

## Standard Article

J Vet Intern Med 2016

## Hyperhomocysteinemia in Greyhounds and its Association with Hypofolatemia and Other Clinicopathologic Variables

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**Background:** Folate and cobalamin are essential cofactors for homocysteine (HCY) metabolism. Hyperhomocysteinemia, a multifactorial condition, may reflect B vitamin deficiency and is associated with increased risk of cardiovascular disease, thrombosis, and neurodegenerative and chronic gastrointestinal diseases in humans. Hyperhomocysteinemia has been reported in Greyhounds with suspected chronic enteropathy.

**Objectives:** To evaluate the frequencies of and the association between hypofolatemia and hyperhomocysteinemia in Greyhounds.

**Animals:** Data and serum samples from 559 Greyhounds.

**Methods:** Nested case-control study. The frequency of hypofolatemia in Greyhounds was determined by a laboratory database search. The relationship between hyperhomocysteinemia (measured by gas chromatography-mass spectrometry) and hypocobalaminemia and hypofolatemia was evaluated, and its frequency compared between healthy Greyhounds and Greyhounds with thrombosis or chronic diarrhea.

**Results:** Hypofolatemia was identified in 172 of 423 (41%) Greyhounds and was more common in hypo- than in normocobalaminemic dogs (49% vs. 35%;  $P = .0064$ ). Hyperhomocysteinemia was detected in 53 of 78 (68%) of Greyhounds, being more common in hypo- than in normofolatememic dogs (88% vs. 59%;  $P = .0175$ ). All healthy Greyhounds, 21 of 30 (70%) of dogs with chronic diarrhea and 6 of 8 (75%) of those with thrombosis, were hyperhomocysteinemic. Serum HCY concentrations were inversely correlated with serum folate concentration ( $\rho = -0.28$ ;  $P = .0386$ ) and were positively associated with serum albumin concentration ( $\rho = 0.66$ ;  $P = .0022$ ).

**Conclusions and Clinical Relevance:** Hyperhomocysteinemia occurs frequently in the Greyhound population. Its association with hypofolatemia suggests decreased intracellular availability of B vitamins, but the functional implications warrant further investigation. Hyperhomocysteinemia in Greyhounds potentially may serve as a spontaneous canine model to further investigate hyperhomocysteinemia in humans.

**Key words:** Dog; Hypocobalaminemia; vitamin B<sub>12</sub>; vitamin B<sub>9</sub>.

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This study was not supported by a grant or otherwise.

Part of the data were presented at the Annual Congress of the 23rd European College of Veterinary Internal Medicine—Companion Animals (ECVIM-CA), Liverpool, UK (12–14 September, 2013); and the 33rd Annual Meeting of the American College of Veterinary Internal Medicine (ACVIM), Indianapolis, IN, USA (June, 3–6, 2015).

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Submitted July 3, 2016; Revised August 22, 2016; Accepted September 13, 2016.

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DOI: 10.1111/jvim.14597

## Abbreviations:

CI	confidence interval
HCY	homocysteine
IQR	interquartile range
OR	odds ratio
OSU	Ohio State University
RI	reference interval
SD	standard deviation
TAMU	Texas A&M University

A recent large database survey indicated that hypocobalaminemia is frequently observed in Greyhounds (40% compared to 21% in dogs of other breeds).<sup>1</sup> It is unknown whether intestinal malabsorption of cobalamin or other micronutrients (e.g. folate) as a consequence of chronic gastrointestinal disease may play a causative role for this finding in the breed. Cobalamin (vitamin B<sub>12</sub>) and folate (vitamin B<sub>9</sub>) are absorbed in the distal and proximal small intestine, respectively, and decreased concentrations of both B vitamins are usually a reflection of chronic gastrointestinal disease associated with these intestinal segments.<sup>2</sup>

In humans, hypocobalaminemia and hypofolatemia have been associated with an increase in serum concentrations of homocysteine (HCY), a sulfurated intermediate amino acid that is synthesized from dietary methionine and then either remethylated to methionine or metabolized to cysteine.<sup>3–5</sup> The B vitamins folic acid

(vitamin B<sub>9</sub>), cobalamin (vitamin B<sub>12</sub>), and pyridoxine (vitamin B<sub>6</sub>) are essential cofactors for enzymes required for HCY metabolism<sup>3–6</sup> and for redox-methylation balance (methoxistasis).<sup>7</sup> Hyperhomocysteinemia thus reflects a lack of intracellular availability of cobalamin, folic acid, or both for the synthesis of methionine<sup>4,5</sup> and represents a sensitive marker for the detection of B vitamin deficiency<sup>5,6,8</sup> and perturbation of methoxistasis.<sup>7</sup>

In humans, hyperhomocysteinemia is an incompletely understood multifactorial condition.<sup>3–5</sup> There is evidence indicating that HCY has direct toxic effects on neurons and endothelial cells<sup>5,8</sup> and that HCY can induce DNA-strand damage, oxidative stress, and apoptosis.<sup>9</sup> In line with these findings, hyperhomocysteinemia has been shown to be associated with cardiovascular disease and with increased risk of venous and arterial thrombosis.<sup>4–6</sup> Neurodegenerative diseases (e.g. cognitive impairment, stroke), chronic gastrointestinal disease, and metabolic and endocrine disorders have also been associated with hyperhomocysteinemia in humans.<sup>4,5,8,10,11</sup>

Recently, cardiovascular and thrombotic diseases<sup>12–19</sup> and an increased frequency of hypocobalaminemia<sup>1</sup> also have been described in Greyhounds. Furthermore, hyperhomocysteinemia in dogs with suspected chronic gastrointestinal disease is associated with hypocobalaminemia,<sup>20</sup> which in turn has been linked to hypoalbuminemia.<sup>2,21</sup> Thus, hypoalbuminemia may affect systemic HCY concentrations, as has been shown previously in people<sup>11</sup> and dogs.<sup>22</sup> It is unknown whether low concentrations of cobalamin, folate, or both as well as hyperhomocysteinemia, are present in Greyhounds with chronic gastrointestinal disease, Greyhounds with thrombotic disease, or healthy Greyhounds.

The aims of our study were to evaluate (1) the frequency of hypofolatemia in hypocobalaminemic Greyhounds and (2) serum HCY concentrations in hypocobalaminemic and hypofolatememic Greyhounds. As a secondary aim of this study (3), serum HCY, cobalamin, and folate concentrations were evaluated in Greyhounds with chronic diarrhea or thrombotic disease as well as in a group of healthy Greyhounds. We hypothesized that hypofolatemia is frequently observed in Greyhounds and that hypocobalaminemia, hypofolatemia, or both are associated with increased HCY concentrations in this breed. We further hypothesized that, compared to healthy Greyhounds, hyperhomocysteinemia would be more common in those dogs with chronic gastrointestinal disease or thrombotic disease.

## Materials and Methods

### Ethics Approval

According to the guidelines of the clinical research review committee (2007–2011) and the Institutional Animal Care and Use Committee (2011–2013) at Texas A&M University (TAMU), formal ethical approval of this study and written owner consent were not needed because all dogs included in the study had been sampled during routine diagnostic investigation and no additional samples had been obtained or interventions performed for the purpose of the study.

### Study Populations and Sampling

**Prevalence of Hypofolatemia.** Sample submissions from Greyhounds (n = 423) to the Gastrointestinal Laboratory at TAMU between March 2006 and February 2010 (48 months) for analysis of serum cobalamin and folate concentrations were retrospectively reviewed. Hypocobalaminemic Greyhounds (serum cobalamin concentration <251 ng/L; reference interval [RI], 251–908 ng/L<sup>a</sup>) were identified and investigated for the proportion of dogs with concurrent hypofolatemia (serum folate concentration <7.7 µg/L; RI, 7.7–24.4 µg/L<sup>a</sup>). Data extracted from the laboratory database included breed, age, sex, and serum concentrations of cobalamin and folate. Information about the submitting veterinarian and the name of the dog and owner was used to exclude multiple submissions from the same dog.

**Frequency of Hyperhomocysteinemia.** For the second part of the study, surplus serum samples from 82 Greyhounds, submitted to the Gastrointestinal Laboratory at TAMU between October 2012 and March 2013 (6 months), were used to measure serum HCY concentrations (RI, 5.0–22.1 µmol/L<sup>20</sup>) in addition to the serum concentrations of cobalamin and folate. Data extracted from the laboratory database included breed, age, sex, and the serum concentrations of cobalamin and folate. Information about the veterinarian and the name of the dog and owner was used to ensure that serial results were removed and only 1 sample included from each dog. Furthermore, all owners or veterinarians of the dogs included in this part of the study were contacted to verify the signalment for each dog.

**Serum HCY Concentrations in Greyhounds with Thrombosis or Chronic Diarrhea and from Healthy Greyhounds.** For the third part of the study, serum samples from Greyhounds with a history of thrombotic events (n = 8; 6 dogs with thrombotic events involving the central nervous system and 1 dog each with a thrombotic event involving a central blood vessel [aorta] or smaller peripheral vessels [blood supply of a limb]), Greyhounds with chronic diarrhea (n = 30), and healthy control Greyhounds enrolled in a blood donor program (n = 16) were collected at the Ohio State University (OSU; n = 27) and Texas A&M University (TAMU; n = 27) between January 2007 and September 2013 (80 months). Serum samples obtained from Greyhounds at OSU were shipped as 1 batch to TAMU overnight on ice packs, and all serum specimens were stored at –80°C until further analysis of serum HCY, cobalamin, and folate at the TAMU Gastrointestinal Laboratory.

Information about the Greyhounds recruited at OSU (n = 27; all 3 groups of Greyhounds) was extracted from the dogs' medical records. Retrieval of information about the dogs recruited at TAMU (n = 27; only Greyhounds with chronic diarrhea) was by phone contact with the veterinarian and return of a completed standard study questionnaire (by fax or email) designed to verify the dog's signalment and evaluate its medical history and current health status. Health status in the control group of Greyhounds was determined on the basis of historical information and the results of a complete physical examination and routine blood work (hematology<sup>b</sup> and serum biochemistry profile<sup>c</sup>).

### Quantification of Serum Cobalamin, Folate, and HCY Concentrations

Serum cobalamin and folate concentrations were measured by means of validated automated solid-phase chemiluminescence assays.<sup>d,e</sup> The lower detection limits of the assays are 150 ng/L and 1.0 µg/L, respectively.<sup>23</sup>

Serum HCY concentrations were measured by use of an established and validated stable isotope dilution gas chromatography-mass spectrometry (GC/MS) assay as previously described.<sup>24</sup> Briefly, standard calibration solutions (200, 100, 50,

25, 12.5, 6.3, and 3.13  $\mu\text{mol/L}$ ) were prepared fresh daily with nonisotopic homocystine<sup>f</sup> in 1 M HCl, and deuterium-labeled homocystine isotope<sup>g</sup> (100  $\mu\text{mol/L}$ ) served as an internal standard. After incubation of serum samples in deuterated homocystine: NaOH (3.4 mM): dithiothreitol (34% [w/v]): ddH<sub>2</sub>O (26.7: 3.3: 3.4: 66.6) for 1 hour at 37°C, HCY was extracted with a chromatography column<sup>h</sup> packed with an ion exchange resin<sup>i</sup> and conditioned with methanol/ddH<sub>2</sub>O. After elution of HCY with 0.4 M acetic acid in methanol, samples were vacuum-dried in nitrogen at 64°C for 30 minutes. Homocystine then was derivatized by silylation with N-methyl-N-tert-butyltrimethylsilyl-trifluoroacetamide: acetonitrile (50 : 50) for 30 minutes at 64°C. Samples were analyzed in a gas chromatograph<sup>j</sup> with a mass-selective detector,<sup>k</sup> with samples being injected into a capillary column<sup>l</sup> at a temperature of 250°C and helium used as carrier gas. The starting temperature was 140°C and was followed by a linear temperature ramp of 30°C/min to 300°C, then 20°C/min to 325°C, and a hold time of 62 seconds. The mass spectrometer source was operated at 230°C, and HCY and its deuterated isotope were quantified by the ions at  $m/z$  420 and 424, respectively. All samples were extracted, derivatized, and analyzed in batches of 20 samples each. This assay has a lower detection limit of 5.0  $\mu\text{mol/L}$ .

### Statistical Analyses

Measurements for continuous variables were first investigated for normality of their distribution by a Shapiro-Wilk  $W$  test. Summary statistics for continuous variables are presented as medians and interquartile ranges (IQR) for nonparametric data and as means  $\pm$  standard deviations (SD) for parametric data. Categorical variables are presented as proportions or percentages.

A Fisher's exact test, with calculation of the odds ratio (OR) and 95% confidence interval (95% CI), was used to test the possibility of an association between: (1) hypocobalaminemia and concurrent hypofolate, (2) hyperhomocysteinemia and either hypocobalaminemia or hypofolate alone, and (3) hyperhomocysteinemia and concurrent hypocobalaminemia and hypofolate. A Mann-Whitney  $U$ -test was utilized to compare serum HCY concentrations in hypocobalaminemic and hypofolatem Greyhounds with those in normocobalaminemic and normofolatem Greyhounds.

Age and serum HCY, cobalamin, and folate concentrations were compared among the 3 groups of Greyhounds by an ANOVA or a nonparametric Kruskal-Wallis test, as applicable. Proportions of categorical variables were compared among the different groups of dogs by use of a chi-square test. Serum HCY concentrations also were assessed with respect to other continuous variables. Correlation analysis was performed to test for any possible correlation between the serum concentration of HCY and the 2 B vitamins (folate and cobalamin) in all Greyhounds and between serum HCY concentrations and 14 clinicopathologic variables (RBC count, hemoglobin concentration, platelet count, WBC count, serum concentrations of BUN, creatinine, phosphorus, total protein, albumin, globulin, and cholesterol, serum Ca $\times$ P product, and serum ALT and AST activities) previously shown to have breed-specific deviations from generic canine reference intervals<sup>25</sup> in 19 Greyhounds by calculating a Spearman rank-sum correlation coefficient  $\rho$  for nonparametric data. For all testing, significance was set at a  $P < .05$ , and the cutoff for statistical significance was adjusted according to the number of correlations ( $n = 14$ ) from  $P < .05$  to  $P < .0035$  by a Bonferroni correction for multiple statistical comparisons.<sup>m</sup> A commercially available software package<sup>n</sup> was used for all statistical analyses.

## Results

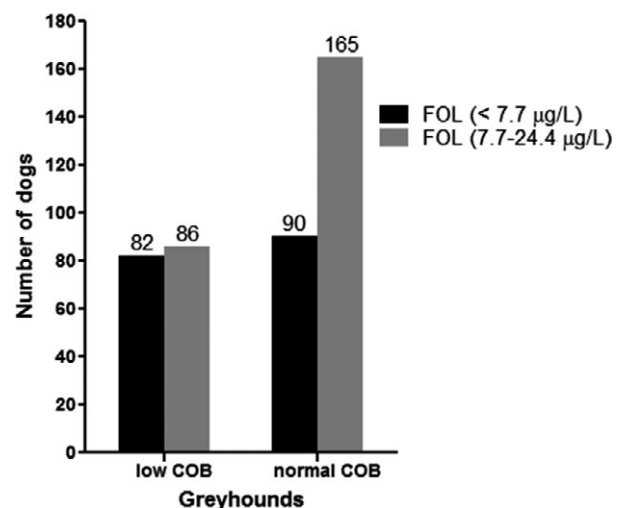
### Prevalence of Hypofolate

In the database review, hypofolate was identified in 172 of the 423 serum samples (41%) from Greyhounds that were submitted for serum cobalamin and folate analysis over a 48-month period; hypofolate was more frequently observed in hypocobalaminemic Greyhounds (82/168, 49%) than in normocobalaminemic Greyhounds (90/255, 35%; odds ratio [OR] [95% CI]: 1.8 [1.2–2.6];  $P = .0064$ ; Fig 1).

### Frequency of Hyperhomocysteinemia

Four of the 82 dogs considered for inclusion in this part of the study were identified as Italian Greyhounds and thus were excluded from further analyses. Hyperhomocysteinemia was identified in 53 of the 78 serum samples (68%) from Greyhounds that were submitted for cobalamin and folate analysis over a 6-month period; hyperhomocysteinemia was detected in 11 of 12 (92%) hypocobalaminemic and hypofolatem Greyhounds and in 28 of 46 (61%) normocobalaminemic and normofolatem Greyhounds ( $P = .0806$ ). Although not statistically significant, serum HCY concentrations were numerically higher in hypocobalaminemic and hypofolatem Greyhounds ( $n = 12$ ; median, 42.6  $\mu\text{mol/L}$ ; interquartile range [IQR], 33.7–55.0  $\mu\text{mol/L}$ ) compared to normocobalaminemic and normofolatem Greyhounds ( $n = 46$ ; median, 30.8  $\mu\text{mol/L}$ ; IQR, 15.0–56.0  $\mu\text{mol/L}$ ;  $P = .1476$ ).

Regardless of the serum folate concentration, hyperhomocysteinemia was identified in 15 of 20 (75%) hypocobalaminemic Greyhounds and in 38 of 58 (64%) normocobalaminemic Greyhounds ( $P = .5808$ ). If only serum folate concentrations were considered for the

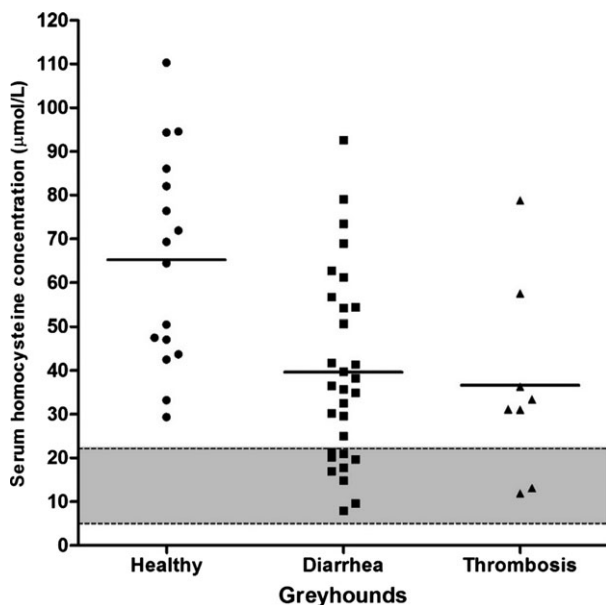


**Fig 1.** Prevalence of hypofolate in Greyhounds ( $n = 423$ ). Shown are the proportions of hypofolatem ( $n = 172$ , 41%; black bars) or normofolatem Greyhounds ( $n = 251$ , 59%; gray bars) divided by concurrent hypocobalaminemia (low COB) or normocobalaminemia (normal COB).

classification of dogs (regardless of the serum cobalamin concentration), hyperhomocysteinemia was identified more frequently in hypofolatememic Greyhounds (21/24, 88%) than in Greyhounds with normofolatememia (32/54, 59%; OR [95% CI], 4.8 [1.3–18.1];  $P = .0175$ ). Neither age ( $P = .6105$ ) nor sex ( $P = .1031$ ) had an effect on serum HCY concentrations, although serum concentrations were numerically higher in males (median, 40.9  $\mu\text{mol/L}$ ; IQR, 25.0–60.9  $\mu\text{mol/L}$ ) compared to female Greyhounds (median, 30.6  $\mu\text{mol/L}$ ; IQR, 13.0–53.3  $\mu\text{mol/L}$ ). Serum HCY concentrations were all  $<9.2 \mu\text{mol/L}$  (median, 8.0  $\mu\text{mol/L}$ ; IQR, 7.1–8.4  $\mu\text{mol/L}$ ) in the 4 Italian Greyhounds identified that were excluded from the study.

### Comparison of Serum HCY Concentrations between Diseased Groups and Healthy Greyhounds

In the group comparison part of the study, serum HCY concentrations differed among the 3 different groups of Greyhounds ( $P = .0012$ ) (Table 1), with healthy Greyhounds having significantly higher serum HCY concentrations than Greyhounds with diarrhea ( $P < .01$ ) or thrombosis ( $P < .05$ ; Fig 2). All healthy Greyhounds had serum HCY concentrations above the upper limit of the previously reported non-breed-specific RI ( $>22.1 \mu\text{mol/L}$ ), whereas 21 of 30 (70%) of the dogs with chronic diarrhea and 6 of 8 (75%) of those with



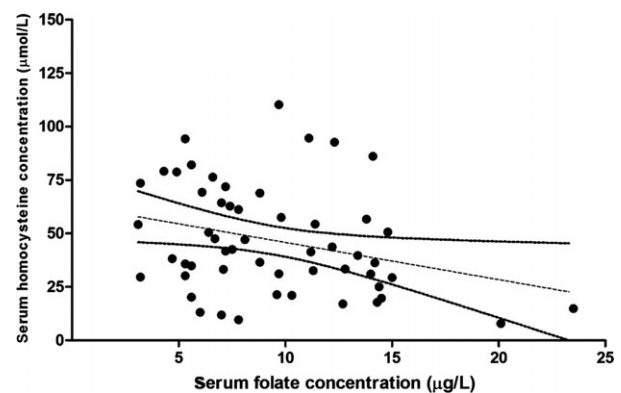
**Fig 2.** Serum homocysteine (HCY) concentrations in 3 groups of Greyhounds ( $n = 54$ ). Serum HCY concentrations were significantly higher in healthy Greyhounds (mean  $\pm$  sd:  $65.2 \pm 24.2 \mu\text{mol/L}$ ) compared to those in Greyhounds with diarrhea ( $39.7 \pm 21.7 \mu\text{mol/L}$ ;  $P < .01$ ) or thrombosis ( $36.7 \pm 22.1 \mu\text{mol/L}$ ;  $P < .05$ ). Solid lines: means; symbols (●, ■, and ▲): serum HCY concentrations in individual dogs; gray-shaded area between dashed lines: previously reported non-breed-specific reference interval (5.0–22.1  $\mu\text{mol/L}$ ).

thrombosis were found to be hyperhomocysteinemic in relation to that RI.

Serum cobalamin and folate concentrations did not differ among the 3 groups of Greyhounds (both:  $P > .05$ ). Serum cobalamin concentrations were within the RI in all healthy Greyhounds, whereas 10 of 30 (33%) of the Greyhounds with chronic diarrhea and 1 of 8 (13%) of those dogs with thrombosis were hypcobalaminemic. Hypofolatememia was associated with hypcobalaminemia ( $P = .0459$ ) and was detected in 10 of 16 (63%) healthy controls, 11 of 30 (37%) Greyhounds with chronic diarrhea, and in 3 of 8 (38%) Greyhounds with thrombosis.

No correlation was observed between the serum HCY and cobalamin concentrations ( $P = .6008$ ), but an inverse correlation was identified between the concentrations of HCY and folate in serum ( $\rho = -0.28$ ;  $P = .0386$ ) when samples from dogs of all groups were analyzed together (Fig 3). Serum HCY concentrations also were negatively correlated with age ( $\rho = -0.42$ ; 95% CI,  $-0.62$  to  $-0.17$ ;  $P = .0020$ ), but Greyhounds with thrombosis were significantly older in this study than those dogs in the control group ( $P < .01$ ). Serum HCY concentrations were numerically higher in male dogs ( $n = 23$ ; median, 56.7  $\mu\text{mol/L}$ ; IQR, 33.6–75.4  $\mu\text{mol/L}$ ) than in female dogs ( $n = 31$ ; median, 35.8  $\mu\text{mol/L}$ ; IQR, 29.5–52.5  $\mu\text{mol/L}$ ), but the difference did not reach statistical significance ( $P = .0643$ ).

Relevant clinicopathologic variables in 19 of the 54 Greyhounds are summarized in Table 2. Evaluation of the relationship between serum HCY concentrations and selected clinicopathologic variables showed a positive association between the serum concentrations of HCY and albumin ( $\rho = 0.66$ ; 95% CI, 0.28 to 0.86;  $P = .0022$ ) but no other significant correlations (for all:  $P > .0035$ ). Only 1 Greyhound (with thrombosis) was found to be hypoalbuminemic (serum albumin



**Fig 3.** Relationship between the serum concentration of homocysteine (HCY) and folate in Greyhounds with chronic diarrhea or thrombosis ( $n = 38$ ) and healthy Greyhounds ( $n = 16$ ). An inverse correlation was detected between the concentration of HCY and folate in serum (Spearman  $\rho = -0.28$ ; 95%CI:  $-0.52$  to  $-0.01$ ;  $P = .0386$ ). Symbols (●): serum HCY and folate concentrations in individual dogs; dotted line: regression line; solid lines: 95% confidence interval of the regression line.

**Table 1.** Characteristics of healthy Greyhounds and Greyhounds with chronic diarrhea or thrombosis (n = 54).

Characteristic	Group			P-value <sup>‡</sup>
	Healthy Controls	Diarrhea	Thrombosis	
Total number, n	16	30	8	–
Age in years, median (IQR) <sup>¶</sup>	5 (4–7) <sup>A</sup>	8 (5–10) <sup>A,B</sup>	10 (9–11) <sup>B</sup>	.0055 <sup>§</sup>
Sex, male/female	8/8	19/11	4/4	.6159
Serum HCY concentration (in $\mu\text{mol/L}$ ), median (IQR) <sup>¶</sup>	66.9 (46.3–83.1) <sup>A</sup>	36.1 (21.1–54.4) <sup>B</sup>	32.3 (26.5–41.6) <sup>B</sup>	.0012 <sup>§</sup>
Serum folate concentration (in $\mu\text{g/L}$ ), median (IQR)	7.1 (6.6–10.0)	9.2 (5.6–13.2)	9.7 (6.7–13.1)	.6907
Serum cobalamin concentration (in $\text{ng/L}$ ), median (IQR)	362 (331–449)	316 (246–435)	339 (280–408)	.2554

n, count; IQR, interquartile range; HCY, homocysteine.

<sup>‡</sup>Global P-value.

<sup>§</sup>Significant difference among the 3 groups of Greyhounds.

<sup>¶</sup>For each parameter, medians (IQR) not sharing a common superscript are significantly different at  $P < .05$ .

concentration, 2.6 g/dL; RI, 2.9–4.2 g/dL). This dog was panhypoproteinemic (serum globulin concentration, 2.0 g/dL; RI, 2.2–2.9 g/dL) and was 1 of the 2 dogs in this group with a normal serum HCY concentration. Hypoglobulinemia was found in 11 of the 19 Greyhounds (58%) from which a serum biochemistry panel was performed. Serum concentrations of HCY were not associated with serum creatinine concentrations ( $P = .2212$ ).

## Discussion

Our study evaluated serum folate, cobalamin, and HCY concentrations in a large population of Greyhounds. Similar to hypocobalaminemia,<sup>1</sup> hypofolatemia also was found to occur frequently in Greyhounds, with combined B hypovitaminoses being more commonly seen than isolated hypocobalaminemia or hypofolatemia. Although this finding may reflect small intestinal malabsorption of both B vitamins as a result of chronic disease,<sup>2</sup> increased demand for these vitamins, or the possibility that systemic concentrations of these vitamins may not reflect their intracellular levels, is a possible alternative explanation.

Although hyperhomocysteinemia was observed in hypofolatemetic and hypocobalaminemic Greyhounds in our study, it also was present in Greyhounds with normal serum concentrations of both B vitamins. Our study did identify an association between hypofolatemia and hyperhomocysteinemia, whereas hypocobalaminemia and hyperhomocysteinemia were not statistically related. These findings also suggest that hyperhomocysteinemia in Greyhounds occurs as a consequence of the lack of these 2 B vitamins, in particular vitamin B<sub>9</sub> (folate), at the intracellular level rather than merely the systemic level.

In keeping with the finding that hyperhomocysteinemia is associated with folate status and oxidative stress,<sup>4,6,7</sup> the inverse correlation observed in our study between serum concentrations of HCY and folate, but not cobalamin, agrees with findings in people with hypertension,<sup>26</sup> cerebral venous thrombosis,<sup>27</sup> epilepsy,<sup>28</sup>

and inflammatory bowel disease.<sup>29,30</sup> However, these findings are in contrast with results in dogs with systemic inflammatory response syndrome or sepsis,<sup>23</sup> dogs with chronic enteropathy,<sup>31</sup> and in some people with inflammatory bowel disease.<sup>11</sup>

The relationship with hypofolatemia in our study suggests that hyperhomocysteinemia in Greyhounds could be a cause or consequence of pathologic conditions (e.g, decreased methylation capacity). However, whether the hyperhomocysteinemia is associated with an increased risk of clinically relevant pathology (such as an increased risk of vascular dysfunction,<sup>32</sup> thrombosis, or other cardiovascular disease) or represents a peculiarity in the Greyhound breed, similar to other breed-specific clinicopathological characteristics likely associated with the unique physiology of this breed,<sup>25,33</sup> cannot be evaluated by the results of our study and requires further investigation. Thus, the clinical relevance and potential need for B vitamin supplementation (particularly folic acid) in Greyhounds requires further study because clinical trials of HCY-lowering treatment in people produced mixed results and did not indicate a clear clinical benefit for all outcomes evaluated.<sup>4,5,7,8</sup> Nevertheless, the finding of hyperhomocysteinemia in all healthy Greyhounds in this study suggests that this breed might be explored as a novel spontaneous canine model to further investigate hyperhomocysteinemia in humans. However, further research is needed to characterize this model.

The transsulfuration (vitamin B<sub>6</sub>-dependent) pathway presents a way to compensate for decreased remethylation of HCY to methionine. However, vitamin B<sub>6</sub> concentrations or the individual methylation potential<sup>7</sup> was not determined as part of our investigation. Whether the transsulfuration pathway plays a role in the hyperhomocysteinemia observed in Greyhounds warrants further study. Also, evaluation of potentially synergistic genetic variations (e.g, enzymes involved in the metabolism of HCY: methylenetetrahydrofolate reductase, methionine synthase, methionine synthase reductase, or cystathionine  $\beta$ -synthase and their cofactors) as well as

**Table 2.** Relevant clinicopathologic data in 19 Greyhounds.

Parameter	Median (IQR)	Reference Interval (RI) <sup>b</sup>	Values Below RI	Values Above RI
<b>Hematology<sup>a</sup></b>				
RBC count ( $\times 10^{12}/L$ )	7.9 (7.3–8.3)	4.8–8.1	0 (0%)	5 (39%)
Hb concentration (g/dL)	18.3 (17.9–19.5)	12.1–18.8	0 (0%)	6 (46%)
Platelet count ( $\times 10^9/L$ )	171 (153–186)	108–433	0 (0%)	0 (0%)
WBC count ( $\times 10^9/L$ )	5.6 (4.3–6.4)	4.1–15.4	3 (23%)	0 (0%)
<b>Clinical chemistry</b>				
BUN (mg/dL)	21.0 (17.5–22.5)	5.0–20.0	0 (0%)	10 (53%)
Creatinine (mg/dL)	1.7 (1.5–2.1)	0.6–1.6	0 (0%)	11 (58%)
Phosphorus (mg/dL)	3.3 (3.1–3.7)	3.2–8.1	9 (47%)	0 (0%)
Ca $\times$ P product (mg <sup>2</sup> /dL <sup>2</sup> )	33.2 (30.5–36.6)	$\leq 60$	–	1 (5%)
Total protein (g/dL)	5.7 (5.6–6.1)	5.1–7.1	3 (16%)	0 (0%)
Albumin (g/dL)	3.6 (3.3–3.7)	2.9–4.2	1 (5%)	0 (0%)
Globulin (g/dL)	2.1 (1.9–2.3)	2.2–2.9	11 (58%)	0 (0%)
Cholesterol (mg/dL)	174 (144–190)	80–315	0 (0%)	0 (0%)
ALT activity (U/L)	46 (39–62)	10–55	0 (0%)	7 (37%)
AST activity (U/L)	42 (32–49)	12–40	0 (0%)	10 (53%)

IQR, interquartile range; Hb, hemoglobin, RBC, red blood cell, BUN, blood urea nitrogen, ALT, alanine aminotransferase; AST, aspartate aminotransferase.

<sup>a</sup>Only available for n = 13 dogs.

<sup>b</sup>OSU Clinical pathology RI (not breed-specific).

nutritional (e.g, dietary protein content, supplementation) and environmental factors is warranted in Greyhounds with hyperhomocysteinemia.

Despite relative hypocoagulability,<sup>25,34</sup> the activity of plasma antithrombin has been shown to be decreased and von Willebrand factor collagen binding to be increased in some Greyhounds,<sup>25</sup> potentially rendering homeostasis a delicate balance between a state of “downregulated” or “upregulated” coagulation in this breed. A procoagulant effect of hyperhomocysteinemia (e.g, induction of tissue factor, activation of factor V, and inhibition of protein C activation and von Willebrand factor secretion) has been identified in people,<sup>35–38</sup> but routine hemostasis or platelet function testing, thromboelastograms, or evaluation of specific coagulation factors or coagulation inhibitors were not included in this study. Thus, the possibility of an association between hyperhomocysteinemia and markers of coagulation, fibrinolysis, and endothelial cell activation and vascular dysfunction in Greyhounds needs to be further investigated. Also, a possible effect of systemic arterial hypertension on serum HCY concentrations in Greyhounds has not been evaluated and warrants further study.

An unexpected finding in our study was a higher frequency of hyperhomocysteinemia in healthy Greyhounds compared to Greyhounds with chronic diarrhea or thrombotic disease. The positive correlation of serum HCY with serum albumin concentration in Greyhounds observed in our study agrees with a previous investigation by this group in dogs with hypcobalaminemia suspected to be associated with chronic enteropathies<sup>22</sup> and in people with end-stage renal disease<sup>39</sup> or nephrotic syndrome,<sup>40</sup> but it contrasts with findings in people with inflammatory bowel disease.<sup>11</sup> A possible explanation for these findings is that the majority (approximately 90%) of systemic

total HCY has been shown to be bound to albumin, with the remainder being bound to globulins.<sup>41</sup> Thus, protein-losing diseases (e.g, protein-losing enteropathy or nephropathy), which were also shown to create a hypercoagulable state,<sup>42,43</sup> may be associated with a lower degree of hyperhomocysteinemia than expected. Nevertheless, the percentage of hyperhomocysteinemic Greyhounds with chronic diarrhea in this study (70%) was similar to that in people in inflammatory bowel disease (50–60%).<sup>11,44</sup>

In humans, hyperhomocysteinemia also can be associated with decreased glomerular filtration rate in patients with renal insufficiency.<sup>5,45</sup> Although staging of possible chronic kidney disease was not included in our study, a correlation between serum HCY and serum creatinine concentration was not observed, and healthy Greyhounds previously were reported to have increased glomerular filtration rates.<sup>46</sup> Furthermore, our study showed that both age and sex do not appear to affect serum HCY concentrations in Greyhounds, which is dissimilar to studies in people where a predominance of hyperhomocysteinemia has been reported in older people and in males.<sup>5,8</sup> However, the possibility must be considered that the significant age differences seen among the 3 groups of Greyhounds may have had a confounding effect on the relationship between aging and serum HCY concentrations. Also, with the large percentage of neutered Greyhounds included in our study (approximately 90%), an effect of sex hormones on serum HCY concentrations may have been masked. A high frequency of concurrent hypoglobulinemia and normoalbuminemia as seen in our study also is consistent with previous reports in Greyhounds.<sup>33</sup>

We acknowledge that our study suffered from some limitations. First, the frequency of hypofolatemia, and also hypcobalaminemia, may have been overestimated because of selection bias with the use of data and

samples from a laboratory database. The specimens from Greyhounds had been submitted to the laboratory for cobalamin and folate analysis, but the specific indication in individual dogs could not be evaluated. Thus, the proportion of hypofolatememic or hypocobalaminemic dogs or both may not reflect the general Greyhound population. Second, the possibility of B vitamin supplementation in individual dogs cannot be excluded. Third, different underlying conditions leading to thrombosis (e.g. protein-losing diseases) could present a confounding factor for the group of dogs with thrombotic events. Consequently, the correlation with hyperhomocysteinemia in this study could be coincidental. Fourth, the intra- and interindividual biologic variation as well as the minimum critical difference or reference change value for serum HCY concentrations has not been evaluated and is unknown in dogs. Lastly, quantification of serum methylmalonic acid would have been useful to evaluate the cellular availability of cobalamin but could not be performed in this retrospective study because of the poor long-term stability of methylmalonic acid in serum samples.

In conclusion, hypofolatememia in Greyhounds was associated with hypocobalaminemia, and increased serum HCY concentrations were observed in hypofolatememic and hypocobalaminemic Greyhounds, but also in Greyhounds with normal serum concentrations of both B vitamins, suggesting a lack of these vitamins at the intracellular rather than the serum concentration. The functional implication of these findings in Greyhounds warrants further study. Healthy Greyhounds had higher serum HCY concentrations than Greyhounds with chronic diarrhea or thrombotic disease, and all healthy Greyhounds were hyperhomocysteinemic, suggesting that hyperhomocysteinemia in healthy Greyhounds might represent a novel spontaneous canine model to further investigate hyperhomocysteinemia in humans. Additional studies are warranted to characterize the potential of this model.

## Footnotes

- <sup>a</sup> Gastrointestinal Laboratory at Texas A&M University (available at: <http://vetmed.tamu.edu/gilab/service/assays/b12folate>)
- <sup>b</sup> Cell-Dyn 3500 Automated Hematology Analyzer, Abbott Diagnostics, Lake Forest, IL
- <sup>c</sup> Cobas<sup>®</sup> c501 Clinical Chemistry Analyzer, Roche Diagnostics, Indianapolis, IN
- <sup>d</sup> Immulite<sup>®</sup>2000, Vitamin B<sub>12</sub>, Siemens Healthcare Diagnostics Inc., Deerfield, IL
- <sup>e</sup> Immulite<sup>®</sup>2000, Folic Acid, Siemens Healthcare Diagnostics Inc., Deerfield, IL
- <sup>f</sup> L-homocystine, Sigma Chemical Co, St. Louis, MO
- <sup>g</sup> DL-Homocystine-3,3,3,3,4,4,4,4-d<sub>8</sub>, C/D/N Isotopes Inc, Pointe-Claire, QC, Canada
- <sup>h</sup> Poly-Prep<sup>®</sup> disposable chromatography column, Bio-Rad, Hercules, CA
- <sup>i</sup> AG<sup>®</sup>1-MP ion exchange resin, Bio-Rad, Hercules, CA
- <sup>j</sup> Agilent model 7890A gas chromatograph, Agilent Technologies, Santa Clara, CA

<sup>k</sup> Agilent 5975C Series mass-selective detector, Agilent Technologies, Santa Clara, CA

<sup>l</sup> DB-1 ms 100% dimethylpolysiloxane column, Agilent Technologies, Santa Clara, CA

<sup>m</sup> Bonferroni correction for multiple statistical comparisons (<http://www.quantitativeskills.com/sisa/calculations/bonfer.htm>)

<sup>n</sup> GraphPad Prism v6, GraphPad Software, La Jolla, CA

## Acknowledgments

Serum samples included in the study were collected at the Gastrointestinal Laboratory at Texas A&M University or the College of Veterinary Medicine at the Ohio State University. Data analysis and manuscript writing were carried out at the College of Veterinary Medicine at the University of Leipzig, Texas A&M University, and the Ohio State University. We acknowledge support from the German Research Foundation (DFG) and Universität Leipzig within the program of Open Access Publishing.

*Conflict of Interest Declaration:* Authors declare no conflict of interest.

*Off-label Antimicrobial Declaration:* Authors declare no off-label use of antimicrobials.

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