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## **Vitamin D, Innate Immunity and Outcomes in Community Acquired Pneumonia**

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## **Summary at a Glance**

We investigated the associations between vitamin D status, the antimicrobial peptides cathelicidin and beta defensin-2 and outcomes in community acquired pneumonia. In hospitalised patients with community acquired pneumonia, vitamin D deficiency but not antimicrobial peptide levels were associated with increased 30-day mortality.

Vitamin D was not associated with levels of the antimicrobial peptide cathelicidin or beta defensin-2.

## **Abstract**

### *Background and objective*

Vitamin D regulates the production of the antimicrobial peptides cathelicidin and beta defensin-2, which play an important role in the innate immune response to infection. We hypothesised that vitamin D deficiency would be associated with lower levels of these peptides and worse outcomes in patients admitted to hospital with community acquired pneumonia.

### *Methods*

Associations between mortality and serum levels of 25-hydroxyvitamin D, cathelicidin, and beta defensin-2 were investigated in a prospective cohort of 112 patients admitted with community acquired pneumonia during winter.

### *Results*

Severe 25-hydroxyvitamin D deficiency (<30nmol/L) was common in this population (15%) and was associated with a higher 30-day mortality compared to patients with sufficient 25-hydroxyvitamin D(>50nmol/L) (OR 12.7, 95% CI 2.2-73.3, p=0.004). These associations were not explained by differences in age, co-morbidities, or the severity of the acute illness. Neither cathelicidin nor beta defensin-2 levels predicted mortality, although there was a trend towards increased mortality with lower cathelicidin (p=0.09). Neither cathelicidin nor beta defensin-2 levels correlated with 25-hydroxyvitamin D.

### *Conclusions*

25-hydroxyvitamin D deficiency is associated with increased mortality in patients admitted to hospital with community acquired pneumonia during winter. Contrary to our hypothesis, 25-hydroxyvitamin D levels were not associated with levels of cathelicidin or beta defensin-2.

**Key words**

Vitamin D, Innate immunity, Pneumonia, Cathelicidin, beta-Defensins

**Short Title**

Vitamin D and Immunity in CAP.

## Introduction

Vitamin D has long been known to have bactericidal, bacteriostatic and bacteriolytic properties *in vivo* and *in vitro*.<sup>1</sup> More recently, associations between low serum 25-hydroxyvitamin D levels and frequency of respiratory tract infections have been demonstrated in Finnish and American populations.<sup>2, 3</sup>

Classically thought to be primarily responsible for calcium and bone homeostasis, vitamin D is now known to regulate the transcription of more than 900 target genes.<sup>4, 5</sup>

Vitamin D response elements are present in promoter regions of the genes encoding for the antimicrobial peptides cathelicidin and beta defensin-2, suggesting that vitamin D plays a role in regulating their expression.<sup>6</sup> Antimicrobial peptides are endogenously synthesized molecules found on mucosal and epithelial surfaces of all multicellular organisms. These molecules act as the first line of defence against bacterial and viral infections, in addition to having many other immunomodulatory functions. The 2 major families of antimicrobial peptides are the defensins, of which there are 6 alpha and 2 beta subclasses, and cathelicidins, of which only 1 subtype exists in humans, the human cathelicidin antimicrobial peptide hCAP18.<sup>7</sup>

Antimicrobial peptide production by epithelial cells and leukocytes is initiated by binding of pathogen-associated molecular patterns to a subclass of pattern recognition receptors embedded in the plasma membranes of phagocytic cells, also known as Toll-like receptors.<sup>8</sup> Toll-like receptor activation in human macrophages is associated with up-regulation of the vitamin D receptor and the CYP27B1 enzyme, which acts

locally to hydrolyse the circulating form of vitamin D, 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, its physiologically active form. Binding of 1,25-dihydroxyvitamin D to the vitamin D receptor leads to increased production of the antimicrobial peptides cathelicidin and defensin beta-2.<sup>6, 9, 10</sup> *In vitro* studies have shown that low 25-hydroxyvitamin D levels are associated with reduced production of cathelicidin by stimulated human macrophages in response to toll-like receptor activation with *Mycobacterium tuberculosis* and that supplementation with 25-hydroxyvitamin D restores cathelicidin production.<sup>10</sup>

Sunlight exposure is the primary source of Vitamin D for most people.<sup>11</sup> Hence vitamin D deficiency is common during the winter months in temperate regions including New Zealand.<sup>12</sup> It is plausible that this contributes to the increased prevalence of respiratory infections such as pneumonia during winter.<sup>13</sup> In a series of small studies of patients admitted with pneumonia, serum defensin levels were 2-3 times higher than healthy controls, and normalised after completion of antibiotics.<sup>14, 15</sup> Smoking has been associated with reduced levels of beta defensin-2 in sputum and pharyngeal washings in acute pneumonia.<sup>16</sup> There is otherwise a paucity of data about vitamin D and antimicrobial peptide levels during respiratory infections and how they contribute to the severity and outcome of these conditions.

We prospectively studied the relationship between serum 25-hydroxyvitamin D and antimicrobial peptide levels in the setting of community acquired during the New Zealand winter. We hypothesized that vitamin D levels would influence the severity

and outcomes of these conditions and that this would be mediated through differences in the levels of the antimicrobial peptides cathelicidin and beta defensin-2.

## **Methods**

This was a prospective, descriptive study undertaken during the winter months of July to October, 2008, at the only acute-care hospital in Hamilton, New Zealand. All adult patients with community acquired pneumonia in this hospital are admitted to the Respiratory Medicine service. These patients were invited to participate in the study. All patients provided written informed consent. The study was approved by the Northern Y Regional Ethics Committee, New Zealand. (Ref NTY/08/05/045)

Community acquired pneumonia was diagnosed by admitting physicians according to British Thoracic Society definitions.<sup>17</sup> Clinical management followed local standards of practice and was not altered by participation in the study. Baseline demographic information including age, ethnicity, sex, smoking history and domicile status was recorded. The Charlson Index score, a validated marker of the burden of co-morbid illnesses,<sup>18,19</sup> and CURB65 score, a prognostic marker for patients admitted with pneumonia were also recorded.<sup>20</sup>

Blood samples were collected within 24 hours of admission. These were analysed for serum 25-hydroxyvitamin D levels and C Reactive Protein (CRP). Serum was stored at -70C, and transferred to a separate laboratory for cathelicidin and beta defensin-2 analysis. Patients taking vitamin D supplements were included, but not if they were

taking calcitriol (1,25-vitamin D3), which is not measured by the 25-hydroxyvitamin D assay used and likely to influence vitamin D status.

In a convenience sample of 41 patients who attended out-patient clinic follow-up, typically 6 weeks after discharge, a second blood sample was obtained and analysed for serum cathelicidin.

Serum 25-hydroxyvitamin D levels were measured using a competitive electrochemiluminescence assay kit (Elecsys, Roche Diagnostics, UK) and quantitative analysis of antimicrobial peptides in serum, was measured using the Human BD-2 ELISA Development Kit (PeproTech Inc, 900-K172, USA) and Human LL-37 ELISA Test Kit (Hycult Biotechnology, HK321, The Netherlands) according to manufacturer instructions. All ELISA assays were performed in duplicate and included internal standards used to construct standard curves for analyte concentration assessment.

Our sample size was constrained by the number of patients admitted with pneumonia during the study period. However, based on previous reports<sup>12</sup> we anticipated that 35% of patients would have severe vitamin D deficiency (<30nmol/L) during the winter months. Predicting that there would be at least 100 admissions over the study period this provided 80% power to detect a difference in 30 day mortality of 5% and 25% with sufficient and severe vitamin D deficiency respectively with a two sided alpha of 0.05. These mortality rates are similar to those found with low (0-1) and high (3-5) CURB65 scores.

Serum 25-hydroxyvitamin D, cathelicidin, and beta defensin-2 had skewed distributions and associations between the levels of these were assessed using Spearman's (non-parametric) correlations. Levels of 25-hydroxyvitamin D were categorised as severely deficient (<30nmol/L), deficient (30-49nmol/L), and sufficient ( $\geq$ 50nmol/L) according to previously published cut points.<sup>11</sup> Cathelicidin and beta defensin-2 levels were categorised by tertiles. The levels were also log-transformed to approximate normal distributions for further analyses.

The primary outcome measure for this analysis was 30 day mortality. Associations between mortality and 25-hydroxyvitamin D levels used stepwise logistic regression to adjust for potential confounding variables including age, sex, diagnosis (pneumonia or COPD), co-morbidities using the Charlson Index, the severity of the acute illness using the CURB65 score, and the systemic inflammatory response measured by CRP. The analysis started with 25-hydroxyvitamin D as a predictor of mortality and additional variables were entered to the model if they were associated with mortality at  $p < 0.05$ . The analysis was also run as a backward stepwise logistic regression starting with all the variables and removing those not significant at  $p < 0.05$ . These analyses were repeated for cathelicidin, and beta defensin-2. Statistical analyses were done using STATA 10 (Stata Corporation, College Station, Texas

## **Results**

128 eligible patients were assessed during the study period and 112 patients were included (Figure 1). A scatter plot of serum 25-hydroxyvitamin D, cathelicidin and beta defensin-2 levels are shown in Figure 2. 25-hydroxyvitamin D levels were

available for all included patients. After routine tests were done, 12 and 9 patients had insufficient serum for beta defensin-2 and cathelicidin analyses respectively.

Characteristics of the study population are shown in table 1. The 18 patients who were taking vitamin D supplements had higher 25-hydroxyvitamin D levels than those who were not (median values 85 nmol/L and 51 nmol/L respectively,  $p=0.002$  by Wilcoxon rank-sum test). Overall, 44% of patients were deficient in 25-hydroxyvitamin D with levels  $<50$ nmol/L, and 15% were severely deficient with levels  $<30$ nmol/L. Vitamin D levels tended to be lower among patients admitted earlier in the study (during July compared to October).

25-hydroxyvitamin D levels were not correlated with either serum cathelicidin ( $\rho=0.002$ ,  $p=0.99$ ) or beta defensin-2 ( $\rho=-0.05$ ,  $p=0.65$ ). Nor were cathelicidin levels correlated with beta defensin-2 levels ( $\rho=0.02$ ,  $p=0.82$ ).

Patients with severe 25-hydroxyvitamin D deficiency ( $<30$ nmol/L) had higher 30 day mortality than patients with mildly deficient (30-49nmol/L) or sufficient ( $\geq 50$ nmol/L) 25-hydroxyvitamin D levels (Figure 3). Forward and backward stepwise logistic regression models provided the same results. Of the potential covariates, only CURB65 scores were significantly associated with mortality and the association between low 25-hydroxyvitamin D and 30 day mortality persisted after adjusting for these (Table 2). Adjusting for the month of admission made no material difference to the results.

Crude 30-day mortality defined by categories of 25-hydroxyvitamin D, cathelicidin, CURB65 and beta defensin-2 are presented in Figure 3. Neither serum cathelicidin nor beta defensin-2 levels were significantly associated with 30 day mortality, although there was a trend to higher mortality in the lowest tertile of serum cathelicidin ( $p=0.08$ ) (Figure 3) This lack of association remained after adjustment for covariates in the logistic regression models, in which CURB65 score was the only significant predictor of mortality (Table 3-4).

Serum cathelicidin levels in 41 patients who provided blood samples at their clinical follow-up were not significantly different to those obtained on admission (median level on admission and follow-up = 83.1 ng/ml and 87.3 ng/ml respectively,  $p=0.52$  by paired t test).

## **Discussion**

In this prospective cohort of patients admitted with community acquired pneumonia, severe 25-hydroxyvitamin D deficiency was associated with higher mortality within the first 30 days after admission. These associations were independent of patient age, sex, co-morbidities, the systemic inflammatory response, and other prognostic factors including CURB65 scores.

The relationship between 25-hydroxyvitamin D concentrations and mortality was not linear. An increased risk of mortality was only apparent in those with severe deficiency (Figure 3). Further exploration of the data by categorising patients by

deciles of 25-hydroxyvitamin D levels confirmed that the threshold for increased mortality was around the 30nmol/L cut off for severe deficiency. To our knowledge this is the first prospective study to explore the association between 25-hydroxyvitamin D, antimicrobial peptide levels and the severity or prognosis of community acquired pneumonia in an adult population. In children with acute lower respiratory tract infection, low 25-hydroxyvitamin D levels have been associated with admission to intensive care.<sup>21</sup> In our cohort, 3 of the 4 patients who were admitted to intensive care had severe 25-hydroxyvitamin D deficiency ( $p=0.011$  by Fisher's exact test).

We did not find any significant correlations between serum 25-hydroxyvitamin D, cathelicidin and beta defensin-2 levels. Hence our hypothesis that 25-hydroxyvitamin D would influence the outcome of community acquired pneumonia through differences in antimicrobial peptide levels was not supported. For cathelicidin, this may be because we measured the active peptide component LL-37 of cathelicidin and not the prepropeptide, which must be proteolytically cleaved to generate the mature active LL-37 peptide. There may also be many other factors that influence antimicrobial peptide concentrations. For example, the uptake of 25-hydroxyvitamin D into target cells and its subsequent 1-alpha-hydroxylation may influence antimicrobial peptide production to a greater extent than serum 25-hydroxyvitamin D levels. In epithelial membranes and other extrarenal sites, 1-alpha hydroxylase activity is regulated locally by toll-like receptor activation, cytokines, growth factors and remains incompletely understood.<sup>22</sup> Serum levels of immature or mature antimicrobial peptides may not correlate with airway concentrations, although

previous studies have shown positive correlations between plasma and bronchoalveolar lavage fluid defensin levels.<sup>14, 15</sup> It is also possible that serum 1,25-hydroxyvitamin D concentration is a stronger determinant of cathelicidin production than 25-hydroxyvitamin D, since it is the main active metabolite of vitamin D and has previously been shown to correlate with serum cathelicidin levels.<sup>23</sup> However, measurement of 25-hydroxyvitamin D is regarded as the most accurate method of determining vitamin D status because it has a serum half-life of weeks compared to the half-life of 1,25-dihydroxyvitamin D, which is less than 4 hours. Moreover, 25-hydroxyvitamin D deficiency results in a compensatory increase in parathyroid hormone secretion, which increases renal 1-alpha hydroxylase activity, maintaining 1,25-dihydroxyvitamin D levels in the normal or even elevated range.<sup>11</sup>

Lower values of serum cathelicidin showed a non-significant trend to an association with higher 30 day mortality. This appears to be consistent with research showing that cathelicidin supplementation is protective in murine models of sepsis.<sup>24</sup> In addition, lower serum cathelicidin levels predict increased mortality due to infections over the following year in patients undergoing haemodialysis.<sup>23</sup> An unexpected finding was that serum cathelicidin levels did not fall after recovery from the acute admission. This contrasts with previous studies of antimicrobial peptide levels in pneumonia, which showed trends to higher defensin levels during the acute phase of illness and a fall after completion of therapy, although these were small studies with sample sizes of less than 20.<sup>14, 15</sup> In our cohort, there was a strong correlation between cathelicidin levels on admission and at follow up (Spearman's rho = 0.59, p<0.001) and it seems

likely that cathelicidin levels are determined by factors other than acute respiratory infection.

Serum beta defensin-2 did not predict 30 day mortality in this cohort. This may be because serum levels of beta defensin-2 may not reflect local concentrations, as it is mainly produced by epithelial leukocytes. In contrast, cathelicidin is produced by circulating neutrophils and myeloid cells in the bone marrow as well as epithelial cells. Therefore serum cathelicidin may be a better systemic marker of innate immunity than beta defensin-2.<sup>7</sup>

As an observational study we cannot establish causal associations between 25-hydroxyvitamin D deficiency and mortality in these patients. It is possible that these are simply serum markers of frailty and poor prognosis. Vitamin D deficiency may also reflect poor underlying nutritional status.<sup>25</sup> However, few foods in nature are rich in vitamin D, and for most people, 90 percent of their vitamin D requirements are met by the conversion of cholesterol precursors in the skin to vitamin D<sub>3</sub>, by solar UV-B radiation.<sup>11</sup> 25-hydroxyvitamin D was not associated with the Charlson co-morbidity index or other indicators of frailty such as age or whether the patients lived in residential care. These covariates did not predict 30 day mortality in this study and do not explain the associations between low 25-hydroxyvitamin D levels and 30 day mortality.

A further limitation of this study is the relatively small sample size. This limits the complexity of the analyses and the extent to which we can adjust for multiple potential confounding factors at the same time. However, of the covariates tested, only CURB65 scores contributed to mortality and adjusting for this did not materially alter the associations between 25-hydroxyvitamin D deficiency and mortality. Adjusting for each of the other risk factors individually also made little difference to the findings: severe 25-hydroxyvitamin D deficiency remained a significant predictor of mortality.

This was a hospital based cohort and although participation in the study was high, we cannot generalise these findings to patients treated in the community, nor can we determine the role that vitamin D may have on primary prevention of infection. Nevertheless, the findings support our hypothesis that 25-hydroxyvitamin D levels may influence the severity and outcomes of community acquired pneumonia, although we did not find the expected association between 25-hydroxyvitamin D and these peptides. There was also a trend towards increased 30 day mortality in patients with lower cathelicidin levels, although this did not reach statistical significance, possibly due to the small number of deaths. These observations raise the possibility that vitamin D supplements and cathelicidin could have a therapeutic role in acute infections. The role of vitamin D supplements in the primary prevention of these diseases also needs to be explored. In the meantime, 25-hydroxyvitamin D deficiency and serum cathelicidin levels may prove to be useful prognostic indicators in severe infections.

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**Table 1. Demographic and Baseline Clinical Characteristics**

Variable	Community Acquired pneumonia n = 112
Age - median yrs(range)	76 (16 – 97)
Ethnicity -- European	88
Maori	22
Other	2
Current smoker	18 (17%)
Ex Smoker	51 (48%)
Never smoked	37 (35%)
Charlson Comorbidity Index	
Score 0-1	57 (51%)
Score 2-3	38 (34%)
Score 4-7	17 (15%)
CURB65 Score 0-1	32 (29%)
CURB65 Score 2	32 (29%)
CURB65 Score 3-5	48 (43%)
Domicile Status	
Living independently	59 (53%)
Requiring assistance	33 (30%)
Residential Care	19 (17%)
25-hydroxyvitamin D median (range) nmol/L	54 (10, 140)
Beta defensin-2 median (range) pg/mL	262 (14, 1734)
Cathelicidin median (range) ng/mL	69 (13, 263)
CRP median (range) mg/mL	170 (2, 550)

**Table 2. Association between low serum 25-hydroxyvitamin D and 30 day mortality (n=112)**

	OR	95% CI	<i>P</i>
Severe 25-hydroxyvitamin D deficiency	13.5	2.6 to 69.1	0.002
CURB65 score	2.23	1.13 to 4.40	0.021

Analyses by stepwise logistic regression (entry method) using 30 day mortality as the dependant variable and 25-hydroxyvitamin D deficiency as the main predictor. Sex, age, CURB65 score, Charlson Index, CRP level, and living in residential care were entered into the model if they were significantly associated with mortality at  $p < 0.05$ . Odds Ratios (OR) represent the odds of death in the 30 days following admission associated with low vitamin D status, and for each increment in the CURB65 score. 17 subjects had low vitamin D (<30nmol/L). Subjects with higher vitamin D values >30nmol/L are the reference category (n=95)

**Table 3. Association between cathelicidin and 30 day mortality (n=103)**

	OR	95% CI	<i>P</i>
Cathelicidin	0.35	0.07 to 1.60	0.173
CURB65 score	2.11	1.06 to 4.19	0.031

Analyses by stepwise logistic regression (entry method) using 30 day mortality as the dependant variable and (log-)cathelicidin as the main predictor. Sex, age, admission diagnosis (COPD or pneumonia), CURB65 score, Charlson Index, CRP levels, and living in residential care were entered into the model if they were significantly associated with mortality at  $p < 0.05$ . Odds Ratios (OR) represent the odds of mortality at 30 days associated with each log increment of cathelicidin levels and for each increment in the CURB65 score.

**Table 4. Association between beta defensin-2 and 30 day mortality (n=100)**

	OR	95% CI	<i>P</i>
Beta defensin-2	1.76	0.63 to 4.93	0.279
CURB65 score	2.08	1.07 to 4.04	0.030

Analyses by stepwise logistic regression (entry method) using 30 day mortality as the dependant variable and (log-)beta defensin-2 as the main predictor. Sex, age, admission diagnosis (COPD or pneumonia), CURB65 score, Charlson Index, CRP levels, and living in residential care were entered into the model if they were significantly associated with mortality at  $p < 0.1$ . Odds Ratios (OR) represent the odds of mortality at 30 days associated with each log increment of beta defensin-2 levels and for each increment in the CURB65 score.

## Figure Legends

**Figure 1** Patient enrolment and completion of study

**Figure 2** Scatter plot of serum 25-hydroxyvitamin D, cathelicidin and beta defensin 2 levels.

**Figure 3** Unadjusted 30 day mortality by categories of 25-hydroxyvitamin D, cathelicidin, CURB-65 score and beta defensin-2. (Numbers above bars indicate deaths in each group. P values are for trend.)

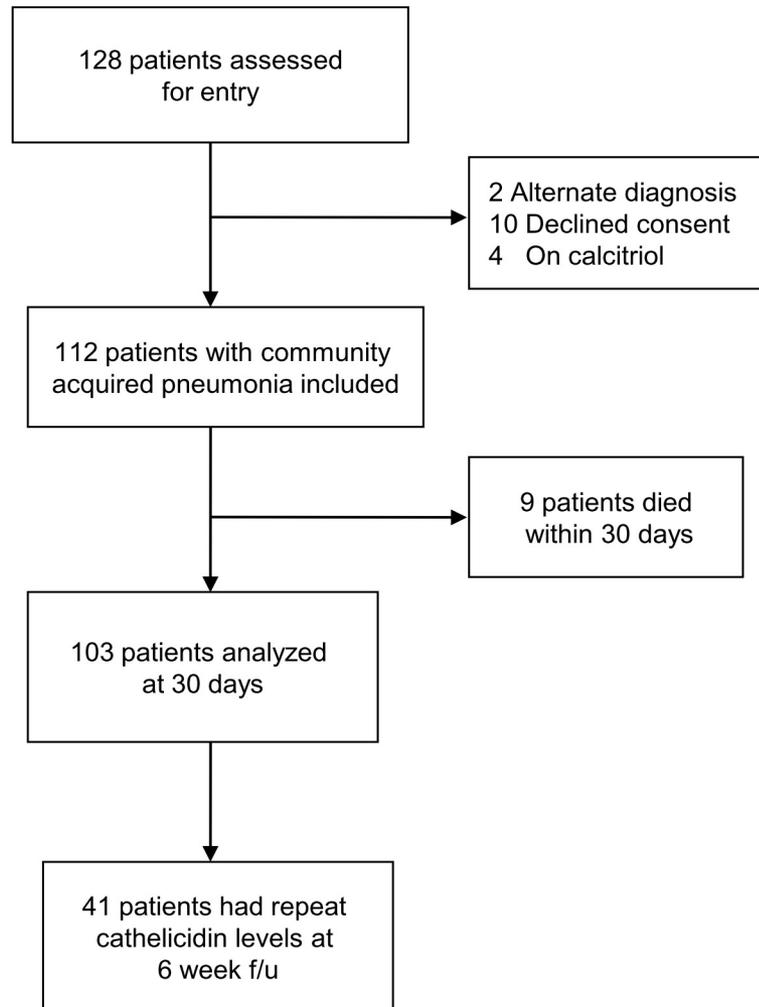


Figure 1

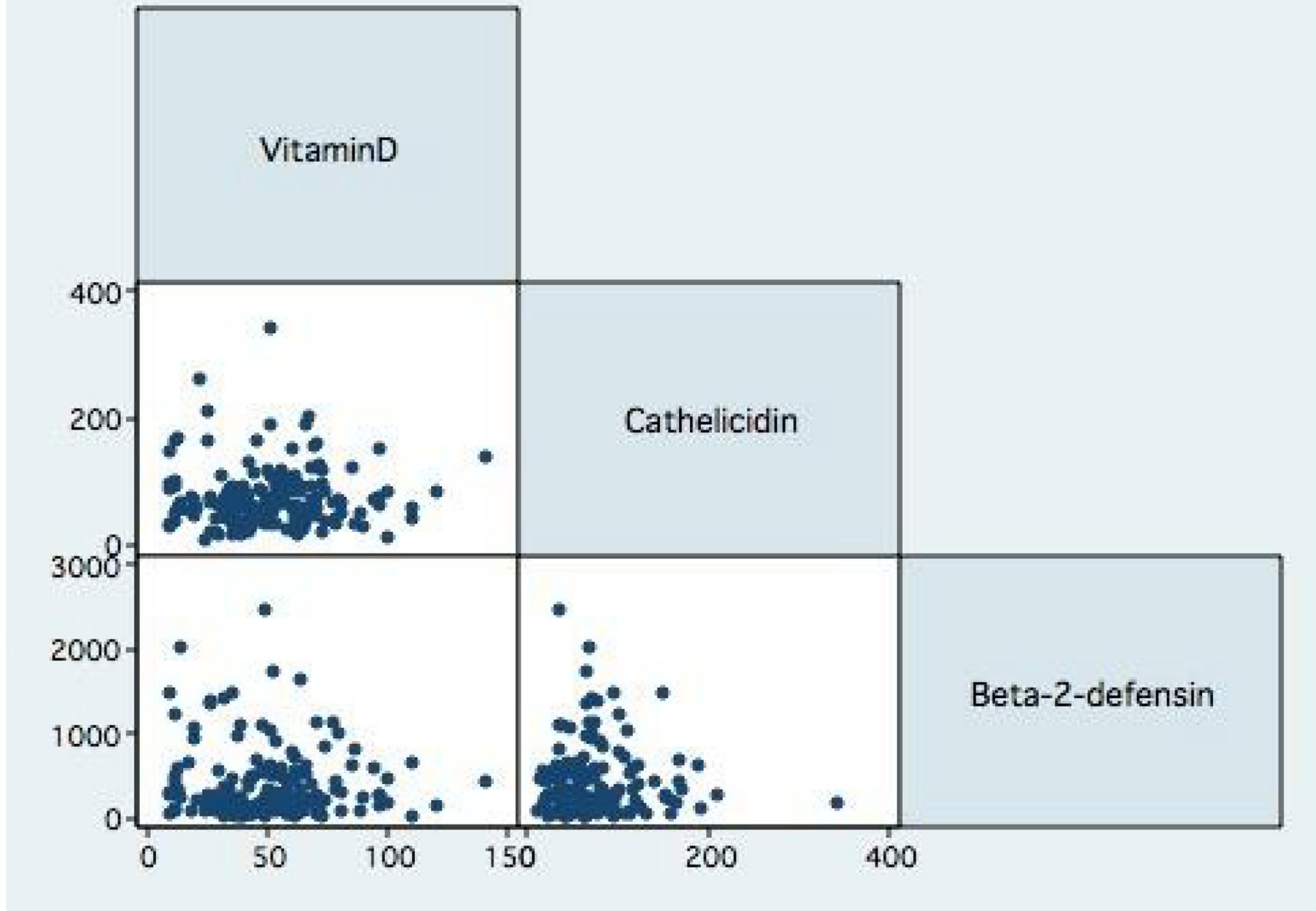


Figure 2

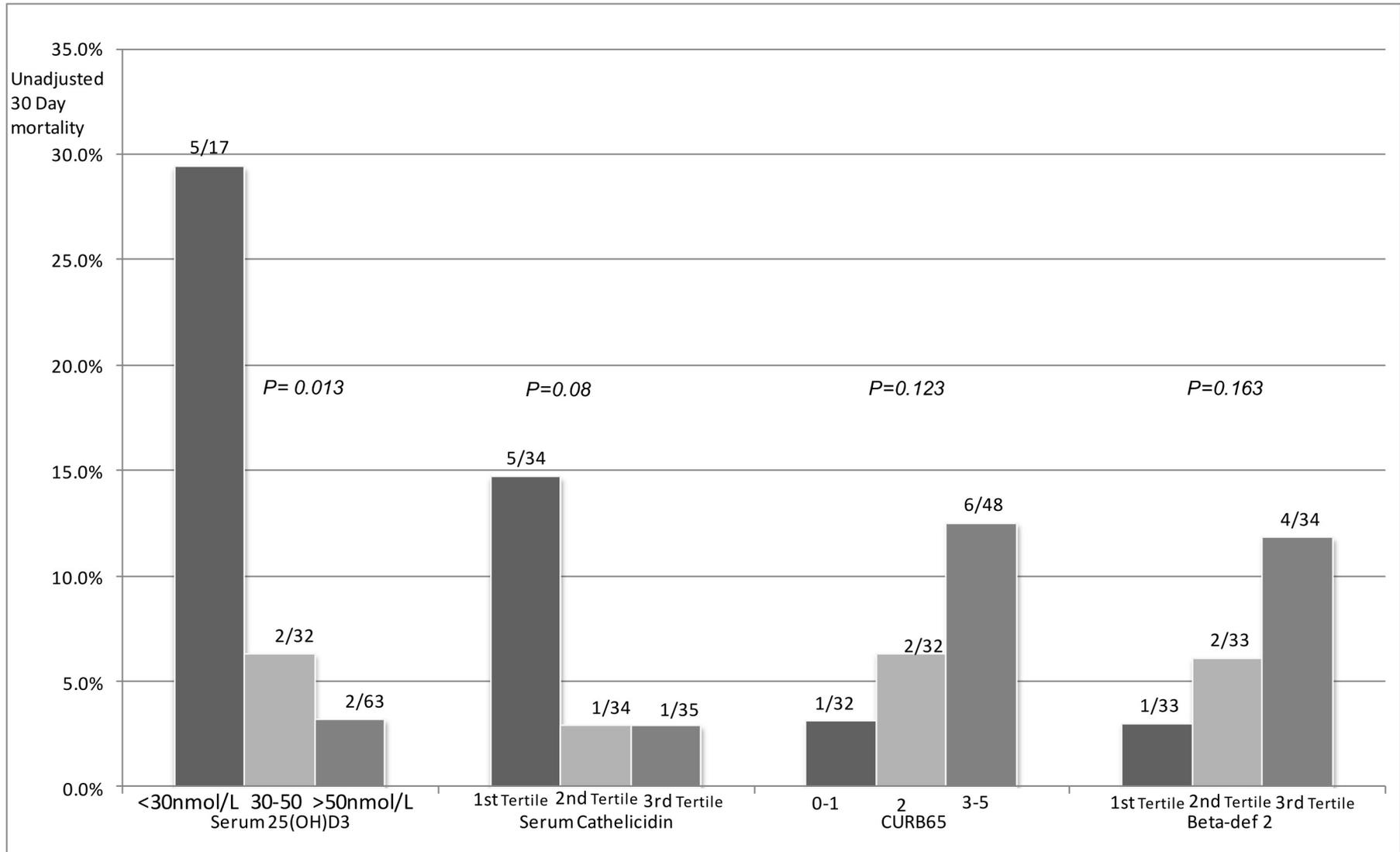


Figure 3