ANTIOXIDANT INTERVENTIONS AND PULMONARY OUTCOMES: THE IMPACT OF NUTRITION AND GENE EXPRESSION ON THE LUNG

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ANTIOXIDANT INTERVENTIONS AND PULMONARY OUTCOMES: THE IMPACT OF NUTRITION AND GENE EXPRESSION ON THE LUNG

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The aims of this research were to determine how gene expression and chronic obstructive pulmonary disease (COPD) susceptibility are related to antioxidant nutritional status and to determine if antioxidant supplementation prevents COPD disease processes. Although cigarette smoking is the primary risk factor for COPD a minority of smokers develop disease thus other factors must play a role in disease susceptibility and progression. The studies conducted for this dissertation address the hypotheses that dietary supplementation with antioxidant vitamins can augment the lung's antioxidant defenses thereby preventing disease development and that antioxidant-related genes are dysregulated by COPD disease processes. The first project was a large-scale randomized clinical intervention trial of vitamin E dietary supplements in which the association of supplementation and risk of COPD was assessed. Women randomly assigned to take vitamin E supplements during a 10-year intervention were 10% less likely to develop COPD during the study period. The effect of the intervention was similar in both smokers and non-smokers and was not changed by other factors measured in the study. The second study was a small-scale clinical intervention trial that investigated how antioxidant supplementation altered concentrations of nutrients systemically and in the lung compartment. For intervention trials assessing whether the supplement is reaching the target tissue is critical and no prior

studies show direct evidence that supplemental antioxidants reach the lung. In this study bronchioalveolar lavage fluid concentrations of vitamin E and selenium were measured before and after intervention with vitamins E and C and selenium and nutrient concentration increased in response to supplementation, providing evidence that antioxidant supplements were delivered to the lung where they could combat oxidative stress that causes lung tissue damage. The third study assessed antioxidant status in plasma and lung tissue and gene expression in lung tissue of COPD patients with different levels of disease severity. Twelve antioxidant-related genes were differentially expressed in patients with more severe disease. Taken together the findings from these studies suggest that intervention with antioxidant nutrients increases lung nutrient status and decreases COPD risk and the mechanism may be in part modulation of antioxidant-related gene expression.

BIOGRAPHICAL SKETCH

Anne Hermetet Agler was raised in Bloomington, Illinois by David and Kathleen, parents who never became frustrated with the endless questions she asked. She did “experiments” in her room throughout her childhood, often pilfering things from around the house to see how they floated, or melted, or burned, or dissolved. When David and Kathleen permitted this kind of exploration they never realized they were raising a scientist. Fortunately, the local public school teachers saw Anne’s love of science and encouraged her pursuits.

Her love of experimentation took Anne to Illinois State University where she majored in Chemistry with a minor in French. She was fortunate to have an intensive undergraduate research experience under the supervision of Dr. Karen Goldberg. Dr. Goldberg’s influence can still be seen today in Anne’s love of problem-solving and her deeply rooted scientific intuition, which was carefully fostered by Dr. Goldberg. Following her B.S. degree, Anne continued on at Illinois State University, pursuing an M.S. in Inorganic Chemistry under the direction of Dr. Douglas X. West. Dr. West taught Anne the art of productivity and helped her develop into a skilled chemist.

Upon graduation, Anne taught general chemistry for one term at Georgia Southern University then went to work as a research scientist at Foto-Wear in Milford, PA, where she worked on product development for high tech paper products. Her work at Foto-Wear resulted in two U.S. Patents. After Foto-Wear outsourced its research operations Anne took a job in New Jersey working with chemical and pharmaceutical companies on product development of specialty chemical products. It was while working with the

pharmaceutical industry that Anne realized that she needed to return to school to complete a doctorate. After a year of careful consideration of PhD programs Anne decided that Cornell University offer the best opportunity for her to study nutrition and epidemiology while making use of her background in chemistry. After she arrived at Cornell, and with much persuasion from her mentor, Dr. Pat Cassano, Anne decided to minor in genomics – which has worked out very well.

Anne is completing her PhD studies in Nutritional Sciences, with minors in Epidemiology and Genomics, in the Spring semester of 2011. She has thoroughly enjoyed her experience at Cornell University and is looking forward to her next adventure.

This work is dedicated to my husband Theo whose unswerving support, excellent cooking, wonderful sense of humor, and passionate love for me have bolstered my spirits, nourished my body and soul, and given me the confidence to live my dreams. It is with you at my side that I am my very best.

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Jason Mezey provided wonderful assistance during my grant writing process and was key in getting me funded and off of a TA appointment. You cannot know how helpful that was – both the grant writing and the escape from TA duties. Thanks for your assistance and I promise I will eventually learn R.

Tim O’Brien was my first genomics chair and he was instrumental in helping me get started in the field. I appreciate the time he spent with me as a newbie in the field and very much miss his presence on campus.

Pat Cassano, there is so much more to say than I could possibly put into words here. You are a wonderful mentor, both as a scientist and as a person. Your guidance has helped me grow beyond what I imagined was possible and your constant encouragement has made it a pleasant process to stretch my limits and find out just how far I can go as a scientist. I appreciate your constant attention to not only the work that needs done but also the scientist and person you are molding. Your dedication to excellence in every area of life shows in all you do. You have truly helped make me who I am today. Thank you so much.

Thanks to all of the members of the Cassano research group (past and present) and my many other friends in the Division of Nutritional Sciences. You have all helped make my time at Cornell fulfilling.

To my husband, Theo, thank you for taking care of me during this process. You never questioned my decision to walk away from a high-paying job to become a lowly student again and I promise I will make it up to you at some point. Your encouragement and love kept me balanced through the rough semesters.

Lastly, thanks to my parents, David and Kathleen, and my sister Sarah. My family supported my somewhat unorthodox decision to come back to school in my mid-30s without hesitation. It was much easier to make the decision knowing that I had the love and support of my family.

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CHAPTER 1

INTRODUCTION

Chronic obstructive pulmonary disease, comprised of emphysema and chronic bronchitis, is a major cause of morbidity and mortality, and is characterized by irreversible airflow limitation.(1) Symptoms of COPD include recurrent respiratory infections, severe cough, and poor overall oxygenation, and at advanced stages COPD commonly progresses to respiratory failure and death. COPD is the fourth leading cause of mortality in the US.(2) The public health burden of COPD worldwide is growing and in 1998 the Global Burden of Disease study predicted that COPD will increase from the 12^{th} to the 5^{th} leading cause of disease burden by 2020.(3) The single most important risk factor for the development of COPD is exposure to cigarette smoke, with current or former smokers constituting 80-90% of COPD patients.(4-6) Despite the clear importance of smoking in the etiology of disease, there is considerable variation among smokers in their response to cigarette smoke exposure. A large cross-sectional study found that only an estimated 15% of the variation in lung function was explained by smoking parameters.(7) While not discounting the paramount importance of cigarette smoke exposure in the development of COPD, clearly other as yet undiscovered factors are important in disease.

The current understanding of COPD pathogenesis suggests a role for exaggerated inflammatory response, protease/antiprotease imbalance, and oxidant/antioxidant imbalance in disease susceptibility. This dissertation focused on oxidant/antioxidant imbalance for the following reasons: firstly, a single puff of cigarette smoke contains 10^{14} radicals and upon contact with the

large surface area of the lung immediately increases the strain on the antioxidant defenses.(8) When antioxidant defenses are overwhelmed, oxidative stress can provoke and enhance both the inflammation and the antiprotease inactivation that are associated with COPD as well as directly contributing to lung tissue damage. Secondly, antioxidant defenses are a compelling COPD research target because they offer the possibility of simple and inexpensive interventions. Finally, observational epidemiologic studies of antioxidants and lung outcomes suggest antioxidants have lung-protective properties.

Observational epidemiological studies of dietary antioxidant intake, serum antioxidant concentration, and lung outcomes suggest that lower levels of antioxidant defense are associated with decreased lung function and higher COPD mortality risk.(9-15) Although a number of studies have investigated the role of antioxidant supplements to prevent COPD only nine randomized controlled trials have been conducted and in each of these COPD was a secondary outcome and the antioxidants studied included vitamins E, C, and beta-carotene and N-acetylcysteine; the majority of studies investigated N-acetylcysteine.(16-23) Prior to this dissertation no clinical trials had assessed the effect of vitamin E supplementation on the primary prevention of COPD. One randomized controlled trial of alpha-tocopherol and beta-carotene found no effect of supplements on COPD symptoms, but the prevalence of COPD at study baseline (prior to intervention) was inversely associated with dietary and serum concentrations of both antioxidants.(20) In a trial where subjects with coronary or occlusive arterial disease or diabetes were supplemented with vitamins E and C and beta-carotene no significant

difference in measures of pulmonary function or number of hospitalizations was seen between the supplemented and placebo groups.(23)

Gene expression patterns offer a snapshot into an individual's adaptive response to a particular exposure, such as smoking. Expression values are a summative measure of all stimuli that promote and suppress transcription, and thus, represent the cellular environment at the time of the assay. In this way, expression patterns represent the amount of oxidant exposure and the level of antioxidant defense together as the "dose" of oxidative stress that reaches the cell in a particular individual. Smokers exhibit altered gene expression compared with nonsmokers and these altered expression patterns are expected to precede biochemical and tissue changes on the pathway to disease development, thus identifying interventions that are successful at the level of transcription will provide strong support for their success as disease preventives or treatments.

The scientific objective of this dissertation is to investigate nutritional, genetic, and environmental factors that increase susceptibility to and/or severity of chronic obstructive pulmonary disease (COPD). Three projects were completed as follows: 1) a randomized study of vitamin E supplementation and risk of chronic lung disease in the Women's Health Study, which was a *post hoc* analysis of the incidence of COPD and asthma in a double-blinded randomized clinical trial of vitamin E and aspirin in women, 2) a study of the effect of supplementary antioxidant vitamins on plasma and airway lining fluid in cigarette smokers, which was a double-blinded randomized clinical trial investigating the effect of dietary antioxidant supplements in the lung compartment (hereafter referred to as ExSEL, for Expression effects of selenium and vitamin E in lung), and 3) a study of the

differential expression of vitamin E and selenium-responsive genes by disease severity in chronic obstructive pulmonary disease (hereafter referred to as LTRC, given the Lung Tissue Research Consortium source of these samples), which was a small study of antioxidant concentrations in plasma and lung tissue in relation to gene expression in lung tissue of COPD patients at varying levels of disease severity.

A molecular epidemiologic model is presented below, showing the purported exposure leading to a biomarker of disease outcome, which in turn leads to disease onset and/or progression. This model provides a framework for understanding how the three proposed projects integrate to form a complementary and inclusive investigation into COPD, its risk factors and biomarkers, and potential dietary and supplementation intervention strategies, including the effect of supplementation on gene expression.

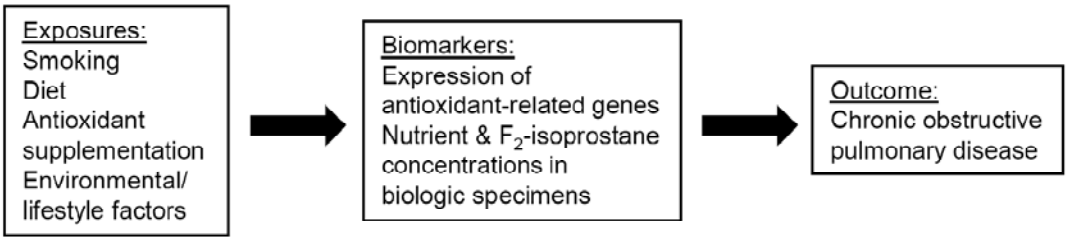

Figure 1.1 Molecular epidemiologic model for risk factors and biomarkers leading to COPD.

Under this framework no single project addressed all aspects of the molecular epidemiologic model, but in combination the projects provide a comprehensive study of the model. The data from the Women's Health Study provided a large and comprehensive study of exposure to supplemental vitamin E and its association with COPD onset, but biomarker information was unavailable. ExSEL was designed to address how antioxidant

supplementation alters systemic and lung-compartment concentrations of antioxidants. ExSEL was not designed to investigate COPD as an outcome because its time-course was far too short for such a determination. The analysis of lung tissue and plasma concentrations of antioxidants and lung tissue gene expression in samples from COPD patients provided insight into how antioxidants concentrations differ by disease severity, and how those differences are related to gene expression, providing clues as to the role of antioxidant status in disease progression. By integrating these three studies a complete model of COPD risk factors, biomarkers, and outcomes emerged with each study contributing an essential piece to the overall understanding of how antioxidants and gene expression are related to COPD risk and progression.

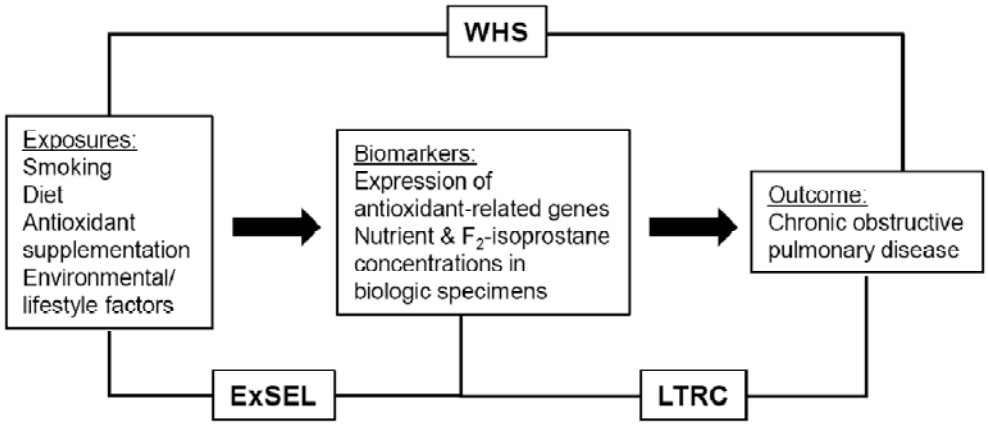

Figure 1.2 Integration of dissertation projects in the molecular epidemiologic model for risk factors and biomarkers leading to COPD; WHS=Women's Health Study; ExSEL= Expression Effects of Selenium and Vitamin E in Lung Study; LTRC=Lung Tissue Research Consortium Study.

The complementary nature of the three proposed projects provided a substantial, well-integrated study of COPD that contributed meaningful new information to the scientific body of knowledge about COPD, its causation and the joint and separate roles of nutrition, gender and lifestyle.

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CHAPTER 2

RANDOMISED VITAMIN E SUPPLEMENTATION AND RISK OF CHRONIC LUNG DISEASE IN THE WOMEN'S HEALTH STUDY*

Abstract

Background: The oxidant/antioxidant balance in lung tissue is hypothesised to contribute to chronic obstructive pulmonary disease (COPD) risk. Observational studies consistently report higher antioxidant status associated with lower COPD risk, but few randomised studies have been reported.

Methods: A post-hoc analysis of 38,597 women without chronic lung disease at baseline was conducted in the Women's Health Study (WHS) to test the effect of vitamin E on risk of incident chronic lung disease. The WHS was a randomised, double-blind, placebo-controlled, factorial trial of vitamin E (600 IU every other day) and aspirin (100 mg every other day) in female health professionals aged ≥45. Using Cox proportional hazards models, the effect of randomised vitamin E assignment on self-reported, physician-diagnosed chronic lung disease was evaluated.

Results: During 10 years of follow-up (376,710 person-years), 760 first occurrences of chronic lung disease were reported in the vitamin E arm compared to 846 in the placebo arm (Hazard Ratio [HR] 0.90; 95% confidence interval [CI] 0.81-0.99; p=0.029). This 10% reduction in the risk of incident

*Agler AH, Kurth T, Gaziano JM, Buring JE, Cassano PA. Randomised vitamin E supplementation and risk of chronic lung disease in the Women's Health Study. Thorax January 21, 2011; doi: 10.1136/thx.2010.155028.

chronic lung disease was not modified by cigarette smoking, age, randomised aspirin assignment, multivitamin use, or dietary vitamin E intake (minimum P for interaction = 0.19). Current cigarette smoking was a strong predictor of chronic lung disease risk (HR 4.17; 95% CI 3.70-4.70; versus never smokers). ***Conclusions:*** In this large, randomised trial, assignment to 600 IU of vitamin E led to a 10% reduction in the risk of chronic lung disease in women.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by progressive, irreversible airflow limitation and comprises a significant public health burden, with increasing trends in incidence and prevalence.(1) COPD prevalence in the U.S. adult population is 3-4% and, worldwide COPD prevalence is about 10%.(1;2) COPD was the 5th leading cause of death in the U.S. in 2001 and is expected to become the 3rd leading cause of death by 2020, largely due to population ageing and increasing cumulative cigarette smoke exposure, the primary risk factor for COPD.(1;3)

Factors that may contribute to rising COPD incidence include: obesity, dietary patterns, environmental and occupational exposures, and improved diagnostic and screening programmes.(1;4-6) Several lines of evidence support the hypothesis that diet plays a role in COPD aetiology.(4;6;7) Observational studies of diet or nutritional status biomarkers and randomised trials of diet or nutritional supplements have investigated the relation of antioxidants, notably vitamin E, and lung outcomes. Observational studies investigating the association of dietary intake and pulmonary function consistently report that higher intake of nutrients with antioxidant properties is associated with better pulmonary outcomes, but causal inferences are limited by concerns about confounding and other biases.(8;9) Studies comparing COPD patients to healthy individuals report lower plasma and peripheral skeletal muscle vitamin E (α–tocopherol) concentrations in patients, and a lower risk of death from respiratory disease with higher serum α-tocopherol concentration, but whether nutrition contributed to the onset of COPD is less clear.(10-12)

Randomised trials of diet change or vitamin E supplements in clinical populations have reported mixed results. COPD patients who increased intake of antioxidant-rich foods had improved pulmonary function over three years while those on usual diets experienced continuous lung function decline.(13) Studies of α-tocopherol treatment in COPD patients report mainly negative results, although conclusions are limited by an incomplete understanding of potential to benefit, the short duration of studies, and case heterogeneity.(14-16) Very few large, randomised studies of non-diseased individuals have been completed. In the Heart Protection Study (HPS), which included participants with coronary disease, other occlusive arterial disease, or diabetes, a post-hoc analysis found no effect of vitamin E supplements on the occurrence of respiratory-related death, on COPD/asthma hospitalization rates, or on pulmonary function measured by spirometry at the end of the study.(17) In the Alpha-Tocopherol and Beta-Carotene (ATBC) study, a study of male cigarette smokers, there was no effect of α-tocopherol on the incidence of chronic bronchitis or COPD symptoms.(16)

Using data from the Women's Health Study (WHS), a large study of apparently healthy women aged $\geq$ 45 years, we tested the hypothesis that supplementation with 600 IUs of α-tocopherol every other day decreases the occurrence rate of chronic lung disease.

Methods

Study Design

The WHS, a randomised, double-blind, placebo-controlled, two-by-two factorial trial assessed risks and benefits of vitamin E supplements (600 IU every other day; Natural Source Vitamin E Association, Washington, DC,

USA) and/or aspirin (100 mg every other day; Bayer AG, Leverkusen, Germany) in the primary prevention of cardiovascular disease and cancer. Full details of the study design are published elsewhere. The study was registered with clinicaltrials.gov (NCT00000479).

Eligibility criteria included: age ≥45 years, healthcare professional, U.S. residence, no previous history of coronary heart disease, cerebrovascular disease, cancer (except non-melanoma skin cancer), or other major chronic illness; no more than weekly use of vitamins E, A, or β-carotene supplements; no history of adverse aspirin effects; less than weekly use of aspirin or non-steroidal anti-inflammatory drug (NSAID), or willingness to forgo; no use of anticoagulants or corticosteroids. A 3-month placebo-only run-in period identified likely long-term compliers. Of these, 39,876 women remained willing and eligible and were randomised into WHS between April 1993 and January 1996.(18)

Questionnaire Data

Mailed questionnaires collected baseline data on anthropometric, demographic, lifestyle, and clinical characteristics. Follow-up questionnaires, completed twice during the first year and annually thereafter, assessed study supplement compliance, new disease occurrence and diagnosis date, personal characteristics and habits, non-study aspirin, vitamin and NSAID use, and side effects. Compliance, defined as taking two-thirds of study supplements, was similar (78.9% and 71.6% at 5 and 10 years, respectively) between active and placebo groups.(19) Non-trial vitamin E supplement use ≥4 days/month was 10.0% and 10.9% at 5 and 10 years, respectively.(19)

Chronic Lung Disease Ascertainment

Chronic lung disease (CLD) was not a pre-specified trial endpoint. Occurrence of self-reported MD-diagnosed CLD was ascertained on questionnaires beginning 12 months after study enrolment. A multipart question asked participants “have you ever been diagnosed by a physician as having any of the following?”, and choices included “other chronic lung disease (e.g. emphysema, chronic bronchitis, bronchiectasis)” as well as “asthma.” For each diagnosis, date of diagnosis was reported. Thereafter, annual questionnaires asked about diagnoses occurring since the prior questionnaire, including diagnosis date. Incident cases were ascertained through March 31, 2004 (scheduled trial end). Prevalent CLD was defined as CLD diagnosis prior to trial enrolment. Women with prevalent CLD were excluded from the analysis (figure 2.1).

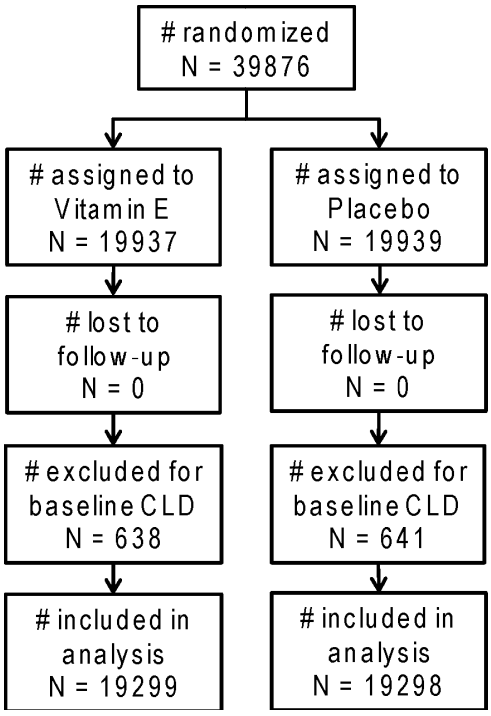

Figure 2.1 Flow diagram of the vitamin E component of the Women’s Health Study chronic lung disease analysis.

Statistical Analysis

1,279 women reported prevalent CLD (638 in vitamin E group; 641 in placebo group), thus 38,597 participants were available for analyses. All analyses followed the intention-to-treat principle. Cumulative incidence of CLD by study arm was assessed using Kaplan-Meier methods and log-rank tests to compare survival curves. Cox proportional hazards models estimated hazard ratios. Further models considered whether the effect of vitamin E on incident CLD was modified by smoking status, age, body mass index, multivitamin use, alcohol intake, baseline asthma, history of cholesterol levels ≥240mg/dl, or randomised aspirin assignment. Effect modification was tested for statistical significance using likelihood ratio tests comparing models with and without interaction terms. Data management and analyses were completed in SAS (SAS Institute Inc., Cary, NC).

Results

Baseline characteristics in the 38,597 participants, summarized in Table 2.1, were balanced between the vitamin E and placebo arms. Thus, participants in both arms were similar on age, smoking, body mass index, multivitamin use, dietary intake of vitamin E, alcohol intake, history of asthma diagnosis, and percent with cholesterol ≥240 mg/dl (Table 2.1). The mean age of study participants was 54.5 years, and women were followed on average for 9.8 years (376,710 person-years; 188,578 person-years in the vitamin E arm, 188,132 person-years in the placebo arm).

Table 2.1 Baseline characteristics of Women's Health Study participants by vitamin E randomisation

Characteristic	Vitamin E (N=19299)* No. (%)‡	Placebo (N=19298)† No. (%)‡
Demographic/lifestyle		
Age, years‡	54.5 (7.0)	54.6 (7.0)
<55	11714 (60.7)	11679 (60.5)
55-64	5654 (29.3)	5677 (29.4)
≥65	1931 (10.0)	1942 (10.1)
Cigarette smoking		
Current	2434 (12.6)	2491 (12.9)
Past	6937 (36.0)	6823 (35.4)
Never	9909 (51.4)	9968 (51.7)
Average duration, years‡§	18.8 (12.5)	18.9 (12.6)
Body mass index, kg/m^2‡	26.0 (5.0)	26.0 (5.0)
<25.0	9598 (50.8)	9670 (51.1)
25.0-<30.0	5880 (31.1)	5837 (30.8)
≥30.0	3404 (18.0)	3411 (18.0)
Nutrition		
Multivitamin Use		
Never	2521 (13.2)	2553 (13.4)
Past only	10927 (57.4)	10982 (57.7)
Current	5574 (29.3)	5499 (28.9)
Vitamin E intake, mg/day		
Diet only‡	6.6 (5.0)	6.6 (5.3)
Diet + Supplements‡	63.2 (143.0)	62.6 (140.9)
Alcohol intake		
Rare/never	8743 (45.3)	8590 (44.5)
1-3/month	2531 (13.1)	2553 (13.2)
1-6/week	6048 (31.4)	6194 (32.1)
1+/day	1970 (10.2)	1959 (10.2)
Medical conditions		
Asthma diagnosis	1104 (5.7)	1105 (5.7)
Cholesterol ≥240 mg/dl	5615 (29.1)	5688 (29.5)
Study aspirin assignment	9638 (49.9)	9654 (50.0)

*N total for each characteristic ranges from 18,882 to 19,299, given missing data in some variables
†N total for each characteristic ranges from 18,918 to 19,298, given missing data in some variables
‡Continuous variables are presented as means (SD)
§Average smoking duration for current and past smokers only

Participants reported 1,606 new diagnoses of chronic lung disease, corresponding to a cumulative incidence of 4.2%. Participants in the vitamin E arm reported 760 incident CLD diagnoses (cumulative incidence, 3.9%) compared to 846 occurrences in the placebo arm (cumulative incidence, 4.4%), corresponding to a statistically significant 10% reduction in risk among participants randomised to vitamin E supplements (hazard rate (HR) 0.90; 95% confidence interval (CI) 0.81-0.99; p = 0.029). Comparing the cumulative CLD incidence by year of follow-up in the vitamin E and placebo groups (figure 2.2) the curves separate at about 1.5 years on study and continue to diverge until about 5 years of supplementation, maintaining a consistent separation thereafter. In contrast, the aspirin intervention had little or no association with risk of chronic lung disease; the hazard ratio was 0.98 (95% CI 0.89-1.08).

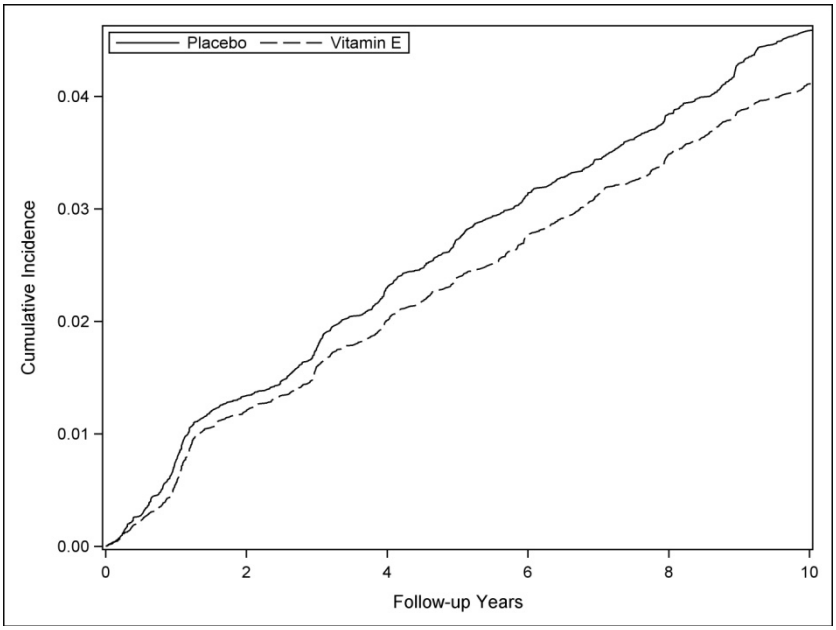

Figure 2.2 Cumulative incidence of chronic lung disease during the randomised component of the Women's Health Study.

Cigarette smoking had a strong association with CLD incidence (current smoker vs. never smoker: HR 4.17; 95% CI 3.70-4.70; p = <0.0001). In addition, other known COPD risk factors were positively associated with the CLD outcome: older age at randomization (age ≥65 years vs. <55 years; HR 2.38; 95% CI 2.07-2.73; p = <0.0001), obesity (BMI ≥30.0 vs. <25.0; HR 1.60; 95% CI 1.41-1.81; p = <0.0001), asthma diagnosis prior to randomization (HR 1.94; 95% CI 1.65-2.28; p = <0.0001), and hypercholesterolemia (HR 1.42; 95% CI 1.28-1.57; p = <0.0001).

There was no statistical evidence that the effect of randomised vitamin E assignment on CLD risk was modified by age (p=0.86), smoking status (p=0.96), body mass index (p=0.25), multivitamin use (p=0.67), baseline asthma history (p=0.89), cholesterol ≥240 mg/dl (p=0.84), or by study aspirin assignment (p=0.19) (figure 2.3). Alcohol intake was borderline statistically significant (p=0.054) as a modifier of the effect of vitamin E on CLD, and women consuming one or more alcoholic drinks per day had the strongest vitamin E protective effect. In additional analyses, there was no evidence of effect modification by race, exercise frequency, hypertension, and baseline dietary intake of either vitamin E or C. For all models, controlling for randomised aspirin assignment did not alter the effect of vitamin E supplement assignment on CLD risk. An additional sensitivity analysis was conducted, censoring women who reported incident asthma from the CLD analysis; the association of vitamin E was similar with a 9% reduction in risk of CLD (HR 0.91, 95% CI: 0.81-1.03).

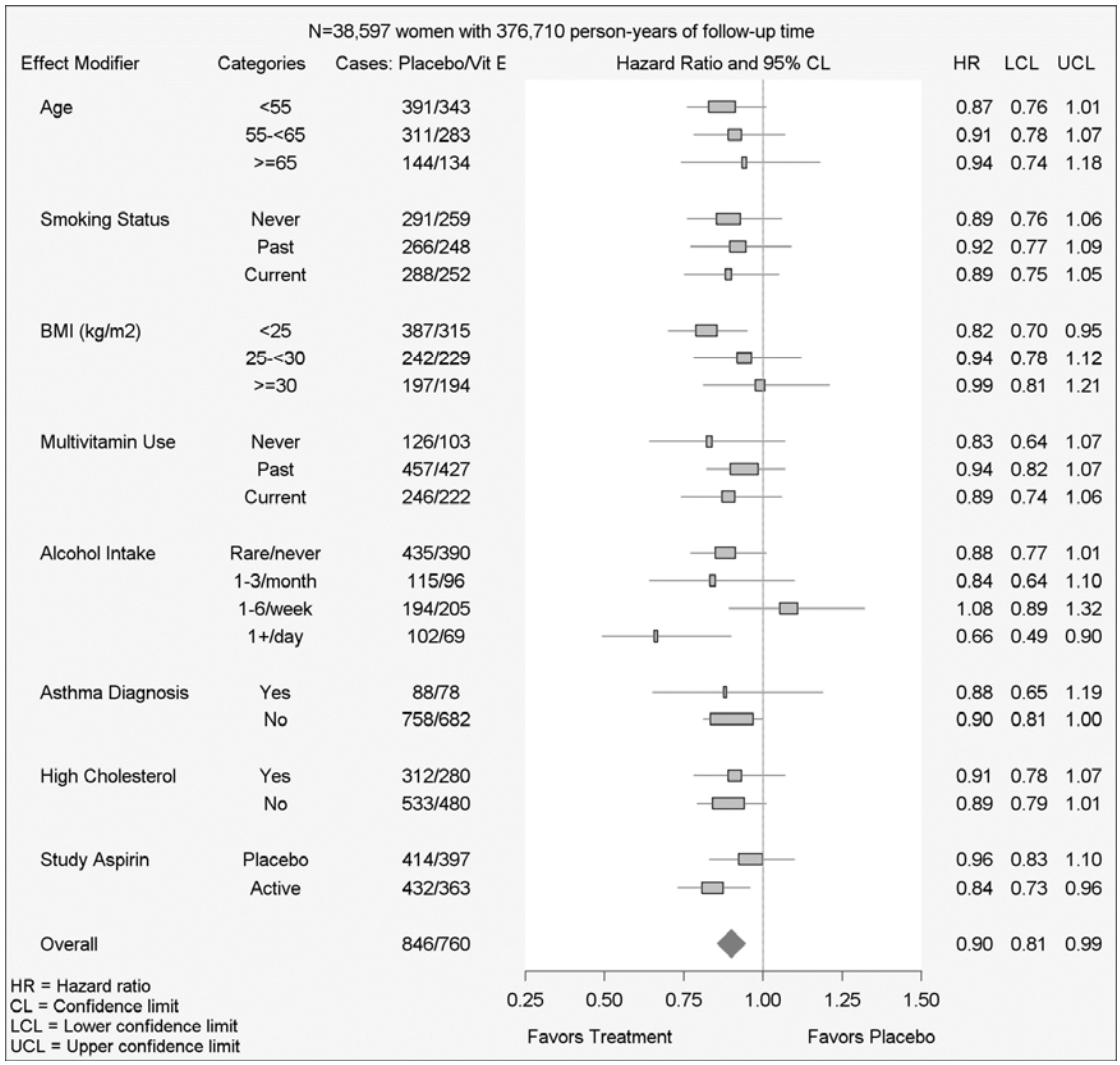

Figure 2.3 Effect modification of the vitamin E/chronic lung disease effect in the Women's Health Study. BMI, Body mass index.

Discussion

In this large, randomised, double-blind, placebo-controlled trial in apparently healthy women 600 IU of vitamin E on alternate days reduced the risk of self-reported newly diagnosed chronic lung disease by approximately 10%. There was no statistically significant difference in the magnitude of the effect of vitamin E by age, smoking status, randomised aspirin assignment,

multivitamin use, or asthma history, and the protective effect was slightly stronger in women consuming ≥ 1 drink/day. Randomised assignment to aspirin had no association with the risk of CLD.

Two prior randomised trials investigated vitamin E supplementation in relation to lung outcomes, and both reported no effect of intervention.(16;17) In the Heart Protection Study (HPS) 20,536 adults aged 40-80 with prevalent coronary artery disease (CAD) were randomised to a combined intervention of 600 IUs of vitamin E, 250 mg vitamin C, and 20 mg β-carotene or placebo daily for 5 years.(17) HPS differed from the WHS in important ways. HPS enroled participants with clinically diagnosed CAD, occlusive arterial disease, or diabetes and 75% of participants were men.(17) WHS comprised women without cardiovascular disease history. Study duration (5 years in HPS vs. 10 yrs in WHS) and supplement formulation (HPS, combined supplement vs. WHS, vitamin E alone) also differed. Finally, the outcomes in HPS, pulmonary function measured by spirometry, death due to respiratory illness, and COPD or asthma-related hospitalizations differed from the WHS, which investigated incidence of chronic lung disease diagnosis.(17)

In the Alpha-tocopherol Beta-carotene Cancer Prevention Study (ATBC) 29,133 male cigarette smokers aged 50-69 were randomised to receive 50 mg vitamin E and/or 20 mg β-carotene or placebo daily for 4 years.(16) ATBC differed substantially from the WHS in the supplement studied, the trial duration and the population studied.(16) ATBC reported no effect of vitamin E on COPD-related symptoms, a substantially different endpoint than incidence of chronic lung disease diagnosis.

Strengths of the WHS include the large number of participants, the large number of self-reported, MD-diagnosed CLD outcomes, high adherence

rate, and high follow-up rate. CLD is associated with ageing, thus the minimum age required for study enrolment, 45 years, yielded a population at risk for incident CLD risk.

Several limitations deserve mention. While the size of this trial was adequate to detect a statistically significant small to moderate effect of vitamin E supplementation on incident CLD, the trial was not specifically designed to test the studied hypothesis. Thus, outcome ascertainment was based solely on self-reported MD-diagnosis, a concern that is partly mitigated by the fact that participants were female health professionals. A validation study of self-reported COPD outcomes in female nurses found that 78% of self-reported COPD cases were confirmed with medical record review, suggesting self-report of lung disease by the female health professionals comprising the WHS is likely to have excellent validity.(20) While the question about outcome occurrence listed bronchiectasis, the low incidence of bronchiectasis in this age range leads to the reasonable assumption that most occurrences reported refer to COPD. Current cigarette smoking was a strong predictor of CLD in these data, providing evidence of face validity for the outcome ascertainment. Finally, if the outcome is misclassified (either by undercounting cases or by including false positives) the misclassification is likely to affect both arms of the trial equally, and the hazard ratio may therefore be an underestimate of the true effect size.

Outcome Definition

The complexity of airway disease phenotypes raises substantial concern about misdiagnosis of COPD and asthma, particularly in women.(21) To address the possibility that women reporting a new asthma diagnosis actually had COPD, a sensitivity analysis was conducted. When women

reporting incident asthma were excluded from the analysis, vitamin E was associated with a 9% reduction in the risk of CLD (HR 0.91, 95% CI: 0.81-1.03), similar to the findings in the full study group. There was little or no effect of vitamin E supplementation on incident self-reported MD-diagnosed asthma (HR 0.99; 95% CI 0.90-1.08; p=0.83). Kurth et al (2008) investigated the effect of randomized aspirin assignment on the risk of incident adult-onset asthma in the Women's Health Study; women in the aspirin arm had a 10% lower risk of incident asthma compared to women in the placebo arm.(22) When women developing chronic obstructive pulmonary disease over follow-up were censored from the analysis, the findings were similar. The effect of aspirin on incident asthma, and the lack of effect of aspirin on incident CLD supports the notion of differentiation in the self-reported diagnoses.(22)

Proposed Mechanisms

Prior studies document the presence of vitamin E in the lung compartment and the mechanisms of delivery of vitamin E to alveolar type II cells in the lung.(23) Vitamin E transport to the type II cells is hypothesised to occur via high-density lipoproteins because type II cells have no physical contact with plasma and interact only with interstitial fluid lipoproteins, which are predominantly HDL lipoproteins.(23) Thus, the concentration of HDL cholesterol in the plasma and in the interstitial fluid predicts the amount of vitamin E available to the lung compartment to combat oxidative stress. Prior studies have reported that HDL cholesterol and apolipoprotein A-I levels are positively associated with FEV_1, even after adjusting for serum antioxidant concentrations, a finding that may reflect the delivery of vitamin E to lung tissues.(24) Thus, a higher HDL cholesterol level is hypothesised to deliver a greater effective dose of vitamin E to the lung compartment.

Given that HDL cholesterol levels are 20-25% higher in women compared to men in all age groups, differences in the biologically effective dose of vitamin E may contribute to the difference in findings between women in WHS and the predominantly male participants in the HPS and ATBC.(25) Sex-related differences in the effect of vitamin E supplementation on all-cause mortality have been reported, with stronger protective effects of low-dose vitamin E supplementation evident in study populations comprised of ≥75% women.(26)

Alcohol intake is proposed to reduce CVD risk by raising HDL cholesterol levels, and a threshold of $\geq$ 1 drink per day is associated with both higher HDL cholesterol levels and attenuation in CVD risk.(27;28) Given the important role of HDL cholesterol in transporting vitamin E to the lung, exploratory analyses considered alcohol intake as a modifier of the effect of vitamin E on CLD. Among variables considered as possible effect modifiers, alcohol intake was marginally statistically significant (p=0.054), and the preventive effect of vitamin E on CLD was greatest in women consuming the highest level of alcohol ($\geq$ 1 drink/day). These preliminary findings are consistent with the hypothesis that delivery of vitamin E to lung tissue may vary by plasma levels of HDL cholesterol.

Under our hypothesis, the effect of antioxidant supplementation was expected to be stronger in participants with a higher oxidative burden, for example, in current cigarette smokers. Contrary to expectation, there was no evidence of effect modification by smoking. If supplementation with vitamin E acts through other systemic mechanisms, for example by improving immune system function, then a general effect of vitamin E would be supported.(29)

Efficacy and Safety Considerations

There has been substantial discussion of the efficacy and safety of vitamin E supplementation in the scientific literature.(30) Potential harmful effects include an increased risk of all cause mortality, susceptibility to bleeding, and haemorrhagic stroke.(31-33) The meta-analysis linking high-dose vitamin E supplementation to increased risk of mortality, however, has been criticised for its methodology and a recent paper suggested that vitamin E has beneficial effects on ischemic stroke risk.(33-35) Thus, the design of future vitamin E supplementation trials must carefully consider information about risks and benefits, and recommendations may need to be tailored to specific populations.

Summary and Conclusion

The WHS was comprised of female health professionals aged $\geq$ 45, the majority of whom were of European descent. Healthy women taking 600 IU vitamin E supplements every other day were 10% less likely to report a new chronic lung disease diagnosis during the study period. Any decisions about use of vitamin E as a preventive must consider information about vitamin E associated risks and bioavailability.(26;30;31;34;35) Given that there are few prevention strategies for emphysema and chronic bronchitis, further study of vitamin E in relation to chronic obstructive pulmonary disease is of public health interest.

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Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.

Ethics Approval

The Women's Health Study was approved by the institutional review board of Brigham and Women's Hospital and monitored by an external data and safety monitoring board.

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CHAPTER 3

EFFECT OF SUPPLEMENTARY NUTRIENTS WITH ANTIOXIDANT PROPERTIES ON PLASMA AND AIRWAY LINING FLUID IN CIGARETTE SMOKERS

Abstract

Background: Consequent to the oxidant stress imposed by smoking, the human airway epithelium marshals both endogenous and exogenous antioxidant defenses. When these defenses are breeched oxidative stress causes cellular damage, contributing significantly to lung tissue damage and the development of COPD. Prior epidemiologic studies demonstrate a direct association between serum markers of nutrients with antioxidant properties and lung outcomes, but no studies have investigated the degree to which supplementation affects nutrient levels in the lung compartment. We hypothesized that oral supplementation with nutrients would alter the lung compartment levels of the nutrients and lower a systemic marker of oxidative stress.

Methods: A placebo controlled, double-blinded study was conducted to assess the effect of 1 month of daily supplementation with vitamin E (400 IU), vitamin C (1000 mg) and selenium (400 µg), or matched placebo, on plasma and bronchioalveolar lavage fluid (BALF) nutrient levels in active healthy smokers. Participants (n=24) were randomized 2:1 (vitamin: placebo), and plasma and BALF were obtained at baseline and 1 month post-supplementation. Nutrients and a marker of oxidative stress were assayed in plasma and BALF via GC/MS, ICP-MS and ELISA methods.

Results: Supplementation increased plasma levels of all nutrients ($p<0.002$, all comparisons pre *vs* post) in participants in the intervention group; levels were unchanged in participants on placebo (all $p \geq 0.172$), with the exception of selenium, which declined slightly ($p=0.01$). Vitamin E and selenium were assayed in BALF, and both nutrients increased with supplementation for the intervention group only ($p<0.02$, for changes in intervention group). F2-isoprostanes, an oxidative stress biomarker, were not significantly reduced by the antioxidant intervention.

Conclusions: Vitamin supplementation increased lung levels of both vitamin E and selenium, thus confirming the delivery of these nutrients to target tissue. While supplementation had no overall effect on a systemic biological marker of oxidative stress, only two participants had pre-supplementation evidence of high oxidative stress. Thus there was limited opportunity to show changes in oxidative stress levels in this study.

Introduction

Chronic obstructive pulmonary disease (COPD), is characterized by airflow limitation that is not fully reversible.(1) The disease progresses insidiously, and by the time the patient becomes symptomatic and the COPD diagnosis is made, lung damage is usually significant.(2-4) COPD comprises a significant health burden, and is predicted to become the 3rd leading cause of death worldwide by 2020.(1;5) Cigarette smoking is the most important risk factor for the development of COPD, with current or former smokers comprising about 90% of patients who develop the disease.(6) Smoking presents a huge oxidant burden to the airways, and each puff of cigarette smoke contains 10^{14} free radicals.(7) When the antioxidant defenses of the airways are overwhelmed, oxidative stress causes cellular damage, contributing significantly to lung tissue damage and the development of COPD.(8;9)

Whereas the lung airway epithelium and the airway epithelial lining fluid have endogenous antioxidant defenses, exogenous antioxidants in the diet also contribute to protecting the lung epithelium from oxidant stress.(10-13) Key nutrients with antioxidant properties include vitamin E, vitamin C and selenium. Lower intake of nutrients and lower serum nutrient levels are associated with lower lung function in both smokers and nonsmokers.(14-20) A seminel study of participants in the Third National Health and Nutrition Examination Survey (NHANES III) found that higher dietary intake of vitamins E and C and serum levels of vitamins E and C, and selenium were positively associated with lung function assessed by spirometry.(21) The joint effect of dietary antioxidant intake was higher than the effect when nutrients were

considered individually, suggesting a synergistic relation among antioxidants in the protection of the lung.(21)

Studies comparing COPD patients to healthy individuals report lower plasma and peripheral skeletal muscle vitamin E (α-tocopherol) concentrations in patients, and a lower risk of death from respiratory disease with higher serum α-tocopherol concentration, but whether nutrition contributes to the onset of COPD is less clear.(15;22;23)

In light of past findings, supplementation of the antioxidant defenses of the lung with exogenous nutrients with antioxidant properties is a logical intervention strategy that is hypothesized to protect the lung from the oxidant stress of smoking. To test this hypothesis, a placebo controlled, double-blinded study was conducted to assess the effect of 1 month of supplementation (daily vitamin C, vitamin E and selenium) or placebo on plasma and bronchioalveolar lavage fluid (BALF) levels of nutrients in active healthy smokers. The study investigated the following hypotheses: (1) supplementation with nutrients raises not only plasma nutrient levels, but also nutrient levels in the lung epithelial lining fluid as assessed by BALF; and (2) increasing antioxidant capacity through exogenous sources reduces a systemic marker of oxidative stress, signaling a potential mechanism through which nutrients act directly in the lung compartment.

Methods

Study Design

Healthy smokers were recruited from the general population through advertisements in the press and on the world-wide web. Participants were evaluated at the Weill Cornell NIH General Clinical Research Center and

Department of Genetic Medicine clinical research facility under protocols approved by the Weill Cornell Medical College Institutional Review Board. All subjects were current smokers by history, with active smoking confirmed by urine metabolites and venous carboxyhemoglobin. Participants were determined to be phenotypically normal on the basis of history, physical examination, routine blood screening tests, urinalysis, chest imaging, electrocardiogram, and pulmonary function testing. All individuals were HIV-negative and had normal alpha1-antitrypsin levels. Inclusion criteria included: good health with no history of chronic lung disease, including asthma and without recurrent or recent acute pulmonary disease, not pregnant, willingness to participate in the study and ability to provide informed consent, no use of antioxidant dietary supplements in the prior 3 months, no allergies to medications used in the brochoscopy procedure. Exclusion criteria included: inability to meet inclusion criteria, current active infection or acute illness of any kind, alcohol or drug abuse within the past 6 months, and evidence of malignancy within the past 5 years. Individuals meeting inclusion/exclusion criteria underwent assessment at baseline for the smoking and clinical variables listed above.

Participants were randomized 2:1 to receive either nutrient supplements (daily vitamin C 1000 mg, vitamin E 400 IU and selenium 400 µg) or matched placebos for one month. All participants were evaluated at study baseline and 1 month after supplementation; evaluations included a blood draw, pulmonary function test, questionnaires, and bronchoscopy to sample lung epithelial lining fluid and the airway epithelium. Compliance was encouraged with a mid-study telephone call and participants were asked to bring their empty pill cases to

the second clinic visit so that unused supplements could be counted and recorded.

Dietary Nutrient Intake Assessment

At randomization participants completed a 12-page food frequency questionnaire (FFQ) to assess usual dietary intake. The FFQ was developed by the Nutrition Assessment Shared Resource (NASR) of the Fred Hutchinson Cancer Research Center (FHCRC). The FFQ included questions on 103 foods and 17 beverages, plus 12 questions on food preparation and 2 questions on fruit and vegetables consumption patterns. The dietary intake of more than 130 nutrients was determined using the Nutrient Data System for Research (NDSR), developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, Food and Nutrient Database (2009 versions of software and database).

Supplements

Vitamin C supplements were provided by NOW Foods (Bloomingdale, IL) and contained ascorbic acid in a formulation with cellulose, croscarmellose sodium, stearic acid and magnesium stearate; matched placebo contained additional cellulose in place of ascorbic acid, but was otherwise identical. In two batches of ascorbic acid supplements the manufacturer's reported contents of active ingredient were 1094.1 mg/tablet and 1122.5 mg/tablet.

Vitamin E supplements were provided by Roche Vitamins (Nutley, NJ) and contained all-*rac*-α-tocopherol suspended in vegetable oil; matched placebos contained only vegetable oil. Roche Vitamins did not provide detailed certificates of analysis for the supplements.

Selenium supplements were provided by Sabinsa, Inc. (Piscataway, NJ) and contained L-selenomethionine and white powder filler; matched

placebos contained only the white powder filler. Sabinsa, Inc. provided certificates of analysis of the supplement content and dose. Average content of the selenomethionine supplements was 215.0 μg per capsule.

The vitamin E and selenium supplements were initially supplied through the pharmacy of The Selenium and Vitamin E Cancer Prevention Trial (SELECT) and then sourced directly though the suppliers. Vitamin E and vitamin C supplements were independently analyzed for content and dosage by Craft Technologies, Inc. (Wilson, NC). Vitamin C supplements from two lots were analyzed and average ascorbic acid contents were 1160 mg/tablet and 1142 mg/tablet, respectively. Vitamin E supplements from two lots contained 547 IU/softgel and 521 IU/softgel, respectively. All supplements and placebo were provided free of charge. Supplements were packaged into 30-day pill cases by the Human Metabolic Research Unit, Cornell University, and labeled for use by the clinical center.

Biologic Specimen Collection

At baseline and one month after active supplementation or placebo, specimens of plasma, urine, large airway epithelium and lung epithelial lining fluid (BALF) were collected.

For plasma levels of nutrients, 50 ml of blood was collected in heparinized tubes (BD Vacutainers, NJ), centrifuged at 1000 g for 30 min at 4°C, aliquoted into cryovials and frozen at -80°C for subsequent analysis. To preserve vitamin C, a 0.7 ml aliquot was added to a cryogenic vial containing 0.7 ml of pre-cooled 10% meta-phosphoric acid solution then immediately frozen at -80°C.

Lung BALF was collected by bronchoalveolar lavage, using 5 x 30 ml aliquots into the segmental bronchus of the right middle lobe.(24) The

bronchoalveolar lavage fluid (BALF) recovered was filtered through gauze to remove debris and mucus and then centrifuged at 1,200 rpm for 5 min at 4°C. The supernatant was separated into multiple aliquots and stored at -80°C. The absolute amount of BALF collected was assumed to be approximately the same on repeated bronchoscopies in the same participant, and thus correction for dilution was not applied.(25-27)

Nutrient Assay Methods

Supplemental methods are in the Appendix, Supplemental Methods A.1.

Vitamin C

Ascorbic acid concentration in plasma samples was determined by HPLC. Briefly, 50 μl of supernatant was mixed with a reducing reagent then incubated. Samples were then acidified and an internal standard was added. Analysis by HPLC with UV detection at 245 nm was conducted. Three known-value serum standards (0.41 μg/ml ± 11.1%, 1.94 μg/ml ± 2.66%, and 14.1 μg/ml ± 1.63%) were concurrently measured in duplicate to assess assay accuracy. Precision was assessed by determining the average coefficient of variation, across all assay runs, which was 5.14% for the six measurements of the three standards. Vitamin C measurements were completed by Craft Technologies (Wilson, NC).

In a recent study, vitamin C supplementation increased ascorbic acid concentrations in nasal lavage fluid for only a few hours post-supplementation, and levels returned to pre-supplementation values within 24 hours of last supplement use.(12;28) Given that the current study did not control the timing of supplementation relative to BALF collection, vitamin C was not assessed in BALF.

Vitamin E

Alpha-tocopherol concentration in plasma and BALF samples was determined by GC-MS. Briefly, for each sample a δ9-α-tocopherol internal standard was added then lipids were extracted with organic solvents and the upper layer was transferred to a vial. The samples were evaporated to dryness under a nitrogen stream in a water bath and the resulting residues were silylated in pyridine with N,O-bis[trimethylsilyl]trifluoroacetimide (Thermo Scientific, Rockford, IL). The vials were tightly capped with nitrogen filling the headspace and placed in a 75ºC dry oven for 25 minutes. For determination of α-tocopherol in the high density lipoprotein (HDL) fraction of the plasma, HDL was separated from the plasma using an HDL cholesterol precipitating reagent (Stanbio, Boerne, TX) prior to the standard sample preparation. α-Tocopherol concentrations in plasma, HDL, and bronchioalveolar lavage fluid samples were determined by GC-MS, using a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 6890 mass spectrometer. Samples were assayed in duplicate and average coefficients of variation were determined for total α-tocopherol and for cholesterol-adjusted total α-tocopherol (where cholesterol was measured with the GC-MS method), respectively, with values as follows: plasma total α-tocopherol CVs were 4.2% and 2.4%, for total and cholesterol-adjusted, respectively; HDL α-tocopherol CVs were13.2% and 4.0%, for total and cholesterol-adjusted, respectively; bronchioalveloar lavage fluid α-tocopherol CVs were 11.6% and 6.2% for total and cholesterol-adjusted, respectively. Vitamin E assays were completed in the Division of Nutritional Sciences, Cornell University, Ithaca, NY.

Selenium

Plasma samples and four reference standards were diluted 20-fold with 2% HNO_3. An Agilent 7500 cs/ce quadrupole ICP-MS equipped with a collision reaction cell was used to determine total selenium concentration in plasma samples. The ion intensity at *m/z* 78 was used to monitor Se and ^{89}Y was used as an internal standard for plasma samples. Plasma sample data were collected using the *Spectrum* mode for direct analysis. Two reference standard solutions, 1 ppb and 5 ppb, were run at regular intervals throughout sample testing and average concentrations were determined to be 1.002 ppb and 5.117 ppb, respectively with standard deviations of 0.023 and 0.073. All four reference materials were determined to have values within the range of acceptable results for the material.

BALF samples were evaporated and reconstituted in 2% HNO_3 spiked with 1ppb Ge to achieve a 5-fold concentration of the original solution. The ion intensity at *m/z* 78 was used to monitor Se and ^{72}Ge was used as an internal standard for BALF samples. BALF sample data was collected using the transient mode and data were individually calculated for each assay. A reference solution containing 1 ppb of Ge and Se in 2% HNO_3 was analyzed at regular intervals throughout sample testing. The BALF was primarily saline solution (i.e., highly dilute BALF) and the sodium contained within the BALF samples interfered with detector response to Ge and Se ions. Interference was quantitated and adjusted by assessing Ge and Se response in the reference solution and comparing it to the reduced response in the samples that were reconstituted in the Ge-spiked solution. Selenium assays were performed by the USDA Plant, Soil, and Nutrition Laboratory, Cornell University, Ithaca, NY.

F2-isoprostane Assay Methods

Plasma F_2-Isoprostanes(29;30)

Briefly, to a 1-3 ml plasma aliquot 1.0 ng of [$^2H_4$]-15-F_{2t}-IsoP ([$^2H_4$]-8-iso-PGF_{2a} (Cayman Chemical, Ann Arbor, MI) was added, serving as an internal standard. The sample was purified using Sep-Pak cartridges and the eluate was collected, dried down and converted to the pentafluorobenzyl (PFB) esters by the addition of 40 µl of a 10% solution of pentafluorobenzyl bromide in acetonitrile and 20 µl of a solution of 10% diisopropylethanolamine in acetonitrile, incubated, then dried under nitrogen. The residue was reconstituted in 30 µl chloroform and 20 µl methanol and the PFB esters and methyl ester of $PGF_{2\alpha}$ were purified in separate lanes by thin layer chromatography. Compounds migrating in the region 1 cm below the $PGF_{2\alpha}$ standard to 1.0 cm above the standard are scraped from the TLC plate, extracted with 1 ml ethyl acetate, and dried under nitrogen. The resulting compounds are converted to trimethylsilyl (TMS) ether derivatives by addition of 20 µl *N,O*-bis(trimethylsilyl)trifluoroacetamide and 10 µl dimethylformamide, incubated then dried under nitrogen. The residue was re-dissolved for GC/MS analysis in 20 µl undecane.

Gas chromatography-negative-ion chemical ionization mass spectrometry (GC/NICI-MS) was carried out on an Agilent 5973 Inert Mass Selective Detector coupled with an Agilent 6890n Network GC system (Agilent Labs, Torrance, CA) interfaced with an Agilent computer. The lower limit of detection of F_2-IsoPs is in the range of 4 pg using an internal standard with a blank of 3 parts per thousand. The precision of this assay in biological fluids is $\pm$6% and the accuracy 94%. Plasma F2-isoprostane measurements were

performed by the Eicosanoid Core Laboratory, Vanderbilt University Medical Center, Nashville, TN.

Urine F2-isoprostanes

Urine was collected using a “clean catch” method into sterile urine collection containers. Urine samples were pipetted into cryovials in 2 ml aliquots then immediately frozen at -80^{o}C. Thawed samples were centrifuged to remove particulates, then 1 ml aliquots were added to microcentrifuge tubes. Repeated sorbent washing procedures achieved initial purification of the specimens and the washed sorbent was then re-suspended in 0.5 ml 95% ethanol (elution buffer) (Fisher Scientific, Pittsburgh, PA) and briefly vortexed. The sample was then centrifuged and the elution buffer was removed and saved. The re-suspension was repeated and the elution buffer washes were all combined and dried down overnight on a speed vacuum. Dried samples were then dissolved in 1 ml of EIA buffer (Cayman Chemicals, Ann Arbor, MI).

F2-isoprostanes in urine samples and 8 standard samples prepared by serial dilution were measured using a competitive enzyme linked immunoassay (ELISA) kit from Cayman Chemicals (Ann Arbor, MI). The tracer and antiserum were each reconstituted with 6 ml of EIA buffer. The standard protocol for plate set up and development provided by Cayman Chemicals was used. The plate was read by spectrophotometry at 405 nm wavelength after 60 minutes and 90 minutes of development.

Control samples were run on each plate and the overall coefficient of variation was 18.7%. Additionally, standard curve correlation values were calculated at two time points and the average R^2 value was 0.9911 at each time point with average R^2 standard deviation of 0.0040.

Urine Creatinine

Creatinine in urine samples was measured on a Siemens Dimension Xpand Plus using standard methods recommended by the manufacturer.

Plasma Cholesterols and Triglycerides Assay Methods

Total, HDL, and low density lipoprotein (LDL) cholesterol and triglycerides were determined using chemiluminescence under standard methods on a Siemens Dimension Xpand, a Centers for Disease Control and Prevention certified instrument. Average within-run and between-run coefficients of variation for the analytes were as follows: total cholesterol, 0.71% and 1.94%; HDL cholesterol, 0.36% and 4.20%; LDL cholesterol, 0.48% and 3.10%; and triglycerides, 0.28% and 2.12%. Two control samples, Biorad Liquid Assayed Multiqual Level 1 and Level 3, were assayed and all values for the lipids were determined to be within the acceptable range.

Urine F2-isoprostanes, urine creatinine, and plasma cholesterol and triglyceride assays were conducted in the Human Metabolic Research Unit, Cornell University, Ithaca, NY.

Data Analysis

All analyses were carried out in SAS 9.2 (Cary, NC). Univariate methods were used to inspect all data, and to make comparisons to known ranges. The pre-post association of nutrients was assessed with a paired t-test—given non-independence of the samples—for plasma and BALF nutrients and oxidative stress markers. All data were plotted for visual inspection of trends. Next, the association of change in plasma compartment with change in lung compartment was assessed with the Pearson product moment correlation coefficient, separately by treatment group, and the magnitude of the effect and variance explained was estimated with ordinary

least squares linear regression. In regression models of change in BALF nutrient predicted by change in plasma nutrient, effect modification was tested by adding product terms to the model. For example, to assess whether the association of BALF changes with plasma changes in each nutrient differed by gender, the gender by plasma change product term was added to the model.

Results

Participant Characteristics

A total of 26 healthy smokers were included in the study; 16 participants received the 'active' intervention, which was a combination of vitamin E, selenium and vitamin C (active intervention group) and 8 participants received matched placebos (placebo group). Principal component analysis of the plasma nutrient levels for all participants' revealed two non-compliant participants in the active group; all plasma nutrient values for these participants at the post-supplementation time point were in the placebo range, thus they were excluded from further consideration and all remaining analyses are based on 24 participants (Appendix Figure A.1).

All participants had normal general physical examinations and no significant prior medical history. Smoking status was confirmed by urine nicotine and cotinine levels. There were no differences between the active and placebo group with regard to gender, ethnicity, pack-year history, urine nicotine and cotinine, venous blood carboxyhemoglobin levels, and pulmonary function tests ($p>0.05$ for all comparisons, Table 3.1).

Table 3.1 Demographics of the study population and descriptive characteristics of biologic measurements

Variable*	Placebo (N=8)	Active (N=16)
Demographic characteristics		
Sex (male; female)	8; 0	13; 3
Age (years)	42.4 (11.2)	43.5 (6.9)
Race (B: W; O)†	3; 4; 1	10; 2; 4
Smoking characteristics		
Smoking history (pack-yr)	31.2 (12.9)	31.1 (16.5)
Urine nicotine (ng/ml)		
Baseline	1760 (3082)	2041 (1636)
Post-intervention	1493 (681)	2239 (1703)
Urine cotinine (ng/ml)		
Baseline	1421 (1263)	2095 (1295)
Post-intervention	1179 (481)	2007 (1221)
Pulmonary function‡		
FVC (% predicted)	107 (13)	110 (13)
FEV1 (% predicted)	107 (14)	104 (14)
FEV1/FVC (%)	82 (6)	77 (6)
TLC (% predicted)	100 (14)	102 (13)
DLCO (% predicted)	99 (14)	94 (13)
Dietary nutrient intake§		
Alpha-tocopherol (mg/day)	13.3 (7.5)	17.9 (12.9)
Vitamin C (mg/day)	125.3 (161.0)	192.9 (174.2)
Selenium (μg/day)	168.4 (51.5)	210.8 (129.4)

* mean (standard deviation) unless otherwise indicated
† B = black, W = white, O = other
‡ FVC - forced vital capacity, FEV_1 - forced expiratory volume in first second of exhalation, TLC - total lung capacity, DLCO - diffusing capacity
§ Dietary nutrient intakes estimated from Food Frequency Questionnaire; placebo n=7, active n=14

Response to Intervention with Antioxidant Supplements

In the main analysis, the mean nutrient levels pre- and post-intervention were assessed, as well as the mean of within-individual change, for both the active and placebo groups (Table 3.2). For participants in the active intervention arm of the study the increases in plasma nutrient levels were statistically significantly for all three nutrients over the 30-day intervention. Plasma α-tocopherol increased 69.4% ($p < 0.0001$, for paired t-test comparing concentration at t_0 to concentration at t_1). Plasma selenium increased 78.0%

Table 3.2 Concentrations of supplemented nutrients and oxidant stress biological marker at baseline of study (t_0) and at 1 month post-supplementation (t_1) for 24 participants in intervention study

Nutrients and Metabolites	Placebo (N=8)				Active (N=16)			
	t_0*	t_1*	Change** (%)	P value	t_0*	t_1*	Change** (%)	P value
Vitamin E								
Plasma total α-toc (cholesterol adjusted); μmol α-toc/mmol cholesterol	12.1 (3.4)	13.6 (1.2)	1.5 (21.3)	0.3094	13.1 (3.3)	21.6 (6.9)	8.6 (69.4)	<0.0001
HDL α-toc; μmol α-toc/mmol cholesterol in HDL	11.8 (2.7)	13.3 (4.3)	1.5 (12.0)	0.1722	12.7 (3.4)	20.5 (6.2)	7.8 (67.1)	<0.0001
Lung BAL α-toc†; nmol α-toc/L	24.2 (12.8)	19.5 (4.8)	-4.7 (-5.8)	0.2648	23.9 (10.6)	36.1 (21.3)	12.2 (64.6)	0.0173
Selenium								
Plasma total selenium; μg Se/L plasma	197.3 (7.3)	188.6 (7.6)	-8.8 (-4.4)	0.0101	193.3 (33.7)	337.9 (65.9)	144.6 (78.0)	<0.0001
Lung BAL selenium; μg Se/L	0.47 (0.12)	0.49 (0.10)	0.02 (5.1)	0.1613	0.41 (0.10)	0.57 (0.13)	0.16 (46.8)	0.0001
Vitamin C								
Plasma ascorbic acid; μg/ml	6.5 (5.7)	7.7 (5.1)	1.2 (116.1)	0.5701	8.4 (3.7)	13.5 (4.5)	5.0 (107.3)	0.0016
F2-isoprostanes								
Plasma F2-isoprostanes‡; pg/ml	0.069 (0.026)	0.057 (0.025)	-0.006 (-5.48)	0.7358	0.066 (0.05)	0.045 (0.02)	-0.018 (-17.4)	0.0876
Urine F2-isoprostanes§; pg/mg creatinine	352.2 (254.8)	324.3 (148.7)	2.43 (22.83)	0.9683	365.8 (168.6)	338.9 (109.7)	-54.33 (-3.7)	0.2099

*Mean (SD)
**average of within-individual change
†7 in placebo group
‡15 in active
§6 in placebo, 14 in active

($p < 0.0001$) and plasma vitamin C increased 107.3% ($p = 0.0016$). HDL α-tocopherol increased 67.1% ($p < 0.0001$). In contrast, in the placebo arm, plasma α-tocopherol increased by 21.3% and plasma selenium declined by 4.4%. Although the slight decrease in selenium was statistically significant ($p = 0.01$) it was in the opposite direction to the supplemented group; measurement precision was excellent in the selenium assays, contributing to this small magnitude of change reaching thresholds of statistical significance. In the placebo group, plasma vitamin C increased by 116.1%, but this result was not statistically significant ($p = 0.57$) and was entirely driven by one person whose plasma vitamin C levels increased dramatically over the 30 days. When the outlier was omitted from the analysis, plasma vitamin C increased an average of 5.3% for all other placebo arm study participants ($p = 0.35$).

In the intervention arm of the study, lung vitamin E and selenium levels increased over the 30-day period of supplementation. α-Tocopherol in BALF increased 64.6% ($p = 0.0173$) and selenium increased 78.0% ($p < 0.0001$). In contrast, there was little or no change in lung nutrient levels in the placebo arm: BALF α-tocopherol decreased 5.8% ($p = 0.2648$) and selenium increased 5.1% ($p = 0.1613$).

Plasma F2-isoprostanes were assayed as a biological marker of oxidative stress, and assays were completed in both plasma and urine samples from study participants. In the active intervention arm of the trial, over the 30-day period of supplementation, plasma F2-isoprostane levels decreased 17.4%, and the decrease was borderline statistically signficant ($p = 0.0876$) and urinary F2-isoprostanes declined 3.7% ($p=0.2099$). Although neither change reached the threshold for statistical significance, the plasma result supports the hypothesis that supplementation reduces systemic markers

of oxidative stress. In the placebo arm of the study plasma F2-isoprostanes declined 5.48% while urinary F2-isoprostanes increased 22.83%, though neither result was statistically significant ($p = 0.7358$ and $p = 0.9683$, respectively).

To examine the consistency of the average change, individual participant data were plotted for plasma total vitamin E, HDL vitamin E, plasma selenium and plasma vitamin C (Figures 3.1 to 3.4). For all nutrients measured in plasma the active intervention group showed substantial increases in nutrient levels after supplementation, though individual variability is apparent, particularly for α-tocopherol. In the placebo group slight variations in concentrations can be seen at the two time points, particularly for α-tocopherol and ascorbic acid, with selenium showing the least variability over time. One participant on placebo had a sharp increase in plasma ascorbic acid concentration over the 30 day supplementation period, contrary to expectation given the participant was assigned to the placebo arm. Indeed, the pattern of change is similar to changes in participants in the active arm, raising the possibility of non-compliance in this participant.

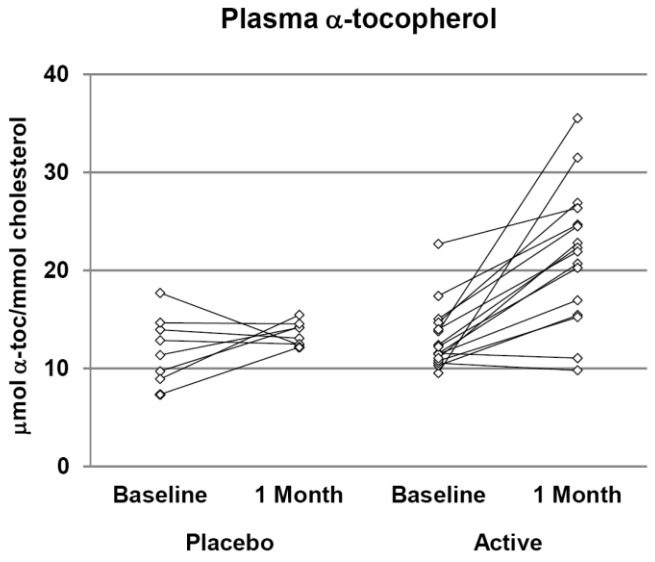

Figure 3.1 Change in plasma α-tocopherol (adjusted for total plasma cholesterol) from baseline to one month for individual study participants.

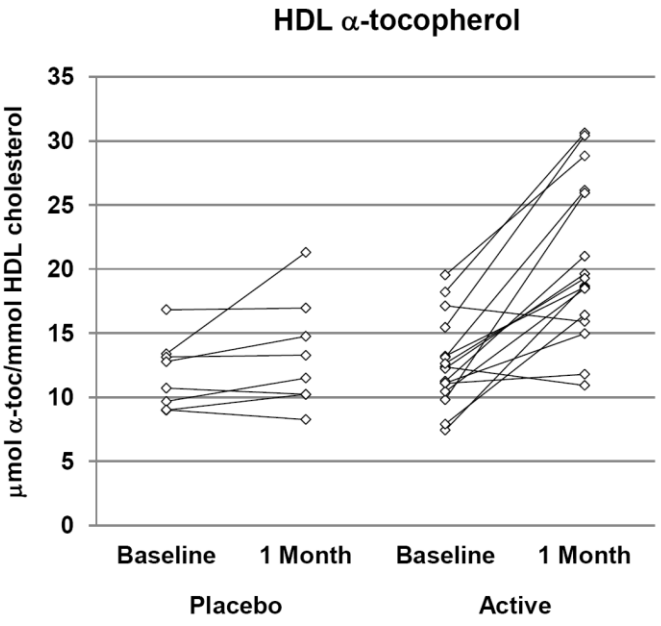

Figure 3.2 Change in high density lipoprotein fraction (HDL) α-tocopherol (adjusted for cholesterol in HDL fraction) from baseline to one month for individual study participants.

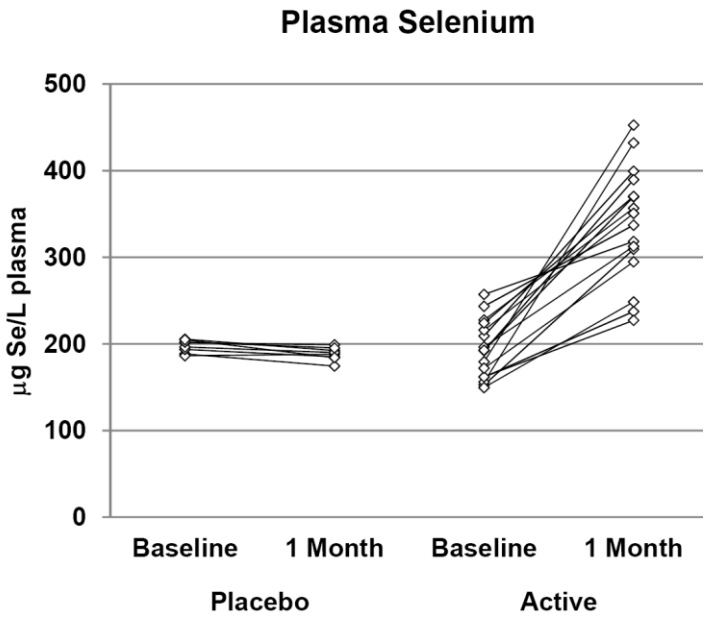

Figure 3.3 Change in plasma selenium from baseline to one month for individual study participants.

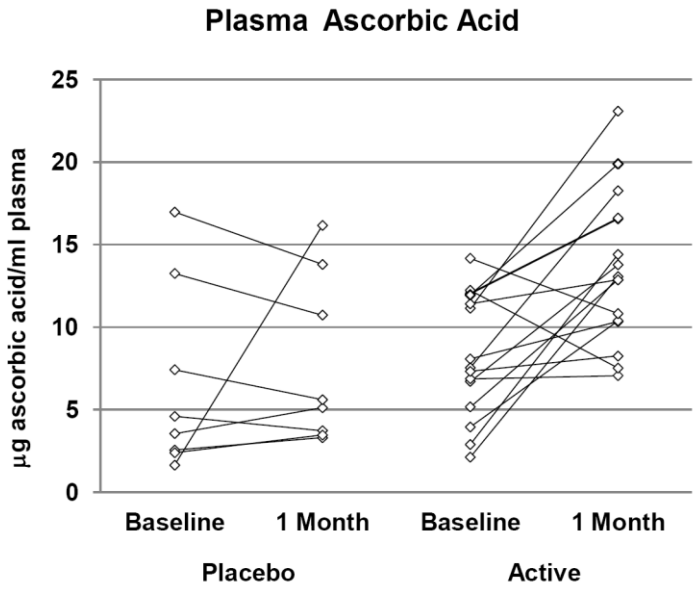

Figure 3.4 Change in plasma ascorbic acid from baseline to one month for individual study participants.

To assess the relation of change in nutrient in the plasma compartment with change in nutrient in the lung compartment (BALF), the pre-post change in nutrients in the BALF was assessed (Figures 3.5 and 3.6). Lung α-tocopherol concentrations increased during the 30-day intervention in most participants in the active supplement arm of the trial and a few participants had marked increases in concentration. In the placebo arm of the trial there was little or no change in α-tocopherol concentrations in the lung over the 30 day supplementation period.

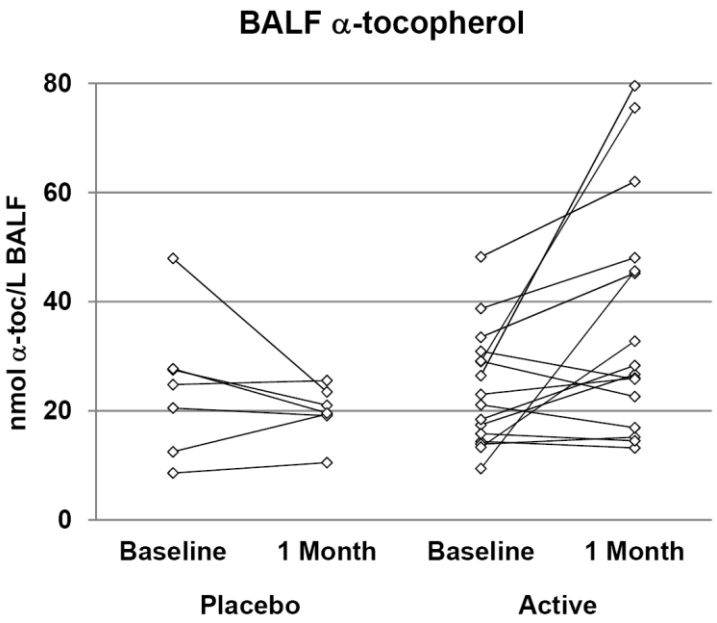

Figure 3.5 Change in BALF α-tocopherol from baseline to one month for individual study participants.

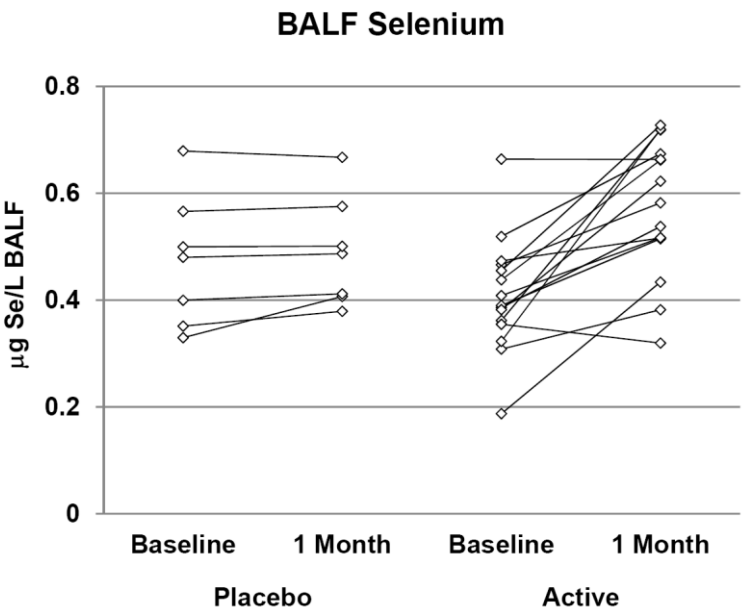

Figure 3.6 Change in BALF selenium from baseline to one month for individual study participants.

The correlation between the change in plasma and the change in BALF nutrients was estimated (Figures 3.7, 3.8, and 3.9). Changes in cholesterol-adjusted total plasma α-tocopherol (μmol α-tocopherol/mmol cholesterol) and BALF α-tocopherol (nmol α-tocopherol/L BALF) were moderately correlated, but the correlation was not statistically significant ($p = 0.1487$; Figure 3.7). Plasma HDL α-tocopherol (μmol α-tocopherol/mmol HDL cholesterol) and BALF α-tocopherol (nmol α-tocopherol/L BALF) were strongly and statistically significantly correlated ($p = 0.0024$; Figure 3.8). Plasma (μg Se/L plasma) and BALF (μg Se/L BALF) selenium were also strongly and significantly correlated ($p = 0.0057$; Figure 3.9). Further consideration of non-parametric models, for example the Spearman rank-order correlation, did not substantially alter these findings.

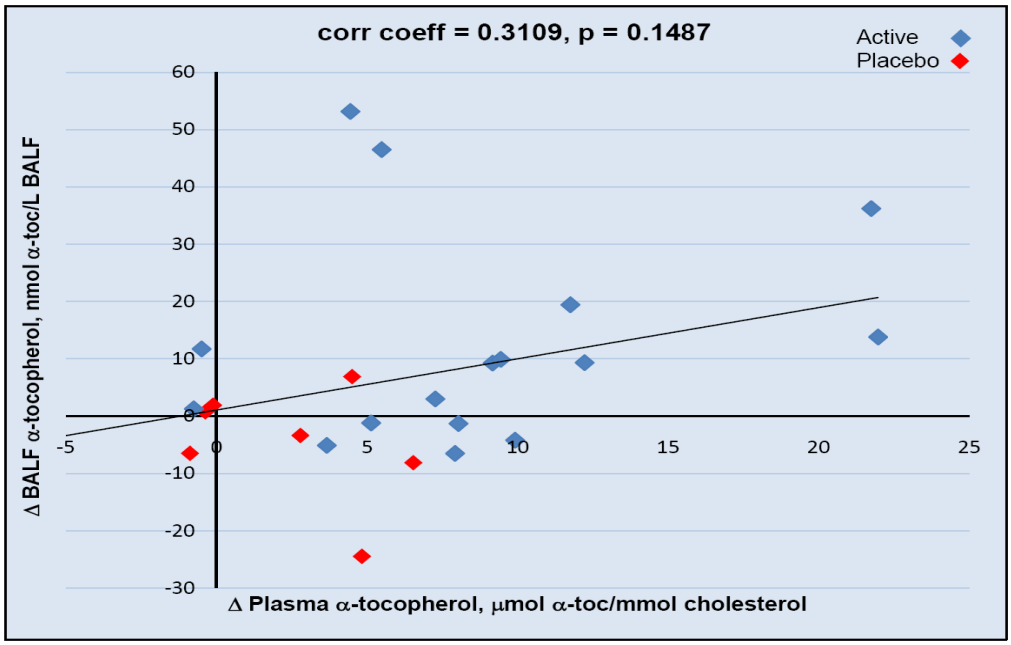

Figure 3.7 Correlation of changes in plasma α-tocopherol (adjusted for total plasma cholesterol) and BALF α-tocopherol concentrations (where change = $\alpha\text{-toc}_{1\ month} - \alpha\text{-toc}_{baseline}$).

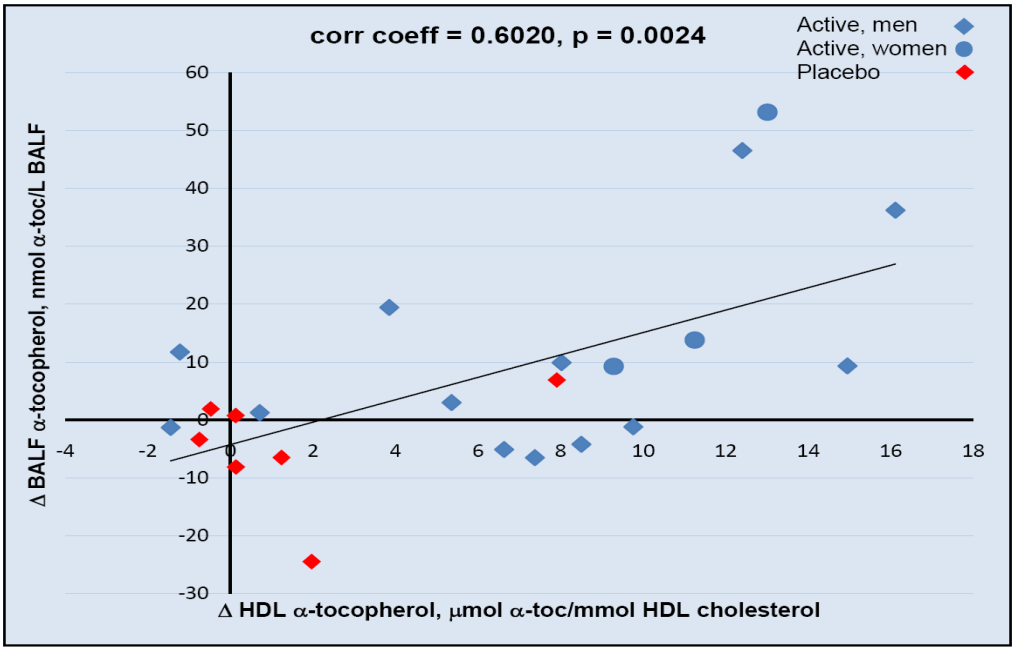

Figure 3.8 Correlation of changes in high density lipoprotein fraction (HDL) α-tocopherol and BALF α-tocopherol concentrations (where change = $\alpha\text{-toc}_{1\ month} - \alpha\text{-toc}_{baseline}$).

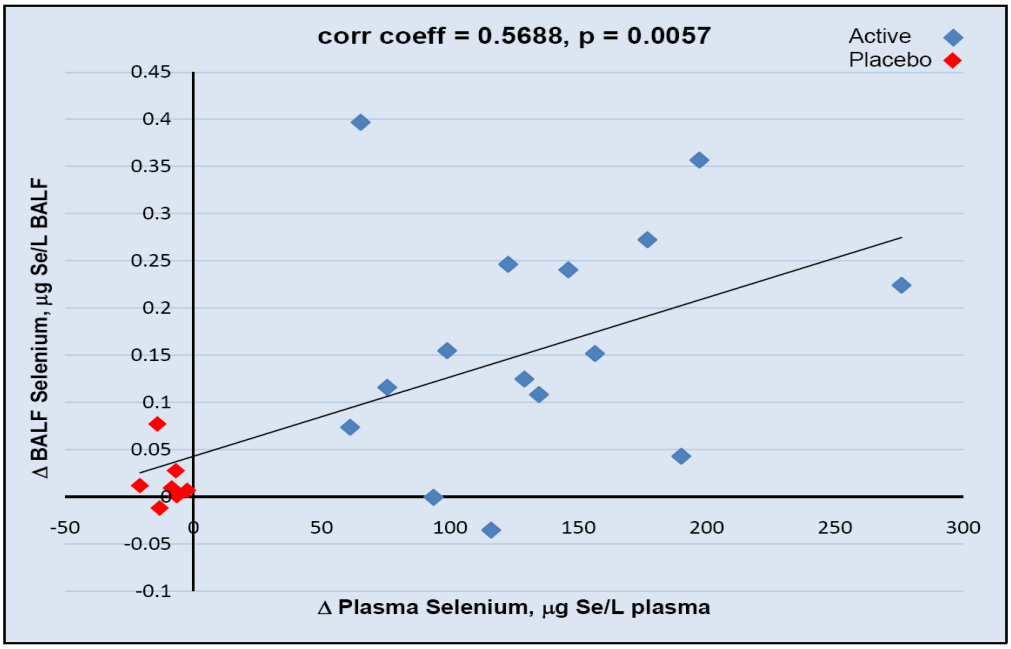

Figure 3.9 Correlation of changes in plasma selenium and BALF selenium concentrations (where change = $Se_{1\ month} - Se_{baseline}$).

In regression models, the change in lung nutrient concentration was regressed on the change in plasma nutrient to quantify the extent to which changes in the systemic circulation compartment predicted changes in the lung compartment (Table 3.3). A one-unit increase in the change in plasma α-tocopherol predicted a 0.89 unit increase (p = 0.1487) in the change in BALF α-tocopherol and 9.7% of the change in BALF α-tocopherol was predicted by the change in plasma α-tocopherol. Given the theory that HDL is the primary lipid penetrating to the lung compartment, we assessed the association of change in HDL α-tocopherol with change in BALF α-tocopherol; change in HDL α-tocopherol explained 36.2% of the change in BALF α-tocopherol and a one-unit increase in the change in HDL α-tocopherol was associated with an increase of about 2 in the change in BALF α-tocopherol (p = 0.0024).

Extending the model, we tested for a differential association of plasma HDLα-tocopherol change with BALF α-tocopherol change by gender. Thus, an interaction term was added to the model (change in HDL α-tocopherol x gender). The regression coefficient for the interaction was borderline statistically significant (p = 0.0678); in men a one-unit increase in change in HDL α-tocopherol predicted a 1.6 unit increase in change in BALF α-tocopherol; in women a one-unit increase in change in HDL α-tocopherol predicted an 11.6 unit increase in change in BALF α-tocopherol.

The association of change in plasma selenium with change in BALF selenium was statistically significant (p = 0.0057) and change in plasma selenium predicted 32.4% of the change in the BALF selenium. A one-unit increase in change in plasma selenium was associated with an 8.4×10^{-4} unit increase in change in BALF selenium, with the size of the coefficient reflecting the overall low concentrations of selenium in BALF.

Table 3.3 Ordinary least squares regression analysis of the change in BALF nutrient regressed on change in plasma nutrient for 16 participants in the active intervention group*

Nutrient	**Regression coefficient**	**Standard Error**	**P value**	**% R-squared**
Plasma total α-tocopherol	0.89	0.596	0.1487	9.7
HDL α-tocopherol	1.94	0.559	0.0024	36.2
Selenium	0.0008	0.0003	0.0057	32.4

*****Unadjusted models

Potential to Benefit

Further exploration of potential to benefit from supplementation was considered by using the median split to divide the participants in the active intervention arm into categories of "high" or "low" baseline values with respect to each of the three nutrients under consideration. Cross-classifying the three nutrients, six participants had low pre-supplementation plasma levels for $\geq$ two nutrients. All six participants were also in the upper half of the distribution on plasma and/or urine F_2-isoprostanes, and 3 of them were of particular interest given their very high F_2-isoprostane levels at study baseline (shown by red lines on Figures 3.10 and 3.11). Plasma and urine F_2-isoprostanes were considered separately because they were only moderately correlated ($r = 0.24$; $p = 0.31$), reflecting the tissue-specific generation of F_2-isoprostanes.(31)

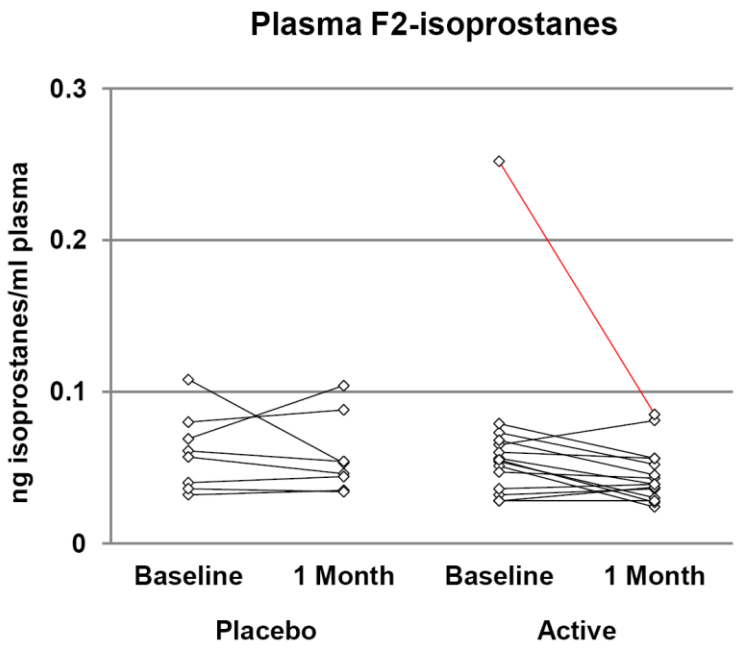

Figure 3.10 Change in plasma F_2-isoprostanes during 30-day intervention; red line indicates greatest potential to benefit from antioxidant supplementation.

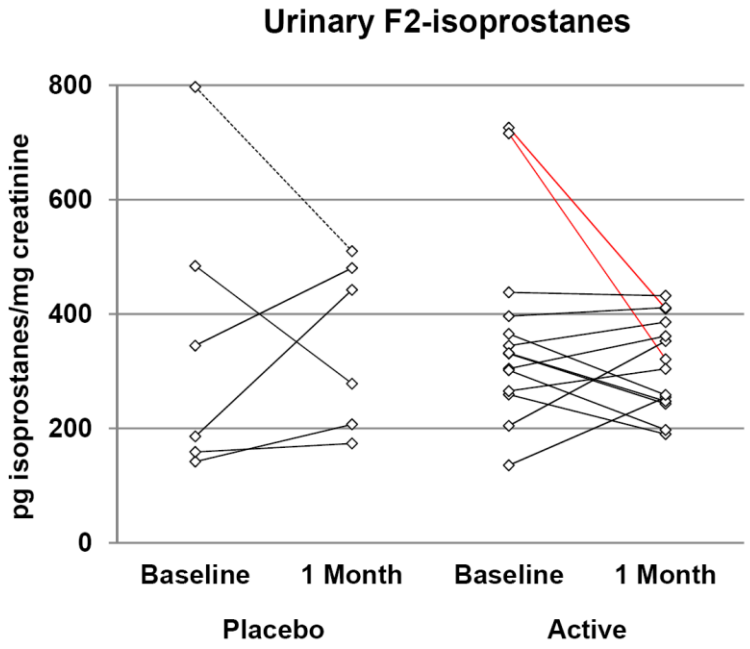

Figure 3.11 Change in urinary F_2-isoprostanes during 30-day intervention; red line indicates greatest potential to benefit from antioxidant supplementation; dashed line indicates a non-compliant placebo participant with a sharp increase in vitamin C intake.

The participant with high starting plasma F_2-isoprostane concentrations (0.252 ng isoprostanes/ml plasma vs. baseline average for all participants 0.064 ng isoprostanes/ml plasma) had the most change with antioxidant supplementation; a 66.3% *decrease* in plasma F_2-isoprostane concentrations was observed.

The two active arm participants with the highest starting concentrations of urine F_2-isoprostanes are also low on plasma antioxidants measured at study baseline. Urine isoprostanes decreased 43.6% and 55.2% with antioxidant supplementation, supporting the hypothesis that participants with

the highest starting level of oxidants have the greatest potential to benefit from intervention.

Discussion

In this small, in-depth study of antioxidant supplementation and systemic and lung-specific compartmentalization of antioxidants, supplementation with vitamins E and C and selenomethionine increased plasma concentrations of all three nutrients and lung concentrations of α-tocopherol and selenium (lung vitamin C was not assayed). The change in plasma selenium was strongly associated with the change in lung selenium, and explained about 32% of the variance in the former. Changes in plasma HDL α-tocopherol concentrations had a strong association with change in lung α-tocopherol concentrations, whereas the change in plasma α-tocopherol was a poor predictor of lung compartment changes; change in plasma HDL α-tocopherol explained four times the variance in the outcome compared to plasma α-tocopherol. This finding agrees well with cell culture and animal studies, which identified HDL cholesterol as the primary transport molecule delivering α-tocopherol to alveolar type II cells in the lung.(32-34) The association was 10-fold stronger in women vs. men, but this finding is based on small number (n=3 women in sample), thus caution is appropriate in considering whether a sex-specific mechanism may be at play in vitamin E transport to the lung.

Prior work suggests that vitamin E supplementation decreases systemic oxidative stress, as represented by F_2-isoprostane concentrations, only when the baseline urine concentrations of the analyte are $\geq$ 400 pg/mg creatinine.(35) In considering this hypothesis, only 3 participants in the current

study had starting levels in the range that identified a stronger potential to benefit. Exploratory analyses confirmed that these 3 participants each had $\geq$ 2 nutrient values that were also low, perhaps indicating a contributing factor to the high oxidant stress load, although cross-sectional data at the baseline preclude a definitive cause-effect statement. Finally, participants with very high starting levels of F_2-isoprostanes, in either plasma or urine, benefitted the most from antioxidant supplement intervention, as evidenced by the greatest pre-post change in F_2-isoprostane.

Several prior studies reported α-tocopherol concentrations in BALF, and there is good agreement with the values reported herein; BALF α-tocopherol concentrations in these studies ranged from 20-26 nmol/L, similar to study baseline and substantially lower than the 36.1 nmol/L concentration attained by active intervention arm participants at the post-supplementation time point.(12;13;36;37) Although one prior supplementation study reported no change in BALF α-tocopherol after supplementation, participants were supplemented with only 100 mg/day of supplementation for one week and no information on baseline BALF α-tocopherol concentration measurements was provided, thus the study design was weak.(12) No prior studies report selenium concentrations in BALF, thus this study adds normative values and novel data on the response of the lung compartment to supplementation.

Tissue-specific data are useful in assessing the extent to which the active substance reaches the target, and in association with less invasive markers may also help identify the most informative systemic biomarkers. Very few studies directly assess tissue-level changes in response to interventions, thus this study is novel in the direct measurement of the lung-compartment changes in nutrient status in response to supplementation of nutrients with

antioxidant properties. The increases in lung nutrient concentrations post-supplementation provide support for the proposed role of dietary and supplemental nutrients with antioxidant properties in lung disease pathogenesis.

Several limitations of this study deserve mention. Firstly, the study was one month long, and a longer intervention period may have yielded larger changes in plasma and BALF nutritional status, particularly for selenium which incorporates into the body's tissue slowly, and is not expected to reach a steady state for 10-12 weeks.(38;39) However, trade-offs in participant retention and length of supplementation had to be balanced and the decision to supplement for one month was considered optimal. The assessment of compliance to study supplement was incomplete, and fewer than 50% of study participants returned the pill case at the end of the study; in all instances, returned cases indicated good to excellent adherence to the supplementation regimen. While it would have been optimal to assay a biomarker of oxidative stress in the lung compartment, for example via exhaled breath condensates, limited resources and subject burden issues prohibited such measurements. Finally, we omitted the two participants who were completely non-compliant to study supplementation from the active arm of the study; although this violates the intent-to-treat principle, in light of the small overall number of participants the "as-treated" analysis is assumed to be less noisy and we were most interested in not missing an effect if one was present.

A number of strengths of this study also deserve mention. Quantitative methods to measure α-tocopherol and selenium were sensitive, accurate and precise. Measurement of plasma **and** lung concentrations of nutrients before and after intervention is a substantial strength of this study and a novel

contribution to the literature in this area. The consideration of potential to benefit provided additional insights into the findings, and while too small to be definitive these analyses help set the stage for further research questions.

In conclusion, oral supplementation with vitamins E and C and selenomethionine leads to increases in the concentrations of nutrients in the plasma and in the lung compartment. The change in HDL α-tocopherol is strongly correlated with the change in BALF α-tocopherol, which provides interesting insights into the transport mechanisms for vitamin E. This study provides evidence to support the hypothesis that supplemental antioxidants may directly protect the lung tissue from oxidative stress, thereby supporting interventions with antioxidants as a means to reduce disease risk.

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CHAPTER 4

DIFFERENTIAL EXPRESSION OF VITAMIN E AND SELENIUM-RESPONSIVE GENES BY DISEASE SEVERITY IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Abstract

***Background*:** Antioxidant nutritional status has been shown to influence chronic obstructive pulmonary disease (COPD) susceptibility and progression. Prior studies document the relation of vitamin E and selenium to gene expression, but there are no data on the specific role of these genes in COPD. We hypothesized that antioxidant status is compromised in patients with COPD in a dose-response pattern with increasing disease severity, and that genes responsive to nutrients with antioxidant function are differentially expressed in a similar pattern.

***Methods*:** Lung tissue samples from COPD patients with varying disease severity were assayed for vitamin E and gene expression, and selenium and vitamin E were determined in corresponding plasma samples. Participants were grouped by disease severity into: 1. moderate, severe, very severe COPD; or, 2.mild COPD and at-risk/normal. Gene expression profiles were compared to identify differential gene expression by group, considering all microarray data in a discovery analysis. Further hypothesis-oriented analyses investigated gene lists as follows: genes differentially expressed in COPD, genes identified by GWAS of COPD, and genes differentially expressed in relation to vitamin E or selenium.

***Results*:** Vitamin E concentrations in the lung tissue of 22 COPD patients were strongly associated with disease stage, and moderate/severe/very severe COPD cases had lower tissue vitamin E levels compared to the mild/at-risk group ($p = 0.0082$); neither plasma vitamin E nor plasma selenium concentrations differed by disease group. In the discovery phase, considering genome-wide array data, no genes were statistically significantly differentially expressed by disease group. In the hypothesis-oriented phase, 16 of the 109 genes investigated were differentially expressed by COPD disease severity group (comparing moderate/severe/very severe COPD group to the mild COPD/at-risk group), with false discovery rate (FDR) q-values < 0.05. Of these 16 genes, six were selenium-responsive genes; four genes were down-regulated (fold-change range: -1.18, -1.39) and two genes were up-regulated (fold-change range: 1.54, 2.25). Six vitamin E-responsive genes were identified; five genes were down-regulated (fold-change range: -1.16, -2.30) and one gene was up-regulated (fold-change: 1.51). Three genes with prior evidence of a relation to COPD were confirmed; two genes were down-regulated (fold-changes: -1.26 and -1.88) and one gene was up-regulated (fold-change: 1.52). A single gene identified as a COPD susceptibility gene in completed GWAS was up-regulated (fold-change: 1.21).

***Conclusions*:** Vitamin E concentration in the lung tissue of COPD patients varied with disease severity and vitamin E-responsive genes were differentially expressed by disease severity. Overall, these findings suggest a causal mechanism for epidemiologic findings, support the hypothesis that targeted nutritional intervention may have therapeutic value, and underscore the importance of lung compartment, tissue-specific antioxidant levels in understanding the disease process.

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 by 2020 COPD is expected to become the third leading cause of death in the United States.(2-4) 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 U.S. in 2000.(5;6)

The primary risk factor for COPD is cigarette smoking, however, substantial evidence points to genetic and genomic risk factors for disease susceptibility and progression.(1;7) Investigations of genetic risk factors for COPD have been conducted using candidate gene studies, genome-wide association studies (GWAS), and gene expression studies.(7) Candidate gene studies and GWAS identify genetic variants associated with COPD risk, and provide clues about mechanisms, and gene expression studies, either genome-wide or candidate-gene, provide complemetary insights into COPD pathogenesis by directly studying the proteome in lung tissue.(7) Gene expression profiling has led to advances in identification of causal mechanisms, identification of potentially informative biomarkers, and new approaches to the classification of disease phenotypes.(8) COPD is a complex chronic disease and new approaches to phenotype classification, beyond measuring pulmonary function, will lead to a deeper understanding of the disease process and ultimately to the identification of novel therapeutic and intervention targets. Recent data show that gene discovery improves when the association of quantitative phenotypic variables with gene expression is assessed in tandem with identifying differential gene expression by disease

category.(8) Information about genetic variation, both at the level of the gene sequence and gene expression, has provided important insights into COPD etiology.(9-22)

An imbalance between oxidants and antioxidants is hypothesized to play an important role in COPD pathogenesis.(23-25) Oxidative stress in the lungs of COPD patients has been demonstrated in many studies.(26-28) Published studies also support an important role for antioxidants, which defend the lung to prevent damage resulting from oxidative stress arising in the context of both endogenous and exogenous oxidants.(28-31) Furthermore, COPD patients have lower antioxidant status compared to non-diseased individuals, and further declines in antioxidant status are associated with acute exacerbations of COPD.(32;33)

The importance of the oxidant/antioxidant balance in COPD susceptibility and pathogenesis is well-established. Complementary research evidence has identified genes responsive to manipulation of antioxidant nutrients.(9;34-46) 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 antioxidant status is compromised in patients with COPD in a dose-response pattern with increasing disease severity, and that genes related to antioxidant function are differentially expressed in COPD by level of disease severity.

Methods

Participants and Specimens

Plasma, frozen lung tissue, and RNAlater-preserved lung tissue specimens were obtained from the Lung Tissue Research Consortium (LTRC), a National Heart, Lung, and Blood Institute-sponsored tissue bank (http://www.ltrcpublic.com). The LTRC collects demographic, radiographic, and physiologic information from COPD or interstitial lung disease (ILD) patients undergoing surgical treatment including lung volume reduction surgery or lung lobectomy/wedge resection surgery. De-identified data and preserved specimens are available to qualified investigators. For this study, twenty-four sample sets were obtained from patients with a major diagnosis of COPD/emphysema, and patient disease status was staged using the Global Obstructive Lung Disease (GOLD) 2001 Initiative guidelines.(47) Gold stage 0 indicates no obstructive lung disease based on pulmonary function testing, but the presence of respiratory symptoms, and is associated with the risk of mortality.(48) Stages 1 through 4 correspond to levels of severity of COPD (Table 4.1) based on objective pulmonary function testing. Although stage 0 indicated persons at-risk of COPD in the 2001 guidelines, recently revised guidelines omit stage 0 given a growing consensus that most individuals do not progress to stage 1. Similarly, there is debate about potential over-diagnosis of COPD using current stage 1 definitions, which use a cut-point for the ratio vs. using the lower limit of normal, and based on these considerations combined with inspection of the characteristics of patients in the stage 1 group, stages 0/1 (indicating absent to mild disease) were combined and compared with stages 2/3/4 (stages 2,3, and 4 indicate clear-cut cases with increasing severity) in all analyses.

Table 4.1 Global Obstructive Lung Disease Classifications (49)

Category	0*	1: mild	2: moderate	3: severe	4: very severe
FEV_1/FVC	> 0.70	< 0.070	< 0.070	< 0.070	< 0.070
FEV_1 %predicted	≥80%	≥80%	50% ≤ and <80%	30% ≤ and <50%	<30% or <50% plus the presence of chronic respiratory failure
Number in study	5	3	4	5	5

*in original GOLD guidelines, stage 0 was defined as "at-risk of COPD", and identified as normal spirometry with ≥ 1 respiratory symptom (breathlessness, cough, and/or sputum production), but 2005 revised guidelines omitted this stage due to uncertainty about progression to next stages.

This study was reviewed by the Institutional Review Board (IRB) of Cornell University, Ithaca, NY. Exempt status was conferred by the IRB based on the de-identification of all samples and specimens provided by the LTRC.

Tissue Nutrient Status Determination

Gas Chromatography Mass Spectrometry (GC-MS) Determination of α-tocopherol

Plasma Total α-tocopherol Sample Preparation

Briefly, 3 μl of a δ_9-α-tocopherol internal standard (99 μmol/L stock solution) was added to 75μl of plasma. 150 μl of ethanol was added, then vortexed. To extract lipids 75 μl of methyl-*tert*-butylether and 1.0 ml hexane were added and sample was shaken for 2 minutes. Samples were centrifuged at 15,000 rpm for 1 minute then the upper hexane phase was transferred to a small vial. The hexane was evaporated to dryness under a nitrogen stream and in a 30°C water bath. The residues were silylated in 40 μl pyridine (Thermo Scientific) and 40 μl N,O-bis[trimethylsilyl]trifluoroacetimide (Thermo

Scientific). The headspace was flushed with nitrogen then samples were tightly capped and placed in a 75°C dry oven for 25 minutes.

High Density Lipoprotein (HDL) α-tocopherol Sample Preparation

20 μl of HDL cholesterol precipitating reagent (Stanbio) was added to 150 μl of plasma and allowed to stand for 5 minutes, then centrifuged at 15,000 rpm for 1 minute. 75 μl of supernatant was transferred to a tube; then the procedure for plasma total α-tocopherol was followed.

Lung Tissue α-tocopherol Sample Preparation

A 30-70 mg piece of lung tissue was sub-sampled from a frozen lung tissue sample without thawing. The frozen sample was weighed in a tared culture tube prior to addition of 2 ml isopropanol, 5 μl of a δ_9-tocopherol internal standard (99 μmol/L stock solution), and 1 ml 0.9% saline solution. The contents of the tube were then homogenized for 15-20 seconds using a Polytron homogenizer. The resulting suspension was transferred to a screw cap tube. The homogenizing tube was rinsed with 1 ml methyl-*tert*-butylether and homogenized for 5-10 seconds. The rinse solvent was added to the screw cap tube, followed by addition of 3 ml hexane. The tube was shaken for 2 minutes then centrifuged at 15,000 rpm for 2 minutes. The solvent phase was transferred to a new tube, concentrated to 1 ml under a nitrogen stream, transferred to a 1.5 ml screw cap vial, and evaporated to dryness under nitrogen in a 30°C water bath. The lipid residue was silylated in 40 μl pyridine (Thermo Scientific) and 40 μl N,O-bis[trimethylsilyl]trifluoroacetimide (Thermo Scientific). The headspace was flushed with nitrogen then samples were tightly capped and placed in a 75°C dry over for 25 minutes.

GC-MS Determination of Tocopherols

Alpha-tocopherol concentrations in plasma (total and HDL) and lung tissue samples were determined by GC-MS, using a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 6890 mass spectrometer. Tocopherols were resolved isothermally at 280°C on an HP-1 capillary column (Agilent Technologies) operated in split injection mode with helium as the carrier gas. Detection was by selected ion monitoring (SIM mode). Tocopherol and free cholesterol (as silyl ethers) were quantitated against the δ_9-α-tocopherol internal standard, adjusting the cholesterol values for pre-determined differences in detector response.

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). HDL α-tocopherol was measured in single samples due to sample volume limitations. The typical CV for the HDL method is 13.2%, and 4.0% after adjusting for cholesterol (as measured within the GC-MS method). Lung tissue samples were run on single samples due to limited tissue sample amounts, and α-tocopherol concentrations were not adjusted for cholesterol due to heterogeneity of lung tissue samples.

All vitamin E assays were completed (by AHA) in the Division of Nutritional Sciences, Cornell University, Ithaca, NY.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Determination of Selenium

Plasma Selenium Sample Preparation

A 2% nitric acid solution was prepared using concentrated nitric acid from a Teflon sub-boiling still and double-deionized water, resistivity > 18 MΩ

cm. Plasma samples were prepared for selenium analysis by adding 5 ml of 2% HNO_3 to 100 μl of plasma (thawed at room temperature). Two reference standard solutions, 1 ppb and 5 ppb, were run at regular intervals throughout sample testing and average concentrations were determined to be 1.002 ppb and 5.117 ppb, respectively with standard deviations of 0.023 and 0.073, respectively. Seronorm Trace Elements, Serum, Levels I and II (Accurate Chemical & Scientific Corp., Westbury, NY) and two NBS standard samples were used as reference materials for method validation and assessment of accuracy and precision. Seronorm and NBS samples were prepared in the same way as the plasma samples and run at regular intervals throughout sample testing. All four reference materials were determined to have values within the range of acceptable results for the material.

ICP-MS Determination of Total Plasma Selenium

An Agilent 7500 cs/ce quadrupole ICP-MS equipped with a collision reaction cell was used to determine selenium concentration in plasma samples. The ion intensity at *m/z* 78 was used to monitor Se and ^{89}Y was used as an internal standard for plasma samples. Data was collected using the *Spectrum* mode for direct analysis. Maximum sensitivity was obtained by daily tuning of the ion lens system, gas flow rates, and other parameters. Samples were run manually with continuous quality monitoring. Table 4.2 summarizes the operating conditions and instrumental parameters.

Sample preparation and analysis was performed at the USDA Plant, Soil & Nutrition Laboratory, Cornell University, Ithaca, NY.

Table 4.2 Inductively Coupled Plasma Mass Spectrometry (ICP-MS) operating conditions and instrumental parameters for plasma selenium determination

Parameter	Value
RF power	1400 Watts
Sample uptake rate	2.5 ml/min
Reaction mode	He or H_2, 5 ml/min, *Spectrum* mode
Gas flow rates:	
Plasma gas	Ar,
Auxillary gas	0 L/min
Carrier gas	Ar, 0.8 L/min
Makeup gas	Ar, 0.45 L/min
Ion sampling depth	8 mm
Torch	Quartz 2.5 mm id fitted with Agilent ShieldTorch system
Nebulizer	Microliter nebulizer, Microglass (Cedar Ridge, CO)
Sample cone	Pt, orifice diameter 1.0 mm
Skimmer cone	Pt, orifice diameter 0.4 mm
Spray chamber	Double cyclonic and water cooled Scott type
Integration time	0.2000 sec
Sampling period	0.6000 sec
Sweeps per reading	40-60

Chemiluminescence Determination of Plasma Cholesterols and Triglycerides

Total, HDL, and LDL cholesterol and triglycerides were determined using chemiluminescence under standard methods on a Siemens Dimension Xpand, a Center for Disease Control and Prevention-certified instrument. Average within-run and between-run CVs for the analytes were as follows: total cholesterol, 0.71% and 1.94%; HDL cholesterol, 0.36% and 4.20%; LDL cholesterol, 0.48% and 3.10%; and triglycerides, 0.28% and 2.12%. Two control samples, Biorad Liquid Assayed Multiqual Level 1 and Level 3, were assayed and all values for the lipids were determined to be within the acceptable range.

Cholesterol and triglyceride measurements were performed in the Francis Johnston and Charlotte Young Human Metabolic Research Unit, Cornell University, Ithaca, NY.

Determination of Gene Expression in Lung Tissue Samples

Extraction of RNA from Lung Tissue Samples

Frozen RNAlater preserved lung tissue samples were thawed at room temperature prior to excising 30 mg subsamples, which were added to tared Eppendorf tubes that were then re-weighed to determine exact sample mass. With the samples on ice at all times 0.9 ml aliquot of TRIzol was added to each tube prior to homogenization with a Kontes Pellet Pestle Motor with a disposable Pellet Pestle. Homogenization was complete when large tissue pieces were no longer visible, typically within 15 seconds. Samples were incubated for 5 minutes at room temperature.

To the homogenized samples, 0.2 ml of choloroform was added then the sample were capped, shaken vigorously by hand for 15 seconds, and allowed to incubate at room temperature for 2-3 minutes. Samples were centrifuged at 10,000 x g for 10 minutes. The upper aqueous phases were transferred to RANse-free 1.5 ml Eppendorf tubes; the remaining interphase and phenol-choloroform phase was retained and stored at -20°C. To the aqueous phases 500 μl of 100% ethanol was added then tubes were capped and gently inverted 4-5 times. For each sample, a 700 μl aliquot was added to an RNeasy MinElute spin column placed atop a 2 ml collection tube (RNeasy MinElute Cleanup Kit, Qiagen) and the column was capped and centrifuged at 14,000 x g for 15 seconds. The flow-through from the collection tube was discarded and the remaining volume of the aqueous phase/ethanol mixture was added to the column, again centrifuging the column and discarding the

flow-through from the collection tube. 500 μl of Buffer RPE was pipetted onto the spin column (RNeasy MinElute kit, Qiagen) and the column was centrifuged at 14,000 x g for 15 seconds, with flow-through discarded. 500 μl of 80% ethanol was added to the spin column, which was then centrifuged at 14,000 x g for 2 minutes followed by discarding of the flow-through and the collection tube. The RNeasy MinElute spin column was placed in a new 2 ml collection tube with the column lid open and then centrifuged at maximum speed for 5 minutes. The flow-through and collection tube were discarded. The column was transferred to a new RNAse free 1.5 ml Eppendorf tube and 18 μl of 1X RNASecure (Ambion) was pipetted onto the gel membrane of the column. The column was centrifuged for 1 minute at maximum speed.

The resulting sample volumes were assessed and recorded. RNA eluate aliquots of 1 μl from each sample were added to new, labeled RNAse free 1.5 ml Eppendorf tubes containing 2 μl of DEPC-treated water. Additional 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 1 patient and one from a GOLD 2 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).

Microarray Preparation and Processing

Using Affymetrix kits (Santa Clara, CA) beginning with the GeneChip One-Cycle cDNA Synthesis Kit, 3 mg of total RNA was used to synthesize double stranded cDNA, followed by cleanup with GeneChip Sample Cleanup

Module, *in vitro* transcription (IVT) reaction using GeneChip IVT Labeling Kit, and clean-up and quantification of the biotin-labeled cDNA yield 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.(50) 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.

RNA extraction and microarray preparation and processing were conducted at Weill Cornell Medical College, Department of Genetic Medicine, New York, NY.

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).

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 2-4 and GOLD 0-1 using the fold-change threshold method.

In addition to a discovery-based genome-wide analysis, four lists of genes were compiled to test for differences in gene expression by disease status based on a hypothesis-oriented approach. The first gene list (Table A2) comprised genes identified by prior studies of expression in COPD patients compared to controls; confirming this list in the current study allowed consideration of the validity of the samples and methods.(18;19) The second gene list (Table A2) comprised genes identified as COPD susceptibility genes in published genome-wide analysis studies (GWAS).(10;11;13;14;51) The third and fourth gene lists (Table A2) related specifically to the hypotheses about oxidant/antioxidant balance and comprised antioxidant-responsive genes. The initial selenium-responsive gene list was prepared by reviewing 17 studies published in the literature, yielding a list of 111 genes. Similarly, review of 12 published studies generated an initial vitamin E-responsive gene list that contained 102 genes. The literature reviews encompassed studies in cell and animal models as well as human intervention trials. Undertaking a gene-by-gene in-depth review, each list was curated for biologic relevance to the present study. Genes were retained if they were known to be expressed in lung tissue, if they were expressed under relevant physiologic concentrations of the nutrient, if expression was *not* limited to highly specific physiologic stress conditions, and if they were not primarily related to cancer processes. The final gene lists (Table A2) comprised 42 selenium-responsive genes from 7 published studies and 42 vitamin E-responsive genes from 6 published studies.(9;34-36;38-46)

Using p-values from the microarray analysis, FDR q-values were estimated for genes in each of the gene lists in SAS. Genes were considered differentially expressed if fold change > 1.15, p-value < 0.05, and q-value < 0.05. Finally, for genes with evidence of differential expression, further analyses assessed the association of gene expression with tissue and plasma nutrient concentrations with the Pearson product moment correlation coefficient.

Gene Set Enrichment Analysis (GSEA) was used to identify whether changes in gene expression were associated with functional changes (Broad Institute, Cambridge, MA). GSEA is based on pre-defined groups of genes that share biological function, regulatory mechanisms, or chromosomal location (Subramanian). The analysis was run using 1000 permutations per gene pathway and gene pathway sizes were restricted to between 15 and 500 genes per pathway. A total of 234 pathways were evaluated, and the FDR q-value, normalized enrichment score (NES), and nominal p-values were used to assess whether the pathway was statistically significantly differentially regulated in lung tissue samples from GOLD stage 2-4 patients compared to GOLD 0-1 patients. Gene pathways with a nominal p-value <0.01 and an FDR q-value <0.10 were considered statistically significant.

Results

Participant Characteristics

High quality RNA samples were obtained from 22 of the 24 lung tissue samples, and thus two patients were not included in further analysis. The remaining 22 participants (Table 4.3) comprised 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 58.6 pack-years of cigarette smoking (1 pack cigarettes/day for one year = 1 pack-year). Participants with severe COPD (GOLD stage 4) tended to be younger than average, which may indicate a genetic predisposition to early onset COPD due to α-1-antitrypsin deficiency.(16)

Table 4.3 Characteristics of twenty-two patients providing tissue samples obtained through the Lung Tissue Research Consortium, National Heart, Lung and Blood Institute, stratified by Global Obstructive Lung Disease (GOLD) Stage

		GOLD Stages					GOLD Stages Grouped	
	Total	0	1	2	3	4	0/1	2/3/4
N=	22	5	3	4	5	5	8	14
Age, years*	66.5 (0.6)	73.8 (8.3)	68.5 (9.0)	71.4 (3.6)	63.4 (10.6)	55.6 (2.8)	71.4 (8.6)	63.5 (9.1)
Sex, male/female	14/8	4/1	3/0	2/2	2/3	3/2	7/1	7/7
Pack-years smoking*	58.6 (38.2)	64.8 (38.9)†	47.8 (25.4)	70.8 (74.4)	42.8 (4.8)	68.6 (9.5)	56.2 (31.7)	60.7 (42.2)
Years since quitting smoking*	8.6 (10.4)	21.0 (14.3)†	7.0 (14.0)	7.0 (8.7)	6.8 (4.0)	3.4 (2.5)	14.0 (15.1)	5.7 (5.6)

*Continuous variables reported as mean (SD)
†One participant was missing smoking data and is not included in the mean or SD

Tissue Nutrient Status

Plasma total selenium, total α-tocopherol, HDL α-tocopherol and lung tissue α-tocopherol levels were measured in all samples (Table 4.4), 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 0-1 (no disease and minimal disease) vs. 2-4 (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 0/1 and lower in GOLD 4. However, when the GOLD 0/1

Table 4.4 Nutritional status of twenty-two patients providing tissue samples obtained through the Lung Tissue Research Consortium, National Heart, Lung and Blood Institute, stratified by Global Obstructive Lung Disease (GOLD) Stage*

		GOLD Stages					GOLD Stages Grouped	
	Total	0	1	2	3	4	0/1	2/3/4
N=	22	5	3	4	5	5	8	14
Plasma Selenium, mg 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	24.4 (11.9)	30.9 (18.8)	21.6 (8.2)	27.4 (13.1)	24.0 (7.5)	17.5 (5.7)	27.4 (15.6)	22.7 (9.4)
HDL α-tocopherol, μmol α-toc/mmol cholesterol	23.5 (8.4)	30.0 (9.5)	16.8 (1.6)	22.7 (8.1)	24.3 (9.2)	20.6 (6.5)	25.1 (10.0)	22.5 (7.5)
Tissue α-tocopherol, nmol α-toc/g lung tissue	39.2 (14.6)	47.9 (12.1)	52.3 (19.7)	36.5 (5.7)	38.5 (13.8)	25.4 (10.0)	49.5 (14.1)	33.2 (11.6)

*All values reported as mean (SD)

group was contrasted with the GOLD 2/3/4 group, the difference in means was not statistically significant for either plasma or HDL α-tocopherol (p=0.38 and p=0.51, respectively), but GOLD 0/1 patients had higher levels of antioxidants on average in comparison to GOLD 2/3/4 patients. Nineteen of 22 plasma samples analyzed by GC/MS for α-tocopherol displayed a peak that is characteristic of dietary supplementation with all-*rac*-α-tocopherol acetate (synthetic α-tocopherol). One participant each in GOLD 2, 3, and 4 had no evidence of synthetic α-tocopherol supplementation. Lung tissue α-tocopherol concentration was similar in GOLD 0 and GOLD 1, and steadily lower with increasing disease severity. When the combined GOLD 0-1 (49.5 nmol/g) and GOLD 2-4 (33.2 nmol/g) categories were compared, tissue α-tocopherol

concentrations were statistically significantly different (p=0.0082). The clear separation in lung tissue α-tocopherol concentration supported combining GOLD 0-1 and GOLD 2-4 into two discrete categories for further investigation of gene expression differences, particularly given the interest in antioxidant/oxidant balance in this study. Plasma and tissue α-tocopherol concentrations were moderately correlated (r=0.44; p=0.04).

Analysis of Gene Expression by GOLD Stage Groups

A genome-wide array analysis was conducted first. Five genes were differentially expressed in GOLD 2/3/4 with nominal p-values <0.0001, but the lowest false discovery rate (FDR) q-value was 0.27. The 25 genes with the lowest p-values are shown in Appendix Table A3; none of the top 25 genes were on any of the hypothesis-oriented gene lists.

In the hypothesis-oriented analyses, four gene lists were developed, as described above, including: 1) COPD GWAS genes (6 genes), 2) COPD differential expression genes (22 genes), 3) vitamin E-responsive genes (42), and 4) selenium-responsive genes (42 genes). Overall, the gene lists comprised 109 unique genes, and 3 genes appeared on two lists (Appendix Table A3). *SERPINE2* was on both the COPD GWAS list and the COPD differential gene expression list. *COL1A1* and *TNF* were on both the COPD differential gene expression list and the vitamin E-responsive gene list.

One gene from the COPD GWAS list, three genes from the COPD differential gene expression list, and six genes each from the vitamin E-responsive and selenium-responsive lists were differentially expressed in GOLD 2/3/4 lung tissue samples compared to GOLD 0/1 with FDR q-value <0.05 (Table 4.5).

Table 4.5 Genes differentially expressed in GOLD Stage 2/3/4 lung tissue compared to GOLD Stage 0/1 lung tissue, FDR q-value < 0.05

Gene list	Gene symbol	Gene name	Fold change*	Raw p-value	FDR q-value†
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 famly 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
	GSTO1	Glutathione S-transferase omega-1	-1.23	0.010	0.0347

*Log_2 fold change
†Estimated for each gene list individually

The *BICD1* gene from the COPD GWAS gene list was significantly up-regulated in GOLD 2-4 lung tissue. Two other genes, which have generated significant interest in recent GWAS, *HHIP* and *SERPINE2*, had an FDR q-value < 0.05 and fold-changes of -1.32 and -1.44, respectively, but nominal p

values did not meet preset criteria (p values were 0.118 and 0.1396, respectively; Table 4.5). In the COPD gene expression list, *PLAUR* and *VEGFB* were down-regulated and *MMP2* was up-regulated in the GOLD 2/3/4/ group vs. GOLD 0/1 (Table 4.5).

Five out of six vitamin E-responsive genes with evidence of differential expression were down-regulated in GOLD 2/3/4 lung tissue samples compared to GOLD 0/1; *KRT1* was up-regulated with fold-change of 1.51. Fold-changes of down-regulated vitamin E-responsive genes ranged from -1.16 to -2.30 (Table 4.5). Two selenium-responsive genes, *NR2F1* and *MS4A1,* were up-regulated in GOLD 2/3/4 lung tissue samples compared to GOLD 0/1, with fold-changes of 1.54 and 2.25, respectively. The remaining four selenium-responsive genes with statistically significant evidence of differential expression by GOLD stage were down-regulated; fold-changes ranged from -1.18 to -1.39 (Table 4.5).

For the 16 genes that were differentially expressed by GOLD stage disease severity groups, the association of nutrient concentrations in lung tissue and plasma with gene expression was assessed (Table 4.6). For four genes, expression was correlated with more than 1 nutrient ($p<0.05$). 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 ($r=0.66$; $p=0.0008$), respectively. *PPARG*, peroxisome proliferator-activated receptor gamma, which was documented to be responsive to vitamin E in prior studies, 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).

Table 4.6 Pearson Product Moment Correlations for the relation of lung tissue gene expression with nutritional status in lung tissue and plasma for subset of genes differentially expressed by GOLD Stage.

Gene list	Gene symbol	Tissue α-tocopherol		Plasma α-tocopherol		Plasma Selenium	
		r *	P value	r *	P value	r *	P value
COPD GWAS	*BICD1*	-.011	0.62	-0.15	0.50	0.31	(0.16)
COPD gene expression	*PLAUR*	0.46	0.03**	0.66	0.0008**	$1.5x10^{-2}$	0.95
	VEGFB	$-4.8x10^{-2}$	0.83	$-8.5x10^{-2}$	0.71	0.29	0.19
	MMP2	$-9.1x10^{-2}$	0.69	$3.6x10^{-2}$	0.87	-0.33	0.13
Vitamin E responsive	*TNNI2*	0.25	0.25	0.29	0.18	0.19	0.40
	KRT1	-0.12	0.63	-0.10	0.65	0.36	0.10
	SRA1	$8.1x10^{-2}$	0.72	$6.3x10^{-3}$	0.98	-0.42	0.049**
	PPARG	0.20	0.36	0.46	0.03**	0.24	0.29
	SULT2B1	$-6.3x10^{-2}$	0.78	$-8.4x10^{-2}$	0.71	0.27	0.23
	KRT4	$5.9x10^{-2}$	0.80	0.34	0.12	0.34	0.12
Selenium responsive	*NR2F1*	-0.21	0.34	-0.21	0.35	$-2.2x10^{-2}$	0.92
	SELT	0.22	0.33	-0.15	0.52	-0.23	0.30
	P4HA1	0.30	0.18	0.41	0.055**	$2.8x10^{-2}$	0.90
	MS4A1	-0.17	0.45	-0.29	0.20	-0.17	0.44
	GNGT2	0.65	0.0010**	0.53	0.01**	$3.2x10^{-2}$	0.89
	GSTO1	$9.5x10^{-3}$	0.97	$-1.3x10^{-2}$	0.95	$-2.8x10^{-2}$	0.90

*Correlation coefficient and associated P value
**P < 0.05

Gene Set Enrichment Analysis (GSEA)

GSEA was used to identify up- or down-regulated pathways using cutoffs of nominal p<0.05 and FDR q-value <0.10. We identified a single up-regulated pathway and 21 down-regulated pathways, 8 of which had an FDR q-value <0.05 (Table 4.7). Figure 4.1 shows the enrichment plot for the IL7 pathway. Genes to the left of the peak contribute the most to enrichment of the pathway, and thus to the differential expression between disease severity groups. Of the down-regulated pathways, 11 are related in some way to

Table 4.7 Gene set enrichment analysis showing pathways of genes differentially expressed in lung tissue from patients with GOLD Stages 2/3/4 disease versus GOLD 0/1

Pathway	# Genes	ES*	NES*	Nominal p-value	FDR* q-value
Up-regulated pathways					
IL7	16	0.681	1.99	0.002	0.052
Down-regulated pathways					
ATP synthesis	21	-0.641	-2.00	0.002	0.006
Type III secretion system	21	-0.641	-2.01	0	0.006
Flagellar assembly	21	-0.641	-1.98	0	0.006
Pyruvate metabolism	37	-0.551	-2.03	0	0.007
Oxidative phospholylation	58	-0.514	-2.04	0	0.007
Photosynthesis	22	-0.63	-2.04	0.002	0.015
Proteasome pathway	20	-0.598	-1.86	0.002	0.020
RNA transcription reactome	36	-0.513	-1.85	0	0.022
Glycolysis	52	-0.438	-1.72	0.002	0.055
Inflam pathway	29	-0.498	-1.70	0.006	0.056
Gluconeogenesis	52	-0.438	-1.71	0	0.056
Porphyrin and cholorphyll metabolism	19	-0.579	-1.74	0.006	0.056
Proteasome	17	-0.582	-1.73	0.006	0.059
Ubiquinone biosynthesis	15	-0.595	-1.74	0.011	0.059
Stem pathway	15	-0.565	-1.64	0.034	0.073
Propanoate metabolism	31	-0.484	-1.66	0.009	0.073
Glycolysis and gluconeogenesis	42	-0.442	-1.66	0.009	0.075
Hytrophy model	17	-0.556	-1.65	0.017	0.075
Krebs TCA cycle	29	-0.476	-1.64	0.006	0.076
Mitochondrial fatty acid betaoxidation	15	-0.575	-1.62	0.027	0.077
Cholesterol biosynthesis	15	-0.558	-1.60	0.030	0.088

*ES – Enrichment score for the gene set; reflects the extent of over-representation of the gene set at the top or bottom of the ranked list; NES – Normalized enrichment score; normalized ES accounting for the size of the gene set; FDR – False discovery rate

cellular energy synthesis and metabolism, including the most strongly down-regulated pathway, the ATP synthesis pathway. Pyruvate metabolism, oxidative phosphorylation, glycolysis, gluconeogenesis, and Krebs TCA cycle pathways are closely related to ATP production and metabolism. In addition, ubiquinone biosynthesis and proteasome pathways are ATP-dependent and thus would down-regulate in response to down-regulation of ATP synthesis. Down-regulation of the inflammatory pathway, which contains many interleukin genes, was observed with more severe disease.

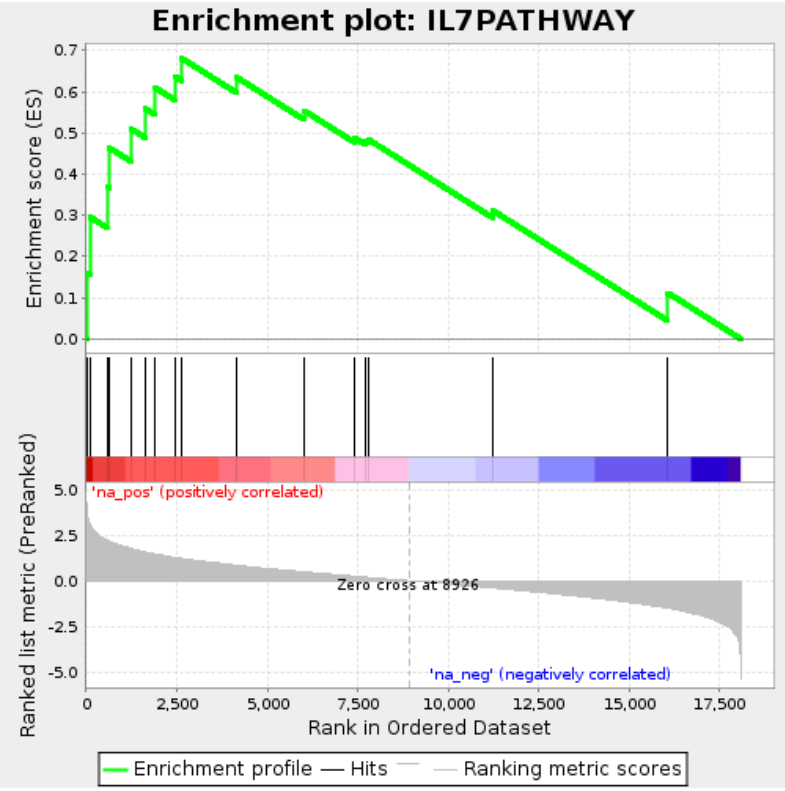

Figure 4.1 Enrichment plot of IL7 pathway from Gene Set Enrichment Analysis (GSEA); genes to the left of the Enrichment Score peak contribute most to the enrichment pathway, reflecting differential expression in the pathway between GOLD 2/3/4 lung tissue and GOLD 0/1 lung tissue

Discussion

Based on prior studies showing differential plasma status of antioxidants in COPD patients compared to controls, we hypothesized that a lung-tissue-specific measure of antioxidant status would be correlated with COPD severity as assessed by GOLD stage diagnosis.(31;52) Lung tissue α-tocopherol varied by disease stage, and levels were about 33% higher in GOLD 0/1 patients compared to GOLD 2/3/4 patients, with a clear trend. Trends for plasma and HDL α-tocopherol were less clear, which suggests the importance of considering compartment-specific nutrient levels. Among all

participants, the use of supplemental vitamin E was common, as demonstrated by the presence of an analyte unique to supplements (unpublished data). Plasma selenium levels were similar by GOLD stage groups, and selenium could not be assayed in lung tissue due to limited tissue volume. Lower lung tissue levels of α-tocopherol with increasing disease severity are consistent with two hypotheses. First, COPD is hypothesized to lead to lowered α-tocopherol in lung tissue either because the nutrient is rapidly utilized in the COPD lung and/or because the transport and/or uptake of α-tocopherol in the lung are impaired in diseased individuals. An alternative hypothesis, which cannot be ruled out in the current design, is that lowered lung tissue α-tocopherol levels is a causal factor in disease development, and the lowered nutrient status contributes, in part, to the pathophysiology. While this study cannot distinguish between the two explanations, both are interesting in terms of identifying possible intervention targets (one preventive and the other therapeutic).

Nutrients with antioxidant properties may play a role in regulating genes, and there is mounting evidence for this in vitamin E and selenium-responsive genes.(9;34-40) Thus, we further hypothesized that gene expression would be differentially regulated in patients with different tissue levels of antioxidants. When patients with GOLD stage 2/3/4 disease were compared to patients with GOLD stage 0/1 disease in a genome-wide array analysis, no genes were significant at genome-wide thresholds for statistical significance. These findings are limited by the small sample size in this study, and effect sizes for genome-wide significance would have had to be very large in light of the small sample size. The further analyses that pursued expression

differences using hypothesis-oriented gene lists identified several expression differences of interest.

The *BICD1* gene, bicaudaul D homolog 1, which was identified as a COPD susceptibility gene in a recent GWAS of COPD, was up-regulated in moderate/severe/very severe COPD cases.(10) 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.(10;53;54) The differential expression of *BICD1* in lung tissue from COPD patients could be due to single-nucleotide polymorphisms (SNPs), gene-environment interactions, or other mechanisms. Two other genes, *HHIP* and *SERPINE2*, also identified by GWAS studies as COPD susceptibility genes, had FDR q-values < 0.05.(11;14;16) The finding that genes identified by prior GWAS studies are differentially expressed in this study of lung tissue from COPD patients provides complementary evidence supporting a causal role for these genes in COPD pathogenesis.

Based on a literature review of studies of differential gene expression associated with COPD, a list of genes was identified for testing. 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 2/3/4 patients (vs. GOLD 0/1), in contrast to a prior report that *PLAUR* expression was inversely associated with FEV_1 (higher expression associated with lower lung function).(18) 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.(18) *VEGFB,* vascular endothelial growth factor B, was down-regulated (fold-change -1.25) in GOLD 2/3/4 vs. GOLD 0/1. Gosselink et al reported on a

related *VEGF* gene, which was dysregulated in small airways, but the finding did not reach statistical significance thresholds thus the direction of fold-change was not reported.(18) The matrix metalloproteinase 2 gene, *MMP2*, was up-regulated in GOLD 2/3/4 cases compared to GOLD 0/1 (fold-change 1.52). Contrary to our findings, a prior study reported that *MMP2* expression in small airways tissue (both the alveolar tissue and surrounding tissue) was directly associated with FEV_1 (decreased protein associated with lower lung function).(18) However, 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).(55) Our finding supports the immunohistochemical study by Baraldo et al and Gosselink's findings are at odds with both Baraldo and the results reported herein.(18;55)

Six differentially expressed genes were identified on the vitamin E-responsive gene list when comparing expression in lung tissue from patients with GOLD stages 2/3/4 compared to GOLD 0/1; five were down-regulated and one was up-regulated. A particularly interesting gene from the vitamin E-responsive list is peroxisome proliferator-activated receptor gamma, *PPARG*; prior studies report *PPARG* is upregulated by α-tocopherol.(44) In lung tissue samples, *PPARG* expression was lower in patients with GOLD stages 2/3/4 and lung tissue from these patients was also lower in α-tocopherol concentrations, consistent with prior findings in other tissues.(44) *PPARG* is implicated in cell signaling and cell-cycle regulation and may be important in management of inflammatory conditions.(44) In a recently completed study of asymptomatic cigarette smokers supplemented with vitamins E and C and

selenomethionine for 30 days, the pre-post supplementation $\log_{10}$ fold-change expression of *PPARG* in large airway epithelial cells was 119.3 (compared to -37.9 fold-change in the placebo group; unpublished data), supporting the finding that *PPARG* expression is responsive to vitamin E.*TNNI2,* troponin 1 type 2, was down-regulated, 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.(41)

Six genes in the selenium-responsive list were differentially expressed by disease group. 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 2/3/4 patients compared to GOLD 0/1. A prior study reported that *NR2F1* was down-regulated by supplemental selenomethionine.(34) Although plasma selenium levels do not differ much by GOLD groups, we were unable to measure selenium concentrations in lung tissue. If lung tissue selenium follows the pattern of α-tocopherol, then GOLD 2/3/4 patients are expected to have lower levels of lung tissue selenium, which would fit with the up-regulation of *NR2F1* observed. The membrane spanning 4 domains-subfamily A-member 1 gene, *MS4A1*, had the greatest fold-change; *MS4A1* expression was 2.25-fold higher in GOLD 2/3/4 samples compared to GOLD 0/1. 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 2/3/4 tissue is depleted in selenium relative to GOLD 0/1 tissue.(34) Expression of *MS4A1* was inversely associated with plasma selenium, consistent with the prior reports, although the correlation was not statistically significant.

Strengths of this study include rigorous methods to quantify selenium and α-tocopherol and high quality gene expression microarray data using a well-validated Affymetrix platform. Given the small sample size, the gene subset approach was an important investigative tool, particularly because our interest was in genes specifically related to antioxidant response and COPD. We examined the correlation of gene expression with tissue and plasma nutrient as a complementary method to test the hypothesis that genes related to antioxidant function are differentially expressed, and this led to supportive data for the contrast by COPD stage in many instances.

Some limitations of this study have already been noted, including the small sample size. Although the hypothesis-oriented genes lists for vitamin E- and selenium-responsive genes were developed post-hoc, which could be viewed as a weakness, these lists were carefully curated to identify findings for further confirmation. As in any study of this kind, the disease groupings must relate to actual differences in clinical manifestations, and the two GOLD stage groupings could be criticized. However, the two groups were consistently different on descriptive lung function data, supporting the classification used. Indeed, this classification also yielded the most distinct difference in lung tissue vitamin E levels, lending support to the hypothesis that antioxidant status may play a role in disease progression. An additional limitation was the lack of tissue to assay selenium concentrations in lung.

In summary, α-tocopherol concentrations in lung tissue were strongly associated with COPD GOLD stage diagnostic level. 16 of 109 genes related to COPD, vitamin E- or selenium were differentially expressed by disease severity. Given the importance of antioxidant status in protecting the lung it is important to understand the relation between lung antioxidants and gene

expression in lung tissue as therapeutic targets may emerge from understanding these pathways and the interactions of antioxidant nutrients with individual genes.

In conclusion, the vitamin E concentration in lung tissue of COPD patients was correlated with disease severity: patients with more severe disease had lower concentrations of vitamin E in lung tissue. A number of genes that are responsive to vitamin E and selenium were differentially expressed in lung tissue of COPD patients with moderate to very severe disease. Overall, these findings suggest a causal mechanism for epidemiologic observations that vitamin E is associated with better lung function and lowered risk of COPD.(31;56-58) The findings support the hypothesis that nutrients contribute to variation in gene regulation, and a further understanding of these associations may identify therapeutic value for vitamin E and/or selenium.

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CHAPTER 5

SUMMARY AND CONCLUSION

Overview

The projects completed for this dissertation were designed and conducted to provide a comprehensive, integrated study of chronic obstructive pulmonary disease (COPD). The following three projects were completed for this dissertation: 1) a randomized study of vitamin E supplementation and risk of chronic lung disease in the Women's Health Study, which was a *post hoc* analysis of the incidence of COPD and asthma in a double-blinded randomized clinical trial of vitamin E and aspirin in women (referred to as WHS), 2) a study of the effect of supplementary antioxidant vitamins on plasma and airway lining fluid in cigarette smokers (referred to as ExSEL), which was a double-blinded randomized clinical trial investigating the effect of dietary antioxidant supplements in the lung compartment, and 3) a study of the differential expression of vitamin E and selenium-responsive genes by disease severity in chronic obstructive pulmonary disease (referred to as LTRC), which was a small study of antioxidant concentrations in plasma and lung tissue in relation to gene expression in lung tissue of COPD patients at varying levels of disease severity.

The WHS analyzed of the effect of randomized vitamin E supplementation on risk of incident chronic lung disease. In the molecular epidemiologic model of disease, the WHS studied exposure to vitamin E in relation to the COPD outcome, but without investigating biomarkers of antioxidant status or genetic information. The ExSEL was a small randomized controlled trial of the effect of antioxidant supplements on systemic and lung-

specific concentrations of antioxidants. Thus, ExSEL linked exposure to nutrients with antioxidant properties to compartment-specific biomarkers of nutritional status and oxidative stress, but the study design precluded consideration of disease outcomes. The LTRC provided that last link in the molecular epidemiologic model by investigating biomarkers of antioxidant status and gene expression in relation to COPD severity, but the intake of nutrients with antioxidant properties was not assessed. The three projects was a whole were a complete study of the relation of antioxidants with COPD and the mediation of that relation through gene expression, which would have been difficult to achieve with a single study.

The integrated nature of the three projects was deliberate. COPD is a complex chronic disease, and studying risk factors, pre-clinical and disease biomarkers, and changes in gene expression associated with disease would be impossible in a single study. Complex chronic diseases have a multifactorial etiology, and this is certainly true for COPD. In addition, COPD, like other complex chronic diseases, develops over a long period of time, thus studying time-varying exposures in relation to disease state over the full natural history would be challenging. Finally, COPD is a complex phenotype, which is thought to comprise subgroups of more homogenous pathophysiology, also adding to the challenges in study design. An integrated approach, combining several types of studies, offered the best approach to understanding how one aspect of diet, nutrients with antioxidant properties, relates to biomarkers and genetic regulation that influences COPD susceptibility and progression. A brief summary of the findings from each project is presented below followed by a short discussion of common themes that emerged from this work.

WHS Summary

The Women's Health Study was a randomized clinical intervention trial of 38,597 women health care professionals who were supplemented with vitamin E for 10 years. We hypothesized that supplementation with vitamin E decreases the occurrence rate of chronic lung disease. We performed a *post hoc* survival analysis of the WHS investigating the effect of vitamin E supplementation on chronic lung disease risk. During the 10-year follow-up of the study, vitamin E supplementation led to a 10% reduction in risk of developing chronic lung disease (376,710 person-years of follow-up). There were 760 new occurrences of chronic lung disease in the women assigned to the vitamin E arm of the trial compared to 846 new occurrences in women assigned to the placebo arm of the trial. The reduction in risk was statistically significant (HR 0.90; 95% CI 0.81 to 0.99). We hypothesized that smoking would modify the effect of the supplements, but no statistically significant modifiers of the effect on vitamin E supplementation on chronic lung disease risk were identified.

Two prior studies investigated antioxidant supplements and COPD-related outcomes and reported no relation of supplements to outcomes, but the studies were substantially different from the WHS. The Heart Protection Study (HPS) was 75% men and all participants had prevalent coronary artery disease. The Alpha-tocopherol and Beta-carotene (ATBC) study was comprised entirely of men, all of whom were cigarette smokers.(1;2) In these trials the outcomes considered included death due to respiratory illness, pulmonary function, and COPD-related hospitalizations. Neither trial considered **primary prevention** of COPD, nor were the studies conducted on women, particularly women in good general health.

The findings from the WHS provide important new information about the relation of vitamin E to chronic lung disease occurrence in women. In light of the prior reports of no effect in studies of male participants, this thesis advanced a hypothesis to account for the differential effect of vitamin E in women compared to men. On average, women have higher levels of high density lipoprotein (HDL) cholesterol; higher HDL leads are hypothesized to lead to greater delivery of supplemental vitamin E to the lung compartment in women, which would explain a greater protective effect of vitamin E in women. This hypothesis is based on *in vitro* and *in vivo* research showing that the high-density lipoproteins in the interstitial fluid deliver vitamin E to alveolar type II cells and that because type II cells have no contact with plasma, vitamin E delivery must occur through the HDLs contained in the interstitial fluid.(3;4) Thus, the naturally higher HDL cholesterol levels in women may allow for delivery of a greater biologically effective dose of vitamin E to the lung compartment and this difference may explain part of the sex-difference in findings related to vitamin E supplementation and COPD.

This hypothesis is supported by the analysis of effect modification presented in Chapter 2; alcohol intake was a borderline statistically significant effect modifier ($p=0.054$) of the relation of vitamin E to COPD incidence. As alcohol is proposed to increase HDL (the mechanism for lowering cardiac risk (5;6), we speculated that stronger effects of vitamin E in moderate alcohol consumers may be mediated by delivery of vitamin E to lung tissue. Although this proposed mechanism cannot be proven with the WHS data the preventive effect of vitamin E in the WHS population and the effect modification by alcohol intake is intriguing and worthy of follow-up. The lack of effect modification by smoking was a surprising finding, and may offer some clues

about the mechanism by which vitamin E alters COPD susceptibility. One hypothesis is that vitamin E is acting systemically, perhaps by improving immune function.(7)

There is concern about high dose vitamin E supplementation given potential harmful effects and these results must be interpreted with caution before making public health recommendations. Making recommendations for higher dose vitamin E supplementation in men to achieve the purported "effective dose" achieved by women would require substantial investigation with particular attention given to increased risks of stroke, bleeding, and other negative health outcomes. It is important to consider carefully the overall risk benefit profile of using dietary supplements prior to making any policy recommendations; furthermore, studies designed to address this question specifically would be needed. However, given the paucity of prevention strategies for chronic lung disease, the 10% reduction in risk demonstrated in this study is of interest and meritorious of further investigation.

ExSEL Summary

Our study of cigarette smokers and the effect of antioxidant supplements on plasma and airway lining fluid, ExSEL, was a double-blind placebo controlled trial investigating the ability of supplementation with vitamins C and E and selenium to augment the lungs' antioxidant defenses thereby attenuating oxidative stress imposed by smoking. We hypothesized that oral supplementation with antioxidants would alter lung compartment concentrations of those nutrients and lower a systemic marker of oxidative stress. We conducted a one-month clinical intervention with the dietary supplements in 26 study participants. Plasma and bronchioalveolar lavage

fluid (BALF) measurements were made to assess change in antioxidant status caused by the intervention. In addition, urine and plasma biomarkers of oxidative stress were assayed to determine whether or not the antioxidant altered oxidative stress caused by smoking.

We found that antioxidant intervention increased concentrations of supplemented nutrients in the plasma and BALF. In response to supplementation, vitamin E increased 69.4% in the plasma, 67.1% in HDL, and 64.6% in the BALF, compared to changes in the placebo group of 21.3%, 12.0% and -5.8%, respectively. Selenium increased 78.0% in plasma and 46.8% in BALF due to the supplementation, while in the placebo group it changed -4.4% and 5.1% respectively in these fluids. These findings are important in establishing that the nutrients are reaching the lung compartment, the tissue target of the intervention. Often the assessment of whether or not an intervention is reaching the target tissue is overlooked in clinical trials, to the detriment of understanding the effectiveness of a treatment. In addition, no prior studies had investigated whether supplementation with antioxidants reached the lung compartment by directly measuring fluid from the lung, and, in fact, no prior work had assessed selenium in the BALF.

The association of change in vitamin E concentrations in the plasma with change in the BALF was interesting. The correlation between change in plasma and BALF selenium was 0.57 ($p = 0.0057$). The correlation coefficient for change in plasma and BALF α-tocopherol was 0.31 ($p = 0.1487$. Change in HDL and plasma α-tocopherol were strongly correlated, ($r = 0.60$; $p = 0.0024$) suggesting that HDL vitamin E concentration is much more important in the transport of vitamin E to the lung. Regression models confirmed these findings. Additional exploration of potential to benefit investigated how starting

antioxidant status and level of oxidative stress affected response to supplementation, and while numbers were small for these analyses the findings were in expected directions.

Prior to the ExSEL study, no prior studies measured plasma and lung concentration of antioxidants before and after intervention, and no literature reports of selenium in BALF were identified. Thus, the findings from ExSEL comprise substantial new information regarding the efficacy of nutritional supplements to alter lung antioxidant concentrations; these findings suggest preventive and therapeutic possibilities for COPD.

LTRC Summary

The final study of my dissertation, the LTRC study, incorporates information about plasma and lung nutrient status and differential gene expression to investigate differences between disease severity groups in patients with COPD. We hypothesized that a lung-tissue-specific measure of antioxidant status would be correlated with COPD severity. We analyzed 22 samples from patients with COPD at all 5 GOLD stages of disease severity (0-4). Samples from 8 participants in GOLD stages 0/1 (at-risk [essentially disease free] and mild COPD, respectively) were compared to 16 samples from participants in GOLD stages 2/3/4 (moderate, severe, very severe). Lung tissue vitamin E concentrations decreased with increasing disease severity ($p=0.0082$). Plasma vitamin E and selenium concentrations were not correlated with disease severity.

We conducted a three-part investigation of the gene expression data, and all comparisons considered whether gene expression differed by GOLD stage group (GOLD 2-4 vs. GOLD 0-1). In the genome-wide array analysis no

genes were differentially expressed at genome-wide significance levels. We also conducted a hypothesis-oriented analysis testing four lists of genes: COPD susceptibility genes, genes with prior evidence of differential expression in COPD, genes responsive to vitamin E, and genes responsive to selenium. We found 16 of 109 genes investigated (across the four lists) were differentially expressed in GOLD 2/3/4 compared to GOLD 0-1 with FDR q-values <0.05. Of the 16 differentially expressed genes, one was a previously identified COPD susceptibility gene, 3 were previously identified as differentially expressed in COPD, six were related to vitamin E, and six were related to selenium.

Differentially expressed genes were analyzed further for associations between antioxidant status and gene expression. Four genes were found to have expression significantly correlated with one or more antioxidant biomarkers. Of these genes peroxisome proliferator-activated receptor gamma, *PPARG*, was particularly interesting because it had been previously identified to be responsive to vitamin E and was associated with plasma vitamin E concentrations. Earlier reports of the regulatory effect of vitamin E on *PPARG* indicate that the gene is upregulated in response to vitamin E.(8) In our samples lung tissue from patients with more severe disease contained less vitamin E and this was associated with lower levels of *PPARG* gene expression.

Our findings support the hypothesis that lung nutrient status is lower in patients with more severe disease and that the differential expression of genes in lung tissue is associated with variation in tissue and plasma levels of antioxidant nutrients. These findings suggest a causal mechanism for

epidemiologic reports of COPD prevention via antioxidants and support the idea that targeted nutritional inventions may have therapeutic value.

Emerging Themes and Future Directions

Several common themes emerged from the three studies conducted for my dissertation research. First, supplementation with antioxidants may provide a useful COPD prevention, but the effect may differ by sex. The sex-based effect of vitamin E on disease prevention seen in the WHS gained additional support from the findings from ExSEL. Firstly, in ExSEL we found that the change in HDL vitamin E was more predictive of change in lung vitamin E than was overall change in plasma vitamin E. The importance of HDL cholesterol in delivery of vitamin E to the lung and the proposed link between HDL and the differential effect by sex is interesting and novel.

Differential expression in lung tissue of genes related not only to COPD but also to antioxidants provides additional support for our hypothesis that nutritional status is an important part of the disease process in COPD. Identification of *PPARG* as a differentially expressed gene in GOLD 2/3/4 compared to GOLD 0/1 was very interesting because it has been shown to be upregulated in response to vitamin E.(8) Furthermore, *PPAR*s have been shown to be involved in lipid biosynthesis and glucose metabolism and they have some anti-inflammatory and immunomodulatory properties mediated through down regulation of pro-inflammatory genes.(9) For this reason, *PPAR*s have recently been proposed as therapeutic targets in inflammatory lung disease, with the goal being to upregulate these genes to enhance their anti-inflammatory properties.(9) Our work from the LTRC study provides preliminary evidence that vitamin E could act as an expression modifier of

PPARG. A supporting line of evidence, from unpublished data in the ExSEL study, shows that *PPARG* is upregulated in epithelial cells of participants taking active vitamin E supplements compared to participants on placebos. While these data require fuller analysis, the finding is intriguing and provides interesting directions for exploring possible therapeutic and preventive roles for vitamin E in relation to COPD. Pharmacologic investigations of *PPARG* agonist drugs, such as rosiglitazone for asthma, are underway.(9)

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APPENDIX

Supplemental Methods A.1

Detailed Assay Methods

HPLC Determination of Plasma Vitamin C

Preservation of plasma samples for vitamin C determination by HPLC

To preserve the ascorbic acid, a 0.7 ml aliquot of plasma was added to a cryogenic vial containing 0.7 ml of pre-cooled 10% meta-phosphoric acid (MPA) solution then immediately frozen at -80°C.

HPLC determination of plasma vitamin C

Upon thawing, samples were mixed and centrifuged at 4°C, 10,000 rpm for 5 min. A 50 μl portion of supernatant was mixed with 150 μl dipotassium phosphate containing tris[2-carboxyethyl]phosphine hydrochloride (TCEP) to reduce any dehydroascorbic acid to ascorbic acid. Samples were incubated 1 hour at room temp. Next samples were acidified with 50 μl 40% MPA and 50 μl of hypoxanthine, added as internal standard, and mixed prior to transferring to autosampler vials. Twenty microliters was injected for analysis by HPLC. A Synergi Hydro RP, 250 x 4.6 mm, 4 μm column was used with UV detection at 245 nm (5 cm light-pipe flowcell), EC 200 mV, 10 nA. The mobile phase was 2% methanol in 25 mM monochloroacetic acid, 2 mM EDTA, pH 3.0. The flow rate was 0.8 ml/min with column temperature of 32°C and sample temperature of 15°C.

Three known-value serum standards (0.41 μg/ml ± 11.1%, 1.94 μg/ml ± 2.66%, and 14.1 μg/ml ± 1.63%) were concurrently measured in duplicate to assess assay precision. The average percent standard deviation was 5.14% for the six measurements of the three standards.

Vitamin C measurements were completed by Craft Technologies, Wilson, NC.

Gas Chromatography Mass Spectrometry (GC-MS) Determination of α-tocopherol

No special sample preservation methods were required for vitamin E assays.

Plasma total α-tocopherol sample preparation

Briefly, 3 μl of a δ_9-α-tocopherol internal standard (99 μmol/L stock solution) was added to 75μl of plasma. 150 μl of ethanol was added, then vortexed. To extract lipids 75 μl of methyl-*tert*-butylether and 1.0 ml hexane were added and sample was shaken for 2 minutes. Samples were centrifuged at 15,000 rpm for 1 minute then the upper hexane phase was transferred to a small vial. The hexane was evaporated to dryness under a nitrogen stream and in a 30ºC water bath. The residues were silylated in 40 μl pyridine (Thermo Scientific) and 40 μl N,O-bis[trimethylsilyl]trifluoroacetimide (Thermo Scientific). The headspace was flushed with nitrogen then samples were tightly capped and placed in a 75ºC dry oven for 25 minutes.

Plasma HDL cholesterol α-tocopherol sample preparation

20 μl of HDL cholesterol precipitating reagent (Stanbio) was added to 150 μl of plasma and allowed to stand for 5 minutes, then centrifuged at 15,000 rpm for 1 minute. 75 μl of supernatant was transferred to a tube; then the procedure for plasma total α-tocopherol was followed.

Bronchioalveolar lavage fluid α-tocopherol sample preparation

3 μl of a δ_9-tocopherol internal standard (99 μmol/L stock solution) was added to 4 ml of BAL fluid. 10 ml of a 90:10 hexane/methyl-*tert*-butylether and

30 μl of butylhydroxytoluene solution (10 mmol/L in 90:10 hexane/MTBE) was added to the tube, covered with nitrogen then tightly capped and shaken for two minutes. Samples were centrifuged for 5 minutes at 1000 rpm. The hexane phase was removed to a new tube, evaporated to 1-2 ml volume under a nitrogen stream and in a 30ºC water bath. The reduced volume was transferred to a small vial, evaporated to dryness under nitrogen in the water bath. The residue was silylated in 40 μl pyridine (Thermo Scientific) and 40 μl N,O-bis[trimethylsilyl]trifluoroacetimide (Thermo Scientific). The headspace was flushed with nitrogen then samples were tightly capped and placed in a 75ºC dry over for 25 minutes.

GC-MS determination of tocopherols

α-Tocopherol concentrations in plasma (total and HDL) and bronchioalveolar lavage fluid samples were determined by GC-MS, using a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 6890 mass spectrometer. Tocopherols were resolved isothermally at 280ºC on an HP-1 capillary column (Agilent Technologies) operated in split injection mode with helium as the carrier gas. Detection was by selected ion monitoring (SIM mode). Tocopherol and free cholesterol (as silyl ethers) were quantitated against the δ_9-α-tocopherol internal standard, adjusting the cholesterol values for predetermined differences in detector response.

Samples were assayed in duplicate and average coefficients of variation were determined for total α-tocopherol and for cholesterol-adjusted total α-tocopherol (where cholesterol was measured within the GC-MS method), respectively, with values as follows: plasma total α-tocopherol (4.2% and 2.4%), plasma HDL cholesterol α-tocopherol (13.2% and 4.0%), and bronchioalveloar lavage fluid α-tocopherol (11.6% and 6.2%).

Vitamin E assays were completed in the Division of Nutritional Sciences, Cornell University, Ithaca, NY.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Determination of Selenium

No special sample preservation methods were required for selenium assays.

Plasma selenium sample preparation

A 2% nitric acid solution was prepared using concentrated nitric acid from a Teflon sub-boiling still and double-deionized water, resistivity > 18 MΩ cm. Plasma samples were prepared for selenium analysis by adding 5 ml of 2% HNO_3 to 100 μl of plasma (thawed at room temperature). Two reference standard solutions, 1 ppb and 5 ppb, were run at regular intervals throughout sample testing and average concentrations were determined to be 1.002 ppb and 5.117 ppb, respectively with standard deviations of 0.023 and 0.073, respectively. Seronorm Trace Elements, Serum, Levels I and II (Accurate Chemical & Scientific Corp., Westbury, NY) and two NBS standard samples were used as reference materials for method validation and assessment of accuracy and precision. Seronorm and NBS samples were prepared in the same way as the plasma samples and run at regular intervals throughout sample testing. All four reference materials were determined to have values within the range of acceptable results for the material.

Bronchioalveolar lavage fluid selenium sample preparation

BALF samples were prepared for selenium analysis by adding 0.5 ml of BALF to a small vial then evaporating to dryness under a nitrogen stream and in a 30ºC water bath. Immediately prior to analysis the samples were

reconstituted in 50 μl of 2% HNO_3 spiked with 1 ppb germanium (Ge) as an internal standard. A reference solution containing 1 ppb of Ge and Se in 2% HNO_3 was analyzed at regular intervals throughout sample testing. The BALF was primarily composed of saline solution and the sodium contained within the BALF samples interfered with detector response to Ge and Se ions. Interference was quantitated and adjusted for by assessing Ge and Se response in the reference solution and comparing it to the reduced response in the samples that were reconstituted in the Ge-spiked solution.

ICP-MS determination of total plasma and BALF selenium

An Agilent 7500 cs/ce quadrupole ICP-MS equipped with a collision reaction cell was used to determine selenium concentration in plasma samples. The ion intensity at *m/z* 78 was used to monitor Se and ^{89}Y was used as an internal standard for plasma samples and ^{72}Ge for BALF samples. For plasma samples data were collected using the *Spectrum* mode for direct analysis. For BALF samples data were collected using the transient mode. Maximum sensitivity was obtained by daily tuning of the ion lens system, gas flow rates, and other parameters. Samples were run manually with continuous quality monitoring. Table A.1 summarizes the operating conditions and instrumental parameters.

Sample preparation and analysis was performed at the USDA Plant, Soil, & Nutrition Laboratory, Cornell University, Ithaca, NY.

Table A.1 Inductively Coupled Mass Spectrometry (ICP-MS) operating conditions and instrumental parameters for plasma and BALF selenium determination*

RF power	1400 Watts
Sample uptake rate	2.5 ml/min
Reaction mode	He or H_2, 5 ml/min, *Spectrum* mode
Gas flow rates:	
Plasma gas	Ar,
Auxillary gas	0 L/min
Carrier gas	*Plasma*: Ar, 0.8 L/min *BALF*: Ar, 0.45 L/min
Makeup gas	*Plasma:* Ar, 0.45 L/min *BALF:* Ar, 0.75 L/min
Ion sampling depth	8 mm
Torch	Quartz 2.5 mm id fitted with Agilent ShieldTorch system
Nebulizer	Microliter nebulizer, Microglass (Cedar Ridge, CO)
Sample cone	Pt, orifice diameter 1.0 mm
Skimmer cone	Pt, orifice diameter 0.4 mm
Spray chamber	Double cyclonic and water cooled Scott type
Integration time	*Plasma:* 0.2000 sec *BALF:* 0.1000 sec/point
Sampling period	*Plasma:* 0.6000 sec *BALF:* 20.0 sec
Sweeps per reading	*Plasma:* 40-60 *BALF:* Transient

*Unless otherwise noted, the conditions and parameters for plasma and BALF samples were the same.

Mass Spectrometry Determination of F_2-Isoprostanes in Plasma

Purification and preparation of plasma samples

For analysis, to 1-3 ml of plasma was added 1.0 ng of [$^2H_4$]-15-F_{2t}-IsoP ([$^2H_4$]-8-iso-PGF_{2a}; Cayman Chemical, Ann Arbor, MI) internal standard. The solution was adjusted to pH 3 with 1N HCl. The sample was then applied to a C-18 Sep-Pak cartridge that has been prewashed with 5 ml methanol and 5 ml 0.01N HCl. The cartridge was then washed with 10 ml 0.01N HCl, followed by 10 ml heptane, and compounds are then eluted with 10 ml ethyl acetate:heptane (50:50, v/v). The eluate was applied to a silica Sep-Pak cartridge prewashed with ethyl acetate (5 ml). It was rinsed with 5 ml ethyl

acetate and compounds eluted with 5 ml ethyl acetate:methanol (50:50, v/v). The eluate was dried under nitrogen. Compounds were converted to the pentafluorobenzyl (PFB) esters by the addition of 40 µl of a 10% solution of pentafluorobenzyl bromide in acetonitrile and 20 µl of a solution of 10% diisopropylethanolamine in acetonitrile and allowed to incubate for 30 minutes at 37°C. Reagents were dried under nitrogen and the residue reconstituted in 30 µl chloroform and 20 µl methanol and chromatographed on a silica TLC plate to 13 cm in a solvent system of chloroform:methanol (93:7, v/v). The methyl ester of $PGF_{2\alpha}$ was chromatographed on a separate lane and visualized with 10% phosphomolybdic acid in ethanol by heating. The R_f of $PGF_{2\alpha}$ methyl ester in this solvent system was 0.15. Compounds migrating in the region 1 cm below the $PGF_{2\alpha}$ standard to 1.0 cm above the standard were scraped from the TLC plate, extracted with 1 ml ethyl acetate, and dried under nitrogen. Following TLC purification, compounds were converted to trimethylsilyl (TMS) ether derivatives by addition of 20 µl *N,O*-bis(trimethylsilyl)trifluoroacetamide and 10 µl dimethylformamide. The sample was incubated at 37°C for 10 minutes and then dried under nitrogen. The residue was re-dissolved for GC/MS analysis in 20 µl undecane that was stored over a bed of calcium hydride.

Mass spectrometry detection of plasma F_2-isoprostanes

Gas chromatography-negative-ion chemical ionization mass spectrometry (GC/NICI-MS) was carried out on an Agilent 5973 Inert Mass Selective Detector coupled with an Agilent 6890n Network GC system (Agilent Labs, Torrance, CA) interfaced with an Agilent computer. GC was performed using a 15 m, 0.25 µm film thickness, DB-1701 fused silica capillary column (J

and W Scientific, Folsom CA). The column temperature was programmed from 190° to 300°C at 20°C per minute.

The major ion generated in the NICI mass spectrum of the PFB ester, TMS ether derivative of F_2-IsoPs was the m/z 569 carboxylate anion [M-181 (M-$CH_2C_6F_5$)]. The corresponding ion generated by the [$^2H_4$]-15-F_{2t}-IsoP internal standard was m/z 573. Levels of endogenous F_2-IsoPs in a biological sample were calculated from the ratio of intensities of the ions m/z 569 to m/z 573. The lower limit of detection of F_2-IsoPs was in the range of 4 pg using an internal standard with a blank of 3 parts per thousand. The precision of this assay in biological fluids is $\pm$6% and the accuracy 94%.

F2-isoprostane measurements were performed by the Eicosanoid Core Laboratory, Vanderbilt University Medical Center, Nashville, TN.

Determination of F2-isoprostanes in Urine Samples

Purification of urine samples

Urine was collected using a “clean catch” method into sterile urine collection containers. Urine samples were pipetted into cryovials in 2 ml aliquots then immediately frozen at -80°C. Samples were thawed at room temperature, centrifuged to remove particulates, then 1 ml aliquots were added to microcentrifuge tubes. Addition of 200 μl of sorbent to the microcentrifuge tubes was followed by gentle mixing for 45 minutes. The tubes were then centrifuged to sediment out the sorbent and the supernatant was removed and disposed of. The remaining sorbent was washed with 1 ml Nanopure water then centrifuged with removal and disposal of the supernatant. The washing procedure was repeated. The washed sorbent was then resuspended in 0.5 ml 95% ethanol (elution buffer) (Fisher Scientific) and

briefly vortexed. The sample was then centrifuged and the elution buffer was removed and saved. The resuspension was repeated and the elution buffer washes were all combined and dried down overnight on a speed vacuum. Dried samples were then dissolved in 1 ml of EIA buffer (Cayman Chemicals).

Enzyme linked immunoassay measurement of F2-isoprostanes

F2-isoprostanes in urine samples were measured using a competitive enzyme linked immunoassay (ELISA) kit from Cayman Chemicals (Ann Arbor, MI). Dilute EIA and wash buffers were prepared with Nanopure water, per the kit instructions. Eight standard samples were prepared by mixing with EIA buffer with the standard using serial dilutions. The tracer was reconstituted with 6 ml of EIA buffer and the antiserum was reconstituted with 6 ml of EIA buffer.

Plate set up was performed per the Cayman Chemical suggested method, which included at least two different dilutions of each sample. After all standards, samples, tracers and antiserum aliquots were added to the plate, the plate was covered and incubated for 18 hours at 4^{o}C. The plate was developed by reconstituting 250 dtn of Ellman's Reagent with 20 ml of Nanopure water. The plate was washed 5X with wash buffer solution. To each well of the plate, 200 μl of Ellman's Reagent was added and 5 μl of tracer was added to total activity wells. The plate was then covered and the plates were developed on a shaker in the dark. The plate was read at 405 nm wavelength after 60 minutes and 90 minutes of development.

Control samples were run on each plate and the overall coefficient of variation was determined to be 18.7%. Additionally, standard curve correlation values were determined at two time points and the average R^2 value was 0.9911 at each time point with average R^2 standard deviation of 0.0040.

Measurement of urine creatinine

Creatinine was measured on a Siemens Dimension Xpand Plus, a Centers for Disease Control and Prevention certified instrument, using standard methods recommended by the manufacturer.

Chemiluminescence Determination of Plasma Cholesterols and Triglycerides

Total, HDL, and LDL cholesterol and triglycerides were determined using chemiluminescence under standard methods on a Siemens Dimension Xpand, a Centers for Disease Control and Prevention certified instrument. Average within run and between run coefficients of variation for the analytes were, respectively, total cholesterol (0.71% and 1.94%), HDL cholesterol (0.36% and 4.20%), LDL cholesterol (0.48% and 3.10%), and triglycerides (0.28% and 2.12%). Two control samples, Biorad Liquid Assayed Multiqual Level 1 and Level 3, were assayed and all values for the lipids were determined to be within the acceptable range.

Urinary F2-isoprostane, creatinine, cholesterol and triglyceride measurements were performed in the Francis Johnston and Charlotte Young Human Metabolic Research Unit, Cornell University, Ithaca, NY.

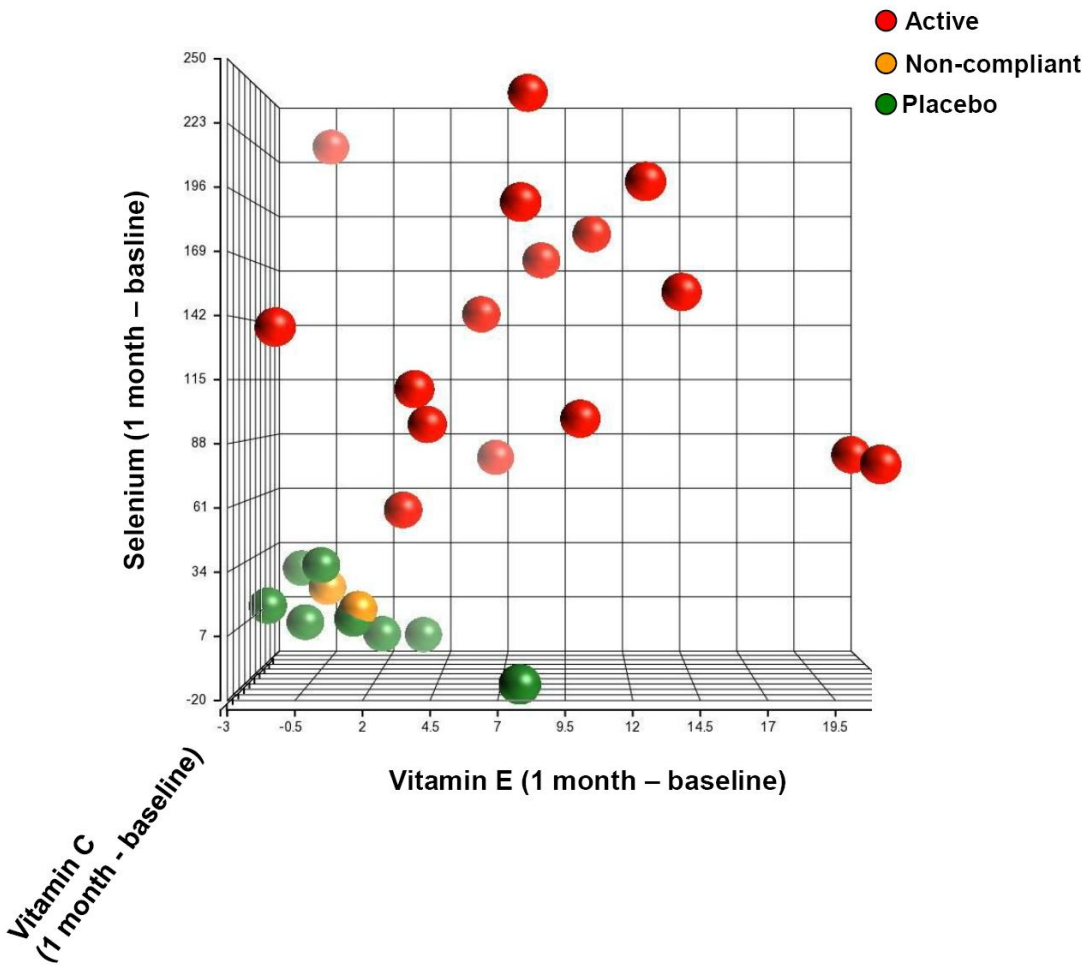

Figure A.1 Principal component analysis (PCA) of plasma nutrient levels of ExSEL participants; two participants assigned to the active supplement arm (yellow markers) were deemed “non-compliant” based on PCA grouping with the participants assigned to the placebo arm of the study.

Table A.2 Gene lists developed by literature review for genes identified in genome-wide association studies (GWAS) of COPD, gene expression studies of COPD, and studies of vitamin E and selenium-responsive genes

Gene Name	Entrez ID	Description
A.2.a. COPD GWAS gene list; N = 6		
BICD1	2671	bicaudal D homolog 1 (Drosophila)
CHRNA3	5927	cholinergic receptor, nicotinic, alpha 3
CHRNA5	11108	cholinergic receptor, nicotinic, alpha 5
FAM13A	17489	family with sequence similarity 13, member A
HHIP	5024	hedgehog interacting protein
SERPINE2	5270	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
A.2.b. COPD differential expression gene list; N = 22		
ADAM33	80332	ADAM metallopeptidase domain 33
CAT	847	catalase
COL1A1	1277	collagen, type I, alpha 1
COL3A1	1281	collagen, type III, alpha 1
EGR1	1958	early growth response 1
FN1	2335	fibronectin 1
GSTP1	2950	glutathione S-transferase pi 1
IL13	3596	interleukin 13
IL1B	3553	interleukin 1, beta
IL8	3576	interleukin 8
MMP10	4319	matrix metallopeptidase 10 (stromelysin 2)
MMP2	4313	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
MMP9	4318	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
PLAU	5328	plasminogen activator, urokinase
PLAUR	5329	plasminogen activator, urokinase receptor
SERPINE2	5270	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
TGFB3	7043	transforming growth factor, beta 3
TIMP1	7076	TIMP metallopeptidase inhibitor 1
TIMP2	7077	TIMP metallopeptidase inhibitor 2
TNF	7124	tumor necrosis factor
VEGFA	7422	vascular endothelial growth factor A
VEGFB	7423	vascular endothelial growth factor B
A.2.c. Vitamin E responsive gene list; N = 42		
ACTA1	7227	actin, alpha 1, skeletal muscle
ALOX12	7198	arachidonate 12-lipoxygenase
ALOX5	3473	arachidonate 5-lipoxygenase
CD36	16861	CD36 molecule (thrombospondin receptor)
CEBPG	3904	CCAAT/enhancer binding protein (C/EBP), gamma
COL1A1	1277	collagen, type I, alpha 1
CTGF	7738	connective tissue growth factor
CYP1A1	8315	cytochrome P450, family 1, subfamily A, polypeptide 1
CYP2A13	5312	cytochrome P450, family 2, subfamily A, polypeptide 13
CYP3A4	13090	cytochrome P450, family 3, subfamily A, polypeptide 4
CYP4F2	6541	cytochrome P450, family 4, subfamily F, polypeptide 2
CYP7B1	5894	cytochrome P450, family 7, subfamily B, polypeptide 1
GFI1	3508	growth factor independent 1 transcription repressor

Table A.2 Gene lists developed by literature review for genes identified in genome-wide association studies (GWAS) of COPD, gene expression studies of COPD, and studies of vitamin E and selenium-responsive genes *(Continued)*

Gene Name	Entrez ID	Description
A.2.c. Vitamin E responsive gene list; N = 42 *(continued)*		
GP2	17066	glycoprotein 2 (zymogen granule membrane)
GSTM3	11498	glutathione S-transferase mu 3 (brain)
HMGCR	7930	3-hydroxy-3-methylglutaryl-CoA reductase
HMGCS2	7851	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)
IGF1	15830	insulin-like growth factor 1 (somatomedin C)
IL17D	4115	interleukin 17D
IL3	6311	interleukin 3 (colony-stimulating factor, multiple)
KRT1	2469	keratin 1
KRT13	11968	keratin 13
KRT15	8523	keratin 15
KRT4	2999	keratin 4
KRT7	3709	keratin 7
LPL	7891	lipoprotein lipase
MMP1	10851	matrix metallopeptidase 1 (interstitial collagenase)
MMP19	3598	matrix metallopeptidase 19
MYH1	6344	myosin, heavy chain 1, skeletal muscle, adult
NOX3	5315	NADPH oxidase 3
NOX5	5470	NADPH oxidase, EF-hand calcium binding domain 5
PGD	3980	phosphogluconate dehydrogenase
POR	6508	P450 (cytochrome) oxidoreductase
PPARG	2477	peroxisome proliferator-activated receptor gamma
SRA1	2473	steroid receptor RNA activator 1
SRD5A1	8341	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)
SULT2B1	2718	sulfotransferase family, cytosolic, 2B, member 1
TNF	7124	tumor necrosis factor
TNNI2	2067	troponin I type 2 (skeletal, fast)
TPM1	8667	tropomyosin 1 (alpha)
TPM2	6127	tropomyosin 2 (beta)
TTPA	19077	tocopherol (alpha) transfer protein
A.2.d. Selenium responsive gene list; N = 42		
CDH5	3236	cadherin 5, type 2 (vascular endothelium)
DPYSL2	3902	dihydropyrimidinase-like 2
ELF3	10820	E74-like factor 3 (ets domain transcription factor, epithelial-specific)
FOSL1	4385	FOS-like antigen 1
FOXC1	19967	forkhead box C1
G6PD	5681	glucose-6-phosphate dehydrogenase
GLRX	4654	glutaredoxin (thioltransferase)
GNGT2	2145	guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2
GPX1	9761	glutathione peroxidase 1
GPX2	4135	glutathione peroxidase 2 (gastrointestinal)
GPX3	6707	glutathione peroxidase 3 (plasma)
GPX4	10100	glutathione peroxidase 4 (phospholipid hydroperoxidase)
GPX7	5452	glutathione peroxidase 7

Table A.2 Gene lists developed by literature review for genes identified in genome-wide association studies (GWAS) of COPD, gene expression studies of COPD, and studies of vitamin E and selenium-responsive genes *(Continued)*

Gene Name	Entrez ID	Description
A.2.d. Selenium responsive gene list; N = 42 *(continued)*		
GRIK1	16028	glutamate receptor, ionotropic, kainate 1
GRIK3	6560	glutamate receptor, ionotropic, kainate 3
GSR	19141	glutathione reductase
GSS	19906	glutathione synthetase
GSTA1	17259	glutathione S-transferase alpha 1
GSTK1	16237	glutathione S-transferase kappa 1
GSTO1	2178	glutathione S-transferase omega 1
HOMER3	3089	homer homolog 3 (Drosophila)
IGF1R	3620	insulin-like growth factor 1 receptor
MGST1	12928	microsomal glutathione S-transferase 1
MS4A1	2109	membrane-spanning 4-domains, subfamily A, member 1
NR2F1	1914	nuclear receptor subfamily 2, group F, member 1
NR4A2	17279	nuclear receptor subfamily 4, group A, member 2
NR5A1	14212	nuclear receptor subfamily 5, group A, member 1
P4HA1	2076	prolyl 4-hydroxylase, alpha polypeptide I
PTGER2	3333	prostaglandin E receptor 2 (subtype EP2), 53kDa
PXN	7128	paxillin
SELK	3643	selenoprotein K
SELP	5264	selectin P (granule membrane protein 140kDa, antigen CD62)
SELT	1950	selenoprotein T
SEPHS1	5618	selenophosphate synthetase 1
SEPP1	9702	selenoprotein P, plasma, 1
SEPW1	16285	selenoprotein W, 1
SNAP23	11915	synaptosomal-associated protein, 23kDa
SORT1	4920	sortilin 1
TGFA	3468	transforming growth factor, alpha
TXN	7142	thioredoxin
TXNRD1	5684	thioredoxin reductase 1
TXNRD2	10329	thioredoxin reductase 2

Table A.3 Genome-wide microarray results; the 25 genes of highest statistical significance in analysis of differential expression in GOLD Stage 2/3/4 lung tissue compared to GOLD 0/1 lung tissue

Gene Symbol	Gene name	Fold change*	Raw p-value	FDR q-value
C9orf30	chromosome 9 open reading frame 30	-1.40151	0.000144	0.266832
CHRAC1	chromatin accessibility complex 1	-1.25305	0.000156	0.266832
CXCL12	chemokine (C-X-C motif) ligand 12	1.6757	0.000156	0.266832
DSC2	desmocollin 2	-1.39102	9.11E-05	0.266832
GMFB	glia maturation factor, beta	-1.37378	2.05E-05	0.266832
HOXA6	homeobox A6	1.245532	6.46E-05	0.266832
ING2	inhibitor of growth family, member 2	-1.57695	0.000109	0.266832
LOC100289494	hypothetical protein LOC100289494	-1.36152	4.49E-05	0.266832
OPN3	opsin 3	-1.42583	0.00014	0.266832
NR2F1	nuclear receptor subfamily 2, group F, member 1	1.541015	0.002575	0.378227
SELT	selenoprotein T	-1.17592	0.003286	0.378227
A2M	alpha-2-macroglobulin	1.245199	0.00182	0.378227
ABHD13	abhydrolase domain containing 13	-1.25626	0.001518	0.378227
ACYP2	acylphosphatase 2, muscle type	-1.20728	0.002389	0.378227
ANXA2P3	annexin A2 pseudogene 3	-1.23764	0.001282	0.378227
ARAP2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	-1.37831	0.000618	0.378227
ARL13B	ADP-ribosylation factor-like 13B	-1.41753	0.00099	0.378227
ARPP19	cAMP-regulated phosphoprotein, 19kDa	-1.17859	0.003087	0.378227
ATP6V1G1	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G1	-1.14135	0.003383	0.378227
BHLHE41	basic helix-loop-helix family, member e41	-1.34452	0.002968	0.378227
BPNT1	3'(2'), 5'-bisphosphate nucleotidase 1	-1.26504	0.002316	0.378227
BRD8	bromodomain containing 8	1.176533	0.003433	0.378227
BRP44L	brain protein 44-like	-1.18758	0.000572	0.378227
BTG1	B-cell translocation gene 1, anti-proliferative	1.212408	0.001581	0.378227
BTN2A1	butyrophilin, subfamily 2, member A1	1.195579	0.001928	0.378227

*Log_2 fold change

Table A.4 COPD GWAS gene list results of analysis of differential expression in GOLD Stage 2/3/4 lung tissue compared to GOLD 0/1 lung tissue

Gene Symbol	Gene name	Fold change*	Raw p-value	FDR q-value†
BICD1	bicaudal D homolog 1 (Drosophila)	1.210257	0.024929	0.0251
HHIP	hedgehog interacting protein	-1.32122	0.117633	0.0403
SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.437754	0.139622	0.0403
CHRNA3	cholinergic receptor, nicotinic, alpha 3	-1.05504	0.159834	0.0403
CHRNA5	cholinergic receptor, nicotinic, alpha 5	-1.03956	0.443572	0.0894
FAM13A	family with sequence similarity 13, member A	1.027545	0.838441	0.1408

*Log_2 fold change
†Estimated for gene list individually

Table A.5 COPD expression gene list results of analysis of differential expression in GOLD Stage 2/3/4 lung tissue compared to GOLD 0/1 lung tissue

Gene Symbol	Gene name	Fold change*	Raw p-value	FDR q-value†
PLAUR	plasminogen activator, urokinase receptor	-1.88258	0.014997	0.0426
VEGFB	vascular endothelial growth factor B	-1.25792	0.025445	0.0426
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	1.519434	0.034261	0.0426
CAT	catalase	-1.21559	0.073803	0.0653
VEGFA	vascular endothelial growth factor A	-1.22743	0.107217	0.0653
TIMP1	TIMP metallopeptidase inhibitor 1	1.346775	0.12917	0.0653
SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.437754	0.139622	0.0653
COL1A1	collagen, type I, alpha 1	1.492563	0.140078	0.0653
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	1.801175	0.189926	0.0738
TNF	tumor necrosis factor	1.132183	0.198166	0.0738
MMP10	matrix metallopeptidase 10 (stromelysin 2)	1.204124	0.217626	0.0738
ADAM33	ADAM metallopeptidase domain 33	1.074191	0.269388	0.0834
COL3A1	collagen, type III, alpha 1	1.37664	0.290469	0.0834
FN1	fibronectin 1	1.070624	0.373036	0.0877
EGR1	early growth response 1	1.548171	0.379473	0.0877
IL8	interleukin 8	-1.67248	0.401375	0.0877

Table A.5 COPD expression gene list results of analysis of differential expression in GOLD Stage 2/3/4 lung tissue compared to GOLD 0/1 lung tissue *(continued)*

Gene Symbol	Gene name	Fold change*	Raw p-value	FDR q-value†
TGFB3	transforming growth factor, beta 3	1.125656	0.403536	0.0877
IL13	interleukin 13	-1.0517	0.42577	0.0877
PLAU	plasminogen activator, urokinase	-1.15553	0.446406	0.0877
TIMP2	TIMP metallopeptidase inhibitor 2	-1.03375	0.662188	0.1235
IL1B	interleukin 1, beta	-1.06185	0.87487	0.1516
GSTP1	glutathione S-transferase pi 1	1.008482	0.894088	0.1516

*Log_2 fold change
†Estimated for gene list individually

Table A.6 Vitamin E-responsive gene list results of analysis of differential expression in GOLD Stage 2/3/4 lung tissue compared to GOLD 0/1 lung tissue

Gene Symbol	Gene name	Fold change*	Raw p-value	FDR q-value†
TNNI2	troponin I type 2 (skeletal, fast)	-1.34288	0.006746	0.0241
KRT1	keratin 1	1.513615	0.018596	0.0241
SRA1	steroid receptor RNA activator 1	-1.15596	0.018731	0.0241
PPARG	peroxisome proliferator-activated receptor gamma	-1.76395	0.018851	0.0241
SULT2B1	sulfotransferase family, cytosolic, 2B, member 1	-1.42175	0.026493	0.0271
KRT4	keratin 4	-2.29597	0.035531	0.0303
ALOX5	arachidonate 5-lipoxygenase	-1.3354	0.052753	0.0306
GFI1	growth factor independent 1 transcription repressor	1.118384	0.054023	0.0306
MMP19	matrix metallopeptidase 19	-1.67281	0.057065	0.0306
KRT7	keratin 7	1.385154	0.06216	0.0306
CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	-1.13516	0.070046	0.0306
PGD	phosphogluconate dehydrogenase	-1.29376	0.072839	0.0306
IL17D	interleukin 17D	-1.41034	0.077823	0.0306
CYP2A13	cytochrome P450, family 2, subfamily A, polypeptide 13	1.105373	0.13142	0.0421
NOX3	NADPH oxidase 3	-1.08219	0.131562	0.0421
NOX5	NADPH oxidase, EF-hand calcium binding domain 5	-1.06578	0.139037	0.0421
COL1A1	collagen, type I, alpha 1	1.492563	0.140078	0.0421
CYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1	-1.10751	0.158604	0.0422
TPM2	tropomyosin 2 (beta)	1.212658	0.169813	0.0422
IL3	interleukin 3 (colony-stimulating factor, multiple)	-1.07219	0.178549	0.0422
MYH1	myosin, heavy chain 1, skeletal muscle, adult	1.397803	0.180132	0.0422

Table A.6 Vitamin E-responsive gene list results of analysis of differential expression in GOLD Stage 2/3/4 lung tissue compared to GOLD 0/1 lung tissue *(continued)*

Gene Symbol	Gene name	Fold change*	Raw p-value	FDR q-value†
POR	P450 (cytochrome) oxidoreductase	-1.17823	0.187581	0.0422
CYP4F2	cytochrome P450, family 4, subfamily F, polypeptide 2	-1.08543	0.189601	0.0422
TNF	tumor necrosis factor	1.132183	0.198166	0.0422
ALOX12	arachidonate 12-lipoxygenase	-1.06314	0.224057	0.0444
ACTA1	actin, alpha 1, skeletal muscle	-1.0717	0.225743	0.0444
CTGF	connective tissue growth factor	1.340231	0.252021	0.0449
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	-1.42727	0.258687	0.0449
LPL	lipoprotein lipase	-1.14447	0.260949	0.0449
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	-1.1864	0.263609	0.0449
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	-1.0573	0.28408	0.0453
SRD5A1	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	1.100896	0.284933	0.0453
KRT15	keratin 15	1.313472	0.294143	0.0453
TPM1	tropomyosin 1 (alpha)	1.143493	0.301014	0.0453
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	1.492249	0.428372	0.0626
GSTM3	glutathione S-transferase mu 3 (brain)	-1.16204	0.468294	0.0665
KRT13	keratin 13	-1.03788	0.495784	0.0685
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	1.028747	0.562043	0.0757
IGF1	insulin-like growth factor 1 (somatomedin C)	-1.09665	0.733641	0.0962
CD36	CD36 molecule (thrombospondin receptor)	1.034515	0.797892	0.1013
GP2	glycoprotein 2 (zymogen granule membrane)	-1.00742	0.812039	0.1013
TTPA	tocopherol (alpha) transfer protein	-1.00494	0.942894	0.1148

*Log_2 fold change
†Estimated for gene list individually

Table A.7 Selenium-responsive gene list results of analysis of differential expression in GOLD Stage 2/3/4 lung tissue compared to GOLD 0/1 lung tissue

Gene Symbol	Gene name	Fold change*	Raw p-value	FDR q-value†
NR2F1	nuclear receptor subfamily 2, group F, member 1	1.541015	0.002575	0.0347
SELT	selenoprotein T	-1.17592	0.003286	0.0347
P4HA1	prolyl 4-hydroxylase, alpha polypeptide I	-1.3145	0.006994	0.0347
MS4A1	membrane-spanning 4-domains, subfamily A, member 1	2.247885	0.007846	0.0347
GNGT2	guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	-1.39245	0.008919	0.0347
GSTO1	glutathione S-transferase omega 1	-1.2314	0.009757	0.0347
HOMER3	homer homolog 3 (Drosophila)	1.153352	0.038264	0.1048
CDH5	cadherin 5, type 2 (vascular endothelium)	1.43305	0.04402	0.1048
PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	-1.2226	0.047528	0.1048
TGFA	transforming growth factor, alpha	-1.08058	0.052575	0.1048
IGF1R	insulin-like growth factor 1 receptor	1.150651	0.057878	0.1048
SELK	selenoprotein K	-1.11716	0.058934	0.1048
DPYSL2	dihydropyrimidinase-like 2	1.104713	0.07002	0.1149
GPX2	glutathione peroxidase 2 (gastrointestinal)	1.131863	0.078732	0.1200
FOSL1	FOS-like antigen 1	-1.71855	0.088596	0.1260
GLRX	glutaredoxin (thioltransferase)	-1.1981	0.100734	0.1343
SORT1	sortilin 1	-1.1286	0.112609	0.1413
SELP	selectin P (granule membrane protein 140kDa, antigen CD62)	1.467479	0.129206	0.1438
GPX7	glutathione peroxidase 7	1.222907	0.138196	0.1438
SEPHS1	selenophosphate synthetase 1	1.128556	0.145634	0.1438
G6PD	glucose-6-phosphate dehydrogenase	-1.16261	0.148141	0.1438
TXNRD1	thioredoxin reductase 1	-1.19544	0.148331	0.1438
GRIK3	glutamate receptor, ionotropic, kainate 3	-1.06849	0.19071	0.1766
GPX3	glutathione peroxidase 3 (plasma)	1.20025	0.198716	0.1766
PXN	paxillin	1.061279	0.220157	0.1811
TXN	thioredoxin	-1.09975	0.220748	0.1811
SEPP1	selenoprotein P, plasma, 1	1.050406	0.359795	0.2767
GPX1	glutathione peroxidase 1	-1.09406	0.36321	0.2767
GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	-1.06856	0.384233	0.2827
TXNRD2	thioredoxin reductase 2	-1.06666	0.398755	0.2836
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	-1.38783	0.426537	0.2935

Table A.7 Selenium-responsive gene list results of analysis of differential expression in GOLD Stage 2/3/4 lung tissue compared to GOLD 0/1 lung tissue *(continued)*

Gene Symbol	Gene name	Fold change*	Raw p-value	FDR q-value†
SNAP23	synaptosomal-associated protein, 23kDa	1.057051	0.492559	0.3284
MGST1	microsomal glutathione S-transferase 1	-1.06892	0.55232	0.3571
NR5A1	nuclear receptor subfamily 5, group A, member 1	-1.02478	0.63119	0.3960
GRIK1	glutamate receptor, ionotropic, kainate 1	1.017431	0.747055	0.4396
GSTK1	glutathione S-transferase kappa 1	1.018051	0.758947	0.4396
SEPW1	selenoprotein W, 1	-1.02027	0.762453	0.4396
GSTA1	glutathione S-transferase alpha 1	1.17028	0.824007	0.4516
NR4A2	nuclear receptor subfamily 4, group A, member 2	1.116419	0.825587	0.4516
GSR	glutathione reductase	-1.00589	0.946505	0.5048
GSS	glutathione synthetase	1.000382	0.994643	0.5074
FOXC1	forkhead box C1	-1.00031	0.998918	0.5074

*Log_2 fold change
†Estimated for gene list individually