Serum cobalamin, urine methylmalonic acid, and plasma total homocysteine concentrations in Border Collies and dogs of other breeds

Sabina Lutz, med vet; Adrian C. Sewell, Dr; Beat Bigler, Dr med vet; Barbara Riond, Dr med vet; Claudia E. Reusch, Prof Dr med vet; Peter H. Kook, Dr med vet

Objective—To determine reference ranges for serum cobalamin (Cbl), urine methylmalonic acid (uMMA), and plasma total homocysteine (tHcys) concentrations and to compare values for healthy control dogs with values for Border Collies (BCs), a breed in which hereditary cobalamin deficiency has been identified.

Animals—113 BCs, 35 healthy control dogs fed a typical diet, and 12 healthy dogs fed a bone and raw food diet exclusively.

Procedures—Urine and blood samples were obtained from each dog and Cbl, uMMA, and tHcys concentrations were determined.

Results—Reference ranges for Cbl (261 to 1,001 ng/L), uMMA (0 to 4.2 mmol/mol of creatinine), and tHcys (4.3 to 18.4 µmol/L) concentrations were determined. Four BCs had a Cbl concentration lower than the assay detection limit (150 ng/L); median uMMA and tHcys concentrations in these dogs were 4,064 mmol/mol of creatinine and 51.5 µmol/L, respectively. Clinical abnormalities included stunted growth, lethargy, anemia, and proteinuria. Abnormalities improved after administration of cobalamin. Of the 109 healthy BCs with Cbl and tHcys concentrations within reference ranges, 41 (37.6%) had a high uMMA concentration (range, 5 to 360 mmol/mol). Results for dogs fed raw food were similar to those for control dogs.

Conclusions and Clinical Relevance—Hereditary cobalamin deficiency is a rare disease with various clinical signs. The finding of methylmalonic aciduria in healthy eucobalaminemic BCs and BCs with clinical signs of Cbl deficiency was surprising and indicated these dogs may have defects in intracellular processing of Cbl or intestinal Cbl malabsorption, respectively. Studies investigating Cbl absorption and metabolic pathways are warranted.

CoA Coenzyme A
CV Coefficient of variation

Hypocobalaminemia can develop in dogs for several reasons, including pancreatic and intestinal disease. In humans, cobalamin deficiency attributable to selective malabsorption is a rare autosomal-recessive hereditary disorder that can be detected in early childhood. Hereditary cobalamin deficiency has been detected in Giant Schnauzers, Australian Shepherd Dogs, and Chinese Shar-Peis. Moreover, other authors have diagnosed cobalamin deficiency in Border Collies and 1 Beagle. Cobalamin is a cofactor for the conversion of methylmalonyl-CoA to succinyll-CoA via methylmalonyl-CoA mutase and for remethylation of homocysteine via methionine synthase. Cobalamin deficiency causes a reduction in activity of both of these enzymes, resulting in increases in methylmalonic acid and total homocysteine concentrations. Measurement of these metabolites allows assessment of availability of cobalamin for cells and is the test of choice to detect early or mild cobalamin deficiency in humans.
Correlation of urine methylmalonic acid and plasma total homocysteine concentrations with serum cobalamin concentrations in dogs has not been determined, to the authors’ knowledge. In addition, reported reference ranges for serum cobalamin concentrations in dogs have not been compared to reference ranges for urine methylmalonic acid or plasma total homocysteine concentrations. Because we have diagnosed cobalamin deficiency in Border Collies that had nonspecific clinical signs, we believed that cobalamin deficiency might be more prevalent than currently recognized. Thus, the purpose of the study reported here was to establish reference ranges for serum cobalamin, urine methylmalonic acid, and plasma total homocysteine concentrations in healthy dogs and to determine concentrations of these markers of cobalamin metabolism in Border Collies.

Materials and Methods

Animals—From July 2009 through September 2010, 113 purebred Border Collies were tested for cobalamin deficiency at the Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich. Border Collie owners were recruited for participation in the study via advertisements on the Swiss Border Collie club website homepage and in Swiss dog magazines and by referring veterinarians who were informed of the study. Assessment of each Border Collie included obtaining a detailed history and performance of a physical examination, CBC, serum biochemical analysis, and urinalysis.

Thirty-five healthy control dogs were recruited for the study; these dogs were owned by staff and students of the clinic. Inclusion criteria for these dogs were as follows: breeds other than Border Collie or Border Collie crossbred, no history of disease within 12 months prior to recruitment, judged to be healthy by the owner, and unremarkable results of physical examination, CBC, serum biochemical analyses, and urinalysis. Thirty-five healthy control dogs were included in the study; these dogs were owned by staff and students of the clinic. Inclusion criteria for these dogs were as follows: breeds other than Border Collie or Border Collie crossbred, no history of disease within 12 months prior to recruitment, judged to be healthy by the owner, and unremarkable results of physical examination, CBC, serum biochemical analyses, and urinalysis.

An additional 12 healthy dogs that were fed bone and raw food exclusively were included in the study. These dogs included 2 Australian Shepherd Dogs, 1 Jack Russell Terrier, 1 Alaskan Malamute, 1 Tervueren, 1 Airedale Terrier, and 6 mixed-breed dogs. Median age of these dogs was 5 years (range, 1 to 15 years), and median body weight was 12.6 kg (range, 5.1 to 43 kg). These dogs included 9 sexually intact females, 5 sexually intact males, 9 spayed females, and 12 neutered males.

An additional 12 healthy dogs that were fed bone and raw food exclusively were included in the study. These dogs included 2 Australian Shepherd Dogs, 1 Jack Russell Terrier, 1 Alaskan Malamute, 1 Tervueren, 1 Airedale Terrier, and 6 mixed-breed dogs. Median age of these dogs was 5 years (range, 1 to 15 years), and median body weight was 22.7 kg (range, 6.1 to 39.7 kg). These dogs included 1 sexually intact female, 1 sexually intact male, 7 spayed females, and 3 neutered males. The study was approved by the Committee for the Permission of Animal Experimentation, Canton of Zurich, Zurich, Switzerland. Owners provided informed consent for inclusion of their dogs in the study.

Study protocol—Food was withheld from all dogs for 8 to 12 hours prior to obtaining blood samples. Blood (8 mL) was obtained from each dog via jugular venipuncture and placed into blood collection tubes. Blood samples were collected by owners the evening or morning before dogs were brought to the clinic. Owners were instructed to store urine samples at 4°C to 6°C. Paired urine samples were collected by owners from 6 of the dogs immediately before and 8 hours after food consumption for determination of the effect of food intake on urinary methylmalonic acid excretion.

Determination of serum cobalamin, plasma total homocysteine, and urine methylmalonic acid concentrations—For determination of serum cobalamin concentrations, blood samples were allowed to clot at room temperature (approx 22°C) for 20 minutes and then centrifuged at 1,500 g for 10 minutes. Serum was harvested and stored at –80°C until measurement. Serum cobalamin concentrations were measured by use of an automated chemiluminescence assay as previously described. The upper limit of detection of this assay was 1,000 ng of cobalamin/L; serum samples were diluted ≥ 1:2 for analysis if the cobalamin concentration was greater than the upper detection limit. The intra-assay and interassay CVs were 2.1% and 3.4%, respectively. The lower detection limit of the assay was 150 ng of cobalamin/L.

Plasma total homocysteine concentrations were determined by use of high-performance liquid chromatography and fluorometric detection. Blood samples collected into chilled tubes containing sodium citrate were centrifuged immediately after collection at 1,570 g at 4°C for 10 minutes. Plasma was obtained and stored at –80°C until performance of assays. Homocysteine was added to a pooled sample of citrated canine plasma (final homocysteine concentration, 100 µmol/L). This pooled plasma sample was sequentially diluted to prepare standards with homocysteine concentrations of 50, 25, 12.5, 5.0, and 2.5 µmol/L. Aliquots of these diluted standards were stored at –80°C until performance of assays; these standards were used to generate standard curves for each assay. Recoveries were assessed by including 3 standards (25, 12.5, and 5.0 µmol homocysteine/L) as samples in 5 assays during the 3-week testing period. Recoveries were > 96% for each standard tested. Because no quality-control samples were commercially available for plasma total homocysteine concentration assays, a pooled sample of canine plasma was tested for each assay (mean homocysteine concentration, 16.8 µmol/L). The between-assay CV for homocysteine concentration in this plasma sample was < 6%. The within-assay CV was < 3% and < 6% for the 50 and 5 µmol of homocysteine/L standards, respectively. The lower limit of detection of this assay was 2.5 µmol of homocysteine/L.

Urine samples were stored at –80°C until measurement. Urine methylmalonic acid concentrations were determined by use of gas chromatography and mass spectrometry with a lower limit of detection of 0.15 mmol/L. Results were expressed as millimoles of urine methylmalonic acid per mole of urine creatinine. Creatinine concentrations were determined via the Jaffe method with an analyzer. This method had been previously validated for determination of methylmalonic acid concentrations in canine urine samples at the University School of Veterinary Medicine, Giessen, Germany.
many with the same model instrument that was used in the present study.

**Statistical analysis**—Data were analyzed by use of software. Each data set was evaluated for normality with the Kolmogorov-Smirnov test. Within the 2 groups (Border Collies and healthy control dogs), serum cobalamin, urine methylmalonic acid, and plasma total homocysteine concentrations and results of a CBC and serum biochemical analyses were compared by use of the Mann-Whitney U test. The Spearman rank correlation coefficient was used to determine the relationship between urine methylmalonic acid, serum cobalamin, and plasma total homocysteine concentrations for both of those groups of dogs. Values of \( P < 0.05 \) were considered significant. Reference ranges were established by use of the nonparametric percentile method. The 2.5 and 97.5 percentiles were determined to achieve the 95% 2-sided reference interval for serum cobalamin and plasma total homocysteine concentrations. For urine methylmalonic acid concentration, the 95th percentile was used to determine a 1-sided reference range (ie, the lower limit of the reference range was 0 mmol/mol of creatinine; the upper limit of the reference range was the 95th percentile value). Serum cobalamin and urine methylmalonic acid concentrations outside the working range of the assays were assumed to be 149 ng/L and 1.9 mmol/mol of creatinine, respectively. Only values for samples obtained from healthy control dogs were used to calculate the reference limit and ranges.

**Results**

**Healthy control dogs**—Cobalamin concentrations in serum samples obtained from healthy control dogs ranged from 261 to 1,001 ng/L (median, 441 ng/L; mean \( \pm \) SD, 540.5 \( \pm \) 235.5 ng/L; Figure 1). The calculated reference range for serum cobalamin concentration was 261 to 1,001 ng/L.

Methylmalonic acid concentrations in urine samples obtained from healthy control dogs ranged from < 2 to 6.6 mmol/mol of creatinine (median, 1.9 mmol/mol of creatinine; mean \( \pm \) SD, 2.1 \( \pm \) 0.8 mmol/mol of creatinine; Figure 2); 32 of the dogs had a urine methylmalonic acid concentration < 2 mmol/mol of creatinine, and 3 dogs had urine methylmalonic acid concentrations of 2.5, 3.4, and 3.6 mmol/mol of creatinine. The upper reference limit calculated for urine methylmalonic acid concentration was 4.2 mmol/mol of creatinine. Food intake had no effect on methylmalonic acid concentrations in urine samples obtained from the 6 dogs for which paired samples were available; concentration of methylmalonic acid in each of these urine samples was < 2 mmol/mol of creatinine.

Total homocysteine concentrations in plasma samples obtained from healthy control dogs ranged from 4.3 to 18.4 \( \mu \)mol/L (median, 9.1 \( \mu \)mol/L; mean \( \pm \) SD, 10.4 \( \pm \) 4.5 \( \mu \)mol/L; Figure 3). The calculated reference range for plasma total homocysteine concentration was 4.3 to 18.4 \( \mu \)mol/L. No correlations were detected (Spearman rank correlation coefficient) between serum cobalamin concentrations and the other variables.
and plasma total homocysteine concentrations, serum cobalamin and urine methylmalonic acid concentrations, or urine methylmalonic acid and plasma total homocysteine concentrations. Results for the 12 dogs that had been fed bone and raw food exclusively were similar to results for the 33 healthy control dogs.

Healthy Border Collies—One hundred nine Border Collies were determined to be healthy. None of these dogs had received cobalamin during the study from any source other than their typical diet (ie, supplemental cobalamin had not been administered). All of these dogs were in athletic physical fitness, and no abnormalities were detected during physical examination. Results of a CBC, serum biochemical analyses, and urinalysis were unremarkable for each of these dogs. Median age of these dogs was 4 years (range, 0.2 to 14 years), and there were 32 sexually intact males, 30 sexually intact females, 28 spayed females, and 19 neutered males. Median body weight of these dogs was 17.3 kg (range, 2.7 to 29.9 kg). Median cobalamin concentration in serum samples obtained from these dogs was 592 ng/L (range, 150 to 1,855 ng/L; mean ± SD, 641.4 ± 304.5 ng/L; Figure 1), which was not significantly different from that in serum samples obtained from healthy control dogs.

Methylmalonic acid concentrations in urine samples obtained from healthy Border Collies ranged from < 2 to 360 mmol/mol of creatinine (median, 1.9 mmol/mol of creatinine; mean ± SD, 23.7 ± 60.1 mmol/mol of creatinine; Figure 2); 47 of the 109 (43.1%) healthy Border Collies had a urine methylmalonic acid concentration > 2 mmol/mol of creatinine (range, 3.2 to 360 mmol/mol of creatinine), and 41 (37.6%) dogs had a urine methylmalonic acid concentration higher than the upper reference limit (4.2 mmol/mol of creatinine). Methylmalonic acid concentrations in urine samples obtained from healthy Border Collies were significantly (P < 0.001) higher than they were in urine samples obtained from healthy control dogs.

Creatinine concentrations in urine samples obtained from the 41 Border Collies with an elevated urine methylmalonic acid concentration were not significantly different from those in urine samples obtained from the 68 Border Collies with a urine methylmalonic acid concentration within the reference range. Total homocysteine concentrations in plasma samples obtained from the 109 healthy Border Collies ranged from 2.8 to 22.4 µmol/L (median, 8.5 µmol/L; mean ± SD, 9.5 ± 4.0 µmol/L; Figure 3) and were not significantly different from those in plasma samples obtained from healthy control dogs. Five of the healthy Border Collies had a serum cobalamin concentration lower than the reference range (261 to 1,001 ng/L); serum cobalamin concentrations ranged from 150 to 259 ng/L (median, 251 ng/L) in these dogs. These 5 Border Collies had urine methylmalonic acid and total homocysteine concentrations within the reference ranges.

Serum cobalamin and plasma total homocysteine concentrations of the 47 healthy Border Collies with a urine methylmalonic acid concentration > 2 mmol/mol of creatinine were not significantly different from those of healthy control dogs. Spearman rank correlation coefficient analysis did not reveal significant correlations among serum cobalamin, plasma total homocysteine, or urine methylmalonic acid concentrations in healthy Border Collies or in Border Collies with a urine methylmalonic acid concentration higher than the upper reference limit.

Border Collies with cobalamin deficiency—Cobalamin deficiency was diagnosed in 4 of the 113 Border Collies. Median age of these 4 Border Collies was 11.5 months (range, 8 to 42 months), median body weight was 11.6 kg (range, 11 to 12.1 kg), and all of these dogs were sexually intact females. All of these dogs had serum cobalamin concentrations < 150 ng/L (Figure 1); median urine methylmalonic acid concentration for these dogs was 4.064 mmol/mol of creatinine (range, 1,800 to 6,665 mmol/mol of creatinine; Figure 2), and median plasma total homocysteine concentration was 51.5 µmol/L (range, 40 to 81.6 µmol/L; Figure 3). Each of these 4 dogs had been fed a different commercial dog food.


Discussion

To the authors’ knowledge, serum cobalamin concentrations have not been correlated with concentrations of the biomarkers methylmalonic acid and total homocysteine in healthy pet dogs, and methods used to determine reference ranges for these factors have not been reported. Reference ranges determined in the present study were similar to previously reported16 reference ranges for these factors. Although, to the authors’ knowledge, no criterion-referenced method exists for determination of cobalamin status in humans or other animals, a plasma methylmalonic acid concentration within the reference range is typically considered to be supportive of a clinically normal cobalamin status in humans (even when serum cobalamin concentration is low).17

Little information has been published regarding methylmalonic acid concentrations in dogs. Cats with elevated serum methylmalonic acid concentrations have low serum cobalamin concentrations; serum methylmalonic acid concentrations decrease after administration of cobalamin to these cats.18 Similarly, other authors13 recently reported that there is a negative correlation between serum cobalamin and methylmalonic acid concentrations in dogs. Results of that study13 also indicate that measurement of serum methylmalonic acid concentration may be a better diagnostic test for detection of cobalamin deficiency than is measurement of serum cobalamin concentration. Measurement of urine methylmalonic acid concentration has only sporadically been reported, and no reference ranges have been established.9,10,12,15 Measurement of urine methylmalonic acid concentration may have sev-
eral advantages for determination of cobalamin status. Methylymalonic acid concentrations in urine are up to 40-fold as high as they are in serum in humans and are therefore easy to determine.20 In addition, use of the ratio of urine methylmalonic acid to creatinine concentration minimizes influences of hemoconcentration and kidney disease on values.20,21 Additionally, methylmalonic acid is very stable in urine,22 whereas no data have been reported regarding stability of methylmalonic acid in serum, to the authors’ knowledge. As well, free-catch urine samples can be obtained less invasively, compared with collection of blood samples, and they can be easily obtained by owners.

Unexpectedly, urine methylmalonic acid concentrations in healthy Border Collies were significantly higher than those in healthy control dogs in the present study. One cause of elevated urine methylmalonic acid concentration in humans is food intake prior to collection of urine samples, although postprandial urine methylmalonic acid concentrations have only been reported to reach as high as 3 mmol/mol of creatinine.23 It is unlikely that the diet of the dogs influenced results of the present study because conditions under which samples were collected were similar for Border Collies and control dogs. Furthermore, methylmalonic acid concentrations in paired urine samples collected from 6 dogs before and after feeding a standard meal were similar (data not shown). Even if diet of the dogs had a minor impact on elevation of urine methylmalonic acid concentrations in healthy Border Collies, values determined in the present study were much higher than those reported for humans that had not fasted.23

Theoretically, bacterial overgrowth in the small intestine may increase urine methylmalonic acid concentration. An overgrowth of bacteria producing propionic acid, a precursor of methylmalonyl-CoA, could cause an increase in urine methylmalonic acid concentration.24 We cannot exclude this possibility, but consider it unlikely because none of the healthy Border Collies in the present study had a history of digestive problems. Notably, diets did not differ between control dogs and Border Collies.

Extremely high methylmalonic acid concentrations (237, 264, and 360 mmol/mol of creatinine) were detected in urine samples obtained from 3 healthy Border Collies in the present study; these dogs were unrelated to each other and were living in the same household. Each of these dogs were fed a diet of bone and raw food. Because feeding bone and raw food usually requires that food undergo a freeze-thaw cycle, loss of water-soluble B vitamins was suspected. To investigate this possibility, serum cobalamin, plasma total homocysteine, and urine methylmalonic acid concentrations were determined for 12 healthy dogs that were fed bone and raw food exclusively. Results for these dogs were similar to those for control dogs.

It is possible that the healthy eucobalaminemic Border Collies with methylmalonic aciduria in the present study were subclinical carriers of hereditary selective cobalamin malabsorption. Genetic testing would have been required to verify this. However, the lack of difference in serum cobalamin and plasma total homocysteine concentrations between healthy control dogs and the healthy Border Collies indicates it was unlikely that these Border Collies were carriers.

In humans, inborn errors of cellular cobalamin metabolism can cause methylmalonic aciduria.23,24 Intra-cellular cobalamin metabolism involves multiple steps between release of cobalamin from lysosomes and synthesis of adenosylcobalamin in mitochondria (which is required for function of the mitochondrial enzyme methylmalonyl-CoA mutase) and methylcobalamin in cytosol (which is required for function of the cytoplasmic enzyme methionine synthase). Nine defects of this pathway in humans have been identified; these defects cause methylmalonic aciduria, homocysteinemia, or both, depending on the metabolic step that is affected.25,26 In affected humans, serum cobalamin concentrations are typically within the reference range, as was found for healthy Border Collies in the present study. However, in humans, most defects in that cobalamin metabolism pathway cause overt clinical signs and life-threatening disease; humans that have methylmalonic aciduria and are asymptomatic for disease are rarely identified.26

The finding that urine methylmalonic acid concentration was increased in 37.6% of healthy Border Collies in the present study might indicate these dogs had benign methylmalonic aciduria, which, to the authors’ knowledge, has not been identified in dogs. Benign methylmalonic aciduria has been identified in human children with no evidence of cobalamin deficiency; methylmalonic aciduria does not resolve after administration of cobalamin to these children.27 Two siblings in that study27 were found to have a defect in the methylmalonyl-CoA mutase enzyme. Authors of another report28 described benign methylmalonic aciduria in a Turkish family, 3 members of which had serum cobalamin and plasma and urine total homocysteine concentrations within the respective reference limits. Results of extensive biochemical screening for other known causes of methylmalonic aciduria, including function of the methylmalonyl-CoA mutase system, were unremarkable for those family members.29

All Border Collies with cobalamin deficiency in the present study had elevated plasma total homocysteine concentrations; these concentrations were higher than those in any of the healthy control dogs. Homocysteine is the intermediate product of methionine metabolism, and subsequent steps in the metabolic pathway are cobalamin-dependent. Homocysteine concentration is a sensitive indicator of cobalamin status in humans; homocysteine concentrations increase early in the course of cobalamin deficiency and often precede development of clinical signs. Renal disease, hemocoagulation, thyroid disease, folate deficiency, and use of certain medications (eg, omeprazole, cyclosporine, and antiepileptic drugs) are known causes of hyperhomocysteinemia in humans.30 Similarly, increased total homocysteine concentrations are associated with renal and cardiac diseases in dogs.30 None of these causes of elevated homocysteine concentration were detected in the Border Collies with cobalamin deficiency in the present study.

Interestingly, none of the 41 healthy Border Collies in the present study that had an elevated urine meth-

a. Sarstedt, Sevelen, Switzerland.
d. QPSO50A, Shimadzu, Kyoto, Japan.
e. ABAXPenta 400 analyzer, AxonLab, Stuttgart, Germany.
f. GraphPad Prism, version 5.0, GraphPad Software Inc, San Diego, Calif.

References