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Relationship between cobalamin and folate deficiencies and anemia in dogs

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Background: Megaloblastic, nonregenerative anemia is a well-known consequence of cobalamin or folate deficiencies in humans but is not recognized in hypocobalaminemic or hypofolatemic dogs. Establishment of relationships between hypocobalaminemia or hypofolatemia and hematologic disease would encourage vitamin B testing, and potentially supplementation, in anemic dogs.

Objectives: To determine the prevalence of anemia in hypocobalaminemic or hypofolatemic dogs and to report the prevalence of hypocobalaminemia and hypofolatemia and nonregenerative anemia, macrocytosis, and anisocytosis in anemic dogs.

Animals: One hundred and fourteen client-owned dogs with known serum cobalamin and folate concentrations and CBCs and 42 client-owned anemic dogs.

Methods: Retrospective comparison of anemia prevalence in hypocobalaminemic or hypofolatemic and normocobalaminemic or normofolatemic dogs was performed. Prospective measurement of erythrocyte variables and cobalamin and folate concentrations in anemic dogs was carried out; relationships among hypocobalaminemia and regenerative status, mean corpuscular volume, and red cell distribution width were evaluated.

Results: Significant differences in prevalence of anemia between hypocobalaminemic (36%) and normocobalaminemic dogs (26%; P = .23) or between hypofolatemic (31%) and normofolatemic dogs (30%; P = .99) were not detected. Between hypocobalaminemic and normocobalaminemic dogs, no significant differences in prevalence of nonregenerative anemia (69% vs 63%; P = .65), macrocytosis (17% vs 0%; P = .53), or anisocytosis (28% vs 0%; P = .14) were detected. Anemic dogs had high prevalence of vitamin B deficiencies (nonregenerative: 64% hypocobalaminemic, 18% hypofolatemic; regenerative: 57% hypocobalaminemic, 21% hypofolatemic).

Conclusions and Clinical Importance: The association between cobalamin and folate deficiencies and macrocytic, nonregenerative anemia established in humans is not routinely present in dogs.

KEYWORDS

hypocobalaminemia, macrocytosis, megaloblastic, vitamin B12

1 | INTRODUCTION

Cobalamin and folate are dietary water-soluble B vitamins integral to the synthesis of numerous proteins, neurotransmitters, and nucleic acids.[1–3] Cobalamin is a cofactor for methionine synthase and methylmalonyl CoA mutase, and folate is required for the activity of thymidylate synthase, enzymes essential for normal DNA synthesis, hematopoiesis, and neuron myelination.[1,3,4] Increased methylmalonic acid (MMA) concentration can be a more accurate early indicator of hypocobalaminemia on a cellular level than serum cobalamin concentration.[1,5] Cobalamin or folate deficiency can result in substantial gastrointestinal (GI), reproductive, neurological, and hematologic disorders in humans.[1,3,4,6–9]

The most common hematologic manifestation of cobalamin or folate deficiency in human medicine is megaloblastic anemia, a nonregenerative anemia resulting from defective DNA synthesis, ineffective

Abbreviations: GI, gastrointestinal; HCT, hematocrit; IGS, Imerslund-Grasbeck syndrome; IMHA, immune-mediated hemolytic anemia; ITP, immune-mediated thrombocytopenia; MCV, mean corpuscular volume; MMA, methylmalonic acid; RDW-SD, red cell distribution width-standard deviation

hematopoiesis, and erythrocyte precursor maturation arrest.[1,3,9] Macrocytosis results from asynchronous development of the erythrocyte cytoplasm and nucleus.[1,6,9] Increased mean corpuscular volume (MCV) or increased red cell distribution width (RDW) can precede anemia.[1,9,10] Leukopoiesis and megakaryopoiesis can also be disrupted as a result of the bone marrow becoming hypercellular from impaired development and systemic release of hematopoietic cells.[3,11] In severe cases, bone marrow failure and pancytopenia occur.[1,11] Such hematologic abnormalities are rapidly reversible with oral or parenteral supplementation of cobalamin.[1,3,9,12]

An equivalent relationship between hypocobalaminemia or hypofolatemia and hematologic changes in small animal veterinary species has not been established, although reports of anisocytosis, macrocytosis, anemia, or pancytopenia in association with hypocobalaminemia do exist.[13–20] In Giant Schnauzers,[16] Australian Shepherds,[19] Border Collies,[20] and Beagles,[15] there is a congenital disorder analogous to Imerslund-Grasbeck syndrome (IGS) in humans. In dogs with IGS, genetic mutations of the ileal cubam receptor lead to cobalamin deficiency, with subsequent nonregenerative anemia, erythrocyte anisocytosis, megaloblastic bone marrow, leukopenia, neutrophil hypersegmentation, and thrombocytopenia.[15–17] More subtle hematologic abnormalities consistent with dyserythropoiesis occur in Border Collies with congenital cobalamin deficiency, particularly when only an automated blood cell count is done.[21] Cats with hypocobalaminemia might have macrocytosis, pancytopenia, and inverse relationships between serum cobalamin concentration and MCV and between serum MMA concentrations and hematocrit (HCT).[13,14,18,22] These findings suggest a pathophysiology similar to the hematologic abnormalities of cobalamin-deficient people, although this has not been explored.

The primary objectives of our study were (1) to retrospectively determine the prevalence of anemia in a population of dogs that were screened for low cobalamin or folate concentrations and (2) to prospectively determine the prevalence of hypocobalaminemia and hypofolatemia and hematologic changes consistent with cobalamin and folate deficiencies in humans (nonregenerative anemia, macrocytosis, increased RDW) in a population of anemic dogs.

Given the fundamental role that B vitamins play in hematopoiesis, we hypothesized that in our retrospective population, dogs with hypocobalaminemia or hypofolatemia would be more likely to be anemic than normocobalaminemic and normofolatemic dogs, independent of disease states. Furthermore, in the prospectively examined population, dogs with nonregenerative anemia would more often be hypocobalaminemic or hypofolatemic than dogs with regenerative anemia, and hypocobalaminemic and hypofolatemic dogs would exhibit hematologic changes such as increased RDW more frequently than dogs with normal cobalamin and folate concentrations.

2 | MATERIALS AND METHODS

2.1 | Retrospective Study

Medical records of dogs examined between April 2014 and March 2016 from The Animal Medical Center in New York were reviewed retrospectively. All client-owned dogs that had both an IDEXX Laboratories GI panel (including serum cobalamin and folate concentrations) and CBC performed on the same date, and a complete medical record and no history of cobalamin or folate supplementation within the preceding 12 months were included. A complete medical record was defined as having information adequate for the authors to identify the variables of interest and categorize each case accordingly. Serum cobalamin and folate concentrations, as well as HCT, MCV, and reticulocyte count, were recorded for each dog (RDW data were not available). Signalment and clinical diagnoses were also noted for each case.

Comparisons of the prevalence of anemia (based on HCT) and the presence of regeneration (based on reticulocyte count) were made between hypocobalaminemic and normocobalaminemic dogs and between hypofolatemic and normofolatemic dogs. As established by the IDEXX reference range, dogs with HCT <38.3% were categorized as anemic and dogs with HCT ≥38.3% were categorized as nonanemic. Regenerative status was determined by the reticulocyte count, with a count >110 K/μL consistent with regeneration and a count ≤110 K/μL considered nonregenerative. Dogs with serum cobalamin concentration <350 ng/L were categorized as hypocobalaminemic, and dogs with cobalamin ≥350 ng/L were categorized as normocobalaminemic. The serum cobalamin concentration cutoff of 350 ng/L was chosen based on increased likelihood of clinically important cobalamin deficiency below this level, as this value and similar lownormal cobalamin concentrations are commonly used to initiate cobalamin supplementation in veterinary practice.[23,24] For analysis, dogs with serum cobalamin concentration <150 ng/L or >2000 ng/L were recorded as 150 and 2000 ng/L, respectively. As established by the IDEXX reference range, dogs with serum folate concentration <4.8 μg/L were categorized as hypofolatemic and dogs with folate ≥4.8 μg/L were categorized as normofolatemic.

2.2 | Prospective Study

Client-owned dogs with anemia that were presented to The Animal Medical Center with a PCV <35% between March 2016 and February 2017 were prospectively enrolled. PCV was used as an enrollment criterion for this portion of our study instead of HCT, as this result is more immediately available upon hospital presentation. Dogs with a history of cobalamin or folate supplementation within the preceding 12 months were excluded. After obtaining owner consent, all dogs had an IDEXX GI panel and CBC with clinical pathologist review performed, as well as serum MMA concentrations measured by the Texas A&M University Gastrointestinal Laboratory. Serum for cobalamin, folate, and MMA concentrations was collected within 24 hours of CBC collection and before administration of blood products, when feasible. Additional CBCs performed at least 1 week before or after enrollment were also reviewed for all dogs to allow differentiation between preregenerative anemia and true nonregenerative anemia. Microscopic review of CBCs was completed by 1 IDEXX clinical pathologist (A.S.) blinded to vitamin B results. Dogs with known renal disease were excluded, as renal insufficiency can falsely increase serum MMA concentrations in individuals with normal functional cobalamin status.[2,4] Although measurement of renal values was not a specific requirement for enrollment in our study, all

cases did have chemistry panels performed, which allowed us to confirm that none had significant renal disease. Additional recorded information included dog signalment and current or recent historical diagnoses.

Enrolled anemic dogs were categorized into groups based on erythrocyte regenerative status (determined by reticulocyte count), the presence of macrocytosis (determined by elevated MCV), the presence of anisocytosis (determined by increased RDW-SD), and serum cobalamin, folate and MMA concentrations. Comparisons of multiple red blood cell indices (HCT, reticulocyte count, MCV, and RDW-SD) between hypocobalaminemic and normocobalaminemic dogs were made. For categorization into groups based on regeneration, cobalamin, and folate parameters, reference intervals as described for the retrospective data were used. As established by the Texas GI Laboratory reference range, dogs with serum MMA concentrations >1193 nmol/L were categorized as hypermethylmalonic-acidemic and dogs with MMA concentrations ≤1193 nmol/L were categorized as normomethylmalonic-acidemic. Increased RDW-SD was documented as RDW-SD >39.6 fL, and macrocytosis was defined as MCV >76 fL, in accordance with IDEXX reference ranges.

2.3 | Statistical analysis

Signalment characteristics, CBC variables, and cobalamin, folate, and MMA concentrations were compared between groups using a Mann-Whitney U test for continuous data and Fisher's exact test for categorical data, respectively, as the data were not normally distributed, with correction for multiple comparisons where necessary. For evaluation of correlations between cobalamin concentration and HCT and between cobalamin and reticulocyte count, linear regression analysis with calculation of Spearman's rho was used. STATA SE (version 14.2, StataCorp software) was used for statistical analysis.

## 3 | RESULTS

3.1 | Retrospective study

A total of 146 medical records of dogs that had a CBC and GI panel performed through IDEXX Laboratories on the same date were reviewed retrospectively. Thirty-two dogs were excluded, because of a history of cobalamin or folate supplementation (8 dogs), incomplete medical records within the electronic database (23), or enrollment in the prospective study (1). Therefore, 114 dogs were included in the retrospective study. Thirty-four dogs were anemic (29.8%) and 80 (70.2%) were nonanemic, 44 (38.6%) were hypocobalaminemic, and 70 (61.4%) were normocobalaminemic, and 13 were hypofolatemic (11.5%), 100 were normofolatemic (88.5%), and 1 was missing folate data. Only 1 dog in the retrospective population had an increased MCV, and this case had a regenerative anemia.

There were no significant differences in the median age between dogs compared by HCT (anemic: 10.8 years, nonanemic: 7.3 years; P = .65), cobalamin (hypocobalaminemic: 9.8 years, normocobalaminemic: 7.9 years; P = .19), or folate status (hypofolatemic: 6.3 years, normofolatemic: 8.5 years; P = .37). Intact males, castrated males,

intact females, and spayed females were evenly distributed (by HCT, P = .65; cobalamin, P = .42; folate, P = .38). Disease diagnoses included 63 dogs with GI disease (excluding GI neoplasia; 55.3%), 13 dogs with neoplasia (including GI; 11.3%), 18 with infectious or inflammatory disease (15.8%), and 20 with other (eg, endocrine, idiopathic) diseases (17.5%).

The proportion of hypocobalaminemic dogs with anemia (16/44, 36%) was not significantly different from the proportion of normocobalaminemic dogs with anemia (18/70, 26%; P = .23). Similarly, the prevalence of anemia did not differ significantly between hypofolatemic (4/13, 31%) and normofolatemic dogs (30/100, 30%; P = .99). See Figure 1A,B.

All 16 dogs that were both hypocobalaminemic and anemic had a nonregenerative anemia. Only 3 dogs in the retrospective population had a regenerative anemia, and all were normocobalaminemic. The difference in prevalence of regenerative vs nonregenerative anemia by cobalamin status was not statistically significant (P = .23), but this comparison was likely influenced by the small number of dogs with regenerative anemia. No correlation was found between cobalamin concentration and reticulocyte count among anemic dogs (Spearman's rho = 0.16; P = .37) or between cobalamin concentration and HCT among all dogs (Spearman's rho = 0.02; P = .83). See Figure 2A,B.
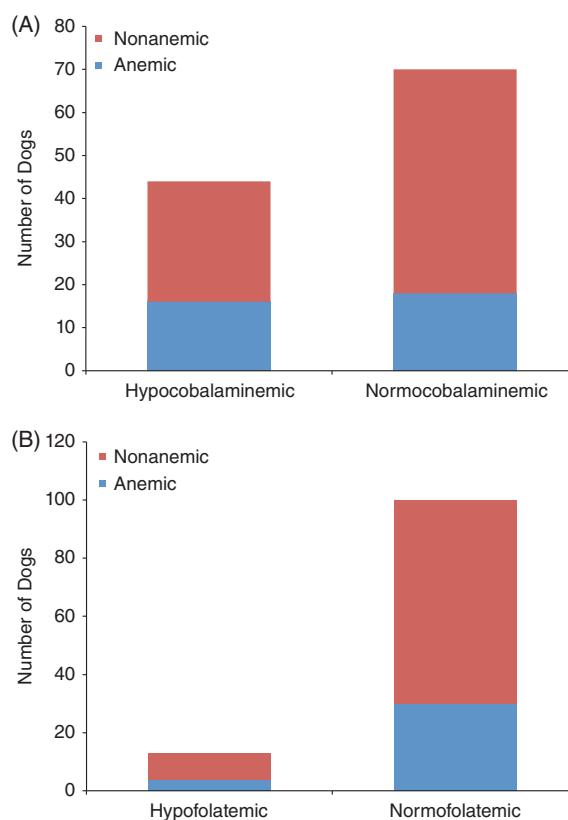
FIGURE 1 (A) Proportion of dogs in retrospective study classified by anemia status and cobalamin status. The prevalence of anemia did not differ significantly between hypocobalaminemic (36%) and normocobalaminemic dogs (26%; P = .23). (B) Proportion of dogs in retrospective study classified by anemia status and folate status. The prevalence of anemia did not differ significantly between hypofolatemic (31%) and normofolatemic dogs (30%; P = .99)
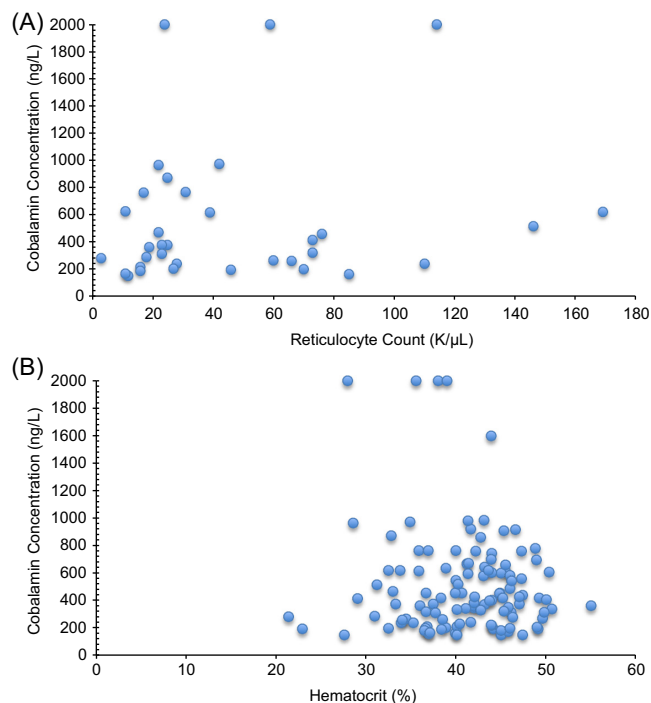
FIGURE 2 (A) Cobalamin concentration compared to reticulocyte count for all dogs with anemia in retrospective study. No correlation was found between cobalamin concentration and reticulocyte count (Spearman's rho = 0.16; *P* = .37). (B) Cobalamin concentration compared to hematocrit (HCT) for all dogs in retrospective study. No correlation was found between cobalamin concentration and HCT (Spearman's rho = 0.02; *P* = .83)

3.2 | Prospective study

Forty-two anemic dogs without a history of cobalamin or folate supplementation were prospectively enrolled; 26 were hypocobalaminemic (62%), 16 were normocobalaminemic (38%), 8 were hypofolatemic (19%), 34 were normofolatemic (81%), 8 had increased MMA concentrations (19%), and 34 had normal or low MMA concentrations (81%). Serum MMA concentrations for all anemic dogs ranged from 366 to 2317 nmol/L, with a median of 682 nmol/L (RI 415-1193 nmol/L). Three dogs with methylmalonic-acidemia (1741, 1692, and 1613 nmol/L) had normal serum cobalamin concentrations (781, 364, and 616 ng/L, respectively), whereas the remaining 5 dogs with increased MMA concentrations had concurrent serum hypocobalaminemia (lowest 150 ng/L). Fourteen dogs had a regenerative anemia (33%), and 28 dogs had a nonregenerative anemia (67%). Eleven dogs had an increased MCV (26%), and 14 dogs had an increased RDW-SD (33%). Among 28 dogs with nonregenerative anemia, 18 (64%) were hypocobalaminemic and 5 (18%) were hypofolatemic, and 8 of 14 dogs with regenerative anemia had hypocobalaminemia (57%) and 3 had hypofolatemia (21%).

There were no significant differences in the median age among dogs based on cobalamin (hypocobalaminemic: 9.0 years, normocobalaminemic: 9.6 years; *P* = .98), folate (hypofolatemic: 7.6 years, normofolatemic: 9.4 years; *P* = .72), or MMA status (increased MMA: 8.8 years, normal MMA: 9.1 years; *P* = .70). Intact males, castrated males, intact females, and spayed females were evenly distributed (by cobalamin status, *P* = .07; folate, *P* = 1.0; MMA, *P* = 1.0). Primary

TABLE 1 Number of dogs in prospective study categorized by disease process and cobalamin status

| Disease process	Hypocobalaminemia	Normocobalaminemia
Immune-mediated disease	10	8
GI disease	2	0
Neoplasia	3	2
Infectious/ inflammatory	8	4
Other	3	2

Of 42 dogs, 18 (43%) had immune-mediated disease, 2 (5%) had GI disease, 5 (12%) had neoplasia, 12 (29%) had infectious/inflammatory disease, and 5 (12%) had other diseases.

diagnoses in the prospective population included 18 dogs with immune-mediated disease (43%), 2 with GI disease (5%), 5 with neoplasia (including GI, 12%), 12 with infectious or inflammatory disease (29%), and 5 with other diseases (eg, endocrinopathy, acute toxicity, 12%). See Table 1.

Of 26 hypocobalaminemic dogs, 8 had regenerative anemia (31%) and 18 had nonregenerative anemia (69%). The proportion of hypocobalaminemic dogs with nonregenerative anemia (18/26, 69%) was not significantly different from the proportion of normocobalaminemic dogs with nonregenerative anemia (10/16, 63%; *P* = .65). See Figure 3.

Of 18 hypocobalaminemic dogs with nonregenerative anemia, only 3 (17%) had macrocytosis and 5 (28%) had increased RDW-SD, consistent with anisocytosis. None of the 10 normocobalaminemic dogs with nonregenerative anemia had macrocytosis (0%) or increased RDW-SD (0%). Prevalence of macrocytosis and elevated RDW among hypocobalaminemic dogs with nonregenerative anemia was not significantly different from the respective prevalence among normocobalaminemic dogs with nonregenerative anemia (macrocytosis: *P* = .53; anisocytosis: *P* = .14), although the small numbers in each group likely
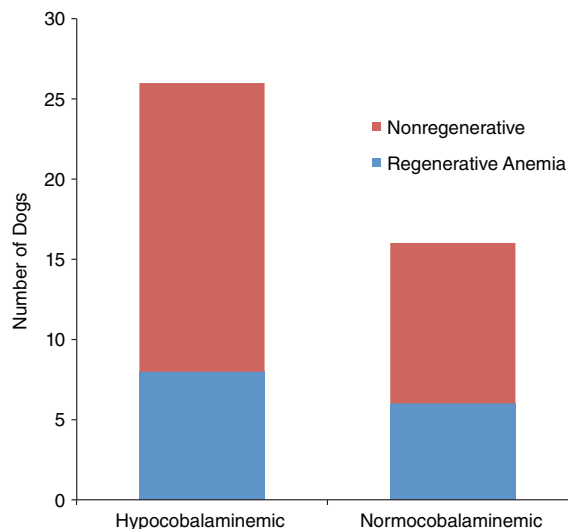

FIGURE 3 Proportion of dogs in prospective study classified by cobalamin status and erythrocyte regeneration status. The prevalence of nonregenerative anemia was not significantly different between hypocobalaminemic (69%) and normocobalaminemic dogs (63%; *P* = .65)

influenced these comparisons. When these analyses were repeated to include 3 dogs with increased MMA concentrations but normal serum cobalamin concentrations within the hypocobalaminemic group (to incorporate dogs with functional cobalamin deficiency), still no significant differences in the prevalence of nonregenerative anemia ($P = .73$), macrocytosis ($P = .54$), or elevated RDW ($P = .28$) were found between groups.

No correlation between cobalamin concentration and reticulocyte count (Spearman's rho = 0.22; $P = .16$) or between cobalamin concentration and HCT (Spearman's rho = 0.049; $P = .76$) was found for the prospective data. See Figure 4A,B.

4 | DISCUSSION

This observational, 2-part study documented the prevalence of anemia and screened for characteristic human hematologic changes in dogs with hypocobalaminemia, hypofolatemia, or both. Our goal was to identify whether the presence of anemia and certain red blood cell abnormalities may be used as indicators of vitamin B status in dogs. If confirmed, the results could affect clinical practice by providing support for measurement of cobalamin and folate concentrations and consideration of vitamin B supplementation in anemic dogs. Despite a high prevalence of hypocobalaminemia and hypofolatemia among anemic dogs in both portions of our study, no significant relationships between cobalamin or folate concentrations and the examined hematologic parameters were found. The results indicate that, in contrast
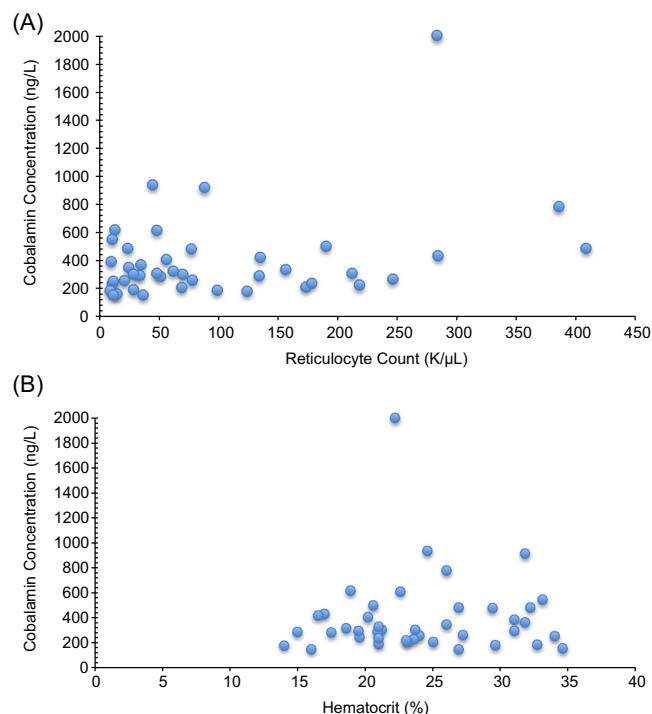
FIGURE 4 (A) Cobalamin concentration compared to reticulocyte count for all dogs in prospective study. No correlation was found between cobalamin concentration and reticulocyte count (Spearman's rho = 0.22; $P = .16$). (B) Cobalamin concentration compared to hematocrit (HCT) for all dogs in prospective study. No correlation was found between cobalamin concentration and HCT (Spearman's rho = 0.049; $P = .76$)

to human medicine, these hematologic changes are not a reliable consequence of hypocobalaminemia or hypofolatemia in dogs, suggesting that B vitamins might play a less prominent role in canine than in human erythropoiesis.

We evaluated retrospectively a population of 114 dogs that were screened for hypocobalaminemia or hypofolatemia, because of their high proportion (over 50%) of presumptive or confirmed primary GI disease. Although the underlying etiology was not confirmed in all cases, prevalence of hypocobalaminemia was high (38.6%), and more than 10% of dogs were hypofolatemic. This result matches with that of a previous study documenting hypocobalaminemia in 36% of dogs with chronic GI disease.[5] In our 42 prospectively enrolled dogs, GI disease was reported in only a small proportion (less than 5%). However, the prevalence of hypocobalaminemia was higher than in the retrospective group, approaching two-thirds of the population (62%), and hypofolatemia occurred in 22%. This could indicate a higher true prevalence of GI disease than was detected or the presence of other disease processes (eg, exocrine pancreatic insufficiency), medications (eg, antacids), or differences in diet that could have contributed to cobalamin and folate deficiencies in our prospective population.[2]

Hematologic changes, with megaloblastic anemia being the most well-recognized abnormality, have been widely documented as important complications of cobalamin and folate deficiencies in humans.[1,3,9] Macrocytosis might precede or occur with nonregenerative anemia in these patients; however, concurrent iron deficiency or chronic inflammatory disease can lead to anisocytosis without overt macrocytosis.[1,3,9,10] A causal relationship between vitamin B deficiencies and anemia has not been established in small animals.[2] An exception in certain dog breeds is congenital hypocobalaminemia (eg, Giant Schnauzers with IGS), resulting in hypocobalaminemia and marked hematologic changes from genetic mutations of ileal receptors, although macrocytosis has not been reported.[15–17] Pancytopenia and increased MCV associated with severe cobalamin deficiency have been recognized rarely in cats.[13,14,18,23]

Despite these findings in humans and specific small animals, the prevalence of anemia did not differ by cobalamin or folate status in our retrospective group. Furthermore, hypocobalaminemia was not significantly associated with nonregenerative anemia, and cobalamin concentration did not correlate with HCT or reticulocyte count. In contrast to our hypotheses, these results did not support a direct relationship between hypocobalaminemia or hypofolatemia and anemia.

We also prospectively examined several hematologic parameters known to arise from hypocobalaminemia in anemic people. No significant association was demonstrated between cobalamin concentration and the presence of erythrocyte regeneration or macrocytosis, and, as in the retrospective group, cobalamin concentration did not correlate with HCT. Because previous studies have shown anisocytosis but not macrocytosis in cobalamin-deficient dogs and because concurrent iron deficiency or chronic disease could mask detection of macrocytosis in humans, we also evaluated RDW in this group.[10,15,17] The prevalence of increased RDW among dogs with nonregenerative anemia was not significantly different between those with hypocobalaminemia and normocobalaminemia. Because iron status was not evaluated, it is possible that iron deficiency prevented appreciation of increased

MCV or RDW in some cases. Overall, these results suggest that hypocobalaminemia is not associated with megaloblastic, nonregenerative anemia in dogs.

Although our focus was on the subset of dogs with nonregenerative anemia, it is worth noting the unexpectedly high prevalence of vitamin B deficiencies among dogs with regenerative anemia in our prospective population (57% hypocobalaminemic, 21% hypofolatemic). In particular, dogs with immune-mediated hemolytic anemia (IMHA) or thrombocytopenia (ITP) had a similar prevalence of deficiencies (56% hypocobalaminemic, 22% hypofolatemic) as the overall prevalence (62% hypocobalaminemic, 19% hypofolatemic). Because dogs with immune-mediated hematologic disease comprised a large portion of our prospective population (18/42 cases), this unanticipated finding may have influenced our results. Rarely hemolytic anemia has been reported to occur in cobalamin-deficient humans as a result of erythrocyte fragility from dyserythropoiesis and increased homocysteine concentrations.[9,25,26] To our knowledge, there is no known association between hypocobalaminemia and immune-mediated hematologic disease in dogs, but these results encourage future investigation into vitamin B status in dogs with IMHA or ITP.

Several potential explanations for our statistically nonsignificant results exist. The first is that cobalamin and folate deficiencies do not reliably result in analogous erythrocyte changes in dogs as are seen in humans. Although the important role of cobalamin and folate in normal hematopoiesis is established in human medicine, unique, incompletely understood factors may regulate canine erythropoiesis. As mentioned above, iron status and chronic disease may have a stronger influence on erythrocyte size and regenerative status than does cobalamin concentration in dogs. In dogs with congenital IGS, anisocytosis but not true macrocytosis has been reported, and despite not reaching significance, only the hypocobalaminemic dogs (and no normocobalaminemic dogs) had increased RDW, macrocytosis, or both among all patients with nonregenerative anemia in our prospective group. This finding suggests increased variability of erythrocyte size in hypocobalaminemic dogs, but the lack of significant association with any of the hematologic parameters studied here shows that the impact of cobalamin on hematopoiesis is more complex than hypothesized.

The severity or chronicity of cobalamin or folate deficiency also likely affects the odds of developing appreciable erythrocyte changes. Dogs with IGS that develop hematologic disease have severe, chronic hypocobalaminemia.[15,17] The chronicity of vitamin B deficiency was not evaluated in our study, but there was a wide range of cobalamin and folate concentrations and severity of anemia; therefore, perhaps, more severe cases of hypocobalaminemia would have more dramatic hematologic changes. Furthermore, none of our dogs had bone marrow evaluation, and we did not analyze other cell lines. Hence, it is possible that other manifestations of hematologic disease are more common than megaloblastic anemia.

Another important explanation for our results is that inclusion of dogs with multiple different diseases might have diluted any associations between hypocobalaminemia or hypofolatemia and hematologic changes, as different disease processes can have multifactorial effects on measured erythrocyte variables. We enrolled 2 distinct populations to attempt to minimize this theoretical confounding factor, and a high prevalence of hypocobalaminemia and hypofolatemia was documented

in both groups despite the range of diseases included. However, selection of a more homogeneous population (eg, all dogs with GI disease or only dogs with anisocytosis) should be considered in future studies to more purely evaluate for a causal link between cobalamin or folate deficiencies and hematologic disease.

There are a few additional limitations to our study. One was the relatively small sample sizes, particularly notable when subgroups of dogs were compared for specific hematologic parameters. For example, small groups prevented analysis of retrospective data for the prevalence of macrocytosis between hypocobalaminemic and normocobalaminemic nonregenerative anemic dogs and appeared to impact analysis of anisocytosis and macrocytosis in nonregenerative dogs in the prospective population.

Seven dogs in the prospective study categorized as having nonregenerative anemia were determined on follow-up CBCs to in fact have had a preregenerative anemia at the time of study enrollment. It was elected to keep these dogs in the nonregenerative anemia group for analysis given that many of these dogs were given cobalamin supplementation, blood products, or other interventions before follow-up CBCs were performed that may have affected the hematologic profile, as well as our assessment that our results would have been equivalent with redistribution or exclusion of these dogs. However, it must be considered that these dogs with preregenerative anemia represented a distinct population from the truly nonregenerative anemic dogs, and this choice of classification could have prevented the documentation of a real correlation between our hematologic variables of interest and vitamin B deficiencies (contributing to a type II error). Nonregenerative and preregenerative anemic dogs were also not differentiated in our retrospective group.

The decision to define hypocobalaminemia in both study populations as <350 ng/L rather than using the lower end of the IDEXX reference interval (<284 ng/L) or severe hypocobalaminemia (<150 ng/L) could have also impacted our results, particularly if lower cobalamin concentrations have a more pronounced effect on erythrocyte parameters and HCT. Prior sources have used varying serum cobalamin concentrations to initiate supplementation, as low-normal cobalamin concentrations theoretically could have important clinical consequences, such as GI signs and weight loss.[23,24] In people with borderline hypocobalaminemia (defined as near the lower limit of the reference range), cobalamin deficiency remains possible, and additional testing through MMA and homocysteine concentrations is warranted, as cobalamin concentrations can fluctuate at different time points and tissue stores may not correlate well with serum cobalamin concentrations.[27–29]

We elected to measure serum MMA concentrations in addition to cobalamin and folate in the prospective group to increase detection of cases with functional, cellular hypocobalaminemia. Because cobalamin is a necessary cofactor for methylmalonyl CoA mutase-mediated conversion of L-methylmalonyl CoA to succinyl CoA, increased MMA concentrations can be a more accurate, early indicator of functional hypocobalaminemia on a cellular level.[1,5,13,23,30] In our population, increased MMA was an uncommon finding (8/42 patients). Only 3 of 8 dogs (24%) with methylmalonic-acidemia had normal cobalamin concentrations, and of the remaining 5 dogs with increased MMA concentrations, all 5 cases had cobalamin concentrations below the

reference interval. These findings are consistent with previous canine studies that have documented hypocobalaminemia without cellular cobalamin deficiency (increased MMA concentrations), although a negative correlation between serum MMA and cobalamin concentrations is typically expected.[5,23] Low cobalamin can also cause functional folate deficiency because of impaired activation by cobalamin-dependent methionine synthase of folate to tetrahydrofolate, so that measurement of homocysteine concentrations in addition to MMA might have helped differentiate functional from true folate deficiency.[3,30]

In conclusion, the results of our study indicate that the association between cobalamin and folate deficiencies and macrocytic, non-regenerative anemia established in humans does not appear to exist routinely in dogs. Despite the high prevalence of hypocobalaminemia and hypofolatemia among anemic dogs in this study, our results demonstrate no evidence-based support for standard testing for cobalamin and folate deficiencies in dogs with macrocytic anemia. The expense and additional serum required for cobalamin and folate measurement may therefore remain reserved for anemic dogs with concurrent clinicopathologic findings suspicious for vitamin B deficiency. The importance of the high prevalence of B vitamin deficiencies among dogs with immune-mediated hematologic disease noted here should be further investigated. Future prospective, interventional studies are indicated to further evaluate the impact, if any, of cobalamin or folate deficiency followed by cobalamin or folate supplementation on hematologic variables in dogs.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

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

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