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Prospective long-term evaluation of parenteral hydroxocobalamin supplementation in juvenile beagles with selective intestinal cobalamin malabsorption (Imerslund-Gräsbeck syndrome)

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Background: Prospective studies on maintenance treatment for Beagles with hereditary selective cobalamin (Cbl) malabsorption (Imerslund-Gräsbeck syndrome, IGS) are lacking. In our experience, measurement of methylmalonic acid (MMA), a Cbl-dependent metabolite, seems more helpful to monitor Cbl status as compared with serum Cbl concentrations.

Objectives: To evaluate a standardized Cbl supplementation scheme in Beagles with IGS. We hypothesized that a single parenteral dose of 1 mg hydroxocobalamin (OH-Cbl) would maintain clinical and metabolic remission for up to 2 months.

Animals: Six client-owned juvenile Beagles with genetically confirmed IGS and 28 healthy control dogs.

Methods: Prospective study. Monthly IM OH-Cbl (1 mg) supplementation was done over a median of 9 months (range, 6-13) in 6 dogs, followed by bimonthly (every 2 months) injections in 5 dogs over a median of 6 months (range, 3-10). Health status was assessed by routine clinical examinations at injection time points and owner observations. Voided urine samples were collected immediately before OH-Cbl injections for measurement of MMA-to-creatinine concentrations using a gas-liquid chromatography-tandem mass spectrometry (GC-MS) method.

Results: All dogs were clinically healthy while receiving monthly and bimonthly OH-Cbl supplementation. Urinary MMA results in healthy dogs ranged from 1.3 to 76.5 mmol/mol creatinine (median, 2.9). Median urinary MMA concentrations did not differ between dogs with IGS receiving monthly (n = 49; 5.3 mmol/mol creatinine; range, 2.3-50.4) and bimonthly (n = 31; 5.3 mmol/mol creatinine; range, 1.6-50) injections.

Conclusions and Clinical Importance: A maintenance parenteral dose of 1 mg OH-Cbl monthly or bimonthly appears adequate in Beagles with IGS monitored by metabolic testing.

KEYWORDS
dogs, methylmalonic acid, urine, vitamin B12

1 | INTRODUCTION

Metabolic derivatives of cobalamin (Cbl), or vitamin B12, act as an essential cofactor of methylmalonyl-CoA mutase, which converts methylmalonyl-CoA to succinyl-CoA. A decrease in activity of this intracellular enzyme results in increases in methylmalonic acid (MMA).[1] Dogs are dependent on intestinal uptake of dietary Cbl bound to intrinsic factor (IF). The Cbl–IF complex subsequently binds to cubam, a heteromeric, multiligand, endocytic receptor, consisting of 2 proteins, amnionless (AMN) and cubilin (CUBN).[2] In humans, mutations in either the AMN or CUBN genes lead to selective Cbl malabsorption known as Imerslund-Gräsbeck syndrome (IGS).[3,4] In dogs, selective intestinal Cbl malabsorption, comparable to IGS in humans, has been described in Australian Shepherds,[5,6] Beagles,[7–12] Border Collies,[13–16] and Giant Schnauzers.[17] It is an autosomal recessive trait caused by 2 distinct AMN mutations in Giant Schnauzers[17] and Australian Shepherds.[5] Our group and others recently identified 2 independent CUBN mutations in Border Collies[18,19] and Beagles.[10,20] Although the disease is rare, substantial carrier frequency has been noted in affected breeds[20] and thus disease occurrence is more common regionally.[8] Clinical signs of IGS usually occur in the juvenile period as fetal Cbl stores gradually diminish. Typical clinical signs include failure to thrive, anorexia, and lethargy. Similar to infants, dogs may also be presented with more subtle clinical signs such as intermittent pyrexia, glossitis, aphthous stomatitis, and paresthesia.[12,15] Laboratory abnormalities consist of neutropenia, mild to moderate normocytic anemia, hyperammonemia, increased liver enzyme activities, and mild proteinuria.[8–16] Left untreated, IGS is life-threatening as a result of metabolic derangements and immunodeficiency.[9–11,16] Treatment consists of parenteral Cbl supplementation, which leads to rapid and complete clinical remission when administered sufficiently early in the course of disease, and the long-term prognosis is excellent with adequate lifelong treatment.[6,8–10,12] In humans with IGS, clinical signs and biochemical variables such as MMA are monitored and usually normalize when treatment is adequate.[21–23] In dogs with IGS, long-term monitoring (including regular assessment of markers of cellular Cbl availability) has not been carried out. Treatment regimens based on anecdotal reports vary widely and include different doses of Cbl administered weekly to every 4–5 months.[6,7,9,10,13–15,17,24] In our experience, measurement of serum Cbl concentration is not helpful for determining dosage frequency and response to treatment; serum Cbl concentrations were found to be persistently subnormal even though the health status of dogs was unremarkable and the results of periodic measurements of MMA were normal. In terms of practicality, most owners of dogs with IGS in our caseload eventually switched to monthly or bimonthly IM injections of 1 mg OH-Cbl, which appeared to be effective in the long term when assessed periodically. In Europe, genetic testing for IGS in dogs became available commercially in 2014 (www.laboklin.de) and for the first time allowed primary care veterinarians to diagnose the disorder before critical illness occurred.[9–11,16] Our continuing education articles on IGS (http://www.zora.uzh.ch/id/eprint/61072/; http://www.zora.uzh.ch/id/eprint/112847/; Lutz S, Bigler B, Sewell A, Riond B, Reusch CE, Kook PH. Ein Vitaminmangel mit weitreichenden Folgen. Hunde 2012(2):86–89.) prompted primary care veterinarians and owners of dogs with IGS to contact us for advice on metabolic monitoring of Cbl status. This gave rise to the idea of monitoring urine concentrations of MMA in dogs newly diagnosed with IGS. We hypothesized that a single parenteral dose of 1 mg Cbl could maintain clinical and metabolic remission for up 2 months in Beagles with genetically confirmed IGS.

2 | MATERIALS AND METHODS

2.1 | Animals

The clinical and metabolic health of six client-owned purebred Beagles with IGS was monitored for a minimum of 6 months. All six dogs had been diagnosed with IGS in private practice using a commercial genetic test (www.laboklin.de) for the CUBN mutation. The median age at the time of genetic testing was 8 months (range, 2–11 months). There were 3 female (1 spayed) and 3 male (2 neutered) dogs. The 2 youngest dogs (1 male, 1 female; each 2 months old) were littermates. Median body weight at the beginning of monthly Cbl supplementation was 7 kg (range, 5–13 kg). At the time of diagnosis, 4 of 6 dogs had clinical signs of IGS, which included failure to thrive (4/4), lethargy (4/4), anorexia (4/4), small stature (3/4), recurrent diarrhea (2/4), intermittent vomiting (1/4), and episodic fever and generalized pain (1/4). The 2 youngest dogs (littermates) had no clinical signs of IGS. Serum Cbl concentration at the time of diagnosis was below the detection limit (74 pmol/L) of the assay (Laboklin Bad Kissingen, Germany. B12 ECLIA on Cobas 8000 e602.) in the four older dogs and subnormal (IDEXX GmbH Ludwigsburg, Germany. Cbl chemiluminescence assay on ADVIA Centaur XP) in the two youngest dogs. Laboratory abnormalities in the clinically affected dogs consisted of mild neutropenia (3), mild normocytic anemia (1), and mild proteinuria (dipstick analysis). The four dogs with clinical signs had been treated with cyanocobalamin (CN-Cbl), administered IM, before beginning monthly supplementation. The amount and frequency of administration of CN-Cbl in the initial replenishment period differed considerably and was 100 μg q7d (n = 2), 1500 μg q7d (n = 1), or 1000 μg, q4wk (n = 1) before the first urine sample (t0) was collected. All six dogs were considered to be free of clinical signs by their owners and the primary care veterinarians at the time of the baseline urine evaluation.

2.2 | Study design

2.2.1 | Beagles with IGS

A baseline voided urine sample was collected before the first (t0) injection of 1 mg hydroxocobalamin (OH-Cbl; VITAMIN B 12 Depot Rotexmedica Injektionslösung, 22946 Trittau, Germany). Thereafter, the dogs received 1 mg OH-Cbl (VITAMIN B 12 Depot Rotexmedica Injektionslösung, 22946 Trittau, Germany), administered IM, once monthly, by the primary care veterinarian. History and physical examination were carried out at each scheduled appointment for OH-Cbl administration. After the collection of urine samples and OH-Cbl injections were done in the same manner each time, and all urine samples were stored at −20°C for analysis later. Our protocol called for a decrease in the interval between OH-Cbl injections if health problems attributable

to Cbl deficiency occurred. The definition of health problems was deliberately kept broad because IGS can manifest itself in a variety of clinical signs. The injection interval was changed from once monthly to once every 2 months (bimonthly) when the clinical well-being of the dogs and the results of urinary MMA measurements indicated adequate Cbl supplementation. The dosing schedule was changed to bimonthly injections after 6 monthly injections in 2 dogs and after 12 monthly injections in 3 dogs. The owner of 1 of the dogs was not willing to collect urine samples after 6 months and thus the dosing schedule remained monthly. Serum Cbl concentrations have been shown to be of limited value for the assessment of Cbl status in dogs with IGS,[17] and thus the decision to include this variable along with urinary MMA concentration was ultimately left to the discretion of the primary care veterinarian and owner. When included, serum samples for Cbl determination were taken immediately before OH-Cbl injections. All urine samples were shipped on dry ice and by a commercial carrier (n = 3) or brought to our clinic by the owner (n = 3).

2.2.2 | Healthy control dogs

Urinary MMA concentrations were determined in 28 clinically healthy dogs that belonged to staff or students and had normal routine laboratory (CBC, serum biochemistry, and urinalysis) results and normal serum Cbl concentrations. There were 5 purebred Beagles (all tested negative for IGS), 4 Labrador Retrievers, 2 Borzois, as well as 12 other breeds and 5 mixed-breed dogs. Immediately frozen voided urine samples were used for analysis in all dogs. Owners of the 28 control dogs reported that all dogs remained clinically healthy after urine collection for a minimum of 20 months (the time of manuscript preparation). Banked surplus serum samples from 20 healthy client-owned dogs (mixed-breed dog [10], Labrador Retriever [2], Nova Scotia Duck Tolling Retriever [1], German Shorthaired Pointer [1], Sibirian Husky [1], Entlebucher Mountain dog [1], West Highland White Terrier [1], Standard Poodle [1], Harrier [1], Rottweiler [1]), collected as part of a different study on laboratory reference values were used to measure serum MMA concentrations.

2.3 | Analyses

2.3.1 | Serum Cbl concentration

Serum samples for Cbl analysis were submitted to 1 of 2 commercial veterinary laboratories (Laboklin Bad Kissingen, Germany. B12 ECLIA on Cobas 8000 e602; IDEXX GmbH Ludwigsburg, Germany. Cbl chemiluminescence assay on ADVIA Centaur XP.) by the primary care veterinarians (n = 12) or samples were frozen and shipped together with urine samples to our laboratory (n = 12). Both commercial veterinary laboratories used chemiluminescence assays for Cbl determination, and the reference intervals (RI) were 221–590 pmol/L (74 pmol/L, lower limit of detection; Laboklin Bad Kissingen, Germany. B12 ECLIA on Cobas 8000 e602) and 173–599 pmol/L (33 pmol/L, lower limit of detection; IDEXX GmbH Ludwigsburg, Germany. Cbl chemiluminescence assay on ADVIA Centaur XP). An automated competitive binding chemiluminescence assay (Immulite 2000, vitamin B12, Siemens Healthcare Diagnostics Inc, Newark, DE) with intra- and inter-assay

coefficients of variation (CV) of 2.1% and 3.4%, respectively, RI of 146–721 pmol/L and lower limit of detection of 111 pmol/L[25] was used in our laboratory.

Urine and serum MMA concentrations were analyzed at the Division of Clinical Chemistry of the University Children's Hospital Zurich according to accredited methods.[26] In brief, samples were supplemented with an internal standard, precipitated, and analysis was done by gas-liquid chromatography-tandem mass spectrometry (GC-MS) on an Ultimate 3000 XRS UHPLC system (Dionex, Thermo Scientific, Waltham, MA) with a SCIEX5500 mass spectrometer (SCIEX, Framingham, MA) using multiple reaction monitoring. The lower limit of quantification for this method was 25 nmol/L in serum and 651 nmol/L in urine. Results for urine MMA were expressed as mmol MMA per mol creatinine. Creatinine concentrations were determined using a kinetic Jaffe method on a DxC600 clinical chemistry analyzer (Beckman Coulter International S.A. Nyon, Switzerland) using commercial reagents. The interassay CVs of the analyses were 5.8% for MMA in serum and urine and 2.7% for creatinine in urine.

2.4 | Statistical analysis

Data were tested for normal distribution using a D'Agostino and Pearson omnibus test. For urine and serum MMA, results of healthy dogs are presented as range and median values. Differences between urinary MMA results obtained during monthly injections were tested using the Friedman test, and differences between monthly and bimonthly urinary MMA concentrations were tested using the Mann-Whitney test. Data were analyzed by use of commercial software (GraphPad PRISM for Windows).

3 | RESULTS

3.1 | Healthy control dogs

Urinary MMA concentrations were not normally distributed in healthy control dogs and ranged from 1.3 to 76.5 mmol/mol creatinine (median, 2.9 mmol/mol creatinine). Serum MMA concentrations in healthy dogs ranged from 539 to 1140 nmol/L (median, 730 nmol/L).

3.2 | Response to monthly Cbl supplementation

3.2.1 | Clinical signs

Dogs received monthly OH-Cbl injections for a median of 9 (range, 6–13) months. All dogs were considered free of clinical signs and described as active and alert by owners and whereas veterinarians receiving monthly OH-Cbl supplementation. Body weight had increased as appropriate for growing dogs from 7 kg to a median of 9 kg (range, 8–13 kg) after 6 months of OH-Cbl supplementation. Four dogs were considered to be athletic based on appearance and activity level. The results of periodic hematologic evaluations undertaken by the primary care veterinarians were normal in all dogs. The owners of dog 5 (Figure 2, yellow squares) were not willing to collect urine samples after 6 months but continued with monthly OH-Cbl injections. Follow-up information was available for another 23 months, and the dog remained clinically normal.

3.2.2 | Serum Cbl concentration

Serum Cbl concentrations were periodically evaluated in 5 of 6 dogs with IGS. In dog 1 (Figure 2, blue circles) serum Cbl concentrations were available for all 6 monthly supplementation time points, and were all below the detection limit of the assay (Immulite 2000, vitamin B12, Siemens Healthcare Diagnostics Inc, Newark, DE). For 4 dogs, 1 serum Cbl measurement each was available. Serum Cbl concentration was low-normal with 212 pmol/L (IDEXX GmbH Ludwigsburg, Germany. Cbl chemiluminescence assay on ADVIA Centaur XP) before the 3rd injection in dog 4 (Figure 2, red squares), and subnormal before the 3rd Cbl injection in dog 5 (Figure 2, yellow squares), before the 4th Cbl injection in dog 6 (Figure 2, green diamonds), and before the 12th Cbl injection in dog 3 (Figure 2, purple triangles).

3.2.3 | Urinary MMA concentrations

Fifty-five urinary MMA results were available from 6 dogs with IGS over a median of 9 (range, 6–12) months. The median baseline urinary MMA concentration before monthly injections was 9.6 mmol/mol creatinine (range, 1.8–77.3; healthy control dogs, median 2.9, range 1.3–76.5). The 2 highest baseline urinary MMA concentrations (77.3 and 20.6 mmol/mol creatinine) were recorded in the two 2-month old littermates (dogs 2 and 3) that had not yet received Cbl supplementation. Results from dog 4 (Figure 2, red squares) differed insofar as all 12 urinary MMA concentrations determined during monthly Cbl supplementation were higher (median, 36.3 mmol/mol creatinine; range, 28.2–50) than the results of the other dogs with IGS, which had urinary MMA of 2 to 13.5 mmol/mol creatinine. Urinary MMA concentrations during monthly OH-Cbl injections did not differ among time points ($P = .74$).

3.2.4 | Additional measurement of serum MMA concentrations in dog 1

Serum MMA concentration was measured in surplus sera from the first 6 time points of Cbl supplementation in dog 1(Figure 2, blue circles). Results ranged from 520 to 900 nmol/L (median, 670 nmol/L; RI, 539–1140 nmol/L).

3.3 | Response to bimonthly Cbl supplementation

3.3.1 | Clinical signs

Five dogs that received bimonthly Cbl injections for a median of 6 (range, 3–10) months were without clinical signs of Cbl deficiency or other diseases.

3.3.2 | Serum Cbl concentrations

Four dogs had serum Cbl concentrations measured. In dog 1 (Figure 2, blue circles) serum Cbl concentrations were available for all 5 bimonthly injections; only the result at the 1st time point was in the low-normal range (162 pmol/L), the subsequent 4 Cbl concentrations were below the detection limit of the assay (Immulite 2000, vitamin B12, Siemens Healthcare Diagnostics Inc, Newark, DE). Subnormal serum Cbl concentrations were measured before the 3rd bimonthly injection in dogs 6 (Figure 2, green diamonds) and 3 (Figure 2, purple triangles), and before the 4th bimonthly injection in dog 2 (Figure 2, orange triangles).

3.3.3 | Urinary MMA concentrations

Thirty-one urinary MMA results were available in 5 dogs, which were on a bimonthly Cbl supplementation schedule, over a median of 6 (range, 3–10) months. Results ranged from 1.6 to 10.5 (median, 4.9) mmol/mol creatinine in 4 dogs, whereas higher urinary MMA concentrations (45.7 to 50 mmol/mol creatinine) were detected in dog 4 (Figure 1, red squares). Urinary MMA concentrations did not differ between bimonthly and monthly Cbl supplementation ($P = .58$).

3.3.4 | Additional measurement of serum MMA concentrations in dog 4

In dog 4, additional serum samples were available at the time of the 2nd and 3rd bimonthly OH-Cbl injections, and analysis of serum MMA concentrations detected 657 and 591 nmol/L, respectively (range in healthy dogs, 539–1140 nmol/L).

4 | DISCUSSION

Our findings provide evidence that the schedule for administration of parenteral OH-Cbl can be adjusted to include longer intervals between dosing in Beagles with IGS provided that patients are regularly monitored. Intervals of up to 2 months between IM injections of 1 mg OH-Cbl appear reasonable and therefore can be recommended. All dogs with IGS were deemed clinically normal by the primary care veterinarians and owners throughout the Cbl supplementation period. A similar approach is used in human medicine wherein the frequency of Cbl injections relies primarily on the health status of the patient, and thus the interval between injections can be increased for maintenance treatment in infants with various inborn errors of cellular Cbl metabolism.[4,23,27,28] Monthly IM injections of 1 mg OH-Cbl constitute the most common maintenance regime in children with IGS.[4,22,29–31] To date, 3 original studies have investigated the long-term outcome of maintenance schedules in humans with IGS,[23,28,32] and IM injections of 1 mg Cbl up to every 6 months[23] provided adequate treatment. In addition, a few case reports describe similar maintenance schedules for long-term treatment that include monthly,[33,34] bimonthly,[35] or trimonthly injections of 1 mg OH-Cbl.[36] The situation is quite different in dogs with IGS based on the information provided in case studies, in which dosing intervals were highly variable with injections being done weekly,[9] every 2 weeks,[6,7,10,12,14,15] once monthly,[10,13,15,17] or bimonthly,[13] and in 1 case every 4–5 months.[24] However, regular long-term evaluation of metabolic variables for determining the adequacy of maintenance treatment was not done in any of these cases. Although all published data describe the use of CN-Cbl for treatment of dogs with IGS, OH-Cbl is considered the treatment of choice for children with IGS[4,21,23,32,37] and other types of hereditary Cbl deficiency[38,39] because it is the natural form of Cbl[40] and IM injection of OH-Cbl is thought to cause less pain than CN-Cbl.[37] For these reasons, we replaced CN-Cbl with OH-Cbl
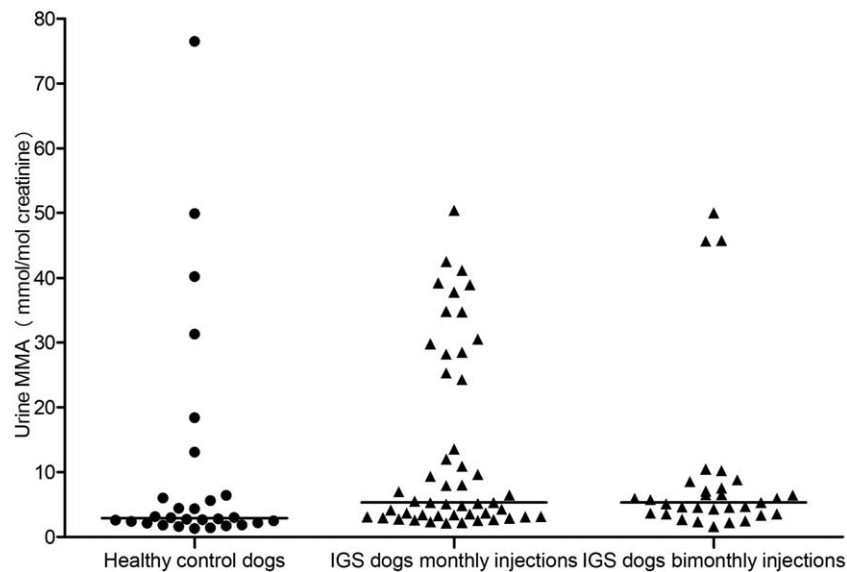
FIGURE 1 Median (horizontal lines) and individual urinary MMA concentrations (mmol/mol creatinine) from urine samples of 28 healthy control dogs, 6 dogs with IGS undergoing monthly, and 5 dogs with IGS undergoing bimonthly supplementation with 1 mg hydroxocobalamin, administered intramuscularly

several years ago for Cbl supplementation in dogs. However, studies that compare the clinical benefits of CN-Cbl and OH-Cbl have not been reported.

Determination of urinary MMA generally is considered a reliable and non-invasive first-line diagnostic and monitoring tool in children with disorders of Cbl metabolism.[21] A survey among European metabolic centers recently showed urinary MMA to be the most valuable variable for predicting outcome in children with inborn errors of Cbl metabolism.[38] We chose to determine MMA in urine rather than in serum or plasma because collection of voided urine samples is less invasive and more convenient for dog owners. In addition, we were concerned that owner compliance would suffer if consecutive blood sampling was required, especially when all dogs were free of clinical signs throughout the study period.

There are currently no guidelines with regard to urinary MMA cut-off points for IGS in humans, and positive responses to Cbl supplementation have been defined only for infants with defects of the mitochondrial enzyme, methylmalonyl-CoA mutase.[38] Very little is known about biochemical responses to Cbl supplementation in dogs with IGS. Although it is well known that urinary MMA concentrations at the time of diagnosis in untreated ill dogs are usually in the thousands,[6,7,9,10,13,14,17,24,25] responses to Cbl supplementation using urinary MMA as a marker of cellular Cbl availability have only been documented in 2 Border collies,[13,14] 2 Beagles,[7,9] and 1 family of Giant Schnauzers.[17,24] Normal urinary MMA concentrations were noted with weekly[9] or biweekly[7] IM injection of various doses of Cbl as well as with parenteral administration of 1 mg Cbl every 1–2 months.[13]

The use of urinary MMA for monitoring the biochemical response to treatment of IGS was described in more detail in a family of Giant Schnauzer puppies.[17] A single IM injection of 1 mg CN-Cbl was found to be sufficient for preventing relapse of clinical and laboratory abnormalities for 2 months despite persistently subnormal serum Cbl

concentrations. The same puppies also had urinary MMA concentrations measured using GC-MS, and urine MMA ranged from 14 to 113 mmol/mol creatinine during the 2 months post-treatment. Age-matched control dogs excreted < 34 mmol/mol creatinine.[17] The affected puppies remained healthy and grew to normal size with regular monthly IM injections of 1 mg CN-Cbl, although this was not monitored long term with MMA measurements.[17]

Variation in urinary MMA concentrations was seen after initiation of monthly OH-Cbl supplementation in our study in agreement with the ranges observed in Giant Schnauzers with IGS.[17] For instance, all urinary MMA results from dog 4 (Figure 2, red squares; median, 38.9 mmol/mol creatinine; range, 28.2–50.4) and 2 urinary MMA results from dog 2 (Figure 2, orange triangles; 24.3 and 31.1 mmol/mol creatinine at t5 and t9, respectively) were higher than most of recorded urinary MMA concentrations, which ranged from 1.6 to 13.5 mmol/mol creatinine. Comparable variation in urinary MMA concentration were found also in healthy control dogs (Figure 1). Differences in urine organic acids profiles also have been reported in populations of healthy infants, with the highest median urinary MMA (95th percentile) of 5.2 (49) mmol/mol creatinine and 29.9 (78.9) mmol/mol creatinine seen at 1 and 6 months of age.[41,42] Concentrations tend to decrease with age because of the steady increase in creatinine excretion as infants develop, especially up to the age of 6 months.[41] The effect of creatinine excretion on urinary MMA concentration may be similar in dogs, but this factor has not been assessed. However, lower muscle mass cannot serve as an explanation for higher urinary MMA results recorded in 3 healthy control dogs (German longhaired pointer, Weimaraner, Borzoi) or in dog 4 (Figure 2, red squares) because they were all muscular dogs. Variation in intestinal bacterial metabolism, especially production of D-lactate and 3-hydroxypropionate, is another well-known cause of discrepancies in urinary MMA excretion in humans.[21,41] An effect of prior food intake on urinary MMA seems less
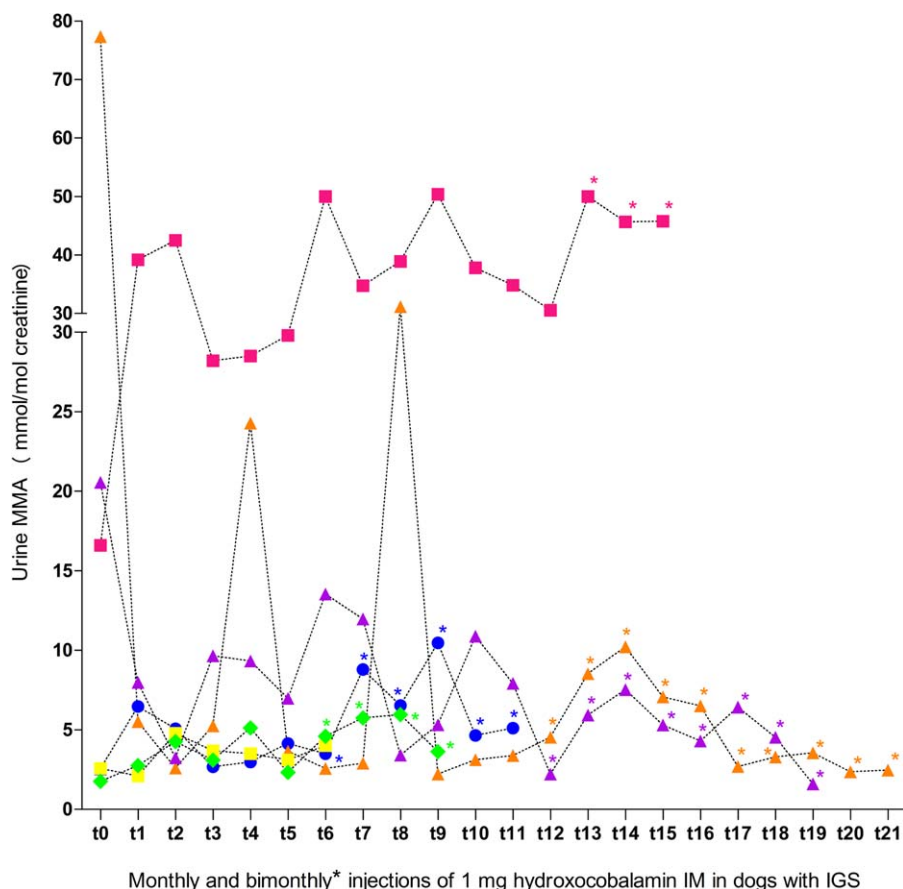
FIGURE 2 Urinary MMA concentrations (mmol/mol creatinine) in 6 dogs with IGS during monthly, and in 5 dogs with IGS during bimonthly (*) supplementation with 1 mg OH-Cbl, administered intramuscularly

likely because all dogs were fed commercial dog foods and postprandial urinary MMA concentrations have been shown only to reach 3 mmol/mol of creatinine in humans.[43] Because ranges in urinary MMA concentration were similar in IGS and healthy control dogs and all urine samples were spontaneously voided, the scattered urinary MMA concentrations might have well been caused by bacterial biosynthesis of MMA.[44,45] Probably the strongest indicator for adequate metabolic Cbl supplementation was the simultaneously low serum MMA concentrations in dog 4, when receiving bimonthly OH-Cbl supplementation. Serum MMA results from dog 1 (with low urine MMA results; Figure 2, blue circles) were equally low during the first 6 months of monthly OH-Cbl injections, further corroborating our theory of bacterial contamination of urine as a source of urinary MMA. Although we are currently in the process of establishing a more robust RI for serum MMA in dogs, our serum MMA results (539–1140 nmol/L) established to date in 20 healthy dogs compare favorably to what has been published recently using similar methodology in 43 healthy dogs (414.7–1192.5 nmol/L).[46]

It has been our long-term experience that serum Cbl concentration can be subnormal or low-normal in treated dogs with IGS that are clinically healthy and have normal MMA or homocysteine concentrations, and thus we did not use serum Cbl concentration as a marker for monitoring Cbl status. Subnormal Cbl concentrations also have been reported in dogs with IGS that were receiving long-term Cbl

supplementation and were clinically normal.[7,14,15,17] Our results showed that only 2 of 24 random serum Cbl measurements were in the low-normal range, and all other concentrations were subnormal (n = 11) or even below the detection limit of the assay (n = 11). The two normal Cbl concentrations were recorded in dog 4 (Figure 2, red squares) immediately before the 3rd monthly Cbl injection when urinary MMA was 42 mmol/mol creatinine and in dog 1 (Figure 2, blue circles) immediately before the first bimonthly Cbl injection when urinary MMA concentration was 8.8 mmol/mol creatinine. The reason for the discrepancy between serum Cbl and urinary MMA concentrations currently is unclear. It is unlikely that pre-analytical factors played a role because samples were routinely processed and it was recently shown that the effects of extended light exposure and room temperature on serum Cbl concentrations were very small.[47] It is possible that the half-life of Cbl bound intracellularly as a coenzyme is longer than the residence time of Cbl in the circulation, which would mean that Cbl continues to be active in tissues and Cbl-dependent metabolism remains normal even when serum concentrations are low. This theory seems plausible given the excellent health status of all IGS dogs, and their MMA concentrations in the range of healthy control dogs. Another possibility is that the lower limit of the serum Cbl RI does not adequately reflect the cellular Cbl status of all dogs. Similar results have been published recently in a study on associations between serum Cbl and MMA concentrations in dogs. More than one-third of 72 dogs

with undetectable serum Cbl concentrations had normal serum MMA results.[46] This dilemma could be solved by measuring the concentration of circulating Cbl bound to its transport protein (holotranscobalamin) and its saturation index.[48] Holotranscobalamin is the biologically active Cbl fraction that can be delivered to all DNA-synthesizing cells, and its measurement appears to better reflect recent Cbl absorption in humans.[49] However, these assays currently are not available for dogs.

Our investigation had several limitations. Simultaneous measurement of serum MMA along with all urinary MMA measurements would have been useful for assessing the clinical relevance of some of the higher urinary MMA results. In addition, urine samples collected by cystocentesis as well as voiding would have helped to determine whether bacterial contamination was a factor affecting urinary MMA concentrations. However, doing so was not possible with the study design, and because all dogs did well clinically, additional blood sampling and repeated cystocentesis were deemed inappropriately invasive.

At the time of writing, all dogs had been monitored for more than 30 months and were doing well clinically with normal hematologic results. In addition, 2 dogs are now on trimonthly OH-Cbl injections, and Cbl supplementation has been changed to the PO route (1 mg once daily; B12 Ankermann Wörwag Pharma GmbH & Co. KG, 71034 Böblingen, Germany) in 3 dogs. We continue to monitor biochemical variables and plan to publish the results later. We have had positive experiences with PO supplementation in older Beagles and Border Collies with IGS from our clinic caseload that did not tolerate the injections anymore. These dogs are clinically normal and have normal hematologic results. Similarly, successful treatment with PO Cbl (1 mg biweekly) also has been reported in humans with IGS, but the frequency of PO intake is still debated.[50]

In conclusion, a maintenance dosage of 1 mg OH-Cbl administered IM appears adequate for maintaining normal clinical status and urinary MMA concentrations for up to 2 months in Beagles with IGS. Our findings suggest that bimonthly parenteral supplementation with OH-Cbl is safe and efficacious for long-term treatment of Beagles with IGS caused by a mutation in the CUBN gene. Further studies are needed to assess if comparable results can be achieved when treating dogs of other known breeds with IGS.

CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflict of interest with the contents of this article.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Authors declare no IACUC or other approval was needed.

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