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# Impact of High-Dose Vitamin $D_3$ on Free Plasma 25-Hydroxyvitamin D Concentrations and Antimicrobial Peptides in Critically Ill Mechanically Ventilated Adults

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## Abstract

**Objectives—**High-dose vitamin $D_3$ increases plasma total 25-hydroxyvitamin D [25(OH)D] in critically ill, ventilated patients, but the impact on plasma levels of free (non-protein bound) 25(OH)D, has not been investigated in critical illness. Moreover, the relationship of free 25(OH)D and regulation of endogenous antimicrobial peptides (AMPs) is unknown.

**Research Methods & Procedures—**In a double blind, randomized controlled trial, critically ill ventilator-dependent adults (n=30) received enteral vitamin $D_3$ (250,000 or 500,000 IU total over 5 days) or placebo. Plasma was obtained serially for concentrations of free 25(OH)D, cathelicidin (LL-37), human beta-defensin-2 (hBD-2) and expression of peripheral blood mononuclear cell (PBMC) human cationic antimicrobial protein (hCAP18) mRNA. Total 25(OH)D and LL-37 concentrations and alveolar macrophage phagocytosis were determined in bronchoalveolar lavage fluid (BALF).

**Results—**Plasma concentrations of free 25(OH)D over time were correlated with total 25(OH)D levels ($\rho$=0.82, $p< 0.001$). The increase in free 25(OH)D was greatest with the 500,000 IU vitamin

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$D_3$ dose compared to the lower dose. The percent change in mRNA expression of hCAP18 was positively associated with percent change in free 25(OH)D at day 7 and 14 (ρ=0.48, p=0.04 and ρ=0.59, p=0.03, respectively). In addition, plasma LL-37 levels correlated with the percentage of alveolar macrophages exhibiting phagocytosis (ρ=0.51, p = 0.04).

**Conclusions—**We found a dose-related increase in plasma free 25(OH)D levels, which was associated with increasing circulating mRNA expression of hCAP18 over time. There were no correlations between changes in total and free 25 (OH)D, and plasma LL-37 and hBD-2 concentrations. Larger studies appear warranted to determine the impact of high-dose vitamin D3 administration on endogenous AMPs.

### Keywords

Antimicrobial peptides; critical care; LL-37; respiratory failure; vitamin D

## INTRODUCTION

The potential benefit of vitamin D administration in critically ill patients is being investigated given the high rate of vitamin D deficiency in this patient population (1–5) and its strong association between low blood levels of total 25-hydroxyvitamin D [25(OH)D] and adverse clinical outcomes, such as increased risk of mortality (6–13). Recently, several randomized clinical trials (RCT) have reported clinical effects of administration of vitamin D in adult intensive care unit (ICU) patients (5, 14–16). Three of the recent RCTs studied various high-dose regimens of vitamin $D_3$ (cholecalciferol) and reported some beneficial effects on secondary clinical endpoints, including mortality (15) and hospital length of stay (LOS) (5, 16), concomitant with increased total 25(OH)D levels in blood.

Vitamin D administration has been a focus for intervention because of benefits to the immune system. $1,25(OH)_2D$ has pleotropic effects on immune cells (17), and in many studies upregulates expression of the endogenous antimicrobial peptide (AMP) cathelicidin (LL-37) in human skin, plasma, monocytes and macrophages (12, 16, 18–21). LL-37, the C-terminal peptide fragment of human cationic antimicrobial protein (hCAP18) and human beta-defensin 2 (hBD-2) (the most abundant beta-defensin in the lung) is induced in respiratory and other epithelia and immune cells in response to infection and inflammation (22–26). LL-37 and other AMPs, such as defensins, exhibit direct antimicrobial effects against microorganisms and also modulate various innate and adaptive immune functions, including chemotaxis and phagocytosis (24, 27). hBD-2 is up-regulated in epithelia during infections and/or inflammation (24, 26). Studies in humans with respiratory infections demonstrate upregulation of these AMPs in lung (28–30). Limited data suggests that $1,25(OH)_2D$ may upregulate hBD-2 (17, 31, 32). This is relevant to studies of vitamin D administration in ICU patients because induction of hBD-2 is impaired in patients with severe sepsis (33). In addition, no studies have explored the relationship between circulating AMPs and lung macrophage phagocytosis.

In catabolic patients, some studies have associated increased blood total 25(OH)D levels with upregulated LL-37 protein and mRNA expression of hCAP18, suggesting a possible mechanistic relationship between vitamin D status and infection (1, 14, 16, 34). However,

other studies in patients with infections or lung disease did not show a relationship between blood total 25(OH)D and LL-37 concentrations (35–37). Thus far, the strongest evidence supporting vitamin D administration in critically ill patients are from the large randomized controlled trial of Amrein et al (15) and a meta-analysis of vitamin D trials in ICUs by Putzu et al (38). Taken together, these data suggest that administration of high-dose vitamin D may be associated with reduced mortality in some patient subgroup (e.g. those with frank vitamin D deficiency) without improvement in other clinical outcomes; thus, vitamin D therapy in this setting remains controversial and there is a need for further investigations (15, 38). In our recent RCT in mechanically ventilated adults, high-dose vitamin $D_3$ increased plasma total 25(OH)D levels by 2 to 3-fold, but did not alter plasma LL-37 concentrations (5).

Total 25(OH)D concentrations in blood reflects 25(OH)D tightly bound to vitamin D binding protein (DBP), the major 25(OH)D carrier protein, the rest is loosely bound to albumin or is free in circulation, defined as bioavailable 25(OH)D (39, 40). Recently, immunoassays have been developed to accurately and directly measure free (non-protein bound) 25(OH)D in human blood (39). Direct measurement of free 25(OH)D may be advantageous in ICU patients, in whom the catabolic response results in a marked decline in blood albumin concentrations and circulating levels of DBP (1,41). Thus far, no studies in ICU patients have evaluated the impact of vitamin D administration on directly measured free 25(OH)D levels or relationships to AMP expression in blood. Here we determined in critically ill adults with respiratory failure: 1) the impact of our previous high-dose regimens of vitamin $D_3$ on free 25(OH)D concentrations, 2) the relationship of free 25(OH)D with circulating LL-37 and hBD-2, and 3) associations between plasma levels of free 25(OH)D and these AMPs to alveolar macrophage phagocytosis function.

## MATERIALS AND METHODS

### Trial Design

The parent RCT was approved by the Emory University Institutional Review Board (www.clinicaltrials.gov NCT01372995) and published previously (5). Written informed consent was obtained from all subjects or their legally authorized representative. Subjects were enrolled at two Emory University School of Medicine teaching hospitals, Emory University Hospital Midtown and Emory University Hospital. Full details of trial design, inclusion and exclusion criteria, safety criteria and other methodological details are provided in the previous publication (5).

### Participant Selection

Briefly, major inclusion criteria were 1) age > 18 years; 2) respiratory failure requiring mechanical ventilation for at least 72 hours after study entry; and 3) anticipated stay in the ICU for at least 96 hours after entry. Major exclusion criteria were 1) use of high-dose vitamin $D_3$ supplementation (≥ 50,000 IU a week) to treat vitamin D deficiency within the prior 6 months; 2) history of medical disorders associated with hypercalcemia, chronic renal failure requiring dialysis, cirrhosis or HIV infection; and 3) hypercalcemia (albumin-corrected serum calcium > 10.8 mg/dL or ionized calcium > 5.2 mg/dL).

Study subjects were block-randomized into the respective double-blind treatment groups according to hospital study site and Acute Physiology and Chronic Health Evaluation II score (APACHE II) score >15 or ≤ 15 (42).

## Intervention

After baseline sample collection (see below), either a daily dose of 50,000 IU vitamin $D_3$, 100,000 IU vitamin $D_3$, or matching placebo was administered enterally via nasal tube for 5 consecutive days by the primary nurse. Thus, the two high-dose vitamin $D_3$ regimens provided a total dose of 250,000 IU or 500,000 IU. Capsules containing 50,000 IU of vitamin $D_3$ were manufactured from Tischon (Westbury, NY) and Biotech (Fayetteville, AR). Bioavailability testing by an independent commercial laboratory showed vitamin $D_3$ content within ± 10% of the expected dose.

## Measurements

**Clinical and demographic data collection:** Details on methodology used are outlined in the parent RCT publication (5).

**Blood sampling:** Twenty mL of venous blood for isolation of plasma was collected at baseline just prior to study drug administration and again 7 and 14 days later while subjects were still hospitalized.

**Bronchoalveolar lavage (BAL) collection:** Conventional methods to obtain BAL fluid (BALF) at baseline in intubated subjects were performed. BAL involved bronchoscopy and instillation of 30 mL aliquots of normal saline into a pulmonary segment followed by suction (5). Bronchoscopy was performed by either JEH or GSM in 19 of the 30 subjects.

**Plasma free and total 25(OH)D:** Free concentrations of 25(OH)D in serial plasma samples was measured with a competitive enzyme-linked two-step immunosorbent assay (ELISA), calibrated against a symmetric dialysis method (DIAsource ImmunoAssays, Louvain-la-Neuve, Belgium). Total 25(OH)D (representing DBP-bound, albumin-bound and free fractions) was measured using a chemiluminescent-based automated method (IDS-iSYS; Immunodiagnostic Systems, Scottsdale, AZ) (5).

**Plasma LL-37 and hBD-2:** ELISA kits were used to measure plasma concentrations of LL-37 (Hycult Biotech; Uden, The Netherlands) and hBD-2 (Phoenix Pharmaceuticals, Inc.; Burlingame, CA).

**BALF LL-37 and total 25(OH)D:** BALF was concentrated 5- to 10-fold and analyzed by ELISA for LL-37 and total 25(OH)D concentrations (5). To control for dilution by the lavage procedure, the LL-37 and total 25(OH)D concentrations were normalized using the urea method (43). Urea nitrogen was measured in the plasma and BALF supernatant by a quantitative colorimetric assay (Pointe Scientific, Canton, MI) and dilution of the BALF was calculated from ($[urea]_{plasma}/[urea]_{BALF}$) (44).

**mRNA expression of hCAP18:** Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using cell preparation tubes containing Ficoll (Becton-Dickinson and Co., Franklin Lakes, NJ) and stored in RNA preservation solution at −80°C before RNA isolation by standard methods. Expression of hCAP18 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in PBMCs were quantitated by real-time polymerase chain reaction (RT-qPCR). GAPDH was used as a reference gene to calculate the fold change of gene expression using the AACT method. Primers used for RT-qPCR reactions were: hCAP18;CTTCACCAGCCCGTCCTTC and CCAGGACGACACAGCGTCA, GAPDH; CTTAGCACCCCTGGCCAAG and TGGTCATGAGTCCTTCCACG. TaqMan probes were: hCAP18; 6-FAM CAG AGG ATT GTG ACT TCA MGBNFQ, GAPDH; VIC-CAT CCA TGA CAA CTT TGG TA MGBNFQ. We were unable to detect hBD-2 mRNA in PBMCs using specific primers for this defensin.

**Alveolar macrophage percent positive analysis and phagocytosis index:** Macrophage phagocytosis was determined using methods as previously described in humans (44). Briefly, BALF was centrifuged and total cell counts determined in the cell pellets with a hemocytometer. Diff-Quick staining (Andwin Scientific, Addison, Ill) was used to determine cellular differential counts from 300 consecutive cells. Cell pellets with <85% alveolar macrophage purity were excluded from further analysis (n=3), leaving a total of 16 subjects with macrophage phagocytosis data.

Fluorescence of phagocytized *S. aureus* was determined by quantitative computer analysis. Macrophages with any internalized bacteria were considered positive for phagocytosis. Phagocytosis was quantified by the percentage (%) of cells positive for phagocytosis and phagocytic index, defined the % of cells positive for phagocytosis multiplied by the mean relative fluorescence units of *S. aureus* per cell (44).

## CALCULATIONS

Descriptive statistics were performed by treatment group. Group differences in continuous variables at baseline were assessed with analysis of variance (ANOVA) or a Kruskal-Wallis test depending on the normality of the distribution, and differences in categorical variables were assessed with a $Chi^2$ test. Data are reported as mean (± SD) for normally distributed variables and median (IQR) for non-normally distributed variables. Differences by treatment, over time, and the treatment by time interaction (treatment × time) for free 25(OH)D were assessed with mixed-model repeated measures ANOVA with Tukey's post-hoc analyses; normal probability plots of residuals were visually assessed to ensure normal distributions. Percent changes in variables were compared by group using the Kruskal-Wallis test. Bivariate relationships were assessed using Spearman or Pearson correlations based on the distributions of the variables. Analyses were performed with the JMP® program (version 12.0.1, SAS Institute Inc., Cary, NC) using 2-sided tests. P-values < 0.05 were considered statistically significant.

## RESULTS

The CONSORT diagram, clinical outcomes and concentrations of total 25(OH)D and LL-37 in plasma over time and in baseline BALF are previously published (5). For illustration purposes, selected baseline demographic characteristics of the same subjects in this sub-study, and the baseline vitamin D and AMP endpoints from plasma and BALF are shown in Table 1. Baseline clinical, demographic indexes, baseline plasma total 25(OH)D and LL-37 concentrations were similar between groups at baseline, as previously reported (5). There were no differences between groups for baseline plasma free 25(OH)D, hBD-2 concentrations, and for PBMC hCAP18 mRNA expression (Table 1). BALF concentrations for total 25(OH)D and LL-37 concentrations obtained at baseline were also similar between groups (Table 1).

### Baseline plasma free 25(OH)D and total 25(OH)D concentrations

There was a strong correlation between baseline plasma free 25(OH)D and total 25(OH)D concentrations in the 30 study subjects ($\rho = 0.82$, $p < 0.001$) (Figure 1).

### High-dose vitamin $D_3$ on plasma free 25(OH)D concentrations over time

In the three study groups, repeated measures ANOVA showed significant effects of treatment, time and treatment × time interaction on plasma free 25(OH)D concentrations (each $p<0.001$) (Figure 2). Free 25(OH)D levels in plasma remained unchanged in the placebo group. In contrast, both high-dose vitamin $D_3$ regimens significantly increased plasma free 25(OH)D levels from baseline values by day 7. Free 25(OH)D levels rose further by day 14 in the group administered 500,000 IU vitamin $D_3$. The magnitude of the plasma free 25(OH)D was dose-responsive. With the lower 250,000 IU dose, levels rose approximately 2-fold from baseline by day 7 and then decreased slightly by day 14. The plasma free 25(OH)D response to the higher 500,000 IU dose was more robust; levels rose more than 4-fold from baseline by day 7 and were nearly 5-fold greater than baseline by day 14 (Figure 2). The percent change from baseline to day 14 in free and total 25(OH)D in the three groups were highly correlated in the 19 subjects in whom plasma was available for analysis at both time points after placebo or vitamin $D_3$ administration (Figure 3).

### Plasma hBD-2 and PBMC hCAP18 mRNA expression

In our previous report, we found no change over time in plasma LL-37 concentrations despite a 2-3-fold increase in plasma total 25(OH)D levels (5). Despite the marked increase in free 25(OH)D concentrations with high-dose vitamin $D_3$ administration in this study, there was similarly no change in plasma levels of hBD-2 or hCAP18 mRNA expression from baseline between groups over time (not shown). There was also no difference between groups for percent change in values from baseline to day 7 for plasma hBD-2 concentrations and hCAP18 mRNA expression (Table 2). Similarly, there was no difference between groups for percent change in plasma LL-37 concentrations at days 7 and 14 (Table 2).

### Correlation analysis

Spearman correlation analysis showed no statistically significant association between baseline plasma free 25(OH)D levels to plasma LL-37 ($\rho=0.002$, $p=0.99$) and plasma free

25(OH)D levels to plasma hBD-2 (ρ=−0.22, p=0.28). There was no difference in the correlation of percent change in plasma free or total 25(OH)D levels and percent change in plasma hBD-2 or plasma LL-37 levels at either days 7 or 14 (Table 3). In contrast, the percent increase in plasma free 25(OH)D, but not total 25(OH)D, was significantly associated with the percent increase in PBMC hCAP18 mRNA expression at both days 7 and day 14 (Table 3).

We evaluated associations between vitamin D, hBD-2 and LL-37 measures and the two indices of alveolar macrophage phagocytosis function in 16 subjects with adequate BALF-derived macrophages isolated for analysis (Table 4). There were no correlations between concomitant plasma total 25 (OH)D, free 25(OH)D, plasma hBD-2, PBMC hCAP18 mRNA expression, BALF LL-37, BALF total 25(OH)D and either of the alveolar macrophage phagocytosis function indices. However, there was a significant positive correlation between plasma LL-37 concentrations and the percent positive alveolar macrophages undergoing phagocytosis (ρ= 0.51, p = 0.04), and a trend for a positive association between plasma LL-37 levels and the alveolar macrophage phagocytosis index (ρ= 0.44, p= 0.09) (Table 4).

## DISCUSSION

This pilot study was designed to demonstrate the impact of high-dose vitamin D3 administration (either 250,000 or 500,000 IU versus placebo) on serial plasma free 25(OH)D concentrations. At baseline (prior to vitamin $D_3$ or placebo administration), plasma values ranged between 4.1–5.8 pg/mL, similar to serum free 25(OH)D levels in our recent report in healthy adults (4.7 pg/mL) and non-critically ill adults with cystic fibrosis (4.6–5.9 pg/mL) (39). Our three study groups were well matched at entry into the study. Baseline and change from baseline to day 14 plasma free 25(OH)D concentrations were highly correlated with total 25(OH)D levels, consistent with our recent report of serum levels in healthy and non-critically ill adults with cystic fibrosis (39). This suggests that changes in blood levels of total 25(OH)D, currently considered the best biomarker of vitamin D status (45, 46), may reflect changes in free 25(OH)D levels.

We found a robust increase in plasma free 25(OH)D levels at day 7 with both high-dose vitamin $D_3$ regimens. A dose-response was clearly evident; these kinetic changes in free 25(OH)D plasma concentrations over time contrast with changes we found in plasma total 25(OH)D levels in the same subjects, where there was no statistically significant dose-response of plasma total 25(OH)D after vitamin $D_3$ administration (5). Therefore, these pilot data suggest that higher doses of exogenous vitamin $D_3$ supplementation in adult ICU patients increase free 25(OH)D to a greater extent than total 25(OH)D plasma concentrations.

A significant proportion (estimated to be 85–90%) of 25(OH)D is tightly bound to DBP. The combined albumin-bound and smaller free 25(OH)D fractions, is considered the “bioavailable” circulating 25(OH)D pool (45–47). Bioavailable 25(OH)D levels are estimates calculated using equations incorporating blood levels of total 25(OH)D, DBP and albumin that were developed in healthy subjects and thus may not be completely applicable to ICU and other types of catabolic patients. In critical illness, blood levels of both albumin

and DBP may be altered due to insufficient dietary protein intake, accelerated protein breakdown, malabsorption, decreased synthesis, leakage of blood proteins into the interstitial space, changes in urinary excretion and/or fluid status, thus affecting calculated bioavailable 25(OH)D estimates (1, 41, 47–50). Further, ethnic differences in DBP affinity for 25(OH)D binding due to genetic DBP polymorphisms may obscure true vitamin D bioavailability in some subjects (39, 50).

In a pilot study similar to ours, Quraishi *et al* administered a one-time enteral dose of vitamin $D_3$ (either 200,000 IU or 400,000 IU) or placebo in adults with new onset of severe sepsis or septic shock (16). Compared to baseline values, these investigators showed a modest ≈ 50% to 70% increase in plasma total 25(OH)D levels from baseline to day 5 of study with the one-time 200,000 IU and 400,000 IU vitamin $D_3$ doses, respectively. However, no change from baseline occurred in calculated bioavailable 25(OH)D from baseline to day 5 with the lower vitamin $D_3$ dose, while the 400,000 IU dose resulted in a significant 60% increase in calculated bioavailable 25(OH)D over this time frame (16). The reasons for the more robust response of total and free 25(OH)D over time in our study, compared to the magnitude of change in total and bioavailable 25(OH)D levels in the previous study (16) are unclear, but may be related to decreased plasma albumin and/or DBP levels in the septic patients or other differences in clinical characteristics. Also, the timing of serial blood sample collection and vitamin $D_3$ bioavailability comparing a one-time dose by Quraishi (16) versus 5 days in the current study may have resulted in different circulating vitamin D responses.

In contrast to the reports cited earlier, some studies in critically ill patients, including our previous report, show no link between total 25(OH)D levels and rates of sepsis or infection (51, 52). This may reflect the impact of altered circulating albumin and DBP on total 25(OH)D measurements (47, 53). Although limited data suggest that free 25(OH)D levels in blood may more accurately reflect changes in vitamin D-mediated metabolic responses (46, 54), further studies are required to determine whether circulating free 25(OH)D values are superior to total 25(OH)D levels as a marker of vitamin D status and metabolic responses in ICU patients.

Given the associations between activated vitamin D and AMP expression, in particular LL-37 (17–19, 55), another goal was to explore potential relationships between high-dose vitamin $D_3$ administration and free plasma 25(OH)D with circulating AMPs over time. We report here serial plasma levels of the major lung defensin, hBD-2, and hCAP18 mRNA expression in PBMCs. There was no difference between groups over time in hBD-2 protein, hCAP18 mRNA expression, plasma LL-37, hBD-2 or in percent change from baseline. However, the percent change from baseline in plasma free 25(OH)D concentrations was positively and significantly associated with the percent change in PBMC hCAP18 mRNA expression at both days 7 and 14 after study entry. This result lends credence to the prevailing concept that vitamin D may regulate LL-37, although these pilot data should be considered hypothesis-generating. We were also unable to detect hBD-2 mRNA in PBMCs or hBD-2 protein in BALF; this may be due to hBD-2 being primarily expressed in epithelia (24, 26), although monocytes may also express hBD-2 (17).

The AMP data in this report nonetheless complement and extend earlier data. Leaf *et al* (14) gave a single 2 μg intravenous dose of $1,25(OH)_2D$ (calcitriol) to 36 adults with severe sepsis compared to 31 subjects who received placebo injections. Blood samples were obtained for LL-37 protein levels by ELISA and whole blood leukocyte LL-37 mRNA at 6, 24 and 48 h after calcitriol. No changes in LL-37 protein occurred in this acute period of observation; in contrast there was a significant (≈ 3-fold) increase in leukocyte LL-37 mRNA expression at the 24-h time point in the calcitriol-treated group (14). In the study of high-dose vitamin $D_3$ in adult septic patients, the higher dose of 400,000 IU significantly increased LL-37 protein levels by ≈30% from baseline to day 5 (16). In that study there was no significant correlation between the change in plasma total 25(OH)D levels and the change in plasma LL-37 levels; however, there was a significant correlation between the change in calculated bioavailable 25(OH)D levels and the change in plasma LL-37 levels (16).

This pilot RCT also evaluated alveolar macrophage phagocytosis indices given that several lines of evidence link AMP expression and vitamin D status with human monocyte/macrophage phagocytosis (17, 18, 27, 56). However, surprisingly little data is available on lung macrophage phagocytosis in human critical illness (24, 44). Our group and others have previously demonstrated that alveolar macrophage function is impaired in humans with poorly controlled asthma (44, 57) and in adults with chronic obstructive lung disease prone to frequent exacerbations (58). MRNA expression of hCAP18 significantly correlated with the percentage of isolated BALF alveolar macrophages exhibiting phagocytosis. No correlation was found between plasma free 25(OH)D levels, plasma levels of hBD-2, PBMC mRNA expression of hCAP18, BALF LL-37 or BALF total 25(OH)D levels and either of the alveolar macrophage phagocytosis endpoints. Nonetheless, these hypothesis-generating data are novel and support the concept that plasma LL-37 levels may impact alveolar macrophage phagocytic function.

The major limitation of this RCT is the small number of patients enrolled, such that the overall study should be considered hypothesis-generating. Our serial endpoint measures were obtained at baseline and days 7 and 14 after entry; earlier and more frequent determination of our endpoints would provide needed insight into both vitamin D and AMP responses to our dosing regimens. As outlined in our parent RCT paper (5), we followed subjects for longer than 14 days, but the increasingly diminished subject availability over time preclude meaningful analysis of the longer-term data.

## CONCLUSIONS

We here show the first data on directly measured, non-protein bound, free 25(OH)D levels in response to high-dose vitamin $D_3$ in critically ill adult patients with respiratory failure. Additionally we relate the administration of vitamin D3 to changes in circulating anti-microbial molecules that may have an impact in critical illness infectious and inflammatory outcomes. Additionally, we find that plasma LL-37 levels may impact alveolar macrophage function. Taken together, our results suggest that additional research should focus on the impact of high-dose vitamin D regimens on clinical outcomes, coupled with changes in vitamin D availability and on anti-microbial peptide responses.

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## Abbreviations

| | |
|---|---|
| **25(OH)D** | 25-hydroxyvitamin D |
| **AMP** | Antimicrobial peptide |
| **APACHE II** | Acute Physiology and Chronic Health Evaluation II score |
| **BALF** | Bronchoalveolar lavage fluid |
| **DBP** | vitamin D binding protein |
| **hBD-2** | Human beta-defensin-2 |
| **hCAP-18** | human cationic antimicrobial protein |
| **IQR** | Inter-quartile range |
| **LL-37** | cathelicidin |
| **PBMC** | Peripheral blood mononuclear cells |

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## Highlights

- First study to determine free vitamin D [25(OH)D] level in critical illness.
- Plasma free 25(OH)D increased with vitamin $D_3$ treatment in a dose-response manner.
- Plasma free 25(OH)D levels correlated with percent change in mRNA expression of human cationic antimicrobial protein (hCAP-18).
- Plasma total 25 (OH)D did not correlate with antimicrobial peptide expression.
- Plasma cathelicidin (LL-37) levels significantly correlated with alveolar macrophage phagocytosis.

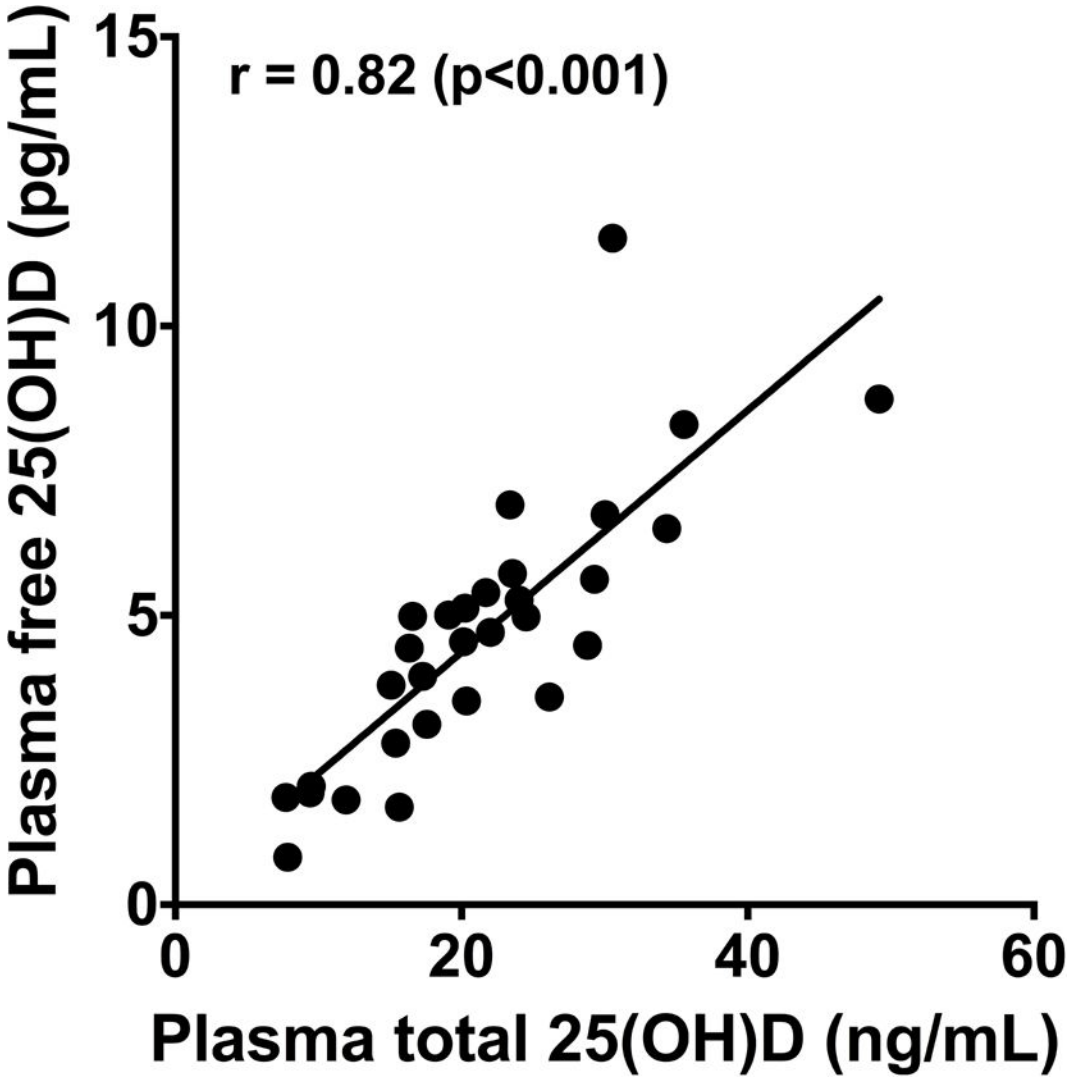


**Figure 1. Baseline Correlation between Free and Total 25-hydroxyvitamin D (25(OH)D)**

At baseline (prior to study drug administration), there was a strong positive correlation between plasma free 25(OH)D and total 25(OH)D concentrations in 30 critically ill adult patients with respiratory failure, $\rho=0.82$, $p<0.0001$. $p<0.05$ considered statistically significant.

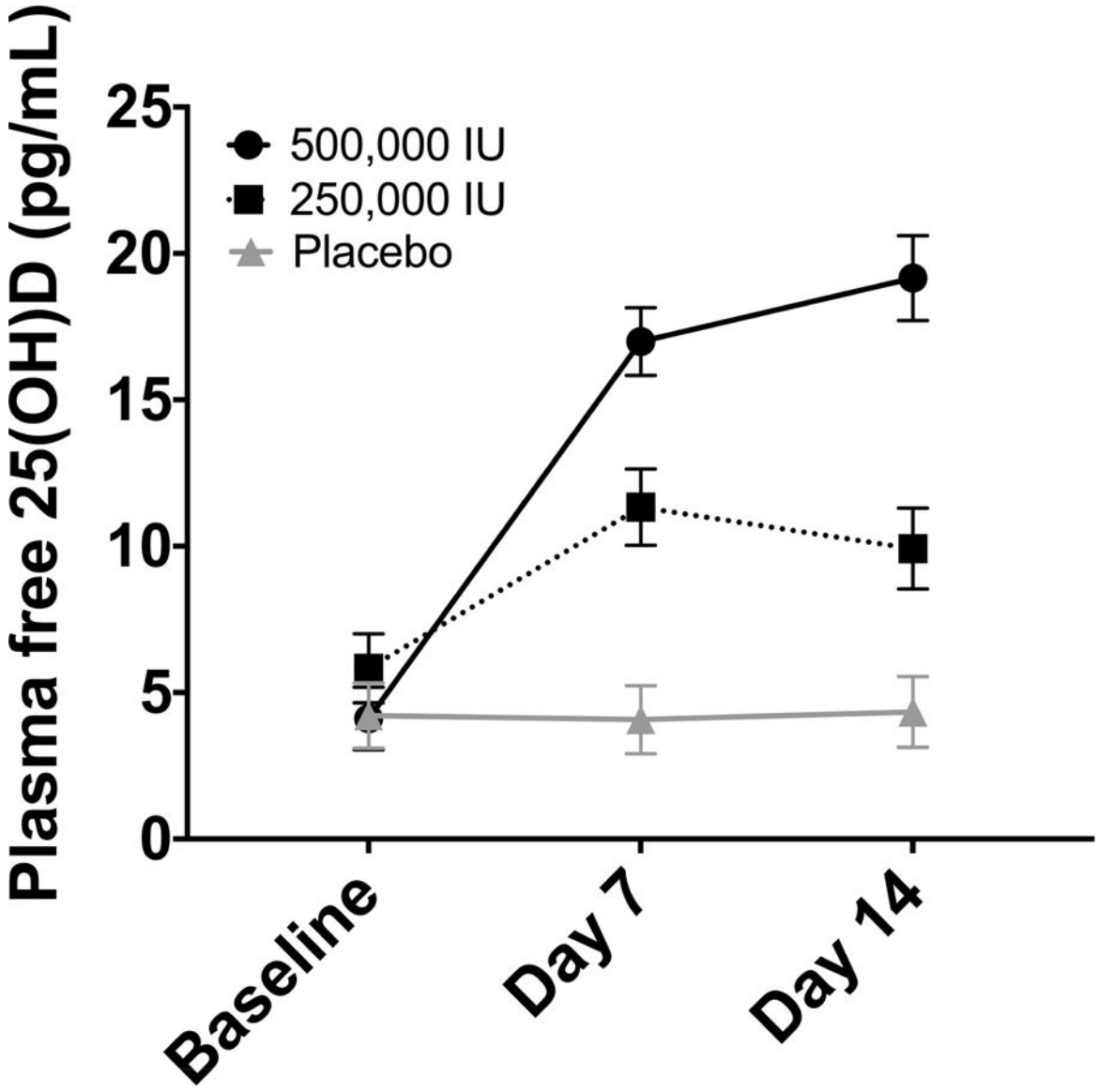


**Figure 2. Dose-Related Effect of High-Dose Vitamin $D_3$ Administration on Free 25(OH)D**

Significant effects of treatment, time and treatment × time interaction on plasma free 25(OH)D concentrations were demonstrated (each $p<0.001$). With the lower 250,000 IU vitamin $D_3$ dose, plasma free 25(OH)D levels rose approximately 2-fold from baseline by day 7 and then decreased slightly by day 14. In contrast, with the 500,000 IU dose, plasma free 25(OH)D concentrations were more than 4-fold greater than baseline values by day 7 and nearly 5-fold greater by day 14.

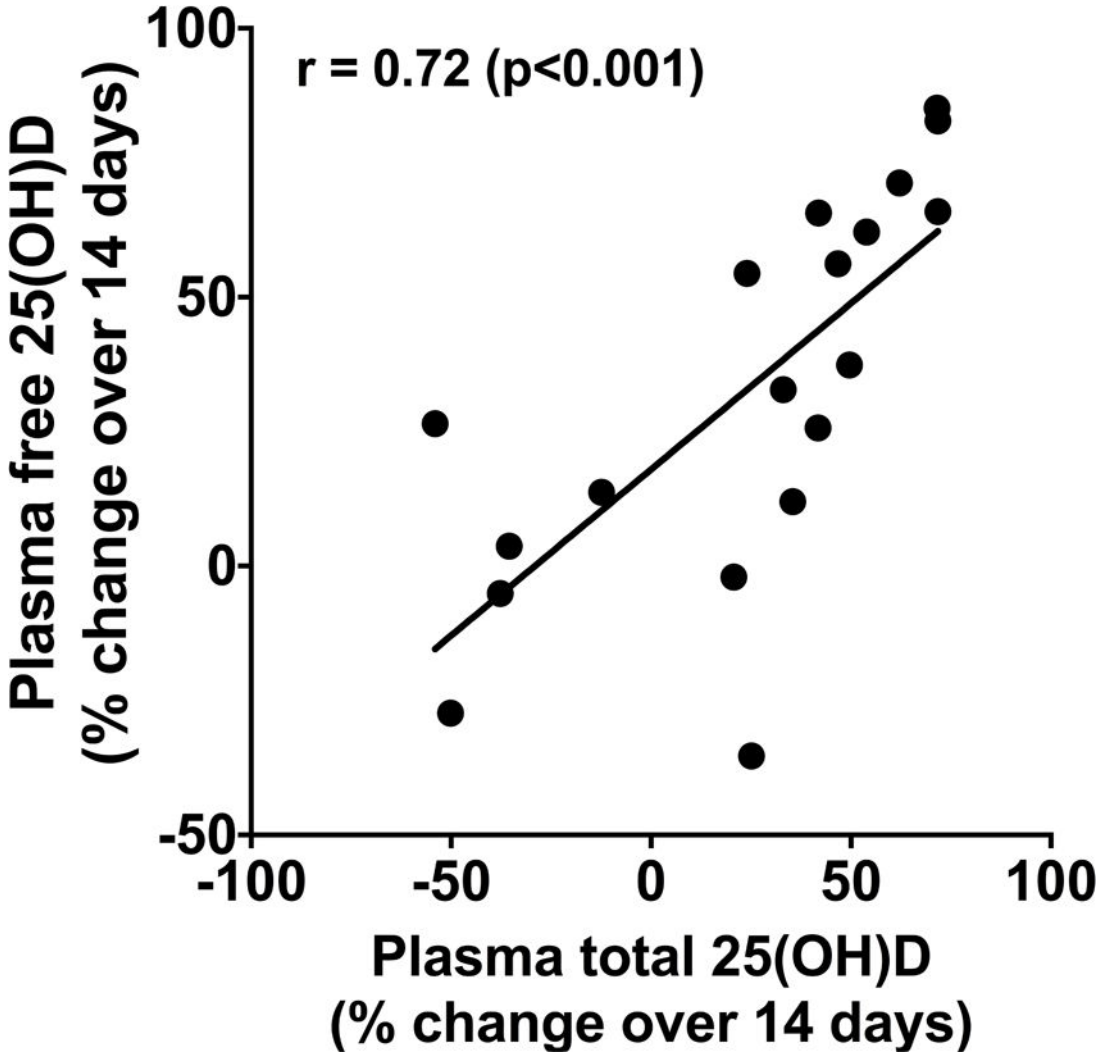


**Figure 3. Correlation of Changes in Serial Concentrations of Free and Total 25(OH) after High-dose Vitamin $D_3$ Administration**

Data were derived from 19 adults with ventilator-dependent critical illness in whom plasma was available for analysis for both vitamin D indexes at baseline and 14 days after placebo or vitamin $D_3$ administration, $\rho$=0.72, $p<0.001$.

## Table 1

Baseline Demographics, 25 (OH)D and Antimicrobial Peptide Indexes

| Variables | Placebo N=10 | Vitamin $D_3$ 250,000 IU N=9 | Vitamin $D_3$ 500,000 IU N=11 | p-value |
|---|---|---|---|---|
| Age (yr) | 64.8 ± 17.5 | 56.4 ± 15.4 | 68.1 ± 18.6 | 0.33 |
| Male | 6 (60%) | 5 (56%) | 8 (73%) | 0.72 |
| African American | 4 (40%) | 7 (78%) | 3 (27%) | 0.09 |
| Surgical ICU | 3 (30%) | 5 (56%) | 8 (73%) | 0.16 |
| BMI, ($kg/m^2$) | 28.2 ± 9.9 | 33.4 ± 6.3 | 30.2 ± 6.1 | 0.62 |
| APACHE II score at entry | 19 ± 7 | 20 ± 10 | 23 ± 9 | 0.55 |
| SOFA Day 0, mean (SD) | 8.6 (4.3) | 8.9 (3.6) | 9.1 (3.1) | 0.47 |
| Plasma total 25(OH)D, (ng/mL) | 21.5 ± 1.2 | 23.2 ± 7.8 | 20.0 ± 7.3 | 0.75 |
| Plasma free 25(OH)D, (pg/mL) | 4.2 ± 2.4 | 5.8 ± 2.8 | 4.1 ± 1.5 | 0.20 |
| Plasma hBD-2, (pg/mL) * | 2212 (669, 8520) | 1489 (348, 2762) | 2310 (708, 6953) | 0.27 |
| Plasma LL-37, (ng/mL) * | 58 (37, 97) | 46 (41,77) | 58 (37, 284) | 0.42 |
| hCAP18 mRNA ¥ | 1.3 (1.1, 1.6) | 1.2 (1.1, 1.4) | 1.1 (1.0, 1.3) | 0.44 |
| BALF LL-37, (ng/mL) † | 0.26 (0.19, 1.32) | 0.31 (0.17, 0.44) | 0.15 (0.12, 0.76) | 0.29 |
| BALF total 25(OH)D, (ng/mL) # | 14.6 ± 9.0 | 13.0 ± 7.0 | 10.4 ± 2.0 | 0.63 |

Data reported N (%) and as mean ± standard deviation (SD) for normal distributions, or median (25%–75% inter-quartile range) for non-normal distributions. P<0.05 = statistically significant. 25 (OH)D= 25-hydroxyvitamin D, APACHE II = Acute Physiology and Chronic Health Evaluation II score, BMI=Body Mass Index, BALF = bronchoalveolar lavage fluid; hBD-2 = human beta-defensin-2;ICU = intensive care units

* placebo N=8; 250,000 IU N=9; 500,000 IU N=10

¥ placebo N=9; 250,000 IU N=8; 500,000 IU N=7

† placebo N=7; 250,000 IU N=2; 500,000 IU N=7

# placebo N=7; 250,000 IU N=5; 500,000 IU N=7

## Table 2

Percent Change from Baseline in Circulating Antimicrobial Peptide Expression

| Percent (%) change from baseline to: | Placebo | 250,000 IU $D_3$ | 500,000 IU $D_3$ | p-value |
|---|---|---|---|---|
| Plasma hBD-2 Day 7 * | −47.9 (−651.4, 9.8) | 18.6 (−193, 59.9) | 3.9 (−9.7, 37.8) | 0.19 |
| hCAP18 mRNA Day 7 ¥ | −14.9 (−40.6, 8.3) | −2.2 (−14.0, 8.4) | 11.4 (−10.5, 15.7) | 0.16 |
| Plasma LL-37 Day 7 ± | −11.5 (−34.2, 30.7) | 9.13 (−21.3, 22.3) | −22.4 (−31.3, 40.5) | 0.48 |
| Plasma LL-37 Day 14 # | 0.8 (−17.9, 27.2) | −12.3 (−52.6, 25.7) | −10.8 (−33.9, 34.7) | 0.85 |

Reported as median (25%–75% inter-quartile range) analyzed using Kruskal-Wallis test. hBD-2 = human beta-defensin-2.

* Placebo N=7; 250,000 IU N=7; 500,000 IU N=9

¥ Placebo N=8; 250,000 IU N=6; 500,000 IU N=6

± Placebo N=9; 250,000 IU N=7; 500,000 IU N=9

# Placebo N=8; 250,000 IU N=6; 500,000 IU N=5

## Table 3

Correlations Between Changes in Total and Free 25(OH)D and Circulating Antimicrobial Peptides.

| | Total 25(OH)D | | Free 25(OH)D | |
|---|---|---|---|---|
| | **Day 7** | **Day 14** | **Day 7** | **Day 14** |
| Plasma hBD-2 | 0.40 (0.06) | 0.17 (0.52) | 0.32 (0.14) | −0.04 (0.88) |
| Plasma LL-37 | 0.07 (0.75) | 0.11 (0.65) | −0.10 (0.62) | −0.07 (0.76) |
| hCAP18 mRNA | 0.36 (0.14) | 0.31 (0.30) | **0.48 (0.04)** * | **0.59 (0.03)** * |

Reported as ρ (p-value) using Spearman's rank test. Percent changes from baseline to day 7 and 14, in all subjects with respective values.

*P<0.05=statistically significant. 25 (OH)D= 25-hydroxyvitamin D; AMP= antimicrobial peptide; hBD-2= human beta-defensin 2

## Table 4

Baseline Correlations Between Plasma Free and Total 25(OH)D, Antimicrobial Peptides and Alveolar Macrophage Phagocytosis

| Variable | Percent (%)of cells phagocytosis-positive | Phagocytosis Index |
|---|---|---|
| Plasma Total 25(OH)D (ng/mL), N=16 | 0.18 (0.51) | 0.23 (0.40) |
| Plasma Free 25(OH)D (pg/mL), N=16 | −0.08 (0.76) | 0.26 (0.32) |
| Plasma hBD-2 (pg/mL), N=15 | 0.37 (0.17) | 0.19 (0.49) |
| Plasma LL-37 (ng/mL), N=16 | **0.51 (0.04)** * | 0.44 (0.09) |
| hCAP18 mRNA, N=13 | −0.15 (0.63) | −0.26 (0.39) |
| BALF LL-37 (pg/mL), N=9 | 0.30 (0.43) | 0.23 (0.55) |
| BALF total 25(OH)D (ng/mL), N=10 | −0.19 (0.60) | −0.14 (0.70) |

Reported as rho (p-value) using Spearman's rank test.

* P<0.05=statistically significant. BALF = bronchoalveolar lavage fluid; N= number of subjects studied

Phagocytosis index = % of cells positive for phagocytosis × mean relative fluorescence units of S. *aureus* per cell.