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Increased α-tocopherol metabolism in horses with equine neuroaxonal dystrophy

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Abstract

Background: Equine neuroaxonal dystrophy/equine degenerative myeloencephalopathy (eNAD/EDM) is an inherited neurodegenerative disorder associated with a vitamin E deficiency within the first year of life. Vitamin E consists of 8 isoforms metabolized by the CYP4F2 enzyme. No antemortem diagnostic test currently exists for eNAD/EDM.

Hypothesis/Objectives: Based on the association of α-tocopherol deficiency with the development of eNAD/EDM, we hypothesized that the rate of α-tocopherol, but not γ-tocopherol or tocotrienol metabolism, would be increased in eNAD/EDM-affected horses.

Animals: Vitamin E metabolism: Proof of concept (POC) study; eNAD/EDM-affected (n = 5) and control (n = 6) horses. Validation study: eNAD/EDM-affected Quarter Horses (QHs; n = 6), cervical vertebral compressive myelopathy affected (n = 6) horses and control (n = 29) horses. CYP4F2 expression and copy number: eNAD/EDM-affected (n = 12) and age- and sex-matched control (n = 11-12) horses.

Abbreviations: CI, confidence interval; CSF, cerebrospinal fluid; CVCM, cervical vertebral compressive myelopathy; CYP, cytochrome P450; ddPCR, droplet digital PCR; EDM, equine degenerative myeloencephalopathy; eNAD, equine neuroaxonal dystrophy; EPM, equine protozoal myeloencephalitis; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDL, low density lipoproteins; PM, postmortem; QH, Quarter Horse; qRT-PCR, quantitative reverse-transcriptase PCR; RNA, ribonucleic acid; RRR-α-TP, natural (or -d) α-tocopherol; TOH, tocopherol; TOT, tocotrienol; TTPA, tocopherol-associated transfer protein (alpha); UDP-glucuronosyltransferase, uridine 5′-diphospho-glucuronosyltransferase; VitE, vitamin E; VLDL, very low-density lipoproteins; α-TOH, α-tocopherol.

Methods: The rates of α-tocopherol/tocotrienol and γ-tocopherol/tocotrienol metabolism were assessed in equine serum (POC and validation) and urine (POC only) using liquid chromatography tandem mass spectrometry (LC-MS/MS). Quantitative reverse-transcriptase PCR (qRT-PCR) and droplet digital (dd)-PCR were used to assay expression and genomic copy number of a *CYP4F2* equine ortholog.

Results: Metabolic rate of α-tocopherol was increased in eNAD/EDM horses (POC, $P < .0001$; validation, $P = .03$), with no difference in the metabolic rate of γ-tocopherol. Horses with eNAD/EDM had increased expression of the *CYP4F2* equine orthologue ($P = .02$) but no differences in copy number.

Conclusions and Clinical Importance: Increased α-tocopherol metabolism in eNAD/EDM-affected QHs provides novel insight into alterations in vitamin E processing in eNAD/EDM and highlights the need for high-dose supplementation to prevent the clinical phenotype in genetically susceptible horses.

KEYWORDS

ataxia, cytochrome P450, equine degenerative myeloencephalopathy, genetics, vitamin E

1 | INTRODUCTION

Equine neuroaxonal dystrophy/equine degenerative myeloencephalopathy (eNAD/EDM) is an inherited neurodegenerative disease associated with vitamin E (vitE) deficiency during the first year of life.[1,2] It can be prevented in genetically susceptible foals by supplementing dams with high doses of water-soluble RRR-α-tocopherol (α-TOH), a form of vitE, during the last trimester of gestation and supplementing foals through the first 2 years of life.[3] Clinical signs include symmetric ataxia (≥grade 2/5), wide-base stance at rest, proprioceptive deficits, and decreased serum α-TOH concentrations.[2-4] Even as they age, serum α-TOH concentrations typically remain decreased in eNAD/EDM-affected horses compared to age-matched healthy controls.[5] These clinical signs mimic those of cervical vertebral compressive myelopathy (CVCM), trauma and equine protozoal myeloencephalitis (EPM). Although EPM[6] and trauma can be diagnosed using antemortem clinical tests, eNAD/EDM and CVCM may be difficult to distinguish if cervical radiographs and myelography are inconclusive.[4,7] Currently, the only way to conclusively diagnose eNAD/EDM is by postmortem histologic evaluation of the brainstem and spinal cord.[2] Although a recently developed biomarker test for phosphorylated neurofilament heavy chain (pNfH) has demonstrated some specificity for eNAD/EDM diagnosis using serum, overall sensitivity is low.[8] Additionally, the sensitivity for a diagnosis of eNAD/EDM may be breed specific, with Quarter Horses (QHs) more likely to have increased serum pNfH concentrations than Warmbloods.[8] A biochemical test based on vitE metabolism would help clients and veterinarians make management decisions, while providing useful insight into the underlying disease etiology.

Vitamin E is a class of fat-soluble vitamers subdivided into 2 groups, tocopherols (TOH) and tocotrienols (TOT), each consisting of multiple enantiomers (α, β, γ, and δ). Vitamin E metabolism has been studied extensively in humans and in mouse models.[9] Four vitE family members (α- and γ-TOH and -TOT) are mainly metabolized by cytochrome P450 family 4 subfamily F member 2 (CYP4F2), which catalyzes ω-hydroxylation of the phytyl side chain.[10] The remainder of the side chain is metabolized by β-oxidation.[10] Eventually, the water-soluble metabolites 5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid (α-CMBHC) and α- and γ-carboxyethyl hydrochroman (CEHC) are produced and excreted in the urine.[10,11] In healthy humans, there is a higher urinary excretion rate of γ-CEHC than α-CEHC, indicating the body's preference of retaining α-TOH.[12] These metabolites are excreted in the urine in conjugated and unconjugated forms. Endogenous compounds are subject to phase II metabolism by the enzymes uridine 5′-diphospho (UDP)-glucuronosyltransferases and sulfotransferases to create more water soluble, easily excreted metabolites in urine.[13] We recently developed quantification methods using liquid chromatography coupled tandem mass spectrometry (LC-MS/MS)[14,15] to measure α-CMBHC, α-CEHC, γ-CEHC, α-TOH, α-TOT, γ-TOH, and γ-TOT and associated metabolites simultaneously in equine serum, cerebrospinal fluid (CSF) and urine.

In humans, ataxia with vitamin E deficiency (AVED), an inherited disease caused by genetic mutations in tocopherol transfer protein alpha (*TTPA*), shares clinicopathologic features with eNAD/EDM.[16,17] In healthy individuals, α-CEHC is excreted in the urine after a concentration of 30 to 40 μmol/L of α-TOH is reached in the plasma.[18,19] In patients with AVED, urinary α-CEHC concentrations are increased even in the face of severe α-TOH deficiency.[19] Because hepatic α-TOH is not transported to the remainder of the body because of loss-of-function of TTP, this additional hepatic α-TOH could be metabolized by CYP4F2, resulting in aberrant vitE metabolism.[19] Although eNAD/EDM is not associated with genetic mutations in *TTPA*,[20] and previous work has demonstrated that vitE absorption[21] and transport to target tissues[3,22] are not altered, vitE metabolism has

yet to be evaluated. We aimed to measure the metabolic rate of vitE, specifically α- and γ-TOH and TOT, in eNAD/EDM-affected horses. We hypothesized that eNAD/EDM-affected horses would have higher rates of hepatic metabolism of α-TOH relative to controls. Additionally, increased expression of *CYP4F2* was hypothesized to be associated with eNAD/EDM.

2 | MATERIALS AND METHODS

2.1 | Animals

All animal procedures were approved by the UC Davis Institutional Animal Care and Use Committee (protocol #18376) and all assayed horses were owned by the UC Davis Center for Equine Health. Tissue samples for molecular work were from a biorepository of eNAD/EDM-affected and control horses, with consent obtained for all postmortem samples from client-owned horses.

2.2 | Proof of concept study (2015-2016)

2.2.1 | Animals

Horses were maintained in dry lots without access to pasture for at least 6 months before the study began. All were fed grass hay at 1.5% body weight during the study, with no additional grain or nutritional supplements. The hay had approximately 10% moisture content with 16 ppm vitE, so that every horse received approximately 180 IU/day of vitE from hay, which is below the recommended 400 to 500 IU/day for the average adult horse.[23] Samples were collected from 5 eNAD/EDM-affected horses, including 4 QHs and 1 Warmblood cross, and 6 age- and sex-matched healthy controls, including 2 QHs, 2 Thoroughbreds and 2 Thoroughbred crosses (Table S1).

All horses underwent neurologic evaluation using the modified Mayhew scale[24] before enrollment. Controls had ataxia scores of 0/5 and no known history of neurologic signs. Four of the eNAD/EDM-affected horses had postmortem-confirmed affected full siblings (horse #5) or offspring (horses #1, 2, and 3) and ataxia scores >2/5.[24] Three of these were euthanized at the end of the study (horses #1, 2, and 5) and diagnosed with eNAD/EDM based on histologic evaluation. Horse #4 had an ataxia score of 2/5, normal cervical radiographs and CSF cytology, was negative for EPM (serum and CSF), and had a pedigree that supported a diagnosis of eNAD/EDM based on postmortem-confirmed relatives.

2.2.2 | Metabolism sample collection

Serum, plasma, and CSF were collected over 9 weeks (5 eNAD cases, 6 controls; Table S1). Baseline samples were collected 1 week before supplementation. Horses were not fasted and were supplemented PO

with 10 IU/kg of liquid RRR-α-TOH (Elevate WS, Kentucky Equine Performance Products LLC, Versailles, Kentucky) every 24 hours for 4 weeks before a 4-week depletion period (Figure 1). To ensure feasibility, each horse was randomly assigned by coin toss to 1 of 2 sampling groups: 5 horses (2 cases, 3 controls) sampled in 2015 and 6 horses (3 cases, 3 controls) sampled in 2016.

On days −7 and 28, CSF was collected from anesthetized animals by atlanto-occipital tap.[3] Horses were premedicated using 1.1 mg/kg xylazine (VetOne, MWI Animal Health, Boise, Idaho); anesthesia was induced using 2.2 mg/kg ketamine (Ketaset, Fort Dodge Animal Health, Overland Park, Kansas) and 0.04 mg/kg midazolam (West-Ward, Eatontown, New Jersey) via IV catheter. Samples taken at −7 and 0 days were considered baseline samples, with 1 week allowed after anesthesia. After blood and urine collection on day 0, RRR-α-TOH was administered and blood was collected from the IV catheter and urine by sterile urinary catheterization at 0, 6, 12, and 24 hours (Figure 1). Sedation with xylazine (0.5 mg/kg IV) was used to facilitate urinary catheterization. For subsequent time points during the supplementation (days 7, 14, and 21) and depletion (days 35, 42, and 56) phases, blood was collected by direct venipuncture and urine collected by sterile catheterization. On day 28, blood was collected before and after induction of anesthesia and urine and CSF were collected under anesthesia. Samples were light-protected after collection. Urine, EDTA blood, and CSF were placed on ice until centrifugation. Blood and CSF were centrifuged at 2000*g* for 10 minutes at 4°C to separate cellular components from plasma, serum, or CSF, aliquoted, flash frozen within 2 hours of collection and stored at −80°C until chemical analysis (within 6-12 months).

2.3 | Validation study (2018)

2.3.1 | Animals

Horses were maintained and fed as described above. Samples were collected from 6 eNAD/EDM-affected QHs, including 3 repeated from the proof of concept (POC) (horses #1-3), 6 CVCM-affected and 29 age- and sex-matched healthy controls, of which 11 were QHs (Table S2).

As for the POC, horses underwent neurologic evaluation before enrollment. Controls had ataxia scores of 0/5 and no known history of neurologic signs. The 3 additional eNAD/EDM-affected horses included 2 that were subsequently confirmed postmortem (horses #4 and 5) and 1 (horse #6) that had an ataxia score of 2/5, normal cervical radiographs and CSF cytology, was negative for EPM on both serum and CSF, and had a pedigree that supported a diagnosis of eNAD/EDM based on postmortem-confirmed relatives. For the 6 CVCM cases, 5 were euthanized and CVCM was confirmed. The sixth (horse #12) was a half-sibling to a postmortem-confirmed case (horse #10), had a grade 3/5 ataxia, had normal CSF cytology, was negative for EPM on both serum and CSF and had moderate remodeling of the articular process joints of the caudal cervical vertebrae (ie, likely type 2 CVCM).[25] Because horse #12 was not postmortem
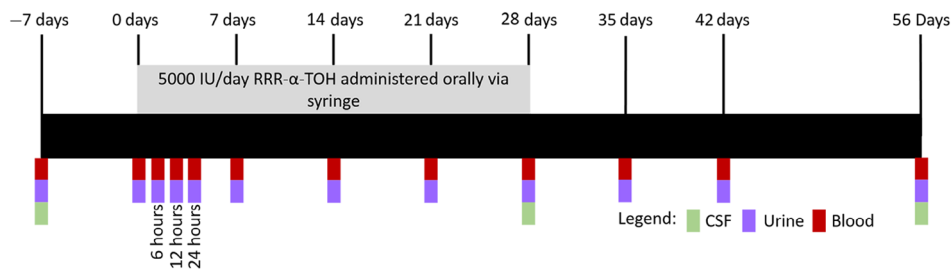
FIGURE 1   Proof of concept metabolism study design: The black bar represents the 9 weeks of the study. Baseline samples were collected at −7 days. Subsequent samples were collected immediately prior to, and every 6 hours following supplementation. For the remainder of the study, samples were collected once a week. At the 28-day sampling, blood samples were collected prior to anesthesia and during anesthesia. Sample types are indicated by colored bars below the black line

confirmed, data analyses were performed both with and without this case and results remained unchanged. Thus, horse #12 was included.

2.3.2 | Sample collection

Based on the POC study, only serum samples were collected over a shortened 24-hours time period after 1 supplementation dose. An IV catheter was placed aseptically before the start of the study for sampling. All sampling was performed from the catheter after removal of 6 mL of blood. Baseline samples (time = 0 hour) were collected directly before supplementation. Horses then were supplemented PO with 10 IU/kg of liquid RRR-α-TOH Elevate WS, Kentucky Equine Performance Products LLC, Versailles, Kentucky once. Subsequent serum samples were collected at 4, 6, 8, 12, 18, and 24 hours. All samples were light-protected after collection and processed as those of the POC study. All horses were sampled in 2018 and sampling was randomized per day using a coin toss, with at least 1 eNAD/EDM and 1 CVCM horse sampled per every 4 to 5 controls.

2.4 | Chemical analysis

Samples in both studies, including serum (POC and validation studies) and plasma and CSF (POC study) underwent processing to isolate α-TOH, α-TOT, γ-TOH, γ-TOT, and their primary and secondary metabolites, α-CMBHC, α-CEHC, and γ-CEHC. Analysis and quantification were carried out using LC-MS/MS as previously described.[14,15] Because α-CMBHC, α-CEHC, and γ-CEHC are excreted in the urine in 2 forms, conjugated (by the addition of the polar glucuronide and sulfate conjugates to the hydroxyl group of the chromanol ring,[26] creating a more water-soluble metabolite) and unconjugated,[12] both were assayed for the POC study. Unconjugated metabolites were assayed using acid hydrolysis.[15] Concentrations of all compounds were calculated in ng per mL of matrix (serum, plasma, CSF, and urine).

2.5 | Metabolism data analysis

Normality was tested on sample sets using a Shapiro-Wilk test, with $\log_{10}$ transformation for nonnormally distributed data. Data that could

not be transformed were analyzed using nonparametric tests (Spearman correlation or Wilcoxon's rank); normally distributed data were analyzed using parametric methods (linear modeling, Pearson correlations, t-tests, and 2-way analysis of variance). Post hoc testing of each time point was performed when significant differences were obtained. For the POC study, sampling year was tested and found to be insignificant. Therefore, all samples from 2015 and 2016 were analyzed together. The effect of anesthetic drug administration on vitE vitamers and metabolites was evaluated in the POC study using serum results pre- and postanesthesia. Alpha-TOH and γ-TOH concentrations were compared from day −7 to day 28 in the serum and CSF of all horses using t-test and Wilcoxon's rank tests in the POC study.

For both studies, serum metabolic ratios were used to determine if eNAD/EDM-affected horses differed in their metabolism of each vitamer. Alpha-metabolic ratio was calculated using the formula (α-CEHC + α-CMBHC)/(α-TOH + α-TOT). The γ-metabolic ratio was calculated using the formula γ-CEHC/(γ-TOH + γ-TOT). Significance was set at $P < .05$.

2.6 | Quantitative RT-PCR

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed on liver cDNA generated from a separate set of 24 horses (12 eNAD/EDM-affected horses and 12 age- and sex-matched controls [Table S3] that underwent complete postmortem central nervous system histological evaluation). Disease status at necropsy was diagnosed by a board-certified veterinary pathologist. Ribonucleic acid (RNA) was isolated using Trizol chloroform phase separation followed by cleanup (Zymo columns, Zymo Research, Irvine, California) and cDNA synthesized (SuperScriptIII, Thermo Firsher, Waltham, Massachusetts). Two loci had conserved sequence similarity to human *CYP4F2* using NCBI tBLASTn (https://blast.ncbi.nlm.nih.gov/; *LOC100062102* and *LOC100147344*). Primer3Plus https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi was used to design primers. Primers could only be designed for *LOC100062102* (XM_014734745.2; Table S4). The second locus with sequence similarity, *LOC100147344*, was too repetitive for primer design. Three housekeeping genes: *GAPDH*, *ACTβ*, and *HPRT1* were evaluated for liver expression based on efficiencies (Table S4). Samples were amplified (Brilliant III SYBR-green qPCR master mix, Agilent, Santa Clara, California) and the standard DNA

binding dye protocol run (AriaMX, Agilent, Santa Clara, California). All samples were run in triplicate, and fold change was calculated using $\Delta\Delta$Ct. An unpaired t-test was performed using Prism 8 (GraphPad, San Diego, California).

2.7 | Droplet digital PCR

Genomic DNA from the qRT-PCR horses (12 cases, 11 controls; genomic DNA from horse #15 not available) was used in this relative-quantification assay. To provide an accurate assessment of *LOC100062102* genomic copy number, a droplet digital PCR (ddPCR) assay was designed around exon 3 of *LOC100062102* genomic DNA (Table S4). Primers and probes (with 3′ Iowa Black FQ and 5′6-FAM) were designed using Integrated DNA Technologies' PrimerQuest Tool (www.idtdna.com/primerquest/home/index) and ordered from Eurofins Genomics (Louiseville, Kentucky) and Integrated DNA Technology (Coralville, Iowa), respectively. The ETS Proto-Oncogene 1, Transcription Factor (*ETS1*; Bio-Rad, Hercules, California) was used as the diploid reference for assessing copy number variation in the *LOC100062102* target assay. Reactions consisted of *ddPCR Supermix for Probes* (no dUTP), *ETS1* reference primer/probe (final concentrations of 900 and 250 nM, respectively), *LOC1000062102* target primer/probe (final concentrations of 1000 and 250 nM, respectively), *HindIII-HF* restriction enzyme (1.5 U/rxn; New England BioLabs, Ipswich, Massachusetts), and varying concentrations of DNA template in a final reaction volume of 20 μL. Droplets were generated using a QX200 Droplet Generator (Bio-Rad, Hercules, California) before PCR

amplification with an annealing temperature of 56°C, 44 amplification cycles, and 2°C/second ramp rate, quantified on the QX200 Droplet Reader (Bio-Rad, Hercules, California) and analyzed using QuantaSoft software (Bio-Rad, Hercules, California). Copy number differences were tested using an unpaired t-test (GraphPad Prism 8, San Diego, California).

3 | RESULTS

3.1 | POC study

3.1.1 | VitE vitamers and metabolites in serum vs plasma

In the POC study, serum and plasma concentrations for α-TOH (Figure 2A), α-CMBHC (Figure 2B), and α-CEHC (Figure 2C) were analyzed to determine the best matrix for measuring vitE and its metabolites. Results were highly correlated between each matrix type (r = 0.87, 0.8, and 0.7, respectively; $P < .0001$). Additionally, α-TOT, γ-CEHC, and γ-TOH measurements were moderately correlated (r = 0.38, 0.42, and 0.57, respectively; $P < .0001$; data not shown). Gamma-TOT was the only metabolite not well correlated between the 2 sample types (r = −.1, $P = .21$; data not shown). Alpha-TOH and α-CMBHC concentrations were slightly higher in the serum compared to plasma (Figure S1). Therefore, only serum results were used for the analysis of metabolic ratios and the effect of the anesthetic drugs on vitE metabolites.

FIGURE 2 Proof of concept study—Correlation between serum and plasma: Scatter plots showing significant positive correlations between serum and plasma in (A) α-TOH, (B) α-CMBHC, (C) α-CEHC. Correlation r and P-values calculated using a Pearson (A) or Spearman (B and C) correlation test. All units are in ng per mL of matrix
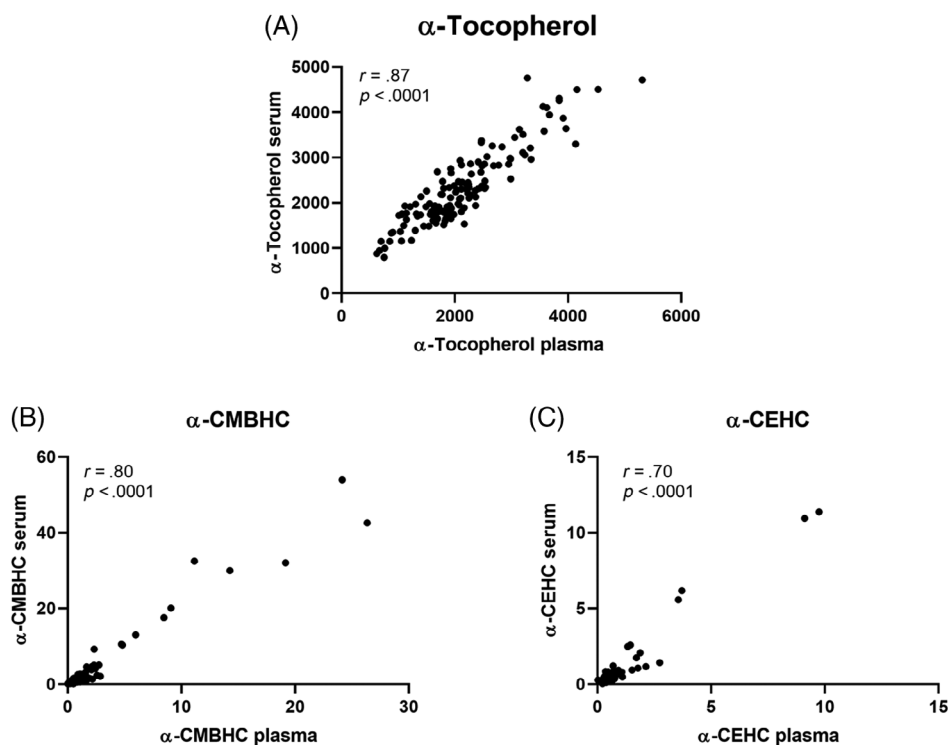
3.1.2 | Effect of anesthesia

Serum samples were used to evaluate the effect of anesthesia on vitamers and their metabolites. Gamma-TOT was undetectable in any of the pre- or postinduction 28-day samples and was excluded from analysis. The α-TOH (Figure 3A) and α-CEHC (Figure 3C) results were highly correlated between pre- and postinduction (Pearson r = 0.95, P < .0001; Spearman r = 0.89, P = .0002, respectively). Alpha-CMBHC values were moderately correlated after drug administration (Spearman r = 0.67; P = .02; Figure 3B). Gamma-TOH also was highly correlated before and after anesthesia (Spearman r = .85; P = .008; data not shown). Gamma-CEHC and α-TOT were not significantly correlated when measured pre- and postinduction (Spearman r = 0.52, P = .09; r = 0.42, P = .24; data not shown). Because of the high correlation between pre- and postinduction α-TOH and metabolite concentrations, preinduction results were used for the remainder of the analysis.

3.1.3 | Supplementation increases serum vitamer concentrations

Serum α-TOH and γ-TOH concentrations were normally distributed. All horses had significant increases in α-TOH and γ-TOH during the study (P < .0001; Figure 4A,B, respectively). For α-TOH, this increase was only present during the supplementation period. No effect of disease status was detected on circulating α-TOH or γ-TOH concentrations (P = .46 and .43, respectively). In the CSF, α-TOH (P = .02) but not γ-TOH (P = .16) concentrations significantly increased with time

in both eNAD/EDM-affected and control horses, with no effect of disease status (P = .72 and .88; Figure 4C,D, respectively). In the post hoc analysis, CSF α-TOH concentration tended to increase within 28 days in controls (P = .08) but not eNAD/EDM-affected horses (P = .23).

3.1.4 | Serum vitE metabolic ratios

Alpha-metabolic ratios were not normally distributed and subsequently were log$_{10}$ transformed. They changed significantly with time (P < .0001), disease status (P = .0013), and interaction of disease status and time (P < .0001; Figure 5A). Post hoc testing identified a significant association between eNAD/EDM disease status and α-metabolic ratio at 6, 12, and 24 hours postinitial supplementation (P < .0001). Gamma metabolic ratios were normally distributed and changed significantly with time (P < .0001) but were not affected by disease status (P = .27; Figure 5B) nor was a significant interaction found between disease status and time (P = .17). Alpha-metabolic ratios in CSF were not different between the 2 groups at either time point, or within the groups at both time points (data not shown). The CSF γ-ratios were not calculated because most CSF samples had γ-CEHC concentrations below the detectable range.

3.1.5 | Urinary metabolites

One eNAD/EDM-affected horse (Table S1, horse #4) could not be catheterized at multiple time points and was excluded from the
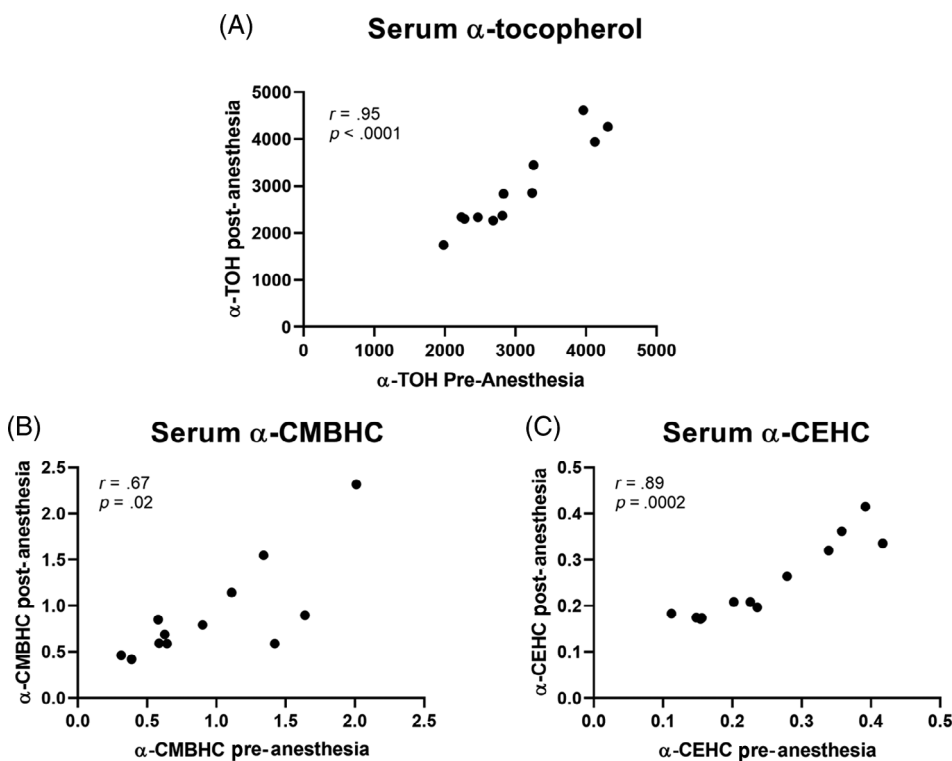
FIGURE 3 Proof of concept study—Anesthesia and measured compounds: Scatter plots showing the significant positive correlation between pre- and postinduction (A) α-TOH, (B) α-CMBHC, (C) α-CEHC. Correlation r values calculated from a Pearson (A) or a Spearman correlation (B and C). All units are in ng per mL of matrix

FIGURE 4 Proof of concept study—Circulating α- and γ-tocopherol concentrations in eNAD/EDM and control horses: Both serum α-TOH (A) and γ-TOH (B) levels increased significantly during supplementation ($P < .0001$), with no effect of disease ($P = .46$). A significant increase was observed in CSF α-TOH (C; $P = .02$), but not γ-TOH (D) concentrations between time points but not disease conditions. Post hoc analysis showed that CSF α-TOH tended to increase in the 28-day control horses only ($P = .08$). Supplementation began at 0 days. The 0.25, 0.5, and 1-day increments represent time points within the first 24 hours (ie, 6, 12, and 24 hours)
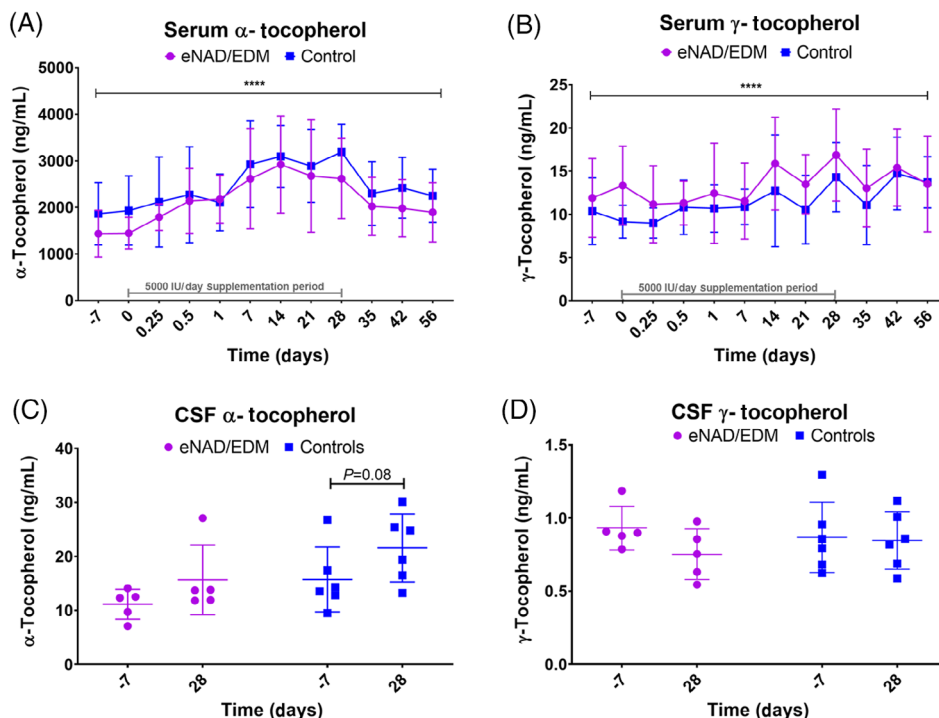
analysis. Two metabolites (α-CEHC and γ-CEHC) were successfully quantified in both hydrolyzed and nonhydrolyzed samples. Alpha-TOH and α-CMBHC were below the limits of quantification (10 and 25 ng/mL, respectively) for most samples and excluded from further analysis.

3.1.6 | Effect of anesthesia on urinary metabolites

Because samples at 28 d were taken pre- and postinduction to determine if anesthesia had any effects, correlation was assessed between unconjugated and conjugated urinary α-CEHC and γ-CEHC metabolites. Unlike serum results, unconjugated urinary α-CEHC and γ-CEHC were poorly correlated pre- and postinduction (Spearman's r = 0.68 and 0.56, respectively; $P > .05$; data not shown). Similar results were obtained for conjugated urinary α-CEHC and γ-CEHC samples pre- and postinduction (Spearman's r = 0.3 and 0.2, respectively; $P > .05$; data not shown). To match sample collection postsedation (ie, xylazine) with other time points, preinduction results were used for comparison of data in the POC study.

3.1.7 | Unconjugated urinary vitE metabolites

Unconjugated vitE metabolite concentrations were not normally distributed and subsequently were log_{10} transformed. No significant differences were found at baseline for unconjugated urinary α-CEHC and γ-CEHC concentrations between eNAD/EDM-affected and control horses. Over the 56 days, a significant effect of time ($P = .005$) but not disease ($P = .26$) was found on unconjugated urinary α-CEHC

concentrations, with an increase noted 24 hours after supplementation (Figure 6A). When evaluating the first 24-hours period of supplementation, which had more intermediary time points (times 0, 6, 12, and 24 hours), time remained significant ($P = .002$) and, although disease was not ($P = .13$), a significant time * disease interaction ($P = .004$) was found, with higher unconjugated urinary α-CEHC concentrations in eNAD/EDM-affected horses (Figure 6B). No single time points were significant after post hoc testing.

For unconjugated urinary γ-CEHC, no significant effect of time ($P = .17$) or disease ($P = .78$) was found over the 56-day course of the study (Figure 6C). Within the first 24-hours period, time and disease remained nonsignificant ($P = .59$ and $P = .66$, respectively; Figure 6D).

3.1.8 | Conjugated urinary vitE metabolites

Conjugated vitE metabolite concentrations were not normally distributed and were log_{10} transformed. No significant differences at baseline were found for conjugated urinary α-CEHC and γ-CEHC concentrations between eNAD/EDM and control horses. Over the 56 days, a significant effect of time ($P = .01$) but not disease ($P = .32$) was found on conjugated urinary α-CEHC concentrations, with an increase noted within the first 24 hours after supplementation (Figure 7A). However, when evaluating the first 24-hours period of supplementation, which had more intermediary time points (times 0, 6, 12, and 24 hours), time ($P = .16$), disease ($P = .31$), and time * disease ($P = .99$) were not significant (Figure 7B).

For conjugated urinary γ-CEHC, no significant effect of time ($P = .28$) or disease ($P = .052$) was found but time * disease was
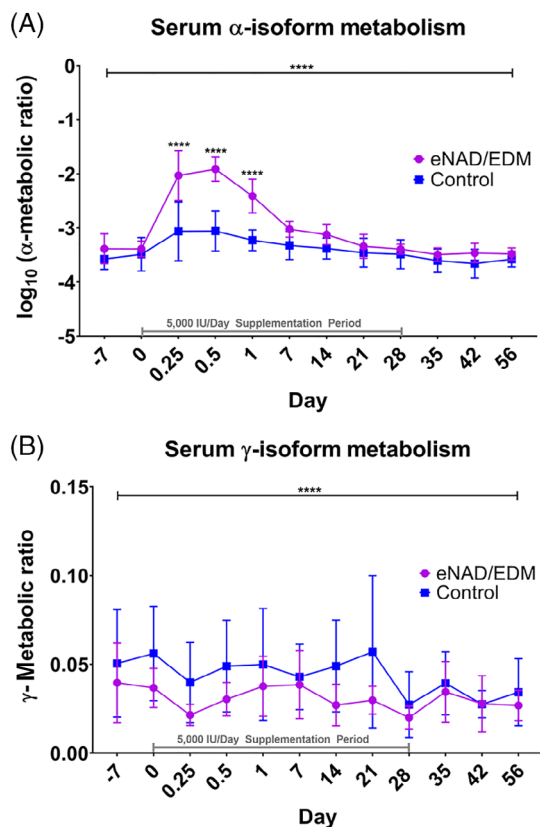
FIGURE 5 Proof of concept study—Metabolic ratios change with supplementation and α-metabolic ratio differs between eNAD/EDM and control horses: A, Alpha-metabolic ratio changed significantly over time in all groups (****$P < .0001$). Post hoc testing revealed an increase in α-metabolic ratio in eNAD/EDM-affected horses at 0.25, 0.5, and 1 day (ie, 6, 12, and 12 hours) (****$P < .0001$). Data analyzed via ANOVA after log transformation with post hoc contrasts between group means. B, The γ-metabolic ratio had significant changes over time ($P < .0001$), but there were no significant time point differences between cases and controls ($P = .27$). Supplementation began at 0 days. The 0.25, 0.5, and 1-day increments represent time points within the first 24 hours

significant ($P = .01$) over the 56-day course of the study (Figure 7C). Within the first 24 hours, time was not significant ($P = .26$), but eNAD/EDM-affected horses had significantly less conjugated γ-CEHC metabolites in their urine ($P = .04$) and a time * disease was significant ($P = .02$; Figure 7D). No single time points were significant after post hoc testing.

Together, these results suggest altered vitE isoform metabolism in eNAD/EDM-affected horses, with higher excretion of urinary free (unconjugated) α-CEHCs and lower excretion of urinary conjugated γ-CEHCs.

3.1.9 | Correlation of serum α-TOH with unconjugated urinary α-CEHCs

To compare our results from eNAD/EDM-affected horses to those identified in patients with ataxia with vitamin E deficiency (AVED),[19]

we analyzed the correlation between serum α-TOH concentrations and unconjugated urinary α-CEHC concentrations at all matched time points for eNAD/EDM-affected and control horses. Poor and comparable correlations between vitamer and metabolite were identified in control (Spearman r = 0.22; $P = .10$) and eNAD/EDM-affected (Spearman r = 0.22; $P = .13$) horses. Despite similar correlations, eNAD/EDM-affected horses had higher urinary α-CEHC concentrations at similar serum α-TOH concentrations (Figure S2).

3.2 | Validation study

Because the results from our POC study identified a significant effect of eNAD/EDM disease status on serum α-metabolic ratios at 6, 12, and 24 hours after initial supplementation, we performed a validation study that included only serum sampling within a 24-hour time window postsupplementation (time points 0, 4, 6, 8, 12, 18, and 24 hours).

3.2.1 | Serum vitE metabolic ratios

Alpha- and γ-metabolic ratios were not normally distributed and were log_{10} transformed. Alpha-metabolic ratios changed significantly with time ($P < .0001$) and disease status ($P = .03$), with no interaction of disease status and time ($P = .12$; Figure 8A). Post hoc testing identified significant differences between eNAD/EDM and CVCM at 18 and 24 h postsupplementation ($P = .03$ and $P = .02$, respectively). No significant differences were found between CVCM and control horses ($P = .13$). Gamma metabolic ratios did not differ significantly with time ($P = .23$) or disease status ($P = .46$; Figure 8B). No significant interaction between disease status and time was identified in the γ-metabolic ratios ($P = .98$). Interestingly, serum α-TOH concentrations were higher at baseline in validation study horses and increased less steeply with time (Figure S3).

Because all eNAD/EDM-affected horses in the validation study were QHs, we analyzed α-metabolic ratios in eNAD/EDM QHs (n = 6) vs a subset of 11 control QHs, with similar results (time; $P < .0001$; disease; $P = .02$) and a significant interaction was identified between time and disease ($P = .02$). Lastly, to confirm no breed effect on α-metabolic ratios, we compared control QHs to all other breeds and identified no effect of breed ($P = .29$).

3.2.2 | CYP4F2 quantitative RT-PCR

Based on differences in the α-isoform metabolic ratio, *CYP4F2* expression was evaluated in the experimental groups. Of the 3 housekeeping genes, *HPRT1* had the least variability and was selected for *LOC100062102* relative quantification. A 1.63-fold increase in hepatic expression of *LOC100062102* was identified in eNAD/EDM-affected horses compared to controls ($P = .02$; Figure 9A).

FIGURE 6 Proof of concept study—Unconjugated urinary α-, but not γ-metabolites, differ between eNAD/EDM and control horses: A, Alpha-CEHC concentrations changed significantly over time in all groups (**$P < .01$) during the 56-day trial period, with no effect of disease. However, within the first 24-hours time period (B), urinary α-CEHC concentrations demonstrated a significant interaction between time × disease (time **$P < .01$) and time × disease (**$P < .01$). C, There was no significant effect of time or disease on unconjugated urinary γ-CEHC concentrations over the entire 56-day study period or (D) within the first 24-hours period. Supplementation began at 0 days. The 0.25, 0.5, and 1-day increments represent time points within the first 24 hours

Unconjugated urinary α-CEHC

Unconjugated urinary γ-CEHC
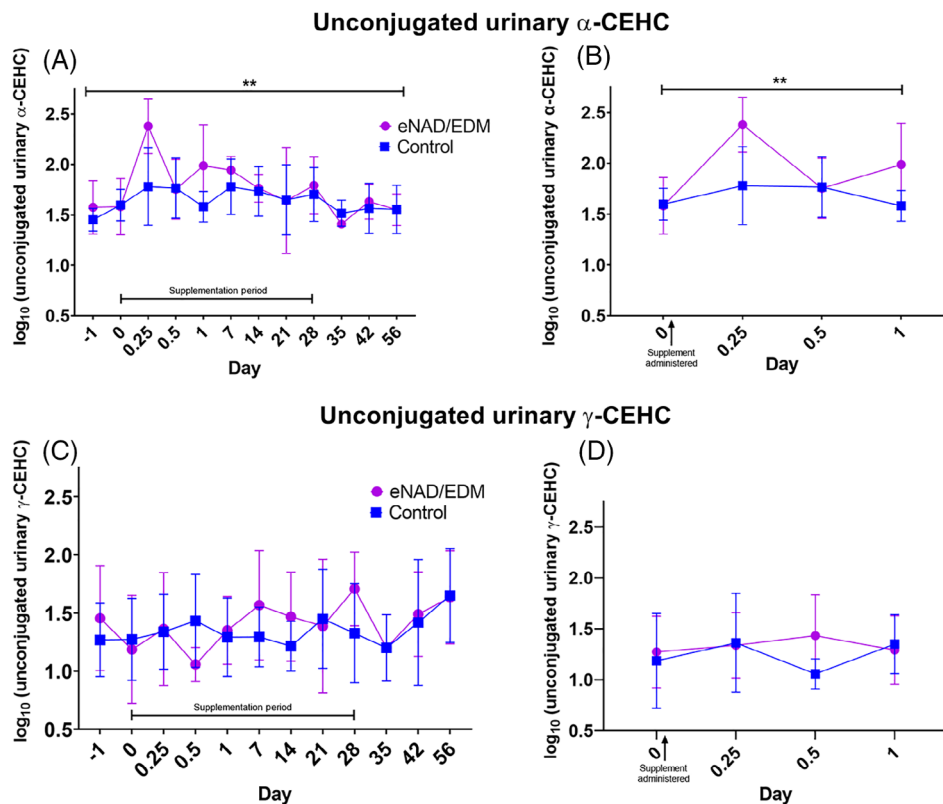
3.2.3 | CYP4F2 droplet digital PCR

To explore the possibility of a copy number variant underlying the change in *CYP4F2* expression, we conducted ddPCR of *LOC100062102* in genomic DNA. Two to 6 copies of the gene were found in both cases and controls ($P = .60$; Figure 9B).[27]

4 | DISCUSSION

Despite the long-standing association of vitE deficiency with eNAD/EDM,[27] it remains unclear why only a subset of foals maintained on a vitE deficient diet develop eNAD/EDM.[3] To date, attempts to define the mechanism by which these horses are more susceptible to vitE deficiency have failed.[20-22] We identified aberrant vitE metabolism in QHs with eNAD/EDM, defined primarily by increased α-TOH metabolism in serum and urine, after a PO dose of RRR-α-TOH. Similar to results obtained in human patients with AVED,[19] eNAD/EDM-affected horses typically had higher urinary α-CEHC concentrations at similar serum α-TOH concentrations. Additionally, increased hepatic expression of *CYP4F2*, the major metabolizer of vitE, was observed in eNAD/EDM-affected horses across breeds. We determined that this increased expression is not a result of *CYP4F2* copy number variations. Rather, it is most likely secondary to the primary genetic defect, similar to findings in AVED.[19] Enhanced metabolism of α-TOH would lead to a higher α-TOH requirement in eNAD/EDM-affected horses. This hypothesis is further supported clinically, because eNAD/EDM

can be prevented, or at least minimized, by supplementing pregnant mares and genetically susceptible foals with α-TOH.[1,2,28]

Studies in humans have used serum and plasma to measure vitamers and metabolites.[29-31] However, our previous study reported that quantification differs between equine serum and plasma and should be accounted for when setting reference ranges.[14] Here, we confirmed the correlation between serum and plasma concentrations and used serum concentrations to analyze the effect of disease status.

Metabolism of vitE, as well as many pharmaceutical compounds, is carried out by CYP450 family members. *In vitro* assays also have shown CYP4F2 to be involved in the metabolism of vitE.[10] Ketamine,[32] xylazine,[33] and midazolam[34] are metabolized by CYP3A family members. In our study, serum metabolite and vitamer concentrations were well-correlated for all but γ-TOT and α-TOT. Gamma-TOT was undetectable in any postinduction samples, and α-TOT was only detected in 7/22 samples. Therefore, serum samples can be evaluated for quantifiable vitE vitamers and metabolites, even in sedated horses. Although horses were sedated with xylazine for urine sampling, the addition of the anesthetic induction drugs ketamine and midazolam significantly impacted urinary vitE metabolites. Some horses experienced an increase in metabolites postinduction and others a decrease. As such, any urinary vitE metabolite profiling should be conducted in nonanesthetized horses to obtain an accurate metabolite profile.

Supplementation with α-TOH resulted in increased circulating vitamer concentrations for all horses that was not significantly mirrored in the CSF. Previous studies of horses have shown that
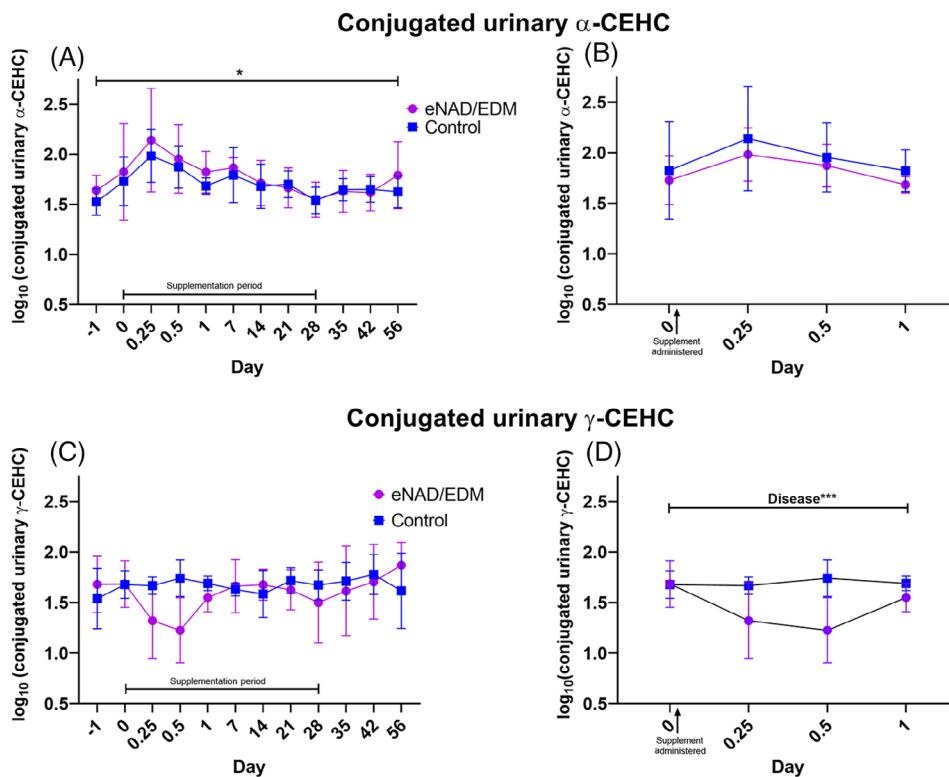
FIGURE 7 Proof of concept study—Conjugated urinary γ-, but not α-metabolites, differ between eNAD/EDM and control horses: A, Conjugated urinary α-CEHC concentrations changed significantly over time in all groups (*P < .05) during the 56-day trial period, with no effect of disease. B, Within the first 24-hours time period, conjugated urinary α-CEHC concentrations did not significantly differ over time or between disease states. C, For conjugated urinary γ-CEHC, there was no significant effect of time or disease but a significant interaction was observed for time × disease (P = .01) over the 56-day course of the study (D) Within the first 24-hours period, time was not significant, however eNAD/EDM-affected horses had significantly less conjugated γ-CEHC metabolites in their urine (P = .04) and a significant interaction was observed between time * disease (P = .02)
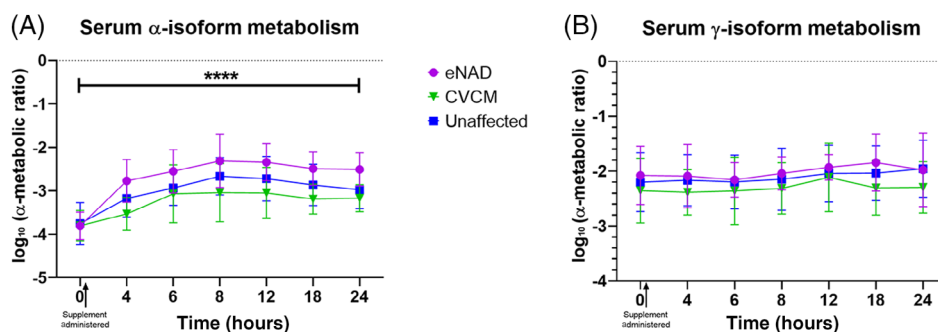
FIGURE 8 Validation study—Metabolic ratios change with supplementation and α-metabolic ratio differs between eNAD/EDM, CVCM, and control horses: A, Alpha-metabolic ratios changed significantly with time (P < .0001) and disease status (P = .03), with no interaction of disease status and time (P = .12). Post hoc testing revealed significant differences between eNAD/EDM and CVCM at 18 and 24 hours postsupplementation (P = .03 and P = .02, respectively). B, Gamma metabolic ratios did not differ significantly with time (P < .23) or disease status (P = .46). Data analyzed via ANOVA after log transformation with post hoc contrasts between group means. ****P < .0001

supplementation with RRR-α-TOH increases CSF α-TOH within 14 days.[35,36] Within our 28-day period, CSF α-TOH concentrations increased nonsignificantly in the controls (P = .08) and in the eNAD/EDM group (P = .23). Our nonsignificant findings in the controls are likely a consequence of individual variability within our small group (n = 6) compared to previous studies.[35-37]

The increased α-metabolic ratio in eNAD/EDM-affected horses was most pronounced in the POC study between 6- and 24-hours postsupplementation. We therefore selected this narrower period in the larger validation study. Although an increase in γ-TOH metabolism was identified in the POC study, this increase was not verified in the validation cohort. The α-metabolic ratio, however, remained
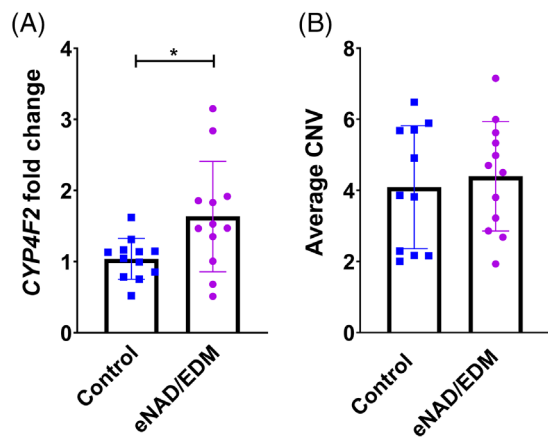
FIGURE 9 eNAD/EDM-affected horses have increased expression of *LOC100062102* but there is no significant difference in copy number: A, Scatter plot showing mean and SD of delta-Ct of *LOC100062102* between eNAD/EDM cases and control horses. All horses were postmortem confirmed for disease status. Expression differences analyzed using an unpaired *t*-test with a Welch's correction, log-fold change was 1.63-fold ($P = .02$). B, Copy number for *LOC100062102* was not significantly different ($P = .60$) between eNAD/EDM cases and control horses. All horses were postmortem confirmed for disease status

significantly higher in eNAD/EDM-affected vs control and CVCM horses, although the distinction was less pronounced than in the POC study. Although the increased number of controls assayed in the validation study (n = 29) vs the POC study (n = 6) may have contributed, we postulate that the clearer distinction in the POC assessment of α-metabolic ratios was related to inadvertent short-term fasting after administering the RRR-α-TOH supplement. The POC study horses were sedated using xylazine for urinary catheterization and supplemented PO after urine collection. As is standard with sedated horses, feed was withheld for 30 to 45 minutes after sedation. Therefore, although horses were not intentionally fasted before supplementation, there may have been an effect of this short-term decreased feed intake in the POC study. In support of this hypothesis, serum α-TOH concentrations started lower and increased much more steeply in both eNAD/EDM-affected and control horses in the POC study. However, it is unclear if short-term fasting in horses would have any clinically relevant effect in rate of α-TOH absorption from the small intestine. Although studies in humans differ in whether or not fasting is performed,[29,38] future metabolic profiling of vitE metabolism in horses should be conducted after an overnight fast.

In our vitE metabolism studies, eNAD/EDM-affected horses consisted primarily of QHs (4/5 in POC study and 6/6 in validation study). Although eNAD/EDM has been reported across breeds, the disease may be genetically heterogeneous. To confirm that our finding of increased α-metabolic ratio was not a breed effect, we reanalyzed our validation results using only the cohort of QH controls and found similar significance. Additionally, we found no difference in α-metabolic ratio between control QHs vs controls from other breeds. Thus, eNAD/EDM significantly alters vitE metabolism in QHs and future

studies are required to determine if these effects occur in other affected breeds.

Although vitE supplementation with α-TOH is known to decrease circulating γ-TOH in humans,[29,38] this effect was not observed in control serum γ-metabolite ratios or urine γ-CEHCs in our cohort. Studies in humans used almost twice the dosage of RRR-α-TOH (approximately 19.3 IU/kg) once a day for 28 to 60 days, which increased serum α-TOH concentrations 200% to 400% by 14 to 60 days.[29,38] We administered 10 IU/kg RRR-α-TOH once a day for 28 days, which resulted in a significant increase in serum α-TOH concentrations, but most concentrations barely only doubled in controls. Therefore, our findings that α-TOH supplementation did not affect γ-TOH is likely related to dosing rather than species differences.

Equine NAD/EDM typically affects horses during the first few years of life.[4] We included mostly older horses with clinical signs documented since 1 to 2 years of age in our cohorts, postulating that an inherited defect in vitE metabolism should exist for the life of the horse, similar to patients with AVED.[29] This notion was further supported by the identification of increased α-metabolite ratios in eNAD/EDM adult horses. From a clinical standpoint, this observation would permit the assay to be used in suspected eNAD/EDM-affected horses of any age. However, because of the overlap in α-metabolite ratios between eNAD/EDM and unaffected horses in the validation study, the assay may have low sensitivity. Profiling of additional horses after an overnight fasting period will be necessary to potentially increase diagnostic accuracy. Additionally, our population of horses was maintained in a vitE deficient environment for 6 months before the study began, with the goal of controlling for baseline serum α-TOH concentrations before supplement administration. Thus, this assay requires further evaluation as a diagnostic test for eNAD/EDM in horses with normal baseline α-TOH concentrations before clinical use, because many horses with suspected eNAD/EDM already may be receiving α-TOH supplementation.

When assessing equine *CYP4F2* using comparative genomics approaches, 2 incompletely annotated transcripts (*LOC100062102* and *LOC100147344*) were identified as equine orthologues. Because of primer design limitations and repetitive DNA, only 1 of these transcripts was assayed using qRT-PCR (*LOC100062102*). Although differential expression between eNAD/EDM-affected and control horses was observed, quantification of the other plausible orthologue (*LOC100147344*) warrants further investigation. The results from these assays suggest that increased hepatic *CYP4F2* expression may occur in eNAD/EDM even though genetic mutations in *TTPA* are not causative. We only profiled gene expression and not protein expression or enzymatic activity of *CYP4F2*. However, if eNAD/EDM is caused by a variant in a gene associated with α-TOH transport, it is hypothesized that *CYP4F2* expression would upregulate, similar to the mechanism for AVED.[19]

In conclusion, we have identified an increase in α-isoform metabolism in eNAD/EDM-affected QHs, providing novel insight into alterations in vitE metabolism with eNAD/EDM. A change in the expression of an equine *CYP4F2* orthologue is a likely consequence of the underlying genetic etiology of eNAD/EDM.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the University of California, Davis, IACUC, protocol number 18376.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.