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Longitudinal Evaluation of Vitamin D, Parathyroid Hormone, Antimicrobial Peptides, and Immunomodulatory Genes in Hospitalized Foals

Ahmed M. Kamr[1,2] | Celine Bartish[1] | Jamie Summers[1] | Julia Horton[1] | Laura D. Hostnik[1] | Kindra Orr[3] | Nimet Browne[4] | Katarzyna A. Dembek[5] | Caroline Saliba[3] | Diego E. Gomez[6] | Ramiro E. Toribio[1]

[1]College of Veterinary Medicine, The Ohio State University, Columbus, Ohio, USA | [2]Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt | [3]Rood and Riddle Equine Hospital, Lexington, Kentucky, USA | [4]Hagyard Equine Medical Institute, Lexington, Kentucky, USA | [5]College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA | [6]Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

Correspondence: Ramiro E. Toribio (toribio.1@osu.edu)

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ABSTRACT

Background: Information about the association of antimicrobial peptides with hypovitaminosis D in hospitalized foals is lacking.

Hypothesis/Objectives: We aimed to longitudinally determine the association of serum concentrations of vitamin D metabolites, vitamin D binding protein (DBP), and parathyroid hormone (PTH) with antimicrobial peptides (β-defensin-1 and cathelicidin-1) and the mRNA expression of the vitamin D receptor (VDR), 1α-hydroxylase (CYP27B1), 24-hydroxylase (CYP24A1), toll-like receptor-4 (TLR-4), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), disease severity, and mortality in hospitalized foals. We hypothesized that hypovitaminosis D would be associated with decreased serum concentrations of antimicrobial peptides, disease severity, and mortality in hospitalized foals.

Animals: One hundred nine foals ≤72 h of age divided into hospitalized ($n = 83$; 60 septic, 23 sick nonseptic [SNS]) and healthy ($n = 26$) foals.

Methods: Blood samples were collected on admission (0), and 24, 48, and 72 h after admission from healthy and hospitalized foals. Data were analyzed by repeated measure methods.

Results: Serum 25(OH)D, 1,25$(OH)_2$D, DBP, β-defensin-1, and cathlicidin-1 concentrations were significantly lower, whereas PTH concentrations were higher in hospitalized compared to healthy foals at different times during hospitalization ($p < 0.05$). Septic foals had lower VDR and CYP27B1, but higher TLR-4, TNF-α, and IL-1β mRNA expression than in healthy foals ($p < 0.05$). Decreased serum 25(OH)D, β-defensin-1, and cathelicidin-1, and high PTH concentrations were associated with higher odds of death in hospitalized foals ($p < 0.05$).

Conclusions and Clinical Importance: Decreased vitamin D metabolite concentrations and decreased antimicrobial peptide concentrations suggest that vitamin D has important immunomodulatory functions in newborn foals.

Abbreviations: 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; B, regression coefficients; CI, confidence interval; CYP24A1, 24-hydroxylase; CYP27B1, 1α-hydroxylase; DBP, vitamin D binding protein; FG-23, fibroblast growth factor 23; IL-1β, interleukin-1β; OR, odds ratios; PTH, parathyroid hormone; RS, Spearman's rank coefficient; SE, standard error; SNS, sick non-septic; TLR-4, toll like receptor-4; TNF-α, tumor necrosis factor-α; VDR, vitamin D receptor.

1 | Introduction

Sepsis is a life-threatening condition in newborn foals with high fatality rates [1–3]. Recently, hypovitaminosis D and increased parathyroid hormone (PTH) concentrations have been documented in hospitalized foals [4, 5].

Parathyroid hormone is an essential regulator of calcium and phosphorus homeostasis through its actions in bone and kidney [3, 6, 7]. Vitamin D is important for calcium and phosphorus regulation, bone remodeling, immunomodulation, and epithelial integrity [8–10]. There are two forms of vitamin D: ergocalciferol of fungal and plant origin and vitamin D3 (cholecalciferol) produced in the skin from sunlight exposure or supplied in feed [11, 12]. Cholecalciferol or ergocalciferol (vitamin D) are converted into 25-hydroxyvitamin D (25(OH)D) by 25α-hydroxylase in the liver and then converted into 1,25-dihydroxyvitamin D ($1,25(OH)_2D$; calcitriol), the active form of vitamin D in the kidneys by 1α-hydroxylase (CYP27B1) [10]. Renal 1α-hydroxylase is important for calcium and phosphorus homeostasis and is regulated by PTH, phosphorus, $1,25(OH)_2D$, and fibroblast growth factor 23 (FGF-23) [7, 13, 14]. However, 25(OH)D also can be converted to $1,25(OH)_2D$ in extrarenal tissues, including leukocytes and epithelial cells, to function as an autocrine and paracrine factor to modulate immunity, epithelial integrity, and energy homeostasis [15]. This conversion by extrarenal 1α-hydroxylase is regulated locally and not involved in calcium homeostasis under physiological conditions. The actions of $1,25(OH)_2D$ in target organs are mediated by the vitamin D receptor (VDR) [16]. Vitamin D is inactivated in target organs by 24-hydroxylase (CYP24A1) to avoid excessive concentrations and adverse effects [17]. Information about vitamin D signaling in healthy and hospitalized foals is lacking.

Vitamin D binding protein (DBP), also known as GC-globulin, is a relatively small (59 kDa) plasma protein produced in the liver [18]. Vitamin DBP is the primary carrier of vitamin D metabolites in circulation to their metabolizing and target organs [18]. Low DBP concentrations are linked to hypovitaminosis D in critically ill humans and mice [19–21]. Because of its small size, conditions that cause hypoproteinemia, including enteropathies and nephropathies, often result in low DBP concentrations [22]. However, DBP concentrations have not been evaluated in hospitalized foals.

Cathelicidin-1 and β-defensin-1 are antimicrobial peptides produced by different tissue types and immune cells in response to infections [23, 24]. These peptides also stimulate chemokines to attract leukocytes to infection sites [24–26]. Vitamin D regulates the expression and synthesis of cathelicidin-1 and β-defensin-1 [27]. The association between hypovitaminosis D and antimicrobial peptides in hospitalized foals has not been investigated. Therefore, we aimed to measure serum 25(OH)D, $1,25(OH)_2D$, DBP, and PTH concentrations longitudinally and determine their association with serum β-defensin-1 and cathelicidin-1 concentrations, mRNA expression of genes of interest, as well as disease severity and nonsurvival of hospitalized foals. We hypothesized that decreased vitamin D metabolite and increased PTH concentrations would be linked to reduced serum concentrations of β-defensin-1 and cathelicidin-1, and regulatory enzyme mRNA expression, as well as increased cytokine and TLR-4 mRNA expression, disease severity, and mortality in critically ill foals.

2 | Materials and Methods

2.1 | Animals and Inclusion Criteria

A total of 109 neonatal foals ≤ 72 h of age of any breed and sex were included. Foals were categorized into healthy foals ($n = 26$) and hospitalized ($n = 83$). Hospitalized foals were admitted to three equine referral hospitals (The Ohio State University Equine Center, Rood and Riddle Equine Hospital, and Hagyard Equine Medical Institute). Healthy foals were evaluated at a nearby breeding farm (Midland Acres) or the hospitals. They were considered healthy based on normal physical examination, hematology, serum biochemistry, serum IgG concentrations (> 800 mg/dL), and sepsis scores < 4. Hospitalized foals were classified into septic ($n = 60$) and sick non-septic (SNS; $n = 23$) based on their sepsis scores [28]. Septic foals were those admitted for different conditions (e.g., pneumonia, enteritis, colitis, omphalitis) and diagnosed based on positive blood culture or sepsis score ≥ 12 or both. The SNS foals were those with illnesses other than sepsis (e.g., orthopedic conditions, retained meconium), that had negative blood cultures, and sepsis score ≤ 11 [28]. Hospitalized foals were further classified into survivors ($n = 57$) and nonsurvivors ($n = 26$). Survivors were foals released alive from the hospital, whereas nonsurvivors were foals that died or were euthanized because of a grave medical prognosis. Foals euthanized for nonmedical reasons were not included in the study. The study was approved by The Ohio State University Institutional Animal Care and Use Committee and carried out following institutional and United States Department of Agriculture guidelines on the use of animals in veterinary research.

2.2 | Clinical Information

Case history obtained on admission included pregnancy duration, dystocia, maternal illnesses, expected foaling date, problems at parturition, and medications (mare and foal). Clinical and laboratory information, including physical examination, CBC, serum biochemistry, and IgG concentrations, were obtained from all foals in the study. Blood cultures were not performed in all hospitalized foals. Sepsis scores were calculated based on clinical history, physical examination, and laboratory findings [28].

2.3 | Sampling

Blood samples were collected upon admission (0 h), and 24, 48, and 72 h after admission into serum clot and EDTA-containing tubes from hospitalized and healthy foals during routine physical examination. Serum was allowed to clot at room temperature for 1 h and EDTA tubes were refrigerated. Subsequently, tubes were centrifuged at 2000*g* for 10 min at 4°C, and serum and plasma were aliquoted into smaller volumes and stored at

−80°C until analysis. Buffy coats from EDTA tubes were harvested and mixed with an RNA stabilizing solution (RNAlater, Thermo Fisher Scientific, Waltham, MA, USA) at a ratio of 1:2 and stored at −80°C until RNA extraction for mRNA gene expression.

2.4 | Serum Vitamin D Metabolites, DBP, PTH, and Antimicrobial Peptides, and Leukocyte Gene mRNA Expression

Serum 25(OH)D and 1,25$(OH)_2$D concentrations were measured using human-specific ELISA immunoassays (Immunodiagnostic Systems, Gaithersburg, MD, USA) previously validated for samples from horses [5, 10]. These assays measure both forms of vitamin D (D2 and D3). Serum DBP concentrations were determined using an equine-specific ELISA (MyBiosource, San Diego, CA, USA) at 500-fold dilutions, with inter and intra-assay coefficients of variation of 3% and 5%, respectively, and good linearity at dilution parallelism of 1:1000, 1:2000, and 1:3000 ($R^2 = 0.99$). Serum intact PTH concentrations were measured using a human-specific ELISA assay (Monobind Inc., Lake Forest, CA, USA) that was validated for equine samples in our laboratory based on dilution parallelism of 1:2, 1:4, and 1:8, with a sensitivity of 0.49 pg/mL, and inter and intra-assay coefficients of variation 4% and 2%, respectively. Serum cathelicidin-1 concentrations were measured using an equine-specific ELISA (MyBiosource, San Diego, CA, USA) that was validated for samples from horses in our laboratory, with a sensitivity of 9.4 pg/mL, and inter and intra-assay coefficients of variation of 9.3% and 8.6%, respectively. Serum β-defensin-1 concentrations were measured using an equine-specific ELISA (Genorise Scientific Inc., Glen Mills, PA, USA), with a sensitivity of 1.8 pg/mL, and inter and intra-assay coefficients of variation of 7.2% and 7.5%, respectively [29]. To assess the potential immunomodulatory actions of vitamin D in peripheral leukocytes, the mRNA gene expression of VDR, CYP27B1, CYP24A1, TLR-4, TNF-α, and IL-1β was determined in the buffy coats of admission EDTA blood samples.

2.5 | RNA Extraction, cDNA Synthesis, and mRNA Expression

The RNA extraction from white blood cell frozen buffy coats was performed using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). NanoDrop One/One (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure RNA purity and concentrations at 260/280 nm optical densities. Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with 2 μg of total RNA as a template per 20 μL reaction. Real-time qPCR was performed using QuantStudio 3 (Applied Biosystems, Foster City, CA, USA) set at 40 cycles of denaturing at 94°C for 15 s, with annealing temperature of 60°C for 30 s, and extension at 72°C for 30 s. Each reaction mixture contained 2 μL of 10 ng template cDNA, 2 μL of forward and 2 μL of reverse primers at 10 μM concentrations, 4 μL of nuclease-free water, and 10 μL 2× PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Relative mRNA gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA using the 2^-ΔΔCT method. Primers for VDR, GAPDH, TLR-4, and inflammatory cytokines were validated in previous studies [30–32]. We further validated all primers by assessing amplicon size and specificity by gel electrophoresis. Primer sequences are listed in Table S1.

2.6 | Statistical Analyses

A convenience foal sample size was calculated based on changes in 25(OH)D concentrations on admission as the main outcome of interest with a power of 80% and a significance of 5% (α), using statistical software (PASS 16.0.4, NCSS LLC, Kaysville, UT). Data normality was assessed using D'Agostino-Pearson omnibus normality test and Shapiro–Wilk statistic and were not normally distributed. Therefore, values are expressed as medians with ranges. Repeated measures analysis of variance (ANOVA) with Tukey's multiple comparisons test was carried out between groups. Correlations were only generated for the hospitalized group and within hospitalized foals, which included septic and SNS foals, using Spearman's rank coefficient (rs). Serum 25(OH)D, 1,25$(OH)_2$D, PTH, cathelicidin-1, and β-defensin-1 concentrations were classified into low, normal, and high groups based on the 5%–95% confidence intervals (CI) from healthy foals. Crude odds ratios (OR) for nonsurvival were generated using univariate logistic regression. Nonsurvival was the dependent variable for the regression analysis. The data fit the model according to the Hosmer-Lemeshow goodness-of-fit test ($p = 0.9$). The Wald test was used to compare models based on their best-fit criteria in the context of logistic regression [33]. The receiver operating characteristic curve (ROC) was used to estimate the sensitivity and specificity for nonsurvival in hospitalized foals. The program IBM SPSS Statistics 25.0 (IBM Corporation, Armonk, NY, USA) was used for univariate regression analysis, whereas plots were generated using Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA). Significance was set at $p < 0.05$.

3 | Results

3.1 | Study Population

Of hospitalized foals, 72.3% (60/83) were categorized as septic and 27.7% (23/83) as SNS. Hospitalized foals included 59 colts, 23 fillies, and 1 of unrecorded sex. Breeds of hospitalized foals consisted of Standardbred ($n = 31$), Quarter Horse ($n = 16$), Thoroughbred ($n = 14$), Percheron ($n = 3$), Shetland Pony ($n = 2$), Friesian ($n = 2$), American Paint Horse ($n = 2$), American Saddlebred ($n = 2$), Gypsy Vanner ($n = 2$), Holsteiner ($n = 1$), Belgian ($n = 1$), Haflinger ($n = 1$), Arabian ($n = 1$), Rocky Mountain ($n = 1$), Morgan ($n = 1$), Mixed ($n = 1$), and unknown ($n = 1$). Healthy foals ($n = 26$) consisted of Standardbred ($n = 13$), Thoroughbred ($n = 4$), Quarter Horse ($n = 4$), Percheron ($n = 2$), Arabian ($n = 1$), and unknown ($n = 2$).

For hospitalized foals, the median range of sepsis score was 13 (range, 5–24), for septic foals was 14 (range, 12–24), and for SNS foals was 7 (range, 5–11). For hospitalized foals, the median age was 24 h (range, 5–72 h), for septic was 24 h (range, 5–72 h), for SNS was 24 h (range, 6–72 h) and for healthy was 30 h (range, 24–48 h). Age was not significantly different between foal groups ($p = 0.2$). In hospitalized foals, 31% (26/83) were nonsurvivors, and 69% (57/83) were survivors. In septic foals, 38% (23/60) were

nonsurvivors, and 62% (37/60) were survivors. In SNS foals, 13% (3/23) were nonsurvivors, and 87% (20/23) were survivors.

3.2 | Serum 25(OH)D, 1,25(OH)$_2$D, PTH, DBP, β-Defensin-1 and Cathelicidin-1 Concentrations and Ratios Based on Disease Severity

On admission, serum 25(OH)D concentrations were significantly lower in hospitalized compared with healthy foals ($p = 0.03$), but no differences were detected between these groups over time ($p = 0.2$; Table 1). Similarly, serum 25(OH)D concentrations were significantly lower in septic foals at admission compared with healthy foals ($p = 0.04$), but no differences between groups were identified during hospitalization ($p = 0.5$; Figure 1A). No other differences in serum 25(OH)D concentrations were detected between groups (Figure 1A).

Serum 1,25(OH)$_2$D concentrations were not significantly different between hospitalized and healthy foals at any time point ($p > 0.05$; Table 1). Serum 1,25(OH)$_2$D concentrations were not significantly different between septic and healthy foals at admission and 24 h but were significantly lower in septic foals at 48 and 72 h after admission compared with healthy foals ($p = 0.03$; Figure 1B). No other differences in serum 25(OH)D concentrations were detected between groups (Figure 1B).

In hospitalized foals, serum DBP concentrations were lower on admission and over time than in healthy foals ($p = 0.003$; Table 1). On admission and over time, serum DBP concentrations were lower in septic compared with healthy foals ($p = 0.004$; Figure 1C). On admission, serum DBP concentrations were not different between SNS and healthy foals ($p = 0.2$), but were lower over time in SNS compared to healthy foals ($p = 0.004$; Figure 1C). On admission, serum PTH concentrations were higher in hospitalized foals compared with healthy foals ($p = 0.004$; Table 1). Septic and SNS foals had higher PTH concentrations than healthy foals ($p = 0.001$; Figure 1D). No other differences were detected in serum PTH concentrations.

Hospitalized foals had lower β-defensin-1 concentrations on admission and over time compared with healthy foals ($p = 0.001$; Table 1). Septic and SNS foals had lower β-defensin-1 concentrations at admission and during hospitalization than healthy foals ($p = 0.001$; Figure 1E). Hospitalized foals had lower serum cathelicidin-1 concentrations on admission and during hospitalization than healthy foals ($p = 0.003$; Table 1). Septic and SNS foals had lower serum cathelicidin-1 concentrations on admission and during hospitalization than healthy foals ($p = 0.001$; Figure 1F). In hospitalized foals, β-defensin-1/1,25(OH)$_2$D ratio was lower at admission and at 24 h than in healthy foals ($p = 0.02$; Table 1). Cathelicidin-1/1,25(OH)$_2$D ratios were lower at admission and 24 h than in healthy foals ($p = 0.01$; Table 1).

3.3 | VDR, CYP27B1, CYP24A1, TLR-4, TNF-α, and IL-1β mRNA Expression and Disease Severity

On admission, VDR and CYP27B1 mRNA expression was lower in septic and SNS compared with healthy foals ($p < 0.002$; Figure 2A,B). No differences in CYP24A1 mRNA

TABLE 1 | Serum 25(OH)D, 1,25(OH)$_2$D, PTH, β-defensin-1, and cathelicidin-1 concentrations and ratios in healthy and hospitalized foals. Values are expressed as median with range.

Times	Variables	Healthy ($n = 26$)	Hospitalized ($n = 83$)
25(OH)D (ng/mL)			
0		7.9 (4.3–20.3)	6.7 (2.1–11.2)[a]
24		6.3 (3.8–9.7)	6.9 (4.2–13.9)
48		6.2 (3.4–7.2)	6.6 (4–11.6)
72		5.5 (4.5–8.5)	7.3 (3.6–12.8)
1,25(OH)$_2$D (pmol/L)			
0		7.8 (5.1–14.8)	9.1 (4–21)
24		8.4 (6.9–19.1)	8.8 (3.8–22.7)
48		13.1 (8.2–25.2)	8.7 (3.5–21.6)
72		13.8 (6.3–20.8)	8.6 (5.4–34.4)
DBP (μg/mL)			
0		30.9 (14.4–71.4)	9.3 (2.9–48.3)[a]
24		33.4 (8.1–50.1)	11.9 (0.3–47.1)[a]
48		41.1 (28.8–49.2)	13.3 (1.2–41.5)[a]
72		40.2 (12.1–50.8)	12.5 (1.6–49.5)[a]
PTH (pg/mL)			
0		44.7 (0.5–544)	89.8 (10.7–1373)[a]
24		47.7 (7.3–371.6)	77.1 (10.8–1069)
48		42.8 (13.1–479)	58.8 (10.5–501.7)
72		46.4 (4.2–185.4)	40.8 (7.2–199.4)
β-Defensin-1 (pg/mL)			
0		142.9 (2.1–1540)	5.4 (0.1–932)[a]
24		375.1 (8.1–1478)	14.2 (2.1–909)[a]
48		229.1 (4.9–1480)	13.9 (0.6–881.7)[a]
72		201.8 (8–1418)	25.1 (0.4–819.5)[a]
Cathelicidin-1 (pg/mL)			
0		1091 (238.2–1665)	286.2 (60.2–1451)[a]
24		1074 (265.8–1649)	393.9 (98.1–1710)[a]
48		1085 (284.5–1571)	367.3 (85–1575)[a]
72		1087 (357.3–1545)	323 (116.1–1681)[a]

(Continues)

TABLE 1 | (Continued)

Times	Variables	Healthy ($n=26$)	Hospitalized ($n=83$)
β-Defensin-1/1,25(OH)$_2$D ratio			
0		16.5 (0.5–198.3)	0.6 (0.01–150.8)[a]
24		41.5 (6.5–213.1)	0.9 (0.2–136.4)[a]
48		19.6 (4.3–111.1)	1.2 (0.2–125.3)
72		20.4 (6.3–67.8)	2.5 (0.03–84.8)
Cathelicidin-1/1,25(OH)$_2$D ratio			
0		146 (21.8–207.3)	34.7 (5.2–307)[a]
24		143 (72.2–198.4)	48.8 (9.3–249.7)[a]
48		102.3 (55.5–119.1)	47.4 (8.1–286.9)
72		104.4 (72.3–147.9)	41.2 (5–253.3)

Abbreviations: 1,25(OH)$_2$D, 1,25-dihydroyvitamin D; 25(OH)D, 25-hydroxyvitamin D; DBP, vitamin D binding protein; *n*, number; PTH, parathyroid hormone; SNS, sick nonseptic.
[a]Indicates statistically different between the same time points of different groups, $p<0.05$.

expression were identified on admission in any group comparison (Figure 2C). On admission, septic foals had higher TLR-4 and TNF-α mRNA expression than healthy and SNS foals ($p<0.003$; Figure 2D,E). On admission, IL-1β mRNA expression was significantly higher in septic than healthy foals ($p=0.04$; Figure 2F).

3.4 | Correlations Between Serum 25(OH)D, 1,25(OH)$_2$D, DBP, PTH, β-Defensin-1 and Cathelicidin-1 Concentrations in Hospitalized Foals

In hospitalized foals, no significant correlations were identified among 1,25(OH)$_2$D, DBP, and PTH concentrations but β-defensin-1 concentrations were positively correlated with 25(OH)D concentrations at 24 h (rs = 0.4; $p=0.04$). In septic foals, β-defensin-1 concentrations were positively correlated with 25(OH)D concentrations at 24 h (rs = 0.41; $p=0.04$), whereas serum cathelicidin-1 concentrations were positively correlated with 25(OH)D concentrations 72 h after admission (rs = 0.5; $p=0.03$).

In septic foals, VDR mRNA expression was negatively associated with TLR-4 and TNF-α mRNA gene expression at admission (rs = −0.5; $p=0.03$; rs = −0.4; $p=0.04$, respectively). No significant correlations were found among 1,25(OH)$_2$D, DBP, and PTH concentrations in septic foals. No significant correlations were identified in SNS foal between serum 25(OH)D, 1,25(OH)$_2$D, DBP, PTH, β-defensin-1 and cathelicidin-1 concentrations and mRNA gene expression.

3.5 | Serum 25(OH)D, 1,25(OH)$_2$D, DBP, PTH, β-Defensin-1, and Cathelicidin-1 Concentrations and Ratios in Nonsurviving and Surviving Hospitalized Foals

On admission, but not during hospitalization, nonsurviving foals had lower serum 25(OH)D and cathelicidin-1

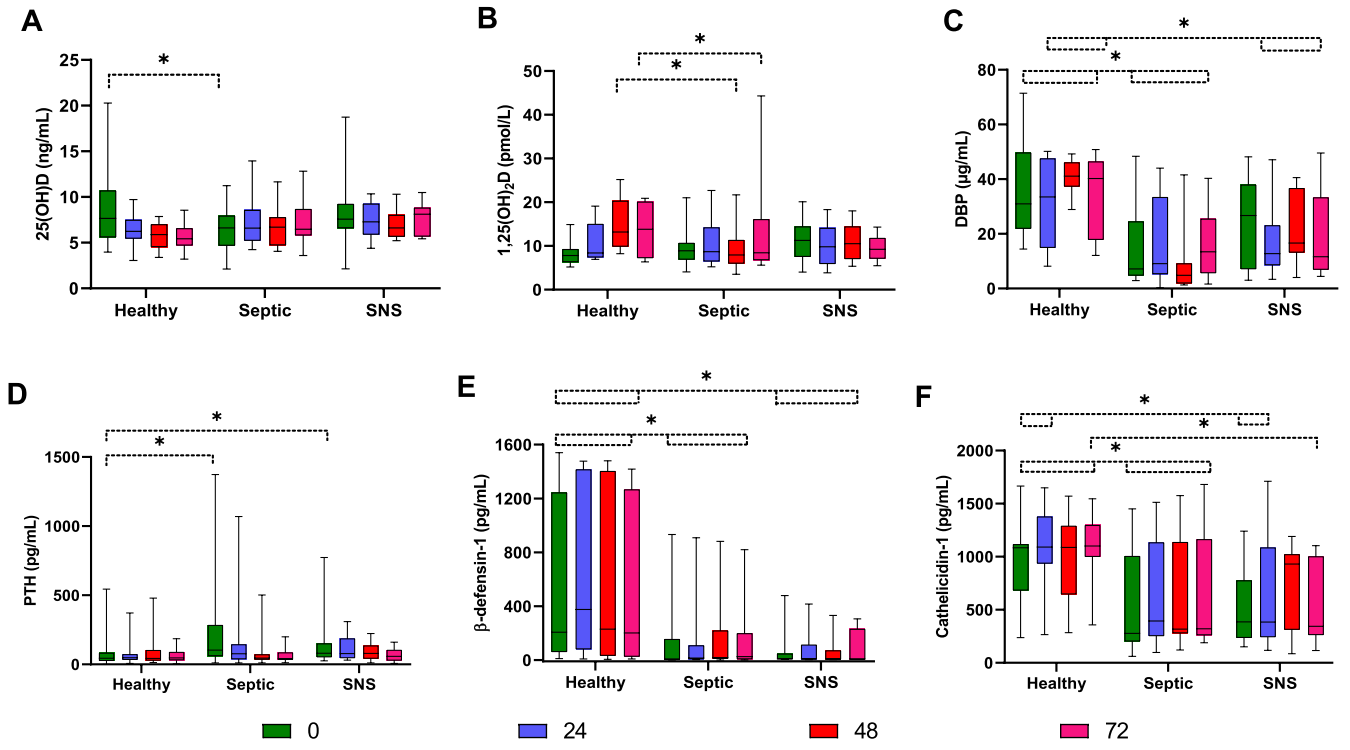

FIGURE 1 | (A–F) Serum 25(OH)D, 1,25(OH)$_2$D, DBP, PTH, β-defensin-1, and cathelicidin-1 concentrations in healthy, SNS, and septic foals. Values are expressed as median and range. * Indicates significance from healthy foals overtime. $p<0.05$. 25(OH)D, 25-hydroxyvitamin D, 1,25(OH)$_2$D;1,25-dihydroxyvitamin D; DBP, vitamin D binding protein; PTH, parathyroid hormone.

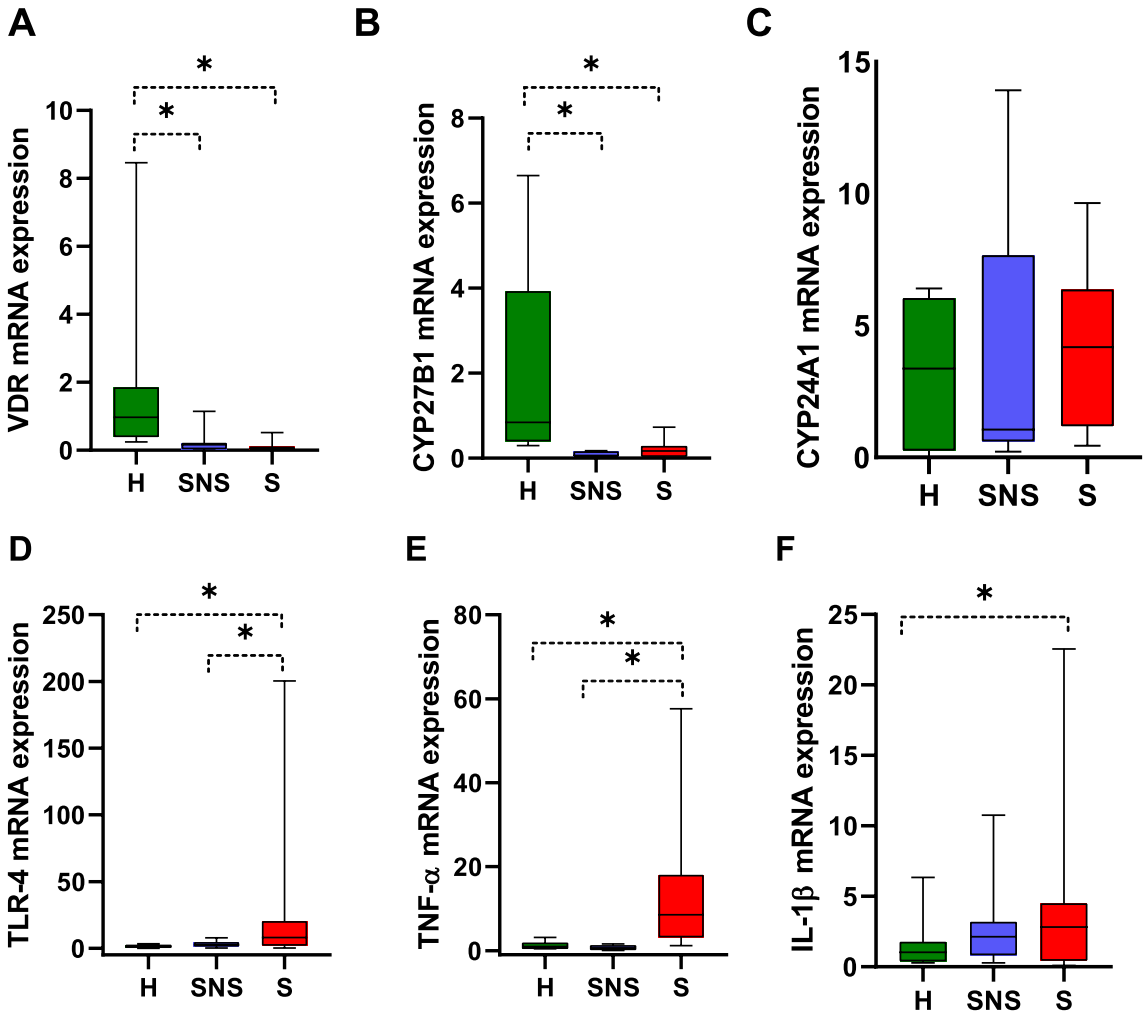

FIGURE 2 | Admission values of VDR, CYP27B1, CYP24A1, TLR-4, TNF-α, and IL1β mRNA expression relative to GAPDH in healthy, sick nonseptic (SNS) and septic foals. Values are expressed as median and range. (A, B) Septic and SNS foals had significantly lower VDR and CYP27B1 mRNA expression than healthy foals. (C) CYP24A1 mRNA expression was not different between groups of foals. (D, E) Septic foals had higher TLR-4 and TNF-α mRNA expression than healthy and SNS ones. (F) IL-1β mRNA expression was significantly increased in septic foals than healthy foals. *$p < 0.05$. CYP24A1, 24-hydroxylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase;H, healthy; IL-1β, interleukin-1β; S, septic, VDR, vitamin D receptor, CYP27B1, 1α-hydroxylase; SNS, sick nonseptic; TLR-4, toll-like receptor-4; TNF-α, tumor necrosis factor-α.

concentrations than survivors ($p = 0.04$; Table 2). On admission, but not during hospitalization, nonsurviving foals had higher PTH concentrations than survivors ($p = 0.02$; Table 2), but differences were not detected between groups during hospitalization ($p = 0.3$; Table 2). Serum $1,25(OH)_2D$, DBP, and β-defensin-1 concentrations and ratios were not different on admission or during hospitalization between survivors and nonsurvivors (Table 2).

On admission, but not during hospitalization, septic nonsurviving foals had lower serum 25(OH)D concentrations ($p = 0.04$; Table 2) and higher PTH concentrations than surviving foals ($p = 0.01$; Table 2). At 24 h of hospitalization, nonsurviving septic foals had lower serum β-defensin-1 concentrations than surviving foals ($p = 0.01$; Table 2).

Nonsurviving septic foals had lower serum cathelicidin-1 concentrations at admission and 24 h than surviving foals ($p = 0.03$; Table 2). Nonsurviving septic foals had lower β-defensin-1/$1,25(OH)_2D$ ratios at admission and over time than surviving foals ($p = 0.003$; Table 2). Cathelicidin-1/$1,25(OH)_2D$ ratios were not significantly different between nonsurviving septic foals and surviving ones ($p = 0.3$; Table 2).

3.6 | Sensitivity and Specificity of 25(OH)D, $1,25(OH)_2D$, DBP, PTH, β-Defensin-1, and Cathelicidin-1 Concentrations and Ratios to Differentiate Surviving From Nonsurviving Foals

Serum 25(OH)D concentrations < 6.7 ng/mL had 72% sensitivity and 60% specificity to discriminate surviving from nonsurviving hospitalized foals (area under the curve [AUC] = 0.67, $p = 0.02$; Table 3), whereas serum PTH concentrations > 88.1 pg/mL had 65% sensitivity and 60% specificity to discriminate surviving from nonsurviving foals (AUC] = 0.69, $p = 0.04$; Table 3). The sensitivity and specificity of serum $1,25(OH)_2D$, DBP, β-defensin-1, and cathelicidin-1 concentrations and ratios to discriminate surviving from nonsurviving foals are presented in Table 3.

TABLE 2 | Serum 25(OH)D, 1,25(OH)$_2$D, DBP, PTH, β-defensin-1, and cathelicidin-1 concentrations and ratios in surviving and nonsurviving hospitalized and septic foals. Values are expressed as median with range.

Variables	Hospitalized ($n = 83$)		Septic ($n = 60$)	
	Survivors	Nonsurvivors	Survivors	Nonsurvivors
Time	($n = 57$)	($n = 26$)	($n = 37$)	($n = 23$)
25(OH)D (ng/mL)				
0	7.3 (3.5–18.7)	5.3 (2.1–10.9)[a]	6.9 (3.5–11.2)	5.1 (2.1–10.9)[a]
24	7.1 (4.2–10.3)	6.2 (4.6–13.9)	6.5 (4.2–10.2)	6.8 (4.6–13.9)
48	6.6 (4.1–11.6)	7.2 (4.1–8.3)	6.6 (4–11.6)	7 (4.1–8.3)
1,25(OH)$_2$D (pmol/L)				
0	9 (4–21)	9.3 (4.2–20.1)	8.9 (4–21)	9.1 (4.2–18.5)
24	9.1 (3.8–22.7)	8.6 (6.8–16.5)	9 (5.2–22.7)	8.6 (6.8–16.5)
48	8 (3.5–21.6)	9.4 (4.4–14.4)	7.7 (3.5–21.7)	9.1 (4.4–12.2)
DBP (μg/mL)				
0	10.6 (3.2–48.3)	7.8 (2.9–40.7)	9.3 (3.2–48.3)	7.5 (2.9–40.7)
24	10.9 (0.3–47.1)	20.7 (4.9–42.8)	12.7 (3.3–47.1)	21.1 (14.5–42.8)
48	13.8 (1.2–41.5)	10.4 (2.1–34.7)	23.9 (1.2–41.5)	16.8 (4–34.7)
PTH (pg/mL)				
0	81.4 (10.7–1179)	166 (25.4–1373)[a]	83.4 (10.7–1179)	169.1 (25.4–1373)[a]
24	80.5 (10.8–1069)	60.9 (24.8–266.5)	81.4 (10.8–1069)	59.5 (24.8–226.5)
48	59.4 (10.5–501.7)	42.8 (30–118.8)	49.6 (10.6–501.7)	40.5 (30–118.8)
β-Defensin-1 (pg/mL)				
0	6.1 (0.1–932)	4.5 (1.8–646.3)	7.2 (0.4–932)	4.6 (1.8–646.3)
24	17.9 (2.1–909)	6.6 (3–34.4)	39.2 (2.1–909)	6.7 (3–34.4)[a]
48	15.4 (0.6–881.7)	12.2 (5.5–470.4)	25.3 (0.6–881.7)	15 (5.5–470.4)
Cathelicidin-1 (pg/mL)				
0	330.4 (63.3–1240)	233.6 (60.2–1451)[a]	319.6 (63.3–1196)	220.3 (60.1–1451)[a]
24	424.3 (117.7–1710)	363.5 (98.1–1448)	955.6 (124.4–1512)	346.9 (98.1–1148)[a]
48	367.3 (85–1575)	492.1 (198.7–1145)	319.8 (120.4–1575)	296.1 (198.7–1145)
β-Defensin-1/1,25(OH)$_2$D ratio				
0	0.6 (0.01–150.8)	0.5 (0.2–63.6)	3.5 (0.1–150.8)	0.6 (0.2–63.6)[a]
24	1.3 (0.2–136.4)	0.7 (0.2–3.9)	5.3 (0.3–136.4)	0.8 (0.2–3.9)[a]
48	1.2 (0.2–125.3)	1.5 (0.5–38.2)	9.2 (0.4–125.3)	2.2 (0.5–38.1)[a]
Cathelicidin-1/1,25(OH)$_2$D ratio				
0	36.4 (5.2–307)	28.4 (6.8–111.7)	37.1 (5.2–258.6)	28.4 (6.8–111.7)
24	54.8 (10.1–249.7)	48 (9.3–166.9)	44.3 (10.1–249.7)	39.2 (9.3–166.9)
48	47.2 (8.1–286.9)	56.2 (19.9–130.5)	40.8 (12.4–286.9)	64.9 (19.9–130.5)

Abbreviations: 1,25(OH)$_2$D, 1,25-dihydroyvitamin D; 25(OH)D, 25-hydroxyvitamin D; DBP, vitamin D binding protein; *n*, number; PTH, parathyroid hormone.
[a]Indicates statistically different between the same time points of different groups, $p < 0.05$.

TABLE 3 | Sensitivity, specificity, and ROC-AUC of biomarkers to discriminate surviving from nonsurviving hospitalized foals and septic foals.

Biomarker	Cut off	Sensitivity (%)	Specificity (%)	AUC	95% CI	*p*
Hospitalized foals						
25(OH)D (ng/mL)	< 6.7 ng/mL	72	60	0.67	41.2–86.6	0.02
$1,25(OH)_2D$ (pmol/L)	< 9.2	50	52	0.5	40.1–65.2	0.9
DBP (μg/mL)	< 10.6	57	56	0.63	36.7–74.2	0.1
PTH (pg/mL)	> 88.1	65	60	0.69	44.1–68.2	0.04
β-Defensin-1 (pg/mL)	< 5.2	55.5	59.1	0.59	45.1–71.7	0.2
Cathelicidin-1 (pg/mL)	< 254.7	52.3	59.2	0.62	32.3–71.6	0.06
β-Defensin-1/$1,25(OH)_2D$ ratio	< 0.56	43	52	0.59	23.1–66.8	0.2
Cathelicidin-1/$1,25(OH)_2D$ ratio	< 34.8	58	53	0.61	36.1–78.2	0.1
Septic foals						
25(OH)D (ng/mL)	< 7.01	78.9	60	0.68	54.7–87.5	0.03
$1,25(OH)_2D$ (pmol/L)	< 8.8	51.2	55	0.51	36.3–65.2	0.8
DBP (μg/mL)	< 9.8	58.3	53.4	0.59	34.6–73.1	0.4
PTH (pg/mL)	> 145.2	67	65	0.69	41.3–78.6	0.02
β-Defensin-1 (pg/mL)	< 6.1	69	64	0.71	44.7–79.2	0.01
Cathelicidin-1 (pg/mL)	< 266.2	75	64	0.69	51.3–84.2	0.03
β-Defensin-1/$1,25(OH)_2D$ ratio	< 0.8	73	60	0.67	49.7–81.9	0.04
Cathelicidin-1/$1,25(OH)_2D$ ratio	< 33.8	59.8	58.6	0.6	47.2–74.4	0.2

Abbreviations: $1,25(OH)_2D$, 1,25-dihydroyvitamin D; 25(OH)D, 25-hydroxyvitamin D;AUC, area under the curve; CI, confidence interval; DBP, vitamin D binding protein; PTH, parathyroid hormone; ROC-AUC, receiver operating characteristic area under the curve.

The sensitivity and specificity of 25(OH)D, $1,25(OH)_2D$, DBP, PTH, β-defensin-1, and cathelicidin-1 concentrations and ratios to differentiate surviving from nonsurviving septic foals are presented in Table 3.

3.7 | VDR, CYP27B1, CYP24A1, TLR-4, and Cytokine mRNA Expression in Hospitalized Nonsurviving and Surviving Foals

The mRNA expression for VDR, CYP24A1, IL-1β, and CYP27B1 was not significantly different between hospitalized nonsurviving and surviving foals on admission ($p = 0.1$, $p = 0.5$, $p = 0.8$, and $p = 0.2$, respectively). However, nonsurviving hospitalized foals had higher mRNA expression of TLR-4 ($p = 0.03$, Figure 3A) and TNF-α ($p = 0.001$, Figure 3B).

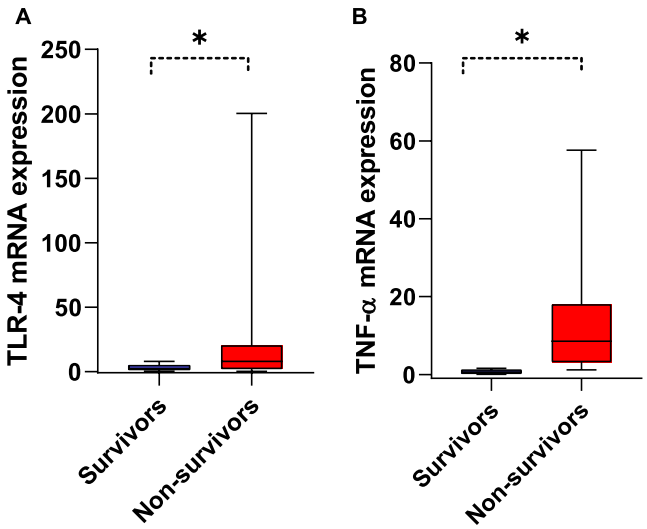

FIGURE 3 | Admission values of TLR-4 and TNF-α mRNA expression in hospitalized survivors and nonsurvivors. (A) Hospitalized nonsurvivors had higher TLR-4 mRNA expression than survivors. (B) Hospitalized nonsurvivors had higher TNF-α mRNA expression than survivors. Values are expressed as median with range. *$p < 0.05$. TLR-4, toll-like receptor-4; TNF-α, tumor necrosis factor-α.

3.8 | Association of Serum 25(OH)D, $1,25(OH)_2D$, DBP, PTH, β-Defensin-1 and Cathelicidin-1 Concentrations and Ratios With Mortality

In hospitalized foals, serum 25(OH)D, PTH, β-defensin-1, and cathelicidin-1 concentrations were associated with nonsurvival (Table 4). Serum β-defensin-1/$1,25(OH)_2D$ and cathelicidin-1/$1,25(OH)_2D$ ratios were significantly associated with nonsurvival in hospitalized foals (Table 4).

In septic foals, no associations were found between serum 25(OH)D, $1,25(OH)_2D$, DBP, and PTH concentrations and the likelihood of death (Table 5). Serum β-defensin-1 and

TABLE 4 | Univariate logistic regression of the measured variables to determine the likelihood of mortality in hospitalized foals.

Variable (units)	Range	OR for nonsurvival	95% CI	*p*
25(OH)D (ng/mL)	5.2–20.1	Referent		
	< 5.2	3.2	1.1–9.5	0.03
$1,25(OH)_2D$ (pmol/L)	5.1–14.2	Referent		
	< 5.1	0.8	0.1–11.6	0.9
DBP (μg/mL)	18.2–69.3	Referent		
	< 18.2	0.3	0.1–1.3	0.6
PTH (pg/mL)	2.2–441	Referent		
	> 441.5	3.4	1.2–13.3	0.04
β-Defensin-1 (pg/mL)	10.4–1538	Referent		
	< 10.4	4.5	1.1–22.8	0.04
Cathelicidin-1 (pg/mL)	238.2–1552	Referent		
	< 238.2	2.6	1.02–7.7	0.04
β-Defensin-1/$1,25(OH)_2D$ ratio	4.4–153	Referent		
	< 4.4	7.7	1.3–64.5	0.02
Cathelicidin-1/$1,25(OH)_2D$ ratio	21.8–179	Referent		
	< 21.8	3.5	1.1–11.2	0.03

Abbreviations: $1,25(OH)_2D$, 1,25-dihydroyvitamin D;25(OH)D, 25-hydroxyvitamin D; B, regression coefficients; CI, confidence interval; DBP, vitamin D binding protein; OR, odds ratios; PTH, parathyroid hormone; S.E, standard error.

TABLE 5 | Univariate logistic regression of the measured variables to determine the likelihood of mortality in septic foals.

Variable (units)	Range	OR for nonsurvival	95% CI	*p*
25(OH)D (ng/mL)	5.2–20.1	Referent		
	< 5.2	2.3	0.7–7.2	0.2
$1,25(OH)_2D$ (pmol/L)	5.1–14.2	Referent		
	< 5.1	1.5	0.1–26.4	0.8
DBP (μg/mL)	18.2–69.3	Referent		
	< 18.2	0.5	0.1–2.1	0.3
PTH (pg/mL)	2.2–441	Referent		
	> 441.5	3	0.8–13	0.1
β-Defensin-1 (pg/mL)	10.4–1538	Referent		
	< 10.4	5.3	1.03–27.5	0.04
Cathelicidin-1 (pg/mL)	238.2–1552	Referent		
	< 238.2	3.9	1.2–13.5	0.02
β-Defensin-1/$1,25(OH)_2D$	4.4–153	Referent		
	< 4.4	10	1.1–88.2	0.03
Cathelicidin-1/$1,25(OH)_2D$	21.8–179	Referent		
	< 21.8	2.7	0.8–10.2	0.2

Abbreviations: $1,25(OH)_2D$, 1,25-dihydroyvitamin D;25(OH)D, 25-hydroxyvitamin D; B, regression coefficients; CI, confidence interval; DBP, vitamin D binding protein; OR, odds ratios; PTH, parathyroid hormone; S.E, standard error.

cathelicidin-1 concentrations were associated with mortality of septic foals (Table 5). Low serum β-defensin-1/1,25(OH)$_2$D ratio was associated with death in septic foals (Table 5).

4 | Discussion

We showed that hospitalized foals had decreased concentrations of vitamin D metabolites, DBP, β-defensin-1, and cathelicidin-1, and increased PTH concentrations. Foals with the lowest concentrations of 25(OH)D, β-defensin-1, and cathelicidin-1, and highest PTH concentrations had more severe disease and were more likely to die. We found that foals with decreased concentrations of vitamin D metabolites and DBP often had decreased concentrations of antimicrobial peptides, suggesting a protective role for vitamin D in equine neonates.

The decreased vitamin D metabolite and antimicrobial peptide concentrations in hospitalized foals were similar to those reported in critically ill humans [34, 35]. Mechanisms leading to decreased concentrations of vitamin D metabolites in hospitalized newborn foals are likely multifactorial, including low DBP concentrations, intestinal or renal losses, increased proinflammatory cytokines, PTH resistance, increased FGF-23 concentrations, increased 24-hydroxylase activity, decreased hepatic synthesis of 25(OH)D, and decreased renal production of 1,25(OH)$_2$D [5, 14, 36–38].

In critically ill humans, low plasma DBP concentrations are linked to sepsis, organ failure, hypovitaminosis D, and mortality [36, 39]. Similarly, we showed that serum DBP concentrations were lower in hospitalized foals. Vitamin D binding protein is relatively small, and low blood concentrations are reported in humans with hypoproteinemia from gastrointestinal disease or acute kidney injury [22], which are also common in sick foals [40, 41]. In addition to transporting vitamin D metabolites, DBP also has protective actions. It binds monomeric actin during cell injury to prevent its polymerization and subsequent endothelial damage, vascular obstruction, and organ dysfunction [42, 43]. Vitamin D binding protein also binds endotoxins frequently present in the circulation of critically ill hospitalized foals and horses [2, 3, 44], suggesting that low DBP concentrations could worsen disease severity in hospitalized foals.

Excessive actions of vitamin D metabolites are prevented by inactivation at the target cells by 24-hydroxylase (CYP24A1) [38]. The mRNA expression of CYP24A1 did not differ between healthy and hospitalized foals, suggesting that decreased 1,25(OH)$_2$D synthesis rather than increased inactivation is a major factor for low 1,25(OH)$_2$D concentrations in hospitalized foals. Upregulation of inflammatory cytokines in hospitalized foals could alter the expression of enzymes involved in the synthesis of vitamin D metabolites [45, 46]. Leukocytes from hospitalized foals had lower VDR and CYP27B1 but higher TLR-4, TNF-α, and IL-1β mRNA expression than healthy foals. This finding suggests that increased proinflammatory cytokines could contribute to decreasing VDR and CYP27B1 mRNA expression and interfere with the immunomodulatory actions of vitamin D [47, 48]. The contribution of leukocytes and epithelial cells to 1,25(OH)$_2$D concentrations in foals is unknown because it is also produced by the kidneys.

Low vitamin D concentrations likely worsen critical illness in foals because vitamin D decreases expression of proinflammatory cytokines [49, 50]. Decreased vitamin D concentrations were associated with increased TLR-4 and inflammatory cytokine expression in human patients and in vitro, which is similar to our findings, supporting the hypothesis that hypovitaminosis D could worsen systemic inflammation by increasing TLR-4 and inflammatory cytokine gene expression in sick foals [51–53].

In hospitalized foals upon admission, increased PTH concentrations along with decreased 1,25(OH)$_2$D concentrations could indicate PTH resistance. Factors contributing to this potential resistance could be abnormal renal function, hyperphosphatemia, hypomagnesemia, increased FGF-23/klotho axis activity, and pro-inflammatory factors [54]. These abnormalities have been reported in hospitalized foals [5, 14]. However, we did not explore PTH signaling in the affected foals. The increased PTH concentrations in the sick foals of our study were likely a response to hypocalcemia, a condition previously reported in critically ill foals [5, 7]. Additionally, it is unlikely that 1,25(OH)$_2$D contributed to the increased PTH concentrations, because no significant differences in 1,25(OH)$_2$D concentrations were identified upon admission in the hospitalized foals, whereas a previous study had documented hypovitaminosis D in hospitalized septic foals [5].

Low concentrations of β-defensin-1 and cathelicidin-1 in sick foals could result from low vitamin D concentrations and increased proinflammatory cytokines [27, 55]. Vitamin D through the VDR binds to vitamin D-responsive elements upstream of the β-defensin and cathelicidin-1 genes to indirectly promote its antibacterial actions [27, 55]. In our study, serum concentrations of β-defensin-1, cathelicidin-1, and 25(OH)D were lower in hospitalized foals, suggesting that hypovitaminosis D likely led to decreased β-defensin-1 and cathelicidin-1 synthesis. Inflammatory cytokines decrease the mRNA expression of VDR and 1α-hydroxylase (CYP27B1), potentially impairing antimicrobial peptide synthesis by immune cells. This mechanism may explain findings in the hospitalized foals in our study, which had increased mRNA expression of proinflammatory cytokines along with lower mRNA expression of VDR and 1α-hydroxylase [45, 56]. A recent study in horses showed that the VDR-vitamin D axis modulates pulmonary immunity [57], providing evidence for the immunomodulatory role of vitamin D in this species.

Similar to our results, hypovitaminosis D has been associated with mortality in sick foals, cats, and dogs [5, 54, 58], implying that vitamin D is protective against neonatal illnesses in horses [5, 59]. Several clinical trials in humans have demonstrated the benefits of vitamin D supplementation during critical illness [60, 61]. However, the therapeutic effects of vitamin D supplementation in sick foals are yet to be determined.

5 | Conclusion

In hospitalized foals, decreased serum concentrations of vitamin D metabolites, DBP, antimicrobial peptides, and increased serum PTH concentrations are linked to disease severity and outcome. Low serum concentrations of vitamin D metabolites and antimicrobial peptides suggest that vitamin D has protective actions against perinatal diseases in horses. Increased expression

of TLR-4, TNF-α, and IL-1β, coupled with decreased VDR and CYP27B1 mRNA expression in peripheral leukocytes indicates that cytokine-mediated disruption in vitamin D signaling in immune cells may interfere with its protective actions. These findings suggest that evaluating the therapeutic potential of vitamin D supplementation in sick foals could have clinical value.

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Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

Approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University (Protocol # 2008A0170-R4). Authors declare human ethics approval was not needed for this study.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.