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Inflammatory phenotype, clinicopathologic variables, and effects of long-term methylene blue in dogs with hereditary methemoglobinemia caused by cytochrome b_5 reductase deficiency

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OBJECTIVE
To determine whether dogs with cytochrome b_5 reductase (CYB5R) deficiency have a constitutive proinflammatory phenotype, characterize hematologic and serum chemistry results, and describe changes in methemoglobin (MetHb) levels and serum C-reactive protein (CRP) concentrations after long-term per os (PO) methylene blue (MB) therapy.

ANIMALS
21 client-owned dogs (CYB5R deficient, n = 10; healthy controls, 11).

PROCEDURES
In this prospective, case-control study, methemoglobin levels were measured using a blood gas analyzer with co-oximetry. Plasma tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-10 (IL-10) concentrations were measured using a canine-specific multiplex bead-based assay. Serum CRP concentrations were measured with a canine-specific commercial ELISA kit. Serum CRP concentration and MetHb levels were measured in 6 dogs with CYB5R deficiency after ≥ 60 days of PO MB therapy.

RESULTS
As expected, MetHb levels were higher in dogs with CYB5R deficiency compared to controls ($P < .001$). Plasma TNF-α, IL-6, IL-10, and serum CRP concentrations were no different between CYB5R-deficient and control dogs. Dogs with CYB5R deficiency had lower absolute lymphocyte ($P = .005$) and eosinophil counts ($P = .04$) and higher alanine transaminase ($P = .04$) and alkaline phosphatase activity ($P = .02$) than controls, but these changes were not clinically relevant. Methemoglobin levels decreased after PO MB therapy ($P = .03$).

CLINICAL RELEVANCE
These results suggest that otherwise healthy dogs with CYB5R deficiency do not have a constitutive proinflammatory phenotype and clinically relevant abnormalities in hematologic and serum chemistry panels are not expected. Dogs with decreased quality of life attributed to methemoglobinemia from CYB5R deficiency might benefit from PO MB therapy.

Methemoglobin (MetHb) forms when iron moieties in heme are oxidized to the ferric state (Fe^{3+}), which cannot bind oxygen. An estimated 2% to 3% of hemoglobin is oxidized to MetHb daily by natural oxidants produced during metabolism and from the spontaneous autoxidation of oxyhemoglobin.[1] The cytochrome b_5 reductase (CYB5R)/cytochrome b_5 redox pathway maintains MetHb levels at < 2% of total hemoglobin.[1–3] Methemoglobinemia results from a hereditary defect in this enzyme system that impairs function, or more commonly, it can be acquired with exposure to exogenous oxidants that saturate the reductive capacity of this pathway.[2]

Cytochrome b_5 reductase deficiency is the most common type of hereditary methemoglobinemia in dogs.[4] This erythrocyte enzymopathy is largely attributable to 2 missense variants in the *CYB5R3* gene resulting in either Arg219pro or Ile194Leu substitutions.[4–8] Methemoglobin levels reported in dogs with CYB5R deficiency range from 7.3% to 48.4%.[1,4–8] Methemoglobin stimulates various cell types in vitro to produce proinflammatory cytokines in a concentration-dependent manner through ligation of Toll-like receptor-4 (TLR-4).[9–12] To the authors' knowledge, there have been no investigations into the systemic inflammatory phenotype in dogs or humans with CYB5R deficiency, despite chronic and often severe elevations in MetHb levels.

Dogs with CYB5R deficiency commonly develop a compensatory erythrocytosis.[4] However, there is

no published information on serum chemistry results or the remaining hematologic parameters from dogs with this disorder. These data are necessary to provide a clinical context for the interpretation of hematologic and serum chemistry results in dogs with CYB5R deficiency. Methylene blue (MB) is an effective therapy to reduce MetHb levels in dogs with CYB5R deficiency.[5,6] However, there are only 2 single case reports[5,6] that describe long-term management with per os (PO) administration of MB in dogs with CYB5R deficiency.

Our study had the following 3 objectives: (i) to determine whether dogs with CYB5R deficiency have a constitutive proinflammatory phenotype by comparing plasma levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), and serum C-reactive protein (CRP) concentrations between CYB5R-deficient dogs and unaffected healthy controls; (ii) to characterize hematologic and serum chemistry results in CYB5R-deficient dogs; and (iii) to describe changes in MetHb levels and serum CRP concentrations after PO MB therapy. A secondary aim of this study was to describe the owner-perceived clinical response to the long-term administration of PO MB. We hypothesized that CYB5R-deficient dogs would have a constitutive proinflammatory phenotype and that PO MB administration would decrease MetHb levels and serum CRP concentrations. Further, we hypothesized that 1 or more hematologic or serum chemistry parameters would be different between CYB5R-deficient dogs and controls.

Materials and Methods

Criteria for selection of cases

A prospective case-control study was performed. Dogs with a historical diagnosis of CYB5R deficiency between March 2020 and July 2020 were eligible for inclusion. Dogs were included in the study after obtaining informed owner consent. This study was conducted in accordance with guidelines for clinical studies and approved by the Midwestern University Institutional Animal Care and Use Committee (protocol No. 2963). Eighty percent (8/10) of the included dogs were previously reported in a separate study[4] that investigated the clinical, metabolic, and molecular genetic characterization of hereditary methemoglobinemia caused by CYB5R deficiency in dogs. A diagnosis of CYB5R deficiency was confirmed in dogs with persistent unexplained cyanosis in the absence of cardiopulmonary disease with the identification of a causal variant in the *CYB5R3* gene, reduced erythrocytic CYB5R enzyme activity, or both.[4] Dogs with CYB5R deficiency were excluded if a clinically relevant comorbid condition not expected to be related to their hereditary erythrocyte enzymopathy was identified. A second population of unaffected healthy control dogs was included. Control dogs were considered healthy based on physical examination and a review of clinical history.

Baseline sample and data collection

Medical records were reviewed for each dog enrolled. The age, sex, and breed were recorded for each. Molecular genetic results were recorded for CYB5R-deficient dogs. In addition, the following clinical information regarding MB treatment in CYB5R-deficient dogs was obtained when available: dosage, adverse effects, and response to therapy. Baseline samples were collected from all dogs. Dogs with CYB5R deficiency were located in various geographic regions within the United States. Primary care veterinarians were provided detailed instructions on sample acquisition, packaging, and shipping. As such, primary care veterinarians collected and allocated blood samples into EDTA-anticoagulated tubes, lithium heparin–anticoagulated tubes, and serum separator tubes. Blood in the serum separator tube was centrifuged, and serum was placed in additive-free tubes. One milliliter of the lithium heparin–anticoagulated blood was centrifuged, and plasma was placed in additive-free tubes. Serum and plasma were harvested within 30 minutes of blood collection. Overall, 4 samples were prepared from each dog and included (i) EDTA-anticoagulated whole blood, (ii) lithium heparin–anticoagulated whole blood, (iii) serum, and (iv) plasma. The samples were packaged in a small polystyrene foam container with icepacks and shipped overnight to the Companion Animal Clinic at the Midwestern University College of Veterinary Medicine (CAC-MWU). Blood samples from unaffected controls and CYB5R-deficient dogs enrolled onsite at the CAC-MWU were collected and processed using the same protocol. Samples from these locally enrolled dogs were packaged in a small polystyrene foam container with icepacks for 24 hours to minimize potential confounding related to shipping.

Hematology and serum chemistry panels were performed by a commercial reference laboratory (Antech Diagnostics). Methemoglobin was analyzed with a veterinary benchtop blood gas analyzer with co-oximetry (Stat Profile Prime Plus Vet; Nova Biomedical) using lithium heparin–anticoagulated whole blood. Methemoglobin was reported as the percentage of total hemoglobin concentration. Throughout the study period, the blood gas analyzer underwent daily quality control and routine maintenance as instructed by the manufacturer. Serum and plasma were transferred to freezer-resistant conical microcentrifuge tubes and stored at −80 °C for batch analysis of plasma cytokine and serum CRP concentrations.

Sample collection after methylene blue therapy

Blood samples were collected from 6 dogs with CYB5R deficiency that were treated with PO MB and allocated into lithium heparin–anticoagulated tubes and serum separator tubes. Blood in the serum separator tube was centrifuged and serum was placed in additive-free tubes within 30 minutes of collection. Samples were packaged and shipped with ice

packs overnight to the CAC-MWU. Samples from dogs located near the CAC-MWU were packaged in a small polystyrene foam container with ice packs for 24 hours. Methemoglobin, hemoglobin, and hematocrit were analyzed with the same benchtop blood gas analyzer using lithium heparin–anticoagulated whole blood. Serum was transferred to freezer-resistant conical microcentrifuge tubes and stored at −80 °C for batch analysis of CRP concentrations. The primary care veterinarian, not the research investigators, made MB treatment and therapeutic monitoring decisions. Samples were acquired after ≥ 60 days of PO MB administration. The timing of sample collection coincided with a planned recheck evaluation with each patient's primary care veterinarian and was not determined by the research investigators. Information regarding clinical response to long-term PO MB administration was gathered from the medical record or direct communication with the owner.

C-reactive protein concentration

Serum CRP was measured with a commercially available canine-specific ELISA (Abcam) as previously described.[13] Samples were measured in duplicate with concurrent standard curves using kit-provided canine standards; the lower limit of detection was 1.1 ng/mL. The optical density of the samples was determined with a Biotek Cytation 3 microplate reader (Biotek) set to a wavelength of 450 nm. Background absorbance was measured at 700 nm and subtracted from sample absorbance. Sample CRP concentration was determined by plotting the kit standards using a linear curve.

Constitutive cytokine concentration

Plasma samples were thawed, and then TNF-α, IL-6, and IL-10 were quantified with a canine cytokine-specific multiplex bead-based assay (Milliplex MAP; EMD Millipore Corp) as described previously.[14] The median fluorescence intensity and cytokine concentration in each sample were measured in duplicate with appropriate controls and associated data analysis software (Milliplex Analyst version 5.1; EMD Millipore Corp). The lower limit of detection for TNF-α and IL-6 was 48.8 pg/mL. The lower limit of detection for IL-10 was 195.3 pg/mL.

Statistical analysis

Statistical analyses were performed using commercial software (SigmaPlot; SyStat Software Inc). Continuous data are presented as median, interquartile range (IQR), and range. Categorical data are presented as proportions. Two group comparisons of continuous variables from baseline data were performed using the Mann-Whitney rank sum test. Comparisons of variables "before" and "after" PO MB treatment in the 6 CYB5R-deficient dogs were made using Wilcoxon signed rank test. Variables included in the "before" and "after" comparisons included MetHb, CRP, hematocrit, and hemoglobin data. Hematocrit and hemoglobin data used in the "before" and "after" PO MB treatment comparisons were taken from the blood gas results, as this analyzer was utilized to obtain the data at both time points. Hematology panels performed by the commercial reference laboratory were only available at the baseline time point. When the measured cytokine or CRP concentration fell below the lower limit of detection for the respective assay, data were recorded at the lower limit of detection for statistical purposes. A *P* value of < .05 was considered significant.

Results

Animal cohort

Eleven dogs with CYB5R deficiency were eligible for enrollment, but 1 dog was excluded due to the identification of a chronic hepatopathy, leaving 10 CYB5R-deficient dogs included in the study. Eleven dogs were included as unaffected healthy controls. There was no difference in age (*P* = .10) or sex (*P* = .39). A complete summary of demographic data (i.e., age, sex, and breed) can be found **(Table 1)**. Five dogs (CYB5R deficient, n = 1; controls, 4) were located near the CAC-MWU and enrolled onsite. The remaining 16 dogs (CYB5R deficient, n = 9; controls, 7) were located in other geographic regions within the United States.

The molecular genetic cause for CYB5R deficiency in 8 of the CYB5R-deficient dogs was previously reported.[4] Briefly, 4 dogs were homozygous for the Ile194Leu variant; 3 were homozygous for the Arg219Pro variant; and 1 dog was heterozygous for the Arg219pro variant. A molecular genetic diagnosis was also achieved in the 2 dogs that were not included in the previously published cohort. One of these dogs was homozygous for the Arg219Pro variant while the other was homozygous for the Ile194Leu variant.

Table 1—Demographic data for 10 dogs with cytochrome b_5 reductase deficiency and 11 unaffected healthy control dogs.

Variable	CYB5R deficient	Control	*P* value
Age (y)[a]	5.6 (5.3)	4.0 (2.5)	.10
Sex (MN, FS)	5, 5	3, 8	.39
Breed	Pit Bull Terrier (4), Pomeranian (3), mixed-breed dog (2), Decker Rat Terrier	Pit Bull Terrier (4), mixed-breed dog (3), Catahoula Hound, Golden Retriever, Border Collie, Pomeranian	—

CYB5R = Cytochrome b_5 reductase. FS = Female spayed. MN = Male neutered.
— = Not performed.
[a]Data are presented as median (interquartile range).

Hematology and serum chemistry

Three dogs with CYB5R deficiency and 2 healthy control dogs had hematocrit levels above the reference interval. The median (range) of these results in CYB5R-deficient dogs was 73% (64% to 80%), while the 2 control dogs each had hematocrit results of 61% **(Table 2)**. No dogs in either group had abnormal serum alanine transaminase (ALT) or gamma-glutamyl transferase (GGT) enzyme activities **(Table 3)**. One control dog had a mild increase in serum aspartate aminotransferase (AST) activity (74 IU/L; reference interval, 15 to 66 U/L). Likewise, 1 CYB5R-deficient dog had a mild increase in serum alkaline phosphatase (ALP) activity (274 U/L; reference interval, 5 to 131 U/L). Dogs with CYB5R deficiency had lower absolute lymphocyte (P = .005) and eosinophil counts (P = .04) and higher ALT (P = .04) and ALP activities (P = .02) than healthy controls. There were no significant differences in the remaining hematologic and serum chemistry parameters (P > .05).

Methemoglobin, c-reactive protein, and cytokines

Methemoglobin levels were higher in dogs with CYB5R deficiency compared to healthy controls (P < .001; **Figure 1**). There was no difference in serum CRP concentration between CYB5R-deficient dogs (median [IQR], 29.2 ng/mL [57.9]) and healthy controls (38.3 ng/mL, 42.5, P = .38). Plasma constitutive TNF-α, IL-6, and IL-10 concentrations were below the limit of detection in most dogs, regardless of group. Specifically, TNF-α, IL-6, and IL-10 concentrations were below the lower limit of detection in 76% (CYB5R deficient, n = 8; controls, 8), 81% (CYB5R deficient, 8; controls, 9), and 95% (CYB5R deficient, 10; controls, 10) of dogs, respectively. There was no difference in plasma constitutive TNF-α, IL-6, or IL-10

Table 2—Comparison of hematology results in 10 dogs with cytochrome b_5 reductase deficiency and 11 unaffected healthy control dogs.

Variable	CYB5R deficient	Control	Reference interval	*P* value
Hematocrit (%)	57 (12.5, 50–80)	53 (10, 36–61)	36–60	.10
MCV (fL)	69.5 (9.8, 62–80)	74 (2, 71–81)	58–79	.06
MCHC (g/dL)	32 (2, 30–34)	34 (3, 31–41)	30–38	.03
Hemoglobin (g/dL)	18.3 (3.7, 16.8–24.3)	17.8 (3.4, 14.9–19.7)	12.1–20.3	.36
WBC (X 10^3 cells/μL)	8.8 (3.3, 6.1–11.6)	9.2 (4.6, 5.5–12.1)	4.0–15.5	.67
Neutrophils (X 10^3 cells/μL)	5.9 (3.4, 4.1–9.1)	4.9 (3.2, 3.1–8.3)	2.1–10.6	.50
Monocytes (X 10^3 cells/μL)	0.4 (0.3, 0.2–1.0)	0.4 (0.2, 0.1–1.2)	0.0–0.8	.75
Lymphocytes (X 10^3 cells/μL)	1.7 (0.6, 0.8–2.3)	2.6 (0.9, 1.7–4.9)	0.7–4.5	.005
Eosinophils (X 10^3 cells/μL)	0.2 (0.4, 0–0.8)	0.5 (0.2, 0.2–1.0)	0.0–1.2	.04
Platelets (X 10^3 cells/μL)	259 (89, 167–529)	291 (113, 142–893)	170–400	.65

Data are presented as median (interquartile range, range).
CYB5R = Cytochrome b_5 reductase. MCHC = Mean corpuscular hemoglobin. MCV = Mean cell volume. WBC = white blood cell.

Table 3—Comparison of serum chemistry results in 10 dogs with cytochrome b_5 reductase deficiency and 11 unaffected healthy control dogs.

Variable	CYB5R deficient	Control	Reference interval	*P* value
Albumin (g/dL)	3.8 (0.7, 3.3–4.3)	3.7 (0.6, 3.2–4.1)	2.7–4.4	.78
Globulin (g/dL)	2.9 (0.8, 2.4–3.4)	2.5 (0.5, 2.2–3.2)	1.6–3.6	.10
AST (U/L)	32.5 (14.5, 20–64)	25 (15, 19–74)	15–66	.31
ALT (U/L)	61 (19.5, 34–93)	35 (15, 24–97)	12–118	.04
ALP (U/L)	72.5 (48.5, 25–274)	38 (33, 19–70)	5–131	.02
GGT (U/L)	3 (1.3, 1–5)	4 (2, 1–8)	1–12	.20
Total bilirubin (mg/dL)	0.1 (0, 0.1–0.2)	0.1 (0.1, 0.1–0.3)	0.1–0.3	.16
BUN (mg/dL)	19 (13.3, 10–36)	17 (8, 10–31)	6–31	.60
Creatinine (mg/dL)	1.0 (0.2, 0.6–1.5)	1.0 (0.2, 0.8–1.3)	0.5–1.6	.59
Glucose (mg/dL)	102 (21.2, 85–123)	96 (7, 79–111)	70–138	.14
Phosphorus (mg/dL)	4.3 (1.7, 2.8–6.6)	4.1 (1.1, 3.6–7)	2.5–6	.75
Calcium (mg/dL)	10.3 (1.1, 9.4–12)	10.2 (0.8, 9.5–10.7)	8.9–11.4	.62
Magnesium (mEq/L)	2.0 (0.3, 1.8–2.6)	1.9 (0.3, 1.6–2.2)	1.5–2.5	.16
Sodium (mEq/L)	149 (3.3, 147–151)	148 (3.0, 144–151)	139–154	.22
Potassium (mEq/L)	4.5 (0.8, 3.8–5.1)	4.4 (0.5, 4.1–5.2)	3.6–5.5	.94
Chloride (mEq/L)	112 (2.3, 109–114)	112 (3.0, 110–115)	102–120	.35
Triglycerides (mg/dL)	144.5 (286.3, 45–522)	62 (19, 46–112)	29–291	.14
Cholesterol (mg/dL)	259 (87, 193–366)	250 (98, 179–368)	92–324	1.00
CK (U/L)	178.5 (159, 64–566)	93 (80, 48–386)	59–895	.14

Data are presented as median (interquartile range, range).
ALP = Alkaline phosphatase. ALT = Alanine transaminase. AST = Aspartate aminotransferase. BUN = Blood urea nitrogen. CK = Creatine kinase. CYB5R = Cytochrome b_5 reductase. GGT = Gamma-glutamyl transferse.

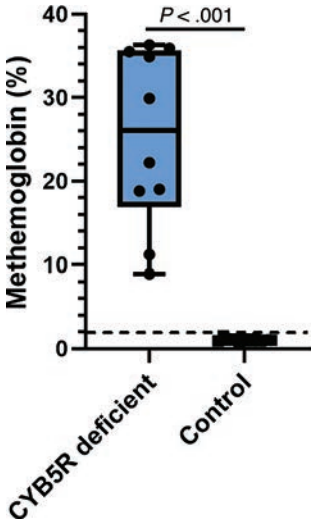

Figure 1—Box and whisker plot comparing methemoglobin levels in dogs with cytochrome b_5 reductase (CYB5R) deficiency (n = 10) and apparently healthy control dogs (11). The top and bottom of the boxes represent the 75th and 25th quartiles, respectively, with the black horizontal line representing the median. The whiskers represent the range of data. Closed circles represent individual dog data. The dashed line represents the normal cutoff for methemoglobin level (< 2%).

concentrations between CYB5R-deficient dogs and healthy controls (*P* > .05; **Supplementary Table S1**).

Methylene blue treatment

Sixty percent (6/10) of dogs with CYB5R deficiency were treated long term with PO MB. At the time of sample acquisition, dogs were treated with MB for a median of 109.5 days (range, 68 to 146 days). Four dogs received MB once every 24 hours at a median dose of 3.0 mg/kg (range, 3.0 to 4.2 mg/kg). One dog received MB at 1.5 mg/kg once every other day. The final dog was administered MB at 1.5 mg/kg once every 24 hours for 2 consecutive days, and then a day was skipped (treatment cycle, X 2 days, then skip a day). The only reported adverse effect was blue to green discoloration of urine in all dogs without clinical signs associated with urinary tract disease **(Supplementary Figure S1)**. A subjective improvement in quality of life was reported for all treated dogs characterized by improved or resolved cyanosis **(Figure 2)**, increased energy, activity, playfulness, and stimulated mentation (i.e., brighter and more interactive). Methemoglobin levels decreased after long-term administration of PO MB (*P* = .03; **Figure 3**). Individual dog responses to MB therapy can be found **(Supplementary Table S2)**. There were no differences in hematocrit, hemoglobin, or serum CRP after long-term PO MB administration (*P* > .05; **Supplementary Table S3**).

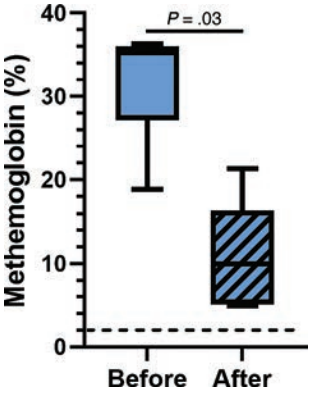

Figure 3—Box and whisker plot comparing methemoglobin levels in dogs with cytochrome b_5 reductase deficiency (n = 6) before and after per os methylene blue therapy. The top and bottom of the boxes represent the 75th and 25th quartiles, respectively, with the black horizontal line representing the median. The whiskers represent the range of data. The dashed line represents the normal cutoff for methemoglobin level (< 2%).

Figure 2—Representative images that illustrate cyanosis in 2 dogs with cytochrome b_5 reductase deficiency before (A and B) and improvement or resolution after per os methylene blue therapy (C and D).

Discussion

This prospective case-control study evaluated the systemic constitutive inflammatory phenotype, complete hematologic and serum chemistry results, and long-term effects of PO MB therapy in dogs with CYBR deficiency. The results from this study suggest that dogs with CYB5R deficiency do not have a constitutive proinflammatory phenotype nor do they have clinically relevant hematologic or serum chemistry abnormalities. Finally, long-term PO MB administration decreased MetHb levels and improved owner-perceived quality of life without meaningful adverse effects.

There was no difference in systemic inflammatory phenotype between CYB5R-deficient and control dogs as determined by plasma constitutive TNF-α, IL-6, IL-10, and serum CRP concentrations. One possible explanation for the lack of shift in the inflammatory milieu is the chronicity of methemoglobinemia in dogs with CYB5R deficiency. Previous studies that investigated the inflammatory response of MetHb were more representative of acute rather than chronic methemoglobinemia. Specifically, in vitro experiments[9–12] incubated various cell types with MetHb that had never been exposed to the molecule, and the effects of long-term, repeated exposures were not determined. Moreover, the study[12]

that assessed microglial TLR-4 and TNF-α expression in rats after infusion of MetHb into the subarachnoid space did so after 24 hours, and thus long-term effects were not investigated.

Chronic methemoglobinemia in dogs with CYB5R deficiency might result in a phenomenon similar to endotoxin tolerance, an adaptive mechanism designed to protect the host from inflammatory injury caused by repeated activation of the TLR-4 pathway. This cellular reprogramming results in downregulation and altered signaling of TLR-4, which dampens the production of proinflammatory cytokines and increases anti-inflammatory cytokines after repeated activation.[15,16] This theory would suggest that CYB5R-deficient dogs might have altered immune responses after exposure to endogenous and exogenous activation of the TLR-4 pathway. Future ex vivo studies investigating leukocyte production of cytokines after exposure to inflammatory stimuli in CYB5R-deficient dogs would provide more insight into this theory.

Dogs with CYB5R deficiency had lower absolute lymphocyte and eosinophil counts and higher ALT and ALP activities than control dogs. Despite being statistically significant, these results were not clinically relevant. There were no dogs with CYB5R deficiency that had clinically important abnormal serum liver enzyme activities. Interestingly, we did not find statistically significant differences between hematocrit or hemoglobin concentrations between CYB5R-deficient and control dogs. This was unexpected, as humans and dogs with CYB5R deficiency commonly develop a compensatory erythrocytosis.[4,17–19] The lack of difference in our study was likely the result of being underpowered (i.e., type II error). Results from this small study suggest that an erythrocytosis is likely to be the only expected abnormality on routine hematology and serum chemistry panels in otherwise healthy dogs with CYB5R deficiency.

Cytochrome b_5 reductase deficiency is characterized by 2 distinct clinical phenotypes that are dependent on which of the CYB5R isoforms are affected. Type I CYB5R deficiency is limited to erythrocytes (soluble form) and in humans results in cyanosis alone or in conjunction with 1 or more of the following symptoms: exertional breathlessness, fatigue, dyspnea, tachypnea, headache, or syncope.[17–24] Type II CYB5R deficiency has not been reported in dogs but has a severe clinical course in humans with a global loss of enzyme function (soluble and membrane-bound isoforms) that causes cyanosis as well as severe impairment of brain development and neurological abnormalities.[25]

Type I CYB5R deficiency in dogs and humans shares a similar clinical spectrum. The most commonly reported clinical signs in dogs include cyanosis alone or in combination with 1 or more of the following: exercise intolerance, lethargy, fatigue, low energy, and occasionally, syncope.[1,4–6] Like humans, otherwise healthy dogs with type I CYB5R deficiency typically adapt well to elevated MetHb levels and have normal life expectancies.[1,4,25] A recent study[4] utilized a questionnaire to ascertain owner-perceived quality of life in dogs with CYB5R deficiency. The overall median quality-of-life scores were low (low scores = high quality of life), indicating that dog owners perceived their dogs to be minimally affected.[4]

Currently, there are no long-term treatment recommendations for otherwise healthy dogs or humans with type I CYB5R deficiency. Logically, this is because clinical signs are generally mild and well tolerated. However, some dogs and humans have clinical signs that benefit from chronic management aimed at reducing MetHb levels.[5,6,18,19,21,26–28] Methylene blue is one of the most commonly used long-term therapy in humans and is the only known effective option in dogs with CYB5R deficiency.[5,6,21,27,28] The NADPH-reductase system is an alternative redox pathway that converts MB to leukomethylene blue, the reducing agent responsible for the reduction of MetHb to hemoglobin.

In our study, the long-term use of PO MB decreased MetHb levels and owners perceived a subjective improvement in quality of life without clinically important adverse effects. Commonly reported clinical abnormalities in dogs that were treated included cyanosis and 1 or more of the following: decreased ability to exercise or play, lethargy, and complaints that dogs appeared generally tired, fatigued, or had low energy. Clinical signs were compounded by complications associated with hyperviscosity syndrome in 1 dog with severe erythrocytosis (hematocrit, 80%). The hematocrit in the aforementioned dog decreased to 61% after 146 days of PO MB and no other intervention. Similar clinical improvement was noted in the only other 2 reported dogs with CYB5R deficiency after long-term PO MB therapy.[5,6] Methylene blue has a narrow therapeutic index because of the potential to saturate the reductive capacity of the NADPH-reductase pathway leading to an accumulation of MB and with it, oxidative damage manifesting as worsened methemoglobinemia and hemolysis.[2] There have been no veterinary or human studies that have determined optimum treatment monitoring recommendations in patients with CYB5R deficiency treated long term with PO MB. Given the possible adverse effects of PO MB, the authors recommend serial evaluations that include hematology panels with clinicopathologic review to look for increased Heinz body formation, and MetHb measurements are warranted in dogs treated long-term with PO MB. Future studies are needed to determine optimal dosing and safety in dogs.

The decision to provide prophylactic therapy to mitigate MetHb levels in otherwise healthy dogs with CYB5R deficiency remains controversial; however, treatment is recommended in humans with comorbid disorders that decrease oxygen delivery or when exposure to exogenous or endogenous oxidants causes acute decompensation.[29] The presence of comorbid conditions such as cardiopulmonary disease or anemia will compound an already reduced total blood oxygen content that could exacerbate clinical signs associated with hypoxia. Clinicians should be prepared to treat CYB5R-deficient dogs

with MB that might decompensate during periods of oxidative stress (e.g. infections, inflammation, toxicosis, certain drugs). Moreover, foods (e.g., garlic, onions, leeks) and drugs (e.g., benzocaine, lidocaine, prilocaine, metoclopramide, nitrofuran, sulfonamides, fluoroquinolones) known to increase oxidative stress should be avoided.

Our study had several limitations that must be considered. The surrogate markers for an inflammatory phenotype used in this study were TNF-α, IL-6, IL-10, and CRP. It is possible that aberrations in the inflammatory milieu of CYB5R-deficient dogs might have been identified if a more expansive panel of cytokines and inflammatory biomarkers had been used. The cytokines investigated in this exploratory study were specifically chosen because activation of the TLR-4 pathway increases immune cell production of TNF-α, IL-6, and IL-10.[30,31] Similarly, CRP is a positive acute phase protein that is commonly used in dogs as a sensitive but nonspecific biomarker of inflammation.[32] In addition, this study focused solely on investigating the constitutive inflammatory phenotype in CYB5R-deficient dogs and did not account for possible changes to immune responses that might occur after challenges such as inflammatory stimuli or exercise. Therefore, our results cannot be extrapolated to infer a comprehensive understanding of the inflammatory phenotype in these dogs. Next, quality-of-life questionnaires were not utilized to assess clinical response in the subset of dogs that received long-term PO MB. In addition, the lack of a placebo group and subjective outcome assessment could have led to biased results. Our investigatory efforts on the long-term effects of PO MB were not an initial objective at the onset of the study but rather, an opportunity that presented itself later on as it became clear many dog owners elected to pursue therapy. The use of a questionnaire or visual analog score before and after therapy could have provided a more detailed assessment of treatment response. Dogs that were treated with long-term PO MB had no clinically relevant adverse effects. However, it is possible that some of these dogs could have had subclinical hemolysis secondary to increased Heinz body formation. The primary care veterinarian, not the research investigators, made therapeutic monitoring decisions and thus serial assessments of hematologic panels with clinicopathologic review were not consistently performed.

The findings of the present study indicate that otherwise healthy dogs with CYB5R deficiency do not have a constitutive proinflammatory phenotype and are not expected to have clinically relevant hematologic or serum biochemical abnormalities. Some dogs may demonstrate a compensatory erythrocytosis. Finally, some dogs with CYB5R deficiency might benefit from long-term treatment with PO MB but careful monitoring is warranted. Treatment monitoring of dogs treated with PO MB must include serial evaluations of hematologic panels with reticulocyte counts and clinicopathologic review to look for increased Heinz body formation and MetHb measurements.

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Supplementary Materials

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