-certified instrument. Average within-run and between-run CVs were 0.71% and 1.94%, respectively. Two control samples were assayed and all values were within acceptable ranges throughout the assay time period.

Determination of gene expression in lung tissue samples

RNA extraction and purification from thawed, 30 mg subsamples of RNAlater preserved lung tissue was completed with TRIzol and RNeasy MinElute Cleanup Kits (Qiagen). Aliquots of each RNA sample were assessed to visualize and quantify the degree of RNA integrity using an Agilent Bioanalyzer (Aglient Technologies, Palo Alto, CA). Two samples, one from a GOLD I patient and one from a GOLD II patient, produced RNA of insufficient quality for use on the microarrays; these samples were removed from the microarray preparation pipeline. RNA concentrations for the remaining 22 samples were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Affymetrix kits (Santa Clara, CA) were used to synthesize double stranded cDNA, and cleanup and label samples, which were quantified by spectrophotometric analysis. Hybridization to test chips and the microarrays were performed according to Affymetrix protocols, using Affymetrix microarrays HG-U133 Plus 2.0 (54,675 probe sets). Microarrays were processed by the Affymetrix fluidics station and scanned with the Affymetrix GeneChip Scanner 3000 7G. Quality of microarrays was assessed by the following criteria: 1) RNA Integrity Number (RIN) ≥ 6.0; 2) 3'/5' ratio for GAPDH ≤ 3; and 3) scaling factor ≤ 10.0 (52, 53). Using Bioconductor version 2.7 (R version 2.12.0) the Microarray Suite version 5.0 (MAS 5.0) algorithm (Affymetrix) was used to analyze the captured images and assess microarray quality.

Statistical analysis

Analytical measurements for nutrients are reported as means with standard deviations and group means were compared using *t*-tests. All data management and analysis was conducted in SAS version 9.2 (SAS Institute, Cary, NC).

GeneChip Robust Multi-Array (GC-RMA) normalization of the expression data was performed using quantile normalization with expression estimates calculated with the empirical Bayes estimate for non-specific binding. Processed images from the microarrays were used to redefine probe sets by using up-to-date databases to annotate probes and assign unique gene identifiers (Entrez IDs). Using the Bioconductor Limma package, differentially expressed probe sets were identified using linear models that apply moderated *t*-statistics that implement empirical Bayes regularization of standard errors. Comparisons were made between GOLD II-IV and GOLD 0-I using the fold-change threshold method.

In addition to a discovery-based genome-wide analysis, several lists of genes were compiled, based on a literature review, primarily to test for differences in gene expression by disease status based on hypotheses about nutrient-responsive genes. One list comprised genes identified in prior studies of expression in patients with COPD compared to controls; confirming this list in the current study allowed consideration of the validity of the samples and methods (19, 20). Another list comprised genes identified as COPD susceptibility genes in published genome-wide association studies (GWAS) (11, 12, 14–16, 54). Finally, two other lists related to primary hypotheses about oxidant/antioxidant balance and comprised antioxidant-responsive genes; vitamin E-responsive and selenium-responsive genes were both investigated. Each nutrient-responsive gene list was curated for biologic relevance to the present study, and genes were retained if they were: known to be expressed in lung tissue, expressed under relevant physiologic concentrations of the nutrient, expression *not* limited to highly specific physiologic stress conditions, and *not* primarily related to cancer pathogenesis (10, 36–48).

Using *p*-values from the microarray analysis, adjusted *p*-values, i.e., false discovery rate (FDR) q-values, were estimated for genes in each of the gene lists. Genes were considered differentially expressed if fold-change was > 1.15, nominal $p < 0.05$, and $p_{adjusted} < 0.05$. Finally, for genes with evidence of differential expression, further analyses assessed the association of gene expression with tissue and plasma nutrient concentrations using the Pearson product moment correlation coefficient. qPCR confirmation of the microarray findings was not possible due to limitations in sample volume and availability.

Data from an expression quantitative trait loci (eQTL) study (55), using lung small airway epithelium tissue samples from a diverse cohort of 116 individuals including smokers and non-smokers of different genders and ancestries, were used to investigate evidence for eQTL associations for genes with differential expression by COPD stage/severity. Briefly, SNPs were assayed using Affymetrix 500K arrays, and SNPs with minor allele frequency > 0.1 and within 100 kb of the candidate genes were tested for association with gene expression using PLINK v1.07. Quantile-quantile (Q-Q) and Manhattan plots were generated in R to visually examine *p*-value distributions.

Results

Participant characteristics

High quality RNA samples were obtained from 22 of the 24 lung tissue samples, (Table 2) comprising 14 males and 8 females, and all but two participants were non-Hispanic White (1 African American male, 1 Hispanic male). All participants were past or current cigarette smokers with an average of 61.0 pack-years of cigarette smoking (standard deviation 39.2). Participants with severe COPD (GOLD stage IV) were younger. There were no data on dietary intake or use of supplements.

Table 2. Descriptive characteristics of 22 patients provided by the Lung Tissue Research Consortium, National Heart, Lung and Blood Institute

	Total	GOLD Stages					GOLD Stage Group	
		0	I	II	III	IV	0/I	II-IV
Number	22	5	3	4	5	5	8	14
Age, years[a]	65.5 (9.4)	73.8 (8.3)	65.0 (7.0)	70.5 (3.5)	63.4 (10.6)	55.6 (2.8)	70.5 (8.6)	62.6 (8.9)
Sex, male/female	14/8	4/1	3/0	2/2	2/3	3/2	7/1	7/7
Pack-years smoking[b]	61.0 (39.2)	64.8 (38.9)[b]	56.0 (23.6)	74.5 (85.3)	42.8 (4.8)	68.6 (9.5)	61.0 (31.0)	61.1 (43.8)
Years since quitting smoking[b,c]	7.3 (9.8)	21.0 (14.3)[b]	0	4.8 (8.2)	6.8 (4.0)	3.4 (2.5)	12.0 (15.1)	5.0 (5.0)

[a]Continuous variables reported as mean (SD).
[b]One participant was missing smoking data and is not included in the mean or SD.
[c]For those participants who quit smoking.

Tissue nutrient status

Plasma total selenium, total α-tocopherol, HDL α-tocopherol and lung tissue α-tocopherol levels were measured in all samples (Table 3) and were examined for trends by GOLD stage. There was little variation in average plasma selenium levels by GOLD stage, and the mean selenium in GOLD 0-I (no disease and minimal disease) vs. II-IV (moderate/severe/very severe) was not statistically significantly different ($p = 0.24$). Plasma α-tocopherol and HDL α-tocopherol varied by GOLD stage such that α-tocopherol concentrations were higher in GOLD 0/I, and lower in GOLD II-IV.

Comparing the GOLD 0/I group with the GOLD II-IV group the difference in means was not statistically significant for either plasma or HDL α-tocopherol ($p = 0.38$ and $p = 0.51$, respectively). For 19 of 22 study participants GC-MS analysis of plasma revealed a metabolic peak consistent with past intake of all-*rac*-α-tocopherol, i.e.,synthetic α-tocopherol, which indicates use of dietary supplements. The evidence for use of supplements by most participants makes it less likely that differences in supplement use by disease stage explain differences in nutrient status. GOLD 0/I patients had higher levels of antioxidants, on average, in comparison to GOLD II-IV patients. Although plasma and tissue α-tocopherol concentrations were moderately correlated (r = 0.44; $p = 0.04$), lung tissue α-tocopherol concentration was similar in GOLD 0 and I, and decreased steadily with increasing disease severity. GOLD 0-I tissue α-tocopherol concentrations (24.9 nmol/g) were statistically significantly higher ($p = 0.0082$) than GOLD II-IV tissue concentrations (16.7 nmol/g).

Gene expression by GOLD stage

A genome-wide array analysis identified five genes that were differentially expressed in GOLD II-IV with nominal p-values <0.0001, but the lowest $p_{adjusted}$ was 0.27 (Supplemental Table 2).

In the hypothesis-driven analyses, 4 gene lists were investigated: 1) COPD GWAS-identified genes (6 genes), 2) COPD differentially expressed genes (22 genes), 3) vitamin E-responsive genes (42 genes), and 4) selenium-responsive genes (42 genes). Overall, the gene lists comprised 109 unique genes (Supplemental Tables 3, 4, 5 and 6), and the overlap included: *SERPINE2,* which was on the COPD GWAS list and the COPD expression list; *COL1A1* and *TNF,* which were on the COPD expression list and the vitamin E-responsive list.

Using a q-value threshold of $p_{adjusted}$ <0.05 (Table 4), 16 genes were identified as differentially expressed by

Table 3. Nutritional status of 22 patients; Samples provided by the Lung Tissue Research Consortium, National Heart, Lung and Blood Institute[a]

	Total	GOLD Stages					GOLD Stage Group	
		0	I	II	III	IV	0/I	II-IV
N =	22	5	3	4	5	5	8	14
Plasma Selenium, µg Se/L plasma	181.8 (29.5)	165.3 (17.6)	182.6 (59.9)	187.8 (37.8)	182.7 (19.3)	192.2 (23.0)	171.8 (35.8)	187.5 (25.0)
Plasma α-tocopherol, µmol α-toc/mmol cholesterol	12.1 (6.0)	15.5 (9.5)	10.9 (4.1)	13.8 (6.9)	12.1 (3.8)	8.2 (2.3)	13.8 (7.9)	11.2 (4.8)
HDL α-tocopherol, µmol α-toc/ mmol cholesterol	14.9 (5.3)	19.1 (6.1)	10.7 (1.0)	14.5 (5.2)	15.5 (5.9)	13.1 (4.1)	16.0 (6.4)	14.4 (4.8)
Tissue α-tocopherol, nmol α-toc/g lung tissue	19.7 (7.4)	24.1 (6.1)	26.3 (9.9)	18.3 (2.9)	19.4 (6.9)	12.8 (5.0)	24.9 (7.1)	16.7[b] (5.8)

[a]All values reported as mean (SD).
[b]$p = 0.0082$; all other p-values are >0.24.

Table 4. Genes differentially expressed ($p_{adjusted} < 0.05$) in lung tissue from GOLD Stage II-IV patients compared to GOLD Stage 0/I patients

Gene list	Gene symbol	Gene name	Fold-change[a]	Nominal p-value	$p_{adjusted}$[b]
COPD GWAS	*BICD1*	Bicaudaul D homolog 1	1.21	0.025	0.0251
COPD gene expression	*PLAUR*	Plasminogen activator, urokinase receptor	–1.88	0.015	0.0426
	VEGFB	Vascular endothelial growth factor	–1.26	0.025	0.0426
	MMP2	Matrix metalloproteinase 2	1.52	0.034	0.0426
Vitamin E responsive	*TNNI2*	Troponin 1 type 2	–1.34	0.007	0.0241
	KRT1	Keratin 1	1.51	0.019	0.0241
	SRA1	Steroid receptor RNA activator 1	–1.16	0.019	0.0241
	PPARG	Peroxisome proliferator-activated receptor gamma	–1.76	0.019	0.0241
	SULT2B1	Sulfotransferase family cytosolic 2B member 1	–1.42	0.026	0.0271
	KRT4	Keratin 4	–2.30	0.036	0.0303
Selenium responsive	*NR2F1*	Nuclear receptor subfamily 2 group F member 1	1.54	0.003	0.0347
	SELT	Selenoprotein T	–1.18	0.003	0.0347
	P4HA1	Prolyl 4-hydroxylase alpha polypeptide	–1.31	0.007	0.0347
	MS4A1	Membrane spanning 4 domains subfamily A member 1	2.25	0.008	0.0347
	GNGT2	Guanine nucleotide-binding protein subunit gamma 2	–1.39	0.009	0.0347
	GST01	Glutathione S-transferase omega-1	–1.23	0.010	0.0347

[a]Log_2 fold-change.
[b]FDR q-value, estimated for each gene list separately.

COPD stage: 1 on COPD GWAS list, 3 on COPD expression list, and 6 on each of the nutrient-responsive lists.

The *BICD1* gene, identified in prior GWAS of the COPD phenotype, was statistically significantly up-regulated in GOLD II-IV lung tissue. Two other genes, *HHIP* and *SERPINE2*, had fold-changes of -1.32 and -1.44, respectively, but nominal *p*-values did not meet preset criteria (p = 0.118 and 0.1396, respectively). Among genes with prior evidence of differential expression by COPD, *PLAUR* and *VEGFB* were down-regulated and *MMP2* was up-regulated in the GOLD II-IV group vs. GOLD 0/I (Table 4).

Six of the 42 vitamin E-responsive genes had evidence of differential expression by disease stage. Five genes were down-regulated in GOLD II-IV lung tissue samples compared to GOLD 0/I, with fold-changes ranging from -1.16 to -2.30; *KRT1* was up-regulated by 1.51-fold (Table 4). Similarly, 6 of the 42 selenium-responsive genes were differentially expressed by disease stage; *NR2F1* and *MS4A1* were up-regulated in GOLD II-IV lung tissue samples compared to GOLD 0/I, with fold-changes of 1.54 and 2.25, respectively. Four selenium-responsive genes were down-regulated in GOLD II-IV lung tissue samples compared to GOLD 0/I, with fold-changes between -1.18 and -1.39 (Table 4).

Across the 4 gene lists, 16 out of 109 genes were differentially expressed by GOLD stage disease severity group; the association of nutrient concentrations in lung tissue and plasma with gene expression was assessed (Supplemental Table 7). For 4 genes, expression was statistically significantly correlated ($p < 0.05$) with more than 1 nutrient. The expression of plasminogen activator urokinase receptor (*PLAUR*) was positively associated with lung tissue vitamin E (r = 0.46; p = 0.03) and plasma vitamin E (r = 0.66; p = 0.0008). *PPARG*, peroxisome proliferator-activated receptor gamma, was associated with vitamin E plasma levels (r = 0.46; p = 0.03). Several genes on the selenium-responsive list were associated with vitamin E nutriture, as follows: guanine nucleotide-binding protein subunit gamma 2, *GNGT2*, (r = 0.65, p = 0.0010 for plasma; r = 0.53, p = 0.01 for lung); propyl 4-hydroxylase alpha polypeptide, *P4HA1* (r = 0.41, p = 0.055 for plasma).

eQTL Results

All 16 genes differentially expressed by GOLD stage disease severity group (Table 4) were queried in the eQTL database, but only 15 genes had available gene expression data (no data available for *MS4A1*). A highly statistically significant *cis* eQTL association was identified for vitamin E-responsive gene *KRT1* (lowest nominal p-value is 8.88×10^{-7} for rs1567757), demonstrating that sequence variation in the *KRT1* gene region affects *KRT1* expression. Supplemental Figure 1 shows the location and association *p*-values of all SNPs within 100KB up and downstream of *KRT1*. Supplemental Figures 2 and 3 show genome-wide Q-Q and Manhattan plots for all SNPs in association with *KRT1* gene expression.

Discussion

Prior studies reported lower plasma antioxidant concentrations in patients with COPD or patients with lower pulmonary function compared to controls (34, 56).

These prior data suggested the hypothesis that lower endogenous antioxidants contribute to COPD progression and/or severity. In support of this hypothesis, we found that lung tissue α-tocopherol levels were about 33% lower in GOLD II-IV patients compared to GOLD 0/I patients, with a clear trend. Although the trends for plasma α-tocopherol and HDL α-tocopherol were less clear, compartment-specific nutrient levels may provide more relevant data. Plasma selenium concentrations were similar by GOLD stage, and selenium could not be assayed in lung tissue due to limited tissue mass.

Prior studies also show that vitamin E and selenium contribute to gene regulation (10,36–41,48), hence we explored whether differences in nutrient status by disease stage had functional consequences by investigating whether genes responsive to vitamin E were differentially expressed by disease stage. Six of 42 vitamin E-responsive genes, namely *KRT1, PPARG, TNNI2, KRT4, SULT2B1,* and *SRA1,* were differentially expressed by disease stage/severity, and there was evidence that genetic variation directly alters expression of the *KRT1* gene in lung tissue.

The average non-cholesterol-adjusted plasma α-tocopherol concentration in the LTRC samples was 52.4 μmol/L, corresponding to a concentration that would be expected in the presence of α-tocopherol supplementation, as reported for the U.S. National Health and Nutrition Examination Survey (NHANES) participants (57). Because biopsy of lung tissue in healthy individuals is rare there are few reports of usual tissue-specific α-tocopherol concentrations, however, α-tocopherol concentrations in lung tissue have been reported for several disease states (58, 59). In patients undergoing lung surgery for tumors, α-tocopherol concentrations in lung tissue were 22.3 nmoles/g tissue (58), which agrees closely with values reported herein. The NHANES reported mean serum selenium concentrations of 136.7 μg/L (60), approximately 25% lower than concentrations reported herein. Specific use of selenium supplements is not common, but we have no data to confirm or refute such use in the participants studied; such use could contribute to higher than expected selenium concentrations.

COPD-related genes

One gene identified in prior GWAS studies of COPD was differentially expressed in lung tissue from COPD GOLD stage II-IV patients compared to at-risk/normal controls. The *BICD1* gene, bicaudaul D homolog 1, was up-regulated in the GOLD II-IV group. Variants in *BICD1* are associated with telomere length and the hypothesized mechanism linking *BICD1* SNPs to COPD risk is accelerated aging, which may contribute to the pathogenesis of emphysema (11,61,62). The evidence of differential expression in lung tissue by COPD severity provides further evidence for a causal role of this gene in COPD pathogenesis.

Three genes with prior evidence of differential expression in COPD patients compared to controls were replicated herein. The plasminogen activator urokinase receptor gene, *PLAUR,* was down-regulated (fold-change -1.88; $p = 0.015$; $q = 0.0426$) in lung tissue from GOLD II-IV patients (vs. GOLD 0/I), in contrast to a prior report that *PLAUR* expression was inversely associated with FEV_1 (higher expression associated with lower lung function) (19). The PLAUR protein assists in the conversion of latent matrix metalloproteinases, such as MMP1—which is implicated in the pathogenesis of emphysema—by converting plasminogen to plasmin (19).

VEGFB, vascular endothelial growth factor B, was down-regulated (fold-change -1.25) in GOLD II-IV vs. GOLD 0/I. Several prior studies reported on a related *VEGF* gene; one study reported dysregulation in small airways, but the direction of fold-change was not reported (19), yet a second study reported bronchiolar VEGF was decreased in smokers with COPD compared to healthy smokers (63). *VEGF* has been implicated in the regulation of new blood vessel growth and vascular leakage (64), both important processes in the pathophysiology of COPD.

Finally, matrix metalloproteinase 2, *MMP2*, was up-regulated in GOLD II-IV patients compared to GOLD 0/I (fold-change = 1.52). Consistent with our findings, an immunohistochemical analysis of MMP2 staining in human lung tissue reported more protein in relation to increasing disease severity, and *MMP2* expression was inversely associated with the ratio of FEV_1/FVC (higher expression associated with lower ratio) (65). Imbalances of metalloproteases and anti-metalloproteases have been shown to lead to inflammatory states and destruction of lung parenchyma, leading to development of emphysema (66).

Vitamin-E responsive genes

Six of 42 vitamin E-responsive genes had statistically significantly differences in expression in lung tissue from GOLD stages II-IV patients compared to GOLD 0/I patients. Notably, *PPARG* expression was lower in patients with GOLD stages II-IV, and lung tissue from these patients was lower in α-tocopherol concentrations; our findings are consistent with a prior study reporting regulation of *PPARG* by α-tocopherol (45). *PPARG* is implicated in cell signaling and cell-cycle regulation and may be important in management of inflammatory conditions (45). In a study of asymptomatic cigarette smokers, 30 days of supplementation with vitamin E led to a +119.3-fold-change in *PPARG* expression ($\log_{10}$ fold-change expression) in airway epithelial cells compared to a negligible change in the placebo arm (-37.9-fold-change; personal communication). Polymorphisms of *PPARG* have been shown to alter COPD susceptibility in a case-control study (67).

TNNI2, troponin 1 type 2, was down-regulated in patients with more severe COPD, with a fold-change of -1.34; *TNNI2* is related to cytoarchitecture and contributes to the formation of capillaries, though information is sparse on vitamin E regulation of this gene (42).

Down-regulation of *KRT4* and coincident up-regulation of other keratin genes, including *KRT1*, led to aberrant epithelial morphology (68). We found down-regulated *KRT4* and up-regulated *KRT1* with more severe COPD. Additionally, genetic variants in the *KRT1* gene region were strongly associated with *KRT1* gene expression in lung tissue in the eQTL analysis, suggesting that genetic variation may be associated with disease status through effects on gene expression.

Selenium-responsive genes

There were no differences in plasma selenium concentration by disease stage/severity, and lung tissue could not be assayed for selenium. However, 6 of 42 genes in the selenium-responsive list were differentially expressed by disease severity. The nuclear receptor subfamily 2 group F member 1 gene, *NR2F1*, was up-regulated and expression levels were 1.54-fold higher in lung tissue samples from GOLD II-IV patients compared to GOLD 0/I. A prior study reported that *NR2F1* was down-regulated by supplemental selenomethionine (36), but expression had little or no association with plasma selenium in the present study.

The membrane spanning 4 domains-subfamily A-member 1 gene, *MS4A1*, had the greatest fold-change; *MS4A1* expression was 2.25-fold higher in lung tissue from GOLD II-IV patients compared to GOLD 0/I patients. Prior studies show that supplementation with selenomethionine down-regulates expression of *MS4A1*, thus the higher expression levels we observed are consistent with the hypothesis that GOLD II-IV lung tissue is depleted in selenium relative to GOLD 0/I (36). Expression of *MS4A1* was inversely associated with plasma selenium, consistent with prior reports, although the correlation was not statistically significant.

P4HA1 codes a key enzyme for collagen synthesis and folding and is important for remodeling of the extracellular matrix (69). We found down-regulation of *P4HA1* with more severe disease, consistent with the hypothesis that less expression inhibits remodeling and increases COPD progression.

Limitations of this study included the small sample size, lack of tissue to assay selenium concentrations in lung, and the inability to confirm mRNA levels for the target genes due to sample limitations. Also, if supplement use patterns differed by disease stage, this would confound the identified associations; although the LTRC did not collect data on dietary supplement use, the assay data on plasma alpha-tocopherol (presence of a supplement-associated peak) are consistent with almost universal use of supplements in the studied participants. Finally, while the classification of disease severity could be attempted in different ways, the studied groups manifested clear differences in phenotype, as exemplified by the descriptive lung function data, thus supporting the classification used.

There are clear strengths of this study including rigorous methods to quantify selenium and α-tocopherol and high quality gene expression microarray assays using a well-validated Affymetrix platform. Given the small sample size, the use of hypothesis-driven gene lists was an important investigative tool, particularly because the primary interest of this study was in genes responsive to nutrients with antioxidant properties. We investigated the correlation of gene expression with tissue and plasma nutrient as a complementary strategy, and this led to supportive data for the contrast by COPD severity group.

Although we could not confirm microarray expression findings by direct assays, an eQTL analysis served as an alternative means of following up microarray expression findings; for the 16 genes differentially expressed by disease status, eQTL data were available on 15 genes, and 1 gene had a highly statistically significant eQTL. This analysis is a strength because it both confirms the expression of these genes in lung tissue in an independent sample, and provides evidence that genetic variation affects gene expression in one of the vitamin E-responsive genes.

In conclusion, α-tocopherol concentrations in lung tissue were associated with COPD GOLD stage such that more severe cases had lower tissue α-tocopherol, and 6 vitamin E responsive genes were also differentially expressed by disease stage. Despite small numbers, these cross-sectional findings show differences in antioxidant status and antioxidant-responsive gene expression by disease stage/severity. The findings are consistent with two alternative hypotheses, which cannot be distinguished in this cross-sectional study: lower lung tissue α-tocopherol concentrations may contribute to COPD pathophysiology and/or COPD may lead to lower lung tissue α-tocopherol either because the nutrient is rapidly utilized in the COPD lung, because the transport and/or uptake of α-tocopherol in the lung is impaired in diseased individuals, or because disease severity is associated with general malnutrition including compromised vitamin E. Further longitudinal and/or intervention studies are needed to confirm the causal direction. Given the proposed role of nutrients with antioxidant properties in protecting the lung, investigating the relation between lung-specific antioxidants and lung tissue gene expression may identify causal mechanisms and ultimately, novel therapeutic targets.

Declarations of Interest Statement

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