THE ROLE OF VITAMIN D IN PULMONARY FUNCTION AND LUNG GENE EXPRESSION

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Brian John Reardon

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Brian John Reardon, Ph. D.

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Decline in lung function is a risk factor for chronic obstructive pulmonary disease (COPD). Vitamin D may protect against loss of lung function by modulating inflammation and tissue remodeling. We investigated the association of serum 25-hydroxyvitamin D (25-OH-D) and genetic variants in vitamin D metabolic pathway genes with prognostic measures of obstructive lung disease including forced expiratory volume in the first second (FEV_1) and the ratio of FEV1 to forced vital capacity (FEV_1/FVC) using data from the Health Aging and Body Composition cohort study of adults aged 70-79. We also obtained the first evidence of differential gene expression in lung epithelial cells associated with serum 25-hydroxyvitamin D in free-living humans. In regression models, there was a significant cross-sectional association of 25-OH-D with FEV_1. We detected no association between 25-OH-D and rate of FEV_1 decline over 10 years. The longitudinal results suggest that vitamin D supplementation in non-deficient individuals is unlikely to prevent decline in lung function in this age range. However, results are also compatible with a beneficial effect of vitamin D earlier in life, which may

explain the cross-sectional association of 25-OH-D with lung function. Single nucleotide polymorphisms (SNPs) in vitamin D metabolic pathway genes were tested in linear regression models stratified by race. In African-Americans, rs3886163 and 2 haplotypes in *CYP24A1* were associated with FEV_1, and rs11168293 in *VDR* was associated with FEV_1/FVC, after correction for multiple testing. Two gene-environment interactions, between serum 25-OH-D and SNPs in *RXRA,* in European-Americans were significant with false discovery rate (FDR) < 0.2. Microarray analysis was used to investigate gene expression in small airway epithelial cells associated with serum 25-OH-D in a separate study of 26 healthy, adult never-smokers. Analysis was restricted to 156 candidate genes with prior evidence of vitamin D-modulated gene expression *in vitro* and at least 1 predicted vitamin D response element. Thirteen genes had significant differences in expression, and 3 genes (*KCNS3*, *FSTL1*, and *DAPK1*) were significant with FDR < 0.2. Gene ontology and literature analysis of differentially expressed genes supported plausible mechanisms for functional roles in asthma, COPD, cancer, and response to infection in lung. The physiological range of 25-OH-D is associated with functional differences in molecular outcomes in lung, implying mechanisms that explain and strengthen the plausibility of population-level studies showing associations of vitamin D with lung health.

BIOGRAPHICAL SKETCH

Brian John Reardon was born in the Philippines and grew up moving with his father's Navy career in Puerto Rico, Colorado, Rhode Island, Massachusetts, and Maine. He attended the University of Maine and earned a B.S. in Microbiology in 1996. After college, he moved to Boston and began working at a biotechnology start-up company that, through a series of buyouts, moved him to San Francisco and ultimately became part of Celera Genomics. After three years, he joined another start-up called Exelixis, where he worked for five years before moving to Genentech in his final two years in industry. His work over this decade in biotechnology was primarily in target discovery and drug development for cancer, giving him a wide variety of laboratory experience, from *C. elegans* genetics to molecular cell biology and functional genomics. He moved across country once again to go to Cornell for graduate school, where he became interested in "dry lab" approaches and joined the research group of Patricia Cassano. A nutritional biochemistry course introduced him to the fascinating biological effects of vitamin D, and left him enthusiastic about its potential importance in human health. Professor Cassano encouraged him to pursue this interest, resulting in the studies described in this dissertation.

For Holly and Helen

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CHAPTER 1

INTRODUCTION TO VITAMIN D AND LUNG HEALTH

Vitamin D Sources and Requirements

Vitamin D is a prohormone that can be obtained either through diet/supplements or through biosynthesis in skin upon exposure to solar ultraviolet rays (UVB) [1,2]. Ultraviolet rays from the sun can convert 7-dehydrocholesterol in skin into pre-vitamin D, which is subsequently converted into vitamin D [1]. Good dietary sources of vitamin D include fatty fish, supplements, and fortified foods such as milk and margarine [2]. Recently, the Institute of Medicine (IOM) increased the Recommended Dietary Allowance (RDA) to 600 IU per day for adults and children age one and above (http://www.iom.edu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D.aspx).

Vitamin D Metabolism

Vitamin D from diet or produced through solar exposure is transported to the liver, where it is enzymatically converted by 25-hydroxylase (encoded by *CYP2R1*) into the major circulating form, 25-hydroxyvitamin D [3]. The largely unregulated hepatic conversion of both dietary and sunlight-derived vitamin D into 25-OH-D [4] makes serum 25-OH-D a good measure of vitamin D nutriture and the primary biomarker for vitamin D exposure [5]. Vitamin D is highly hydrophobic and requires the carrier molecule, Vitamin D binding protein (also known as DBP, encoded by *GC*), to circulate in the bloodstream. 25-hydroxyvitamin D is thus carried to the kidney, where it is

enzymatically converted to the more active form, 1,25-dihydroxyvitamin D, by 1α-hydroxylase (*CYP27B1*). In addition to endocrine activity of kidney-derived 1,25-dihydroxyvitamin D, evidence suggests a role for autocrine or paracrine 1,25-dihydroxyvitamin D in regulation of immune function, cell growth, as well as many other functions [6,7]. This active metabolite is not used as a serum biomarker for vitamin D nutriture because it has a short half-life, is tightly regulated, and can be generated in localized tissues on demand [8]. Removal of the active form is accomplished when 1,25-dihydroxyvitamin D is degraded by the 24-hydroxylase (*CYP24A1*) to form 1,24,25-trihydroxyvitamin D.

Vitamin D as a Steroid Hormone

The cellular mechanism of action for vitamin D is through its ability to directly modulate gene expression, which has been clearly demonstrated *in vitro* [9,10]. Vitamin D influences gene expression through the binding of 1,25-OH_2-D to the vitamin D receptor (VDR) in the cytoplasm of the target cell, as depicted in Figure 1.1. The 1,25-OH_2-D:VDR complex, a heterodimer, enters the nucleus and can bind the retinoid X receptor (RXR). The newly formed complex is able to recognize and bind target DNA sequences called vitamin D response elements (VDRE) [10,11]. VDR and RXR affect the ability of 1,25-OH_2-D to bind VDREs and thus have an important role in expression of VDRE containing genes [10,11]. VDREs are frequently located within the promoter regions of genes, and binding to a VDRE leads to regulation of transcription and thus regulation of the gene's protein product [9,12]. VDRE-containing genes are involved in a diverse array of physiological functions, including inflammation and immunomodulation [9,13,14].

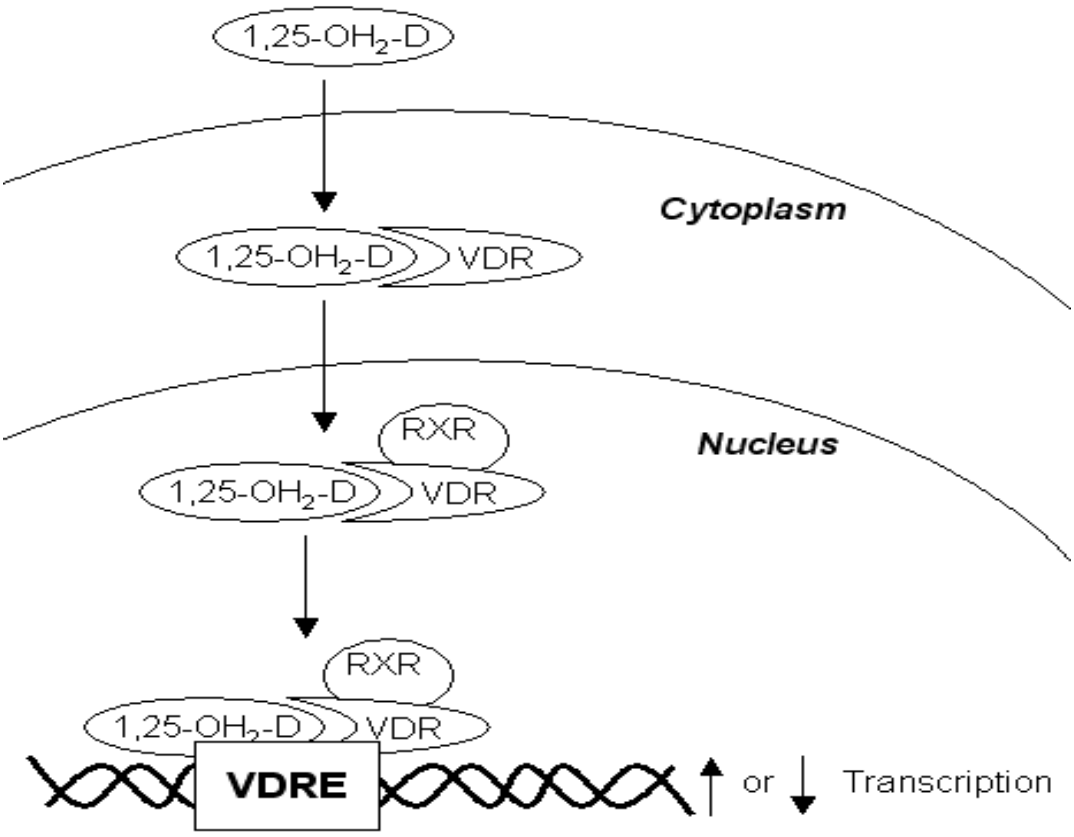

Figure 1.1. Vitamin D regulation of transcription through binding vitamin D responsive elements

Previous work in cell lines has led to the identification of genes directly up or down regulated by 1,25-OH_2-D, and data mining has generated lists of genes with putative DR3 or ER6 hexameric VDRE sequences in their promoter regions [9].

COPD

Chronic Obstructive Pulmonary Disease (COPD) is the fourth leading cause of death in the United States and has been diagnosed in more than 12 million Americans [15,16]. The obstruction in the small airways of the lungs that characterizes COPD is typically diagnosed by using information from pulmonary function tests, which measure forced expiratory volume in the first second (FEV_1) and maximum expiratory air volume when emptying the lungs (forced vital capacity, or FVC). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines mild or stage 1 COPD according to the ratio of FEV_1 to FVC (FEV_1/FVC): a ratio less than 0.7 and an $FEV_1 \geq 80\%$ predicted (by age, race, gender, and height); similarly, other levels of COPD are defined by a ratio less than 0.7 and lower values for % predicted FEV_1[17]. A steep decline in lung function is characteristic of COPD, and is also associated with increased risk of all-cause mortality in the elderly[18]. Pulmonary function decreases with age [19], although age-related decreases are typically not steep enough to lead to the loss of lung function that characterizes COPD. Cigarette smoking is the single most important risk factor for steep declines in FEV_1, however not all smokers develop COPD, and about 15% of COPD-related mortality arises in persons who have never smoked [20]. It is unclear why some non-smokers develop COPD and why some smokers never develop COPD.

There are likely additional environmental and/or genetic factors affecting the trajectory of lung function decline that have not yet been identified [21,22]. Mechanisms contributing to COPD in non-smokers and susceptibility factors in smokers are of great interest as they could suggest new targets for prevention efforts [19].

The inflammatory and tissue-remodeling processes of the cellular immune system can influence risk for obstructive lung disease. These processes in the lung contribute to irreversible limitations of airflow [23]. Chronic inflammation within the small airways is a key characteristic of COPD pathology. This inflammation occurs when exogenous substances enter the lung; in COPD sufferers, the most common exogenous exposure is cigarette smoke. The effects of cigarette smoke have been demonstrated in animal models; for example, mice exposed to cigarette smoke for 24 weeks had an increased presence of inflammatory cells (neutrophils, macrophages, and lymphocytes) in small and large airways [24]. Dendritic cells have also been shown to infiltrate COPD airways, and their accumulation is proportional to disease severity [25]. Exposure to cigarette smoke activates dendritic cells and upregulates the expression of major histocompatibility complex II (MHC II) and co-stimulatory molecules, CD40 and CD86 [24]. These effects on dendritic cells are part of the chronic inflammatory response to smoking that is observed in COPD [26]. Several studies suggest a relation between biomarkers of systemic inflammation and COPD, although results are not consistent. A cross-sectional analysis in the Health, Aging, and Body Composition Study (Health ABC) found an inverse association between IL-6 and FEV_1 in elderly participants with or without obstructive lung disease (regression coefficients: $\beta = -5.3$ and -3.1, respectively; $p = .09$) [27]. Patients with stable COPD had elevated serum levels of TNF-α and IL-6

[28], and a case control study of men with COPD reported a significant association of C-reactive protein with COPD risk, but little or no association of either IL-6 or TNF-α [5,28].

Tissue remodeling is understood to be another key factor in COPD pathology. The process of tissue remodeling affects the airways in COPD lungs through a combination of tissue destruction, reducing elasticity, and thickening of the walls of the small airways, which reduces airway diameter [29,30]. Thus, environmental or genetic factors producing individual differences in tissue remodeling capacity or efficiency is postulated to be related to disease risk.

Vitamin D and lung health

Although vitamin D is known primarily as a hormone responsible for regulation of serum calcium and phosphorous, preliminary evidence suggests a role for vitamin D in the occurrence and progression of COPD, as well as several other diseases affecting lung. Low serum 25-hydroxyvitamin D was associated with higher risk for severe exacerbation of mild-to-moderate persistent asthma in children [31], and a higher risk for active tuberculosis [32]. The possible role of vitamin D in lung cancer is controversial, but low serum 25-hydroxyvitamin D was a risk factor for lung cancer in women and young people [33], and high serum 25-hydroxyvitamin D was associated with improved survival in non-small cell lung cancer patients [34]. In contrast, cancer mortality was associated with both very low and very high vitamin D levels [35]. Vitamin D is associated with both asthma and COPD, but whether effects are causal is unknown, and mechanisms are not well understood [36].

Recent findings from the National Health and Nutrition Examination Survey show a direct association between serum 25-OH-D concentration and pulmonary function (FEV_1 and FVC); these associations were strongest in the oldest age group studied (≥ 60 years) [37]. However, a recent cross-sectional reported no association between serum 25-hydroxyvitamin D and lung function [38]. A prospective case-control study, comparing smokers with rapid rate of decline in FEV_1 to smokers with shallow decline, reported no difference in plasma vitamin D at the beginning of the study period [39], but the analysis did not consider confounding and/or interactions with race, and the study did not include non-smokers. Despite conflicting epidemiological evidence, studies in cell culture and animal models have shown that 1,25-dihydroxyvitamin D affects expression of genes related to inflammation, cell proliferation, and cell migration [40,41], which suggests plausible causal mechanisms for vitamin D associations with asthma, COPD, and lung cancer. The limited epidemiologic data highlighted the need for further studies of these associations.

Numerous studies connect vitamin D with modulation of chronic inflammation and airway remodeling [6,40,42,43,44,45]. For example, vitamin D inhibits the differentiation and maturation of dendritic cells, causing downregulation of MHC II, CD40, and CD86 [41,46], suggesting a possible role for vitamin D in preventing or mitigating the responses of dendritic cells to chronic cigarette smoke exposure. Other evidence suggests that vitamin D plays a role in suppression of inflammation through its effects on T helper 1 cells (Th_1). Increased Th_1 levels have been found in the bronchoalveolar lavage of COPD patients [47]. Th_1 cells contribute to the immune process primarily by increasing production of both CD8+ T cells and cytokines, including interferon-gamma

(IFN-γ) and tumor necrosis factor-alpha (TNF-α) [48]. Vitamin D acts as an immunosupressive by impairing the production of key inflammatory Th_1 cytokines. The active form of vitamin D, 1,25-dihydroxyvitamin D, inhibits secretion of the Th_1 cytokines interleukin-2 (IL-2) and IFN-γ [49], and the serum biomarker, 25-hydroxyvitamin D, is associated with levels of these cytokines [49,50,51,52]. Additionally, 1,25-dihydroxyvitamin D can inhibit myelocyte secretion of IL-12 [53], which is an essential cytokine in the differentiation of naïve T helper cells into Th_1 cells [54]. Vitamin D is also associated with biological markers of systemic inflammation. In the Baltimore Hip Studies (a cohort study of women ≥ 65 years of age with hip fractures in the previous year), study participants with vitamin D deficiency (serum 25-OH-D ≤ 15 ng/ml) had increased levels of IL-6 ($p = 0.02$), supporting the notion that vitamin D deficiency is proinflammatory [50]. Similarly, a study in healthy women (age 25 - 82 years) reported an inverse association between serum 25-OH-D and TNF-α ($p = 0.0463$) [51].

The evidence that vitamin D plays a key role in tissue remodeling is mounting. A recent study of gene expression in cultured bronchial smooth muscle cells found more than 400 genes that were up or down-regulated in response to 1,25-dihydroxyvitamin D treatment; most of these genes were associated with cell proliferation and cell migration, hence likely to be important in the process of tissue remodeling and perhaps in carcinogenesis [40]. The matrix metalloproteases (MMPs) are involved in tissue remodeling in COPD [55]. Compared to healthy controls, patients with COPD have higher levels of sputum MMP-9 and a higher ratio of MMP-9 to its inhibitor, TIMP1 [56]. Treatment of isolated bronchial smooth muscle cells with 1,25-dihydroxyvitamin D resulted in reduced expression of the metalloproteases MMP-9 and ADAM-33 [44].

Conversely, the metalloprotease inhibitors, TIMP-1 and TIMP-2, were found to be induced by 1,25-dihydroxyvitamin D in other cellular models [30,57]. Gene expression profiles of COPD lungs revealed increased expression of a protease, urokinase plasminogen activator (uPA), and its receptor, uPAR, both of which have been implicated in cell migration and extra-cellular matrix degradation [58]. Increased uPAR expression was associated with lower pulmonary function ($FEF_{25-75\%}$) [58]. In breast carcinoma cells, uPA expression was downregulated by 1,25-dihydroxyvitamin D in a dose-dependent manner [30]. Taken together, these findings are provocative, and point to a plausible biological mechanism linking vitamin D nutriture and COPD risk: this link, namely a role of vitamin D in tissue remodeling, has not been investigated directly to date. Only one intervention study has reported the effect of vitamin D supplementation on inflammation and tissue remodeling biomarkers: in vitamin D deficient British Bangladeshi men, vitamin D supplementation (four intramuscular injections of 500 IU each over one year) decreased circulating serum levels of MMP-9 (68%) and CRP (23%) in relation to pre-supplementation values. Vitamin D intervention was the only predictor of serum MMP-9 concentrations [59].

Interestingly, preliminary evidence suggests that SNPs in the gene encoding the vitamin D binding protein, or *GC*, are associated with COPD risk. A study comparing the relative risk for COPD associated with the three most common *GC* alleles, *GC1F*, *GC1S*, and *GC2* (alleles each having different non-synonomous polymorphisms) suggested a protective effect for the homozygous *GC2* genotype (OR = 0.17, 95% CI = 0.03 to 0.83) [60]. Similar results were observed in previous studies: the homozygous *GC2* genotype was protective, with relative risks of 0.2 and 0.7 for COPD [61,62]. A

separate study in 88 healthy, Japanese smokers identified homozygous *GC1F* as an increased COPD risk genotype (OR = 2.3; 95% CI = 1.2 to 4.6) [63]. A study comparing COPD smokers with asymptomatic smokers in Han Chinese found a higher proportion of the *GC1F* allele in COPD smokers, with an odds ratio of 3.5 for the homozygous *GC1F* genotype. This study also found a higher proportion of the *GC2* allele in asymptomatic smokers than in COPD smokers [64]. A proposed mechanism for the functional effects of the *GC* alleles stems from their effect on vitamin D binding affinities: differences are reported among the three common *GC* alleles. Of the three common alleles, *GC1F* had the lowest affinity for vitamin D, with a Ka of 1.12 ± 0.13 nM and 1.80 ± 0.21 nM for 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, respectively. The *GC1S* Ka was 0.60 ± 0.15 nM and 0.64 ± 0.10 nM for 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, respectively. The *GC2* genotype had the highest affinity for vitamin D, with a Ka of 0.36 ± 0.10 nM and 0.42 ± 0.11 nM for 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, respectively [65]. A difference in binding affinity could produce a difference in concentration of vitamin D reaching cells; measures of gene expression at the target cell would reveal this relationship, but no published research has studied this directly. Polymorphisms in *VDR* are also associated with lung phenotypes, including sarcoidosis [66], asthma [67,68], pulmonary tuberculosis [69], and non-small cell lung cancer [34]. If the associations with vitamin D are causal, the public health implications are significant because the consequences of vitamin D insufficiency are amenable to a simple intervention.

Factors Affecting Vitamin D Status

Genetics may also affect levels of circulating vitamin D; the SUNLIGHT consortium

reported significant contribution of transport and metabolism genes to serum 25-hydroxyvitamin D concentrations [9], and high heritability of serum vitamin D concentrations [70]. Skin color also affects vitamin D levels because skin pigmentation interferes with vitamin D synthesis in response to sunlight [5]. An estimated 32% of African Americans are deficient (<12 ng/mL serum 25-hydroxyvitamin D) in vitamin D [71]. Aging Americans are also more likely to have insufficient levels of serum 25-hydroxyvitamin D, in addition to being at risk of lower pulmonary function [72,73]. The prevalence of vitamin D deficiency has been shown to increase with age [72]; the elderly (> 65 years of age) are most at risk for vitamin D deficiency due to a combination of biological and lifestyle factors, including reduced production of vitamin D in skin, reduced outdoor activity, and fewer sources of vitamin D in the diet [73]. Because inadequate vitamin D levels are common and frequently overlooked in preventive medical care [74], this modifiable environmental factor could affect a large proportion of the at-risk population for COPD and other lung disorders.

Unresolved Issues in the Field

Observational studies of vitamin D share an important limitation in that status of this nutrient is often correlated with general health; moreover, because illness may lead to reduced outdoor activity and sun exposure, reverse causation may be at play in cross-sectional studies. In addition, association studies of vitamin D typically rely on a single measurement of serum 25-hydroxyvitamin D, which may not be an accurate proxy for an individual's average status over a lifetime, from the prenatal period through adulthood. Attempts to adjust statistically for seasonal variation of serum 25-hydroxyvitamin D levels are also imperfect.

The recent IOM specification of the RDA for vitamin D disappointed some critics who claimed that requirements were not high enough; controversy remains because some researchers in the field maintain that at least 75 nM 25-hydroxyvitamin D (30 ng/mL) should be the cut-point for adequacy, whereas the IOM recommendations are based on dietary amount needed to attain 50 nM (20 ng/mL) serum 25-hydroxyvitamin D [75]. The IOM panel used only bone health to develop the dietary reference intakes; evidence for other outcomes, such as cancer, respiratory infections, and asthma, was rejected based on dubious causality (as described above for observational studies), contradictory results (as described above for cancer), or insufficient data to establish a dose-response relationship [75]. The latter problem is especially relevant to lung outcomes, in which the majority of evidence is based on effects of 1,25-dihydroxyvitamin D, without any known correspondence of these doses to levels of the biomarker, 25-hydroxyvitamin D. Thus, studies designed to bridge the gap between effects of 1,25-dihydroxyvitamin D *in vitro* and associations of 25-hydroxyvitamin D *in vivo* are especially needed.

Summary of Research Approach

The studies described in this dissertation seek to create a connection between *in vitro* and animal model experimentation and observational evidence in humans. Although our work is observational in nature, the studies were designed to strengthen causal inferences, and to demonstrate mechanisms of vitamin D action in free-living humans. A conceptual framework of the hypothesized mechanisms for the associations being studied in this dissertation is depicted in Figure 1.2.

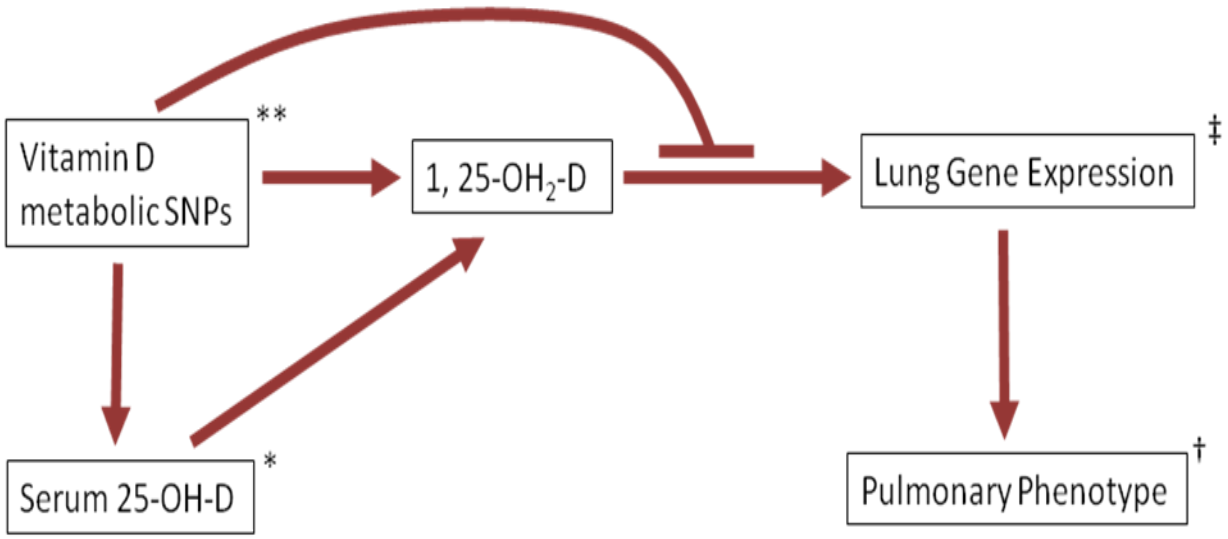

Figure 1.2. Hypothesized mechanism of action for vitamin D and vitamin D metabolic SNPs in lung health.

The first study investigates the hypothesis that higher serum 25-OH-D is associated with attenuation in the rate of decline in FEV_1 using data available from the Health, Aging and Body Composition Study (Health ABC). Health ABC is a prospective study of 3,075 healthy (free from disability and functional limitation in daily living) people aged 70 to 79 at baseline focused on changes in body composition and decline in function in healthy, older persons. The study has approximately as many male as female participants, with African Americans representing 33% of male and 46% of female participants. By examining both cross-sectional and longitudinal relationships, we aim to provide evidence less susceptible to reverse causality than previous epidemiologic studies of vitamin D and lung function.

The second study investigates the association of genetic variation in vitamin D metabolic pathway genes with lung function (FEV_1 and FEV_1/FVC) using data from the Health ABC 1 Million marker genome-wide association study. We examine all genes that function in the pathway, from 25-hydroxyvitamin D synthesis to degradation of 1,25-dihydroxyvitamin D. Relationships of individual single nucleotide polymorphisms (SNPs), haplotypes, and gene-environment interactions of serum 25-hydroxyvitamin D level and genotype are examined. Genetic evidence represents the cumulative effect of a lifetime of altered vitamin D functionality; as such, it is free from the lifestyle confounding and measurement issues that may be problematic in association studies based on 25-hydroxyvitamin D concentration. Positive associations found in this study will greatly support a role for vitamin D in lung function.

The third study investigates the hypothesis that low/inadequate serum 25-OH-D is

associated with significant changes in gene expression in small airway lung epithelial cells, and that these changes occur in genes from functional categories potentially relevant to pathogenesis of COPD and other lung diseases. This study reveals underlying mechanisms for vitamin D effects in lung, and establishes a relationship between function at the molecular level and circulating concentration of 25-hydroxyvitamin D in free-living humans.

Conclusion

Preliminary evidence for a possible role for vitamin D in maintaining lung health has already engendered a call for a vitamin D intervention study in COPD [76]. However, even if the conflicting epidemiological evidence is to be disregarded, not enough data exists to determine optimal vitamin D levels or dosing, or the appropriate age group to receive such an intervention. Moreover, the mechanisms underlying an effect of vitamin D in lung tissue have not been fully elucidated.

The studies presented here attempt to replicate cross-sectional findings of an association of vitamin D with lung function, and extend those findings to longitudinal effects in an elderly population. These results may aid in determination of optimal age ranges for preventive intervention strategies. We also investigate the role of vitamin D in lung health more directly, through a study of SNPs in genes that control vitamin D metabolism. Understanding gene regulation by vitamin D in lung epithelium could also provide further evidence for functional mechanisms of protection against COPD, which must be better understood if improved treatments are to be found.

No studies to date have examined whether genes regulated by vitamin D in cell models are similarly regulated in free-living humans. In view of the current controversy over recommendations for optimal serum 25-hydroxyvitamin D [77], a method to assess dose-dependent function via gene expression is important to the broader field of vitamin D research and public health. Furthermore, characterization of changes in gene expression associated with low serum 25-hydroxyvitamin D will help to demonstrate that differences in serum 25-hydroxyvitamin D are associated with meaningful physiological changes in gene regulation. These studies have the potential to identify genetic markers and new drug targets related to lung function decline and COPD risk, and to inform preventive medicine and clinical care by providing evidence relevant to lifestyle interventions to optimize vitamin D levels, which may be important in extending the healthy lifespan.

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CHAPTER 2
ASSOCIATION OF SERUM VITAMIN D WITH LUNG FUNCTION IN THE HEALTH, AGING AND BODY COMPOSITION (HEALTH ABC) STUDY

Abstract

Decline in lung function is a risk factor for chronic obstructive pulmonary disease (COPD). Vitamin D may protect against loss of lung function through modulation of inflammation and tissue remodeling processes. We investigated the cross-sectional and longitudinal association of serum 25-hydroxyvitamin D (25-OH-D) with prognostic measures of obstructive lung disease, FEV_1 and FEV_1/FVC. Analysis was conducted in the Health ABC study of well-functioning adults aged 70-79. The association of 25-OH-D and FEV_1 and FEV_1/FVC was estimated in regression models, which were adjusted for age, sex, height, race, smoking, season, and study site. The cross-sectional association of 25-OH-D with FEV_1 was statistically significant, positive, and non-linear ($p<0.0001$ and $p=0.0012$ for linear and quadratic model terms, respectively). Participants with 10 ng/mL serum 25-OH-D (in the range defined as deficient) had a mean baseline FEV_1 132 mL lower than participants with 30 ng/mL 25-OH-D (in the range defined as adequate). In the initial longitudinal analysis, higher serum 25-OH-D was associated with a steeper FEV_1 decline. However, further inspection showed that the shallower rate of FEV_1 decline in participants with low 25-OH-D was due to increased missed clinic visits and subsequent selective loss of FEV_1 measurements in these participants over 10 years of follow-up. When analyses were limited to the upper 2 tertiles of serum 25-OH-D (20.6 ng/mL - 75 ng/mL), there was no association between 25-OH-D and rate of FEV_1 decline. The longitudinal results suggest that vitamin D

supplementation in non-deficient individuals is unlikely to prevent decline in lung function in this age range. However, results are also compatible with a beneficial effect of vitamin D earlier in life, which may explain the cross-sectional association of 25-OH-D with lung function.

Introduction

A steep decline in lung function is the hallmark of chronic obstructive pulmonary disease (COPD), and also a predictor of an increased risk of all-cause mortality in the elderly[1]. COPD is the third leading cause of death in the United States, and an understanding of mechanisms contributing to decline in lung function is a critical public health concern[2,3]. While cigarette smoking is the single most important risk factor for a steeper rate of decline in lung function and the primary risk factor for COPD, about 15% of COPD arises in persons who have never smoked, and not all smokers are susceptible [4]. Factors contributing to COPD in non-smokers and susceptibility factors in smokers are of great interest as they could suggest new targets for prevention efforts [5]. Age-related decreases in pulmonary function are typically not steep enough to lead to the loss of lung function that characterizes COPD. Thus, aside from smoking, other environmental and/or genetic factors are hypothesized to affect the trajectory of lung function decline[6,7]. Currently available drugs treat symptoms, but do not cure COPD; thus, research to identify factors associated with lung function decline may yield clues for future drug development and/or lifestyle interventions to prevent disease and/or to slow disease progression.

The obstruction in the small airways of the lungs that characterizes COPD is diagnosed by pulmonary function testing, and more specifically by the forced expiratory volume in the first second (FEV_1) and the forced vital capacity (FVC). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines mild or stage 1 COPD as an FEV_1/FVC ratio < 0.7 and an $FEV_1 \geq 80\%$ predicted (by age, race, gender, and height)[8]. Given the hypothesis studied here focuses on obstructive lung disease, the

FEV_1 and FEV_1/FVC ratio were the main outcomes investigated.

Prior research supports a role for vitamin D, as measured by its main circulating form, serum 25-hydroxyvitamin D, in lung health. In adults in the Third National Health and Nutrition Examination Survey (NHANES III) serum 25-hydroxyvitamin D concentration was positively associated with pulmonary function parameters (FEV_1 and FVC), and the association was strongest in the oldest age group[9]. In addition to being at risk of morbidity associated with declining pulmonary function, elderly Americans are more likely to have low serum 25-hydroxyvitamin D[10,11]. Vitamin D is a sunlight-derived hormone that is primarily known for its role in regulating serum calcium and phosphorous, but the association of vitamin D with lung function is hypothesized to reflect a mechanism involving chronic inflammation and airway remodeling[12,13,14,15,16,17]. The hypothesized role of vitamin D as an immunomodulator and as a factor in tissue remodeling suggests plausible biological mechanisms for a causal role of vitamin D in promoting and maintaining lung health over the life course, but the epidemiological data supporting this association remain limited.

To date, the only epidemiologic evidence supporting a role for vitamin D in lung function is cross-sectional[9]. This result has been contradicted by a second cross-sectional study that reported no association between serum 25-hydroxyvitamin D and lung function[18]. A recent case-control study, comparing smokers with rapid rate of decline in FEV_1 to smokers with shallow decline, reported no difference in plasma vitamin D at the beginning of the study period[19], but the analysis did not consider confounding

and/or interactions with race, and the study did not include non-smokers. Prospective studies of diverse populations, including older adults, smokers and non-smokers, and different races, are needed to more fully investigate the role of vitamin D status in lung function decline and pulmonary health.

This study investigates the hypothesis that serum 25-hydroxyvitamin D is inversely associated with the rate of decline in FEV_1 and FEV_1/FVC. Participants in the Health, Aging, and Body Composition prospective cohort study (Health ABC) were studied, and both cross-sectional and longitudinal associations of serum vitamin D and pulmonary function were investigated.

Materials and Methods

Study Population

The Health ABC study, a longitudinal cohort study, tracks a period of rapid health transitions in the elderly. A random sample of Whites and all Black Medicare-eligible persons residing in ZIP codes from the metropolitan areas surrounding Pittsburgh, PA and Memphis, TN were invited to join the study, and the final study comprised 3,075 Black and White men and women (46% of women and 37% of men were Black). Eligibility criteria included: aged 70 to 79 in the recruitment period (March 1997 to July 1998); self-report of no difficulty walking one-quarter of a mile or climbing 10 steps without resting; no difficulty performing basic activities of daily living; no reported use of a cane, walker, crutches or other special equipment to ambulate; no history of active

treatment for cancer in the prior 3 years; and no plan to move out of the area in the subsequent 3 years. Further details of this population are described elsewhere (http://www.nia.nih.gov/ResearchInformation/ScientificResources/HealthABCDescription.htm).

Participants were excluded from this analysis if they were missing measurements or had extreme outlier serum 25-hydroxyvitamin D (>100 ng/mL). Individuals with prevalent COPD were excluded from these analyses given further changes in their lung function are affected by treatment and/or the disease process itself. Prevalent COPD was defined by spirometry values, according to the GOLD-criteria, but modified to use the lower limit of normal (LLN) given age-associated loss in pulmonary function leads to over-diagnosis with the use of definitions based on ratio < 0.70. Thus, participants with an FEV_1/FVC below the lower limit of normal (LLN, defined as the 5^{th} percentile in people of similar age) were excluded. Other exclusion criteria were self-reported physician-diagnosed asthma at baseline, missing smoking history, or no FEV_1 and FEV_1/FVC measurements with acceptable quality scores.

Spirometry and Vitamin D Measurements

FEV_1 and FVC were measured using NIOSH spirometry systems at year 1, 5, 8, and 10 follow-up visits (corresponding to 0, 4, 7 and 9 years on study). Measurement quality was assessed using American Thoracic Society standardization of spirometry guidelines[20,21]. Acceptable quality was defined as having no early coughs, no early termination, limited extrapolated volume, and ≤ 200 ml between the two best FEV_1 measurements at each visit (based on criteria in use at the time of this study). Serum

25-hydroxyvitamin D was assessed in stored serum from the year 2 visit using the Diasorin RIA assay according to manufacturer specifications (November 2006). The inter-assay coefficient of variation for the measurement of 25-hydroxyvitamin D was 6.78% for log-transformed values[22].

Statistical Analysis

The cross-sectional association of 25-hydroxyvitamin D with FEV_1 or FEV_1/FVC was examined using ordinary least squares regression. Age, gender, height, race, current smoking status, Health ABC study site, and season of blood collection were included as covariates in the models, and the non-linearity of the vitamin D—pulmonary function association was considered. The interaction of vitamin D with smoking status, race, and comorbidities (hypertension, cardiovascular disease, all cancers except skin cancer, diabetes) were evaluated by adding product terms to the model.

Longitudinal analysis used linear mixed models to estimate the association of serum 25-hydroxyvitamin D with decline in FEV_1 or FEV_1/FVC. Age, gender, height, race, current smoking status, smoking pack years, Health ABC study site, and season of blood collection were included as covariates in the models, and race and comorbidities were evaluated as effect modifiers of the serum vitamin D—pulmonary function association. To determine the associations of 25-hydroxyvitamin D with rate of decline in FEV_1 or FEV_1/FVC, models included a time variable that quantified time elapsed from study entry to each spirometry measurement; interaction terms between 25-hydroxyvitamin D and the time variable were evaluated to quantify the association of vitamin D with rate of decline. Outlier points for FEV_1 were excluded if the absolute value of conditional

Studentized residual was > 5. Follow-up analysis to detect selection bias included evaluation of percent mortality and missed clinic visits in each tertile of 25-hydroxyvitamin D level. All analyses were carried out in SAS version 9.1 (Cary, NC).

Results

Population Characteristics

A total of 2,015 participants from the Health ABC cohort were included to investigate the cross-sectional associations of FEV_1 and FEV_1/FVC ratio with serum 25-hydroxyvitamin D. Participants with low quality control scores on spirometry, missing smoking status and/or history, missing year 2 serum 25-hydroxvitamin D, report of prevalent doctor-diagnosed asthma, or prevalent spirometry-defined COPD (defined as an FEV_1/FVC ratio below LLN) were excluded from further analyses. Thus, the longitudinal analysis included 1,983 participants (Table 2.1). Participants included in the longitudinal analysis met the same inclusion criteria as in the cross-sectional analysis, but also were required to have data on lifetime, cumulative smoking (pack years). The characteristics of participants at the study baseline were compared between included and excluded participants. Both the cross-sectional and the longitudinal study populations were similar, thus only the cross-sectional comparisons are shown. Excluded participants had a higher proportion of Black participants, higher average smoking pack-years, and lower average FEV_1 at study baseline. Black participants were more likely to be excluded from analyses due to a higher frequency of low spirometry quality control scores; 9.1% of Blacks and 3.1% of Whites were excluded for low quality control scores on spirometry. Additionally, participants excluded due to prevalent COPD and/or asthma had higher smoking pack years and lower FEV_1 at the study baseline, thus

included participants generally had higher FEV_1 and lower smoking pack-years.

The cross-sectional association of serum 25-hydroxyvitamin D with lung function was estimated in regression models, which were adjusted for age, sex, height, race, smoking, season of blood collection, and study site. Physical performance, alcohol consumption, education, and BMI were considered as possible confounders, but were not included as covariates in the models because inclusion had no effect on the coefficient for 25-hydroxyvitamin D, or the variable was potentially causally antecedent to 25-hydroxyvitamin D levels. The association between serum 25-hydroxyvitamin D and FEV_1 was positive and non-linear (Figure 2.1); regression coefficients for the linear and quadratic terms for serum 25-hydroxyvitamin D were 14.75 ($p < 0.0001$) and -0.20 ($p = 0.0012$), respectively. To interpret the coefficients, predicted values for the FEV_1 at different serum vitamin D concentrations were computed based on the model; the average FEV_1 in vitamin D-deficient participants (serum 25-hydroxyvitamin D of 10 ng/mL) was 132 mL lower than the average in participants with 30 ng/mL serum 25-hydroxyvitamin D (note: the 30ng/mL concentration is within the range defined as safe and adequate by the Office of Dietary Supplements and the Institute of Medicine; http://ods.od.nih.gov/factsheets/vitamind/, http://www.iom.edu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D.aspx). While the mean serum 25-hydroxyvitamin D differed by race (20.78 ng/mL and 28.97 ng/mL for Black and White participants, respectively), there was no evidence for an interaction of race and serum 25-hydroxyvitamin D in models predicting FEV_1 ($p = 0.93$ and 0.94 for the interaction coefficients). Further models assessed the interaction of serum 25-hydroxyvitamin D with smoking status ($p = 0.95$ and 0.99), gender ($p = 0.39$ and 0.75), and comorbidities,

but no interactions terms were statistically significant. There was little or no association between serum 25-hydroxyvitamin D and the ratio of FEV_1/FVC ratio (regression coefficient = -0.003 SE = .013; p = 0.7961).

The association of serum 25-hydroxyvitamin D with rate of decline in FEV_1 was estimated in mixed models adjusted for age, sex, height, race, smoking, season of blood collection, and study site. Higher serum 25-hydroxyvitamin D was associated with a steeper rate of FEV_1 decline; thus for every unit increase in serum 25-hydroxyvitamin D the rate of decline increased by 0.30 mL/year ($p < 0.0001$). Comparing participants who were deficient (10ng/mL) to participants in the adequate range for 25-hydroxyvitamin D (30ng/mL), the adequate group had an average rate of decline that was 6mL/year less steep than the rate in the deficient group. No significant longitudinal interactions between serum 25-hydroxyvitamin D and race or comorbidities were observed. There was little or no association of serum 25-hydroxyvitamin D with the trajectory of change in the ratio of FEV_1/FVC ratio ($p = 0.2241$).

The positive association between serum 25-hydroxvitamin D and rate of decline in lung function was unexpected. A possible explanation for this finding is selection bias if participants with lower serum 25-hydroxvitamin D experienced increased morbidity and mortality that led to incomplete pulmonary function testing for the longitudinal analysis. To investigate whether selection bias affected the rate of FEV_1 decline at lower levels of serum 25-hydroxvitamin D, morbidity (clinic attendance) and mortality patterns were examined by tertile of serum 25-hydroxvitamin D (baseline characteristics of tertiles are presented in Supplemental Table 2.1). Participants in the lowest tertile of serum 25-

hydroxvitamin D had lower FEV_1 at study baseline compared to participants in the upper two tertiles (Supplemental Table 2.2). Participants in the lowest tertile of serum 25-hydroxvitamin D were also more likely to die or miss clinic visits over the study follow-up period (Figures 2.2a and 2.2b). Compared to the highest tertile of serum 25-hydroxyvitamin D, participants in the lowest tertile were more likely to have only a baseline FEV_1 measurement and less likely to have all four FEV_1 measurements from baseline and years 5, 8, and 10 (Supplemental Table 2.3). As a result, longitudinal pulmonary function data were less likely to be available on participants with the lowest serum 25-hydroxvitamin D, due to missing year 5, 8, and/or 10 pulmonary function measurements. The selective loss of data from participants at lower 25-hydroxyvitamin D levels may result in overall better trajectories in FEV_1 among the remaining participants with lower 25-hydroxyvitamin D levels creating a non-representative group with repeated measurements of pulmonary function. This selection bias may explain the observation that participants with lower serum 25-hydroxvitamin D had a shallower rate of decline in FEV_1.

To address selection bias, a sensitivity analysis assessed the association of serum 25-hydroxvitamin D with rate of decline in FEV_1 in an analysis limited to participants in the top two tertiles of serum 25-hydroxvitamin D (a range that is not associated with vitamin D deficiency and/or insufficiency). Of note, the serum concentration of 25-hydroxvitamin D currently under scrutiny for determination of optimal levels is included in the highest two tertiles of 25-hydroxvitamin D (20.6 - 74.7 ng/mL) in the study population. In this analysis, there was little or no association of serum 25-hydroxvitamin D with rate of decline in FEV_1 (Table 2.2, regression coefficient = -0.12

mL/year; SE = 0.12; p = 0.28). Similarly, there was little or no cross-sectional association of serum 25-hydroxvitamin D with FEV_1 when analysis was limited to the two highest tertiles of 25-hydroxvitamin D.

Discussion

Serum 25-hydroxyvitamin D is positively associated with FEV_1 in the cross-sectional analysis, but not with the FEV_1/FVC ratio in elderly participants of the Health, Aging, and Body Composition study. This finding is consistent with a prior report from NHANES III, which showed that serum 25-hydroxyvitamin D was positively associated with FEV_1, but not with FEV_1/FVC ratio [9]. The findings contradict a recent finding from the U.K.-based Hertfordshire Cohort Study[18]. Of great interest, in a longitudinal analyses of Health ABC follow-up data there was no evidence for an association of serum 25-hydroxyvitamin D with the trajectory of decline in the FEV_1/FVC ratio.

Although a positive association between serum 25-hydroxyvitamin D and rate of decline in FEV_1 (higher serum vitamin D associated with steeper rate of decline) was observed, further analysis suggests this result is due to selection bias. Thus, participants in the lowest tertile of 25-hydroxyvitamin D experienced both higher mortality and more missed clinic visits, leading to an under-representation of participants with lower serum vitamin D status in the longitudinal study. When analyses were limited to the uppermost tertiles of serum 25-hydroxyvitamin D, where far fewer participants were lost to follow-up, there was little or no association of serum 25-hydroxyvitamin D measured early in follow-up (range 20.6 to 74.7 ng/mL) with the subsequent rate of decline in FEV_1.

The use of the Health ABC cohort may have introduced several limitations, including the selection bias described above arising because missing longitudinal lung function measurements occurred disproportionately in participants with low 25-hydroxyvitamin D levels. In addition, serum 25-hydroxyvitamin D was measured in the second year of the study, while the first measurement of pulmonary function was in year 1. This introduces the possibility that poor health associated with lower lung function could have led to lower 25-hydroxyvitamin D status the following year due to reduced sunlight exposure from reduced outdoor activity. As with all cross-sectional associations of 25-hydroxyvitamin D, there is the possibility that serum 25-hydroxyvitamin D is a proxy for overall health rather than a causal contributor to lung function.

Ideally, longitudinal studies help resolve temporal sequence and reverse causality concerns. Kunisaki et al[19] recently reported a study of 196 smokers with 6 years of follow-up, comparing mean serum 25-hydroxyvitamin D in steep FEV_1 decliners (cases) with shallow FEV_1 decliners (controls); no analyses of baseline cross-sectional associations were presented, statistical models were not adjusted for race despite a significant difference in race between cases and controls, and serum vitamin D was measured late in follow-up (one year before the study ended). There was little or no difference in serum 25-hydroxyvitamin D between steep and shallow FEV_1 decliners, but the limitations of the study preclude definitive conclusions and provide no information about non-smokers.

The Health ABC study reported herein is the first longitudinal analysis of serum 25-hydroxyvitamin D in relation to rate of decline in FEV_1 and the FEV_1/FVC ratio in a large

and racially diverse population that includes a large proportion of healthy non-smokers.

Our cross-sectional analysis support the positive association between serum 25-hydroxyvitamin D and FEV_1 reported by Black, et al.[9], but contradict the recent report by Shaheen, et al.[18]. The longitudinal analysis of decline in FEV_1 and decline in the FEV_1/FVC ratio over 10 years revealed little or no association of serum 25-hydroxyvitamin D with rate of decline, suggesting that supplementing vitamin D in older adults with serum values of 20 ng/mL or higher is unlikely to be beneficial.

Although we had to eliminate the most severely vitamin D deficient participants from the longitudinal analysis, the range of vitamin D nutriture studied represents the range that is most hotly debated in the current controversy over defining sufficient and/or optimal 25-hydroxyvitamin D levels. Although there is no doubt that it is beneficial to supplement vitamin D deficient (< 12 ng/mL according to the Office of Dietary Supplements, http://ods.od.nih.gov/factsheets/vitamind/) patients for reasons that go beyond lung health, the Institute of Medicine has defined current dietary recommendations based on intake required to achieve 20 ng/mL serum 25-hydroxyvitamin D in 97.5% of the population (http://ods.od.nih.gov/factsheets/vitamind/, http://www.iom.edu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D.aspx). Our study suggests further supplementation in older patients with serum 25-hydroxyvitamin D above approximately 20 ng/mL will not mitigate lung function decline. However, results from on-going trials of vitamin D supplementation will provide important further evidence.

Including the study reported herein, 2 studies confirm a cross-sectional association of

25-hydroxyvitamin D status and FEV_1, and 2 studies confirm no association of 25-hydroxyvitamin D with longitudinal FEV_1 decline. As mentioned above, a potential explanation is that the cross-sectional result reflects overall health of the participants and not a casual association. Another possibility, however, is that vitamin D has a causal role in attenuating FEV_1 decline at an earlier stage in life than the age range investigated in studies to date. This is consistent with a cross-sectional difference in FEV_1 by vitamin D status observed in older Americans. A causal role for vitamin D is supported by vitamin D's known role in important lung function processes such as inflammation and tissue remodeling, and cannot be ruled out based on the findings from the Health ABC study.

These issues highlight the need for longitudinal studies in a younger age group, which would also avoid the issues arising from morbidity/mortality-related selection bias. Our results suggest the possibility that an intervention of vitamin D supplementation to improve lung health may only be effective at younger ages, or only in people with true vitamin D deficiency. These results demonstrate the need for further studies to evaluate whether a longitudinal association exists, and to define the optimal timing for intervention.

Tremendous enthusiasm for vitamin D supplementation for lung function has been generated by a single cross-sectional result, which we have replicated here. The study reported herein presents the first longitudinal results examining the association of 25-hydroxyvitamin D with decline in FEV_1 and FEV_1/FVC ratio in a large cohort comprised of both smoking and non-smoking older adults. Participants with serum 25-

hydroxyvitamin D below approximately 20 ng/mL at year 2 had higher rates of missed clinic visits and mortality in subsequent years, suggesting that this level of vitamin D may be a prognostic indicator for overall poor health in later years. However, in participants in this age group, there was no evidence that vitamin D supplementation would attenuate decline in lung function.

Table 2.1. Baseline Characteristics of Health ABC Participants Included* and Excluded from Analyses

	Cross-sectional*	Cross-sectional Excluded	Longitudinal*	Longitudinal Excluded
	(n =2015)	(n = 1060)	(n = 1979)	(n = 1096)
Age, years (SD)	73.6 (2.9)	73.7 (2.9)	73.6 (2.9)	73.7 (2.9)
Females (%)	1024 (51%)	467 (44%)	1002 (51%)	489 (45%)
Blacks (%)	728 (36%)	553 (52%)	720 (36%)	561 (51%)
Memphis, TN site (%)	1036 (51%)	491 (46%)	1022 (52%)	505 (46%)
Former Smokers (%)	930 (46%)	474 (45%)	902 (46%)	502 (46%)
Current Smokers (%)	169 (8.4%)	149 (14%)	166 (8.4%)	152 (14%)
Packyears (SD)	17.4 (26.5)	22.1 (30.4)	17.5 (26.6)	22.0 (30.0)
Height, cm (SD)	166.7 (9.2)	165.3 (9.6)	166.4 (9.1)	165.4 (9.7)
BMI, kg/m^2 (SD)	27.4 (4.6)	27.5 (5.2)	27.4 (4.6)	27.5 (5.2)
FEV_1 at baseline, mL (SD)	2284 (608)	1819 (615)	2282 (609)	1844 (625)
FEV_1 ratio at baseline (SD)	75.71 (5.73)	73.77 (13.6)	75.72 (5.72)	73.83 (13.3)
Serum 25-hydroxyvitamin, ng/mL (SD)	26.0 (10.1)	25.3 (14.2)	26.0 (10.1)	24.5 (14.0)

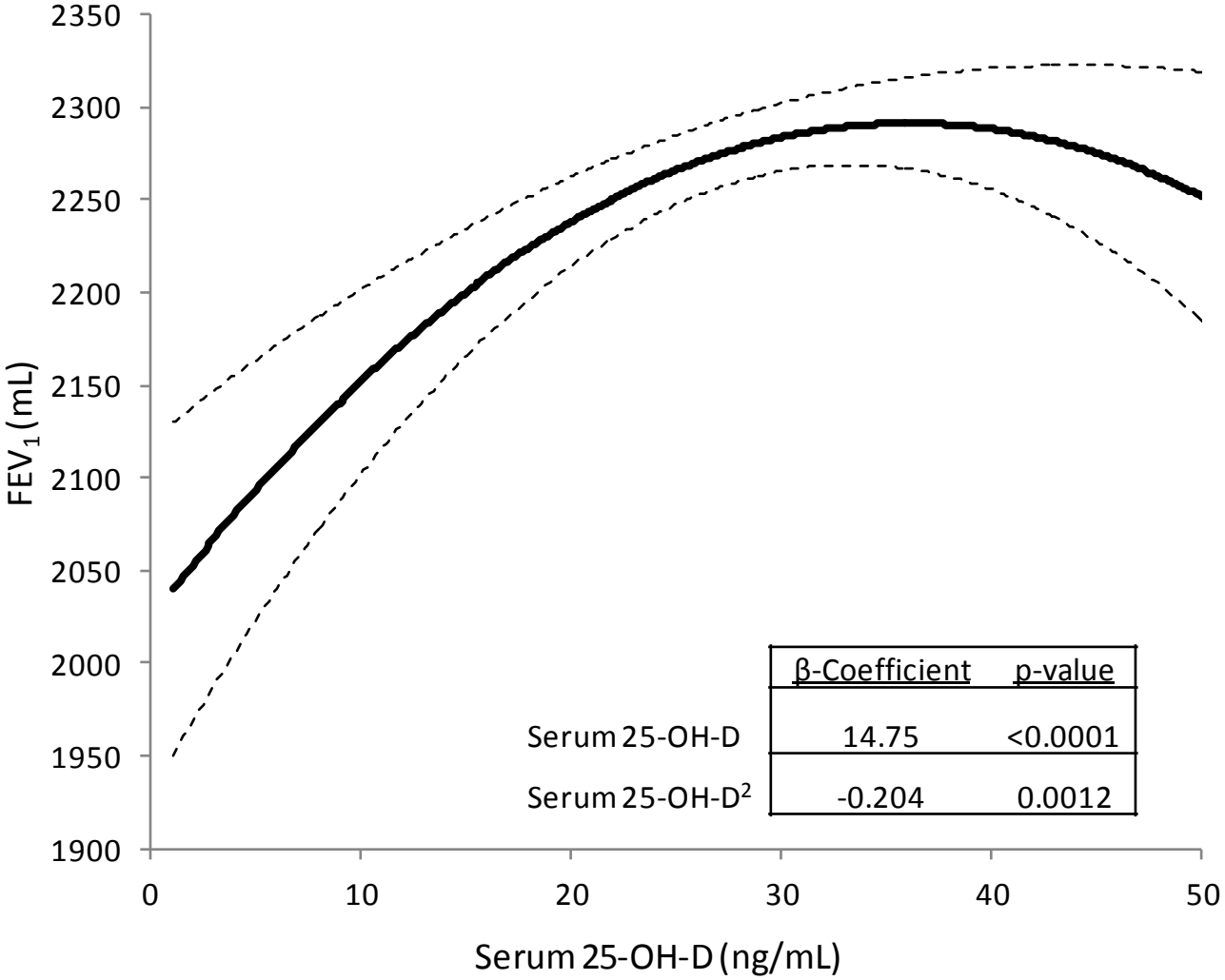

Figure 2.1. Non-linear association of year 2 serum 25-hydroxyvitamin-D with baseline FEV_1 (Dashed line represents the 95% confidence interval)

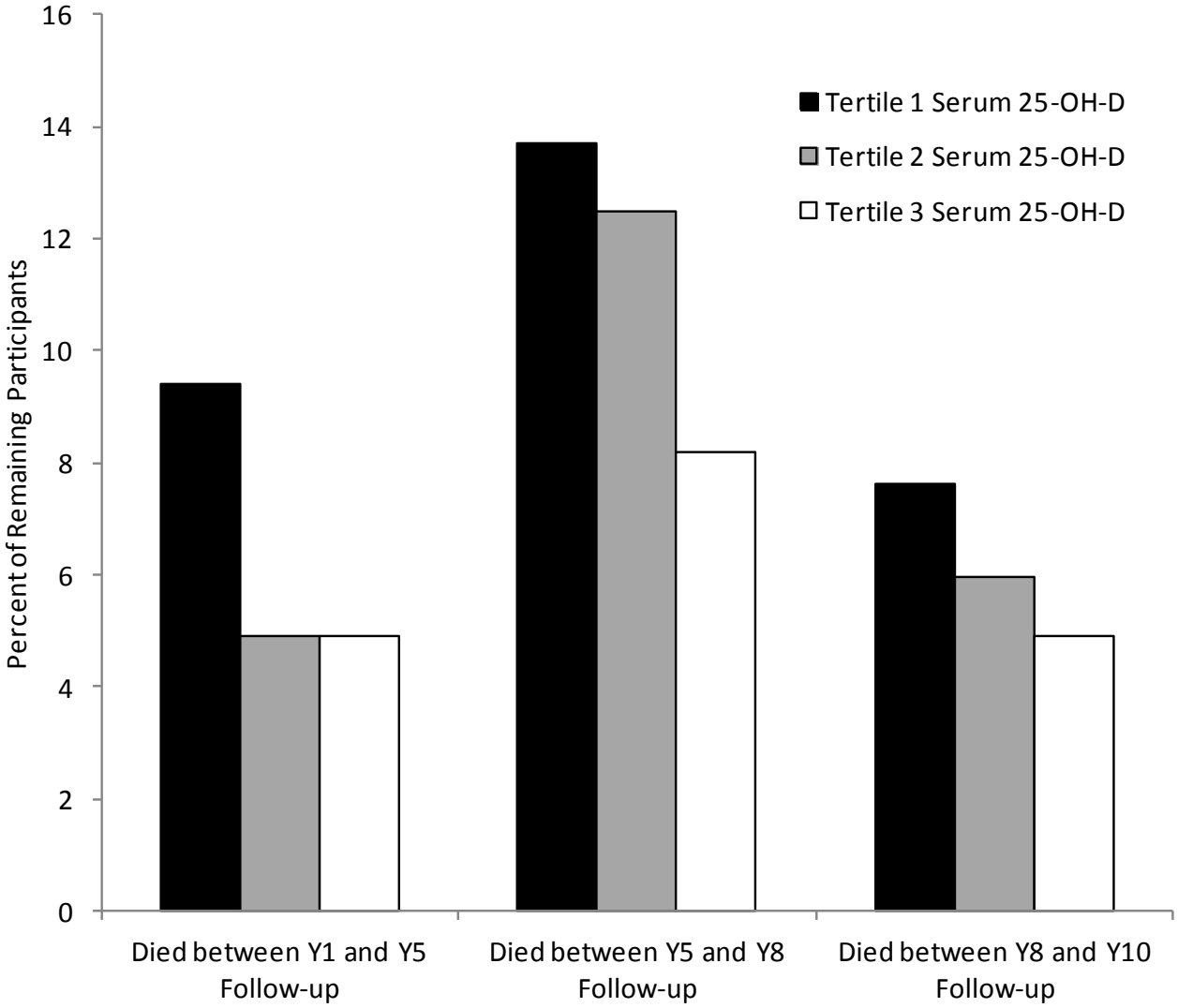

Figure 2.2a. Mortality by tertile of serum 25-hydroxyvitamin D* through 10 years of follow-up.
*Tertile 1: 5.01 – 20.57 ng/mL, Tertile 2: 20.58 – 29.87 ng/mL, Tertile 3: 29.88 – 74.74 ng/mL

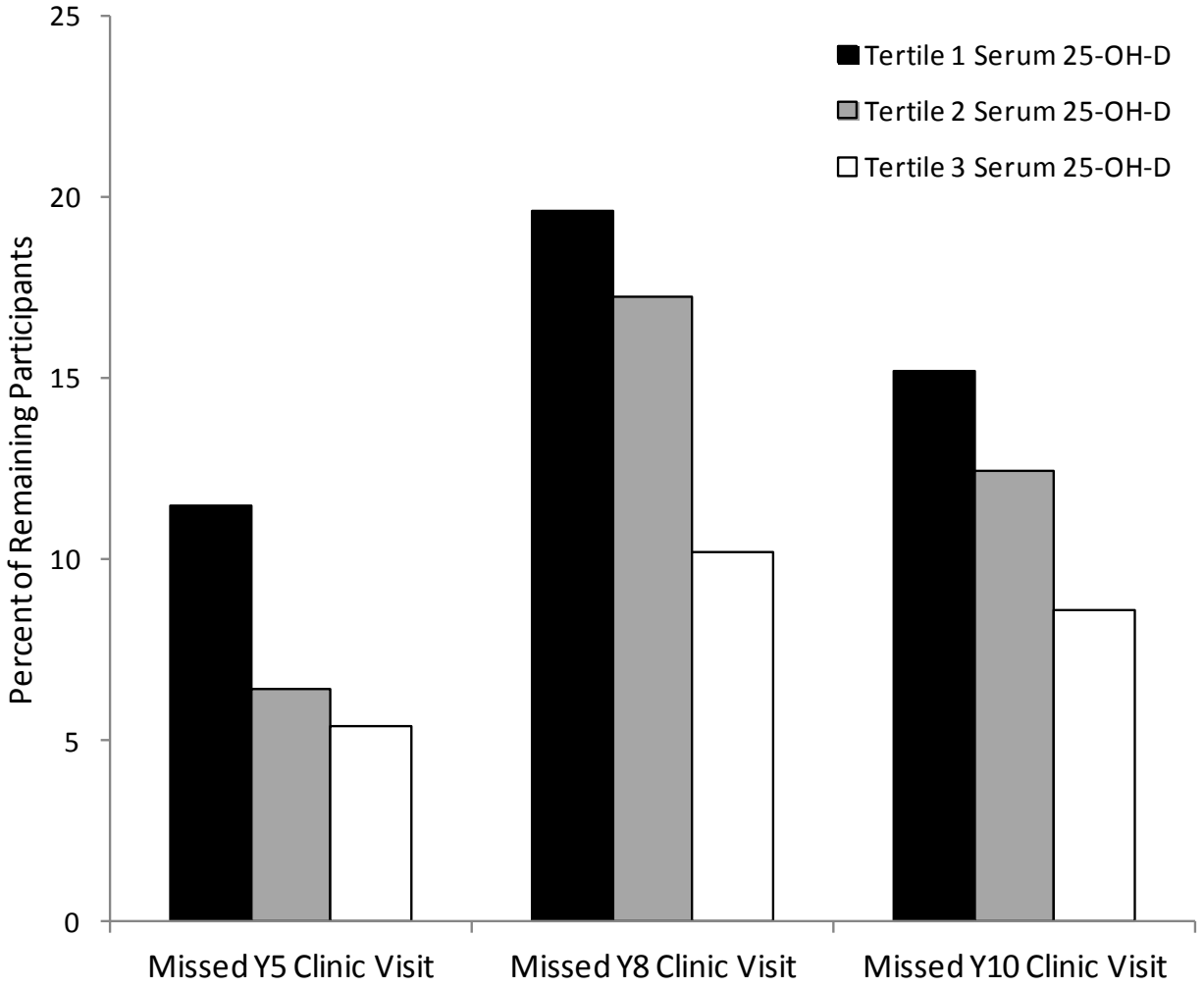

Figure 2.2b. Missed clinic visits by tertile of serum 25-hydroxyvitamin D* through 10 years of follow-up. Missed clinic visits include missed visits for any reason, including mortality.
*Tertile 1: 5.01 – 20.57 ng/mL,Tertile 2: 20.58 – 29.87 ng/mL, Tertile 3: 29.88 – 74.74 ng/mL

Table 2.2. Change in FEV_1 Decline by Tertile of Serum 25-hydroxyvitamin D* Over 10 Years of Follow-up

	β-coefficient**	P-value
All tertiles of serum 25-hydroxyvitamin D	-0.300	<0.0001
Highest 2 tertiles of serum 25-hydroxyvitamin D	-0.120	0.283

* Tertile 1: 5.01 – 20.57 ng/mL, Tertile 2: 20.58 – 29.87 ng/mL, Tertile 3: 29.88 – 74.74 ng/mL

** The β–coefficient represents the difference in FEV_1 decline per each ng/mL increase in serum 25-hydroxyvitamin D

Supplemental Table 2.1. Baseline Characteristics of Health ABC Participants by Tertile of Serum 25-hydroxyvitamin D*

	Tertile 1	Tertile 2	Tertile 3
	(n =671)	(n = 672)	(n = 672)
Age, years (SD)	73.5 (2.9)	73.7 (2.9)	73.6 (2.8)
Females (%)	318 (47%)	352 (52%)	354 (53%)
Blacks (%)	411 (61%)	207 (31%)	110 (16%)
Memphis, TN site (%)	354 (53%)	340 (51%)	342 (51%)
Former Smokers (%)	298 (44%)	306 (46%)	326 (49%)
Current Smokers (%)	88 (13%)	149 (6.5%)	166 (5.5%)
Packyears (SD)	17.7 (25.7)	16.8 (25.5)	17.9 (28.4)
Height, cm (SD)	166.1 (9.2)	167.0 (9.0)	167.1 (9.3)
BMI, kg/m^2 (SD)	28.8 (5.2)	27.2 (4.3)	26.1 (3.8)
FEV_1 at baseline, mL (SD)	2135 (573)	2321 (613)	2397 (610)
FEV_1 ratio at baseline (SD)	75.92 (6.01)	75.85 (5.63)	75.36 (5.52)
Serum 25-hydroxyvitamin D, ng/mL (SD)	15.3 (3.69)	25.2 (2.64)	37.5 (6.34)

* Tertile 1: 5.01 – 20.57 ng/mL, Tertile 2: 20.58 – 29.87 ng/mL, Tertile 3: 29.88 – 74.74 ng/mL

Supplemental Table 2.2. Cross Tabulation of Tertile of Serum 25-hydroxyvitamin D and of Tertile of FEV_1

Tertile of serum 25-hydroxyvitamin D	Tertile 1 of FEV_1 (620 – 1964mL)	Tertile 2 of FEV_1 (1965 – 2518 mL)	Tertile 3 of FEV_1 (2521 – 4432 mL)
Tertile 1 (5.01 – 20.57 ng/mL)	276	234	161
Tertile 2 (20.58 – 29.87 ng/mL)	209	224	239
Tertile 3 (29.88 – 74.74 ng/mL)	186	214	272

Supplemental Table 2.3. Number of Participants with Only Baseline FEV_1 or all Four FEV_1 Measurements by Tertile Serum 25-hydroxyvitamin D

Tertile of serum 25-hydroxyvitamin D	# participants with only baseline FEV_1 (%)	Number of participants with four FEV_1 measurements (%)
Tertile 1 (5.01 – 20.57 ng/mL)	160 (23.9%)	254 (37.9%)
Tertile 2 (20.58 – 29.87 ng/mL)	113 (16.8%)	306 (45.5%)
Tertile 3 (29.88 – 74.74 ng/mL)	89 (13.2%)	331 (49.3%)

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CHAPTER 3

GENETIC VARIANTS IN VITAMIN D METABOLIC PATHWAY GENES ASSOCIATED WITH PULMONARY FUNCTION

Abstract

Vitamin D is hypothesized to play role in lung health as reflected by pulmonary function, but evidence from observational studies is mixed. The association of genetic variants in vitamin D metabolism genes with lung function (FEV_1 and FEV_1/FVC) was investigated in the Health, Aging, and Body Composition (Health ABC) study. Single nucleotide polymorphisms (SNPs) in *GC, CYP2R1, CYP27B1, VDR, RXRA*, and *CYP24A1* were tested in linear regression models stratified by race. In African-American participants, rs3886163 in *CYP24A1* was associated with FEV_1, and rs11168293 in *VDR* was associated with FEV_1/FVC, at a false discovery rate (FDR) < 0.2. Two haplotypes in *CYP24A1* were associated with FEV_1 in African-Americans with permuted P < 0.05. An interaction between serum 25-hydroxyvitamin D and two SNPs in *RXRA* in European-Americans reached the FDR threshold (< 0.2). Participants homozygous for the minor allele on rs6537944 (vs. wild type homozygotes and heterozygotes) had a lower FEV_1/FVC ratio only in the presence of low serum 25-hydroxyvitamin D (< 20 ng/mL); at higher levels of serum vitamin D there was little or no association of the SNP with the ratio phenotype. These findings provide novel evidence supporting a role for vitamin D in lung function relevant to obstructive disease. This study identifies genetic variants and gene-environment interactions that may increase risk for compromised pulmonary function.

Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the United States and over 12 million Americans have prevalent disease [1]. COPD is characterized by poor lung function, and is diagnosed using the forced expiratory volume in the first second (FEV_1) and the forced vital capacity (FVC). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defines mild or stage I COPD as an FEV_1/FVC ratio < 0.7 and an $FEV_1 \geq 80\%$ predicted (by age, race, gender, and height) [1]. Smoking is the primary risk factor for COPD, but not all smokers develop the disease, and about 15% of COPD-related mortality arises in persons who have never smoked [2]. Thus, other genetic and/or environmental factors are postulated to affect lung function and to contribute to COPD risk. Research is needed to identify these contributing factors in order to inform COPD prevention and treatment efforts.

Several lines of evidence suggest a role for vitamin D as an environmental factor in the maintenance of lung function. Inadequate vitamin D levels are common and often overlooked in routine medical visits [3]; if a connection with lung function is established, this modifiable environmental factor could affect a large proportion of the at-risk population for COPD. Vitamin D is formed naturally in the skin from exposure to sunlight, or can be consumed through diet and supplements [4,5]. In the liver, the 25-hydroxylase (*CYP2R1*) converts vitamin D to the major circulating form, 25-hydroxyvitamin D [6]. In plasma, 25-hydroxyvitamin D is transported via the vitamin D binding protein (encoded by *GC*). 25-hydroxyvitamin D is converted by 1-alpha-hydroxylase (*CYP27B1*) to the more active form, 1,25-dihydroxyvitamin D, which is formed in the kidney, and is hypothesized to form in individual tissues for endocrine and

paracrine/autocrine activity, respectively [7]. In addition to its more commonly known role in calcium homeostasis, the active form of vitamin D acts as a steroid hormone, directly modulating gene expression by interaction with the vitamin D receptor (*VDR*) and its heterodimer partner, the retinoid X receptor (*RXRA*), with subsequent binding to vitamin D response elements in DNA [8,9]. Finally, the active form of vitamin D is degraded by the 24-hydroxylase (*CYP24A1*) to form 1,24,25-trihydroxyvitamin D. Serum concentrations of 25-hydroxyvitamin D are more stable over time than the active form, and this metabolite is used as the primary biomarker of vitamin D nutritional exposure in epidemiological studies [10].

A positive association between serum 25-hydroxyvitamin D and pulmonary function (FEV_1 and FVC) was reported in a cross-sectional study of adult participants in the Third National Health and Nutrition Examination Survey (NHANES III) [11]. However, a recent cross-sectional study in the Hertfordshire Cohort reported no association between serum 25-hydroxyvitamin D and lung function [12]. A recent case-control study reported no difference in initial serum 25-hydroxyvitamin D comparing smokers with subsequent rapid decline in FEV_1 to smokers with slow decline, but the study did not account for race or other confounding factors [13]. The conflicting results of the observational studies leave the role of vitamin D in lung health an open question, and raise the possibility that vitamin D levels may be a proxy for general good health and not a causal player.

Genotype is unaffected by 'lifestyle' confounding that affects the vitamin D—disease association in traditional epidemiologic studies. In the case that genotype is a key determinant of exposure status and only a direct cause of disease through the exposure pathway, the use of genetic data as an independent measure of exposure

status strengthens causal inference [14]. The SUNLIGHT consortium reported significant contribution of variation in transport and metabolism genes to circulating 25-hydroxyvitamin D concentrations [15], and high heritability of serum vitamin D concentrations [16], supporting the use of genetic proxies for exposure status in epidemiologic studies. Moreover, some genetic variants may work by altering net vitamin D function independent of serum 25-hydroxyvitamin D concentrations. Several studies reported that single nucleotide polymorphisms (SNPs) in the gene encoding the vitamin D binding protein (*GC*) were associated with COPD risk [17,18,19,20,21]. These variants alter binding affinity for circulating forms of vitamin D, which could affect delivery to target cells [22].

The present study investigated the hypothesis that variants in candidate genes in the vitamin D metabolic pathway, which produce sustained and long-term differences in vitamin D nutriture, predict lung function phenotypes. The hypothesis was studied using data from the Health, Aging, and Body Composition (Health ABC) Study, and considering single SNP associations, haplotypes, and interactions with serum 25-hydroxyvitamin D concentrations.

Materials and Methods

Study Population

The Health ABC study, a longitudinal cohort study that tracks a period of rapid health transitions in the elderly, comprises 3,075 African-American and European-American men and women (46% of women and 37% of men were African-American). A random sample of European-American and all African-American Medicare-eligible persons residing in ZIP codes from the metropolitan areas surrounding Pittsburgh, PA

and Memphis, TN were invited to join the study. Eligibility criteria included: aged 70 to 79 years in the recruitment period (March 1997 to July 1998); self-report of no difficulty walking one-quarter of a mile or climbing 10 steps without resting; no difficulty performing basic activities of daily living; no reported use of a cane, walker, crutches or other special equipment to ambulate; no history of active treatment for cancer in the prior 3 years; and no plan to move out of the area in the subsequent 3 years. Further details of this population are described elsewhere (http://www.nia.nih.gov/ResearchInformation/ScientificResources/HealthABCDescription.htm).

Pulmonary Function Measurement

FEV_1 and FVC were measured using NIOSH spirometry systems at year 1, 5, 8, and 10 follow-up visits (corresponding to 0, 4, 7 and 9 years on study). Measurement quality was assessed using American Thoracic Society standardization of spirometry guidelines [23,24]. Acceptable quality was defined as having no early coughs, no early termination, limited extrapolated volume, and ≤ 200 ml between the two best FEV_1 measurements at each visit.

Vitamin D Measurement

Serum 25-hydroxyvitamin D was assessed in stored serum from the year 2 visit using the Diasorin RIA assay according to manufacturer specifications (assayed in November 2006). The inter-assay coefficient of variation for the measurement of 25-hydroxyvitamin D was 6.78% for log-transformed values [25].

Genotyping

Genomic DNA was extracted using the PUREGENE® DNA Purification Kit from buffy coat obtained at the baseline exam visit. Genotyping was performed by the

Center for Inherited Disease Research (CIDR) using the Illumina Human1M-Duo BeadChip system. Samples were not included in cases of sample failure, genotypic sex mismatch, and first-degree relative of an included individual based on genotype data. A total of 1,151,215 SNPs were genotyped for genome-wide association studies in 2,802 unrelated individuals (1,139 African-Americans and 1,663 European-Americans). For this study, a total of 190 SNPs in African-Americans and 152 SNPs in European-Americans in *GC, CYP2R1, CYP27B1, VDR, RXRA*, and *CYP24A1* were examined for association with pulmonary function (Supplementary Tables 3.1 and 3.2).

Data Analysis

Analysis of the association of SNPs with pulmonary function was stratified by race and included 737 African-Americans and 1,259 European-Americans from the Health ABC cohort. Participants with low spirometry quality control scores, missing smoking status and/or history, prevalent COPD by spirometry (defined as FEV_1 and FEV_1/FVC ratio below the lower limit of normal), report of prevalent doctor-diagnosed asthma, or missing the first two principal components accounting for genetic substructure were excluded from analysis (Table 3.1). For vitamin D interaction analysis, participants missing serum 25-hydroxyvitamin D measurements were also excluded.

All associations (single SNP, haplotype, and interactions) were adjusted for gender, smoking status, height, study site, and the first two principal components of genetic substructure, as covariates. To study single SNP associations with FEV_1 and FEV_1/FVC at study baseline, linear regression analyses considered additive, dominant, or recessive models using PLINK v1.07 [26]. Individuals missing more than 30% of genotypes or SNPs missing more than 10% of genotypes were excluded, and only

SNPs with a minor allele frequency > 1% (or, > 5% in recessive models) and in Hardy-Weinberg equilibrium were studied. Associations were considered significant at a false discovery rate (FDR) of < 0.2 to account for multiple testing.

For SNPs with a nominal $p < 0.01$, further analyses considered haplotypes. Phase was imputed and haplotypes were identified in the African-American and European-American Health ABC populations using Haploview version 4.2 [27]. In Haploview, linkage disequilibrium blocks were defined by the confidence interval-based algorithm of Gabriel et al [28]. Haplotype associations were assessed using PLINK linear regression with an additive model, with the same covariates as above. The max(T) permutation procedure was used with 10,000 permutations to correct for multiple testing. Haplotype allele frequencies < 5% were excluded from analysis, and all other exclusions were the same as for the single SNP analysis.

The association of serum 25-hydroxyvitamin D concentration and SNP genotype was assessed using PLINK. For genes downstream of 25-hydroxyvitamin D production, the interaction of gene variants and serum 25-hydroxyvitamin D level in relation to lung phenotypes was considered, using product terms added to linear regression in SAS version 9.2 and considering the same covariates and exclusion criteria as above.

Results

Population Characteristics

A total of 737 African-American and 1,259 European-American participants from the Health ABC cohort were included to investigate the associations of SNPs in vitamin D metabolic pathway genes with measures of pulmonary function related to obstructive lung disease (Table 3.1). Excluded from analysis were 544 African-American and 535

European-American participants with low spirometry quality control scores, missing smoking status and/or history, report of prevalent doctor-diagnosed asthma, missing principal component, or prevalent spirometry-defined COPD (Supplemental Table 3.3). Characteristics of included and excluded study participants were similar. As expected due to exclusion of participants with lung disease, excluded African-American and European-American participants had lower FEV_1 than included participants.

Single SNP Associations

Analysis of the associations of SNP variants in vitamin D metabolic pathway genes with FEV_1 and FEV_1/FVC revealed two significant associations in African-American study participants with false discovery rate (FDR) < 0.2. An intronic SNP in *CYP24A1*, rs3886163, was associated with FEV_1 (beta coefficient = 116.1, p = 0.001, FDR = 0.125; dominant model). An exonic (or 5' UTR depending on splice variant) SNP in *VDR*, rs11168293, was associated with FEV_1/FVC (beta coefficient = 4.867, p = 0.002, FDR = 0.174; recessive model). Neither of these SNPs was statistically significantly associated with serum levels of 25-hydroxyvitamin D concentrations (Supplemental Table 3.4).

In African-American participants, a total of seven SNPs with nominal p < 0.01 were selected for further haplotype analysis (two SNPs in *CYP24A1* for FEV_1 and five SNPs in *VDR*, *CYP24A1*, and *GC* for FEV_1/FVC; Table 3.2). In European-American participants, no SNPs reached the preset FDR threshold of < 0.2, but a total of four SNPs with nominal p < 0.01 (two SNPs in *GC* and *CYP24A1* for FEV_1 and two SNPs in *VDR* for FEV_1/FVC) were selected for further haplotype analysis (Table 3.3).

Haplotype Analysis

Haplotype blocks in regions surrounding SNPs with nominal $p < 0.01$ were tested for association with FEV_1 and FEV_1/FVC in African-American and European-American participants. In African-American participants, 7 SNPs were the starting point to construct 4 haplotype blocks in 3 genes. In European-American participants, 4 SNPs were the starting point to construct 4 haplotype blocks in 3 genes. In both populations the *GC*, *CYP24A1*, and *VDR* genes were investigated in the haplotype analysis. From 43 haplotypes in 8 haplotype blocks, 2 haplotypes in *CYP24A1* were associated (permuted p-value < 0.05) with FEV_1 in African-Americans (Figure 3.1). Haplotypes in two separate blocks in *CYP24A1* were statistically significantly associated (permuted p-value < 0.05) with FEV_1 (Table 3.4). Haplotype block 4 of *CYP24A1* consisted of two SNPs and three separate haplotypes. This block contained rs3886163, which was significantly associated with FEV_1 in single SNP analysis. The 'AT' haplotype in block 4 of *CYP24A1* (vs. all other block 4 haplotypes, combined) was associated with higher FEV_1 (102 mL, p = 0.001, permuted p = 0.006) (Table 3.4.) The 'CA' haplotype in block 7 of *CYP24A1* (vs. all other block 7 haplotypes, combined) was associated with lower FEV_1 (-83.3mL, p = 0.005, permuted p = 0.024). No other haplotypes were statistically significantly associated with FEV_1 or FEV_1/FVC in African-American or European-American participants.

Vitamin D Interactions with SNP Genotype

Two statistically significant interactions between SNP genotypes in *RXRA* and serum 25-hydroxyvitamin D were detected in European-American participants. Thus, the combination of a lower serum 25-hydroxyvitamin D concentration and a 'CC' or 'CT'

genotype for rs6537944 was associated with decreased FEV_1 (p = 0.002, FDR = 0.114) (Figure 3.2a, Supplemental Table 3.5). European-American participants with a 'CC' genotype for rs4842196 had a lower FEV_1/FVC ratio only in the presence of a lower concentration of serum 25-hydroxyvitamin D (p = 0.001, FDR = 0.042) (Figure 3.2b, Supplemental Table 3.6). For both SNPs, the difference in lung function between major and minor allele carriers disappeared as serum levels of 25-hydroxyvitamin D increased, thus the SNP effects were attenuated with increasing vitamin D nutriture.

Discussion

Genetic variants in the vitamin D metabolic pathway, specifically in the *VDR*, *CYP24A1* and *RXRA* genes, were associated with pulmonary function. In African-American participants, a SNP in *VDR* was significantly associated with FEV_1/FVC at a false discovery rate cutoff of 0.2. No haplotypes in *VDR* were significantly associated with lung function, but genotyping in this study may not be dense enough to estimate haplotype effects accurately. A SNP in *CYP24A1* was associated with FEV_1 (FDR < 0.2), also in African-American participants. Haplotype analysis of the *CYP24A1* gene revealed one haplotype that was statistically significantly associated (permuted p < 0.05) with higher FEV_1, and a second haplotype that was associated with lower FEV_1. No SNPs or haplotypes were statistically significantly associated with pulmonary function in European-American participants, but serum 25-hydroxyvitamin D levels modified the association of SNPs in the *RXRA* with FEV_1 and FEV_1/FVC in this population.

Despite the limited and conflicting epidemiological evidence on the association of serum vitamin D and lung function, molecular studies consistently support a plausible

role for vitamin D in pulmonary function. The decline in lung function that is a key feature in the pathogenesis of COPD is hypothesized to occur through processes of chronic inflammation and tissue remodeling [29,30]. Molecular evidence shows that vitamin D modulates the expression of genes involved in both processes, both *in vitro* and in animal models [7,31,32,33]. Only one study has examined molecular outcomes following vitamin D supplementation in humans: British Bangladeshi men supplemented with vitamin D (four intramuscular injections of 500 IU in one year) had decreased circulating levels of biomarkers of inflammation and tissue remodeling in relation to pre-supplementation values [34], supporting the molecular evidence on mechanisms whereby vitamin D may affect non-bone outcomes.

Putative Mechanisms for CYP24A1Variants

Elevated *CYP24A1* expression is reported for many cancers, including cancer of the lung, and is associated with poor prognosis; the hypothesized mechanism is that higher expression leads to more removal of 1,25-dihydroxyvitamin D from regions surrounding tumors, with detrimental consequences [35]. No other associations of *CYP24A1* with lung phenotypes have been reported. CYP24A1 degrades the active form of vitamin D, thus a genetic variant affecting activity or expression of the enzyme is hypothesized to lead to differences in 1,25-dihydroxyvitamin D levels, and in turn, may affect phenotype. Dominant negative-acting splice variants for *CYP24A1* that are catalytically inactive, but retain substrate binding ability, have been reported in several cell types [36,37]; thus, another potential mechanism for SNP effects is via alteration of binding activity or expression of these splice variants.

CYP24A1 degrades both 1,25-dihydroxyvitamin D and circulating 25-

hydroxyvitamin D [38], so genetic variants affecting CYP24A1 activity may lead to lower concentrations of the circulating biomarker. In a candidate gene analysis, the SUNLIGHT Consortium identified a SNP near *CYP24A1* (rs6013897) that was associated with serum 25-hydroxyvitamin D concentration [15]. In the current study, there was little or no association between serum 25-hydroxyvitamin D concentration and *CYP24A1* variants; this negative finding is consistent with no true association or with SNP effects on 25-hydroxyvitamin D concentration that are subtle and within the noise of day-to-day variation. The possibility remains that *CYP24A1* variants alter 1,25-dihydroxyvitamin D levels, and that even small changes may have large effects due to the higher potency of the active form.

Putative mechanisms for VDR variants

Polymorphisms in *VDR* were associated with lung phenotypes in prior studies, including sarcoidosis [39], asthma [40,41], pulmonary tuberculosis [42], and non-small cell lung cancer [43]. Genetic variants in *VDR* are expected to be independent of 25-hydroxyvitamin D levels, since such variants likely affect function of 1,25-dihydroxyvitamin D as a regulator of gene expression. Thus, the effects of these genetic variants would not be detected in an epidemiologic study using 25-hydroxyvitamin D as a biomarker for vitamin D status.

Race Differences in Genetic Associations

It is noteworthy that genetic variants associated with lung function were detected in African-American participants, since this group is already at increased risk for poor vitamin D status. Skin pigmentation interferes with vitamin D formation from exposure

to sunlight [44], and an estimated 32% of African Americans are deficient (<12 ng/mL serum 25-hydroxyvitamin D) in vitamin D [45]. Thus, the study may have more sensitivity to detect genotype—phenotype associations in African Americans if such associations are strengthened in the presence of low vitamin D nutriture. In addition, the African American population has greater genomic variation, and thus the genotype—phenotype associations may be more readily detected in the African-American subgroup. No interactions with serum 25-hydroxyvitamin D were observed in African-American participants, and the left-shifted distribution of serum concentrations may be a contributing factor.

SNP—Serum Vitamin D Interactions: Gene x Environment

In European-American participants, significant interactions between serum 25-hydroxyvitamin D concentration and genotype for two *RXRA* SNPs were observed, one associated with FEV_1 and the other with FEV_1/FVC. Individuals carrying the minor alleles for these SNPs are more likely to have low lung function if they also have relatively low levels of serum 25-hydroxyvitamin D. Carriers of the alleles had lower lung function even at 20 ng/mL 25-hydroxyvitamin D, which is not conventionally considered to be a deficient level (Dietary Reference Intakes for Calcium and Vitamin D, Institute of Medicine: http://www.iom.edu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D.aspx). However, minor allele carriers with the highest 25-hydroxyvitamin D levels had lung function that was no different from participants with the major allele, suggesting that nutritional intervention may be effective at mitigating lung function decline. SNPs in *RXRA* were only significantly associated with lung function in combination with 25-hydroxyvitamin D, which suggests that the mechanism

of action for genetic variants in *RXRA* affecting lung function may be through the vitamin D metabolic pathway. This is important because RXRA has many functions as a regulator of gene expression, and binds other heterodimeric partners besides VDR. However, an interaction with 25-hydroxyvitamin D level is consistent with genetic variation that alters 1,25-dihydroxyvitamin D or VDR binding; if the slope of the linear portion of the binding curve differs due to genetic variation, low serum 25-hydroxyvitamin D levels would affect lung function, with no detectable differences by genotype at relatively high serum 25-hydroxyvitamin D concentrations when binding is saturated.

Observational studies of vitamin D are open to criticism because vitamin D nutriture is often correlated with general health; moreover, the possibility of reverse causation cannot be eliminated in cross-sectional studies, since illness can lead to lifestyle changes preventing sun exposure. In addition, a crucial limitation of association studies of vitamin D is the reliance on a single measurement of serum 25-hydroxyvitamin D. Attempts to adjust statistically for seasonal variation of serum 25-hydroxyvitamin D levels may not be completely effective, and a single measurement may not accurately reflect an individual's average status over the time period of interest (e.g., during adulthood as chronic disease is developing). The approach in the current study addresses these limitations, considering genetic variants as instrumental variables to provide an alternative, and theoretically more accurate, assessment of the cumulative effect of a long-term alterations in vitamin D functionality. The studied genes function downstream of serum 25-hydroxyvitamin D, affecting levels of 1,25-dihydroxyvitamin D and/or its interaction with the VDR/RXR heterodimer. Thus the current study provides strong evidence supporting a role for vitamin D in lung function.

Genetic variants in the vitamin D metabolic pathway genes, specifically in *VDR and CYP24A1,* were associated with lung function phenotypes in African-Americans. Gene-environment interactions, specifically SNP associations that were conditional on serum 25-hydroxyvitamin D concentrations, were identified in European-Americans for the *RXRA* gene. The results support a beneficial role for vitamin D in lung health. These results provide novel insights into molecular mechanisms governing lung function, and may help to identify at-risk individuals, and nutritional and/or pharmacological interventions for COPD.

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Table 3.1. Descriptive Characteristics of Health, Aging and Body Composition Study Participants

Variable:	African-Americans (N = 737)	European-Americans (N = 1259)
Age, years*	73.4 (2.9)	73.8 (2.9)
Females (%)	397 (53.9)	681 (54.1)
Memphis, TN site (%)	331 (44.9)	625 (49.6)
Former Smokers (%)	311 (42.2)	623 (49.5)
Current Smokers (%)	108 (14.7)	69 (5.5)
Packyears of cigarette smoking	15.4 (21.9)	19.0 (28.9)
Height, cm	165.8 (9.3)	167.2 (9.3)
FEV_1, mL	2051 (538)	2408 (617)
FEV_1 ratio	76.4% (6.1)	75.3% (5.5)
Serum 25-hydroxyvitamin D, ng/mL	21.1 (10.9)	29.0 (10.1)

*mean, SD shown unless otherwise indicated

Table 3.2. Statistically Significant SNPs (FDR < 0.2) and SNPs Selected for Haplotype Analysis (p < 0.01) in 737 African-American Participants in the Health ABC Study.

Phen	SNP	Gene	CHR	Location	MAF (%)	Model	B-coeff	P-value	FDR
FEV_1	rs3886163	*CYP24A1*	20	intron	12.3	dominant	116.1	0.001	**0.125***
	rs4809960	*CYP24A1*	20	intron	13.2	additive	-85.2	0.004	0.394
FEV_1/FVC	rs11168293	*VDR*	12	Exon/ 5'UTR	15.0	recessive	4.867	0.002	**0.174***
	rs3890733	*VDR*	12	intron	14.8	recessive	4.695	0.004	0.234
	rs6022999	*CYP24A1*	20	intron	37.6	recessive	1.786	0.006	0.292
	rs222046	*GC*	4	intron	12.9	additive	-1.334	0.004	0.643
	rs4364228	*GC*	4	intron	39.3	dominant	-1.253	0.007	0.692

Abbreviations: Phen, Phenotype; SNP, single nucleotide polymorphism; CHR, chromosome; MAF, minor allele frequency; B-coeff, beta coefficient; FDR, False Discovery Rate.

* FDR < 0.2

Figure 3.1

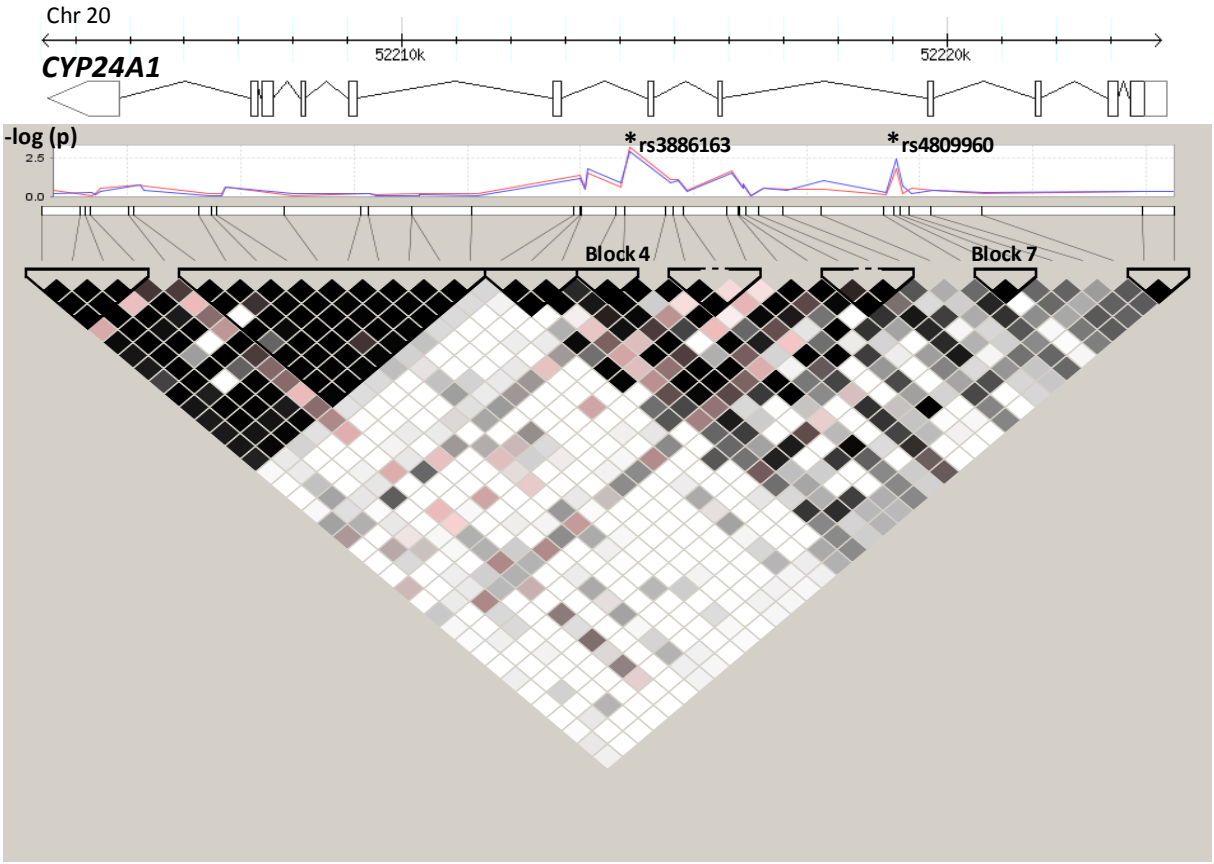

Figure 3.1. Haplotype structure and single SNPs in *CYP24A1*. Linkage disequilibrium map of *CYP24A1*, with black/gray shading representing regions of high LOD and high D' with darker shades representing higher D', and regions with pink shading representing low LOD and high D' with darker shades representing higher LOD. Gene annotation is shown at the top of the figure, with a Manhattan plot in between showing -log(p-value) for the association of single SNPs with FEV_1. SNPs with unadjusted $p < 0.01$ are shown (*), as well as haplotype blocks surrounding these SNPs (brackets, labeled Block 4 and Block 7).

Table 3.3. SNPs Selected for Haplotype Analysis ($p < 0.01$) in 1,259 European-American Participants in the Health ABC Study*.

Phenotype	SNP	Gene	CHR	LOC	MAF (%)	Model	B-coeff	P-value	FDR
FEV_1	rs705120	*GC*	4	intron	47.0	recessive	-77.44	0.010	0.697
	rs6097801	*CYP24A1*	20	3' UTR	27.5	dominant	-72.84	0.007	0.972
FEV_1/FVC	rs2254210	*VDR*	12	intron	34.2	dominant	0.862	0.006	0.455
	rs2853564	*VDR*	12	intron	13.7	dominant	0.8611	0.006	0.455

*Note, no SNPs in European-Americans reached the FDR preset threshold of 0.2

Abbreviations: SNP, single nucleotide polymorphism; CHR, chromosome; MAF, minor allele frequency; LOC, location; B-coeff, beta coefficient; FDR, False Discovery Rate

Table 3.4. Statistically Significant Haplotypes (Permuted P < 0.05) in *CYP24A1* Gene for the FEV_1 Phenotype in 737 African-American Participants in the Health ABC Study.

HAP Block	# SNPs in HAP	SNP 1	SNP 2	HAP	FREQ (%)	B- Coeff*	P-value	Permuted p-value†
4	2	rs6022994	rs3886163	AT	11.7	102	0.001	**0.006 ‡**
				CC	34.4	-31.6	0.140	0.496
				AC	53.9	-13.9	0.495	0.944
7	2	rs4809960	rs2296241	CA	13.3	-83.3	0.005	**0.024 ‡**
				TA	36.3	14.6	0.488	0.941
				TG	50.4	26	0.207	0.649

***** B-coefficient is for each haplotype compared with combination of other haplotypes in haplotype block with an allele frequency ≥5%

† Permuted p-value is the empirical p-value from the max(T) permutation procedure, which controls the family-wise error rate.

‡ Permuted p-value < 0.05

Abbreviations: SNP, single nucleotide polymorphism; HAP, haplotype; FREQ, frequency; B-coeff, beta coefficient

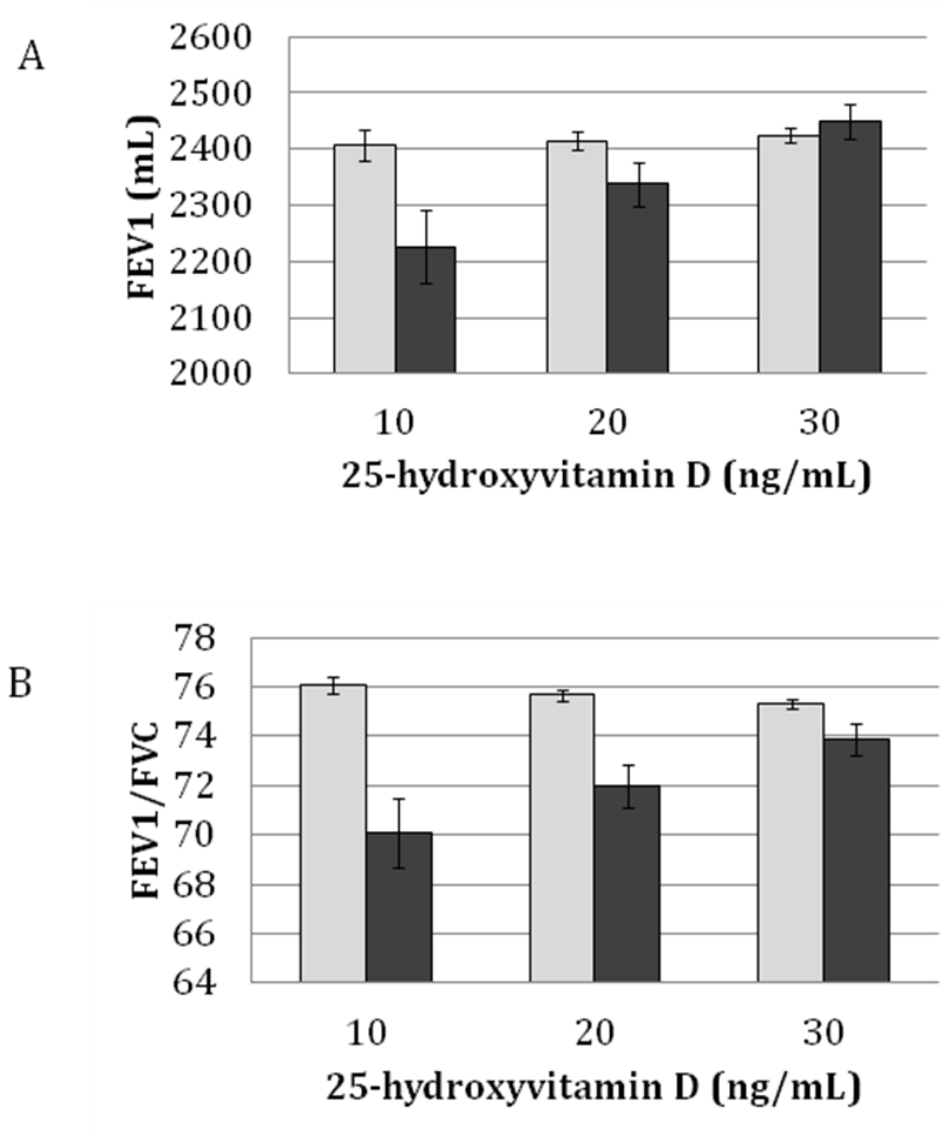

Figure 3.2. Statistically Significant Interaction of SNPs in *RXRA* and Serum 25-hydroxyvitamin D with FEV_1 in European-American Participants. (A) Predicted FEV_1 for each genotype of rs6537944 at different serum levels of 25-hydroxyvitamin D. Under dominant model, black bars comprise minor allele carriers (CC/CT genotypes), and gray bars comprise wildtype homozygotes (TT). **(B)** Predicted FEV_1/FVC for each genotype of rs4842196. Under recessive model, black bars comprise homozygote variant genotype (CC), and gray bars comprise wildtype (CA/AA genotypes).

Supplemental Table 3.1. SNPs in Vitamin D Metabolic Genes Analyzed for Association with Pulmonary Function in African-American Participants of the Health ABC Study.

GC	RXRA	CYP2R1	VDR	CYP27B1	CYP24A1
rs222048	rs11185647	rs11023374	rs2525046	rs11172325	rs6097801
rs705117	rs7855881	rs7935792	rs2525045	rs8176353	rs2762931
rs2282679	rs872298	rs1993116	rs881383	rs8176351	rs2762932
rs3755967	rs10881576	rs10500804	rs11574143	rs8176348	rs6097805
rs222047	rs3818740	rs7129781	rs11574138	rs4646537	rs6068810
rs222046	rs12339187	rs12794714	rs9729	rs8176347	rs6097807
rs16846912	rs10881578	rs2060793	rs7967673	rs4646536	rs16999060
rs1491709	rs10881580		rs7954412	rs11172327	rs4809957
rs705120	rs4917354		rs11574127	rs10877012	rs2762934
rs9016	rs914853		rs3858733	rs703842	rs927650
rs4588	rs11185659		rs3847987		rs1570669
rs7041	rs12348547		rs739837		rs1570670
rs4752	rs7041449		rs731236		rs2296239
rs4364228	rs7853934		rs7975232		rs6022990
rs6835052	rs7048602		rs11574114		rs912505
rs3737549	rs10881582		rs757343		rs6127119
rs222014	rs11185660		rs1544410		rs6097816
rs222016	rs7039190		rs7967152		rs6022993
rs222020	rs34312136		rs2239185		rs6022994
rs222023	rs34399387		rs2239184		rs3886163
rs16847015	rs35079168		rs11168266		rs6068816
rs1491718	rs4501664		rs11168267		rs6013905
rs1155563	rs11102986		rs11168268		rs6022995
rs1352844	rs11103473		rs11574077		rs4809958
rs1352845	rs10776909		rs2248098		rs3787554
rs2298849	rs6537998		rs2239182		rs3787555
rs3733359	rs7869783		rs2107301		rs2244719
rs16847024	rs1805352		rs2283342		rs3787557
	rs3132296		rs2239181		rs2762941
	rs3118529		rs1540339		rs2181874
	rs731516		rs2239179		rs4809959
	rs3118536		rs11574065		rs4809960
	rs7038018		rs12717991		rs2296241
	rs4240705		rs886441		rs2245153
	rs11103462		rs2189480		rs2585428
	rs6537944		rs3819545		rs6022999
	rs12349076		rs2239186		rs2248359
	rs3118571		rs11574048		rs2248461
	rs1536475		rs10735810		
	rs3132294		rs10160907		
	rs877954		rs11574047		
	rs1805343		rs2254210		
	rs4842194		rs11574038		
	GA007945		rs2238136		
	rs1045570		rs2853564		
	rs4842196		rs11574032		
			rs4760648		
			rs11168287		
			rs4328262		
			rs4334089		
			rs4237855		
			rs11574026		
			rs3890733		
			rs11168293		
			rs7136534		
			rs7299460		
			rs11574020		
			rs11574019		
			rs11574017		
			rs11574012		
			rs4516035		

*Gene names are column headings; rs numbers in columns under gene name show SNPs selected for analysis for each gene

Supplemental Table 3.2. SNPs in Vitamin D Metabolic Genes Analyzed for Association with Pulmonary Function in European-American Participants of the Health ABC Study.

GC	RXRA	CYP2R1	VDR	CYP27B1	CYP24A1
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rs2282679	rs872298	rs7935792	rs11574143	rs4646536	rs2762931
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rs222047	rs3818740	rs10500804	rs3858733	rs10877012	rs6097805
rs222046	rs12339187	rs7129781	rs3847987	rs703842	rs6068810
rs1491709	rs10881578	rs12794714	rs739837		rs6097807
rs705120	rs10881580	rs2060793	rs731236		rs4809957
rs4588	rs4917354		rs7975232		rs2762934
rs7041	rs914853		rs11574114		rs927650
rs4364228	rs11185659		rs757343		rs1570669
rs222014	rs7041449		rs1544410		rs1570670
rs222016	rs7853934		rs7967152		rs2296239
rs222020	rs7048602		rs2239185		rs912505
rs222023	rs10881582		rs2239184		rs6127119
rs16847015	rs11185660		rs11168266		rs3886163
rs1491718	rs7039190		rs11168267		rs6068816
rs1155563	rs34312136		rs11168268		rs6013905
rs1352844	rs35079168		rs11574077		rs4809958
rs1352845	rs11102986		rs2248098		rs3787554
rs2298849	rs11103473		rs2239182		rs3787555
rs3733359	rs10776909		rs2107301		rs2244719
	rs1805352		rs2283342		rs3787557
	rs3132296		rs2239181		rs2762941
	rs3118529		rs1540339		rs2181874
	rs3118536		rs2239179		rs4809959
	rs7038018		rs12717991		rs4809960
	rs4240705		rs886441		rs2296241
	rs6537944		rs2189480		rs2245153
	rs3118571		rs3819545		rs2585428
	rs1536475		rs2239186		rs6022999
	rs3132294		rs11574048		rs2248359
	rs877954		rs10735810		rs2248461
	rs1805343		rs2254210		
	rs1805348		rs2238136		
	rs4842194		rs2853564		
	GA026144		rs11574032		
	rs1045570		rs4760648		
	rs4842196		rs11168287		
			rs4328262		
			rs4334089		
			rs4237855		
			rs11574027		
			rs11574026		
			rs3890733		
			rs11168293		
			rs7136534		
			rs7299460		
			rs11574012		
			rs4516035		

*Gene names are column headings; rs numbers in columns under gene name show SNPs selected for analysis for each gene

Supplemental Table 3.3. Characteristics of Excluded Participants, the Health Aging and Body Composition Study.

Variable:	African-Americans (n = 544)	European-Americans (n = 535)
Age, years *	73.5 (2.9)	73.8 (2.9)
Females (%)	212 (39.0)	258 (48.2)
Memphis, TN site (%)	282 (51.8)	310 (57.9)
Former Smokers (%)	187 (34.5)	283 (53.1)
Current Smokers (%)	99 (18.3)	42 (7.9)
Packyears of cigarette smoking	18.3 (26.8)	25.1 (33.2)
Height, cm	164.8 (9.4)	165.9 (9.5)
FEV_1, mL	1755.5 (593)	1949 (636)
FEV_1 ratio	75.3% (13.8)	72.3% (12.8)
Serum 25-hydroxyvitamin D, ng/mL	20.6 (9.04)	29.1 (13.0)

*mean (SD) unless otherwise noted

Supplemental Table 3.4. The Association of SNPs with Serum 25-hydroxyvitamin D Concentration for Vitamin D Metabolic SNPs associated with Pulmonary Function in Prior Models (FDR < 0.2)

SNP	Gene	CHR	LOC	MAF (%)	Model	B-coeff*	P-value	FDR
rs3886163	*CYP24A1*	20	intron	12.3	additive	-1.135	0.138	0.415
rs11168293	*VDR*	12	Exon/ 5' UTR	15.0	additive	0.501	0.462	1

Abbreviations: SNP, single nucleotide polymorphism; CHR, chromosome; LOC, location; MAF, minor allele frequency; B-coeff, beta coefficient; FDR, False Discovery Rate

* The beta-coefficient reflects the difference in serum 25-hydroxyvitamin D concentration (ng/mL) per allele copy.

Supplemental Table 3.5. Full Regression Model for Statistically Significant Interaction of rs6537944S (*RXRA)* and Serum 25-hydroxyvitamin D with FEV_1 in European-American Participants.

Variable	**β-Coefficient***	**Standard Error**	**P-value**
Intercept	-359.15	499.11	0.4719
25-OH-D	0.91	1.29	0.4782
rs6537944	-281.49	100.56	0.0052
25-OH-D * rs6537944	10.21	3.35	0.0023
Principal Component 1	-82.00	543.80	0.8802
Principal Component 2	-3075.01	3957.13	0.4373
Smoking status	-40.45	12.65	0.0014
Age (years)	-27.10	4.14	<.0001
Gender	-443.04	37.70	<.0001
Height (cm)	29.57	2.01	<.0001
Study site	102.06	25.89	<.0001

*β-Coefficients reflect the difference in FEV_1 (mL) for variables as follows:
25-OH-D: FEV1 difference per unit of 25-OH-D (ng/mL), continuous variable
rs6537944: FEV_1 difference in 'CC' and 'CT' genotypes versus the homozygous major allele, 'TT'
25-OH-D * rs6537944: Interaction between rs6537944 genotype and 25-OH-D concentration
Smoking status: FEV_1 difference in current versus former smokers; FEV1 difference in former versus never smokers
Gender: FEV_1 difference in females versus males
Study site: FEV_1 difference in Pittsburgh, PA participants versus Memphis, TN participants

Supplemental Table 3.6. Full Regression Model for Statistically Significant Interaction of rs4842196 (*RXRA)* and Serum 25-hydroxyvitamin D with FEV_1/FVC in European-American Participants.

Variable	**β-Coefficient***	**Standard Error**	**P-value**
Intercept	97.79694640	6.62966348	<.0001
25-OH-D	-0.03636600	0.01626905	0.0256
rs4842196	-8.21227487	2.05614502	<.0001
25-OH-D*rs4842196	0.22553208	0.06501572	0.0005
Principal Component 1	-5.32950821	7.19503491	0.4590
Principal Component 2	-53.61006232	52.31159098	0.3057
Smoking status	-0.76686965	0.16809712	<.0001
Age (years)	-0.17355614	0.05492437	0.0016
Gender	-0.15736494	0.50030684	0.7532
Height (cm)	-0.04748588	0.02667441	0.0753
Study site	0.54069414	0.34345300	0.1157

*β-Coefficients reflect the difference in FEV_1/FVC for variables as follows:
25-OH-D: FEV1 difference per unit of 25-OH-D (ng/mL), continuous variable
rs4842196: FEV_1/FVC difference in the 'CC' genotype versus carriers of the major allele, 'CA' and 'AA'
25-OH-D * rs4842196: Interaction between rs4842196 genotype and 25-OH-D concentration
Smoking status: FEV_1/FVC difference in current versus former smokers; FEV1 difference in former versus never smokers
Gender: FEV_1/FVC difference in females versus males
Study site: FEV_1/FVC difference in Pittsburgh, PA participants versus Memphis, TN participants

CHAPTER 4

ASSOCIATION OF 25-HYDROXYVITAMIN D WITH GENE EXPRESSION IN LUNG EPITHELIUM OF HEALTHY NON-SMOKERS

Abstract

Vitamin D is known to alter gene expression *in vitro*, but effects on gene expression in humans are unclear. The primary biomarker of vitamin D status, serum 25-hydroxyvitamin D, is associated with lung health in epidemiologic studies, but the mechanisms mediating vitamin D—lung outcome associations are poorly understood. The findings presented herein demonstrate, for the first time, differential gene expression in lung epithelial cells associated with serum 25-hydroxyvitamin D in a diverse sample of free-living humans. Microarray analysis investigated the association of gene expression in small airway epithelial cells with serum 25-hydroxyvitamin D in healthy, adult non-smokers. The analysis was restricted to candidate genes with prior evidence of vitamin D-modulated gene expression *in vitro* and at least one predicted vitamin D response element. Of the 156 genes studied, 13 genes had statistically significant differences in expression by serum 25-hydroxyvitamin D status ($p < 0.05$), and 3 genes (*KCNS3*, *FSTL1*, and *DAPK1*) were significant at a false discovery rate cutoff of <0.2. Serum 25-hydroxyvitamin D level explained 40%, 28%, and 14% of variance in gene expression in *FSTL1*, *KCNS3*, and *DAPK,* respectively. Gene

ontology and literature analysis of differentially expressed genes supported plausible mechanisms for functional roles in asthma, COPD, cancer, and response to infection in lung, as well as striking similarities between glucocorticoid responses and vitamin D-associated gene expression. The physiological range of 25-hydroxyvitamin D is associated with functional differences in molecular outcomes in lung, implying mechanisms that explain and strengthen existing associations of 25-hydroxyvitamin D with lung health.

Introduction

Recent evidence suggests plausible mechanisms by which vitamin D might affect lung health, but there is little research translating animal and cell models to results in humans. The majority of experimental work to date has focused on effects of the active metabolite of vitamin D: 1,25-dihydroxyvitamin D. This metabolite is generated in the kidney for systemic circulation, and is hypothesized to be produced locally in many tissues, including lung [1]. Because 1,25-dihydroxyvitamin D has a short half-life, is tightly regulated, and can be generated in localized tissues for autocrine or paracrine effects on demand, it may not be an appropriate serum biomarker for overall vitamin D nutriture [2]. Instead, the precursor, 25-hydroxyvitamin D, is accepted as the primary biomarker for vitamin D exposure in humans [3]. However, it is not yet clear how 25-hydroxyvitamin D levels correspond to active 1,25-dihydroxyvitamin D levels in target tissues, raising the possibility that effects observed in experimental models may be pharmacologic rather than physiologic, due to inappropriate dosing of 1,25-dihydroxyvitamin D. Moreover, effects observed in the relatively controlled environments of cell and animal models might not be detectable in free-living humans experiencing diverse diets and environments. It is not yet established whether ranges of 25-hydroxyvitamin D observed in humans are associated with effects similar to those seen *in vitro* for 1,25-hydroxyvitamin D.

Observational studies support an association of physiological ranges of 25-hydroxyvitamin D with lung outcomes. A study in the Third National Health and Nutrition Examination Survey (NHANES III) reported a positive association between serum 25-hydroxyvitamin D concentration and lung function [4]. Low serum 25-hydroxyvitamin D was associated with higher risk for severe exacerbation of mild-to-

moderate persistent asthma in children [5], and a higher risk for active tuberculosis [6]. Evidence that vitamin D plays a role in lung cancer is controversial, but low 25-hydroxyvitamin D levels were associated with increased lung cancer risk in women and young people [7], and high levels of 25-hydroxyvitamin D were associated with improved survival in non-small cell lung cancer patients [8]. Reports of an association of 25-hydroxyvitamin D with lung outcomes are intriguing, but few studies directly addressed mechanisms, which would strengthen the causal inference of population-level association studies.

Vitamin D is postulated to affect lung health through its function as a steroid hormone, by directly altering gene expression by interaction with the vitamin D receptor (VDR), and subsequent binding to vitamin D response elements in DNA [9]. Cell studies have shown that 1,25-dihydroxyvitamin D affects expression of genes related to inflammation, cell proliferation, and cell migration [10,11], which suggests plausible causal mechanisms for vitamin D associations with asthma, chronic obstructive pulmonary disease (COPD), and lung cancer. Only one clinical intervention study reported the effect of vitamin D supplementation on inflammation and tissue remodeling biomarkers: in vitamin D deficient British Bangladeshi men, injections of non-hydroxylated vitamin D decreased circulating serum levels of matrix metalloprotease-9 (68%) and C-reactive protein (23%) in relation to pre-supplementation values [12].

Here, we investigate the hypothesis that serum 25-hydroxyvitamin D levels affect gene expression in lung epithelial tissues sampled from free-living humans. We examined gene expression by microarray and evaluated gene ontology annotations for functional information. Our results provide evidence that physiological levels of serum 25-hydroxyvitamin D are associated with differences in gene expression in lung tissue,

and that the differences in expression occur in genes from functional categories with high relevance to lung health.

Materials and Methods

Healthy nonsmoker volunteers were recruited yielding 26 individuals who were evaluated at the Weill Cornell Medical College General Clinical Research Center under IRB-approved protocols. Participants underwent physical examinations to confirm the absence of disease as described elsewhere [13].

Frozen serum samples were sent to the Nutritional Biomarkers Branch of the Division of Laboratory Sciences at the Centers for Disease Control and Prevention to be assayed for 25-hydroxyvitamin D by liquid chromatography-tandem mass spectrometry. Samples were evaluated in parallel with NIST standard SRM 972, and results were within 2 standard deviations of NIST target values. External quality assurance was provided through participation in the Vitamin D External Quality Assessment Scheme.

Two individuals from the second tertile of 25-hydroxyvitamin D levels were excluded from further consideration because serum and cell collections occurred in different seasons and more than 120 days apart. The average time between serum and epithelial cell collection was 42 ± 40 days, within the 10-week half-life of serum 25-hydroxyvitamin D [14].

Airway epithelial cells were collected by fiberoptic bronchoscopy, as described elsewhere [13]. Briefly, small airway epithelial cells were collected with gentle brushing, removed from the brush by flicking in ice-cold basal epithelial cell medium, and cells were immediately processed to extract RNA [13].

First and second strand cDNA was synthesized from 6 μg of RNA, *in vitro*

transcribed, and fragmented according to Affymetrix protocols. As a quality control, only labeled RNA producing a 3' to 5' ratio of < 3 on test chips was used. Samples were hybridized to the Affymetrix HG-U133 Plus 2.0 array, washed and treated with reagents by the fluidics station, then scanned in duplicate [13].

Candidate genes for microarray analysis were identified based on literature evidence of regulation by 1,25-dihydroxyvitamin D in squamous epithelial cells [15]. Candidate genes were required to contain at least one predicted binding site for VDR (a DR3 or ER6 response element with up to 1 base mismatch from the consensus sequence) [15]. Image files for the arrays were assessed for quality of hybridization by comparing 3' to 5' intensity of transcripts for actin and GAPDH (ratio < 3). Normalization was carried out by GeneChip Robust Multi-Array Average (GC-RMA) using MADMAX software (https://madmax.bioinformatics.nl). Quality control of normalized data was evaluated using plots of relative log expression and normalized unscaled standard errors to identify array artifacts. Only probe sets with an interquartile range (IQR) of $\log_2$ normalized values < 0.5 were included in analysis.

The statistical significance of fold-changes in expression between the first and third tertile of serum vitamin D was calculated using a t-test with Bayesian correction (Limma). A threshold of nominal P <0.05 was used to highlight findings for further analyses. Q-values taking into account false discovery rate (FDR) were calculated for all candidate genes using SAS version 9.2. Pearson product moment correlation coefficients and the variance (R^2) in gene expression explained by serum 25-hydroxyvitamin D were calculated, and included the full range of vitamin D concentrations. Gene ontology annotations were obtained from the UniProtKb-GOA database (http://www.ebi.ac.uk/QuickGO/), with preference given to IDA annotations

(inferred from direct assay) or TAS (traceable author statement) evidence codes. IEA (inferred from electronic annotation) evidence codes were used if no other information was available.

Results

Healthy, non-smoking adults (n=26) were divided into tertiles of serum 25-hydroxyvitamin D status (Table 4.1). Candidate genes were identified based on literature evidence of 1,25-dihydroxyvitamin D responsiveness and presence of at least one predicted vitamin D response element, as described above. Further filtering for interquartile range (IQR) > 0.5 on $\log_2$ scale yielded a final set of 156 candidate genes (Supplemental Table 4.1).

Differential Expression in High Compared to Low 25-hydroxyvitamin D Tertiles

Among the candidate genes considered, thirteen genes had statistically significant (nominal $p < 0.05$) differences in expression between the first and third tertiles of serum 25-hydroxyvitamin D (Table 4.2). Of these, three genes (*KCNS3, FSTL1*, and *DAPK1*) were significant at a false discovery rate cutoff of < 0.2. Investigating a list of candidate genes selected *a priori* based on prior evidence of vitamin D-regulated expression and VDR binding sites yielded proportionately more hits than a random list of all genes. While the expression of 8.3% of candidate genes (13/156 genes) was associated with serum 25-hydroxyvitamin D level, considering the unfiltered list of all genes passing IQR filtering, only 2.75% (129/4688 genes) were associated with serum vitamin D. Thus, restricting analysis to candidate genes with prior *in vitro* and *in silico* evidence of vitamin D-regulated expression resulted in a three-

fold higher percentage of genes with nominally significant differences in expression between the highest and lowest tertiles of vitamin D status.

To characterize further the relation of serum 25-hydroxyvitamin D level with gene expression, the linear association of gene expression with continuous serum 25-hydroxyvitamin D was assessed for all nominally significant genes (Table 4.3). The percent of variance (R^2, from linear regression analysis) explained by serum 25-hydroxyvitamin D ranged from 8 to 40%; the R^2 for *FSTL1* was 40%.

Further investigation was undertaken to assess the likelihood of a causal association of serum vitamin D with gene expression; literature searches were conducted to identify evidence of modulation of these genes by vitamin D in cell culture or animal models. Among the thirteen genes with nominally significant associations, five genes (*DAPK1, CST6, SLITRK6, EMB,* and *KLF4*) had evidence of regulation by vitamin D in prior studies [16,17,18,19,20].

Functional Categories of Differentially Expressed Genes

To determine known functions, pathways, and subcellular locations for differentially expressed genes, gene ontology categories assigned to each gene in the UniProtKb-GOA database were investigated (Table 4.4). Six genes were integral membrane proteins (*KCNS3, SLITRK6, TMEM40, EMB, PTGER2, SGPP2*), with cell surface or extracellular location specified for 5 genes (*FSTL1, CST6, KAL1, EMB, PTGER2*). The most common functional category was for proteins with metal ion binding or channel activity (*KCNS3, FSTL1, RSAD2,* and *DTX4*). Additionally, two genes were endopeptidase inhibitors (*CST6*, *KAL1*), and two genes were kinases or phosphatases (*DAPK1, SGPP2*). Roughly half of the genes were involved in pathways

of cell fate determination or morphogenesis (*KLF4, DTX4, SLITRK6, KAL1, CST6, FSTL1*), and 3 genes were assigned to pathways of cell proliferation or apoptosis regulation (*PTGER2, KLF4, DAPK1*).

Discussion

In a study of free-living, healthy never smokers, we identified 13 genes with significantly different lung epithelial cell gene expression between high and low tertiles of serum 25-hydroxyvitamin D; three genes (*KCNS3, FSTL1*, and *DAPK1*) were significant at an FDR cutoff of 0.2. The variance in gene expression (R^2) explained by serum vitamin D in linear models exceeded 20% for 8 of the 13 nominally significant genes; *FSTL1* had the highest R^2, at 40%. The primary analysis was limited to an *a priori* selection of 156 candidate genes, all of which had prior evidence of regulation by vitamin D in cultured human squamous epithelial cells and contained putative VDR response elements predicted from consensus sequences. By limiting the analysis to an hypothesis-oriented selected set of genes, causal inferences are strengthened, and the selected genes were about 3-times more likely to be associated with serum vitamin D than an unselected list.

Although associations were observed between serum 25-hydroxyvitamin D levels and gene expression, this cross-sectional study can not establish causality. A potentially confounding factor, race, also differed by tertile of vitamin D, with a higher proportion of European-Americans in the upper portion of the vitamin D distribution and higher proportion of African Americans in the lower portion. Given that skin pigmentation interferes with vitamin D synthesis in response to sunlight [3], the association of race with serum 25-hydroxyvitamin D levels is expected, but because of the small sample

size the analysis cannot be limited to single race groups. Thus, observed associations may be partly explained by race rather than by serum vitamin D levels. A recent microarray study comparing gene expression levels in lymphoblastoid cell lines derived from European and African populations in the International HapMap Project [21] provides evidence that 4 of our 13 nominally significant genes were differentially expressed by race: *SGPP2, EMB, DAPK1*, and *RSAD2*. In all four, the direction of the expression difference was similar between this study and the HapMap results, thus the associations of these genes with serum 25-hydroxyvitamin D may be partly explained by race. However, other literature confirms vitamin D regulation for *EMB* and *DAPK1* in cell or animal models [16,19], thus raising the possibility that both race and vitamin D levels affect expression of these genes.

Functional analysis of the set of 13 differentially expressed genes suggested plausible mechanisms for vitamin D function in lung health. Nearly half of the genes were assigned to gene ontology pathways of cell fate determination or morphogenesis, thus implying roles in both cancer pathogenesis and airway remodeling typical of chronic obstructive pulmonary disease. Two genes were identified as endopeptidase inhibitors, and three were assigned to pathways of cell proliferation or apoptosis regulation, supporting possible roles in airway remodeling and cancer. To further explore the mechanisms by which vitamin D-modulated gene expression affects lung health, the literature evidence for the 13 differentially expressesd genes is reviewed below.

Asthma and COPD

Vitamin D is associated with both asthma and COPD, but mechanisms remain

unclear [22]. Among the set of 13 differentially expressed genes reported herein, several genes were associated with asthma or COPD in past studies, and several genes were reported to play a role in airway remodeling and inflammation, which are common features in both diseases. For example, polymorphisms in *KCNS3* were associated with airway hyperresponsiveness [23], and genetic variation in *PTGER2* was associated with aspirin-intolerant asthma [24]. *PTGER2* was significantly down-regulated in T cells isolated from children with persistent wheeze [25], but was up-regulated in the high vitamin D tertile in the current study. In COPD patients with wasting, *KLF4* was differentially regulated [26]. Thus, emerging evidence supports a role for the identified genes in asthma and COPD.

The differentially expressed gene with the lowest nominal P, *KCNS3*, was up-regulated by the lung growth factor, bone morphogenetic protein (BMP-2, [27]), which plays a role in embryonic lung development and airway branching, and may influence inflammatory processes [28]. The second most statistically significant gene, *FSTL1*, is also part of the BMP signaling pathway in gene ontology analysis. *FSTL1* (down-regulated with high vitamin D) is pro-inflammatory, and leads to up-regulation of the pro-inflammatory cytokines TNF-alpha, IL-6, and IL-1beta [29]. Several studies report that higher serum 25-hydroxyvitamin D level is associated with lower TNF-alpha and IL-6 [30,31,32]. The findings presented herein suggest that *FSTL1* may mediate the association of serum 25-hydroxyvitamin D level with the pro-inflammatory cytokines.

In examining these results, a common pattern of gene regulation was observed. Genes differentially regulated by vitamin D overlapped with the set of genes differentially regulated by glucocorticoids. Dexamethasone, which is used to treat both asthma and COPD, is associated with the expression of many of the genes identified by

the present study, and there were striking similarities in the direction of effect. *KCNS3* and *FSTL1* are down-regulated by dexamethasone [33,34], and they are similarly down-regulated in the highest tertile of serum vitamin D. Glucocorticoid receptor knockout mice had 10-fold up-regulated *SLITRK6* in lung compared to wild type mice, suggesting glucocorticoids normally down-regulate *SLITRK6* [35]; similarly, *SLITRK6* was down-regulated in the highest tertile of serum vitamin D. *EMB* was up-regulated by dexamethasone treatment in alveolar macrophages [36], and *EMB* was similarly up-regulated in the highest tertile of serum 25-hydroxyvitamin D level. The combination of 1,25-dihydroxyvitamin D with dexamethasone has been investigated *in vitro* as an anti-inflammatory treatment [37]; our results suggest the strong possibility of synergistic effects for this treatment combination. Indeed, high vitamin D levels have recently been associated with improved glucocorticoid response [38].

Lung Cancer

Vitamin D is implicated in carcinogenesis, but its role is controversial, and cancer mortality is associated with both very low and very high vitamin D levels [39]. Supporting a preventive role for vitamin D, *RSAD2*, which we found to be up-regulated with high serum 25-hydroxyvitamin D level, is also induced by interferon-gamma (IFN-g, [40]), a cancer treatment. In one study, co-administration of 1,25-dihydroxyvitamin D with IFN-g produced synergistic responses in lung tumors [41]; up-regulation of the same genes by two different mechanisms might explain this synergy. Another gene up-regulated with high 25-hydroxyvitamin D, *CST6*, is a candidate tumor suppressor gene purported to be down-regulated in lung cancer [42]. Similarly, *PTGER2*, which was up-regulated with high serum 25-hydroxyvitamin D level, is down-regulated in 80% of non-small cell lung

cancer (NSCLC) cell lines. Methylation of *PTGER2* was associated with lower gene expression in 58% of NSCLC specimens, compared to only 1% of non-malignant lung samples [43]. Up-regulation of *KLF4*, as observed here in the highest tertile of serum 25-hydroxyvitamin D level, inhibits lung cancer cell invasion in Matrigel assays [8].

The expression of two genes in relation to serum 25-hydroxyvitamin D level suggests a cancer promoting role for vitamin D. *SGPP2* was upregulated in the highest tertile of serum 25-OH-D, and is also upregulated in NSCLC cell lines with somatic mutations in EGFR [44], was associated with vitamin D.*DAPK1*, which was down-regulated in the highest serum 25-hydroxyvitamin D tertile, may be a tumor suppressor gene; *DAPK1* was down-regulated in 72% of squamous cell lung cancer samples [45]. *KAL1*, also down-regulated with high serum 25-hydroxyvitamin D level, was down-regulated in lung adenocarcinomas [46]. Moreover, low expression of *KAL1* was associated with poor prognosis in NSCLC [47]. Thus, the direction of effect of vitamin D on gene expression is inconsistent, and these inconsistencies may contribute to resolving the reports of increased cancer risk with excessive vitamin D supplementation.

Lung Response to Infection

Serum 25-hydroxyvitamin D concentration is associated with native immune response [22], and our results suggest specific mechanisms that could mediate lung response to viral and bacterial infections. For example, the protein product of *RSAD2* (up-regulated with high 25-hydroxyvitamin D levels), is an antiviral protein called viperin, strongly induced in lung immune cells in response to viral or *Pneumocystis* infection [36,48]. *EMB* was up-regulated in the highest tertile of serum 25-hydroxyvitamin D, and *Pneumocystis* infection also up-regulated *EMB* expression in alveolar macrophages

[36]. *SGPP2* was up-regulated with high serum 25-hydroxyvitamin D level, and was also up-regulated in response to cytomegalovirus infection in a human lung fibroblast cell line [49]. These results identify hypotheses for further investigation related to the mechanisms through which vitamin D affects immune functioning and response of the lung to infection.

Conclusions

An abundance of experimental data support effects of 1,25-dihydroxyvitamin D on gene expression *in vitro*. However, virtually no translational studies have linked the often non-physiological concentrations of 1,25-dihydroxyvitamin D used *in vitro* with levels of the commonly used biomarker, 25-hydroxyvitamin D, and molecular effects in humans *in vivo*. We have demonstrated here, for the first time, differential gene expression in lung associated with the physiologic range of 25-hydroxyvitamin D in a diverse sample of free-living humans. The candidate genes considered had prior evidence of modulation by 1,25-dihydroxyvitamin D in cell culture and/or animal models, and the subset of genes with statistically significant differences in expression by serum 25-hydroxyvitamin D levels have known roles in biological processes relevant to lung health. These results suggest mechanisms for the role of vitamin D in cancer, asthma, COPD, and response to infection, and we have identified a physiological range for 25-hydroxyvitamin D at which differential responses occur at the molecular level. The results inform future discussions of public health recommendations for optimal serum 25-hydroxyvitamin D levels, and will help to guide the design of randomized controlled intervention trials for vitamin D in lung disorders.

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Table 4.1. Characteristics of 26 Non-smoking Human Volunteers by Tertile of Serum Vitamin D.

	Serum Vitamin D		
Variable	Tertile 1 (n=9)	Tertile 2 (n=9)	Tertile 3 (n=8)
Serum 25-OH-D, ng/mL (range)	8.99 (2.3 - 11.8)	20.9 (12.7 - 26.7)	33.3 (27.9 - 39.7)
Age, years (median)	36.9 (38)	44.1 (45)	50.6 (46.5)
Males (%)	6 (67%)	6 (67%)	7 (87%)
Race/Ethnicity (%)			
African American	5 (56%)	6 (67%)	1 (13%)
European	1 (11%)	3 (33%)	7 (87%)
Hispanic	2 (22%)	0 (0%)	0 (0%)
Asian	1 (11%)	0 (0%)	0 (0%)

*mean (standard deviation), unless otherwise noted

Table 4.2. Fold Change in Expression and P-value of Nominally Significant (p<0.05) Candidate Genes.

Gene	Gene name	CHR	Fold Change*	P-value	Q-value**
KCNS3	Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3	2	-1.62	0.00084	0.12739
FSTL1	Follistatin-like 1	3	-1.55	0.00163	0.12739
DAPK1	Death-associated protein kinase 1	9	-2.06	0.00381	0.19820
RSAD2	Radical S-adenosyl methionine domain containing 2	2	1.41	0.01103	0.43023
CST6	Cystatin E/M	11	1.79	0.01516	0.47285
KAL1	Kallmann syndrome 1 sequence	X	-1.38	0.01840	0.47828
SLITRK6	SLIT and NTRK-like family, member 6	13	-1.52	0.02482	0.49106
TMEM40	Transmembrane protein 40	3	1.55	0.02518	0.49106
EMB	Embigin	5	1.52	0.03099	0.50707
PTGER2	Prostaglandin E receptor 2 (subtype EP2)	14	1.36	0.03574	0.50707
DTX4	Deltex homolog 4	11	-1.34	0.03812	0.50707
KLF4	Kruppel-like factor 4	9	1.66	0.03901	0.50707
SGPP2	Sphingosine-1-phosphate phosphatase 2	2	1.69	0.04491	0.53897

Abbreviation: CHR, chromosome
*Fold change in high versus low tertile serum 25-hydroxyvitamin D
**FDR adjusted p-value (Calculated with QVALUE software by Alan Dabney and John Storey. http://genomics.princeton.edu/storeylab/qvalue/index.html)

Table 4.3. Linear Regression of Nominally Significant ($p<0.05$) Candidate Genes, Predicting Gene Expression by Serum Vitamin D Concentration (linear variable, including full range of vitamin D)

Gene	Gene Name	Regression Coefficient Beta (SE)	R-squared (%)*
KCNS3	Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3	-0.247	28%
FSTL1	Follistatin-like 1	-21.1	40%
DAPK1	Death-associated protein kinase 1	-0.768	17%
RSAD2	Radical S-adenosyl methionine domain containing 2	1.99	16%
CST6	Cystatin E/M	2.95	20%
KAL1	Kallmann syndrome 1 sequence	-9.20	28%
SLITRK6	SLIT and NTRK-like family, member 6	-11.3	25%
TMEM40	Transmembrane protein 40	0.448	23%
EMB	Embigin	0.482	23%
PTGER2	Prostaglandin E receptor 2 (subtype EP2)	2.32	9%
DTX4	Deltex homolog 4	-12.0	15%
KLF4	Kruppel-like factor 4	1.71	9%
SGPP2	Sphingosine-1-phosphate phosphatase 2	1.09	24%

*R-squared is the proportion of the variance in expression accounted for by serum vitamin D

Table 4.4. Gene Ontology of Nominally Significant Candidate Genes from the UniProtKb-GOA Database (http://www.ebi.ac.uk/QuickGO/)

Gene	Gene Name	Function(s)	Pathway(s)	Location(s)
KCNS3	Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3	delayed-rectifier potassium channel	potassium ion transport, regulation of insulin secretion	Golgi and plasma membrane
FSTL1	Follistatin-like 1	calcium ion binding, heparin binding	Bone morphogenetic protein signaling pathway	Extracellular space
DAPK1	Death-associated protein kinase 1	ATP and calmodulin binding	intracellular protein kinase cascade, apoptosis regulation	actin cytoskeleton
RSAD2	Radical S-adenosyl methionine domain containing 2	iron-sulfur cluster binding, metal ion binding	defense response to virus	endoplasmic reticulum
CST6	Cystatin E/M	cysteine-type endopeptidase inhibitor	anatomical structure morphogenesis	cornified envelope, extracellular region
KAL1	Kallmann syndrome 1 sequence	extracellular matrix structural component, serine-type endopeptidase inhibitor	axon guidance, chemotaxis, cell movement, cell adhesion	cell surface, extracellular space
SLITRK6	SLIT and NTRK-like family, member 6	N/A	axonogenesis	integral membrane protein
TMEM40	Transmembrane protein 40	N/A	N/A	integral membrane protein
EMB	Embigin	N/A	cell adhesion	integral membrane protein
PTGER2	Prostaglandin E receptor 2 (subtype EP2)	G protein coupled receptor for prostaglandin E	GPCR signaling, regulation of cell proliferation	integral to plasma membrane
DTX4	Deltex homolog 4	zinc ion binding	Notch signaling pathway	cytoplasm
KLF4	Kruppel-like factor 4	transcription repressor activity	regulation of cell proliferation, mesodermal cell fate determination	nuclear
SGPP2	Sphingosine-1-phosphate phosphatase 2	sphingosine-1-phosphate phosphatase activity	sphingosine metabolic process	endoplasmic reticulum membrane

Supplemental Table 4.1**.** List of 156 Candidate Genes Examined for Differential Gene Expression

ACAD8	EIF2C2	KMO	SCPEP1
ADAM28	ELP4	KRAS	SDC1
ADRB2	EMB	LAMB3	SEMA4B
AKR1C1	ENTPD3	LGALS8	SERPINB13
ALMS1	ETNK2	LOC113230	SERPINB3
ALOX5	EVI5	LOC157381	SGPP2
ALS2	FAM38A	LOC284837	SIX2
ANTXR2	FAM45B	LYN	SLC30A1
ARL2BP	FAM49A	MAFB	SLITRK6
C14orf149	FGFBP1	MAFF	SMAD7
CA2	FHOD3	MAN1C1	SNCAIP
CAB39	FLRT3	MAN2C1	SOX9
CASP4	FOXF1	MAP3K8	SP100
CD14	FOXQ1	MMP1	SQLE
CD86	FSTL1	MPHOSPH6	ST3GAL6
CFL2	GADD45A	MYO1D	ST8SIA1
CHIC2	GALNT5	NAV3	STAMBP
CHST11	GCH1	NFKBIZ	STAU2
CLC	GCNT1	NSUN6	STK39
CLGN	GEM	OASL	TAF1B
COL16A1	GIMAP8	OLR1	TCF7L1
COL1A1	GREM1	OSTalpha	TM4SF1
COLEC12	HBEGF	OXTR	TM7SF3
CST6	HSD17B2	PAK6	TMEM27
CTSD	IER3	PAX9	TMEM40
CXCL11	IFIT1	PCYOX1	TNFRSF19
DAPK1	IFIT2	PDE4B	TPBG
DCN	IGFBP3	PINK1	TPST1
DNAJC3	INHBA	PODXL	TRAF4
DNER	ITPKB	PRICKLE2	TRIM23
DOCK11	JAG2	PTGER2	ULK1
DOCK4	JUNB	PTPRM	USP40
DTX4	KAL1	PTPRZ1	UST
DUSP5	KCNJ15	RAB3IP	VGLL4
EAF2	KCNS3	RASEF	WNT5A
EFNB2	KIAA1967	RP2	WSB1
EGR3	KIF3A	RRAD	YPEL3
EHBP1L1	KLF4	RSAD2	ZNF165
EHD4	KLK13	S100A8	ZNF318

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

The studies presented in this dissertation have examined the role of vitamin D in lung health using complementary epidemiologic, genetic, and functional genomics approaches. Taken together, the majority of the evidence suggests that vitamin D is important in healthy lung function, and that the mechanism of action involves regulation of gene expression in processes of inflammation and immunomodulation, cell proliferation and cell fate determination.

The first study (Chapter 2) investigated the association of serum 25-hydroxyvitamin D with lung function (FEV_1 and FEV_1/FVC) in Health ABC study participants. The cross-sectional association of serum 25-hydroxyvitamin D with FEV_1 was positive and statistically significant and the effect size was of interest. Counting the study presented in this dissertation, there are now two cross-sectional results showing a positive association of 25-hydroxyvitamin D with FEV_1 [1], and one cross-sectional study reporting no association [2]. We extended the cross-sectional result to examine the longitudinal association of serum 25-hydroxyvitamin D with FEV_1 over the ten year follow-up period. Individuals in the lowest tertile of serum vitamin D at the start of the study were more likely to die or miss clinic visits over the subsequent period, leading to selective loss of lung function measurements in this group. To avoid selection bias, the longitudinal analysis was limited to the upper two tertiles of serum 25-hydroxyvitamin D (20.6 ng/mL - 75 ng/mL), and there was little or no association between vitamin D

concentration and FEV_1 decline. These results suggest that there is no benefit to supplementation of elderly patients with serum 25-hydroxyvitamin D at 20 ng/mL or above. However, if the vitamin D-lung function association in the cross sectional analysis is indeed causal, then one possibility is that vitamin D led to a change in decline in lung function during an earlier stage of life. If this is the case, then future longitudinal studies in younger populations may detect an association of vitamin D nutriture with rate of decline in FEV_1. It should be noted that, by themselves, these results do not rule out the possibility of reverse causation as an alternative explanation for the cross-sectional association.

The second study (Chapter 3) examined genetic associations that are theoretically free from the lifestyle confounding that is problematic in associations with serum 25-hydroxyvitamin D. In African-American participants in the Health ABC study, a SNP and two haplotypes in *CYP24A1* were associated with FEV_1, and a SNP in *VDR* was associated with FEV_1/FVC after correction for multiple testing. In European-Americans, two interactions between SNPs in *RXRA* and serum 25-hydroxyvitamin D were significant at a false discovery rate < 0.2. These results, which characterize vitamin D nutriture via genetic proxies, strongly support a causal role of vitamin D in lung health, since function of these genes is critical for vitamin D activity at the cellular level. Individuals with variants in *CYP24A1* may have altered half-life and/or levels of 1,25-dihydroxyvitamin D, since this gene encodes the enzyme responsible for degrading the active form of vitamin D. A genetic variant altering *VDR* function would affect the ability of vitamin D to modulate gene expression, given *VDR* binding in the cytoplasm is the first step to nuclear localization of the complex for gene regulation. Similarly, RXRA is

crucial for transcriptional regulation by vitamin D; compromised lung function was observed for the combination of low serum 25-hydroxyvitamin D with SNPs in *RXRA*, and with little or no association of the *RXRA* SNPs at high serum 25-hydroxyvitamin D levels. These results strongly suggest that the mechanism of action for these *RXRA* genetic variants is through the participation of RXRA in the vitamin D/VDR heterodimer rather than through another heterodimeric pairing in a different pathway. All three genes with statistically significant associations with pulmonary phenotypes (*CYP24A1, VDR*, and *RXRA*) function downstream of 25-hydroxyvitamin D, and the genetic variants did not have a detectable effect on circulating 25-hydroxyvitamin D concentration. Thus, this study provides evidence complementary to the epidemiologic study of serum 25-hydroxyvitamin D in the Health ABC population. The association of genetic variants in vitamin D metabolic genes with pulmonary phenotypes are hypothesized to represent the cumulative effect of a lifetime of altered vitamin D net functionality, confirming the importance of vitamin D in lung health. Of note, the issue of timing of vitamin D effects is not addressed by the genetic study, as there is no way to deduce from these results whether there is a critical time period for vitamin D nutriture. As with any genetic study, it is possible that the most important time period for vitamin D functionality is during lung development and growth, in the prenatal period or during infancy. However, the current epidemiological evidence, from the NHANES III [1] study and from the results reported herein, supports the inference that adult vitamin D levels are important for maintaining lung function.

The third study (Chapter 4) addresses the question of mechanisms in a study of lung tissue gene expression. In this study, we sought to understand how gene expression

modulated by vitamin D affects lung health by studying the association of serum levels of 25-hydroxyvitamin D with altered function at the molecular level. We found differential gene expression in lung epithelial cells associated with the physiological range of 25-hydroxyvitamin D in a diverse sample of free-living humans. In this hypothesis-driven study, candidate genes were selected for study only if there was evidence of *in vitro* regulation by vitamin D and if the gene contained a vitamin D response element.Thirteen genes had statistically significant changes in expression between low and high serum concentrations of 25-hydroxyvitamin D, and 3 genes were significant at FDR < 0.2. Gene ontology and literature analysis of differentially expressed genes supported plausible mechanisms for functional roles in asthma, COPD, cancer, and response to infection in lung. Many genes were known to function in cell proliferation or cell fate determination, which could affect cancer development and progression, as well as processes of airway remodeling in COPD and asthma. Several genes also had well-established immunomodulatory effects, with known roles either in inflammation (relevant to obstructive lung disease), or response to pathogens (relevant to respiratory infections). In addition, striking similarities between glucocorticoid responses and vitamin D-associated gene expression were observed, suggesting that combination therapies of vitamin D and glucocorticoids may have synergy by producing similar effects through different pathways.

Our results also provide information that may be useful for any future intervention trials studying the use of vitamin D supplementation to maintain lung health. Specifically, changes in the expression of the panel of genes identified here could be used to identify effects of varying doses of dietary vitamin D. Ultimately, monitoring concentrations of

serum 25-hydroxyvitamin D corresponding with changes in expression of these genes may help to establish optimal levels of serum vitamin D for lung outcomes.

It should be noted that there are some limitations inherent to the studies described here. The epidemiologic study cannot rule out the possibilities of reverse causation or lifestyle confounding producing the cross-sectional result, although every effort was made to adjust for potential confounders. The study of genetic associations provides stronger evidence for a role for vitamin D in lung health, but false positives are possible. In addition, variants in *RXRA* may affect some other pathway involving a heterodimeric partner other than VDR; however, this is unlikely given that interactions with 25-hydroxyvitamin D level were detected for *RXRA*. Similarly, false positives are possible in the expression microarray results, although limiting hits to FDR < 0.2 and genes with predicted VDRE's and prior evidence of vitamin D modulation decreases the likelihood of spurious associations. Perhaps a more difficult problem to address fully is whether the changes in expression are physiologically relevant, because it is unclear what degree of fold change is required to affect cell function, and this quantity may vary for each gene.

Future studies may strengthen evidence for a role for vitamin D in lung health by building on these results. Additional longitudinal association studies are needed in younger populations to determine at which life stages vitamin D nutriture is most important for lung function. In vitro experimentation in cells carrying the genetic variants associated with lung function (for example, lymphoblasts from International HapMap project participants) could be used to verify that these genotypes respond differently to

1,25-dihydroxyvitamin D treatment. For example, effects of *RXRA* or *VDR* SNPs on gene expression changes for a representative panel of vitamin D target genes could be evaluated, as well as duration of response for *CYP24A1* variants. Interestingly, in vitro follow-up for the microarray study has essentially already been done by prior studies. Instead, a small proof-of-concept study verifying that vitamin D supplementation alters expression of these genes in lung, with comparison to effects on these genes in a more accessible tissue such as blood cells, would be a helpful first step towards a full-scale randomized controlled trial of vitamin D for COPD. Ideally, experimentation with multiple doses could be used to establish a dose-response relationship between dietary vitamin D, resulting 25-hydroxyvitamin D levels, and molecular changes in lung.

In summary, this dissertation describes the findings of several studies aimed at understanding the role of vitamin D in lung health. We found a cross-sectional association of the serum biomarker of vitamin D with lung function, and genetic variants in genes in the vitamin D metabolic pathway were also associated with lung function. By using complementary approaches, we were able to address the issue of confounding that makes causal inferences especially challenging in vitamin D research. Furthermore, in a study of lung epithelial cells we demonstrated molecular effects associated with concentrations of the serum biomarker of vitamin D, which is a first step to establishing dose-response relationships. Overall, the findings support an important role for vitamin D in maintaining lung health, and vitamin D-regulated gene expression may have a plausible role in processes related to COPD, asthma, cancer, and respiratory infections.

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