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The effects of age and sex on reference intervals for cobalamin, homocysteine, and serum and urinary methylmalonic acid in healthy adult dogs

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Abstract

Background: In dogs, data on reference intervals (RIs) for cobalamin, markers of metabolism (markersB_{12}met), age and sex effects are limited.

Hypothesis/Objectives: Establish RI for serum cobalamin, homocysteine, and methylmalonic acid (sMMA) concentrations, urinary methylmalonic acid-to-creatinine ratio (uMMA:crea), and determine effects of sex and age.

Methods: Prospective study using healthy dogs (1-10 years). Cobalamin and markersB_{12}met were determined using chemiluminescence immunoassay (cobalamin) and liquid chromatography/tandem mass spectrometry (homocysteine, sMMA, uMMA:crea). In dogs with outlying data, changes in health, markersB_{12}met, and onset of gastrointestinal signs were reevaluated after 9-15 months.

Results: Twelve of 120 healthy dogs had abnormal uMMA:crea ratios. No other cobalamin analyte outliers were found. Outlying data re-examination (odRE) was performed in 10/12 dogs. Chronic gastrointestinal signs occurred in 64% of odRE-dogs, whereas 36% remained healthy. In total, 112 dogs (67 females, 45 males; median ages, 3.5 and 3.75 years, respectively) were included in RI analyses. Reference intervals were 178.5-851 pmol/L (cobalamin), 5.8-29.0 µmol/L (homocysteine), 45.3-159.5 µg/L (sMMA), and ≤22.4 mg/g (uMMA:crea). Only age affected cobalamin concentrations (significant decrease). Compared by sex and neuter status, intact male dogs had significantly higher uMMA:crea ratios (median, 13.5; range, 1.9-83.6 mg/g) than the other groups (median, 2.5; range, 0.7-9.7 mg/g; $P < .0001$). Sex-specific RI were ≤58.9 mg/g (intact male) vs ≤5.2 mg/g (females and neutered males).

Abbreviations: CLIA, chemiluminescence immunoassay; CRP, C-reactive protein; CV, coefficient of variation; CV_A, analytical coefficient of variation; CV_I, coefficient of variation within the individual (biological variation); DGGR-lipase, 1,2-o-dilauryl-rac-glycero-3-gludaric acid ester lipase; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; HCy, homocysteineLC-MS/S, liquid chromatography tandem mass spectrometry; HPLC, high pressure liquid chromatography; odRE, outlying data re-examination; SD, standard deviation; sMMA, serum methylmalonic acid concentration; tCBI, total cobalamin concentration; uMMA, urinary methylmalonic acid concentration; uMMA:crea, urinary methylmalonic acid to creatinine ratio; USG, urine specific gravity.

Conclusion and Clinical Importance: Intact male dogs had significantly higher uMMA:crea ratios than the other groups. Thus, sex-specific RI are recommended for uMMA:crea. Because of the wide distribution of uMMA:crea ratios, careful interpretation in intact male dogs is advised.

KEYWORDS
B12 metabolism, canine, serum, sex, urine, vitamin B12

1 | INTRODUCTION

Cobalamin is an essential co-factor of 2 enzymes: methylmalonyl-CoA mutase and methionine synthase. Cobalamin deficiency limits both reactions, leading to increases in the intermediate products methylmalonic acid (MMA) and homocysteine (HCy).[1,2] Hence, HCy and MMA are regarded as markers of intracellular cobalamin deficiency[3,4] or markers of cobalamin metabolism (markersB_{12}met). In human medicine, no gold standard exists to diagnose a patient's cobalamin status because all analytes (total cobalamin concentration [tCbl], MMA, and HCy) lack sensitivity and specificity.[4,5] Hence, because use of a single analyte might lead to misdiagnosis of the true cobalamin status, in humans the combination of tCbl with at least 1 of the markersB_{12}met is recommended.[4,6] In dogs, isolated tCbl still more often is used compared with the combination of tCbl and markersB_{12}met,[5] whereas the latter currently often are limited to research settings. Reference intervals (RIs) in humans for all currently available markersB_{12}met are well established.[7-15] Additionally, the effects of age and sex have been assessed.[13,14,16,17] In contrast, in dogs little data is available.[18-20] Knowledge of HCy and MMA RI in dogs also is hampered by study limitations such as small sample size, breed specific RI, and undefined inclusion and exclusion criteria with a lack of health data for the dogs. Results are conflicting with respect to the effects of age and sex on cobalamin analytes in humans. Some studies report significantly higher tCbl[21-24] and lower HCy concentrations[25,26] in women than men. Also, decreasing tCbl is reported with increasing age and significantly higher HCy and MMA concentrations occur in elderly compared with younger people.[17,21,27,28] Although RI for the evaluation of cobalamin status in dogs exist, little information about the effects of age and sex is available[19] and neither age-specific nor sex-specific RI for dogs have been established. Also, RI for both serum MMA (sMMA) and urinary-MMA-to-creatinine ratio (uMMA:crea) have not been established using the same study samples to address potential differences between sMMA and uMMA:crea.

Thus, our aims were (a) evaluate analytical performance (intra- and inter-assay coefficient of variation [CV]) of tCbl, HCy, sMMA, and uMMA:crea measurements, (b) establish RI for tCbl, HCy, sMMA, and uMMA:crea, (c) evaluate the effect of age and sex on these analytes, and (d) provide sex-specific RI if sex-specific differences occur. It was hypothesized that, similar to humans, tCbl would decrease with age and HCy and MMA would vary by sex.

2 | MATERIALS AND METHODS

2.1 | Assay and analytical performance of cobalamin analytes

This prospective clinical trial was performed between November 2021 and March 2023. It was approved by the Ethics Committee for animal welfare (ethical number: V 54-19 c 20 15 h 01 GI 18/17). All owners gave written consent for inclusion of their dogs in the study.

After collection, sample specimens were immediately frozen and stored at −80°C. Shipping and measurements were performed in 6 batches with a maximum storage time of 2 months until analysis.

2.1.1 | Measurement of cobalamin analytes

Cobalamin was measured using a chemiluminescent enzyme immunoassay (IMMULITE 2000) as previously described[18,19] whereas HCy, sMMA, and uMMA were determined using liquid chromatography/tandem mass spectrometry (LC-MS/MS). Details are provided in Supporting Information. In brief, HCy was analyzed using SCIEX 4000QTRAP, with a Phenomenex C8 precolumn and Thermo Beta-MaxAcidic separation column. Serum MMA and uMMA were measured using SCIEX 4000QTRAP, with a Phenomenex C8 precolumn and Phenomenex Gemini C18 separation column.

2.1.2 | Assessment of intra- and inter-assay coefficients of variation

Intra-assay coefficient of variation (CV) of tCbl was assessed using pooled dog serum whereas commercial control material[1] was used to evaluate inter-assay CV. The CV established for HCy, sMMA, and uMMA were assessed from surplus samples of expected low, medium, and high concentrations from dogs of the study population. Pooled serum, sodium fluoride plasma, and urine specimens were used for determination of sMMA, HCy, and uMMA, respectively, to evaluate intra-assay and inter-assay imprecision. Intra-assay CV was calculated from 20 measurements performed within 1 run for each concentration and analyte. For assessment of inter-assay CV, pooled samples were aliquoted and stored frozen at −20°C until measurement. Measurements were performed once daily on 10 working days.

To determine CVs, mean and standard deviation (SD) were calculated using Excel Office 2019. The CVs were calculated as CV% = (SD/mean) × 100.[29]

2.2 | Determination of reference intervals in the study sample

2.2.1 | Animals

Healthy dogs of all breeds aged 1-10 years were considered. Before scheduling, owners completed a questionnaire including questions about their dog's general housing and wellness (eg, method of feeding, husbandry, vaccination, deworming, travel history) and specific questions about the dog's health (eg, previously diagnosed diseases and medication, changes in appetite and water intake, unintentional weight gain or loss, fecal consistency, frequency of defecation, fecal abnormalities [eg, mucus, blood], vomiting, borborygmi, and flatulence as well as behavioral changes, seizures, voiding abnormalities, pruritus, paw licking, and head shaking).

Health was further assessed by physical examination and basic laboratory testing (hematology, serum biochemistry including concentrations of C-reactive protein [CRP] and activity of 1,2-o-dilauryl-rac-glycero-3-gludaric acid lipase [DGGR lipase], urinalysis including bacterial culture from an aseptically collected urine specimen). Dogs were included if age was within the required age range and general health was established by the questionnaires as well as clinical and laboratory examinations. Exclusion criteria were questionnaire responses suspicious of underlying disease or if abnormalities were found during physical examination or assessment of laboratory test results. Vegetarian or vegan diet, vitamin supplementation, pro- or prebiotics, or systemic antibiotic treatment and any medication used to treat chronic disease (eg, immunosuppressants, gastric protectants, anticonvulsive drugs, analgesics) within 6 months before the study were exclusion criteria.

2.2.2 | Blood samples

Before sampling, dogs were fasted for a minimum of 12 hours. Blood was collected directly into plain tubes (tCbl, sMMA), heparin tubes (biochemistry), sodium fluoride tubes (HCy), and tripotassium ethylenediamin tetraacetic acid (EDTA) tubes (hematology), collected in that order. Hematological analysis[2] and serum biochemistry tests[3] including canine C-reactive protein (CRP)[4] were performed within 4 hours after collection. Sodium fluoride plasma (centrifuged at 10 158.7, 1 minute) was immediately frozen and stored at −80°C. Blood in plain tubes was allowed to clot at room temperature for at least 30 minutes. After centrifugation (10 158.7, 1 minute), the decanted supernatant was frozen and stored at −80°C.

2.2.3 | Urine samples

Urine was collected by cystocentesis, urinary catheterization or as voided sample. An aliquot of 2 mL was immediately frozen at −80°C and stored (plain tube) until uMMA and urinary creatinine[5] concentration measurements. The remainder of the urine sample was used for urinalysis which included urine specific gravity,[6] dip stick chemistry,[7] and manual sediment evaluation. For urine samples collected by cystocentesis or catheterization, bacterial culture also was performed when sufficient sample volume was available. Bacterial culture results were reported as colony forming units/mL (cfu/mL).

2.2.4 | Statistical analysis

Reference intervals of tCbl, HCy, and sMMA were calculated as 2-sided 95% RI including 90% confidence intervals (CIs; Reference Value Advisor 2.1).[30] For uMMA:crea, a 95% right-sided RI was established using MedCalc Statistical Software version 20.112, Ostend, Belgium. The RI of uMMA:crea (mg/g) was calculated as the ratio of uMMA (µg/L) to urinary creatinine (g/L). To facilitate comparison with published data, for each measured uMMA:crea result, the corresponding calculated mmol/mol results are reported.

Before analysis, data distribution of the 4 cobalamin analytes was assessed by visual inspection of histograms and QQ plots in addition to a Shapiro Wilk test using validated software to generate RI (Reference Value Advisor 2.1).[30]

Because of extreme right-skewed data distribution of the uMMA:crea results (Figure 1), dogs with outlying data (odRE-dogs) were scheduled for re-examination after a minimum of 6 months. Outlying data was defined as uMMA:crea results marked as suspected (0.75th percentile + 1.5 × inter quartile range) or definite (0.75th percentile + 3 × inter quartile range) outliers by a Tukey's test result that exceeded the 0.9th percentile (uMMA:crea >30 mg/g [>28.7 mmol/L]).

In odRE-dogs, tCbl and markersB_{12}met were compared between initial and follow-up examinations. Distribution of normality was assessed using the Shapiro-Wilk test. Comparisons were performed using a paired *t* test for tCbl, sMMA, and uMMA:crea, and the Wilcoxon matched-pairs rank test for HCy concentrations.

To compare the proportion of odRE-dogs developing chronic gastrointestinal signs to a control group, control dogs were recruited from dogs of the study population that had no outlying cobalamin analyte results. Control dogs were selected such that each study dog that had its study appointment scheduled right before and after an odRE-dog. Owners of the control group were asked to complete a follow-up questionnaire a minimum of 12 months after their appointments. Clinical follow-up examinations were not possible based on study design and financial restrictions.

The difference of proportions of odRE-dogs and healthy controls that developed chronic gastrointestinal signs was determined using Fisher's exact test (Graph Pad Prism 5, Graph Pad Software Inc., San Diego).

Finally, odRE-dogs that developed gastrointestinal signs (odRE-GI-dogs) after their first examination and dogs that were lost to follow-up were excluded from the calculation of RI.

For group comparisons of the effects of age and sex, distribution of normality was assessed using the Shapiro-Wilk test. Age

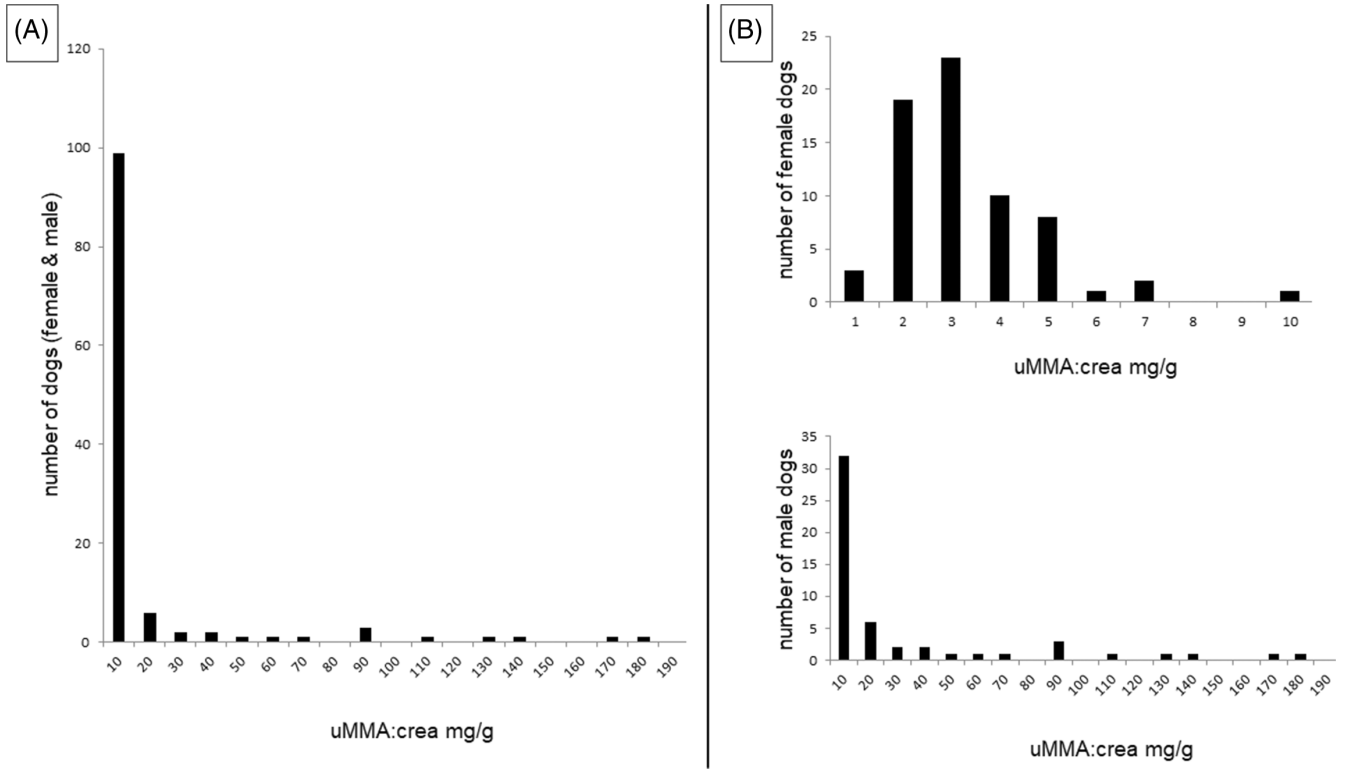

FIGURE 1 Histogram of urinary methylmalonic acid concentrations in 120 healthy dogs. (A) Histogram displays extreme right skewed data distribution. Of 120 dogs, 99 (83%) had urinary methylmalonic acid concentrations given as the ratio to urinary creatinine (uMMA:crea) ≤10 mg/g (9.6 mmol/mol) and 108/120 (90%) dogs showed uMMA:crea ratios ≤30 mg/g (28.7 mmol/mol). (B) Histograms of uMMA:crea ratios (mg/g) of 120 dogs grouped by sex (67 female [upper graph] and 53 male dogs [lower graph]). Note the different scales of the *y*-axes (number of dogs) and *x*-axes (uMMA:crea ratios) of histograms of female and male dogs. All female dogs had uMMA:crea ratios <10.0 mg/g (9.6 mmol/mol) while 32/53 (60%) male dogs had uMMA:crea ratios <10.0 mg/g (9.6 mmol/mol) and 41/53 (77%) had uMMA:crea ratios <30 mg/g (28.5 mmol/mol).

dependency of the final RI was evaluated by polynormal regression analysis after Box-Cox transformation using MedCalc Statistical Software. Additionally, dogs were grouped into 3 age groups (juvenile, <3 years; mature, 3 < 7 years; senior, ≥7 years). Comparisons of the 3 age groups were performed using analysis of variance (ANOVA) and Dunn's multiple comparison test. The effect of sex was evaluated using the Mann-Whitney *U*-test whereas Kruskal-Wallis and Dunn's multiple comparison tests were used for comparison of all 4 sex groups (intact female, spayed female, intact male, neutered male; Graph Pad Prism 5).

For all tests, the level of significance was $P < .05$.

3 | RESULTS

3.1 | Intra- und inter-assay coefficient of variation

Cobalamin low- and high-level intra-assay CVs were 5.1% and 5.3%, respectively, and inter-assay CVs were 6.9% for both low and high levels. Intra-assay CVs for low, medium, and high concentrations of HCy, sMMA, and uMMA were <2.6%. With the exception of the CV of uMMA (CV of 12.4%), all inter-assay CV were <7.7% (Table 1).

3.2 | Initial study sample including dogs with outlying data

Based on responses collected by the questionnaires, 169 dogs were scheduled for study appointments and subjected to clinical and laboratory examinations. In 49 dogs, abnormal clinical examination (mammary gland tumors, SC masses, otitis, skin lesions, heart murmurs, enlarged lymph nodes, poor body condition) or laboratory findings (anemia, thrombocytopenia, thrombocytosis, eosinophilia, increased inflammation markers, hypoalbuminemia, hypercalcemia, increased liver enzyme activities, azotemia, or increased DGGR-lipase activity) led to exclusion. Of all presented dogs, 120 dogs were considered healthy by questionnaire as well as clinical and laboratory examination, and thus were subjected to preliminary RI analysis (Figure 2).

3.3 | Dogs with outlying data

In the preliminary RI analysis, 12/120 uMMA:crea results, all obtained from intact male dogs, were considered outlying data. Ten were marked as suspected outliers and 2/12 as definite outliers by Tukey's test results. None of the odRE-dogs had outlying data for cobalamin or the other markersB_{12}met.

TABLE 1 Intra- and inter-assay coefficients of variation of homocysteine and methylmalonic acid.

	tCbl (pmol/L) low level	tCbl (pmol/L) medium level	tCbl (pmol/L) high level	HCy (µmol/L) low level	HCy (µmol/L) medium level	HCy (µmol/L) high level	sMMA (µg/L) low level	sMMA (µg/L) medium level	sMMA (µg/L) high level	uMMA:crea (mg/g) low level	uMMA:crea (mg/g) medium level	uMMA:crea (mg/g) high level
Sample specimen	Serum	n.a.[a]	Serum	Sodium-fluoride plasma	Sodium-fluoride plasma	Sodium-fluoride plasma	n.a.[b]	Serum	Serum	Urine	Urine	n.a.[b]
Intra-assay												
Number of runs	15	n.a.	15	20	20	20	n.a.	20	20	20	20	n.a.
Mean	357.3	n.a.	796.9	4.8	10.8	25.9	n.a.	86.1	120.6	3376.0	37 825.0	n.a.
SD	18.5	n.a.	41.9	0.1	0.26	0.43	n.a.	2.2	1.7	37.2	405.1	n.a.
CV %	5.1	n.a.	5.3	2.11	2.42	1.70	n.a.	2.55	1.43	1.10	1.07	n.a.
Inter-assay												
Number of runs	15	n.a.	15	10	10	10	10	10	10	10	10	n.a.
Mean	382.5	n.a.	676.9	5.48	17.5	25.3	49.8	96.4	121.9	4407.0	28 030.0	n.a.
SD	26.3	n.a.	41.4	0.42	0.67	1.7	1.26	3.2	7.32	331.0	3466.0	n.a.
CV %	6.9	n.a.	6.1	7.6	3.8	6.7	2.53	3.32	6.0	7.5	12.4	n.a.

Note: Intra- and inter-assay imprecision of tCbl were part of the routine quality performance of the contributing laboratory. Intra-assay coefficient of variation of low and high tCbl concentrations was assessed with pooled dog serum while commercially available control material[c] was used to evaluate inter-assay CV. The CVs established for HCy, sMMA, and uMMA were assessed from surplus samples of dogs enrolled in the current study population. For tCbl concentrations, the assay's lower detection limit was 125 pg/mL. Assay lower detection limits for HCy and MMA (both sMMA and uMMA) were 0.027 µmol/L and 0.3 µg/L, respectively.

Abbreviations: CV, coefficient of variation; HCy, homocysteine; MMA, methylmalonic acid; n.a., not available; SD, standard deviation; sMMA, methylmalonic acid in serum; uMMA:crea, methylmalonic acid in urine, given as the ratio to urinary creatinine.

[a]Assessment of intra- and inter-assay precision of tCbl was part of the corresponding laboratory's routine and was performed prior to the study. This validation routine did not include medium level.

[b]Intra- and inter-assay imprecision of sMMA low level and uMMA:crea high level concentrations were not available due to insufficient sample material from surplus material gained from the dogs included in this study.

[c]Liquicheck Immunoassay Plus control, Bio-Rad Laboratories GmbH, Feldkirchen, Germany.

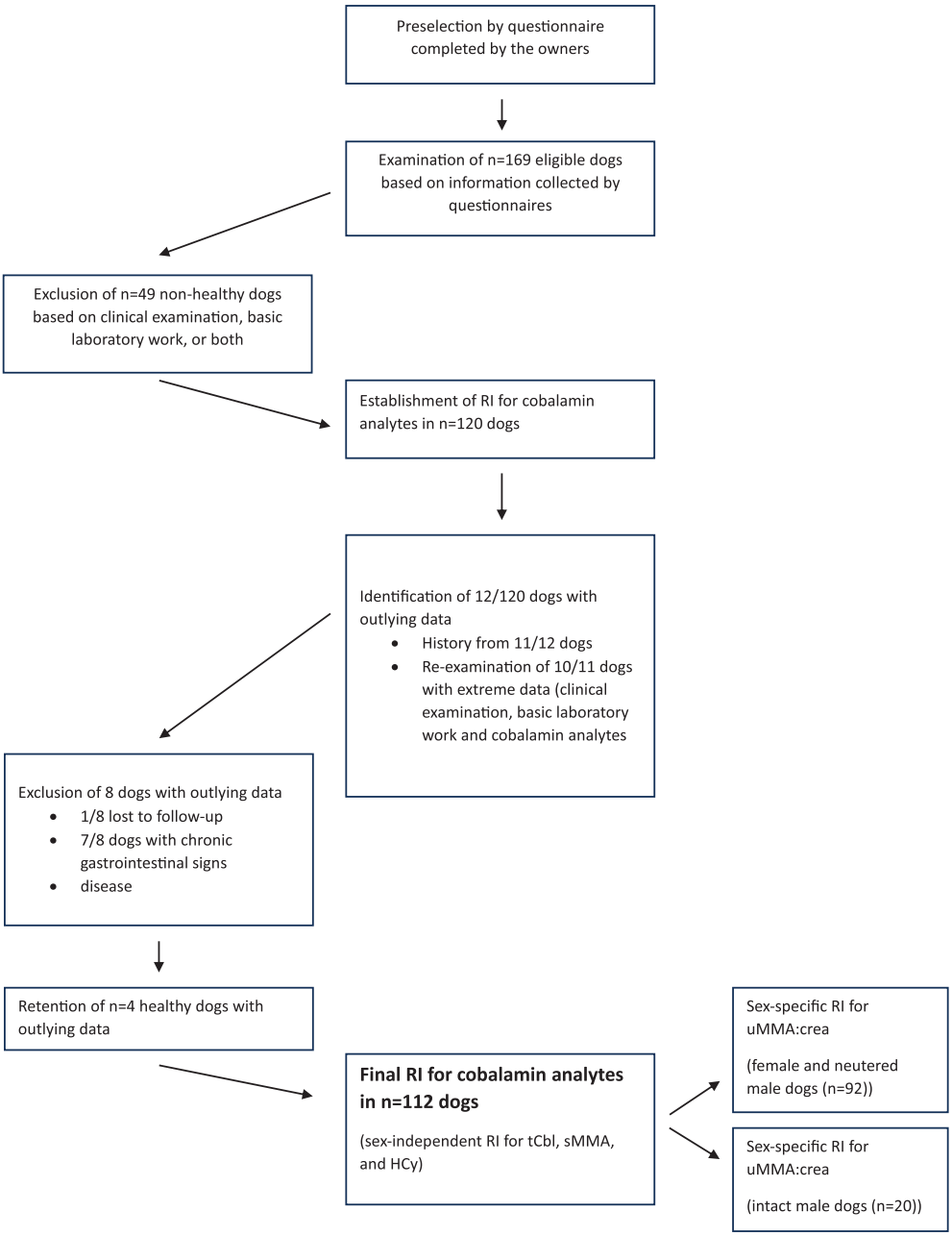

FIGURE 2 Flow chart summarizing the course of inclusion and exclusion of dogs. Of 169 presented dogs, 49 were excluded because of clinical or laboratory abnormalities or both. Samples from 120 dogs fulfilling the inclusion criteria were analyzed and reference intervals (RI) were calculated. When results were identified as outlying data, the respective dogs were re-examined after a minimum of 6 months. Dogs with outlying data of cobalamin analytes developing chronic gastrointestinal signs were excluded whereas outlying data of healthy dogs remained in the final analysis. In total, 112 dogs were used for final RI calculations. Total cobalamin concentration (tCbl), serum methylmalonic acid (sMMA), homocysteine (Hcy), and urinary methylmalonic acid-to-creatinine ratio (uMMA:crea) were compared between male and female dogs. Only uMMA:crea ratios were sex-dependent, and sex-specific RI were calculated.

At the time of initial presentation, urine samples of odRE-dogs were collected by cystocentesis in 9/12 dogs (all with negative bacterial cultures), catheterization in 2/12 dogs (all with negative bacterial cultures), and a voided sample in 1/12 dogs.

Follow-up questionnaire results were available for 11/12 odRE-dogs but only 10/12 odRE-dogs were presented for reevaluation after 9-15 months. One dog was lost to follow-up.

In 7/11 (64%) odRE-dogs, owners reported onset of chronic gastrointestinal signs (eg, increased surface licking or lip smacking, hyporexia, vomiting, or diarrhea on a daily basis or recurrently) weeks to months after their first visit. At reevaluation, none of the 10 odRE-dogs had any abnormalities in their clinical findings or routine laboratory test results. Urine samples at reevaluation were only available as voided samples (collection of samples was performed during the examinations at the clinic). In 7/10 urine samples, sediment examination did not identify inflammatory cells or bacteria. However, in 3/10 samples bacteria were identified microscopically but none of the owners reported voiding abnormalities in their dogs. Comparison of cobalamin analytes between initial and follow-up examination identified no significant differences ($P = .42$, $P = .76$, $P = .42$, and $P = .48$ for tCbl, HCy, sMMA, and uMMA:crea, respectively; Table 2).

To compare the proportion of odRE-dogs with controls, 22 dogs without outlying uMMA:crea data distribution were recruited. Owners of control dogs completed a follow-up questionnaire 13-18 months after their appointments. Of the controls, 4/22 (18%) dogs developed chronic or intermittent gastrointestinal signs after their study participation. A significant difference was identified between the proportion

TABLE 2 Dogs with outlying data in uMMA:crea concentrations.

	Breed	Age first exam (years)	Duration between exams (months)	tCbl 1 (pmol/L)	tCbl 2 (pmol/L)	HCy 1 (μmol/L)	HCy 2 (μmol/L)	sMMA 1 (μg/L)	sMMA 2 (μg/L)	uMMA:crea 1 (mg/g)	uMMA:crea 2 (mg/g)
Patient 1	Mixed breed	7.75	12.5	505.5	649	12	11.5	103	88.5	36.9	31.5
Patient 2	Mixed breed	3.75	12	687.1	726	24.2	18.6	n.a.	109	82.4	99.7[a]
Patient 3	Mixed breed	10.5	12	352	337	11.1	10.6	82.8	44.2	83.6	77.1
Patient 4	Mixed breed	6.5	15.75	476.7	656	13.6	12.9	98	79.3	44.4	36.6
Patient 5	Nova Scotia	2.25	8.5	608.9	545	16	20.7	146	171	168.8	201
Patient 6	Labrador Retriever	1.75	12.25	629.5	520	9.23	8.08	110	112	135.4	156.9
Patient 7	Mixed breed	3.5	12	570	466	11.4	10.1	138	175	86.2	165
Patient 8	Magya Vizsla	1.5	12	572	697	10.5	12.9	84.7	86.5	124.3	135.5[a]
Patient 9	Samoyede	1.5	9.75	420	562	19.7	27	121	105	101.8	96.9
Patient 10	Dachshund	1.5	14	568.3	753	9.42	13	158	137	179	295.2[a]

Note: Signalment, duration of time between initial and follow-up examination, and cobalamin analytes in 10 dogs with outlying data in uMMA:crea ratios. Patients 5-10 developed recurrent gastrointestinal signs after first study examination. All dogs were intact males.
Abbreviations: 1, initial measurement; 2, follow-up measurement; HCy, homocysteine; n.a., not available; RI, reference interval; sMMA, methylmalonic acid in serum; tCbl, total cobalamin; uMMA:crea, methylmalonic acid in urine, given as the ratio to urinary creatinine.
[a]Free catch urine samples with bacteria identified at sediment examination of urinalysis.

of dogs with gastrointestinal signs in the odRE-group and the controls (64% vs 18%, $P = .02$).

According to RI guidelines, odRE-dogs without development of gastrointestinal signs were included in the final RI calculations,[31] whereas odRE-GI-dogs were excluded. In total, 8 dogs (7 odRE-GI-dog, 1 lost to follow-up) were excluded leaving 112 dogs for final calculation of RIs.

3.4 | Study sample for calculation of reference intervals

As seen in Table 3, the included 112 dogs consisted of 67 female (42/67 intact, 25/67 spayed) and 45 male (20/45 intact, 25/45 neutered) dogs. Grouped by sex, median ages of female and male dogs were 3.5 and 3.75 years, and median body weights were 19.8 and 25.1 kg, respectively. Breed predominance was mixed breed in both groups (Table 3).

Serum creatinine and urea concentrations were within normal limits with a median of 87.7 μmol/L (range, 47.6-119.2; RI, 54-122 μmol/L) and 5.5 mmol/L (range, 3.0-8.8; RI, 3.3-9.8 mmol/L), respectively. Urine specific gravity (USG) was ≥1.030 in 91/112 dogs.

In the included 112 dogs, urine was collected by cystocentesis, catheterization, and voiding in 96, 7, and 9 dogs, respectively. Reasons for catheterization or voiding were inability to perform cystocentesis because of moderate to marked restlessness of the dog or small bladder volume. All dogs catheterized were male (2/7 intact, 5/7 neutered) as were 4/9 dogs with voided samples. Two male dogs with voided samples were intact and 2 were neutered. Bacterial culture was performed on all cystocentesis samples and 6/7 catheter samples. Bacterial culture was negative in all but 1 sample (1000 cfu/mL *E. coli*. Intact female dog with no clinical signs of cystitis, normal sediment examination, uMMA:crea 2.5 mg/g [2.4 mmol/L]). In 7/9 voided samples, no inflammatory cells or bacteria were found whereas in 2/9 samples inflammatory cells but no bacteria were detected. The

TABLE 3 Signalment of 112 healthy dogs included into final RI analyses.

Variable	Female (n = 67)	Male (n = 45)
Age (years)	Median: 3.5 Range: 1-10.5	Median: 3.75 Range: 1-10.5
Sex	Intact: 42 Neutered: 25	Intact: 20 Neutered: 25
Body weight (kg)	Median: 19.8 Range: 3.9-47.0	Median: 25.1 Range: 3.8-46.5
Breed distribution	Australian Shepherd: 7 Bolonka Zwetna: 1 Border Terrier: 1 Boxer: 1 Dachshund: 2 English Cocker: 1 French Bulldog: 2 German Longhair: 1 German Shepherd: 1 Golden Retriever: 6 Gordon Setter: 1 Hovawart: 1 Husky: 3 Labrador Retriever: 4 Miniature Poodle: 1 Mixed: 29 Mudi: 1 Nova Scotia: 1 Old German Herding Dog: 1 Standard Poodle: 1 Vizsla: 1	Australian Shepherd: 2 Bernese Mountain Dog: 1 Bichon Frise: 1 Border Collie: 1 Golden Retriever: 2 Groenedale: 1 Irish Setter: 1 Labrador Retriever: 1 Malinois: 1 Maltese: 1 Miniature Poodle: 1 Mixed: 27 Old German herding dog: 1 Old German Shepherd: 1 Standard Poodle: 1 Tibetanian Terrier: 1 Vizsla: 1

owners of both dogs did not report voiding abnormalities. The dogs (both intact males) had uMMA:crea ratios of 12.3 mg/g (11.8 mmol/mol) and 36.9 mg/g (35.3 mmol/L), respectively.

3.5 | Reference intervals and effect of sex and age

Reference intervals are shown in Table 4. Sufficient serum volume for measurement of both tCbl and sMMA was only available in 91/112 dogs.

TABLE 4 Reference intervals for cobalamin and markersB_{12}met.

Analytes	RI after exclusion of dogs with gastrointestinal signs	Statistical method and number of dogs
tCbl (pmol/L)	178.5-805.1	Robust after Box Cox transformation (n = 112)
HCy (μmol/L)	5.8-29.0	Robust after Box Cox transformation (n = 112)
sMMA (μg/L)	45.3-159.5	Robust after Box Cox transformation (n = 91[a])
uMMA:crea (mg/g)	≤22.4[b]	Robust method (n = 112)

Abbreviations: HCy, homocysteine; markersB_{12}met, markers of cobalamin metabolism; RI, reference interval; sMMA, methylmalonic acid in serum; tCbl, total cobalamin; uMMA:crea, methylmalonic acid in urine, given as the ratio to urinary creatinine.

[a]In dogs where the provided volume of serum specimen was insufficient to measure both tCbl and sMMA concentrations, priority treatment was given to tCbl measurement. Thus, sMMA concentrations were only available in 91 dogs.

[b]Corresponding RI for all dogs with units converted to mmol/mol: ≤21.4 mmol/mol.

A significant effect of age was only detected for tCbl with a significant decrease with increasing age ($P = .004$; Figure 3). Comparing the 3 age groups, median tCbl was significantly higher in the juvenile adult group compared with the senior group (450.9 vs 360.1; $P = .05$; Figure 4). There was no effect of age on HCy, sMMA, and uMMA:crea ($P = .85$, $P = .37$, and $P = .33$, respectively).

Male dogs (n = 45) had significantly higher ($P = .0004$) uMMA:crea ratios (median, 3.8 mg/g; range, 0.9-83.6 mg/g [3.6 mmol/L; range, 0.9-79.9 mmol/L]) than females (n = 67; median, 2.5 mg/g; range, 0.7-9.7 mg/g [2.4 mmol/mol; range, 0.7-9.3 mmol/mol]). This difference was caused by the group of intact male dogs that had significantly higher uMMA:crea ratios than both female groups and neutered male dogs ($P < .0001$; Figure 5). Even if the 4 healthy odRE-dogs (all intact male) included in the final RI calculations were excluded from the RI study group, uMMA:crea would still be significantly higher in intact male dogs ($P = .006$). However, no significant effect of sex on tCbl ($P = .84$), HCy ($P = .14$), and sMMA ($P = .65$) was found. Because of the significant difference in uMMA:crea ratios between intact male and the other dogs, sex-specific RI were calculated. In female and neutered male dogs, RI was ≤5.2 mg/g (90% CI; ≤5.0 mmol/mol) whereas RI in intact male dogs was ≤58.9 mg/g (90% CI; ≤56.3 mmol/mol; robust method; Table 5).

4 | DISCUSSION

We determined RI and the effects of age and sex on tCbl and markersB_{12}met (HCy, sMMA, uMMA:crea) in healthy adult dogs. Cobalamin concentrations significantly decreased with increasing age. In contrast, markersB_{12}met were not affected by age. A significant effect

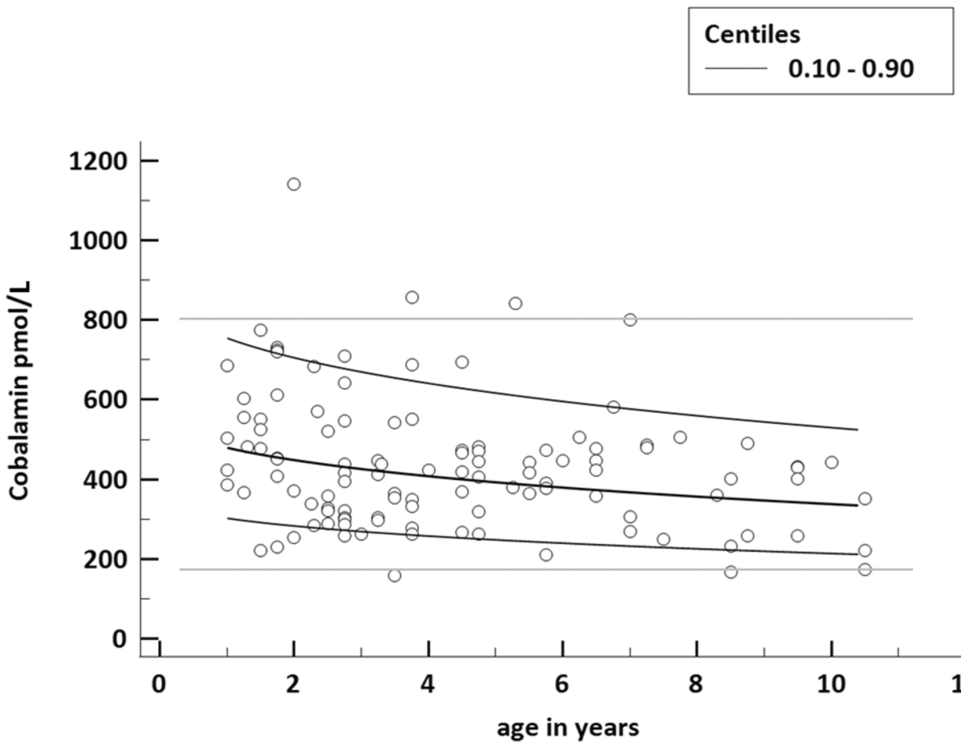

FIGURE 3 Effect of age effect on total cobalamin concentrations. Significant and continuous decrease of total cobalamin concentration (tCbl) occurred with increasing age in adult dogs. The central bold line represents the median and is bound by the 0.01 and 0.90 centiles. The gray lines represent the upper and lower margin of the reference interval for tCbl.

of sex was detected for uMMA:crea ratios: intact male dogs had significantly higher uMMA:crea ratios than females and neutered male dogs, but no effect of sex was found for any other cobalamin analyte. As a consequence, sex-specific RI for uMMA:crea ratios were calculated and RI for intact male dogs were 10-fold higher than for the other sex groups.

For the establishment of new RI, quality standards must be followed.[31] Goals for imprecision are influenced by biological variation within individuals (CV_I). Optimum, desirable, and minimum imprecision goals are given as analytical CVs (CV_A) of <0.25 × CV_I, <0.5 × CV_I, and <0.75 × CV_I, respectively.[32] For dogs, neither intra-individual nor inter-individual biological variations of cobalamin and markersB_{12}met are available. Also, total allowable errors adhering to performance goals given by the American Society of Veterinary Clinical Pathology[29] are not available. Hence, CVs in our study were compared with CVs_A published for dogs and humans. In dogs, cobalamin (Immulite) and HCy (high pressure liquid chromatography [HPLC] and fluorometric detection [sodium-citrate plasma]) inter-assay CVs are reported as 5.5%-7.8% and <6%, respectively. Intra-assay imprecision for HCy ranges between <3% and <6%.[18,33] Coefficients of variation in dogs for sMMA or uMMA have not previously been reported. However, in our study, intra- and inter-assay CVs for both analytes were comparable to CV for human plasma (liquid chromatography electrospray ionization tandem mass spectrometry) and urine (HPLC and LC-MS/MS) with intra-assay CV for plasma MMA of 1.5% and inter-assay CV of 6.7%.[34] Intra-and inter-assay CVs for urine in humans range between 3.1%-8.7% and 2.7%-5.6%, respectively[35,36] and thus, the intra-assay and inter-assay CVs obtained in our study are within published ranges. Thus, imprecision of the test methods in our study was considered acceptable.

Regarding management of outlying data in our study, it generally is not recommended to remove outliers.[31] Nevertheless, the high proportion of suspect and definite outliers in the uMMA:crea data observed in our study was striking. It therefore was considered important that dogs with uMMA:crea ratios >90th percentile were further evaluated in a follow-up examination and compared to controls. Matching of the control dogs was based on the time of presentation of the dogs. In retrospect, matching by sex and neuter status also would have been valuable but was not possible because of limited financial resources.

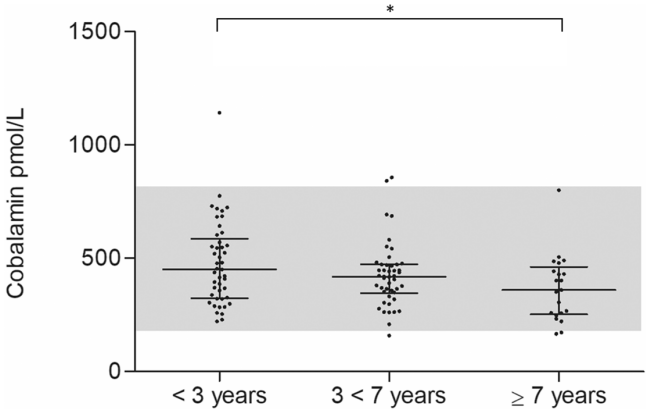

FIGURE 4 Serum cobalamin concentrations in dogs grouped by age. Scatter plot of group comparison of serum cobalamin concentrations between young adult, mature, and senior dogs. Senior dogs had significantly lower total cobalamin concentrations than young adult dogs. Horizontal lines represent the median (central line), the upper and lower interquartile boundary lines. Gray background refers to the upper and lower margins of the reference interval of serum total cobalamin concentrations. The asterisk refers to the level of significance (p=.05).

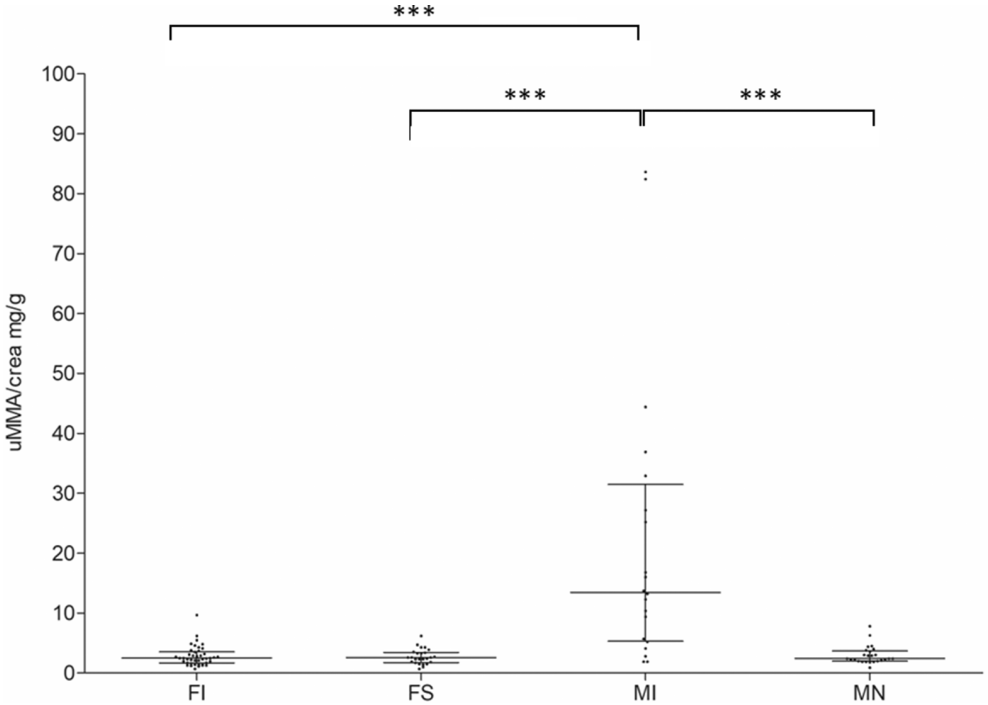

FIGURE 5 Effect of sex and neuter status on urinary methylmalonic acid-to-creatinine ratios. Grouped by sex and neuter status, intact male (MI) dogs had significantly higher ($P = .001$) concentrations of their urinary methylmalonic acid-to-creatinine ratios (uMMA:crea) than neutered male (MN) dogs as well as intact female (FI) and spayed female (FS) dogs. Horizontal lines represent the median (central line), the upper and lower interquartile boundary lines. Asterisks (***) refer to the level of significance (p<.0001).

TABLE 5 uMMA:crea ratios in dogs grouped by sex and neuter status as well as sex-specific reference intervals for uMMA:crea ratios.

	Intact female entire (n = 42)	Spayed female spayed (n = 25)	Neutered male neutered (n = 25)	Intact male (n = 20)
Median	2.5 mg/g (2.4 mmol/mol)	2.6 mg/g (2.5 mmol/mol)	2.4 mg/g (2.3 mmol/mol)	13.5 mg/g (12.9 mmol/mol)
Range	0.7-9.7 mg/g (0.7-9.3 mmol/mol)	0.7-6.2 mg/g (0.7-5.9 mmol/mol)	0.9-7.8 mg/g (0.9-7.4 mmol/mol)	1.9-83.6 mg/g (1.8-79.9 mmol/mol)
Sex-specific RI for uMMA:crea	≤5.2 mg/g (≤5.0 mmol/mol) Robust method (n = 92)			≤58.9 mg/g (56.3 mmol/mol) Robust method (n = 20)

Note: Intact male dogs had significantly higher uMMA:crea ratios compared with neutered male dogs, intact female, and neutered female dogs ($P = .001$).
Abbreviations: RI, reference interval; uMMA:crea, urinary methylmalonic acid-to-urinary-creatinine ratio.

Direct comparison of RI of different study populations is difficult.[29,31] Lack of comparability among studies is caused by limited published data on study methods, different test methods (eg, gas chromatography vs LC-MS/MS [MMA]; gas chromatography or ELISA vs LC-MS/MS [HCy]), and patient demographics (eg, United States of America vs Europe).[31] Nevertheless, RIs for tCbl, HCy, and sMMA obtained in our study are similar to the RI of dogs in other studies.[18-20] There is a difference however between the RI of uMMA:crea in our study sample compared with RIs provided for humans and dogs. In humans, a recently established RI of uMMA:crea ranged from 0.54 to 5.82 mg/g (0.52-5.57 mmol/mol)[36] and most human medical laboratories use a RI for uMMA:crea of <3.8 mg/g (<3.6 mmol/mol).[37-41] Reference intervals of uMMA:crea in humans are also similar to a RI previously established in dogs (uMMA:crea ≤4.4 mg/g [≤4.2 mmol/mol]).[18] Contrary to the published RI in humans and dogs, the RI of uMMA:crea in our study was much higher (uMMA:crea ≤22.4 mg/g [≤21.4 mmol/mol]). However, using our sex-specific RI, the RI of female and neutered male dogs (uMMA:crea ≤5.2 mg/g [≤5.0 mmol/mol]) would be in agreement with published RI, which contrasts the 10-fold higher RI of intact male dogs. Interestingly, in men, uMMA concentrations are significantly lower than in women. Although statistically significant, the difference is very small and not considered clinically relevant.[36] Nevertheless, sMMA concentrations do not differ between men and women,[42,43] which is in agreement with the dogs evaluated in our study. In our study, the impact of sex on uMMA:crea but not sMMA concentrations was striking because generally a similar effect on uMMA and sMMA concentrations would be expected.[44] Even after exclusion of all odRE-dogs from the statistical comparisons, intact male dogs still had significantly higher uMMA:crea ratios than the other sex groups, with a broad range of uMMA:crea values. A group of 30 dogs serving as healthy controls for beagles with Imerslund-Graesbeck syndrome (IGS) had uMMA:crea ratios of 1.4-80 mg/g (1.3-76.5 mmol/mol), which not only match the RI of uMMA:crea of our study population[45,46] but also have a similar distribution of uMMA:crea ratios. Although most dogs had uMMA:crea ratios <10.5 mg/g (<10 mmol/mol), some had ratios as high as 83.7 mg/g (80 mmol/mol). However, sex and neuter status in those healthy dogs were not reported.[46] It thus remains unknown, whether this distribution was caused by inter-individual variation of intact male dogs or other possible reasons such as bacterial MMA biosynthesis in voided urine samples.[45,46] Yet, the extent of the higher uMMA:crea ratios in healthy dogs reported in the previous study and our study population should be viewed with caution. In humans and dogs with inborn errors of MMA metabolism or IGS, uMMA:crea ratios are usually much higher, ranging in the 1000s (mg/g or mmol/mol),[47-51] although patients with lower uMMA:crea ratios are reported.[52,53] Despite exceedingly high uMMA:crea ratios, some human patients remain clinically healthy for years (eg, benign MMA aciduria[53-55]). Also, in dogs with IGS supplemented with cobalamin, weight gain and ability to thrive were observed despite subnormal tCbl concentrations and uMMA:crea ratios ranging up to 118.2 mg/g (113 mmol/mol)[46,47] and thus, smaller increases of uMMA:crea ratios might not be clinically relevant. However, in our study of only healthy dogs higher uMMA:crea ratios were limited to intact male dogs. Thus, a physiologic and most likely hormonal (eg, testosterone) effect seems probable. With respect to normal sMMA in intact male dogs, MMA secretion by the active prostate gland into urine might be possible although no such physiological mechanism was found in the literature. To our knowledge, our study represents the first time MMA concentrations were simultaneously measured in both serum and urine in a large healthy dog population of different breeds. In a small group of dogs with IGS, high uMMA:crea ratios (voided urine specimen) were determined in a single dog with simultaneously normal sMMA concentration. The authors hypothesized potential bacterial biosynthesis of MMA especially because antibiotic treatment in that dog caused a rapid and stable decrease.[45] However, in our study, bacterial synthesis is unlikely because cultures obtained from 103/112 dogs were negative except for 1 sample. Interestingly, this positive bacterial culture was acquired from a female dog with low uMMA:crea ratio. More importantly, in 11/12 odRE-dogs bacterial cultures were negative and thus, bacterial synthesis cannot explain high uMMA:crea ratios. The discordance between sMMA and uMMA in intact male dogs observed here is apparently a novel finding because sMMA and uMMA:crea ratios are expected to have a linear relationship.[44] Apart from cobalamin deficiency, increased sMMA is reported in common conditions such as renal disease[43,56] and small intestinal dysbiosis.[57,58] Increased uMMA:crea ratios were found postprandially[59] in IGS,[47,60] benign MMA aciduria,[53,55] genetic defects in mitochondrial metabolic cobalamin pathways,[61] and as a breed difference.[18] Interestingly, in patients with renal disease and increased plasma cystatin concentrations, uMMA:crea ratios were not increased compared to patients without renal disease.[62] In a more recent study of humans, sMMA was dependent of the glomerular filtration rate, whereas

uMMA:crea ratios were not, and the authors concluded that uMMA:crea ratios are independent of renal function.[56] In our study, uMMA:crea ratios differed between intact male dogs and the remainder of the study population, but sMMA did not. In all dogs, serum creatinine and urea concentrations were within the respective RI, and 91/112 dogs had USG ≥1.030. For the subpopulation of dogs with USG <1.030, interpretation of USG results was hampered because symmetric dimethylarginine concentrations were not assessed because of financial constraints. Also, free access to water was available before the study appointments. However, in the odRE-dogs, serum creatinine and urea concentrations did not increase during the observation period. Thus, renal disease seems an unlikely cause for the increased uMMA:crea ratios in our study population. In contrast to humans,[59] a postprandial effect seems less relevant in dogs.[18] Furthermore, all dogs were fasted for at least 12 hours, which makes a postprandial effect on uMMA:crea concentrations less probable.

In humans, a benign course of chronic methylmalonyl aciduria is described in children who remain clinically unaffected over many years.[53,55] A similar benign effect on methylmalonyl-Co-A mutase pathways would thus be worthy of consideration for the high uMMA:crea ratios in the odRE-dogs because their uMMA:crea ratios did not change over many months. In general, genetic influence on cobalamin analytes in dogs is supported by breed-specific differences. Cobalamin deficient Chinese Shar Pei dogs have higher serum HCy and MMA concentrations than cobalamin deficient dogs of other breeds.[20] Also, healthy Border Collies have significantly higher uMMA:crea ratios compared with healthy dogs of other breeds.[18] Lastly, IGS is a known genetic disorder affecting the cobalamin status in dogs.[60,63] In our study, no breed predisposition was evident and no dog was suspected having IGS. However, a possible genetic (X-linked) effect would not fully explain the discrepancy between intact and neutered male dogs observed in our study. Overall, a hormonal effect on uMMA:crea ratio in intact male dogs in our study population seems to be the most logical explanation for the high uMMA:crea ratios, but future studies focusing on hormonal influences and X-linked genes on uMMA:crea ratios in male dogs are necessary.

Simultaneous increases in HCy and MMA concentrations are considered evidence for cellular cobalamin deficiency,[1,3] which is a common feature of chronic enteropathy.[19,64-66] Compared with controls (4/22, 18%), the significantly higher proportion of odRE-dogs (7/11, 64%) developing chronic gastrointestinal signs was interesting. Increases in MMA concentrations also can be caused by intestinal dysbiosis,[5,67,68] and small intestinal dysbiosis commonly occurs in dogs with chronic gastrointestinal signs.[69-71] Given the significant difference in the onset of chronic gastrointestinal signs between odRE-dogs and controls, small intestinal dysbiosis causing high uMMA:crea ratios could be possible. Because of financial restrictions, analysis of microbiota was not performed in our study. However, in terms of potential underlying chronic enteropathy, alterations in cobalamin analytes (eg, progressive increase of markersB_{12}met accompanying decreasing tCbl concentrations) would be expected in at least some of the odRE-dogs that developed chronic gastrointestinal signs, but neither concentrations of tCbl nor any other markerB_{12}met differed between both examinations in odRE-dogs. Hence, although not excluded, dysbiosis seems a less likely cause of high uMMA:crea ratios in our study population. Overall, with regard to the high uMMA:crea ratios in intact male dogs, a hormonal effect seems the most likely explanation for the identified effect of sex on this analyte.

Data on the effect of age on cobalamin status is scarce in dogs. In 1 study, no effect of age on sMMA was observed.[19] In cats, tCbl decreases with increasing age[72] whereas in humans the effects of age are conflicting. Some studies found a significant decrease in tCBl with increasing age.[16,28] Additionally, higher sMMA[21,27] and HCy[27] were described in elderly compared to younger people. Whether these are true effects of increasing age or rather represent mild underlying chronic gastrointestinal disease in the elderly is not clear yet.[67] In our study, only tCbl significantly decreased with age whereas the other analytes remained unaffected. Therefore, a true age effect seems more likely than an underlying disease causing cobalamin depletion in older dogs.

One major limitation of our study is that follow-up information regarding the healthy control group (n = 22) only consisted of information gathered by a follow-up questionnaire whereas for most of the dogs (n = 87), no follow-up was available. As a consequence, possible later changes in clinical examination, routine laboratory test results, and especially tCbl and markersB_{12}met remain unknown for the majority of dogs. Thus, the true proportion of onset of chronic gastrointestinal signs in our population cannot be determined. Another limitation is that folate measurements were not integrated into the study design. Folate is interconnected with cobalamin by its function as a methyl group donor to the enzyme methionine synthase.[1,73] Although folate derangements in our study population cannot be excluded, a derangement of the folate metabolism would be expected to simultaneously alter MMA in both serum and urine and to also affect HCy concentrations. However, neither sMMA nor HCy were suspicious for folate derangements, and folate likely plays a negligible role here.

In conclusion, age considerably affected tCbl but seems to be of little clinical importance. On the other hand, the effects of sex and neuter status on the uMMA:crea ratios could be clinically relevant. Intact male dogs had significantly higher uMMA:crea ratios than female and neutered male dogs. This difference most likely was caused by a hormonal effect because none of the other cobalamin analytes were affected as would be expected with underlying or developing cobalamin deficiency. Because of the broad distribution of uMMA:crea ratios in intact male dogs, sex-specific RI for uMMA:crea ratios should be used. In diseased intact male dogs, careful test interpretation is advised when uMMA:crea is applied as the only analyte to assess intracellular cobalamin deficiency because a high uMMA:crea ratio may be normal or indicate cobalamin deficiency.

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CONFLICT OF INTEREST DECLARATION

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OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Approved by the ethics committee for animal welfare (Regierungspräsidium Giessen, Hessen). Ethical number: V 54-19 c 20 15 h 01 GI 18/17.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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ENDNOTES

[1] Liquicheck Immunoassay Plus control, Bio-Rad Laboratories GmbH, Feldkirchen, Germany.

[2] ADVIA2120/2120i, Siemes Healthcare GmbH, Erlangen, Germany.

[3] ABX Pentra C400, ABX Horiba, Montpellier, France.

[4] Canine-CRP, Gentian AS, Moss, Norway; performed on ABX Pentra C400, ABX Horiba, Montpellier, France.

[5] ALINITY C CREATININE, Abbott GmbH, Max-Planck-Ring 2, Wiesbaden, Germany.

[6] Refractometer (handheld), Protein 0-12 g/dL, ATC, Euromex Holland, Arnhem, The Netherlands.

[7] Combur 9 Test, Roche Diagnostics (Schweiz) AG, Rotkreuz, Switzerland.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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