Vitamin D Metabolism is Dysregulated in Asthma and Chronic Obstructive Pulmonary Disease

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ABSTRACT

Rationale: Vitamin D deficiency is common in patients with asthma and COPD. Low 25-hydroxyvitamin D (25[OH]D) levels may represent a cause or a consequence of these conditions.

Objective: To determine whether vitamin D metabolism is altered in asthma or COPD.

Methods: We conducted a longitudinal study in 186 adults to determine whether the 25(OH)D response to six oral doses of 3 mg vitamin D_3, administered over one year, differed between those with asthma or COPD vs. controls. Serum concentrations of vitamin D_3, 25(OH)D_3 and 1α,25-dihydroxyvitamin D_3 (1α,25[OH]$_2D_3$) were determined pre- and post-supplementation in 93 adults with asthma, COPD or neither condition, and metabolite-to-parent compound molar ratios were compared between groups to estimate hydroxylase activity. Additionally, we analyzed fourteen datasets to compare expression of 1α,25[OH]$_2D_3$–inducible gene expression signatures in clinical samples taken from adults with asthma or COPD vs. controls.

Measurements and Main Results: The mean post-supplementation 25(OH)D increase in participants with asthma (20.9 nmol/L) and COPD (21.5 nmol/L) was lower than in controls (39.8 nmol/L; P=0.001). Compared with controls, patients with asthma and COPD had lower molar ratios of 25(OH)D_3-to-vitamin D_3 and higher molar ratios of 1α,25(OH)$_2D_3$-to-25(OH)D_3 both pre- and post-supplementation (P≤0.005). Inter-group differences in 1α,25[OH]$_2D_3$–inducible gene expression signatures were modest and variable where statistically significant.

Conclusions: Attenuation of the 25(OH)D response to vitamin D supplementation in asthma and COPD associated with reduced molar ratios of 25(OH)D_3-to-vitamin D_3

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and increased molar ratios of 1α,25$(OH)_2D_3$-to-25$(OH)D_3$ in serum, suggesting that vitamin D metabolism is dysregulated in these conditions.

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INTRODUCTION

Asthma and COPD are major contributors to the global burden of non-communicable disease: collectively they affect more than 500 million people, and were responsible for an estimated 3.6 million deaths in 2015 (1). Prevalent asthma and COPD have been widely reported to associate with vitamin D deficiency, as indicated by low circulating concentrations of the major circulating vitamin D metabolite 25-hydroxyvitamin D (25[OH]D) (2-7), which is used as a biomarker of vitamin D status by virtue of its relatively long half-life. These observations, coupled with data from mechanistic studies suggesting favorable effects of vitamin D on lung development and immune function (8-10), suggest that vitamin D deficiency might represent a reversible risk factor for development of these inflammatory airways diseases (11-13). However, null results from Mendelian randomization studies in asthma (14, 15) and reports that carriage of the Gc2 haplotype of vitamin D binding protein is protective against COPD (16, 17) indicate that genetically determined low levels of 25(OH)D do not increase the risk of developing asthma or COPD.

An alternative explanation for the observed associations between vitamin D deficiency and prevalent asthma and COPD is that low 25(OH)D levels might arise as a consequence of disease, e.g. if disease processes perturb vitamin D metabolism in such a way as to reduce synthesis and/or increase catabolism of 25(OH)D, or if disease causes immobility resulting in less exposure to sunlight. Figure 1 illustrates the major vitamin D_3 oxidation pathways. Vitamin D_3, derived from cutaneous synthesis or absorption in the gastro-intestinal tract from dietary sources, is hydroxylated at C25 to form 25(OH)D. The cytochrome P450 enzymes catalyzing this conversion (CYP2R1, CYP3A4 and CYP27A1) are primarily expressed in the

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liver, but their expression and activity has also been reported in airway epithelium, T cells and dendritic cells (18, 19). 25(OH)D subsequently undergoes a second hydroxylation step at C1 to form the active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 (1α,25$[OH]_2D_3$); this oxidation step is catalyzed by CYP27B1, which is expressed in multiple tissues including the kidney, airway epithelium and myeloid cells (20). Alternatively, 25(OH)D may undergo hydroxylation at C24 or C4 to form the inactive metabolites 24R,25-dihydroxyvitamin D_3 (24R,25$[OH]_2D_3$) and 4β,25-dihydroxyvitamin D (4β,25$[OH]_2D_3$), respectively. 1α,25$(OH)_2D_3$ may also undergo hydroxylation at C24 to form the inactive metabolite 1α,24,25-trihydroxyvitamin D_3 (1α,24,25$(OH)_3D_3$). The enzymes catalyzing 24-hydroxylation (CYP24A1) and 4β-hydroxylation (CYP3A4, CYP3A5) are also expressed in both liver and airway epithelium (21, 22).

Expression of TNF, IL-1β and TGF-β is increased in patients with asthma and COPD (23), and these cytokines have been reported to induce expression of CYP24A1 and CYP27B1 in cultured airway epithelial cells (22, 24). We therefore hypothesized that vitamin D deficiency might arise in patients with asthma and COPD as a result of increased 24-hydroxylation and/or 1α-hydroxylation of 25(OH)D. An opportunity to test this hypothesis recently arose in the context of three clinical trials in which adults with asthma, COPD or neither condition were randomized to receive a standard regimen of vitamin D_3 supplementation vs. placebo for one year (25-27). In this paper, we report results of secondary analyses of biochemical data from participants randomized to the intervention arms of these studies, conducted to determine whether the 25(OH)D response to oral administration of vitamin D_3 differed between individuals with and without inflammatory airways disease, with adjustment for potential phenotypic and genotypic confounders. Having observed that the vitamin D_3-induced increase in

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25(OH)D levels was attenuated in patients with asthma and COPD, we proceeded to measure serum concentrations of the vitamin D metabolites discussed above and to calculate metabolite-to-parent compound molar ratios for individual participants, both before and after vitamin D supplementation. Mean molar ratios were then compared between groups at each time point, to gain insights into potential differences in activity of vitamin D hydroxylases in cases vs. controls, with higher ratios indicating greater activity and vice versa. We also conducted Gene Set Variation Analysis of fourteen publicly available datasets to compare expression of the cytochrome P450 enzymes catalyzing vitamin D oxidation pathways and vitamin D–inducible gene expression signatures in blood, sputum, airway epithelium and lung tissue of adults with asthma or COPD vs. controls.

METHODS

Clinical trial participants and procedures

The 227 adults contributing data to analyses presented in this paper were participants in one of three randomized controlled trials of vitamin D supplementation, conducted in London, UK (ClinicalTrials.gov Identifiers NCT00978315, NCT00977873 and NCT01069874) whose methods are described elsewhere (25-27). The cohort of participants contributing data to longitudinal analysis of 25(OH)D concentrations comprised 88 adults with asthma treated with inhaled corticosteroids (ICS), 79 patients with COPD, and 19 controls who had neither condition. Eligibility criteria and study procedures are detailed in the Online Data Supplement. All individuals contributing data to longitudinal analyses received six oral doses of 3mg vitamin D_3 over a 1-year period, administered at 2-monthly intervals. Administration of doses at baseline, 2 months and 6 months was directly observed. Doses at 4, 8 and 10 months were self-

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administered by study participants during a telephone call with the study team. Serum samples were collected at baseline and at 2 and 12 months thereafter. The group contributing data to cross-sectional analyses included 19 adults with ICS-treated asthma, 17 adults with COPD, and 57 adults with neither condition, selected as above. All studies were approved by East London and The City Research Ethics Committee 1 (refs 09/H0703/67, 09/H0703/76 and 09/H0703/112) and written informed consent was obtained from all participants before enrolment.

Laboratory analyses

For participants contributing data to prospective analyses, serum samples taken at baseline and at 2- and 12-months post-enrolment were sent to the Department of Clinical Biochemistry at Homerton Hospital, London, UK for quantification of 25(OH)D_2 and 25(OH)D_3 concentrations by isotope-dilution liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an Architect ci8200 analyzer (Abbott Diagnostics, Chicago, IL, USA). Concentrations of these metabolites were summed to give total serum 25(OH)D concentration. The limit of detection for 25(OH)D_2 and 25(OH)D_3 for this assay was 10 nmol/L. For participants contributing data to cross-sectional analyses, a baseline (i.e. pre-supplementation) serum sample was sent to the Thummel Laboratory at the Department of Pharmaceutics, University of Washington, Seattle, WA, for measurement of concentrations of vitamin D_3, 25(OH)D3, 1α,25$(OH)_2D_3$, 24R,25$(OH)_2D_3$ and 4β,25$(OH)_2D_3$ using an LC-MS/MS assay as previously described (28). Limits of detection were 0.07 nmol/L for vitamin D_3; 0.25 nmol/L for 25(OH)D_3; 1.87 pmol/L for 1α,25$(OH)_2D_3$; 0.05 nmol/L for 24R,25$(OH)_2D_3$; and 1.70 pmol/L for 4β,25$(OH)_2D_3$. Both laboratories participate in the international vitamin D external quality assurance scheme (www.deqas.org/). Details

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of single nucleotide polymorphism selection, DNA extraction and genotyping are provided in the Online Data Supplement.

Gene expression datasets

Nine datasets from three studies were analyzed to compare gene expression in patients with asthma vs. controls. The Unbiased Biomarkers in Prediction of respiratory disease outcomes (UBIOPRED) study (29) contributed data from analysis of endobronchial biopsies, endobronchial brushings, nasal brushings, blood and sputum samples; the Airways Disease Endotyping for Personalized Therapeutics (ADEPT) study (30) contributed data from analysis of sputum samples; and the study by Woodruff and colleagues (31) contributed data from analysis of blood, airway epithelium and endobronchial biopsies. Five datasets from four studies were analyzed to compare gene expression in patients with COPD vs. controls. The Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE) study (32) contributed data from analysis of blood and sputum samples; the studies by Spira and colleagues and Crystal and colleagues (33) contributed data from analysis of airway epithelium; and the Lung Tissue Research Consortium (LTRC) study (reference set GSE47460) (34) contributed data from analysis of whole lung homogenates. The selection process for genes to be included in vitamin D-inducible signatures is detailed in the Online Data Supplement.

Statistical analyses

Statistical analyses were performed using Stata/IC v12.1 (StataCorp, College Station, TX) and R v3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was inferred where P values were <0.05. Determinants of the 25(OH)D response to vitamin D supplementation were evaluated using linear regression. In

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cross-sectional analyses, serum concentrations of metabolites and metabolite-to-parent compound molar ratios were compared between groups using Mann Whitney tests. Prospective analyses to compare post- vs. pre-supplementation values were conducted using Wilcoxon matched-pairs signed rank tests. For gene expression studies, enrichment scores for individual genes encoding CYP450 enzymes in the vitamin D pathway, and for groups of genes comprising epithelial and PBMC signatures (Table S7, Online Data Supplement) were derived using Gene Set Variation Analysis (GSVA) as previously described (35), and compared between groups using Kruskal-Wallis rank sum tests (where enrichment scores were non-normally distributed) or Student's t-tests (where enrichment scores were normally distributed). Further details of statistical analyses are provided in the Online Data Supplement.

RESULTS

The 25(OH)D response to vitamin D supplementation is blunted in asthma and COPD

A total of 186 adults (88 with asthma, 79 with COPD and 19 controls without either condition) were allocated to the intervention arms of one of three clinical trials (25-27) and recorded as receiving six oral doses of 3 mg (120,000 IU) vitamin D_3 at 2-monthly intervals over one year. Their baseline characteristics are presented in Table S1 (Online Data Supplement). Mean age was highest in the COPD cohort (64.4 years), and lower in the asthma and control cohorts (48.8 and 47.3 years, respectively). Females were in the majority in asthma and control cohorts (60.2% and 84.2%, respectively), but in the minority in the COPD cohort (34.2%). Prevalence of current cigarette smoking was highest in the COPD cohort (49.4%), intermediate in controls

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(15.8%) and lowest in the asthma cohort (4.5%). Mean baseline 25(OH)D was similar for all three groups (46.1 vs. 45.2 vs. 44.3 nmol/L in asthma vs. COPD vs. controls, respectively).

Results of univariate and multivariate analyses evaluating phenotypic determinants of the vitamin D_3-induced increase in 25(OH)D_3 levels at 2 and 12 months post-randomization are presented in Table 1. The mean increase in 25(OH)D at 2 months after a single directly observed oral dose of 3 mg vitamin D_3 was 23.5 nmol/L in controls, 12.3 nmol/L in participants with asthma and 10.5 nmol/L in participants with COPD (adjusted mean difference for asthma vs. controls, -8.0 nmol/L, 95% CI -16.3 to 0.4 nmol/L; adjusted mean difference for COPD vs controls, -9.5 nmol/L, 95% CI -18.0 to -1.0 nmol/L; Figure 2). A reduced increment in 25(OH)D at 2 months post-dose was also seen among participants enrolled between July-January vs. February-June (adjusted mean difference -12.8 nmol/L, 95% CI -7.8 to -17.8 nmol/L) and in those with higher baseline vitamin D status (adjusted mean difference per 1 nmol/L increase in baseline 25(OH)D, -0.5 nmol/L, 95% CI -0.6 to -0.4 nmol/L). Determinants of the 25(OH)D response to six 2-monthly oral doses of 3 mg vitamin D_3, evaluated at one year, were similar to those seen for 2-month follow-up, with the mean 25(OH)D increase among controls (39.8 nmol/L) being almost double that seen in participants with asthma (20.9 nmol/L) and COPD (21.5 nmol/L; Figure 2; adjusted mean difference in 25(OH)D increment for asthma vs. controls, -20.3 nmol/L, 95% CI -32.2 to -8.5 nmol/L; adjusted mean difference for COPD vs. controls, -20.3 nmol/L, 95% CI -32.2 to -8.4 nmol/L). As before, higher baseline vitamin D status was independently associated with a reduced mean 25(OH)D increment post-supplementation (adjusted mean difference per 1 nmol/L increase in baseline 25(OH)D, -0.7 nmol/L, 95% CI -0.8 to -0.5 nmol/L).

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Attenuated 25(OH)D response to vitamin D supplementation in asthma and COPD is not explained by genetic variation in the vitamin D pathway

We next investigated whether genetic variants in the vitamin D pathway previously reported to associate with blunted 25(OH)D response to supplementation were over-represented in patients with asthma and COPD. Participants were genotyped for a panel of 19 single nucleotide polymorphisms in genes encoding vitamin D binding protein (*DBP*, 6 SNPs) and enzymes responsible for reduction of 7-dehydrocholesterol (*DHCR7*, 2 SNPs), 25-hydroxylation of parent vitamin D (*CYP2R1*, 3 SNPs; *CYP27A1*, 1 SNP; *CYP3A4*, 1 SNP), 1-alpha hydroxylation of 25(OH)D (*CYP27B1*, 2 SNPs), 24-hydroxylation of 25(OH)D (*CYP24A1*, 4 SNPs; Figure 1). Genotype frequency was compared between disease conditions, and found to vary for one SNP in *CYP27B1* (rs4646536) and two SNPs in *DBP* (rs2298849 and rs16846876; Supplementary Table S2). However, none of these three SNPs associated with 25(OH)D increment post-supplementation after adjustment for baseline 25(OH)D level and disease status (Supplementary Table S3). Thus, we found no evidence to indicate that blunting of the 25(OH)D response to vitamin D supplementation in patients with asthma and COPD is explained by genetic variation in the vitamin D pathway.

Vitamin D metabolite-to-parent compound molar ratios differ in unsupplemented patients with asthma and COPD vs controls

Next, we reasoned that blunting of the vitamin D_3-induced increase in 25(OH)D levels in patients with asthma and COPD might arise as a consequence of reduced synthesis of 25(OH)D from vitamin D_3 and/or increased catabolism of 25(OH)D to one or more of its dihydroxylated metabolites, and that this might be reflected in reduced molar ratios of 25(OH)D-to-vitamin D_3 and/or increased molar ratios of 1α,25$(OH)_2$D-to-

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25(OH)D, 24R,25$(OH)_2$D-to-25(OH)D and 4β,25$(OH)_2$D-to-25(OH)D. To test this hypothesis, we first determined concentrations of vitamin D_3, 25$(OH)D_3$ and three dihydroxylated vitamin D metabolites (1,25[OH]D_3, 24R,25[OH]D_3 and 4β,25[OH]D_3) in serum of patients with asthma (n=19), patients with COPD (n=17) and controls with neither condition (n=57) who had not received vitamin D supplementation, and compared absolute concentrations and metabolite-to-parent compound molar ratios between groups. Characteristics of participants contributing data to this analysis are presented in Table S4 (Online Data Supplement). Mean age was highest in those with COPD (66.8 years), lowest in those with asthma (47.8 years) and intermediate in controls (59.5 years). Females were in the majority among controls (66.7%) but in the minority among patients with asthma (47.4%) and COPD (41.2%). Prevalence of current cigarette smoking was highest in those with COPD (35.3%), intermediate in controls (29.8%) and lowest in those with asthma (10.5%).

Table 2 presents results of this cross-sectional analysis of baseline data. Both participants with asthma and those with COPD had higher median serum concentrations of vitamin D_3 ($P<0.001$) and 1,25$(OH)_2$D ($P\leq0.04$) than controls. Patients with COPD also had lower 25(OH)D concentrations than controls ($P=0.03$), but those with asthma did not. Molar ratios of 25$(OH)D_3$-to-vitamin D_3 were lower in patients with asthma and COPD vs. controls, while molar ratios of 1α,25$(OH)_2D_3$-to-25$(OH)D_3$ were higher ($P\leq0.005$; Figure 3). No statistically significant differences in median molar ratios of 24R,25$(OH)_2D_3$-to-25$(OH)D_3$ or 4β,25$(OH)_2D_3$-to-25$(OH)D_3$ were seen between groups (Table 2).

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Pre-supplementation metabolite-to-parent compound molar ratios do not differ by disease severity or medication use in patients with asthma and COPD

Having found reduced molar ratios of 25(OH)D-to-vitamin D_3 and increased molar ratios of 1α,25$(OH)_2$D-to-25(OH)D in patients with asthma and COPD vs. controls at baseline, we were interested to determine whether the extent of derangements in these molar ratios among patients with inflammatory airways disease varied according to medication use or measures of disease activity or severity. Results are presented in Supplementary Tables S5 and S6: no statistically significant associations with either ratio were seen for use of long- or short-acting β2-agonists or inhaled corticosteroids in either asthma or COPD. Moreover, neither ratio associated with fractional exhaled nitric oxide concentration in patients with asthma, or with % predicted FEV1 in patients with COPD.

Similar inter-group differences in vitamin D metabolite-to-parent compound molar ratios are seen following vitamin D_3 supplementation

We next proceeded to compare absolute concentrations and metabolite-to-parent compound molar ratios for the same analytes between groups at one year post-enrolment, following ingestion of six oral doses of 3 mg vitamin D_3 administered at

0, 2, 4, 6, 8 and 10 months; blood samples were taken at 12-month follow-up (i.e. 12 months after the first dose and 2 months after the sixth dose). Results are shown in Table 2 and Figure 3. All participants with asthma and COPD included in baseline metabolite profiling also contributed data to this prospective analysis, but post-supplementation metabolite concentrations were only available for a subset of 28/57 controls (the other 29 received a different supplementation regimen). Paired analyses revealed that vitamin D_3 supplementation induced statistically significant increases in

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serum concentrations of vitamin D_3 (P=0.01), 25(OH)D_3 (P<0.001), 1α,25(OH)$_2D_3$ (P=0.002), 24R,25(OH)2D3 (P<0.001) and 4β,25(OH)$_2D_3$ (P<0.001). Inter-group comparisons of follow-up data revealed higher concentrations of vitamin D_3 in both asthma and COPD vs. controls (P≤0.006) and higher 1α,25(OH)$_2D_3$ concentrations in asthma vs. controls (P<0.001). Consistent with results of cross-sectional analyses at baseline, post-supplementation molar ratios of 25(OH)D_3-to-vitamin D_3 and 1α,25(OH)$_2D_3$-to-25(OH)D_3 were higher in both asthma and COPD vs. controls (P≤0.003). Changes in 25(OH)D_3-to-vitamin D_3 and 1,25(OH)$_2D_3$-to-25(OH)D_3 ratios from baseline were calculated for each clinical group: median values of these changes were close to zero for all three groups, indicating that ratios were essentially unchanged by vitamin D supplementation. When median changes in ratios were compared between disease groups vs. controls, no statistically significant differences were seen.

Expression of vitamin D pathway genes in asthma and COPD

Next, we investigated whether altered metabolite-to-parent compound molar ratios were associated with any differences in expression of genes encoding proteins responsible for vitamin D metabolism (*CYP2R1, CYP3A4, CYP3A5, CYP27A1, CYP24A1, CYP27B1, DHCR7*), transport (*DBP*) and signaling pathways (*VDR*, *RXRA*; Figure 1). Table 3 presents expression data for these 10 genes across nine datasets relating to six sample types from three studies in patients with asthma (31, 36, 37), and five datasets relating to four sample types from four studies in patients with COPD (38-41). Small but statistically significant differences in expression were seen for genes encoding the 25-hydroxylase CYP27A1 (decreased in airway epithelium in both asthma and COPD, and in endobronchial biopsy tissue in asthma), the 24-hydroxylase

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CYP24A1 (decreased in nasal brushings, airway epithelium and endobronchial biopsy in asthma), VDR (increased in nasal and endobronchial brushings and sputum in asthma, and in lung homogenate in COPD) and RXRA (decreased in endobronchial brushings in asthma and small airway epithelium in COPD). For other genes, statistically significant differences in expression for patients with asthma or COPD vs. controls were either in opposite directions for different datasets (*CYP2R1*, *CYP3A5*), were seen in one dataset only (*DBP*, *CYP3A4*) or were absent (*DHCR7*, *CYP27B1*).

Expression of vitamin D-inducible signatures in asthma and COPD

Having found that circulating concentrations of $1\alpha,25(OH)_2D_3$ were elevated in asthma and COPD, and that expression of *VDR* was increased in sputum, endobronchial brushings, nasal brushings and lung homogenates from patients with one or other of these conditions, we hypothesized that expression of vitamin D-inducible genes might be increased in blood and airway tissue of patients with asthma and COPD. To explore this possibility, we compared expression of $1,25(OH)_2D_3$-inducible gene signatures in nine datasets relating to seven sample types from three studies in patients with asthma (31, 36, 37) and five datasets relating to four sample types from four studies in patients with COPD (38-41). Results are presented in Table 4 and Figure S1 (Online Data Supplement): in patients with asthma, the epithelial vitamin D signature was modestly upregulated in nasal brushings (one dataset, logFC 0.10, P=0.04), sputum (two datasets, logFC ≤0.18, P≤0.04) and endobronchial biopsies (one dataset, logFC 0.11, P=0.04), and the PBMC signature was modestly upregulated in peripheral blood (one dataset, logFC 0.06, P=0.03). In patients with COPD, the epithelial vitamin D signature was modestly downregulated in whole lung homogenate (one dataset, logFC -0.08,

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P=0.006), and the PBMC signature was modestly downregulated in peripheral blood (one dataset, logFC -0.13, P=0.01).

DISCUSSION

To our knowledge, this is the first study to compare vitamin D metabolism in patients with inflammatory airways disease vs. controls. We found that the 25(OH)D response to vitamin D supplementation was significantly attenuated in patients with asthma and COPD. This phenomenon was associated with reduced molar ratios of 25(OH)D_3-to-vitamin D_3, and increased molar ratios of 1α,25[OH]$_2D_3$-to-25(OH)D, in serum of patients with asthma or COPD vs. controls, both before and after vitamin D supplementation. No statistically significant differences in molar ratios of 24R,25(OH)$_2$D-to-25(OH)D or 4β,25(OH)$_2$D-to-25(OH)D were seen between groups. Analysis of multiple gene expression datasets revealed reduced expression of *CYP27A1* in airway epithelium (asthma and COPD vs. controls) and endobronchial biopsy tissue (asthma vs. controls), reduced expression of *CYP24A1* in nasal brushings, airway epithelium and endobronchial biopsy (asthma vs. controls) and increased expression of *VDR* in nasal brushings, endobronchial brushings and sputum (asthma vs. controls) and lung homogenate (COPD vs. controls). By contrast, no evidence of differences in expression of *CYP27B1* between groups was seen. 1α,25[OH]$_2D_3$–inducible gene expression signatures were modestly upregulated in blood, sputum, nasal brushings and endobronchial biopsies of patients with asthma, but modestly downregulated in whole lung homogenate and peripheral blood of patients with COPD.

Our study yielded several unanticipated results. Findings from an *in vitro* study (22) had led us to expect that 24-hydroxylation of 25(OH)D would be increased in asthma

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and COPD, but we found no evidence of this *in vivo*. Instead, we found that molar ratios of 25(OH)D_3-to-vitamin D_3 were lower in patients vs. controls, and that expression of the 25-hydroxylase *CYP27A1* was reduced in lung tissue. The former observation echoes findings of a recent study reporting reduced 25-hydroxylation of vitamin D_3 in a mouse model of diabetes mellitus (42), although in this case the effect was mediated via reduced hepatic expression of *CYP2R1*. From a clinical perspective, our finding that the 25(OH)D response to vitamin D supplementation is attenuated in patients with asthma and COPD suggests that these patients may require higher doses of vitamin D to attain optimal 25(OH)D levels as compared with healthy controls.

The fact that increases in the ratio of 1α,25$[OH]_2D_3$-to-25(OH)D were not associated with increased expression of *CYP27B1* in human lung tissue is also at variance with findings of animal studies (24), suggesting that lung tissue is unlikely to be the primary site of increased 1α-hydroxylation of 25(OH)D in patients with asthma and COPD. Although inter-group differences in metabolite-to-parent compound ratios may reflect variation in rates of conversion of a parent compound to its metabolite, they may also be explained by removal of the daughter metabolite from the circulation via direct excretion or sequestration into depots such as adipose tissue and muscle. Further studies to evaluate expression and activity of enzymes in the vitamin D metabolic pathway in patients with asthma and COPD are needed to determine whether differences in metabolite-to-parent ratios truly reflect changes in activity of metabolic enzymes.

With regard to downstream effects of 1α,25$[OH]_2D_3$ on gene expression, we were interested to note that 1α,25$[OH]_2D_3$-inducible gene expression signatures were increased in patients with asthma but not in those with COPD: this may reflect reduced

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responsiveness to 1α,25$[OH]_2D_3$ in COPD, possibly mediated at the level of the VDR. Whatever the underlying mechanism for the derangements in metabolite-to-parent compound molar ratios that we report here, our findings suggest that reverse causality might at least partially explain reported associations between prevalent asthma and COPD and vitamin D deficiency (2-7). This possibility does not preclude favorable effects of vitamin D supplementation in reducing risk of exacerbations of asthma and COPD, as previously demonstrated in intervention studies (43, 44); the relationship between airway inflammation and vitamin D deficiency may therefore be bi-directional. Given that we have shown evidence of reduced 25-hydroxylase activity in asthma and COPD, a case could be made for investigating effects of administering 25(OH)D in patients with these conditions (45).

Our study has several strengths. Three out of six doses of vitamin D supplementation were directly observed in all participants and the remaining three doses were supervised by telephone, which ensured a high degree of adherence. Strict case definitions for asthma and COPD, based on spirometric criteria, were applied. Our analyses of gene expression incorporated datasets generated from analysis of diverse clinical samples taken from patients with a range of disease severity recruited in different settings, enhancing the generalizability of our findings. We also had access to detailed information regarding potential phenotypic and genotypic confounders of the association between disease status and metabolite-to-parent compound molar ratios, allowing for comprehensive adjustment in our analyses.

Our study also has some limitations. Controls were recruited on the basis of an absence of a medical record diagnosis of asthma or COPD, and they did not undergo spirometry. This group could theoretically have included individuals with undiagnosed

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inflammatory airways disease. If this was the case, then results would be biased towards the null. Our study would have benefitted from the inclusion of data relating to renal and hepatic gene expression data, given that differences in metabolite-to-parent compound molar ratios were not always associated with consistent patterns of expression of genes encoding CYP450 enzymes in lung tissue, sputum and peripheral blood. Additionally, we draw attention to the fact that we gave intermittent bolus doses of vitamin D_3 during this study: our findings might have been different if a daily or weekly dosing regimen had been employed. Finally, we highlight the fact that we studied a relatively small number of participants; this may have limited the generalizability of our findings, and our power to detect sub-group effects.

In conclusion, we report that the 25(OH)D response to vitamin D supplementation was attenuated in adults with asthma and COPD, as compared with controls with neither condition. This phenomenon was associated with reduced molar ratios of $25(OH)D_3$-to- vitamin D_3 and increased molar ratios of $1\alpha,25(OH)_2D_3$-to-$25(OH)D_3$ in the serum, suggesting that vitamin D metabolism is dysregulated in these conditions. Similar studies should be conducted in patients in other settings to determine whether these intriguing findings can be replicated.

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FIGURE LEGENDS

Figure 1: Major vitamin D_3 oxidation pathways. The cytochrome P450 enzymes catalyzing each conversion are shown in capitals.

Figure 2: Mean change in serum 25-hydroxyvitamin D concentration (Δ25[OH]D) at 2- and 12-month follow-up vs. baseline. Six oral doses of 3 mg vitamin D_3 were administered at 0, 2, 4, 6, 8 and 10 months; serum 25(OH)D levels were determined at baseline (i.e. immediately prior to the first dose), 2-month follow-up (i.e. 2 months after the first dose and immediately prior to the second dose) and at 12-month follow-up (i.e. 12 months after the first dose and 2 months after the sixth dose). Error bars, SEM.

Figure 3: Vitamin D metabolite-to-parent compound molar ratios by disease status, pre- and post-supplementation. A, Pre-supplementation $25(OH)D_3$-to-vitamin D_3 molar ratios in adults with asthma (n=19), COPD (n=17) or neither condition (controls, n=57). B, Pre-supplementation $1,25(OH)_2D_3$-to-$25(OH)D_3$ molar ratios in the same groups. C, Post-supplementation $25(OH)D_3$-to-vitamin D_3 molar ratios in adults with asthma (n=19), COPD (n=17) or neither condition (controls, n=28). D, post-supplementation $1,25(OH)_2D_3$-to-$25(OH)D_3$ molar ratios in the same groups. E, change in (Δ) $25(OH)D_3$-to-vitamin D_3 molar ratios at follow-up vs. baseline in adults with asthma (n=19), COPD (n=17) or neither condition (controls, n=28). F, change in (Δ) $1,25(OH)_2D_3$-to-$25(OH)D_3$ molar ratios at follow-up vs. baseline in adults with asthma (n=19), COPD (n=17) or neither condition (controls, n=28). P values from Mann Whitney tests. Bars show median values in each group.

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Table 1: Phenotypic determinants of the 25(OH)D response to vitamin D_3 supplementation at 2- and 12-month follow-up

			2-month follow-up					12-month follow-up				
		N (%)	**Mean Δ25(OH)D, nmol/L (SD)**	**Unadjusted mean difference (95% CI)**	**P**	**Adjusted mean difference (95% CI) [(1)]**	**P**	**Mean Δ25(OH)D, nmol/L (SD)**	**Unadjusted mean difference (95% CI)**	**P**	**Adjusted mean difference (95% CI) [(2)]**	**P**
Sex	Male	90 (48.4)	12.8 (17.5)	*Referent*	-	-	-	23.0 (24.4)	*Referent*	-	-	-
	Female	96 (51.6)	12.4 (26.6)	-0.4 (-7.0 to 6.1)	0.90	-	-	22.8 (32.4)	-0.2 (-8.5 to 8.2)	0.97	-	-
Age, years	-	186 (100.0)	-	-0.0 (-0.3 to 0.2)	0.84	-	-	-	0.1 (-0.1 to 0.5)	0.25	-	-
Disease status	Controls	19 (10.2)	23.5 (22.9)	*Referent*	-	*Referent*		39.8 (29.8)	*Referent*	-	*Referent*	-
	Asthma	88 (47.3)	12.3 (25.4)	-11.2 (-22.4 to -0.0)	0.05	-8.00 (-16.3 to 0.4)	0.06	20.9 (27.4)	-18.9 (-33.7 to -4.1)	0.01	-20.3 (-32.2 to -8.5)	0.001
	COPD	79 (42.5)	10.5 (18.4)	-13.0 (-24.3 to -1.7)	0.02	-9.5 (-18.0 to -1.0)	0.03	21.5 (28.9)	-18.3 (-33.2 to -3.4)	0.02	-20.3 (-32.2 to -8.4)	0.001
Ethnic origin	White European	159 (85.5)	12.1 (22.9)	*Referent*	-	-	-	22.1 (29.5)	*Referent*	-	-	-
	Other	27 (14.5)	16.0 (20.8)	3.9 (-5.4 to 13.2)	0.41	-	-	27.9 (22.6)	5.8 (-6.1 to 17.8)	0.34	-	-
Body mass index, kg/m^2	-	186 (100.0)	-	-0.2 (-0.7 to 0.3)	0.42	-	-	-	-0.3 (-0.9 to 0.4)	0.42		
Smoking status	Never-/ex-smoker	140 (75.3)	13.7 (21.1)	*Referent*	-	-	-	23.3 (26.4)	*Referent*	-	-	-
	Current smoker	46 (24.7)	9.4 (26.8)	-4.4 (-11.9 to 3.2)	0.26	-	-	21.5 (34.7)	-1.9 (-11.5 to 7.8)	0.71	-	-
Alcohol intake, units/week	-	186 (100.0)	-	0.0 (-0.2 to 0.3)	0.76	-	-	-	-0.1 (-0.4 to 0.2)	0.48	-	-
Month of first dose	Feb-Jun	92 (49.5)	22.1 (21.1)	*Referent*	-	*Referent*	-	26.5 (26.1)	*Referent*	-	*Referent*	-
	Jul-Jan	94 (50.5)	3.4 (20.1)	-18.8 (-24.7 to -12.8)	<0.001	-12.8 (-17.8 to -7.8)	<0.001	19.3 (30.7)	-7.1 (-15.5 to 1.2)	0.09	-	-
Days from first dose to follow-up	-	186 (100.0)	-	-0.7 (-0.6 to 0.4)	0.79	-	-	-	-0.2 (-0.7 to 0.2)	0.34	-	-
Baseline 25(OH)D, nmol/L	-	186 (100.0)	-	-0.5 (-0.6 to -0.4)	<0.001	-0.5 (-0.6 to -0.4)	<0.001	-	-0.7 (-0.8 to -0.5)	<0.001	-0.7 (-0.8 to -0.5)	<0.001

Abbreviations: SD, standard deviation; CI, confidence interval; COPD, chronic obstructive pulmonary disease; Δ25(OH)D, change in serum 25-hydroxyvitamin D_3 concentration at follow-up vs. baseline.
(1) Adjusted for disease status, month of first dose and baseline concentration of 25-hydroxyvitamin D; (2) Adjusted for disease status and baseline concentration of 25-hydroxyvitamin D;

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Table 2: Baseline and 12-month serum concentrations of vitamin D_3 and its metabolites and metabolite-to-parent compound molar ratios by disease status

	Controls (n=57)	**Asthma (n=19)**		**COPD (n=17)**	
Baseline	**Median (IQR)**	**Median (IQR)**	**P(1)**	**Median (IQR)**	**P(2)**
Vitamin D_3, nmol/L	0.07 (0.07 to 1.21)	3.25 (2.44 to 4.14)	<0.001	2.54 (1.37 to 5.49)	<0.001
25(OH)D_3, nmol/L	52.28 (32.11 to 70.30)	43.79 (30.97 to 71.74)	0.97	27.23 (15.47 to 62.37)	0.03
1α,25(OH)$_2D_3$, pmol/L	42.49 (1.87 to 76.50)	88.33 (70.02 to 117.70)	<0.001	77.27 (36.53 to 99.18)	0.04
24R,25(OH)$_2D_3$, nmol/L	2.72 (0.98 to 4.24)	2.27 (1.21 to 3.87)	0.93	1.47 (0.87 to 3.51)	0.33
4β,25(OH)$_2D_3$, pmol/L	120.73 (72.65 to 191.38)	146.75 (85.80 to 230.14)	0.48	63.25 (1.70 to 170.37)	0.04
25(OH)D_3-to-vitamin D_3 ratio	166.51 (38.07 to 700.67)	13.81 (8.86 to 30.73)	<0.001	10.49 (6.91 to 13.54)	<0.001
1α,25(OH)$_2D_3$-to-25(OH)D_3 ratio x10^3	0.79 (0.12 to 1.42)	1.91 (1.23 to 2.75)	<0.001	2.04 (0.80 to 4.39)	0.005
24R,25(OH)$_2D_3$-to-25(OH)D_3 ratio	0.05 (0.03 to 0.07)	0.05 (0.03 to 0.06)	0.72	0.06 (0.05 to 0.08)	0.23
4β,25(OH)$_2D_3$-to-25(OH)D_3 ratio x10^3	2.33 (1.84 to 3.99)	3.14 (2.18 to 3.49)	0.50	2.09 (0.11 to 3.85)	0.23
	Controls (n=28)	**Asthma (n=19)**		**COPD (n=17)**	
12 months	**Median (IQR)**	**Median (IQR)**	**P(1)**	**Median (IQR)**	**P(2)**
Vitamin D_3, nmol/L	1.22 (0.07 3.20)	4.29 (2.68 to 6.64)	0.001	3.71 (2.37 to 6.23)	0.006
25(OH)D_3, nmol/L	91.02 (59.69 to 108.57)	78.00 (63.03 to 91.74)	0.44	62.94 (39.18 to 86.58)	0.10
1α,25(OH)$_2D_3$, pmol/L	74.35 (16.47 to 126.54)	126.54 (87.81 to 150.11)	<0.001	84.13 (57.20 to 115.69)	0.25
24R,25(OH)$_2D_3$, nmol/L	5.72 (2.90 to 8.57)	5.62 (3.67 to 7.42)	0.83	4.31 (3.03 to 6.98)	0.36
4β,25(OH)$_2D_3$, pmol/L	262.07 (86.84 to 396.76)	248.97 (139.60 to 298.14)	0.76	201.72 (71.12 to 274.83)	0.27
25(OH)D_3-to-vitamin D_3 ratio	66.26 (26.10 to 419.32)	18.68 (12.08 to 25.67)	<0.001	14.30 (10.91 to 22.06)	<0.001
1α,25(OH)$_2D_3$-to-25(OH)D_3 ratio x10^3	0.72 (0.27 to 0.89)	1.58 (1.18 to 2.25)	<0.001	1.18 (0.88 to 2.55)	0.003
24R,25(OH)$_2D_3$-to-25(OH)D_3 ratio	0.06 (0.04 to 0.08)	0.07 (0.06 to 0.08)	0.25	0.07 (0.05 to 0.09)	0.30
4β,25(OH)$_2D_3$-to-25(OH)D_3 ratio x10^3	2.66 (1.64 to 3.95)	2.77 (2.24 to 3.82)	0.59	2.95 (1.83 to 3.68)	0.93

Abbreviations: IQR, inter-quartile range; CI, confidence interval; COPD, chronic obstructive pulmonary disease; 25(OH)D_3, 25-hydroxyvitamin D_3; 1α,25(OH)2D_3, 1alpha,25-dihydroxyvitamin D_3; 24R,25(OH)$_2D_3$, 24R,25-dihydroxyvitamin D_3; 4β,25(OH)$_2D_3$, 4β,25-dihydroxyvitamin D_3.
1, P value from Mann Whitney test comparing values for asthma vs. controls. 2, P value from Mann Whitney test comparing values for COPD vs. controls

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Table 3: Expression of vitamin D pathway genes in patients with asthma and COPD vs. controls

				CYP2R1		CYP3A4		CYP3A5		CYP27A1		CYP24A1		CYP27B1		DBP		DHCR7		VDR		RXRA	
	Study	**Specimen**	**Phenotype (N)**	**logFC**	**P**	**logFC**	**P**	**logFC**	**P**	**logFC**	**P**	**logFC**	**P**	**logFC**	**P**	**logFC**	**P**	**logFC**	**P**	**logFC**	**P**	**logFC**	**P**
Asthma	UBIOPRED	Endobronchial biopsy	asthma (n=41) vs controls (n=26)	NA	NA	0.04	0.76	NA	NA	-0.01	0.96	0.13	0.36	NA	NA	NA	NA	NA	NA	0.10	0.48	-0.16	0.21
		Endobronchial brushings	asthma (n=54) vs controls (n=44)	0.05	0.56	0.18	0.03	0.17	0.05	0.00	0.98	-0.02	0.87	0.14	0.23	-0.01	0.95	0.15	0.19	0.22	0.01	-0.25	0.03
		Nasal brushings	asthma (n=32) vs controls (n=25)	0.09	0.55	-0.19	0.14	-0.32	0.03	0.30	0.06	-0.32	0.05	NA	NA	0.19	0.19	0.10	0.52	0.37	0.01	0.11	0.46
		Blood	asthma (n=165) vs controls (n=87)	-0.02	0.71	0.00	0.95	-0.32	<0.0001	0.11	0.17	-0.05	0.55	-0.05	0.53	0.03	0.64	-0.07	0.30	0.01	0.86	0.05	0.52
		Sputum	asthma (n=43) vs controls (n=16)	0.09	0.56	0.29	0.10	NA	NA	-0.26	0.08	NA	NA	NA	NA	NA	NA	NA	NA	0.30	0.04	0.06	0.73
	ADEPT	Sputum	asthma (n=39) vs controls (n=9)	NA	NA	0.03	0.90	NA	NA	-0.11	0.59	NA	NA	NA	NA	NA	NA	NA	NA	0.05	0.80	0.19	0.36
	Woodruff	Blood	mild-moderate asthma (n=23) vs controls (n=20)	-0.10	0.41	0.05	0.46	-0.14	0.21	-0.19	0.38	0.12	0.24	0.03	0.73	0.03	0.80	0.02	0.72	-0.02	0.81	0.03	0.70
		Airway epithelium	mild-moderate asthma (n=62) vs controls (n=43)	0.12	0.10	-0.08	0.03	-0.10	0.007	-0.17	0.02	-0.61	<0.001	0.06	0.12	-0.02	0.69	0.06	0.15	-0.05	0.16	-0.06	0.08
		Endobronchial biopsy	mild-moderate asthma (n=37) vs controls (n=24)	0.14	0.14	-0.08	0.19	-0.17	0.19	-0.19	0.01	-0.41	0.015	0.04	0.57	0.13	0.17	0.10	0.12	0.09	0.10	-0.01	0.76
COPD	ECLIPSE	Blood	COPD (n=151) vs controls (n=47)	0.19	0.05	-0.18	0.07	NA	NA	0.00	0.97	NA	NA	NA	NA	0.06	0.52	NA	NA	-0.04	0.65	-0.17	0.05
		Sputum	COPD (n=104) vs controls (n=32)	-0.06	0.54	-0.13	0.15	-0.11	0.33	0.13	0.27	-0.13	0.27	0.03	0.77	0.10	0.35	0.14	0.22	0.03	0.78	0.07	0.53
	Spira	Airway epithelium	current/former smokers, COPD (n=151) vs controls (n=87)	0.11	0.005	-0.01	0.80	0.24	<0.001	-0.28	<0.001	-0.08	0.42	0.04	0.07	-0.01	0.83	0.06	0.22	0.01	0.84	-0.04	0.19
	Crystal	Small airway epithelium	current smokers, COPD (n=36) vs controls (n=72)	0.13	0.06	-0.01	0.92	0.23	<0.001	0.07	0.38	-0.22	0.18	0.06	0.18	-0.07	0.36	-0.02	0.79	0.11	0.06	-0.09	0.035
	Lung Tissue Research Consortium (GSE47460)	Lung homogenate	COPD (n=215) vs controls (n=105)	-0.11	0.002	NA	NA	-0.52	<0.001	NA	NA	0.36	0.06	0.19	0.09	0.29	0.005	0.08	0.15	0.15	0.008	-0.05	0.09

Abbreviations: COPD, chronic obstructive pulmonary disease; UBIOPRED, Unbiased Biomarkers in Prediction of respiratory disease outcomes; ADEPT, Airways Disease Endotyping for Personalized Therapeutics; ECLIPSE, Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points; CYP-,Cytochrome P450 enzyme; DBP, Vitamin D binding protein; DHCR7, 7-dehydrocholesterol reductase enzyme; RXRA, Retinoid-X receptor-A; VDR, Vitamin D receptor.

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Table 4: Expression of vitamin D-inducible signatures in patients with asthma and COPD vs. controls

				PBMC signature		Epithelial signature	
	Study	**Specimen**	**Phenotype (N)**	**logFC**	**P**	**logFC**	**P**
Asthma	UBIOPRED	Endobronchial biopsy	asthma (n=41) vs controls (n=26)	NA	NA	-0.04	0.43
		Endobronchial brushings	asthma (n=54) vs controls (n=44)	NA	NA	0.00	0.90
		Nasal brushings	asthma (n=32) vs controls (n=25)	NA	NA	0.10	0.04
		Blood	asthma (n=165) vs controls (n=87)	0.06	0.03	NA	NA
		Sputum	asthma (n=43) vs controls (n=16)	NA	NA	0.11	0.04
	ADEPT	Sputum	asthma (n=39) vs controls (n=9)	NA	NA	0.18	0.01
	Woodruff	Blood	mild-moderate asthma (n=23) vs controls (n=20)	0.00	0.99	NA	NA
		Airway epithelium	mild-moderate asthma (n=62) vs controls (n=43)	NA	NA	0.07	0.053
		Endobronchial biopsy	mild-moderate asthma (n=37) vs controls (n=24)	NA	NA	0.11	0.04
COPD	ECLIPSE	Blood	COPD (n=151) vs controls (n=47)	-0.13	0.01	NA	NA
		Sputum	COPD (n=104) vs controls (n=32)	NA	NA	-0.01	0.79
	Spira	Airway epithelium	current/former smokers, COPD (n=87) vs controls (n=151)	NA	NA	0.03	0.20
	Crystal	Small airway epithelium	current smokers, COPD (n=36) vs controls (n=72)	NA	NA	0.01	0.72
	Lung Tissue Research Consortium (GSE47460)	Whole lung homogenate	COPD (n=215) vs controls (n=105)	NA	NA	-0.08	0.006

Abbreviations: logFC, log2 fold change; COPD, chronic obstructive pulmonary disease; UBIOPRED, Unbiased Biomarkers in Prediction of respiratory disease outcomes; ADEPT, Airways Disease Endotyping for Personalized Therapeutics; ECLIPSE, Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points; PBMC, Peripheral blood mononuclear cells. NA, not applicable.

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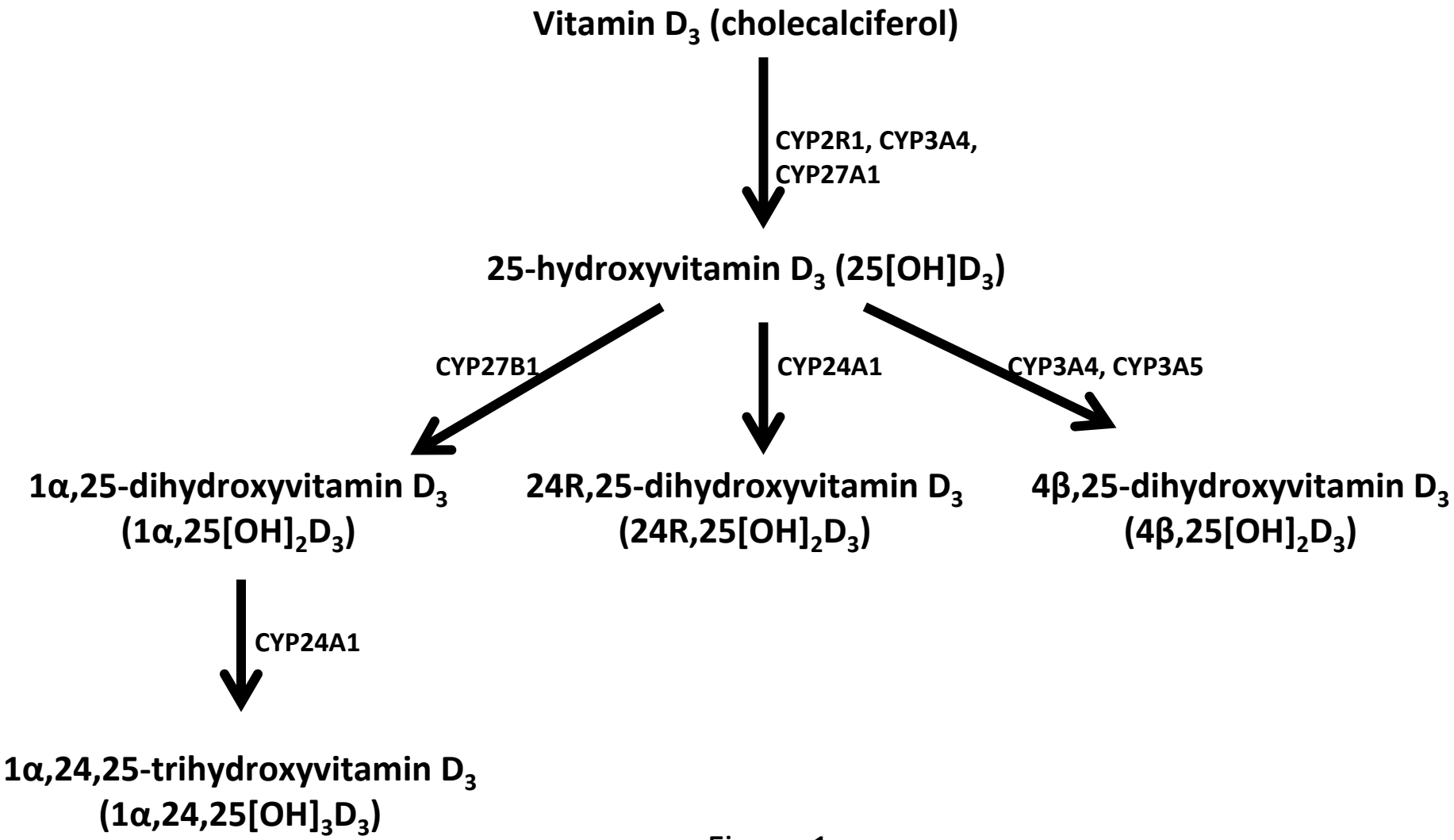

Figure 1

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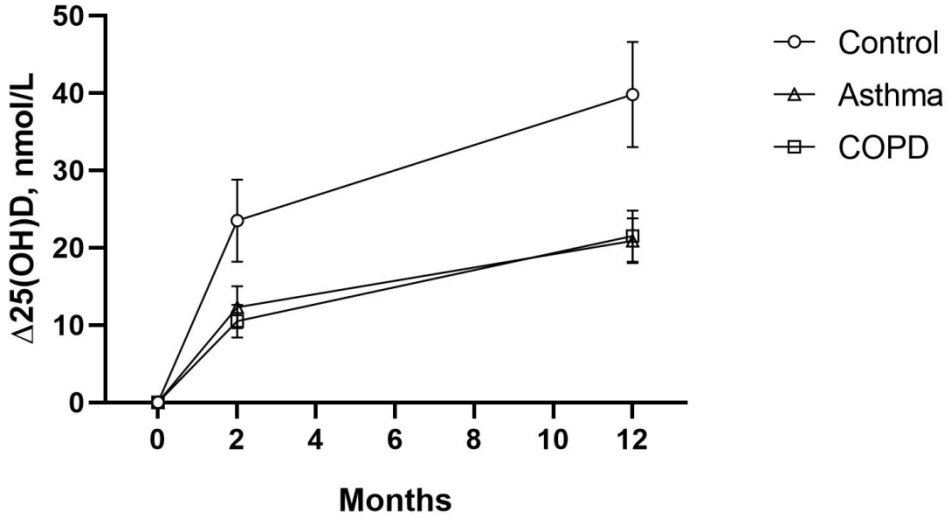

Figure 2: Mean change in serum 25-hydroxyvitamin D concentration (Δ25[OH]D) at 2- and 12-month follow-up vs. baseline. Six oral doses of 3 mg vitamin D3 were administered at 0, 2, 4, 6, 8 and 10 months; serum 25(OH)D levels were determined at baseline (i.e. immediately prior to the first dose), 2-month follow-up (i.e. 2 months after the first dose and immediately prior to the second dose) and at 12-month follow-up (i.e. 12 months after the first dose and 2 months after the sixth dose). Error bars, SEM.

131x76mm (300 x 300 DPI)

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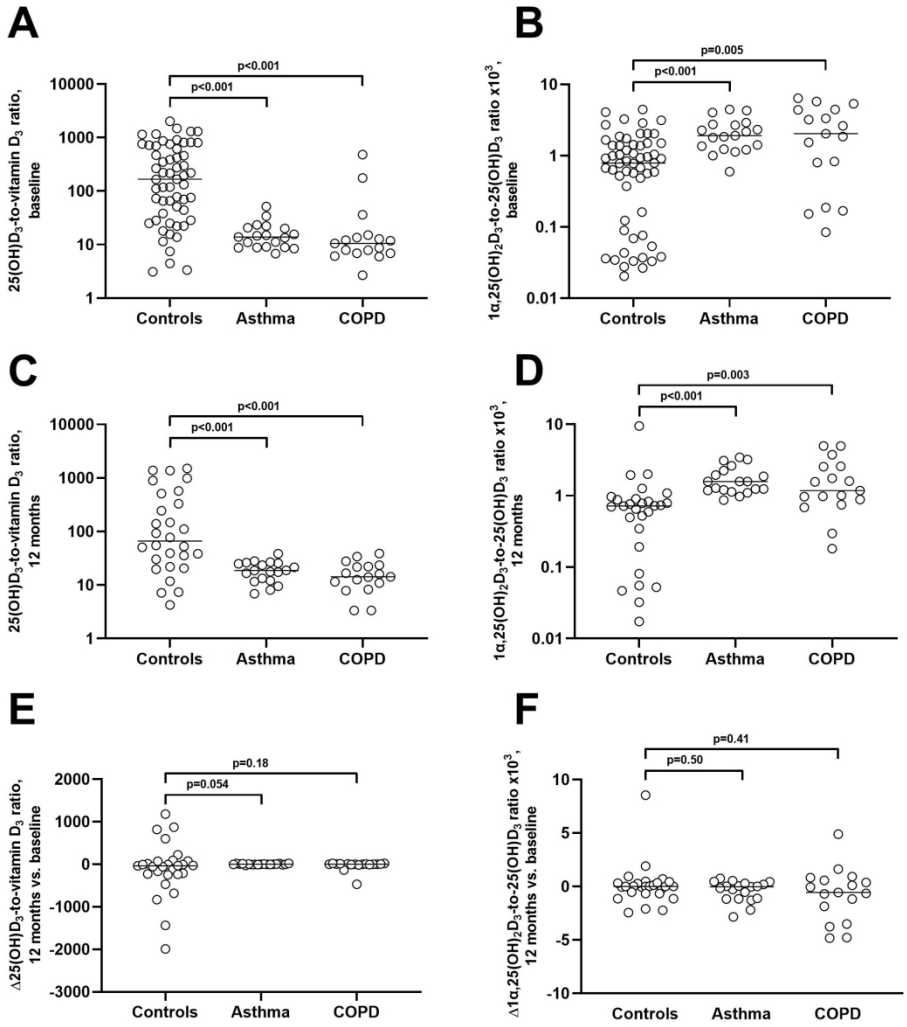

Figure 3: Vitamin D metabolite-to-parent compound molar ratios by disease status, pre- and post-supplementation. A, Pre-supplementation 25(OH)D3-to-vitamin D3 molar ratios in adults with asthma (n=19), COPD (n=17) or neither condition (controls, n=57). B, Pre-supplementation 1,25(OH)2D3-to-25(OH)D3 molar ratios in the same groups. C, Post-supplementation 25(OH)D3-to-vitamin D3 molar ratios in adults with asthma (n=19), COPD (n=17) or neither condition (controls, n=28). D, post-supplementation 1,25(OH)2D3-to-25(OH)D3 molar ratios in the same groups. E, change in (Δ) 25(OH)D3-to-vitamin D3 molar ratios at follow-up vs. baseline in adults with asthma (n=19), COPD (n=17) or neither condition (controls, n=28). F, change in (Δ) 1,25(OH)2D3-to-25(OH)D3 molar ratios at follow-up vs. baseline in adults with asthma (n=19), COPD (n=17) or neither condition (controls, n=28). P values from Mann Whitney tests. Bars show median values in each group.

194x209mm (300 x 300 DPI)

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Vitamin D Metabolism is Dysregulated in Asthma and Chronic Obstructive Pulmonary Disease: Online Data Supplement

Supplementary Methods

Eligibility criteria, clinical trial participants

All participants were selected on the basis that they were not taking any supplemental vitamin D at enrolment, and that adequate volumes of serum for measurement of vitamin D and its metabolites were available. Additionally, asthma patients were selected on the basis that they had a medical record diagnosis of asthma treated with ICS, were aged 16-79 years at enrolment, had a tobacco smoking history ≤15 pack-years, exhibited significant variability / reversibility in airway obstruction (defined as either a ≥12% increase in forced expiratory volume in 1 second (FEV1) after inhalation of 400 µg salbutamol at screening, or ≥ 20% diurnal variability in peak expiratory flow rate) and did not have a medical record diagnosis of COPD. COPD patients were selected on the basis that they had a medical record diagnosis of COPD, were aged ≥40 years at enrolment, had a tobacco smoking history >15 pack-years, had a ratio of forced expiratory volume in one second (FEV1) to forced or slow vital capacity (VC) after inhalation of 400 micrograms salbutamol <70% and did not have a medical record diagnosis of asthma. Controls were selected on the basis that they were employed as carers in a sheltered accommodation scheme, and that they did not have a medical record diagnosis of either asthma or COPD.

Baseline assessment, clinical trial participants

At baseline, participants were asked to complete a lifestyle questionnaire detailing potential phenotypic determinants of serum 25(OH)D concentrations including age, sex, racial/ethnic origin, socio-economic position (SEP, using the National Statistics –

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Socio-Economic Classification [NS-SEC] method) (E1), smoking history, alcohol consumption and medication use. Height and weight were measured using a Telescopic Measuring Rod (Seca, Hamburg, Germany) and column scales (Marsden, Rotherham, UK) respectively.

SNP selection, DNA extraction and genotyping

A literature search of the PubMed database was performed to identify SNPs previously shown to associate with altered vitamin D metabolism (E2). A total of 38 SNPs in 7 genes were identified. TagSNPs (tSNPs) were selected based on linkage disequilibrium (LD) information from the HapMap database (release #27: Phase 1, 2 & 3 - merged genotypes & frequencies); 'Utah residents with Northern and Western European ancestry from the CEPH collection (CEU)' dataset, due to the large proportion of longitudinal cohort participants who reported their ethnic/racial origin to be 'White European' (85.5%). Using Bioinformatics' Haploview program (v.3.3), selecting the 'pairwise tagging only' option, setting the r2 threshold to > 0.8 and accepting a minimum genotype completeness of 75% and a minor allele frequency threshold of 0.04, tagging reduced the number of alleles to be genotyped from 38 to 19 SNPs. Genotyping was conducted in the Genome Centre at Queen Mary University of London. DNA was extracted from whole blood using a salting-out method on the Biomek FX robot (Beckman Coulter), quantified using the Nanodrop spectrophotometer and normalized to 5ng/µl. 10ng DNA were used as template for 2 µl TaqMan assays (Applied Biosystems, Foster City, CA, USA) performed on the ABI 7900HT platform in 384-well format and analyzed with Autocaller software as previously described (E3). Typing for one SNP failed (rs6127118, CYP24A1), reducing the panel for analysis to 19 SNPs.

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Selection of genes for inclusion in vitamin D-inducible signatures

Data from high throughput gene expression studies (E4-11) were reviewed to identify genes that were regulated 2-fold or more in the same direction in response to treatment with $1\alpha,25(OH)_2D_3$. Genes fulfilling these criteria in at least 8/17 epithelial cell datasets were included in the epithelial vitamin D-inducible gene signature, and those fulfilling these criteria in at least 5/6 PBMC datasets were included in the PBMC vitamin D-inducible gene signature. The genes selected for each signature are listed in Table S7.

Power and sample size

Sample sizes for the clinical trials were predicated on power to detect pre-specified effects sizes of the interventions on primary outcomes with 80% power and an α of 0.05 as previously described (E12-14). The number of samples contributing to cross-sectional analyses of biochemical data was based on availability of adequate volumes of serum for measurement of vitamin D and its metabolites. Sample sizes for gene expression datasets were based on power to detect differences in gene expression profiles between clinical groups, as previously described (E15-20).

Statistical analyses

Statistical analyses were performed using Stata/IC v12.1 (StataCorp, College Station, TX) and R v3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was inferred where P values were <0.05. Where the concentration of a given vitamin D metabolite was below the LOD in a sample, a value of the LOD for that metabolite/$\sqrt{2}$ was imputed. In the longitudinal study, phenotypic predictors of the change in 25(OH)D concentration (Δ25(OH)D) in response to vitamin D supplementation at 2 and 12 months after administration of the first dose were

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identified by univariate linear regression to give unadjusted mean differences in Δ25(OH)D, with associated 95% confidence intervals and P values. Independent variables associating with Δ25(OH)D with P<0.05 on univariate analysis were then fitted in multivariate linear regression models to give adjusted mean differences in Δ25(OH)D concentration. Covariates were treated as categorical variables, with the exception of age, body mass index, days from previous dose to sampling and baseline 25(OH)D concentration which were treated as continuous variables. Genotype frequencies were compared between groups using chi-square tests, where there were at least five individuals per genotype. Potential genetic determinants of Δ25(OH)D were analyzed using an additive model. SNP predictors of Δ25(OH)D concentration from baseline to 12 months post-supplementation were identified by multivariate linear regression, correcting for disease status (asthma vs. COPD vs. control) and baseline 25(OH)D concentration (analyzed as a continuous variable). This analysis yielded adjusted mean differences in Δ25(OH)D concentration, with associated 95% confidence intervals for heterozygous and minor homozygous genotypes, taking major homozygous as the referent genotype, and a P value for trend across all three genotypes. SNPs with <5 individuals at any level were not analyzed. Multiple comparison testing was then applied using the Benjamini & Hochberg method with a false discovery rate (FDR) of 5% (E21). In cross-sectional analyses, metabolite-to-parent compound ratios were compared between groups using Mann Whitney tests. In gene expression studies, enrichment scores for individual genes encoding CYP450 enzymes in the vitamin D pathway, and for groups of genes comprising epithelial and PBMC signatures (Table S7, Supplementary Material) were analyzed using Gene Set Variation Analysis (GSVA), an unsupervised method which allows observation of the variation in the activity of a gene or gene list over a sample population. Distributions

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of enrichment scores representing cumulative gene expression were assessed for normality using the Shapiro-Wilk test, and compared between groups using Kruskal-Wallis rank sum tests (where enrichment scores were non-normally distributed) or Student's t-tests (where enrichment scores were normally distributed).

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Table S1: Baseline characteristics of participants receiving vitamin D_3 supplementation and entering prospective analysis of 25(OH)D response (n=186)

		Asthma (n=88)	COPD (n=79)	Controls (n=19)
Sex, n (%)	Male	35 (39.8)	52 (65.8)	3 (15.8)
	Female	53 (60.2)	27 (34.2)	16 (84.2)
Mean age, years (S.D.)	-	48.8 (14.6)	64.4 (8.3)	47.3 (11.4)
Ethnicity, n (%)	White	75 (85.2)	70 (88.6)	14 (73.7)
	Other	13 (14.8)	9 (11.4)	5 (26.3)
Body mass index, kg/m2 (S.D.)	-	28.8 (6.7)	28.2 (6.2)	28.4 (5.4)
Smoking status, n (%)	Never-/ex-smoker	84 (95.5)	40 (50.6)	16 (84.2)
	Current smoker	4 (4.5)	39 (49.4)	3 (15.8)
Mean alcohol intake, units/week (S.D.)(1)	-	8.9 (11.1)	10.9 (15.2)	7.2 (12.7)
Month of enrolment, n (%)	Jul-Jan	44 (50.0)	44 (55.7)	6 (31.6)
	Feb-Jun	44 (50.0)	35 (44.3)	13 (68.4)
BTS step of asthma treatment, n (%)	Mild intermittent asthma	0 (0)	-	-
	Regular preventer therapy	41 (46.6)	-	-
	Initial add-on therapy	35 (39.8)	-	-
	Persistent poor control	11 (12.5)	-	-
	Continuous / frequent use of oral corticosteroids	1 (1.1)	-	-
GOLD stage, n (%)	Mild (FEV1 ≥80% predicted)	-	19 (24.1)	-
	Moderate (50% ≤FEV1 <80% predicted)	-	40 (50.6)	-
	Severe (30% ≤FEV1 <50% predicted)	-	15 (19.0)	-
	Very Severe (FEV1 <30% predicted)	-	5 (6.3)	-
Mean serum 25-hydroxyvitamin D, nmol/L (S.D.)	-	46.1 (23.5)	45.2 (25.7)	44.3 (34.0)

(1) One alcohol unit = 8 g pure alcohol.

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Table S2: Genotype frequency by disease status (n=186)

Gene	SNP	Genotype	Asthma n (%)	COPD n (%)	Controls n (%)	P [1]
CYP24A1	rs2762939	GG	47/86 (54.7)	42/77 (54.6)	8/16 (50.0)	0.80
		CG	29/86 (33.7)	30/77 (39.0)	6/16 (37.5)	
		CC	10/86 (11.6)	5/77 (6.5)	2/16 (12.5)	
	rs2248137	CC	26/86 (30.2)	32/77 (41. 6)	6/19 (31.6)	0.15
		CG	38/86 (44.3)	37/77 (48.1)	9/19 (47.4)	
		GG	22/86 (25.6)	8/77 (10.4)	4/19 (21.1)	
	rs2762934	GG	63/88 (71.6)	57/78 (73.1)	12/19 (63.2)	0.75
		AG	23/88 (26.1)	17/78 (21.8)	6/19 (31.6)	
		AA	2/88 (2.3)	4/78 (5.1)	1/19 (5.3)	
	rs6013897	TT	51/87 (58.6)	53/77 (68.8)	12/19 (63.2)	0.08
		AT	34/87 (39.1)	17/77 (22.1)	5/19 (26.3)	
		AA	2/87 (2.3)	7/77 (9.1)	2/19 (10.5)	
CYP27A1	rs17470271	AA	36/87 (41.4)	27/77 (35.1)	6/19 (31.6)	0.68
		AT	38/87 (43.7)	33/77 (42.9)	8/19 (42.1)	
		TT	13/87 (14.9)	17/77 (22.1)	5/19 (26.3)	
CYP27B1	rs4646536	AA	48/83 (57.8)	30/76 (39.5)	9/18 (50.0)	0.04
		AG	32/83 (38.6)	34/76 (44.7)	6/18 (33.3)	
		GG	3/83 (3.6)	12/76 (15.8)	3/18 (16.7)	
	rs4646537	TT	81/87 (93.1)	69/77 (89.6)	15/19 (79.0)	--[2]
		GT	6/87 (6.9)	8/77 (10.4)	4/19 (21.1)	
		GG	0	0	0	
CYP2R1	rs10500804	TT	32/87 (36.8)	28/78 (35.9)	11/19 (57. 9)	0.10
		GT	43/87 (49.4)	31/78 (39.7)	4/19 (21.1)	
		GG	12/87 (13.8)	19/78 (24.4)	4/19 (21.1)	
	rs2060793	GG	28/84 (33.3)	26/77 (33.8)	6/19 (31.6)	0.14
		AG	42/84 (50.0)	36/77 (46.8)	5/19 (26.3)	
		AA	14/84 (16.7)	15/77 (19.5)	8/19 (42.1)	
	rs10766197	GG	28/83 (33.7)	25/76 (32.9)	9/19 (47.4)	0.23
		AG	43/83 (51.8)	31/76 (40.8)	6/19 (31.6)	
		AA	12/83 (14.5)	20/76 (26.3)	4/19 (21.1)	
CYP3A4	rs2740574	AA	77/87 (88.5)	68/78 (87.2)	14/19 (73.7)	--[2]
		AG	8/87 (9.2)	10/78 (12.8)	4/19 (21.1)	
		GG	2/87 (2.3)	0	1/19 (5.3)	
DBP	rs7041	CC	25/84 (29.8)	20/78 (25.6)	3/18 (16.7)	0.30
		AC	39/84 (46.4)	44/78 (56.4)	8/18 (44.4)	
		AA	20/84 (23.8)	14/78 (18.0)	7/18 (38.9)	
	rs12512631	TT	32/87 (36.8)	27/78 (34.6)	10/19 (52.6)	0.70
		CT	43/87 (49.4)	39/78 (50.0)	7/19 (36.8)	
		CC	12/87 (13.8)	12/78 (15.4)	2/19 (10.5)	
	rs4588	GG	51/88 (58.0)	32/78 (41.0)	12/19 (63.2)	0.20
		GT	31/88 (35.2)	39/78 (50.0)	6/19 (31.6)	
		TT	6/88 (6.8)	7/78 (9.0)	1/19 (5.3)	
	rs2070741	TT	66/86 (76.7)	70/77 (90.9)	12/19 (63.2)	--[2]
		TG	20/86 (23.3)	7/77 (9.1)	6/19 (31.6)	

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Gene	SNP	Genotype	Asthma n (%)	COPD n (%)	Controls n (%)	P [(1)]
		GG	0	0	1/19 (5.3)	
	rs2298849	AA	58/87 (66.7)	55/78 (70.5)	10/18 (55. 6)	0.001
		AG	28/87 (32.2)	21/78 (26.9)	4/18 (22.2)	
		GG	1/87 (1.2)	2/78 (2.6)	4/18 (22.2)	
	rs16846876	AA	46/85 (54.1)	28/76 (36.8)	11/19 (42.1)	0.043
		AT	37/85 (43.5)	40/76 (52.6)	8/19 (42.1)	
		TT	2/85 (2.4)	8/76 (10.5)	0	
DHCR7	rs3829251	GG	69/88 (78.4)	57/77 (74.0)	12/19 (63.2)	--[(2)]
		AG	18/88 (20.5)	19/77 (24.7)	6/19 (31.6)	
		AA	1/88 (1.1)	1/77 (1.3)	1/19 (5.3)	
	rs12785878	TT	41/87 (47.1)	47/78 (60.3)	7/18 (38. 9)	0.15
		GT	37/87 (42.5)	26/78 (33.3)	7/18 (38.9)	
		GG	9/87 (10.3)	5/78 (6.4)	4/18 (22.2)	

Abbreviations: chronic obstructive pulmonary disease; CYP-,Cytochrome P450 enzyme; DBP, Vitamin D binding protein; SNP, single nucleotide polymorphism; *DHCR7,* 7-dehydrocholesterol reductase. (1), P values from chi-square test. (2), SNP not analysed due to <5 individuals with one or more genotypes.

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Table S3: Potential genetic determinants of 25(OH)D response to vitamin D_3 supplementation at 12-month follow-up

Gene	SNP	Genotype	N (%)	Mean Δ25(OH)D, (SD)	Adjusted mean difference in Δ25(OH)D (95% CI) [1]	P
CYP27B1	rs4646536	AA	85 (48.9)	23.4 (25.9)	*Referent*	0.99
		AG	72 (41.4)	23.2 (32.2)	0.4 (-6.9 to 7.6)	
		GG	17 (9.8)	15.0 (27.8)	-0.3 (-12.7 to 12.0)	
DBP	rs2298849	AA	121 (67.2)	19.8 (28.9)	*Referent*	0.38
		AG	52 (28.9)	26.0 (27.4)	5.0 (-2.4 to 12.5)	
		GG	7 (3.9)	42.4 (24.4)	-2.4 (-21.3 to 16.5)	
DBP	rs16846876	AA	83 (46.9)	23.3 (29.6)	*Referent*	0.14
		AT	84 (47.5)	21.9 (28.8)	-4.4 (-11.5 to 2.7)	
		TT	10 (5.6)	21.7 (26.7)	-8.9 (-24.4 to 6.7)	

(1) Adjusted for disease status and baseline 25(OH)D concentration. (2) Did not survive multiple comparison testing, using the Benjamini & Hochberg method and a false discovery rate of 5%. (3) SNP not analysed due to <5 individuals at any one level. Abbreviations: SD, standard deviation; CI, confidence interval; Δ25(OH)D, delta-25-hydroxyvitamin D_3; CYP-,Cytochrome P450 enzyme; DBP, Vitamin D binding protein; DHCR7, 7-dehydrocholesterol reductase enzyme; SNP, single nucleotide polymorphism.

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Table S4: Characteristics of participants undergoing vitamin D metabolite profiling (n=93)

	Asthma (n=19)	COPD (n=17)	Controls (n=57)
Mean age, yrs (SD)	47.8 (3.1)	66.8 (1.4)	59.5 (13.3)
Sex, n (%)			
Male	10 (52.6)	10 (58.8)	19 (33.3)
Female	9 (47.4)	7 (41.2)	38 (66.7)
Mean BMI, n (%)	29.7 (5.5)	27.4 (6.1)	29.2 (5.9)
Ethnicity, n (%)			
White	19 (100.0)	17 (100.0)	50 (87.7)
Black / Black British	0 (0.0)	0 (0.0)	4 (7.0)
Asian / Asian British	0 (0.0)	0 (0.0)	3 (5.3)
Smoking status, n (%)			
Never-/ex-smoker	17 (89.5)	11 (64.7)	40 (70.2)
Current smoker	2 (10.5)	6 (35.3)	17 (29.8)
Mean alcohol intake, units/week (SD)[(1)]	10.9 (11.3)	12.4 (16.7)	5.9 (9.7)
ICS use, n (%)			
Yes	19 (100.0)	12 (70.6)	0 (0.0)
No	0 (0.0)	5 (29.4)	57 (100.0)
Quarter of blood draw, n (%)			
Q1 (January – March)	6 (31.6)	4 (23.5)	19 (33.3)
Q2 (April – June)	7 (36.8)	4 (23.5)	20 (35.1)
Q3 (July – September)	2 (10.5)	7 (41.2)	6 (10.5)
Q4 (October – December)	4 (21.1)	2 (11.8)	12 (21.1)
BTS step of asthma treatment, n (%)			
Mild intermittent asthma	0 (0.0)	-	-
Regular preventer therapy	10 (52.6)	-	-
Initial add-on therapy	6 (31.6)	-	-
Persistent poor control	2 (10.5)	-	-
Continuous / frequent use of oral corticosteroids	1 (5.3)	-	-
GOLD stage, n (%)			
Mild (FEV1 ≥80% predicted)	-	3 (17.6)	-
Moderate (50% ≤FEV1 <80% predicted)	-	8 (47.2)	-
Severe (30% ≤FEV1 <50% predicted)	-	3 (17.6)	-
Very Severe (FEV1 <30% predicted)	-	3 (17.6)	-

(1) One alcohol unit = 8 g pure alcohol. Alcohol consumption not reported in n=1.
Abbreviations: COPD, Chronic Obstructive Pulmonary Disease; SD, Standard Deviation; BMI, Body Mass Index; ICS, Inhaled Corticosteroid; BTS, British Thoracic Society; GOLD, Global Initiative for Chronic Obstructive Lung Disease.

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Table S5: Vitamin D metabolite-to-parent compound ratios by medication use

			25(OH)D_3 : vitamin D_3 ratio		1α,25$(OH)_2D_3$: 25$(OH)D_3$ ratio x10^3	
		N (%)	**Median (IQR)**	**P**	**Median (IQR)**	**P**
Asthma (n=19)	**ICS**[1]					
	≥400 µg/d	9 (47.4)	13.81 (8.76 to 13.81)	0.48	1.80 (1.00 to 2.91)	0.75
	<400 µg/d	10 (52.6)	14.97 (11.15 to 22.37)		1.85 (1.21 to 2.16)	
	LABA					
	Yes	9 (47.4)	13.81 (9.09 to 20.73)	0.87	1.41 (1.23 to 2.75)	0.46
	No	10 (52.6)	14.05 (8.87 to 20.09)		2.22 (1.85 to 2.68)	
	SABD					
	Yes	17 (89.5)	13.47 (8.86 to 20.09)	0.51	2.16 (1.36 to 2.75)	0.14
	No	2 (10.5)	17.27 (13.81 to 20.73)		1.21 (1.00 to 1.41)	
COPD (n=17)	**ICS/LABA**[2]					
	Yes	12 (70.6)	9.60 (7.38 to 12.41)	0.83	1.95 (0.82 to 4.88)	0.92
	No	5 (29.4)	13.54 (5.96 to 15.10)		3.21 (0.19 to 3.35)	
	SABD					
	Yes	11 (64.7)	12.10 (7.85 to 36.26)	0.16	1.54 (0.19 to 4.39)	0.48
	No	6 (35.3)	7.42 (6.91 to 8.71)		2.98 (2.04 to 4.45)	

Abbreviations: IQR, inter-quartile range; ICS, Inhaled Corticosteroids; LABA, Long-acting Beta-agonists; SABD, Short-acting Bronchodilators; COPD, Chronic Obstructive Pulmonary Disease. (1) ICS dose in betamethasone equivalents: 1 µg betamethasone assumed equivalent to 1 µg budesonide, 0.5 mcg fluticasone dipropionate and 0.75 mcg ciclesonide. (2) all patients with COPD who were taking ICS were also taking a LABA, and vice versa

Table S6: Vitamin D metabolite-to-parent compound ratios by fractional exhaled nitric oxide (asthma) and % predicted FEV1 (COPD)

			25(OH)D_3 : vitamin D_3 ratio		1α,25$(OH)_2D_3$: 25$(OH)D_3$ ratio x10^3	
		N (%)	**Median (IQR)**	**P**	**Median (IQR)**	**P**
Asthma (n=19)	**FeNO**					
	>36ppb[1]	10 (52.6)	12.31 (8.81 to 20.09)	0.51	2.04 (1.36 to 1.41)	0.68
	≤36ppb[1]	9 (47.4)	14.62 (9.09 to 22.37)		1.84 (1.23 to 2.68)	
COPD (n=17)	**$ppFEV_1$**					
	>50%	11 (64.7)	7.93 (6.08 to 15.10)	0.31	1.86 (0.17 to 3.35)	0.19
	≤50%	6 (35.3)	12.41 (8.71 to 13.54)		3.70 (0.83 to 5.79	

Abbreviations FeNO, Fractional exhaled Nitric Oxide; $ppFEV_1$, percent predicted Forced Expiratory Volume in one second; CI, confidence interval; COPD, Chronic Obstructive Pulmonary Disease. (1), 36 parts per billion = median value.

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Table S7: Genes contributing to 1α,25(OH)$_2$D$_3$-inducible gene signatures in epithelial tissue and peripheral blood mononuclear cells (PBMC).

Epithelial		PBMC	
Gene Name	**Gene ID**	**Gene Name**	**Gene ID**
CYP24A1	1591	*FBP1*	2203
PADI3	51702	*CYP24A1*	1591
IL1RL1	9173	*G0S2*	50486
SEMA3B	7869	*THBD*	7056
THBD	7056	*CA2*	760
SERPINB1	1992	*CD14*	929
G6PD	2539	*DHRS9*	10170
CLMN	79789	*OSM*	5008
EFL1	79631	*TKTL1*	8277
ARHGEF28	64283	*CAMP*	820
CDA	978	*EDN1*	1906
G0S2	50486	*CD93*	22918
TRIM56	81844	*LAMB3*	3914
ZBTB38	253461	*SEMA6B*	10501
WSB1	26118	*C19orf59*	199675
TCEA2	6919	*PFKFB4*	5210
PDPN	10630	*MAP3K4*	4216
FBLIM1	54751	*TSPAN3*	10099
GRK5	2869	*KIAA1199*	57214
KIF26A	26153	*TREM1*	54210
PIK3R1	5295	*FUCA1*	2517
FTH1	2495	*DDEF2*	8853
KITLG	4254	*ITGAM*	3684
		CRISPLD2	83716
		LOC729049	729049
		EFTUD1	79631
		FAM116B	414918
		BCAT1	586
		CHST7	56548
		HK2	3099
		CLMN	79789
		DIRC2	84925
		IER3	8870
		FPRL1	2358
		CD36	948
		LMNA	4000

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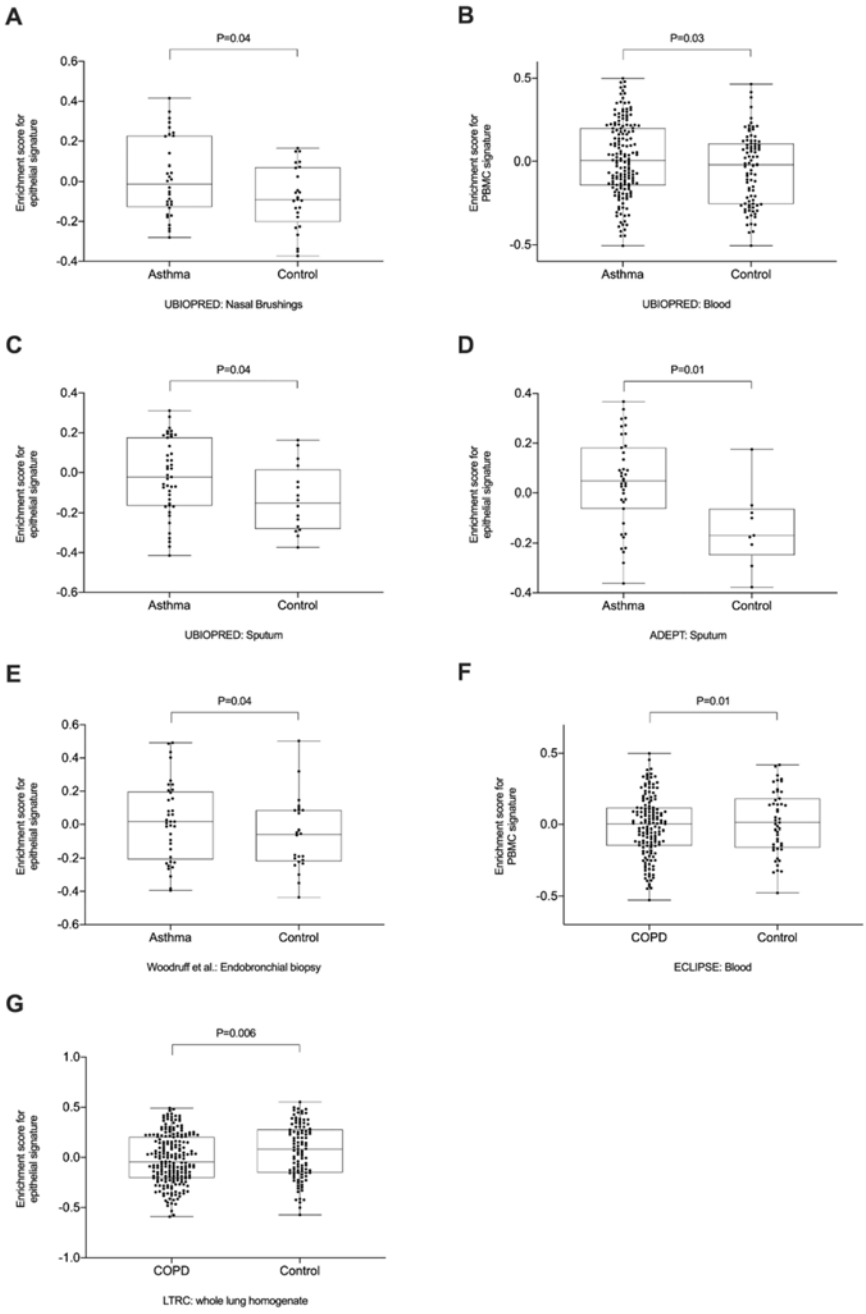
Figure S1: Expression of vitamin D-inducible signatures, patients with asthma and COPD vs. controls. **A**, epithelial signature in nasal brushings, asthma vs. controls (UBIOPRED study). **B**, peripheral blood mononuclear cell (PBMC) signature in peripheral blood, asthma vs. controls (UBIOPRED study). **C**, epithelial signature in sputum, asthma vs. controls (UBIOPRED study). **D**, epithelial signature in sputum, asthma vs. controls (ADEPT study). **E**, epithelial signature in endobronchial biopsy, asthma vs. controls (Woodruff et al). **F**, PBMC signature in peripheral blood, COPD vs. controls (ECLIPSE study). **G**, epithelial signature in whole lung homogenate, COPD vs. controls (Lung Tissue Research Consortium [LTRC]). Enrichment scores from Gene Set Variation Analysis; P values from Kruskal-Wallis rank sum tests (non-normally distributed enrichment scores) or Student's t-tests (normally distributed enrichment scores). Boxes and whiskers show median, interquartile ranges and ranges.



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