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CASE REPORT

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Clinical, metabolic, and genetic characterization of hereditary methemoglobinemia caused by cytochrome b_5 reductase deficiency in cats

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Abstract

Two non-pedigreed male castrated cats had persistent cyanosis over a 3-year observation period. Clinical cardiopulmonary evaluations did not reveal abnormalities, but the blood remained dark after exposure to air. Erythrocytic methemoglobin concentrations were high (~40% of hemoglobin) and cytochrome b_5 reductase (CYB5R) activities in erythrocytes were low (≤15% of control). One cat remained intolerant of exertion, and the other cat developed anemia and died due to an unidentified comorbidity. Whole-genome sequencing revealed a homozygous c.625G>A missense variant (B4:137967506) and a c.232-1G>C splice acceptor variant (B4:137970815) in *CYB5R3*, respectively, which were absent in 193 unaffected additional cats. The p.Gly209Ser missense variant likely disrupts a nicotinamide adenine dinucleotide (NADH)-binding domain, while the splicing error occurs at the acceptor site for exon 4, which likely affects downstream translation of the protein. The 2 novel *CYB5R3* variants were associated with methemoglobinemia using clinical, biochemical, genomics, and in silico protein studies. The variant prevalence is unknown in the cat population.

KEYWORDS

cyanosis, *CYB5R3*, cytochrome b_5 reductase, methylene blue, whole-genome sequencing

1 | CAT 1

A 6-month-old castrated male domestic shorthair cat was presented to South Coastal Animal Health for evaluation of right hind limb lameness. The cat had been adopted, along with a littermate (unaffected),

Abbreviations: CYB5, cytochrome b_5; CYB5R, cytochrome b_5 reductase; FAD, flavin adenine dinucleotide; Gly, glycine; Hb, hemoglobin; His, histidine; Leu, leucine; MB, methylene blue; methb%, methemoglobin percentage; NADH, nicotinamide adenine dinucleotide; Phe, phenylalanine; RBC, red blood cell; RI, reference interval; Ser, serine; Tyr, tyrosine.

at approximately 2 months of age, and lived indoors. At the time of adoption, the owner reported that the cat had pink mucous membranes, was playful, and was comparable in size to the littermate. Decreased energy, stamina, and cyanotic mucous membranes were noted by the owner over the subsequent 4 months. In addition, the kitten had a considerably slower growth rate than the littermate. The kitten had no known access to toxins or drugs, including acetaminophen. This cat and its littermate were fed a dry commercial feline diet. Pertinent physical examination findings included normal heart and respiratory rates, right medial patellar luxation (which was not further investigated), as well as cyanotic oral and preputial mucous membranes and foot pads (Figure 1). Cardiopulmonary auscultation did not reveal abnormalities.

Thoracic radiographs did not reveal abnormalities. Pulse oximetry (OxiMax N-65; Medtronic, Minneapolis, Minnesota) yielded an oxygen hemoglobin (Hb) saturation of 86% (reference interval [RI], 95%–100%) with a fraction of inspired oxygen of 0.21. An echocardiogram and agitated saline contrast study were subsequently performed and revealed normal cardiac structure and function with no intracardiac or extracardiac shunts. A "methemoglobin spot test", exposing fresh blood to air on a filter paper, remained dark compared to the bright red color of a control supporting a clinical assessment of methemoglobinemia (Figure 2).[1] The cat remained well and never exhibited abnormal respiratory rate or effort over a 3-year observation period.

2 | CAT 2

An approximately 4-year-old intact male domestic longhair cat was presented for a wellness examination and persistent cyanosis to the primary care veterinarian. The cat lived outdoors as a stray for 3 years before being adopted and housed indoors. According to the current owner, Cat 2 always appeared to have purplish mucous membranes, nasal planum, and ear pinnae over the entire 3-year observation period, which were confirmed at the time of physical examination. A CBC, serum chemistry, and urinalysis did not reveal abnormalities, and fecal floatation, and feline leukemia virus antigen and feline immunodeficiency virus antibody test (IDEXX Laboratories, Inc, Westbrook, Maine) were negative. A "methemoglobin spot test" indicated the presence of methemoglobinemia.

Cat 2 was evaluated by the primary care veterinarian 1 year later for lethargy, anorexia, polyuria, and polydipsia. Physical examination abnormalities included pale/cyanotic oral mucous membranes, dehydration, and pyrexia (104°F). The cat had a nonregenerative anemia (hematocrit 21%) and leukocytosis, but a blood smear was not examined. Abnormalities detected by biochemistry included an increased blood urea nitrogen (43 mg/dL; RI, 16–36 mg/dL) and creatinine within the RI (1.6 mg/dL; RI, 0.8–2.4 mg/dL). Urine obtained by cystocentesis revealed pyuria, bacteriuria (cocci), and a urine specific gravity of >1.050. The cat was treated with lactated Ringer's solution (100 mL SC once), maropitant (2 mg/kg PO q24h), marbofloxacin (3.1 mg/kg PO q24h), iron dextran (10 mg/kg IM once), vitamin B_{12} (62.5 mg/kg SC once), and capromorelin (1.9 mg/kg PO q24h). Four days later, the cat was evaluated because of progressive labored breathing at which time the anemia had worsened (PCV 17%; total solids of 7.0 g/dL). A blood transfusion was recommended but declined, and the cat died the next day. A specific cause of death in this cat was unknown and was possibly multifactorial, but the methemoglobinemia likely exacerbated the signs of the underlying disease(s).

FIGURE 1 Cyanosis of cat 1 with hereditary methemoglobinemia (A-C) and a healthy non-cyanotic cat (D-F)

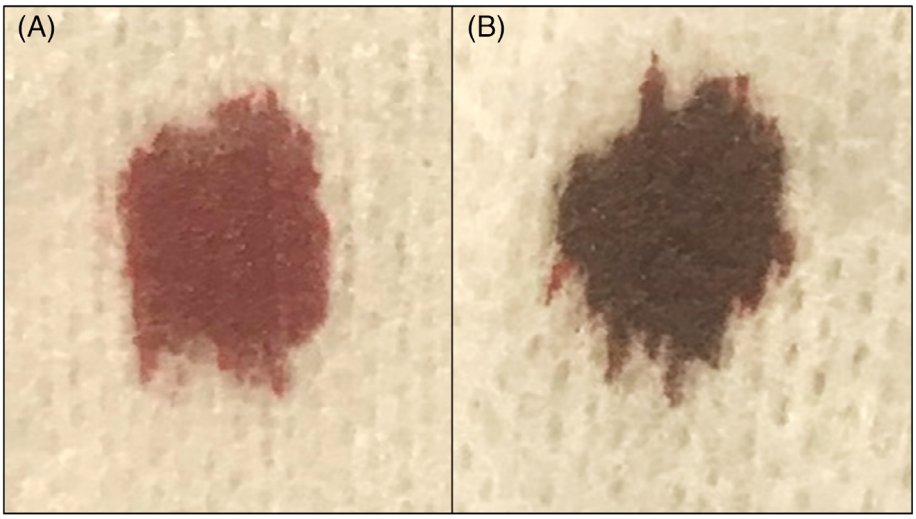

FIGURE 2 "Methemoglobin spot test". A, One drop of blood from a normal unaffected cat placed on a piece of white absorbent paper or gauze will appear bright red. B, A drop of blood from a cat (cat 1) with methemoglobinemia will appear brown when the methemoglobin content is ≥10%

3 | METHEMOGLOBIN AND CYB5R ACTIVITY MEASUREMENTS

The "spot test" was supportive of methemoglobinemia in both cats.[1] The chronicity of cyanosis in the absence of any known oxidative toxin exposure suggested that both cats had hereditary methemoglobinemia caused by an error in the methemoglobin redox pathway. Therefore, fresh ethylenediaminetetraacetic acid-anticoagulated blood samples from both cats were obtained to assess erythrocytic methemoglobin percentage (methb%) spectrophotometrically and CYB5R activity.[2] The methb% of the total hemoglobin was 41% in Cat 1 and 40% in Cat 2 compared to 3% in the healthy controls. The CYB5R activity in erythrocytes from Cat 1 was 15% and Cat 2 was 4% when compared to simultaneously tested control samples (100%), supporting a diagnosis of hereditary CYB5R deficiency.

4 | VARIANT DETECTION BY WHOLE-GENOME SEQUENCING

While a specific candidate gene was suggested by the high methb% and low CYB5R enzyme activities, a whole-genome sequencing approach was opportunistically used to investigate the genetic cause of the methemoglobinemia in both cats. To identify causal genomic DNA variants for each case, ~6 μg DNA was submitted to The McDonnell Genome Institute at Washington University (St. Louis, Missouri) for library preparation and whole-genome sequencing. For Cat 1, a 450 bp library was constructed and sequenced using an Illumina NovaSeq 6000 instrument (San Diego, California). For Cat 2, a 350 bp library was constructed and sequenced using an Illumina HiSeq X Ten instrument (San Diego, California), both using 150 bp paired-end reads and producing ~30× coverage. Sequence data were processed and variants filtered using VarSeq software (Golden Helix, Inc, Bozeman, Montana) as previously described.[3] Each cat was investigated separately and assumed as homozygous for DNA variants that would have a major impact on the protein and would be rare (<2%) to absent in the 99 Lives Cat Genome Sequence data set.[4-7] To be considered a valid variant, alleles were filtered to have at least a 10× depth of coverage, were present in 90% of cats and represented ~50% of reads. All sequence data were submitted to the National Center for Biotechnology Information Short Read Archive under BioProject PRJNA528515.

In Cat 1, 45 protein-altering variants were identified in 40 known genes (Table S1). Twenty-one variants were homozygous, missense, and unique including a c.625G>A in *CYB5R3* leading to a p.Gly209Ser amino acid change in transcript *CYB5R3*-202 (ENSFCAT00000056925) at position B4:137967506. This cat also possessed a more common p.Arg20His variant (Table S2). A clinically healthy and non-cyanotic full sibling of Cat 1 genotyped as heterozygous for this variant. A 378 bp product was generated using 50 ng DNA in a 25 μL volume and following primer pair: sense—5′TGT TTG AGA GCT GGG GTC TC and antisense—5′TTG TAG CAT CGG AGT GAT GC. The PCR and Sanger sequencing of the product were conducted as previously described.[8]

In Cat 2, 48 protein-altering variants were identified in 41 known genes (Table S3) in which 20 unique variants were homozygous, including a putative loss of function splice acceptor variant at c.232-1G>C in the *CYB5R3*-202 transcript (ENSFCAT00000056925) at position B4:137970815 that is in the acceptor site for exon 4, which likely affects downstream translation of the protein. No sample was available to conduct RNA studies to confirm the effect of this splice variant.

5 | IN-SILICO PROTEIN ANALYSIS OF FELINE CYB5R3

A comparative analysis was conducted between the feline and human CYB5R3 proteins using EMBOSS needle,[9] BIOVIA Discovery Studio Visualizer,[10] DUET,[11] PROVEAN,[12] and Human Gene Mutation Database[13] to assess linear and 3-dimensional sequence alignment, modeling, protein stability, and comparison of known variants putatively causing disease in humans. The feline CYB5R protein, inferred to be like the human homolog, is expressed as 2 isoforms, a longer protein containing a transmembrane region and a shorter soluble isoform.

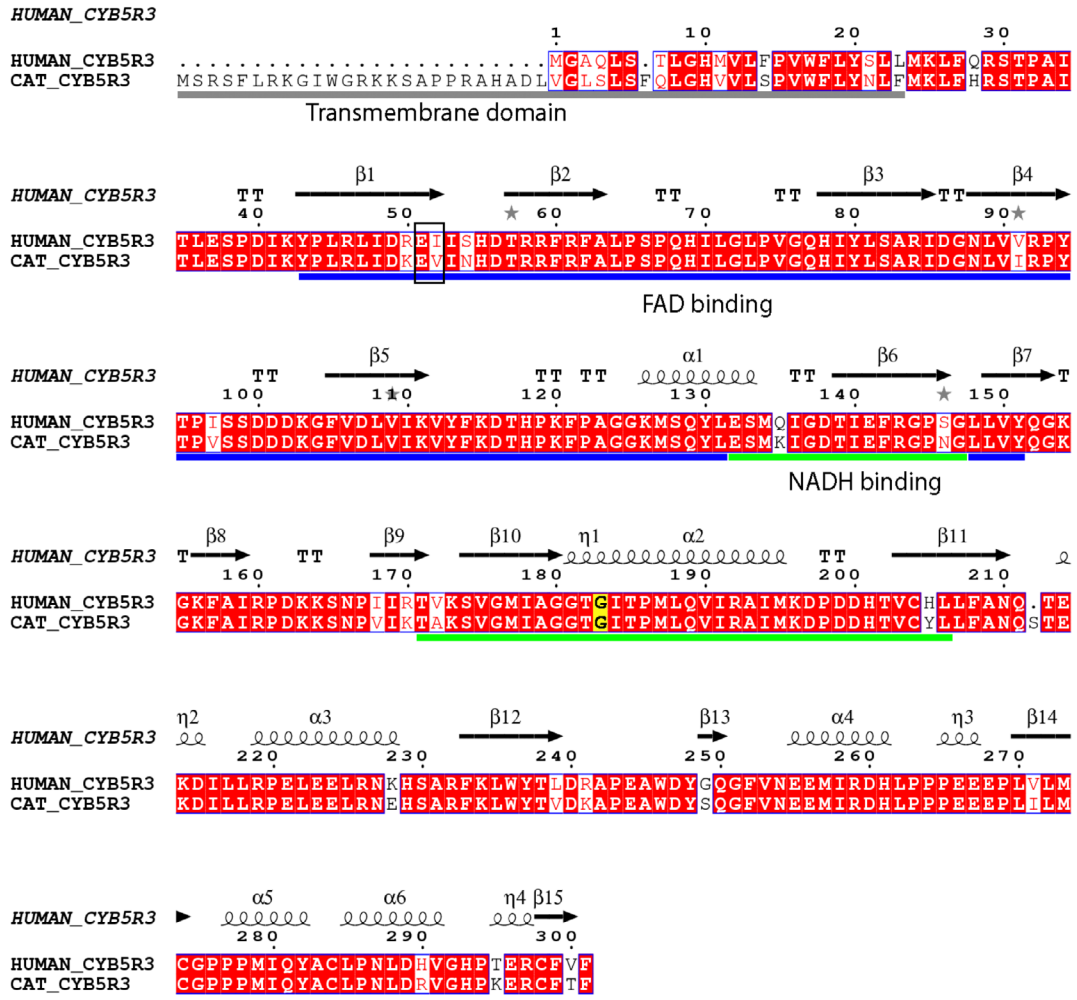

FIGURE 3 Pairwise sequence alignment of normal human and feline *CYB5R3* gene region and from 2 cats with cytochrome b_5 reductase (CYB5R) enzyme activity deficiency. The *CYB5R3* sequences were aligned using the EMBOSS/EMBL-EBI server and visualized by ENDSCRIPT program. Residue numbers are labeled according to the human sequence. The completely identical residues are shaded in red, the p.Gly209 variant is shaded in yellow, and the location of the c.232-1G>C splice junction variant between exons 3 and 4 is indicated by a black box. Secondary elements of *CYB5R3* derived from human crystal structure are drawn above the alignment. The transmembrane (gray), nicotinamide adenine dinucleotide (NADH) (blue), flavin adenine dinucleotide (FAD) (green) domains are indicated by solid lines under the alignment. UNIPROT database accession numbers are P00387 (human) and A0A337RZ16 (feline)

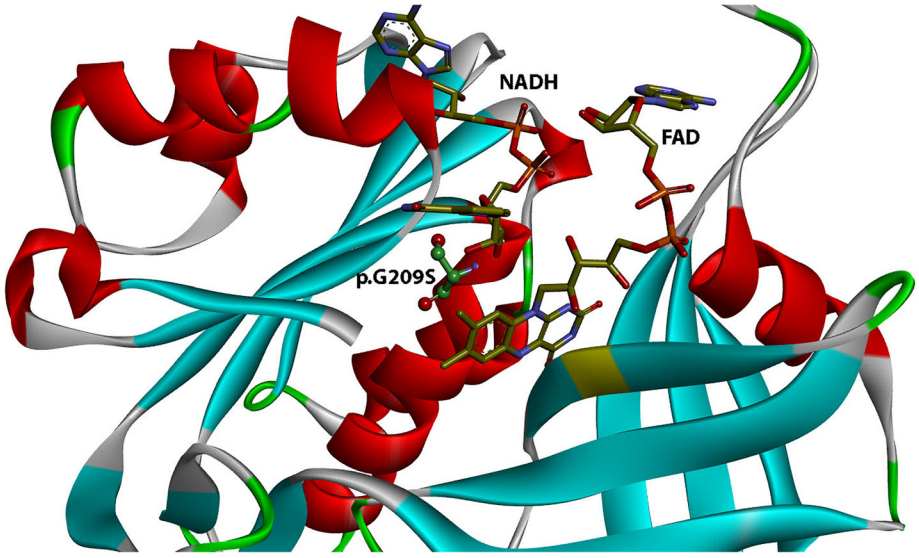

FIGURE 4 Three-dimensional visualization of feline cytochrome b_5 reductase (CYB5R) model. The ribbon diagram displays the p.Gly209Ser mutation and enzyme cofactors flavin adenine dinucleotide (FAD)/nicotinamide adenine dinucleotide (NADH) stylized in a stick representation and annotated. The image was developed by modeling feline CYB5R amino acid sequence against the homologous human (Protein data bank ID: 1UMK) and rat (Protein data bank ID: 1IB0) CYB5R crystal structures using BIOVIA Discovery Studio Visualizer

Variants in the soluble isoform lead to erythrocytic enzyme deficiency and methemoglobinemia rather than developmental/neurological disorders.[14] The feline CYB5R protein shares 92.4% amino acid identity with the human ortholog, with 21 amino acid differences in the core protein, excluding the transmembrane region (Figure 3). Two genetic variants were identified within the feline *CYB5R3* gene: c.625G>A: p.Gly209Ser (Cat 1) and c.232-1G>C; acceptor splice site (Cat 2). The c.625G>A; p.Gly209Ser variant is located in an α-helix which comprises 1-side of the flavin adenine dinucleotide (FAD)/NADH coenzyme binding site. A change from a small glycine (Gly) residue to a polar serine (Ser) residue is predicted (DUET ΔΔG: −1.519; PROVEAN score: −5.910 [neutral threshold >−2.5]) to destabilize protein structure or activity of the enzyme (Figure 4).

In Cat 2, the *CYB5R3* variant c.232-1G>C is predicted to alter the splice acceptor junction between exons 3 and 4. The next predicted acceptor site is located in exon 4, which would truncate the modified protein by 17 amino acids (residues 78–94) and thereby disrupt the FAD/NADH-binding domains.

6 | DISCUSSION

Methemoglobinemia refers to Hb with a ferric iron (Fe^{3+}) in heme, which cannot carry oxygen, instead of ferrous iron (Fe^{2+}). Clinically, cyanosis is noted when methemoglobin fraction of blood Hb is >15%, and is considered fatal when >70%.[15] Cyanosis in cats could be caused by cardiopulmonary diseases or methemoglobinemia. While methemoglobinemia could readily be determined by showing dark blood after exposing blood to air, termed "methemoglobin spot test" (turns red with cardiopulmonary diseases as hemoglobin can bind oxygen), a complete cardiopulmonary work up, as performed in Cat 1 of this report, is frequently first pursued. In both cats, the in-clinic spot blood test suggested methemoglobinemia. Standard pulse oximetry in patients with methemoglobinemia is not diagnostic because methemoglobin absorbs both infrared and red light equally, which interferes with the measured percentage of oxyhemoglobin and deoxyhemoglobin as was seen in Cat 1 (oxygen Hb saturation of 86%).[15] In contrast to standard pulse oximetry, a co-oximeter measures light absorbance at 4 different wavelengths, which allows for characterization of methemoglobin with a peak absorbance of light at 630 nm.[15]

Cats are unique since their Hb is chloride-dependent in releasing oxygen, instead of being 2,3-diphosphoglyceride-dependent,[16] has multiple adult beta-chains,[17] and contains more sulfate groups, which are targets for oxidative damage, as well as influencing Heinz body formation.[18] Furthermore, cats cannot readily metabolize and conjugate certain drugs, thus methemoglobinemia is commonly caused by oxidative drugs.[18] The 2 cats in this report had no known drug or toxin exposure and were fed a commercial feline diet. They had persistent cyanosis without cardiopulmonary signs, suggesting a hereditary methemoglobinemia. Noteworthy, previously reported cats with methemoglobinemia also had minimal clinical signs and might only be detected as an incidental finding during routine wellness examination unless confounded by other illnesses.[19,20] Indeed, the 2 cats in this report had minimal clinical signs other than cyanosis directly related to methemoglobinemia. However, Cat 2 became acutely ill 1 year later and died. The specific cause for anemia in this cat was unknown and was likely multifactorial. However, potential causes related to urosepsis include Heinz body anemia secondary to oxidative stress and anemia of inflammatory disease. The methemoglobinemia likely aggravated the clinical signs in this case. In fact, due to the methemoglobinemia, cats and other animals develop compensatory mechanisms including erythrocytosis (polycythemia) to increase oxygen transport.[21]

Methemoglobinemia can be acquired or have a hereditary cause. Hereditary methemoglobinemia in dogs[22-28] and cats,[19,20,29] like people,[14,30] is most commonly caused by CYB5R deficiency. Both cats of this report had markedly reduced CYB5R activity in their erythrocytes. In addition, alterations in CYB5 function associated with methemoglobinemia are rarely reported in people[31,32] and has not been reported in cats but has been identified in 1 dog.[33] Close to 60 different variants likely cause CYB5R deficiency in people, while only 2 causal variants have been identified in dogs[25-27] and 1 in a cat.[29] Recently, 2 homozygous missense variants, an exon 2 (Phe36Leu) and exon 6 (Tyr179His) missense, in *CYB5R3* were identified in 1 random bred cat from Japan (annotated using human sequence [NM_001171661]).[29] The Phe36Leu variant identified in the Japanese cat was not found in the entire 99 Lives Cat Genome Sequence data set, further supporting the variant as causal for methemoglobinemia (annotated as p.Phe88 in *CYB5R3-202* (056925.1; Figure S1). The Tyr179His in human *CYB5R3* is Tyr231His in the feline *CYB5R3* and is a polymorphic amino acid in the 99 Lives Cat Genome Sequence data set (Table S2) and is also found in both cats of this study.

In Cat 1, a missense variant (c.625G>A; p.Gly209Ser) and in Cat 2 an acceptor splicing variant (c.232-1G>C) are reported here. The Gly209Ser variant seen in Cat 1 closely resembles the *CYB5R3* p.Gly76Ser causal mutation in people which disrupts catalytic enzyme activity.[34] Located in the FAD/NADH-binding domain of CYB5R, the variant likely causes the type I methemoglobinemia observed in Cat 1.[14,34] The Cat 2 variant involves the splice junction (acceptor) between exons 3 and 4, and a similar splice junction (donor) variant is found in human type II methemoglobinemia.[35] In people, the donor-site variant results in extended transcription of exon 3 to cause type II methemoglobinemia, while the feline acceptor-site variant results in a truncated protein to cause type I methemoglobinemia.[14] Although other protein-altering variants were identified in these cats, these variants were not explored because a known and highly likely candidate was identified. Examining the current 99 Lives Cat Genome Sequence data set from 195 cats, the number of unique, homozygous protein coding variants per cat can range from a few variants to hundreds of variants (data not shown), depending on the relatedness of other cats in the data set. Thus, we expect to have "false-positive" variants in different genes in most whole-genome sequencing (WGS) studies and are lucky when a clear candidate is identified.

Cytochrome b_5 reductase is a member of the flavoprotein transhydrogenase family of oxidoreductase enzymes.[36] This enzyme deficiency can manifest clinically in 2 forms. Type I CYB5R deficiency

is restricted to the soluble erythrocyte cytosolic isoform.[14,36] In contrast, type II CYB5R deficiency is a global loss of both membrane-bound and soluble enzymes resulting in cyanosis, progressive encephalopathy, and often premature death in people.[14]

The severity of clinical signs related to methemoglobinemia in people is associated with the degree of methb% (i.e., percent of total blood Hb).[15] Otherwise healthy people with methb% of <20% do not exhibit clinical signs, but concentrations of 20%–50% methemoglobin could cause exercise intolerance, fatigue, and syncope, and levels of ≥50% methemoglobin can result in seizures, coma, and even death.[15,37,38] These clinical predictions are based on the assumption that the human patient has a total Hb concentration of 15 g/dL and lacks comorbid conditions that could decrease arterial oxygen tension or perfusion.[15] Methemoglobinemic human patients with concurrent anemia or cardiopulmonary diseases experience more severe clinical signs for a given methb%.[15] This might have been evident in Cat 2 of this report, which only demonstrated minimal clinical signs for years, but rapidly decompensated and died after an unexplained anemia developed.

First-line treatment for acute symptomatic methemoglobinemia is IV methylene blue (MB).[15] Methylene blue has been used in dogs and cats with hereditary methemoglobinemia (1 mg/kg IV over 20 minutes).[20,34,35] Methylene blue in cats is most commonly used for toxin-associated methemoglobinemia or N-acetylcysteine for methemoglobinemia associated with acetaminophen toxicosis. One study documented the administration of MB (1.5 mg/kg IV once) to cats with sodium nitrite induced methemoglobinemia, as well as healthy control cats, was safe.[39] The administration of MB to cats with methemoglobinemia ± Heinz body anemia should be used with caution, because it has the potential to have an additive effect on Heinz body formation and anemia. Clinicians should assess hematocrit for several days after MB administration to assure a life-threatening anemia does not develop. Severe symptomatic cases of methemoglobinemia in people are treated with blood transfusions, red blood cell (RBC) exchange apheresis, or hyperbaric oxygen (i.e., simple oxygen treatment will not help without methemoglobin reduction).[40-42] Cat 2 in this report could possibly have benefited from either a blood transfusion, MB, or both, which highlights the potential need for clinicians to treat cats with hereditary methemoglobinemia during periods of decompensation with MB, packed RBC transfusion, or both.

In conclusion, using routine clinical and biochemical tests to advanced genomic and in silico protein studies, 2 novel *CYB5R3* variants associated with CYB5R enzyme activity deficiency are reported in 2 cats with persistent methemoglobinemia.

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CONFLICT OF INTEREST DECLARATION

Dr. Giger's laboratory (PennGen) offers hematological and biochemical testing for methemoglobinemia. Dr. Lyons' laboratory has collaborative efforts with various laboratories that provide genetic testing for cats. The authors disclose no other conflict of interest. The production and interpretation of the data was not influenced by any funding program.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

University of Missouri Institutional Animal and Care and Use Committee Protocol Ex-9178. All work was conducted with the full agreement of the cats' owners.

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.

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