PLOS | ONE

RESEARCH ARTICLE

Vitamin D Metabolites and Their Association with Calcium, Phosphorus, and PTH Concentrations, Severity of Illness, and Mortality in Hospitalized Equine Neonates

Ahmed M. Kamr[1,4], Katarzyna A. Dembek[1], Stephen M. Reed[2], Nathan M. Slovis[3], Ahmed A. Zaghawa[4], Thomas J. Rosol[1], Ramiro E. Toribio[1]*

1 College of Veterinary Medicine, The Ohio State University, Columbus, Ohio, United States of America, 2 Rood and Riddle Equine Hospital, Lexington, Kentucky, United States of America, 3 Hagyard Equine Medical Institute, Lexington, Kentucky, United States of America, 4 Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt

* toribio.1@osu.edu

🔓 OPEN ACCESS

Abstract

Background

Hypocalcemia is a frequent abnormality that has been associated with disease severity and outcome in hospitalized foals. However, the pathogenesis of equine neonatal hypocalcemia is poorly understood. Hypovitaminosis D in critically ill people has been linked to hypocalcemia and mortality; however, information on vitamin D metabolites and their association with clinical findings and outcome in critically ill foals is lacking. The goal of this study was to determine the prevalence of vitamin D deficiency (hypovitaminosis D) and its association with serum calcium, phosphorus, and parathyroid hormone (PTH) concentrations, disease severity, and mortality in hospitalized newborn foals.

Methods and Results

One hundred newborn foals $\leq$72 hours old divided into hospitalized (n = 83; 59 septic, 24 sick non-septic [SNS]) and healthy (n = 17) groups were included. Blood samples were collected on admission to measure serum 25-hydroxyvitamin D_3 [25(OH)D_3], 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$], and PTH concentrations. Data were analyzed by nonparametric methods and univariate logistic regression. The prevalence of hypovitaminosis D [defined as 25(OH)D_3 <9.51 ng/mL] was 63% for hospitalized, 64% for septic, and 63% for SNS foals. Serum 25(OH)D_3 and 1,25(OH)$_2D_3$ concentrations were significantly lower in septic and SNS compared to healthy foals (P<0.0001; P = 0.037). Septic foals had significantly lower calcium and higher phosphorus and PTH concentrations than healthy and SNS foals (P<0.05). In hospitalized and septic foals, low 1,25(OH)$_2D_3$ concentrations were associated with increased PTH but not with calcium or phosphorus concentrations. Septic foals with 25(OH)D_3 <9.51 ng/mL and 1,25(OH)$_2D_3$ <7.09 pmol/L were more likely to die (OR=3.62; 95% CI = 1.1-12.40; OR = 5.41; 95% CI = 1.19-24.52, respectively).

Conclusions

Low 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ concentrations are associated with disease severity and mortality in hospitalized foals. Vitamin D deficiency may contribute to a pro-inflammatory state in equine perinatal diseases. Hypocalcemia and hyperphosphatemia together with decreased 1,25(OH)$_2$D$_3$ but increased PTH concentrations in septic foals indicates that PTH resistance may be associated with the development of these abnormalities.

Introduction

Perinatal infections, sepsis in particular, are the main cause of mortality in newborn foals, representing major economic losses due to fatality and hospitalization costs. Critically ill foals often present to veterinary hospitals with fluid, acid-base, and electrolyte disturbances, evidence of systemic inflammation, organ dysfunction, as well as endocrine dysregulation [1]. Hypocalcemia is a common finding in foals with sepsis and horses with enterocolitis, colic, and endotoxemia [2–4]. A number of adult horses with hypocalcemia have parathyroid gland dysfunction, which is characterized by abnormally low concentrations of parathyroid hormone (PTH) [4, 5]. However, the mechanisms leading to low calcium concentrations in hospitalized foals remain unclear. We have shown that most foals with hypocalcemia have increased PTH concentrations indicating that other mechanisms may be involved [3, 6].

Disorders of phosphorus are poorly documented in sick foals. In critically ill children and adult human patients hypophosphatemia is more frequent than hyperphosphatemia [7]. Of interest, a previous study by our group showed that serum phosphorus concentrations were higher in septic compared to healthy foals [3]. It remains to be elucidated whether pathologic processes afflicting critically ill equine neonates (e.g. sepsis, endotoxemia, acidosis, cell injury, endocrine disturbances) alter phosphate dynamics, leading to hyperphosphatemia.

Vitamin D$_3$ is produced in the skin by photolytic cleavage of 7-dehydrocholesterol, which is transported by vitamin D-binding protein (DBP) to the liver where it is hydroxylated to 25(OH)D$_3$ by 25α-hydroxylase [8, 9]. Subsequently, 25(OH)D$_3$ is carried to the kidney by DBP to be converted by 1α-hydroxylase to 1,25(OH)$_2$D$_3$ (calcitriol), the active metabolite of vitamin D$_3$ [8, 9]. Because 25(OH)D$_3$ has a much longer half-life (days) than 1,25(OH)$_2$D$_3$ (hours) and it is poorly regulated by endocrine factors, serum concentrations of 25(OH)D$_3$ are considered the best measure of the vitamin D status [10, 11]. Through the vitamin D receptor, 1,25(OH)$_2$D$_3$ promotes intestinal absorption and renal reabsorption of calcium and phosphorus, bone remodeling, and suppression of parathyroid gland function and PTH secretion [12, 13]. There is also evidence that 1,25(OH)$_2$D$_3$ has roles other than calcium and phosphorus homeostasis, including anti-inflammatory, anti-proliferative and immunomodulatory properties [14]. In addition to the kidney, multiple tissues and cell-types have 1α-hydroxylase activity and the local synthesis of 1,25(OH)$_2$D$_3$ may be responsible for many of the pleiotropic and non-classical functions of vitamin D [15]. Low 1,25(OH)$_2$D$_3$ concentrations could potentially exacerbate a pro-inflammatory state.

An editorial in the New England Journal of Medicine highlights the importance of hypovitaminosis D in the pathogenesis of hypocalcemia in critically ill human patients [16]. The same group found that hypovitaminosis D was highly prevalent in ICUs and associated with adverse outcome [16, 17]. Recent studies have further confirmed that hypovitaminosis D is a common finding during critical illness and that reduced vitamin D levels (both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$) are linked to mortality [16–20]. In critically ill children, the vitamin D status has

been defined as sufficient, insufficient, and deficient if $25(OH)D_3$ concentrations are >20 ng/ml, 10–20 ng/ml, and < 10 ng/ml, respectively [21]. Similar $25(OH)D_3$ ranges are used for adult humans [16]. Under this criteria, up to 60% of critically ill children and over 70% of adult human patients have been found to be vitamin D insufficient [16–22].

Mechanisms for low $25(OH)D_3$ levels include reduced hepatic synthesis, decreased DBP concentrations, and epithelial waste, while a drop in $1,25(OH)_2D_3$ concentrations could be explained by insufficient renal 1α-hydroxylation of $25(OH)D_3$, PTH insensitivity, and low DBP [18, 23]. PTH concentrations are often elevated in septic humans with low $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations [17, 18].

We recently showed that healthy foals have lower $25(OH)D_3$ concentrations compared to horses [9]. However, information on the clinical relevance of vitamin D metabolites and their association with serum calcium, phosphorus, and PTH concentrations, disease severity and outcome in critically ill equine neonates has not been investigated.

The aims of the present study were to measure the blood concentrations of vitamin D metabolites, determine the prevalence of vitamin D deficiency (hypovitaminosis D), and to assess their association with serum calcium, phosphorus, and PTH concentrations, severity of disease, and likelihood of mortality in hospitalized newborn foals. We hypothesized that hypovitaminosis D, hypocalcemia, hyperphosphatemia, and increased PTH concentrations will be frequent in critically ill foals. We also proposed that in hospitalized foals, concentrations of vitamin D metabolites will be inversely associated with severity of illness and odds of mortality.

Materials and Methods

Animals and Inclusion Criteria

A total of 100 neonatal foals of ≤ 72 hours old of any breed and sex evaluated at three equine hospitals (The Ohio State University, Columbus, Ohio; Hagyard Equine Medicine Institute, Lexington, Kentucky; Rood and Riddle Equine Hospital, Lexington, Kentucky) were included in the study. Foals were divided into hospitalized and healthy groups. Hospitalized foals were those admitted with a history of disease and were further classified as septic and SNS based on their sepsis scores [24]. Septic foals were those with a positive blood culture or a sepsis score ≥ 12. SNS foals were those presented for illnesses other than sepsis such as retained meconium, orthopedic conditions and failure of transfer of passive immunity, a negative blood culture and a sepsis score ≤ 11. Healthy foals were assessed at the farms as a part of the routine newborn physical evaluation. Foals with normal physical examination, complete blood count (CBC), serum biochemistry profile, serum immunoglobulin G (IgG) > 800 mg/dL and sepsis score < 4 were considered healthy. Foals discharged from the hospital were classified as survivors. Foals that died or were euthanized due to a grave medical prognosis were defined as non-survivors. Foals euthanized for non-medical reasons were not included in this study.

The study was approved by the Institutional Animal Care and Use Committee of The Ohio State University (Protocol # 2008A0170-R1) and adhered to the principles of humane treatment of animals in veterinary research, as stated by the American College of Veterinary Internal Medicine and the National Institutes of Health Guidelines.

Clinical information

Clinical history obtained on admission included duration of pregnancy, expected foaling date, assisted parturition, dystocia, passing and appearance of fetal membranes, maternal illnesses, and medications (mare and foal). Foal clinical data such as physical examination, CBC, serum biochemistry, and IgG concentrations were included. The sepsis score was calculated for each foal based on clinical history, physical examination, and laboratory findings [24].

Sampling

Blood samples were collected into serum clot tubes from hospitalized foals on admission and from healthy foals during their routine physical examination. Blood was allowed to clot at room temperature for 1 hour, centrifuged at 2,000 × g for 10 minutes at 4°C, serum was aliquoted in smaller volumes, and stored at -80°C until analysis.

Hormone, total calcium and phosphorus measurements

Serum $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations were measured using human-specific enzyme immunoassays (Immunodiagnostic Systems, Gaithersburg, MD, USA) after extraction steps as described by our group [9]. The $25(OH)D_3$ assay has a detection limit of 2 ng/mL, a working range of 2.6–153.6 ng/mL, and inter and intra-assay coefficients of variation for equine samples of < 10% [9]; while the $1,25(OH)_2D_3$ assay has a detection limit of 4 pmol/L, a working range of 4.0–500 pmol/L, and inter and intra-assay coefficients of variation of < 10%. Serum total intact PTH concentrations were measured using a human-specific immunoradiometric assay (Scantibodies Laboratory, Santee, CA, USA) with a detection limit of 0.1 pmol/L, and inter and intra-assay coefficients of variation of 4.7% and 2.5%, respectively [4]. Serum total calcium, phosphorus, total protein and albumin concentrations were measured with the Roche Cobas C501 chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Definition of hypovitaminosis D

For the study reported here, hypovitaminosis D (vitamin D deficiency) was defined as serum $25(OH)D_3$ concentrations below the lowest limit of the 95% confidence interval (CI) from the healthy foals.

Statistical Analyses

Data were assessed for normality using Shapiro-Wilk statistic and D'Agostino-Pearson omnibus normality test and were not normally distributed. Descriptive statistics were calculated and expressed as median and 95% CI. Age is reported as median and range. Comparisons between two groups (e.g. non-survivors and survivors) were carried out by the Mann-Whitney U test. Comparisons between three different groups were performed by Kruskal-Wallis non-parametric ANOVA and Dunn's post-hoc test was used to assess differences between groups. Correlations between variables were determined using Spearman's rank coefficient (r_s). Serum $25(OH)D_3$, $1,25(OH)_2D_3$, PTH, calcium and phosphorous concentrations were categorized based on the 95% CI values from healthy foals into hypovitaminosis D, normovitaminosis D, hypervitaminosis D, hypocalcemia, normocalcemia, hypercalcemia, hypophosphatemia, normophosphatemia, and hyperphosphatemia, and analyzed using univariate logistic regression. Crude odds ratios (OR) for non-survival were calculated. The dependent variable was non-survival. The Hosmer-Lemeshow goodness of fit test indicated that the data fitted the model (P = 0.99). Fisher's exact test was carried out to test the significance of proportions of hypovitaminosis D, hypocalcemia, and hyperphosphatemia within the population of hospitalized, septic and SNS foals. Data were analyzed with IBM SPSS Statistics 22.0 (IBM Corporation, Armonk, NY, USA) and SigmaStat 3.5 (Systat Software, Inc., San Jose, CA, USA). Box plots were created using Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Significance was set at P < 0.05.

Results

Study population

In the hospitalized foal population, 71% (59/83) were classified as septic and 29% (24/83) as SNS. The median sepsis score for all hospitalized foals was 12, for septic foals 13, and for SNS

Table 1. Percent distribution of serum 25(OH)D$_3$, total calcium, and phosphorus concentrations in hospitalized, septic, and SNS foals, based on 95% CI values from healthy foals.

Variables	Hospitalized foals	Septic foals	SNS foals
Hypervitaminosis D	4% (3/83)	2% (1/59)	8% (2/24)
Normovitaminosis D	33% (27/83)*	34% (20/59)*	29% (7/24)
Hypovitaminosis D	63% (53/83)**	64% (38/59)**	63% (15/24)**
Hypercalcemia	18% (15/83)	14% (8/59)	29% (7/24)
Normocalcemia	43% (36/83)**	37% (22/59)**	58% (14/24)**
Hypocalcemia	39% (32/83)**	49% (29/59)**	13% (3/24)
Hyperphosphatemia	48% (40/83)**	58% (34/59)**	25% (6/24)
Normophosphatemia	27% (22/83)	25% (15/59)	29% (7/24)
Hypophosphatemia	25% (21/83)	17% (10/59)	46% (11/24)

*P < 0.05

**P < 0.01; SNS—sick non-septic foals

For vitamin D, * = statistically different from hypervitaminosis D; ** = statistically different from normovitaminosis D and hypervitaminosis D in all foal groups.

For calcium, ** = statistically different from hypercalcemia in hospitalized and septic foals, and statistically different from hypocalcemia in SNS foals.

For phosphorus, ** = statistically different from normophosphatemia and hypophosphatemia.

doi:10.1371/journal.pone.0127684.t001

foals it was 6. The median admission age was 13 hours (1–72 hours) for hospitalized, 10 hours (1–72 hours) for septic, and 13 hours (1–72 hours) for SNS. Healthy foals (n = 17) were 24 hours (14–48 hours) old. Age was not significantly different between foal groups (P = 0.07). In hospitalized foals, 64% (53/83) were survivors, in septic foals, 54% (32/59) were survivors, while in SNS foals, 87.5% (21/24) were survivors.

Prevalence of hypovitaminosis D, hypocalcemia, and hyperphosphatemia in hospitalized, septic and SNS foals

Prevalences of hypovitaminosis D, hypocalcemia, and hyperphosphatemia per foal group are listed in **Table 1**. Hypovitaminosis D (25(OH)D$_3$ < 9.51 ng/mL) was more prevalent than normovitaminosis and hypervitaminosis D in hospitalized, septic, and SNS foals (P < 0.01). Hypocalcemia was more frequent than hypercalcemia in hospitalized and septic foals (P < 0.01), but less common than normocalcemia in SNS foals (P < 0.01). In hospitalized and septic foals, hyperphosphatemia was more prevalent than normophosphatemia and hypophosphatemia (P < 0.01), while hypophosphatemia was more frequent in SNS foals, but this difference was not statistically different (P = 0.22).

Association of serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ concentrations with severity of disease

Serum 25(OH)D$_3$ concentrations were significantly lower in septic (8.22 ng/mL; 3.74–15.12 ng/mL) and SNS (6.94 ng/mL; 2.30–24.17 ng/mL) compared to healthy foals (14.54 ng/mL; 9.51–19.05 ng/mL) (P < 0.0001) (**Fig 1A**). Serum 1,25(OH)$_2$D$_3$ concentrations were significantly lower in septic (6.91 pmol/L; 4.05–13.49 pmol/L) and SNS (6.90 pmol/L; 4.08–18. 74 pmol/L) compared to healthy foals (9.99 pmol/L; 7.09–16.48 pmol/L) (P = 0.037) (**Fig 1B**). Sepsis score was negatively correlated with serum 25(OH)D$_3$ (rs = - 0.23; P = 0.02), but not associated with 1,25(OH)$_2$D$_3$ concentrations (rs = - 0.16; P = 0.19) in all newborn foals.
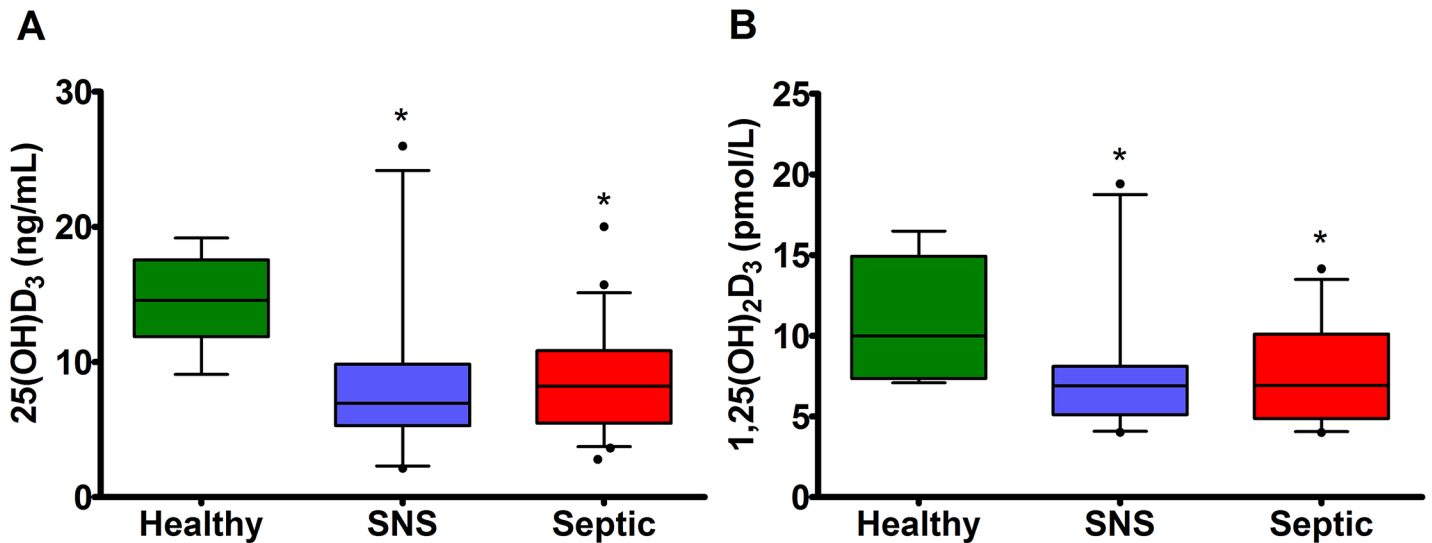
Fig 1. Serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ concentrations in healthy, SNS, and septic foals. Values are expressed as median and 95% CI. (A) Septic and SNS foals had significantly lower serum 25(OH)D$_3$ concentrations compared to healthy foals (P < 0.0001). (B) Septic and SNS foals had significantly lower serum 1,25(OH)$_2$D$_3$ concentrations compared to healthy foals (P = 0.037). * indicates a statistically significant difference from healthy foals.

Serum total calcium, phosphorus, PTH concentrations, and severity of disease

Serum total calcium concentrations were significantly lower in septic (10.90 mg/dL; 8.60–13.10 mg/dL) than healthy (11.60 mg/dL; 10.75–12.65 mg/dL) and SNS foals (11.70 mg/dL; 9.8–15.48 mg/dL) (P = 0.01) (**Fig 2A**). Serum phosphorus concentrations were significantly higher in septic (7.15 mg/dL; 3.7–17 mg/dL) compared to healthy (5.8 mg/dL; 4.65–6.67) and SNS foals (4.65 mg/dL; 3.2–13 mg/dL) (P = 0.02) (**Fig 2B**). Septic foals had significantly higher serum PTH concentrations (12.62 pmol/L; 1.89–41.79 pmol/L) compared to healthy foals (5.47 pmol/L; 1.91–12.30 pmol/L) (P = 0.018), but were not different than SNS foals (6.15 pmol/L; 1.43–50.49 pmol/L) (P = 0.28). In all neonatal foals, sepsis score was negatively correlated with total calcium (rs = - 0.27; P = 0.005) and positively correlated with phosphorus (rs = 0.25; P = 0.01) and PTH concentrations (rs = 0.40; P = 0.0001). Serum total protein and albumin concentrations were not different between foal groups (**S1 Table**).

Serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ in surviving and non-surviving hospitalized foals

Hospitalized foals that died had significantly lower 25(OH)D$_3$ concentrations (7.40 ng/mL; 3.18–12.21 ng/mL) than hospitalized foals that survived (7.96 ng/mL; 3.50–19.19 ng/mL) (P = 0.04). However, serum 1,25(OH)$_2$D$_3$ concentrations were not significantly different between hospitalized survivor and non-survivor foals (P = 0.13). Septic foals that died had significantly lower serum 25(OH)D$_3$ concentrations (7.55 ng/mL; 3.12–12.64 ng/mL) than surviving septic foals (8.67 ng/mL; 4.30–17.43 ng/mL) (P = 0.03) (**Fig 3A**). Similarly, non-surviving septic foals had significantly lower serum 1,25(OH)$_2$D$_3$ concentrations (4.98 pmol/L; 4–13.02 pmol/L) than surviving septic foals (9.1 pmol/L; 5.25–14.14 pmol/L) (P = 0.01) (**Fig 3B**). In SNS foals, serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ concentrations were not statistically different between survivors and non-survivors (P > 0.05).
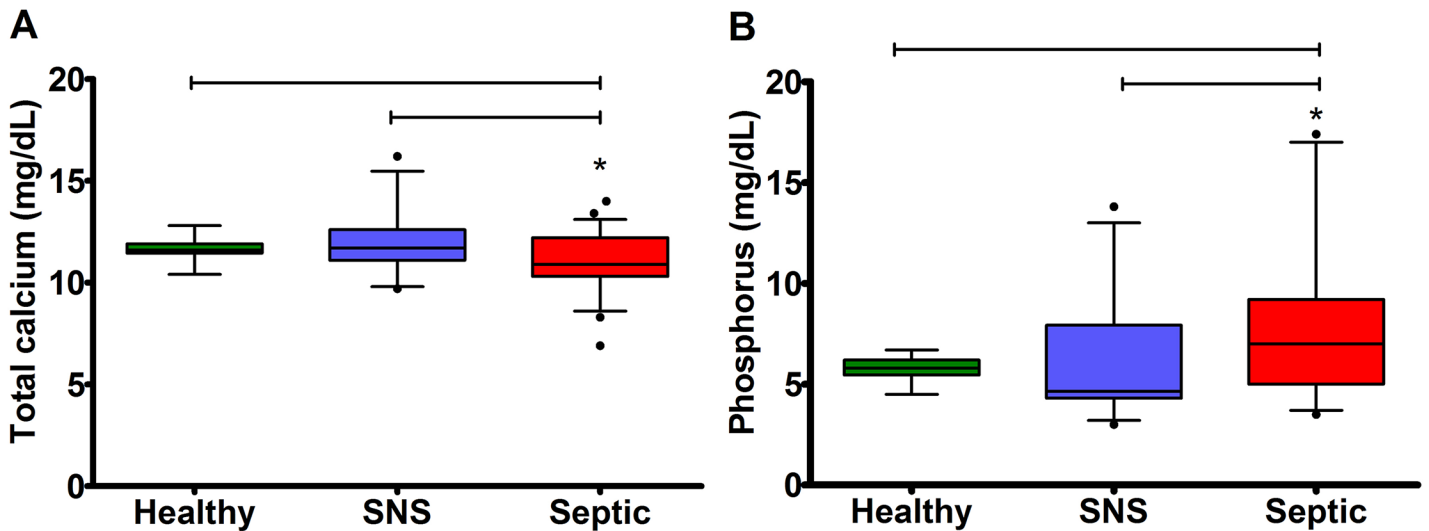
Fig 2. Serum total calcium and phosphorus concentrations in healthy, SNS, and septic foals. Values are expressed as median and 95% CI. (A) Septic foals had significantly lower serum total calcium concentrations compared to healthy and SNS foals (P = 0.01). (B) Septic foals had significantly higher serum phosphorus concentrations compared to healthy and SNS foals (P = 0.02). * indicates a statistically significant difference from healthy and SNS foals.

Association of serum 25(OH)D₃, 1,25(OH)₂D₃, PTH, calcium, phosphorus concentrations, and sepsis score with mortality

Hospitalized foals with serum $25(OH)D_3$ concentrations < 9.51 ng/mL were 3.38 times more likely to die than hospitalized foals with normal $25(OH)D_3$ concentrations (95% CI = 1.11–10.26; P = 0.03) ([Table 2]). However, serum $1,25(OH)_2D_3$ concentrations < 7.09 pmol/L in hospitalized foals were not associated with mortality ([Table 2]). In septic foals, the likelihood of mortality was significantly higher if serum $25(OH)D_3$ concentrations were < 9.51 ng/mL (OR = 3.62; 95% CI = 1.1–12.40; P = 0.03) and $1,25(OH)_2D_3$ concentrations < 7.09 pmol/L (OR = 5.41; 95% CI = 1.19–24.52; P = 0.04) ([Table 3]). In SNS foals, hypovitaminosis D was not associated with likelihood of mortality (P = 0.34).
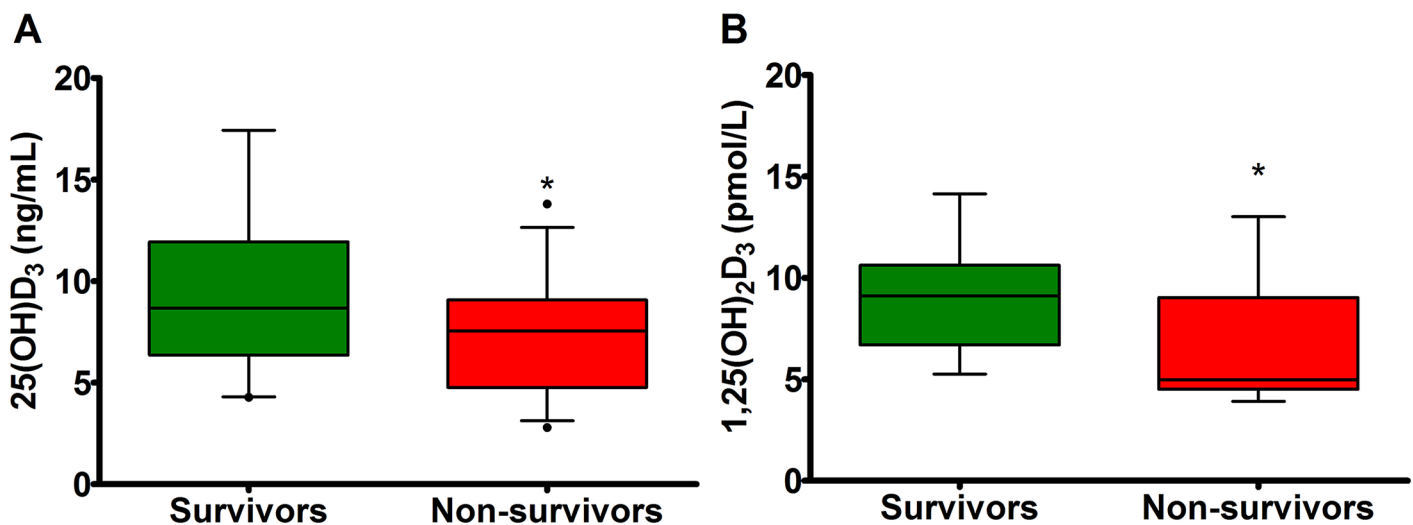

Fig 3. Serum 25(OH)D₃ and 1,25(OH)₂D₃ concentrations in survivor and non-survivor septic foals. Values are expressed as median and 95% CI. (A) Septic non-survivors had lower serum $25(OH)D_3$ concentrations compared to septic survivors (P = 0.03). (B) Septic non-survivors had lower serum $1,25(OH)_2D_3$ concentrations compared to septic survivors (P = 0.01). * indicates a statistically significant difference from septic survivor foals.

Table 2. Univariate analysis for mortality in hospitalized foals.

Variable (units)	Range	OR for non-survival	95% CI	P value
25(OH)D$_3$ (ng/mL)	9.51–19.05	Referent		
	< 9.51	3.38*	1.11–10.26	0.03
1,25(OH)$_2$D$_3$ (pmol/L)	7.09–16.48	Referent		
	< 7.09	1.85	0.61–5.61	0.27
PTH (pmol/L)	1.91–12.30	Referent		
	>12.30	1.95	0.71–5.33	0.19
Total calcium (mg/dL)	10.75–12.65	Referent		
	< 10.75	1.015	0.37–2.75	0.97
	>12.65	1.23	0.35–4.22	0.74
Phosphorus (mg/dL)	4.65–6.67	Referent		
	< 4.65	0.63	0.16–2.41	0.50
	> 6.67	1.83	0.61–5.49	0.27
Sepsis score	4–11	Referent		
	12–15	4*	1.13–15.53	0.04
	16–23	19.25**	3.64–101.77	0.0001

*P < 0.05;
**P < 0.01
25(OH)D$_3$-25 – dihydroxyvitamin D$_3$; 1,25(OH)$_2$D$_3$ – 1,25-dihydroxyvitamin D$_3$; PTH – parathyroid hormone; OR – odds ratio; CI – confidence interval.

doi:10.1371/journal.pone.0127684.t002

In hospitalized and septic foals, serum concentrations of PTH >12.30 pmol/L, total calcium < 10.75 mg/dL, and phosphorus > 6.67 mg/dL, were not associated with mortality (Table 2 and Table 3). There was no difference in serum total protein and albumin concentrations between surviving and non-surviving septic foals (S1 Table). Hospitalized foals with sepsis score ≥ 16 were 19.25 more likely to die than hospitalized foals with sepsis score < 12 (95%

Table 3. Univariate analysis for mortality in septic foals.

Variable (units)	Range	OR for non-survival	95% CI	P value
25(OH)D$_3$ (ng/mL)	9.51–19.05	Referent		
	< 9.51	3.62*	1.1–12.40	0.03
1,25(OH)$_2$D$_3$ (pmol/L)	7.09–16.48	Referent		
	< 7.09	5.41*	1.19–24.52	0.04
PTH (pmol/L)	1.91–12.30	Referent		
	>12.30	1.32	0.39–4.37	0.65
Total calcium (mg/dL)	10.75–12.65	Referent		
	<10.75	0.647	0.209–2	0.45
	>12.65	1.25	0.26–5.93	0.77
Phosphorus (mg/dL)	4.65–6.67	Referent		
	< 4.65	0.85	0.17–4.26	0.85
	> 6.67	1.59	0.46–5.49	0.46
Sepsis score	12–15	Referent		
	16–23	4.81**	1.31–17.36	0.01

*P < 0.05;
**P < 0.01
25(OH)D$_3$ – 25-dihydroxyvitamin D$_3$; 1,25(OH)$_2$D$_3$ – 1,25-dihydroxyvitamin D$_3$; PTH – parathyroid hormone; OR – odds ratio; CI – confidence interval.

doi:10.1371/journal.pone.0127684.t003

CI = 3.64–101.77; P = 0.0001) ([Table 2](#)). Septic foals with sepsis scores $\geq$ 16 were 4.81 times more likely to die than septic foals with sepsis scores from 12–15 (95% CI = 1.31–17.36; P = 0.01) ([Table 3](#)).

Association of serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ with total calcium, phosphorus and PTH concentrations

In hospitalized foals, serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ concentrations were not correlated with serum total calcium and phosphorus concentrations (P > 0.05). However, serum 1,25(OH)$_2$D$_3$ concentrations < 7.09 pmol/L were negatively correlated with serum PTH concentrations (rs = -0.52; P = 0.005). In hospitalized foals, serum PTH concentrations were negatively correlated with total calcium (rs = - 0.34; P = 0.004) and positively correlated with phosphorus concentrations (rs = 0.50; P < 0.0001).

In septic foals, serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ concentrations were not associated with serum total calcium and phosphorus concentrations (P > 0.05); however, 1,25(OH)$_2$D$_3$ concentrations < 7.09 pmol/L were negatively associated with serum PTH concentrations (rs = - 0.61; P = 0.008). Serum PTH concentrations were inversely associated with total calcium (rs = - 0.44; P = 0.003) and positively correlated with phosphorus concentrations (rs = 0.33; P = 0.02).

In SNS foals, serum 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ concentrations were not associated with total calcium and phosphorus concentrations (P > 0.05), and 1,25(OH)$_2$D$_3$ concentrations < 7.09 pmol/L were not associated with PTH. Serum PTH concentrations were positively correlated with phosphorus (rs = 0.504; P = 0.012), but not with total calcium concentrations.

Discussion

In this study we showed that vitamin D deficiency was highly prevalent in hospitalized foals and that those with the lowest concentrations of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ had more severe disease and were more likely to die. We also demonstrated that hypocalcemia and hyperphosphatemia were frequent abnormalities; however, they were not associated with the concentrations of either metabolite of vitamin D. Of interest, serum PTH concentrations were inversely correlated with 1,25(OH)$_2$D$_3$ concentrations. To our knowledge this is the first study investigating the clinical relevance of reduced vitamin D metabolite concentrations in hospitalized foals and their association with calcium, phosphorus, and PTH concentrations, severity of illness, and mortality. Our findings on hypovitaminosis D in hospitalized foals were similar to those reported in critically ill humans in which decreased concentrations of 25(OH)D$_3$ have been associated with disease severity and outcome [16, 25].

Mechanisms leading to decreased concentrations of vitamin D metabolites is sick newborn foals are likely multifactorial. Compared to horses, healthy newborn foals have lower concentrations of 25(OH)D$_3$ and DBP [9, 26], which may predispose them to hypovitaminosis D during illness for a number of reasons, including reduced 25(OH)D$_3$ stores, decreased renal synthesis of 1,25(OH)$_2$D$_3$, and increased renal and gastrointestinal losses of vitamin D metabolites and DBP. In addition, since milk is the main source of vitamin D$_3$ for newborn foals, reduced intake due to disease could further vitamin D deficiency [9, 27]. Inflammatory cytokines may also alter the expression of enzymes involved in the synthesis and catabolism of vitamin D metabolites [28]. Immune cells also express 1α-hydroxylase and leukocyte dysfunction could decrease 1,25(OH)$_2$D$_3$ synthesis [15, 18]. In target tissues, increased 24-hydroxylase activity may decrease 1,25(OH)$_2$D$_3$ concentrations, resulting in vitamin D deficiency [29]. It is also possible that increased concentrations of fibroblast growth factor-23 (FGF-23), a bone-derived

factor that inhibits 1α-hydroxylase activity, contributes to reduced 1,25(OH)$_2$D$_3$ synthesis during illness [30, 31].

Low plasma DBP concentrations have been associated with sepsis, organ failure, hypovitaminosis D, and mortality in critically ill human patients [32–34]. In addition of transporting vitamin D metabolites, DBP has other functions. For example, during cell injury, DBP binds monomeric actin to prevent its polymerization and subsequent endothelial injury, vascular obstruction, and organ failure [33]. It also binds to endotoxins to protect against endotoxemia during sepsis [35]. Because DBP is a small protein (~55 kDa for humans and horses), it is conceivable that inflammatory conditions that alter endothelial and epithelial integrity (renal, intestinal, vascular) could result in protein loss, including DBP waste. From that perspective, we can propose that in septic foals, low DBP could contribute to a pro-inflammatory state, but also exacerbate hypovitaminosis D. This remains to be documented in sick equine neonates.

Other factors to consider in the pathogenesis of hypovitaminosis D are megalin and cubilin [36]. Megalin is a large transmembrane protein (~600 kDa) expressed in the proximal convoluted tubules where it acts as an endocytic receptor to recycle filtered plasma proteins [36]. Cubilin, a 460-kDa protein, is co-expressed and cooperates with megalin in protein uptake [36]. Both proteins are necessary in DBP – 25(OH)D$_3$ endocytosis and subsequent synthesis of 1,25(OH)$_2$D$_3$ [37]. Endotoxemia and acute renal injury, which are common in septic foals, decrease the expression of megalin and cubilin in rodents [38]. Reduced expression of these proteins could lower the concentrations of 25(OH)D$_3$ due to urinary wasting and 1,25(OH)$_2$D$_3$ from decreased renal synthesis.

In the present study, PTH concentrations were elevated in critically foals. This increase in PTH could be explained by hypocalcemia and/or hyperphosphatemia directly stimulating PTH secretion by the parathyroid chief cells [13]. However, it is also probable that low 1,25(OH)$_2$D$_3$ played a role [39]. Calcitriol inhibits parathyroid cell function and PTH secretion and a decline in 1,25(OH)$_2$D$_3$ concentrations could trigger parathyroid chief cell proliferation and PTH secretion [13].

Low serum 1,25(OH)$_2$D$_3$ concentrations in hospitalized and septic foals were associated with elevated concentrations of PTH. Physiologically, PTH stimulates 1,25(OH)$_2$D$_3$ production by increasing renal 1α-hydroxylase activity [13]. Thus, it would be expected that foals with high PTH will have elevated concentrations of 1,25(OH)$_2$D$_3$, which was not the case in the sick foals of this study. This suggests that PTH receptor signaling in critically ill foals may be impaired. As previously mentioned, it is also plausible that increased concentrations of FGF-23 could have suppressed 1,25(OH)$_2$D$_3$ synthesis [30]; however, information on FGF-23 in foals and horses is lacking.

Septic foals in this study had significantly lower total calcium concentrations than healthy and SNS foals. Although it would have been ideal to measure ionized calcium concentrations because it better reflects the active calcium status in the extracellular compartment, unlike total calcium, this is not a routine measurement in most equine hospitals [13]. However, we have already shown that septic foals develop ionized hypocalcemia [3]. The causes of equine neonatal hypocalcemia are poorly understood and likely multifactorial. These include intracellular sequestration, calcium chelation, alkalosis, intestinal losses, renal dysfunction, parathyroid gland dysfunction, hypovitaminosis D, and hypomagnesemia [3, 4, 6, 13, 40]. Total hypocalcemia could have also resulted from hypoproteinemia [1]; however, serum total protein and albumin concentrations were not statistically different between foal groups, making it an unlikely cause of hypocalcemia in the septic foals of this study. Based on previous studies by our group [3], we suggest that a decrease in ionized calcium concentration is a major contributor to the development of total hypocalcemia in critically ill foals. However, this does not explain mechanistically why serum calcium concentrations decrease in foals with evidence of systemic inflammation.

In septic foals, hypocalcemia was not associated with low $1,25(OH)_2D_3$ concentrations, indicating that other factors are involved. It is unlikely that inappropriate PTH secretion is central to the development of equine neonatal hypocalcemia as PTH concentrations were elevated in the foals of this study and we have previously documented that PTH increases in response to illness in septic foals [3]. These findings further support our theory that PTH receptor resistance may be implicated in the pathogenesis of abnormal calcium, phosphorus, and vitamin D homeostasis in sick foals.

Hyperphosphatemia was more prevalent than hypophosphatemia in sick foals, which is contrary to what has been reported in critically ill humans, where hypophosphatemia is more frequent [7]. The pathogenesis of hyperphosphatemia in critically ill foals is unclear, but potential explanations include cell injury, acidosis, magnesium deficiency, hypoparathyroidism, and PTH receptor resistance [41]. Hypervitaminosis D as a cause of hyperphosphatemia is these foals is unlikely. The fact that phosphorus and PTH were positively correlated further supports impaired PTH receptor signaling because an appropriate response to PTH would have been characterized by reduced phosphorus concentrations.

Even though magnesium concentrations were not measured in the foals of this study, hypomagnesemia reduces PTH secretion and action, and has been linked to hypocalcemia, hypovitaminosis D, and hyperphosphatemia [13, 42]. Often critically ill horses and foals develop hypomagnesemia [3, 4].

Another system to take into consideration is the FGF-23/klotho axis [30, 31]. FGF-23 is a hypophosphatemic factor produced by osteoblast/osteocytes, while klotho, which is produced by renal tubular cells, is the co-receptor for FGF-23 [30, 31]. PTH and $1,25(OH)_2D_3$ increase FGF-23 and klotho expression [30, 43], and FGF-23 decreases renal phosphorus reabsorption and 1α-hydroxylase expression, and reduces PTH synthesis and secretion [30, 31]. One might speculate that in addition to PTH resistance, decreased klotho expression from low $1,25(OH)_2D_3$ concentrations could hamper FGF-23 receptor signaling, resulting in hyperphosphatemia and elevated PTH secretion. Although we have no evidence of abnormal FGF-23 and klotho concentrations in sick newborn foals, they should be considered in future equine neonatal studies.

In the study reported here, non-surviving septic foals had significantly lower concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ than septic surviving foals, and those with the lowest metabolite concentrations were more likely to die. Similarly, hypovitaminosis D has been associated with mortality in critically ill people [16–19], suggesting that in addition to calcium and phosphorus homeostasis, vitamin D_3 has other essential functions. In acute illnesses, vitamin D enhances survival by boosting innate immunity, reducing local and systemic inflammation, and by increasing the synthesis of antimicrobial factors such as β-defensin and cathelicidin [44, 45].

In conclusion, low concentrations of vitamin D_3 metabolites is highly prevalent and associated with disease severity and outcome in hospitalized foals. These findings support a protective role for vitamin D_3 against equine perinatal diseases. Based on these results, the therapeutic value of vitamin D_3 in sick foals deserves clinical attention. Recent studies in critically ill human patients have shown benefits of vitamin D supplementation and clinical trials are ongoing [46–48]. Hyperphosphatemia and hypocalcemia, together with hypovitaminosis D and increased PTH concentrations, indicate that PTH resistance may be involved in the development of these abnormalities; however, foal-specific studies will be required to document abnormal PTH receptor signaling in response to systemic inflammatory processes. The importance of DBP, altered hydroxylase activities, and the FGF-23/klotho axis in disorders of vitamin D, calcium, phosphorus, and PTH in newborn foals remain to be explored.

Supporting Information

S1 Table. Serum total protein and albumin concentrations in septic, sick non-septic, healthy, septic survivor and septic non-surviving foals.
(DOCX)

Author Contributions

Conceived and designed the experiments: RET AK. Performed the experiments: AK KAD RET. Analyzed the data: AK KAD RET. Contributed reagents/materials/analysis tools: AK KAD RET NS SR TR AZ. Wrote the paper: AK RET.

References

1. Toribio RE (2011) Endocrine dysregulation in critically ill foals and horses. Vet Clin North Am Equine Pract 27:35–47. doi: 10.1016/j.cveq.2010.12.011 PMID: 21392652

2. Garcia-Lopez JM, Provost PJ, Rush JE, Zicker SC, Burmaster H, Freeman LM (2001) Prevalence and prognostic importance of hypomagnesemia and hypocalcemia in horses that have colic surgery. Am J Vet Res 62:7–12. PMID: 11197564

3. Hurcombe SD, Toribio RE, Slovis NM, Saville WJ, Mudge MC, Macgillivray K, et al. (2009) Calcium regulating hormones and serum calcium and magnesium concentrations in septic and critically ill foals and their association with survival. J Vet Intern Med 23:335–343. doi: 10.1111/j.1939-1676.2009.0275.x PMID: 19210311

4. Toribio RE, Kohn CW, Chew DJ, Sams RA, Rosol TJ (2001) Comparison of serum parathyroid hormone and ionized calcium and magnesium concentrations and fractional urinary clearance of calcium and phosphorus in healthy horses and horses with enterocolitis. Am J Vet Res 62:938–947. PMID: 11400854

5. Toribio RE, Kohn CW, Capen CC, Rosol TJ (2003) Parathyroid hormone (PTH) secretion, PTH mRNA and calcium-sensing receptor mRNA expression in equine parathyroid cells, and effects of interleukin (IL)-1, IL-6, and tumor necrosis factor-alpha on equine parathyroid cell function. J Mol Endocrinol 31:609–620. PMID: 14664720

6. Toribio RE, Kohn CW, Hardy J, Rosol TJ (2005) Alterations in serum parathyroid hormone and electrolyte concentrations and urinary excretion of electrolytes in horses with induced endotoxemia. J Vet Intern Med 19:223–231. PMID: 15822568

7. Santana e Meneses JF, Leite HP, de Carvalho WB, Lopes E Jr (2009) Hypophosphatemia in critically ill children: prevalence and associated risk factors. Pediatr Crit Care Med 10:234–238. doi: 10.1097/PCC.0b013e3181937042 PMID: 19057439

8. Holick MF (1990) The use and interpretation of assays for vitamin D and its metabolites. J Nutr 120 Suppl 11:1464–1469. PMID: 2243289

9. Pozza ME, Kaewsakhorn T, Trinarong C, Inpanbutr N, Toribio RE (2014) Serum vitamin D, calcium, and phosphorus concentrations in ponies, horses and foals from the United States and Thailand. Vet J 199:451–456. doi: 10.1016/j.tvjl.2014.01.002 PMID: 24524849

10. DeLuca HF (2004) Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr 80:1689S–1696S. PMID: 15585789

11. Jones G (2008) Pharmacokinetics of vitamin D toxicity. Am J Clin Nutr 88:582S–586S. PMID: 18689406

12. Norman AW (2008) From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. Am J Clin Nutr 88:491S–499S. PMID: 18689389

13. Toribio RE (2011) Disorders of calcium and phosphate metabolism in horses. Vet Clin North Am Equine Pract 27:129–147. doi: 10.1016/j.cveq.2010.12.010 PMID: 21392658

14. Haroon M, Fitzgerald O (2012) Vitamin D and its emerging role in immunopathology. Clin Rheumatol 31:199–202. doi: 10.1007/s10067-011-1880-5 PMID: 22020389

15. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 96:1911–1930. doi: 10.1210/jc.2011-0385 PMID: 21646368

16. Lee P, Nair P, Eisman JA, Center JR (2009) Vitamin D deficiency in the intensive care unit: an invisible accomplice to morbidity and mortality? Intensive Care Med 35:2028–2032. doi: 10.1007/s00134-009-1642-x PMID: 19756497

17. Nair P, Lee P, Reynolds C, Nguyen ND, Myburgh J, Eisman JA, et al. (2013) Significant perturbation of vitamin D-parathyroid-calcium axis and adverse clinical outcomes in critically ill patients. Intensive Care Med 39:267–274. doi: 10.1007/s00134-012-2713-y PMID: 23064465

18. Nguyen HB, Eshete B, Lau KH, Sai A, Villarin M, Baylink D (2013) Serum 1,25-dihydroxyvitamin D: an outcome prognosticator in human sepsis. PLoS One 8:e64348. doi: 10.1371/journal.pone.0064348 PMID: 23741318

19. Amrein K, Zajic P, Schnedl C, Waltensdorfer A, Fruhwald S, Holl A, et al. (2014) Vitamin D status and its association with season, hospital and sepsis mortality in critical illness. Crit Care 18:R47. doi: 10.1186/cc13790 PMID: 24661739

20. Venkatesh B, Nair P (2014) Hypovitaminosis D and morbidity in critical illness: is there proof beyond reasonable doubt? Crit Care 18:138. doi: 10.1186/cc13863 PMID: 24976501

21. Hebbar KB, Wittkamp M, Alvarez JA, McCracken CE, Tangpricha V (2014) Vitamin D Deficiency in Pediatric Critical Illness. J Clin Transl Endocrinol 1:170–175. PMID: 25580380

22. Aygencel G, Turkoglu M, Tuncel AF, Candir BA, Bildaci YD, Pasaoglu H (2013) Is vitamin d insufficiency associated with mortality of critically ill patients? Crit Care Res Pract 2013:856747. doi: 10.1155/2013/856747 PMID: 24455224

23. Izadpanah M, Khalili H (2013) Potential benefits of vitamin D supplementation in critically ill patients. Immunotherapy 5:843–853. doi: 10.2217/imt.13.84 PMID: 23902554

24. Brewer BD, Koterba AM (1988) Development of a scoring system for the early diagnosis of equine neonatal sepsis. Equine Vet J 20:18–22. PMID: 3366100

25. Rippel C, South M, Butt WW, Shekerdemian LS (2012) Vitamin D status in critically ill children. Intensive Care Med 38:2055–2062. doi: 10.1007/s00134-012-2718-6 PMID: 23052958

26. Madej JP, Nowacki W, Boratynski J, Borkowski J, Wlodarczyk-Szydlowska A, Musial E (2009) The relationship between concentrations of vitamin D-binding protein (DBP) in serum and colostrum of mares and in serum of their foals in the neonatal period. Pol J Vet Sci 12:499–507. PMID: 20169924

27. Salle BL, Delvin EE, Lapillonne A, Bishop NJ, Glorieux FH (2000) Perinatal metabolism of vitamin D. Am J Clin Nutr 71:1317S–1324S. PMID: 10799409

28. Edfeldt K, Liu PT, Chun R, Fabri M, Schenk M, Wheelwright M, et al. (2010) T-cell cytokines differentially control human monocyte antimicrobial responses by regulating vitamin D metabolism. Proc Natl Acad Sci U S A 107:22593–22598. doi: 10.1073/pnas.1011624108 PMID: 21149724

29. Lang CL, Wang MH, Chiang CK, Lu KC (2014) Vitamin D and the immune system from the nephrologist's viewpoint. ISRN Endocrinol 2014:105456. doi: 10.1155/2014/105456 PMID: 24587915

30. Kuro-o M (2010) Overview of the FGF23-Klotho axis. Pediatr Nephrol 25:583–590. doi: 10.1007/s00467-009-1260-4 PMID: 19626341

31. Olauson H, Vervloet MG, Cozzolino M, Massy ZA, Urena TP, Larsson TE (2014) New Insights Into the FGF23-Klotho Axis. Semin Nephrol 34:586–597. doi: 10.1016/j.semnephrol.2014.09.005 PMID: 25498378

32. Jeng L, Yamshchikov AV, Judd SE, Blumberg HM, Martin GS, Ziegler TR, et al. (2009) Alterations in vitamin D status and anti-microbial peptide levels in patients in the intensive care unit with sepsis. J Transl Med 7:28. doi: 10.1186/1479-5876-7-28 PMID: 19389235

33. Speeckaert MM, Speeckaert R, van GN, Delanghe JR (2014) Vitamin D binding protein: a multifunctional protein of clinical importance. Adv Clin Chem 63:1–57. PMID: 24783350

34. Waldron JL, Ashby HL, Cornes MP, Bechervaise J, Razavi C, Thomas OL, et al. (2013) Vitamin D: a negative acute phase reactant. J Clin Pathol 66:620–622. doi: 10.1136/jclinpath-2012-201301 PMID: 23454726

35. Meier U, Gressner O, Lammert F, Gressner AM (2006) Gc-globulin: roles in response to injury. Clin Chem 52:1247–1253. PMID: 16709624

36. Christensen EI, Birn H, Storm T, Weyer K, Nielsen R (2012) Endocytic receptors in the renal proximal tubule. Physiology (Bethesda) 27:223–236. doi: 10.1152/physiol.00022.2012 PMID: 22875453

37. Marzolo MP, Farfan P (2011) New insights into the roles of megalin/LRP2 and the regulation of its functional expression. Biol Res 44:89–105. doi: 10.4067/S0716-97602011000100012 PMID: 21720686

38. Schreiber A, Theilig F, Schweda F, Hocherl K (2012) Acute endotoxemia in mice induces downregulation of megalin and cubilin in the kidney. Kidney Int 82:53–59. doi: 10.1038/ki.2012.62 PMID: 22437417

39. Karefylakis C, Naslund I, Edholm D, Sundbom M, Karlsson FA, Rask E (2014) Vitamin D status 10 years after primary gastric bypass: gravely high prevalence of hypovitaminosis D and raised PTH levels. Obes Surg 24:343–348. doi: 10.1007/s11695-013-1104-y PMID: 24163201

40. Lind L, Carlstedt F, Rastad J, Stiernstrom H, Stridsberg M, Ljunggren O, et al. (2000) Hypocalcemia and parathyroid hormone secretion in critically ill patients. Crit Care Med 28:93–99. PMID: 10667505

41. DiBartola SP, Willard MD. Disorders of phosphorus: hypophosphatemia and hyperphosphatemia. In: DiBartola SP, editor. Fluid, electrolyte and acid-base disorders in small animal practice. Philadelphia: Saunders; 2005. p 195–209.

42. Rude RK, Oldham SB, Singer FR (1976) Functional hypoparathyroidism and parathyroid hormone end-organ resistance in human magnesium deficiency. Clin Endocrinol (Oxf) 5:209–224. PMID: 182417

43. Lavi-Moshayoff V, Wasserman G, Meir T, Silver J, Naveh-Many T (2010) PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. Am J Physiol Renal Physiol 299:F882–F889. doi: 10.1152/ajprenal.00360.2010 PMID: 20685823

44. Watkins RR, Yamshchikov AV, Lemonovich TL, Salata RA (2011) The role of vitamin D deficiency in sepsis and potential therapeutic implications. J Infect 63:321–326. doi: 10.1016/j.jinf.2011.07.002 PMID: 21777617

45. Borella E, Nesher G, Israeli E, Shoenfeld Y (2014) Vitamin D: a new anti-infective agent? Ann N Y Acad Sci 1317:76–83. doi: 10.1111/nyas.12321 PMID: 24593793

46. McKinney JD, Bailey BA, Garrett LH, Peiris P, Manning T, Peiris AN (2011) Relationship between vitamin D status and ICU outcomes in veterans. J Am Med Dir Assoc 12:208–211. doi: 10.1016/j.jamda.2010.04.004 PMID: 21333923

47. Han JE, Ziegler TR (2014) Vitamin D supplementation in sepsis and critical illness: where are we now? Am J Respir Crit Care Med 190:483–485. doi: 10.1164/rccm.201408-1443ED PMID: 25171307

48. Amrein K, Sourij H, Wagner G, Holl A, Pieber TR, Smolle KH, et al. (2011) Short-term effects of high-dose oral vitamin D3 in critically ill vitamin D deficient patients: a randomized, double-blind, placebo-controlled pilot study. Crit Care 15:R104. doi: 10.1186/cc10120 PMID: 21443793