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25-hydroxyvitamin D deficiency, exacerbation frequency and human rhinovirus exacerbations in chronic obstructive pulmonary disease

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Abstract

Background: 25-hydroxyvitamin D deficiency is associated with COPD and increased susceptibility to infection in the general population.

Methods: We investigated whether COPD patients deficient in 25-hydroxyvitamin D were more likely to be frequent exacerbators, had reduced outdoor activity and were more susceptible to human rhinovirus (HRV) exacerbations than those with insufficient and normal levels. We also investigated whether the frequency of *Fok*I, *Bsm*I and *Taq*Iα 25-hydroxyvitamin D receptor (VDR) polymorphisms differed between frequent and infrequent exacerbators.

Results: There was no difference in 25-hydroxyvitamin D levels between frequent and infrequent exacerbators in the summer; medians 44.1nmol/L (29.1 – 68.0) and 39.4nmol/L (22.3 – 59.2) or winter; medians 24.9nmol/L (14.3 – 43.1) and 27.1nmol/L (19.9 – 37.6). Patients who spent less time outdoors in the 14 days prior to sampling had lower 25-hydroxyvitamin D levels (p = 0.02). Day length was independently associated with 25-hydroxyvitamin D levels (p = 0.02). There was no difference in 25-hydroxyvitamin D levels between baseline and exacerbation; medians 36.2nmol/L (IQR 22.4-59.4) and 33.3nmol/L (23.0-49.7); p = 0.43. HRV positive exacerbations were not associated with lower 25-hydroxyvitamin D levels at exacerbation than exacerbations that did not test positive for HRV; medians 30.0nmol/L (20.4 – 57.8) and 30.6nmol/L (19.4 – 48.7). There was no relationship between exacerbation frequency and any VDR polymorphisms (all p > 0.05).

Conclusions: Low 25-hydroxyvitamin D levels in COPD are not associated with frequent exacerbations and do not increase susceptibility to HRV exacerbations. Independent of day length, patients who spend less time outdoors have lower 25-hydroxyvitamin D concentration.

Background

Serum concentrations of 25-hydroxyvitamin D vary with age, race, sex, season and geographic location [1]. In its physiologically active form 25-hydroxyvitamin D is released into the circulation, binds to a carrier protein in the plasma (25-hydroxyvitamin D binding protein (DBP)) and is transported to various target organs where it mediates its biological effects by binding to the 25-hydroxyvitamin D receptor (VDR) [2]. Deficiency in 25-hydroxyvitamin D results from a number of causes and is associated with increased risk of infections including

influenza, TB and pneumonia [3]. VDR dysfunction, linked to 25-hydroxyvitamin D deficiency, is thought to cause a decline in innate immune function that increases susceptibility to infections [4].

COPD is characterised by irreversible expiratory airflow limitation [5]. The disease is interspersed with periods of exacerbation that have important consequences for patients and health care providers [6-10]. Exacerbations are predominantly triggered by infection and the commonest respiratory virus detected in the airways at exacerbation is human rhinovirus (HRV) [11,12]. Some individuals are more susceptible to developing exacerbations and these individuals are termed "frequent exacerbators" [13]. Frequent exacerbators have worse quality of life [6], greater limitation of their daily activity, spend

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less time outdoors [14], have faster disease progression [8,15,16] and greater airway inflammation [17] and increased mortality [18] compared to patients with infrequent exacerbations.

Exacerbations are approximately 50% more likely in the winter [19]. It has been postulated that humans have improved innate immunity in the summer, impaired in the winter and that with 25-hydroxyvitamin D synthesis being so dependent on sunlight exposure, falling winter levels may trigger immune deficiencies [20]. It has also been shown recently that vitamin D deficiency is associated with increased mortality in patients admitted to hospital with community acquired pneumonia [21] and it is possible that vitamin D deficiency in COPD may increase susceptibility to pneumonia at the time of exacerbation. Genetic variants in the 25-hydroxyvitamin D pathway have been associated with chronic obstructive pulmonary disease (COPD) [22-26] and many polymorphisms in the VDR gene [27] have been linked with infection.

Our London COPD cohort, a well characterised group of patients allows us to study various aspects of 25-hydroxyvitamin D deficiency on COPD. We hypothesised that with exacerbations peaking in the winter/early spring when 25-hydroxyvitamin D levels are at their lowest, this deficiency in 25-hydroxyvitamin D may increase exacerbation risk via changes in airway inflammation. As our primary outcome we investigated whether COPD patients deficient in 25-hydroxyvitamin D were more likely to be frequent exacerbators. We also investigated whether they had reduced outdoor activity, and were more susceptible to HRV at exacerbation than those with insufficient or normal levels. We investigated whether 25-hydroxyvitamin D levels correlated with HRV load. We also investigated whether the frequency of VDR polymorphisms differed between frequent and infrequent exacerbators.

Methods

Patient recruitment

Ninety seven COPD patients were studied between 1st April 2006 and 30th March 2009. The recruitment and monitoring of patients in the London COPD cohort has previously been described [6,8,11,12,14,17]. This study was approved by the Royal Free Hospital Research Ethics Committee (Ref: 05/Q0501/126) and patients gave written informed consent.

All patients had COPD as defined by a forced expiratory volume in one second (FEV_1) of $\leq$ 80% and FEV_1 to forced vital capacity (FVC) ratio below 70% with β_2 agonist reversibility of less than 15% or 200ml. Patients were excluded if they had other significant respiratory diseases. Patients were recruited when stable, with no exacerbations reported in the preceding month.

At the initial visit, daily respiratory symptoms, smoking history, drug history and co-morbidities were recorded. Height and weight were measured along with baseline lung function using a volumetric storage spirometer (Vitalograph 2160, Maids Moreton, Buckingham, UK). Blood was collected for 25-hydroxyvitamin D assay. Summer samples were taken in June, July or August, and winter samples in January, February or March. Both samples were collected in each individual in the same 12 month period. In each patient, spontaneously produced sputum when available and a nasopharyngeal swab (NPS) were collected for HRV detection.

Exacerbations

COPD patients completed daily diary cards, recording any increase in daily respiratory symptoms. They were asked to contact the study team if they experienced an increase in their symptoms and were usually reviewed within 24 hours. Major symptoms were increased dyspnoea, sputum volume or sputum purulence and minor symptoms increased cough, wheeze, sore throat or coryzal symptoms. Exacerbations were defined according to our previously validated criteria of two symptoms (at least one major) for two consecutive days, or if in the opinion of the attending clinician, the patient had an exacerbation [17]. Our exacerbation definition has been validated against changes in quality of life [6], inflammatory markers [17], and FEV_1 decline [8]. All exacerbations in this study were treated with antibiotics and steroids. None of the exacerbations required hospitalisation.

At an exacerbation visit information was collected on symptom type. The end of the exacerbation was taken as the last day on which lower airway symptoms were recorded. Spirometry was performed and blood taken for 25-hydroxyvitamin D assay. Sputum was collected if spontaneously produced and a NPS taken. All exacerbations were treated with bronchodilators, antibiotics and/or oral steroids as judged by the clinician. All samples were taken prior to the initiation of treatment. Exacerbation visits were not limited to summer or winter months and results were adjusted for seasonality.

Exacerbation frequency

Exacerbation frequency was determined from diary cards. Patients were defined as 'frequent exacerbators' if they had three or more exacerbations per year, or 'infrequent exacerbators' if they had less than three exacerbations per year [28,29]. 3 exacerbations were chosen as both treated and untreated exacerbations were included in our definition.

Time outdoors and daylength

At the end of each day, patients record on diary cards the number of hours they have spent out of the house that day. The time outdoors for baseline and exacerbation visits was calculated as the average time spent outdoors in the 14 days preceding the clinic visit. Daylength data were obtained from Meteorological Office data (at Heathrow Airport, London) on the day of the visit to clinic.

Patient blood sampling

Seven millilitres of venous blood collected at baseline and exacerbation visits was centrifuged at 224 x g for 10 minutes at 4°C within two hours of collection. The serum was then separated and stored at −80°C for later analysis.

25-hydroxyvitamin D measurement

Samples were assayed using the LIAISON 25-OH 25-hydroxyvitamin D TOTAL (DiaSorin, Italy). The LIAISON 25-OH 25-hydroxyvitamin D TOTAL is a fully automated antibody-based two-step direct competitive chemiluminescence immunoassay (CLIA) in the clinical biochemistry department at the Royal Free Hospital. The assay recognises 100% 25-OH 25-hydroxyvitamin D2 and 25-OH 25-hydroxyvitamin D3 using magnetic micropeptide separation. The limit of detection is ≤ 4.0 ng/ml. 25-hydroxyvitamin D deficiency was defined as <25nmol/L, insufficiency 25 − 75nmol/L and sufficiency >75nmol/L (conversion factor of 2.5 for nmol/L from ng/ml). We chose to measure 25-hydroxyvitamin D as there is a standardised assay for measurement which is widely used and previously published on. The serum concentration of 25-hydroxyvitamin D is typically used to determine vitamin D status as it reflects vitamin D produced in the skin as well as that acquired from the diet, and has a fairly long circulating half-life.

Virus detection in NPS and sputum

Samples were collected and processed according to our previously published methodology [28]. Briefly, patients were instructed to blow their nose prior to the swab being passed gently through the nose towards the posterior nasopharynx. The swab was rotated 5–6 times and allowed to remain in place for 5 seconds. The swab was then immediately placed in an eppendorf containing 0.5ml PBS (phosphate buffered saline) and stored at −80°C until RNA extraction. Sputum samples were examined as soon as possible and within two hours of collection. The sample was separated from contaminating saliva and processed using previously published methods [30].

RNA was extracted from NPS using the High Pure Viral RNA kit (Roche) according to manufacturer instructions. RNA was extracted from sputum using Trireagent LS (Sigma) according to the manufacturer instructions. cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer instructions. Real-time PCR was performed using the ABI Prism 7500 Real Time PCR System (Applied Biosystems). 25μl reaction volumes were set up (12.5μl QuantiTect Probe PCR Master Mix (ROX reference dye, Qiagen), 1μl forward and reverse primers (20μM), 0.35μl probe (20μM), 2.5μl template and 7.65μl RNase free water. PCR conditions: 95°C 15 min, 40 cycles of 95°C 15 sec and 58°C 80 sec. This methodology has been published previously [28].

Genotyping

Venous blood samples (10-20ml) were taken in EDTA tubes at the initial visit for all patients and control subjects and stored at −80°C prior to DNA extraction. DNA extraction was performed using a Gentra® systems Puregene® genomic DNA purification kit (Qiagen Cat no. 158389) following the Whole-Blood-Enhanced Productivity protocol supplied by the manufacturer. The primers and PCR conditions are given in Table 1.

The specific VDR polymorphisms chosen were based on previous published literature as they are associated with functionality.

FokI PCR

The reaction consisted of 12.5μl PCR master mix, 1.0μl Forward primer, 1.0μl Reverse primer, 8.0μl water and 2.5μl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 40 cycles of denaturation 94°

Table 1 Primers for VDR polymorphisms

PCR	Forward primer	Reverse primer
VDR FokI	AGCTGGCCCTGGCACTGACTCTGCTCT	ATGGAAACACCTTGCTTCTTCTCCCTC
VDR TaqI	GGGACGATGAGGGATGGACAGAGC	GGAAAGGGGGTTAGGTTGGACAGGA
VDR BsmI	AACTTGCATGAGGAGGAGCATGTC	GGAGAGGAGCCTCTGTCCCATTTG

C for 30 seconds, annealing 56°C 30seconds, extension 72°C 30 seconds. The final extension was at 72°C for 5 minutes.

Taq1a PCR

The reaction consisted of 12.5μl PCR master mix, 1.0μl Forward primer, 1.0μl Reverse primer, 8.0μl water and 2.5μl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 40 cycles of denaturation 94°C for 30 seconds, annealing 66°C 30seconds, extension 72°C 30 seconds. The final extension was at 72°C for 5 minutes.

BsmI PCR

The reaction consisted of 12.5μl PCR master mix, 1.0μl Forward primer, 1.0μl Reverse primer, 8.0μl water and 2.5μl DNA. PCR machine conditions; denaturation 94°C for 5 minutes, then 30 cycles of denaturation 94°C for 30 seconds, annealing 56°C 30seconds, extension 72°C 30 seconds. The final extension was at 72°C for 5 minutes.

All PCR products were run on a 2% agarose gel with a PCR low ladder. The gel was run at 100mV for 15 minutes (Horizon 58, Biometra).

VDR FokI RFLP

To genotype the samples for the *FokI* polymorphism (rs2228570), a RFLP analysis was carried out by using the *FokI* restriction endonuclease enzyme (R0109S New England BioLabs). The reaction consisted of; 2μl buffer, 7.5μl dH$_2$O, 0.5μl RE (*FokI*) and 10μl PCR template. The reaction digested at 37°C for 3 hours on PCR machine. The product was run on a 3% agarose gel. This resulted in the following bands; FF 265bp, Ff 265, 196 and 69bp, ff 196 and 69bp.

VDR TaqIa RFLP

To genotype the samples for the *TaqIα* polymorphism (rs731236), a RFLP analysis was carried out by using the *TaqIα* restriction endonuclease enzyme (R0149T New England BioLabs). The reaction consisted of; 2μl buffer, 0.2μl BSA, 7.7μl dH$_2$O, 0.1μl RE (*TaqIα*) and 10μl PCR template. The reaction digested at 65°C for 3 hours on PCR machine. The product was run on a 3% agarose gel. This resulted in the following bands; TT 495bp, Tt 495, 290 and 205bp, tt 290 and 205bp.

VDR BsmI RFLP

To genotype the samples for the *BsmI* polymorphism (rs1544410), a RFLP analysis was carried out by using the *BsmI* restriction endonuclease enzyme (R0134S New England BioLabs). The reaction consisted of; 2μl buffer, 7.75μl dH$_2$O, 0.25μl RE (*BsmI*) and 10μl PCR template. The reaction digested at 65°C for 3 hours on PCR

machine. The product was run on a 3% agarose gel. This resulted in the following bands; BB 813bp, Bb 813, 670 and 145bp, bb 670 and 145bp.

Statistical analysis

Data were analysed using SPSS version 15 or STATA 8.2 (Stat Corporation, Texas, USA). The Kolmogorov-Smirnov test of normality was applied. Normally distributed data were expressed as mean and standard deviation (SD), skewed data as median and interquartile range (IQR). Spearman rank was used to assess non-parametric correlations. Wilcoxon and Mann Whitney U tests were used for paired and unpaired non-parametric tests respectively. Adjustment for seasonality and investigation of exacerbation and baseline 25-hydroxyvitamin D levels was done using year period sine and cosine terms. Results were therefore adjusted for repeated measures. The study was powered at 0.90 at a 2-sided 0.05 significance level to detect a difference in 25-hydroxyvitamin D of 10nmol/L between frequent and infrequent exacerbators. This study was powered for the primary outcome.

Results

Baseline patient characteristics

Ninety seven COPD patients were studied, 61 male and 36 female. The patients had a mean FEV$_1$ of 1.19 l or 50.3% predicted. Ten COPD patients were on 25-hydroxyvitamin D supplementation (Calcichew D3), and were not included in the following analysis unless otherwise stated. Each tablet of calcichew D3 contains 10 micrograms of colecalciferol and patients usually take 2 a day. The baseline characteristics are reported in Table 2.

Table 2 Baseline Characteristics of 97 patients

	COPD patients (n = 97)Mean (SD)
Age (years)	71.8 (8.8)
FEV1 (litre)	1.19 (0.54)
FEV1 (% predicted)	50.3 (19.7)
FVC (litre)	2.5 (0.84)
BMI (kgm^{-2})	27.0 (6.0)
Pack years smoking	50.7 (34.2)
SpO$_2$ (%) on air	95 (2)
25-hydroxyvitamin D nmol/L	Median (IQR)
Summer	41.3 (26.8 – 64.8)
Winter	27.8 (19.4 – 44.4)
	Number (%)
Male	61 (62.9)
Frequent exacerbators	28 (28.9)
Current smokers	25 (25.8)

Seasonal variation and 25-hydroxyvitamin D levels in COPD

COPD patients had lower 25-hydroxyvitamin D levels in winter compared to summer; medians 26.7nmol/L (IQR 17.8 – 41.2) and 39.6nmol/L (26.4 – 62.9); $p < 0.001$. Within individuals, summer and winter 25-hydroxyvitamin D levels varied by 10% monthly and by 50% between summer and winter, with summer levels being higher. Patients taking Calcichew D3 did not show the same significant seasonal variation in 25-hydroxyvitamin D levels; winter median 58.1nmol/L (35.2 – 69.6), summer median 56.2nmol/L (33.9 – 73.1; $p > 0.05$. The seasonal difference in 25-hydroxyvitamin D levels between these patients is illustrated in Figure 1. There were no differences in summer 25-hydroxyvitamin D levels between current smokers and ex-smokers, medians; 45.5nmol/L (27.4 – 63.3) and 40.3 nmol/L (26.7 – 66.3) or winter 25-hydroxyvitamin D levels; medians 27.0 nmol/L (14.6 – 47.7) and 28.0 nmol/L (19.9 – 43.7).

25-hydroxyvitamin D levels in frequent and infrequent exacerbators

Figure 2 shows that there was no difference in 25-hydroxyvitamin D levels between frequent exacerbators (1/3 of the cohort) and infrequent exacerbators in the summer; medians 44.1nmol/L (29.1 – 68.0) and 39.4nmol/L (22.3 – 59.2) or winter; medians 24.9nmol/L (14.3 – 43.1) and 27.1nmol/L (19.9 – 37.6). The proportion of patients' deficient, insufficient and sufficient in 25-hydroxyvitamin D was the same in both frequent and

infrequent exacerbators groups. Exacerbation history was available in a subset of 10 patients before and after treatment with Calcichew D3. There was no difference in actual exacerbation number from year 1 to 2; $p = 0.45$, or in exacerbation frequency from year 1 to 2; $p = 0.38$.

Time outdoors and 25-hydroxyvitamin D levels

Figure 3 shows that shorter day length on the day of sampling was associated with lower levels of 25-hydroxyvitamin D (coef 1.65, se 0.69; $p = 0.02$). Patients who spent less time outdoors in the 14 days prior to sampling also had lower 25-hydroxyvitamin D levels coef 2.36, se0.96; $p = 0.02$). This was independent of day length.

25-hydroxyvitamin D levels and exacerbations of COPD

We measured serum 25-hydroxyvitamin D levels in 58 exacerbations; 1 per patient in the study period. Adjusting for seasonality, there was no difference in 25-hydroxyvitamin D levels between baseline and exacerbation; medians 36.2nmol/L (IQR 22.4-59.4) and 33.3nmol/L (23.0-49.7); $p = 0.43$.

25-hydroxyvitamin D and HRV exacerbations

46 exacerbations were tested for the presence of HRV in sputum or NPS. HRV positive exacerbations (n = 12, viral load > 175pfu/ml; [28] were not associated with lower 25-hydroxyvitamin D levels at exacerbation than exacerbations that did not test positive for HRV; medians 30.0nmol/L (20.4 – 57.8) and 30.6nmol/L (19.4 – 48.7). HRV load in sputum or NPS at exacerbation did not
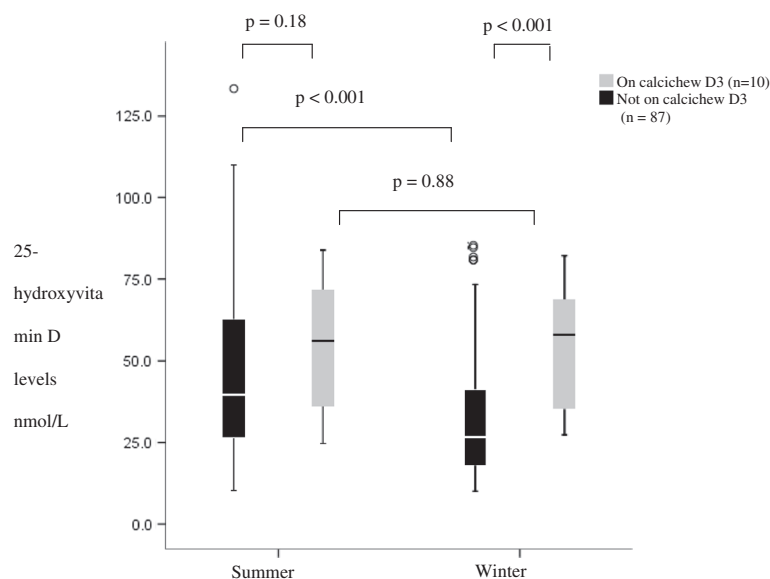

Figure 1 25-hydroxyvitamin D levels in COPD patients in summer and winter in those on and not on calcium and 25-hydroxyvitamin D supplementation. Data are presented as median, with the boxes representing the interquartile range and the whiskers representing SD. O: extreme outliers.
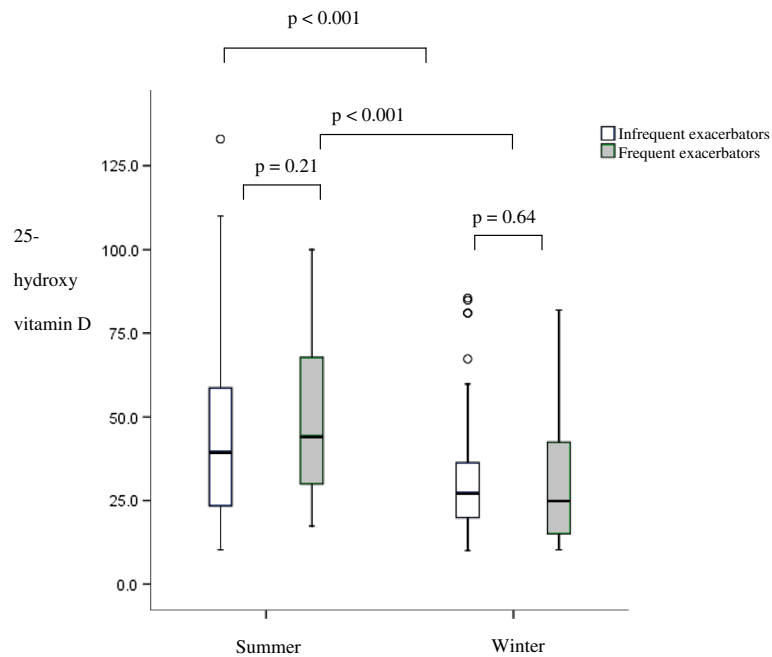
Figure 2 25-hydroxyvitamin D levels in summer and winter in frequent and infrequent exacerbators. Data are presented as median, with the boxes representing the interquartile range and the whiskers representing SD. ○: extreme outliers.

correlate with exacerbation 25-hydroxyvitamin D levels (data not shown). When including all exacerbations per patient taken over the study period tested for HRV, patients deficient in 25-hydroxyvitamin D at baseline did not have an increased proportion of HRV positive exacerbations compared to those insufficient or sufficient. This is illustrated in Table 3. The presence of cold

symptoms at exacerbation was not related to vitamin D deficiency ($p > 0.05$).

Exacerbation length and severity
There was no relationship with 25-hydroxyvitamin D levels either at that exacerbation or the baseline preceding that exacerbation and the length of the exacerbation;
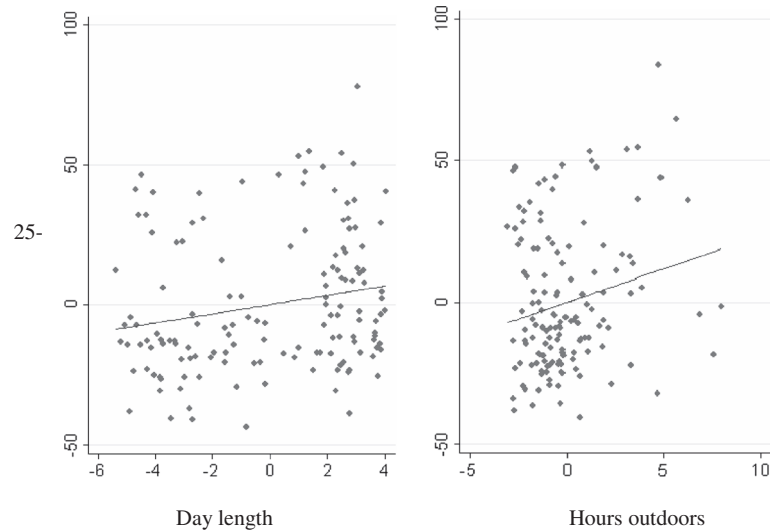

Figure 3 Adjusted partial residual plot of the relationship between 25-hydroxyvitamin D levels and (1) day length (hours), and (2) hours outdoors. The graphs show the effect of each variable on 25-hydroxyvitamin D independently of the other variable. The zero value is the annual average.

Table 3 25-hydroxyvitamin D status and positivity for HRV at exacerbation

25-hydroxyvitamin D status	positive for HRV at exacerbation		Total
	No	Yes	
Deficient	4 (25.0%)	7 (23.3%)	11
Insufficient	10 (62.5%)	19 (63.3%)	29
Sufficient	2 (12.5%)	4 (13.3%)	6
Total	16	30	46

25-hydroxyvitamin D deficiency was defined as <25nmol/L, insufficiency 25 – 75nmol/L and sufficiency > 75nmol/L (conversion factor of 2.5 for nmol/L from ng/ml).

rho = −0.41: p = 0.12 and rho = −0.41: p = 0.12 respectively or time to the next exacerbation; rho = −0.11, p = 0.66 and rho = −0.06, p = 0.81 respectively. All exacerbations recovered.

VDR polymorphisms

All genotypes were within Hardy Weinberg equilibrium. The *Fok*I polymorphism was not related to *Taq*I or *Bsm*I, however the *Bsm*I and *Taq*I polymorphisms were linked (p < 0.001). There was no relationship with genotyping and exacerbation frequency for any of the polymorphisms as illustrated in Table 4.

Discussion

We have investigated several aspects of 25-hydroxyvitamin D deficiency in a well characterised cohort of COPD patients and shown that independent of day length, COPD patients who spend less time outdoors have lower 25-hydroxyvitamin D levels. In this study we found that patients on Calcichew D3 had much higher levels of 25-hydroxyvitamin D than COPD patients not on 25-hydroxyvitamin D supplementation and showed much less seasonal variation in 25-hydroxyvitamin D levels. This is an interesting finding as several studies have investigated the pharmacokinetics of 25-hydroxyvitamin D, and it is thought that the amount of cholecalciferol in Calcichew is far below the amount required to treat deficiency and maintain a stable 25-hydroxyvitamin D level. This suggests that perhaps these patients may differ with respect to underlying sub-phenotype or co-morbidities (e.g. osteoporosis). However with only 10 patients in this group it is not possible to reach firm conclusions.

Outdoor activity and 25-hydroxyvitamin D levels

From this study it appears that 25-hydroxyvitamin D levels are driven primarily by season and outdoor activity, with patients with lower 25-hydroxyvitamin D levels spending less time outdoors in the 14 days preceding sampling. This has implications for pulmonary rehabilitation, patient education and lifestyle modification. Independent of 25-hydroxyvitamin D status, reduced activity

in COPD is related to poorer prognosis [31]. Perhaps outdoor pulmonary rehabilitation should be encouraged in those COPD patients deficient in 25-hydroxyvitamin D.

A large proportion of the elderly population in the USA and Europe are 25-hydroxyvitamin D deficient [32] and those with COPD are at particularly high risk [1]. Studies in asthma linking low 25-hydroxyvitamin D levels with disease severity have postulated the relationship may be secondary to time spent indoors [33]. This is also likely to be important in COPD [14]. 25-hydroxyvitamin D deficiency in the COPD population may reflect poor general health status rather than having particular importance in the natural progression of COPD. However, if it were that simple, patients with COPD living in tropical countries should have milder disease and fewer exacerbations.

25-hydroxyvitamin D levels and exacerbations and exacerbation frequency

Patients deficient in 25-hydroxyvitamin D in the stable state (< 25nmol/L), were not more likely to be frequent exacerbators and did not have a shorter time to their next exacerbation than those insufficient or sufficient in 25-hydroxyvitamin D. 25-hydroxyvitamin D levels were unchanged between baseline and exacerbation in COPD when adjustments were made for seasonality and we did not find a relationship between 25-hydroxyvitamin D deficiency and exacerbation severity. This is in keeping with a recent study which found that low baseline 25-hydroxyvitamin D levels in patients with severe COPD did not predict subsequent exacerbations [34]. A recent RCT has also shown that high dose vitamin D

Table 4 VDR polymorphisms and exacerbation frequency

SNP	Genotype	Frequent exacerbators (n = 28)	Infrequent exacerbators (n = 68)	Chi squared P value
Rs1544410	BB	3 (10.7%)	15 (22.1%)	
Bsml	Bb	12 (42.9%)	26 (38.2%)	0.43
	Bb	13 (46.4%)	27 (39.7%)	
HWE p value		0.93	0.08	
		(n = 26)	(n = 66)	
Rs731236	TT	10 (38.5%)	24 (36.4%)	
Taql	Tt	13 (50%)	29 (43.9%)	0.64
	Tt	3 (11.5%)	13 (19.7%)	
HWE p value		0.69	0.43	
		(n = 28)	(n = 68)	
Rs2228570	FF	10 (35.7%)	21 (30.9%)	
Fokl	Ff	14 (50.0%)	38 (55.9%)	0.87
	Ff	4 (14.3%)	9 (13.2%)	
HWE p value		0.80	0.21	

supplementation in COPD patients did not reduce exacerbation incidence [35]. However the authors of this study in a post-hoc analysis suggested that in patients with the most severe vitamin D deficiency at baseline, supplementation may reduce future exacerbations.

Influenza and other viruses show a distinct predilection for wintertime infectivity, and exacerbations of COPD are significantly more likely to occur in the winter. 25-hydroxyvitamin D deficiency has been associated with self-reported upper respiratory tract infections (URTI) [36,37]. As HRV is the commonest cause of colds and present in over 50% of COPD exacerbations, we chose specifically to investigate a relationship between HRV exacerbations and 25-hydroxyvitamin D deficiency. We did not find a relationship between 25-hydroxyvitamin D deficiency and the presence of HRV at exacerbation or HRV load at exacerbation. In other diseases, 25-hydroxyvitamin D supplementation studies have shown mixed results with regards to preventing viral and bacterial infections [38,39]. We did not have bacterial culture data available in this study and were unable to investigate any association between vitamin D levels and bacterial exacerbations.

VDR polymorphisms

We did not find a relationship between VDR polymorphisms and exacerbation frequency. However, the FokI, and TaqI Vitamin D receptor polymorphisms have been shown to be associated with lower respiratory tract infections in children [40]. Genetic variants in the 25-hydroxyvitamin D pathway have been associated with COPD [1,24]. Although GWAS studies did not find VDR to be an important risk gene for COPD, associations of VDR with risk for infections [40] has been found in investigating interactions between low 25-hydroxyvitamin D levels and the VDR. Many polymorphisms exist in the VDR gene and the influence of these polymorphisms on VDR protein function may influence immunomodulatory responses [27]. To date no study has found a link with VDR polymorphisms and airway infection in COPD although there are several mechanisms by which activated 25-hydroxyvitamin D binding to the VDR could modulate viral lower respiratory tract disease [41-43]. Our study was powered on the primary outcome and our sample size for a genetic study is relatively small. This may explain our negative findings.

There are several strengths to this study. The London COPD cohort is a well characterised cohort of COPD patients with detailed information on exacerbations and exacerbation frequency. In this cohort monitoring visits are predefined at regular time intervals and driven by clinical visits because of exacerbations thus allowing information to be obtained on 25-hydroxyvitamin D at baseline and exacerbation.

We do not actually know what constitutes 25-hydroxyvitamin D deficiency, particularly in the context of its immunomodulatory properties. In terms of calcaemic effects, levels below 50nmol/L are probably deficient [3,44]. With regards to the immunomodulatory mechanisms of 25-hydroxyvitamin D it has even been suggested that levels > 100nmol/L are needed for optimal immune functioning. There is much to be learned about the role of 25-hydroxyvitamin D in COPD and the mechanisms by which increasing 25-hydroxyvitamin D levels into the normal range would influence the natural history of COPD.

Conclusions

In conclusion, we have shown that independent of day length, patients who spend less time outdoors have lower 25-hydroxyvitamin D levels. Low 25-hydroxyvitamin D levels in COPD are not associated with exacerbation frequency and do increase susceptibility to HRV exacerbations.

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Authors' contributions
JKQ, GCD, JRH, NW, MT and JAW contributed to the conception and design of the study. JKQ, to the acquisition of samples, NW to vitamin D processing, JKQ and GCD to data analysis, JKQ, GCD, JRH, NW, MT and JAW to interpretation of data. JKQ wrote the first draft of the manuscript and all authors contributed to subsequent drafts. All authors read and approved the final manuscript.

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