Low serum folate concentrations in dogs with non-associative immune-mediated haemolytic anaemia

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Short running title: Low folate concentrations in canine IMHA

The work was done at the Faculty of Veterinary Medicine, University of Glasgow, Bearsden, Glasgow.

Preliminary results were presented as a clinical research abstract at the ECVIM-CA congress 2007 in Budapest, Hungary.

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Summary

Background: Folate deficiency in people can occur in conditions causing increased demand, including haemolytic anaemia. This has not been investigated in dogs with non-associative immune-mediated haemolytic anaemia (IMHA).

Methods: Cohort study of 15 dogs with non-associative IMHA. Haematocrit and serum folate concentrations were measured at presentation and each subsequent venipuncture performed for monitoring. The relationship between serum folate concentrations and haematocrit was investigated using linear and logistic mixed-effects regression models and in paired samples using a one-tailed paired T-test.

Results: Low serum folate concentrations occurred in 5/15 dogs. In 126 samples, a significant positive relationship was found between haematocrit and corresponding serum folate concentrations. A significant relationship was found between dichotomised folate concentrations (below the reference interval or within/above the reference interval) and haematocrit and between serum folate concentrations and dichotomised haematocrit (less than or equal/above 0.30 l/l). For paired samples (available in 8 dogs), the mean serum folate concentration of samples with the lowest haematocrit was significantly lower than that of samples in which the haematocrit first exceeded 0.30 l/l.

Conclusions: Low serum folate concentrations were observed in some dogs with non-associative IMHA. Further studies are needed to determine the cause and investigate whether folate supplementation would be beneficial.

Key words: Folic acid, canine, IMHA, haemolysis.

Abbreviations:

CBC: complete blood count

DAT: direct antiglobulin test

HCT: haematocrit

IMHA: immune-mediated haemolytic anaemia

RBC: red blood cell

Introduction

Haemolytic anaemia is characterized by a reduced red blood cell (RBC) lifespan.[1] Immune-mediated haemolytic anaemia (IMHA) caused by antibody production against RBCs is the most common cause of haemolytic anaemia in dogs.[2] Recently, a new system of classification in which the disease is categorized as "non-associative" or "associative" has been proposed.[3] The term "associative" is used when a comorbidity that either might have caused the IMHA or might be coincidental is identified, while the term "non-associative" IMHA is used when comorbidities are not identified in the diagnostic evaluation and include "idiopathic" and cryptogenic cases.[3] Non-associative IMHA is diagnosed by marked spherocytosis, the presence of true agglutination, a positive direct antiglobulin test (DAT) or a combination of these findings in the presence of anaemia and the exclusion of known trigger factors such as infections, neoplasms, drugs (including potentiated sulphonamides and cephalosporins), vaccines, and inflammatory processes,[2,3,4] or any other major comorbidities that might have caused the IMHA.[3,5,6]

Haemolytic anaemia is usually markedly regenerative.[7] In patients with non-associative IMHA, a regenerative response is expected to start within 3 to 5 days of disease development, but the precise timing of disease onset is often unknown. Regeneration lasts until normalisation of the haematocrit **(**HCT), but it has been suggested that erythropoietin and reticulocyte production decrease when the HCT exceeds 0.30 l/l.[7,8] Up to 30% of dogs with immune-mediated haemolytic anaemia have non-regenerative anaemia at the time of diagnosis.[9,10] In some cases, this is the result of RBC precursor destruction.[4]

The maintenance of normal serum folate (folic acid or vitamin B9) concentrations depends on the absorption of exogenous folate from the gut.[11] Folate is present in most foodstuffs and is also synthesized by intestinal bacteria; nutritional folate deficiency in dogs is therefore rare.[12] The liver is the organ with highest folate concentration and represents about 50% of the body's folate store. It plays a major role in maintaining folate homeostasis not only because of its relatively high folate content, but also because of its ability to rapidly redistribute stored folate through enterohepatic circulation. The latter process evens out the intermittent nature of dietary folate intake and may account for as much as 50% of the folate that ultimately reaches the tissues.[11]

Low serum folate concentrations have been documented in dogs with reduced folate absorption due to small intestinal disease and, more recently, in a study of dogs with anaemia of various aetiologies.[13,14] In addition, in people, folate deficiency has been documented with dietary deficiency, the use of drugs which interfere with folate absorption or metabolism (methotrexate, trimethoprim, barbiturate anticonvulsants),[15] and in conditions causing increased folate utilization (pregnancy, exfoliative dermatitis, chronic myelofibrosis and active haemopoiesis).[16] Folate deficiency has been shown to occur in people with haemolytic anaemia, including those with immune-mediated forms, particularly when RBC destruction is chronic.[17,18] This is thought to occur due to the increased utilization of folate for DNA synthesis for RBC production,[18,19] and folate deficiency can result despite normal intake, normal intestinal absorption, and normal hepatic folate store redistribution.[20] The resulting low folate concentrations can aggravate the severity of the haemolytic anaemia by slowing regeneration[21] and can, rarely, cause megaloblastic haemopoiesis.[17] Folate supplementation is therefore recommended in these

cases as an adjunct to more specific treatment because it can produce an increased reticulocyte count and HCT within 5 - 7 days. [19,22,23,24]

A recent study showed a high prevalence of vitamin B deficiency among dogs with regenerative anaemia,[14] however low serum folate concentrations have not been specifically studied in dogs with immune-mediated haemolytic disease. The aims of this study were to investigate whether low serum folate concentrations occur in dogs with non-associative IMHA, whether there is a relationship between serum folate concentrations and HCT in these patients, and whether serum folate concentrations are lower in samples collected when the anaemia is expected to be in its most regenerative phase than it is when the HCT exceeds 0.30l/l. Given the increased demand and utilization of folate in haematopoiesis,[19] we hypothesized that low serum folate concentrations would occur in dogs with non-associative IMHA, that there would be a positive relationship between serum folate concentrations and HCT and that serum folate concentrations would be lower in samples collected at the time of expected maximal RBC regeneration than it would be when HCT exceeded 0.30 l/l.

Material and Methods

Dogs

This study was performed with the approval of the University of Glasgow School of Veterinary Medicine Ethics and Welfare Committee. Dogs with non-associative IMHA presented to the University of Glasgow Small Animal Hospital between 1st December 2005 and 31st October 2007 were eligible for entry into this prospective population-based

cohort study. IMHA was diagnosed based on a HCT less than 0.37 l/l (reference interval 0.37 to 0.55 l/l) evidence of haemolysis (e.g., spherocytosis, hyperbilirubinemia, haemoglobinemia / haemoglobinuria) and the presence of marked spherocytosis, true autoagglutination and/or a positive DAT (ImmonO^{TM}, Canine Anti-Globulin Test, MP Biomedicals, Strasbourg, France).[2,3] True agglutination was confirmed by microscopically evaluation of a drop of EDTA blood mixed with 4 drops of NaCl 0.9% at room temperature on a smear slide.[3] The IMHA was considered non-associative if there was no history of administration of medications suspected to trigger IMHA (e.g. potentiated sulphonamides or cephalosporins),[4] no history of vaccination within 30 days prior to anaemia detection, and if investigations ruled out concurrent conditions such as neoplasia, inflammatory or infectious diseases. Moreover, dogs were excluded if they had other physiological or pathological conditions (i.e., pregnancy, clinically significant gastrointestinal disease, severe diffuse dermatitis) or were receiving any medications known in veterinary or human medicine to interfere with folate absorption and metabolism (e.g., barbiturate anticonvulsants, trimethoprim, sulphasalazine or aspirin). For the purpose of this study, dogs were considered not to have clinically significant gastrointestinal disease if they had no history of chronic vomiting or diarrhoea and their gastrointestinal tract had a normal appearance on abdominal ultrasound examination. Severe diffuse dermatitis was ruled out based on clinical examination, while pregnancy was excluded based on lack of a recent history of mating and abdominal ultrasound findings. Cases treated with folate supplementation were also excluded.

At the time of admission, a full clinical history was collected, and all dogs were evaluated by physical examination, complete blood count (CBC) (Cell Dyn 3500R

analyser, Abbott Diagnostic, Abbott Park, IL), blood smear examination, serum biochemistry profile, thoracic radiography and abdominal ultrasonography. Other diagnostic tests (e.g., bone marrow biopsy, faecal analysis, urinalysis, *Leptospira spp*. serology) were performed as considered necessary by the attending clinician. Specific diagnostic tests for *Ehrlichia spp, Leishmania spp, Dirofilaria immitis and Babesia spp* were not performed as these diseases had never been reported in the region where the study was conducted, and none of the dogs had a history of travel.

Residual blood from the time of first venipuncture and at each subsequent venipuncture performed by the attending clinician for monitoring purposes was stored in serum tubes for folate measurement, and the samples' HCT was recorded. Residual samples were collected during hospitalisation and at every revisit after hospital discharge until normalization of the HCT and discontinuation of all immunosuppressive drugs. Longitudinal folate measurements were performed in these dogs to increase the likelihood of collecting samples at the time of maximum regeneration and therefore folate demand.

Cases were managed as deemed appropriate by the clinician in charge of the case, and the timing of revisit appointments was also determined by them; therefore, timing and number of samples collected varied between dogs (table 1). Response to treatment was assessed during hospitalisation and at each revisit with a physical examination, CBC and blood smear examination, and in some cases by repeated DAT, in-saline agglutination tests and serum biochemistry profiles.

Signalment information (age and sex), the presence or absence (and duration) of anorexia, diarrhoea or vomiting during hospitalisation, treatments administered including

details of blood or blood product transfusions, outcome information (date and cause of death or date of last contact for patients that were lost to follow up) were also recorded.

Serum folate measurement

Serum samples were spun and separated within 30 minutes of collection. All samples regardless of serum discolouration (jaundiced, haemolysed or discoloured due to bovine-derived haemoglobin glutamer-200 [Oxyglobin®] transfusion), were included in the study.

Serum folate concentrations were measured at one of two commercial veterinary laboratories using immunoassay techniques validated in dogs.[25] Samples collected between 1st December 2005 and 6th March 2006 (40 samples) were assayed at The TLI Laboratory, University of Liverpool, UK after which time this assay was no longer available. All subsequent samples (86 samples) were assayed by Cambridge Specialist Laboratory Services Ltd, UK. The TLI laboratory, Liverpool used a solid-phase competitive chemiluminescent enzyme immunoassay for the measurement of folate concentrations (Immunolite 1000; reference interval 4.7-11.3 ng/ml), while Cambridge Specialist Laboratory Services Ltd used a dual isotope competitive radioimmunoassay (MP Biomedical; reference interval 3-13 ng/ml). Serum folate results were not made available to the clinician managing the case.

Statistical analysis

Standard descriptive statistics were used to summarize data and to describe whether low serum folate concentrations occurred in non-associative IMHA.

The relationship between serum folate concentrations and HCT was studied with a linear mixed effect model. Sex, body weight, age and laboratory used were included in the analysis to assess their possible effect on the folate concentrations and its relationship with HCT. The linear mixed effect model was fitted with serum folate concentration as the dependent variable; HCT, sex, body weight, age and laboratory used as fixed effects; and dogs' identity as the random effect. This random intercept model took into account correlations among measurements from the same animal for the determination of the serum folate concentrations, assuming an equicorrelated correlation structure. Explanatory variables were then selected using a backward elimination procedure, that is, entering all the independent variables into the equation fist and then removing them one at the time starting from the least statistically significant variable up to removing all the variables with a *P*-value > 0.05. Then, to assess whether lower serum folate concentration was associated with lower HCT, samples were categorised into two groups based on serum folate concentrations: either below the lower limit of the reference interval for the laboratory or within/above the reference interval. A logistic mixed effects model was fitted to investigate whether haematocrits were lower in the group with folate concentrations below the lower limit of the reference interval. The model included HCT as fixed effect and dogs' identity as random effect. Thirdly, to assess whether serum folate concentrations were lower when the anaemia was expected to be in its most regenerative phase, samples were categorised on the basis of the HCT into two groups: less than 0.30 l/l or equal/above 0.30 l/l. A linear effects mixed model was used to assess whether serum folate concentrations were lower in the group with haematocrits less than 0.30 l/l. The model included categorical HCT as the fixed effect and dogs' identity as the random effect. All the diagnostics on the residuals supported the 3 above estimated models.

Finally, samples from dogs in which paired folate and HCT results were available both at the time of the lowest measured HCT and when the HCT first exceeded 0.30 l/l were used to test the null hypothesis that serum folate concentrations were not lower when the anaemia was in its most regenerative phase. Results from samples in which the HCT exceeded 0.30 l/l due to a blood transfusion were not used in this analysis and dogs were included only if both serum folate samples were run in the same laboratory. Following assessment of normality using the Shapiro-Wilk test, a one-tailed paired T-test was used to compare the mean folate concentrations between the two time-points.

Estimations of the linear mixed effects models were obtained using the residual maximum likelihood method of Patterson and Thompson,[26] while the logistic mixed effects model was estimated with the Gauss-Hermite quadrature method.[27] The linear mixed effects models were estimated with the R package nlme that implements the lme function, while the logistic mixed effects model was fitted with the R package lme4 that implements the glmer function (https://www.r-project.org/). The significance level for all analysis was set to $\alpha = 0.05$.

Results

Clinical presentation and treatment

Within the study period, 15 dogs met the inclusion criteria. At the time of diagnosis, ages ranged from 8 months to 12 years 9 months (median 5 years 9 months) and body weights ranged from 6 to 31 kg (median 18 kg). Individual dogs' characteristics are reported in table 1.

The median HCT at admission was 0.13 l/l (interval range 0.04 to 0.24 l/l). At diagnosis, 11 dogs (73.3%) had true autoagglutination, 8 of the 12 dogs (66.6%) in which a DAT was performed had a positive result, 11 dogs (73.3%) presented with spherocytosis on blood smear examination, 9 dogs (60%) had hyperbilirubinemia, and samples from 6 dogs (40.0%) were macroscopically haemolysed. None of the 15 dogs were diagnosed with clinically significant gastrointestinal disease, but self-limiting anorexia and sporadic vomiting were reported in the first few days of hospitalisation in 10 and 2 dogs, respectively. Diarrhoea was never reported in any of the dogs.

All 14 dogs that survived the first 24 hours were treated with immunosuppressive doses of glucocorticoids. The median length of hospitalisation was 6 days (interval range 1 to 15). All 12 discharged dogs were given prednisolone (2 mg/kg PO total daily dose). Other immunosuppressive drugs (azathioprine, cyclophosphamide, cyclosporine and human gammaglobulin), antibiotics, gastroprotectants and crystalloid intravenous fluid therapy were administered in some cases. Thirteen dogs (86.7%) received blood or blood products (whole blood, packed RBC, Oxyglobin®).

Serum folate concentrations, HCT and clinical outcome

One hundred and twenty-six samples in which both HCT and serum folate were measured were obtained (1 to 19 samples per dog; median 7 samples). Of these, 13 (10.3%) had serum folate concentrations below the reference interval for the laboratory and all 13 samples with serum folate concentrations below the reference interval were recorded in samples with a HCT less than 0.30 l/l. Six of these 13 samples (46.2%) were collected within 1 week of presentation, 11 (84.6%) within two weeks, and the remaining two

samples were collected 15- and 17-days post-presentation. Five of the 15 dogs (33.3%) had at least one sample with a low serum folate concentration; however, in 3 of these 5 dogs, only one sample with low serum folate concentration was identified.

Estimates of the regression coefficients from the linear mixed effects model including serum folate concentrations, HCT, sex, body weight, age, and laboratory used are reported in table 2. None of the covariates analysed with the exception of HCT were significant in the final model after backward analysis. The final linear mixed effects model of the HCT and corresponding serum folate concentrations of all 126 samples revealed a significant positive relationship between these two variables with a slope of 7.90 ± 3.66 (t = 2.2, *P* = 0.033; figure 1). The estimated model was: serum folate = 7.48 + (7.90 x HCT).

The logistic mixed effects model showed a significant relationship between dichotomised serum folate concentrations (i.e., below the lower limit of the reference interval [n = 13] or within/above the reference interval [n = 113]) and HCT (OR = 1.15, 95% CI: 1.04 to 1.29; *P* = 0.018). The effect of the two laboratories on the relationship between dichotomised folate and the HCT was not statistically significant (t = -0.805, *P* = 0.420).

Sixty-eight samples (54.0%) had a HCT less than 0.30 l/l while in the remaining 58 samples the HCT was equal to or above 0.30 l/l. The linear mixed effects model showed a significant relationship between serum folate concentrations and dichotomised HCT with a slope of 2.69 ± 0.89. The estimated mean serum folate concentration was 8.37 ± 0.89 ng/ml for samples with a HCT less than 0.30 l/l and 11.06 ± 1.17 ng/ml for samples with a HCT equal to or above 0.30 l/l (t = 3.02, *P* = 0.003; figure 2). The effect

of the two laboratories on the relationship between serum folate concentrations and the dichotomised HCT was not statistically significant (t = -0.548, *P* = 0.721).

Paired samples with the lowest recorded HCT and when the HCT first exceeded 0.30 l/l, were available for 8/15 dogs (53.3%). No dogs were excluded from this analysis because the paired serum folate concentrations were measured in different laboratories. In these 8 dogs, the mean serum folate concentration was significantly lower in the sample with the lowest recorded HCT (6.46 ± 4.04 ng/ml) than in the first sample in which the HCT exceeded 0.30 l/l (10.98 ± 5.97 ng/ml, t = 2.26, *P* = 0.029; figure 3). In 7 out the 8 included dogs (87.5%), the serum folate concentrations in the sample with the lowest recorded HCT was lower than that of the sample in which the HCT first exceeded 0.30 l/l.

Of the 5 dogs in which folate depletion was recorded, 2 achieved a normal HCT and discontinued treatment, 1 achieved a normal HCT but was lost to follow-up while still on treatment (after 69 days) and 2 died of non-associative IMHA or related complications (after 1 and 30 days). The 3 dogs which developed low serum folate and were followed-up until their anaemia resolved had normal serum folate concentrations in the non-anaemic samples. In the other 10 dogs in which folate depletion was not recorded, 2 achieved a normal HCT and discontinued treatment, 4 were lost to follow-up while anaemic and on treatment (after 17, 25, 28, and 161 days) and 4 died while anaemic and on treatment (after 1, 3, 9 and 44 days).

Discussion

316 The first aim of the current study was to describe whether low serum folate
317 concentrations occur in dogs with non-associative IMHA. The results showed that low
318 serum folate occurred on at least one occasion in a third of dogs with non-associative
319 IMHA. It was only detected in samples with a HCT less than 0.30 l/l and occurred in 19%
320 of these samples. The prevalence of low folate detected in this study of dogs with non-
321 associative IMHA is a little higher than the 21% reported in dogs with anaemias, in large
322 part comprised of immune-mediated hematologic diseases, by Stanley and others.[14] Low
323 serum folate concentrations have also been described in humans with haemolytic
324 disorders due to the increased demand of folate during the regenerative response.[17,18,20]
325 This may be an underestimate of the true prevalence of low serum folate for several
326 reasons. Firstly, intravascular haemolysis (manifesting as grossly haemolysed samples)
327 was present in 6 dogs and this may have increased serum folate concentrations. It is likely
328 that even minor degrees of haemolysis (insufficient to cause sample colour change)
329 increase the serum folate due to the very high folate concentration within RBCs in
330 comparison to serum (packed red cells contain 160 to 640 ng/ml versus serum which
331 contains 2 to 15 ng/ml, human data).[18] Secondly, serum folate concentrations may also
332 have been increased in the first samples as these were collected from unfasted, acutely ill
333 animals; however, the magnitude of the effect of recent food intake is unknown and likely
334 subject to many variables. Finally, 3 dogs had small numbers of samples assayed (1 to 3
335 samples) so low serum folate concentrations may have been missed.

336 Further aims of this study were to investigate the relationship between serum
337 folate concentrations and HCT, to determine whether lower serum folate concentrations
338 were associated with lower haematocrits, and whether serum folate concentrations were

lower when the anaemia was expected to be in its most regenerative phase. A significant positive relationship between serum folate concentrations and HCT was found, however the dispersion was high. This high dispersion is probably the result of the complex interaction between the factors influencing folate concentrations and HCT in the course of non-associative IMHA such us food intake, intravascular haemolysis, blood transfusions, etc. Furthermore, samples collected from dogs when serum folate concentrations were low had a significantly higher chance of having a lower HCT than samples collected when serum folate concentrations were normal. Additionally, the estimated mean serum folate concentration was significantly lower in samples with a HCT less than 0.30 l/l than in samples with a HCT greater than or equal to 0.30 l/l. These results in conjunction with the finding, in paired samples, that serum folate concentrations were significantly lower in samples with the lowest recorded HCT than in samples in which the HCT first exceeded 0.30 l/l, suggest that serum folate concentrations in dogs with non-associative IMHA are lower when the HCT is low than when the HCT returns to near normal values. As RBC regeneration is likely to be greater at lower haematocrits than when the HCT is near normal,[7,8] this suggests serum folate concentrations are lower when regeneration is stronger.

Although this study was not designed to investigate the cause of the low serum folate concentrations in dogs with non-associative IMHA, it may be hypothesised that low folate occurred when the HCT was low due to the increased use of folate for DNA synthesis for RBC production at this time, as is the case in humans.[18] It was not possible to correlate serum folate concentrations with the manual reticulocyte count to establish this relationship because the absolute reticulocyte count is calculated by multiplying the

362 percentage of reticulocytes by the total RBC number and the latter is affected by
363 autoagglutination (artefactually reducing RBC number due to clumping) and the
364 administration of blood transfusions (artificially increases the number of mature RBCs).
365 All dogs in this study were either slide agglutination positive or were given one or more
366 blood transfusions. An alternative hypothesis to explain the low folate concentrations is
367 that they were caused by decreased folate intake or failure of folate absorption due to
368 subclinical gastrointestinal disease. Although some dogs showed either inappetence (10
369 dogs) or vomiting (2 dogs) in the acute stage of the disease, these signs were intermittent,
370 short-lived and never associated with diarrhoea. Inappetence lasted no more than 4 days,
371 and is unlikely to have been the sole cause for the low serum folate concentrations as
372 ingestion of a folate deficient diet for a minimum of 8-16 weeks is needed before hepatic
373 stores of this vitamin are depleted in otherwise normal humans and dogs with normal
374 erythropoiesis.[12,17] Further investigations are needed to elucidate the cause of low folate
375 concentrations in dogs with non-associative IMHA. Ideally these studies would include
376 control populations of dogs with non-regenerative anaemia as well as diseased dogs
377 without evidence of anaemia or gastrointestinal disease.

378 The development of low serum folate concentrations in these dogs is potentially
379 of clinical significance. In humans with haemolytic anaemia, the development of folate
380 deficiency has been shown to slow recovery due to impaired regenerative bone marrow
381 activity caused by the low folate.[21] This study was not designed to assess the effect of low
382 serum folate on recovery in these patients however, interestingly, the dog with most
383 sustained low serum folate had the longest hospitalisation time (data not shown). In this
384 dog and the other dogs with low serum folate in which a normal HCT was achieved, the

detected low serum folate was self-limiting and normalised without supplementation. This suggests that: a) normal dietary intake may be sufficient to correct this deficiency when the increased demand is no longer present[23] and b) adequate/normal intestinal absorption of folate was present in these dogs.

In humans, folate deficiency can lead to megaloblastic changes (macrocytosis)[17] however macrocytosis (aside from that associated with polychromasia) was not reported on any of the blood smears examined in this study. Automated measures of mean cell volume would have been affected by blood transfusion, autoagglutination and spherocytosis in these dogs and therefore could not be used as an accurate measure of RBC size in these patients. The absence of detectable macrocytosis in this study agrees with previous reports that canine folate deficiency rarely results in megaloblastic changes or overt macrocytosis.[12,14]

This study has some limitations. Firstly, within the cell, metabolism of vitamin B12 and folate are interconnected in the methionine cycle where homocysteine is converted to methionine.[28] Intracellular lack of vitamin B12 can lead to functional folate deficiency and an increased serum concentration of homocysteine. Intracellular folate deficiency can also lead to increase serum concentration of homocysteine.[29] Without the measurement of these two molecules in dogs with low serum folate concentrations, in this study it is impossible to determine whether or not the depletion was severe enough to cause an intracellular deficiency nor is it possible to determine whether functional folate deficiency also occurred in some of the dogs with normal serum folate concentration. Despite this, it is worth pointing out that vitamin B12 supplementation is already routinely recommended in dogs with serum concentrations just above the lower end of

the reference range and with no evidence of cellular deficiency because these concentrations are already considered suboptimal.[29] The same might also apply for serum folate concentrations at the lower end of the reference interval, given that vitamin B12 and folate are metabolically interconnected and given that folate supplementation in dogs is considered safe (https://vetmed.tamu.edu/gilab/research/folate-information%20/). Secondly, the magnitude of the difference in folate concentrations during and after RBC regeneration has never been previously described, thus it was not possible to calculate the sample size necessary to demonstrate our null hypotheses. We therefore decided to include all the dogs that presented during a pre-fixed period of time. Consequently, the results of our study should be interpreted with care however, they could be used to inform formal power calculations for the design of future studies aiming to validate the current findings. Thirdly, 7/15 cases were lost to follow-up or died before the HCT had exceeded 0.30 l/l, reducing the population size for the comparison of the folate concentrations in the paired samples. Moreover, the loss of cases early in the disease process also affected the number of samples collected from some of the dogs and this, alongside the non-standardised sampling times, may have resulted in the lowest serum folate concentrations going undetected and therefore underestimated the prevalence of low serum folate in dogs with non-associative IMHA.

In conclusion, a low serum folate concentration was identified in at least one sample in one third of dogs with non-associative IMHA during the course of their anaemia. The HCT and serum folate concentrations were positively associated with each other and low serum folate concentrations were only documented in samples with a HCT less than 0.30 l/l, when the anaemia would be expected to be in its most regenerative

phase. Further studies are needed to confirm these findings, to investigate the cause of the low serum folate concentrations and to assess whether folate supplementation would be of benefit in dogs with non-associative IMHA. If these results are confirmed, measurement of serum folate concentrations and supplementation of folate-deficient dogs with non-associative IMHA could become routine clinical practice.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure 1: Scatterplot of HCT versus serum folate concentrations and fitted model.

Figure 2: Boxplots of serum folate concentrations for the 68 samples with a HCT less than 0.30 l/l (left) and the 58 samples with a HCT greater than or equal to 0.30 l/l (right). The bottom and top of the box are the 1st and 3rd quartiles; the median is the band inside the box. The whiskers correspond to the lowest datum still within 1.5 interquartile ranges of the first quartile, and the highest datum still within 1.5 interquartile ranges of the fourth quartile. Circles are outlier values (more than 1.5 interquartile ranges away from the closest end of the box).

Figure 3: Ladder graph showing the serum folate concentrations from the sample with the lowest recorded HCT and the first sample when the HCT exceeded 0.30 l/l in the 8 dogs in which paired samples were available and serum folate concentrations were measured at the same laboratory.

538 **Table 1:** Cohort characteristics and number of samples collected for each dog

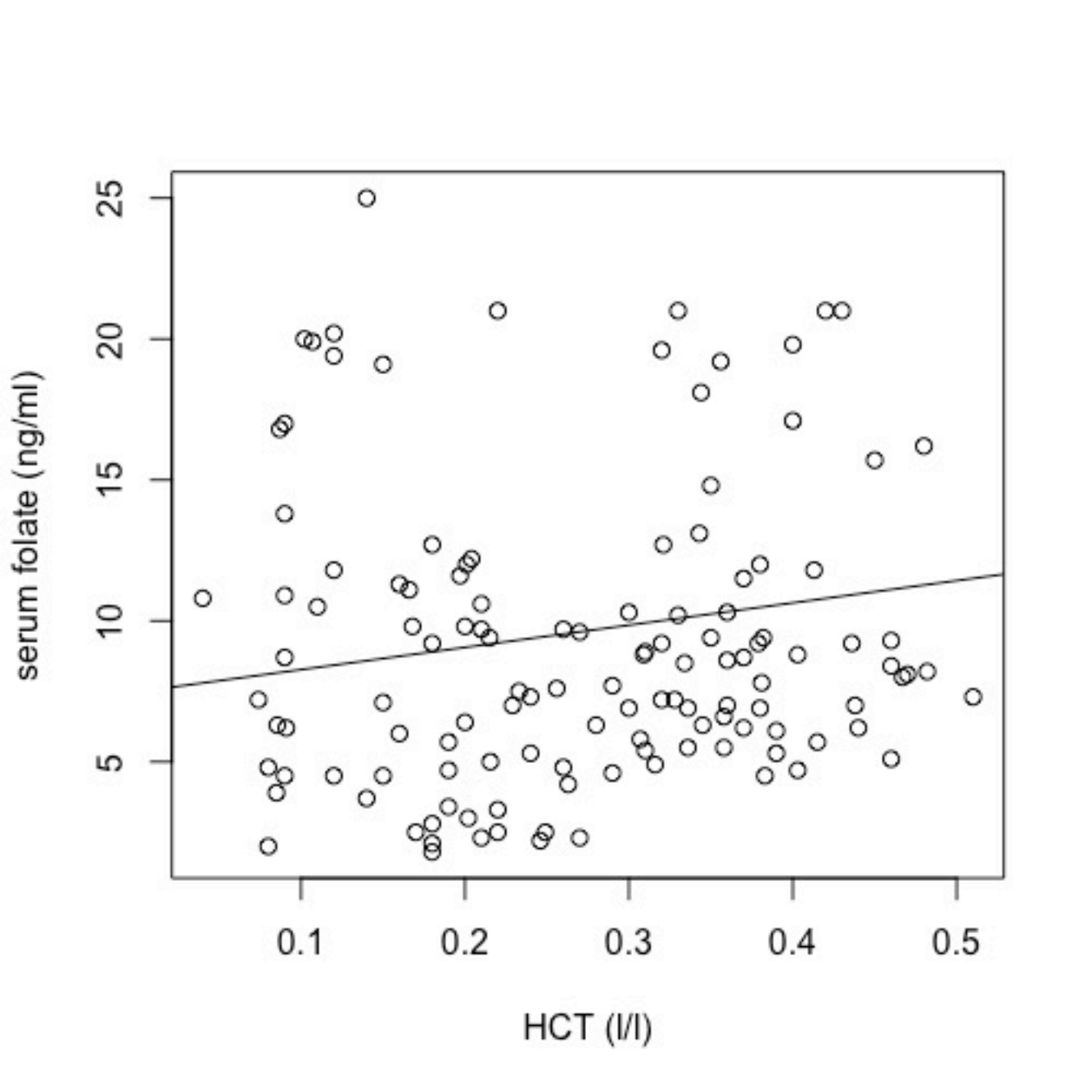
Dog	**Breed**	**BW** (kg)	**Sex**	**Age** (months)	**Nº of samples**	**Days between 1st and last sample**
1	Mongrel	18	ME	72	19	124
2	German Shepherd	28	FE	85	13	71
3	Border Collie	16	FN	120	7	31
4	Border Terrier	12	ME	29	10	137
5	Mongrel	15	FE	30	1	0
6	English Springer Spaniel	25	ME	28	7	17
7	Springer Spaniel	18	FN	68	1	0
8	Border Collie	23	ME	61	6	26
9	Cocker Spaniel	22	MN	84	3	3
10	Cairn Terrier	8	FN	91	7	9
11	Bearded Collie	18	FE	76	10	134
12	Mongrel	13	FN	37	17	264
13	Border Collie	12	ME	8	8	161
14	Toy Poodle	6	MN	153	7	117
15	Labrador	31	FN	71	10	42

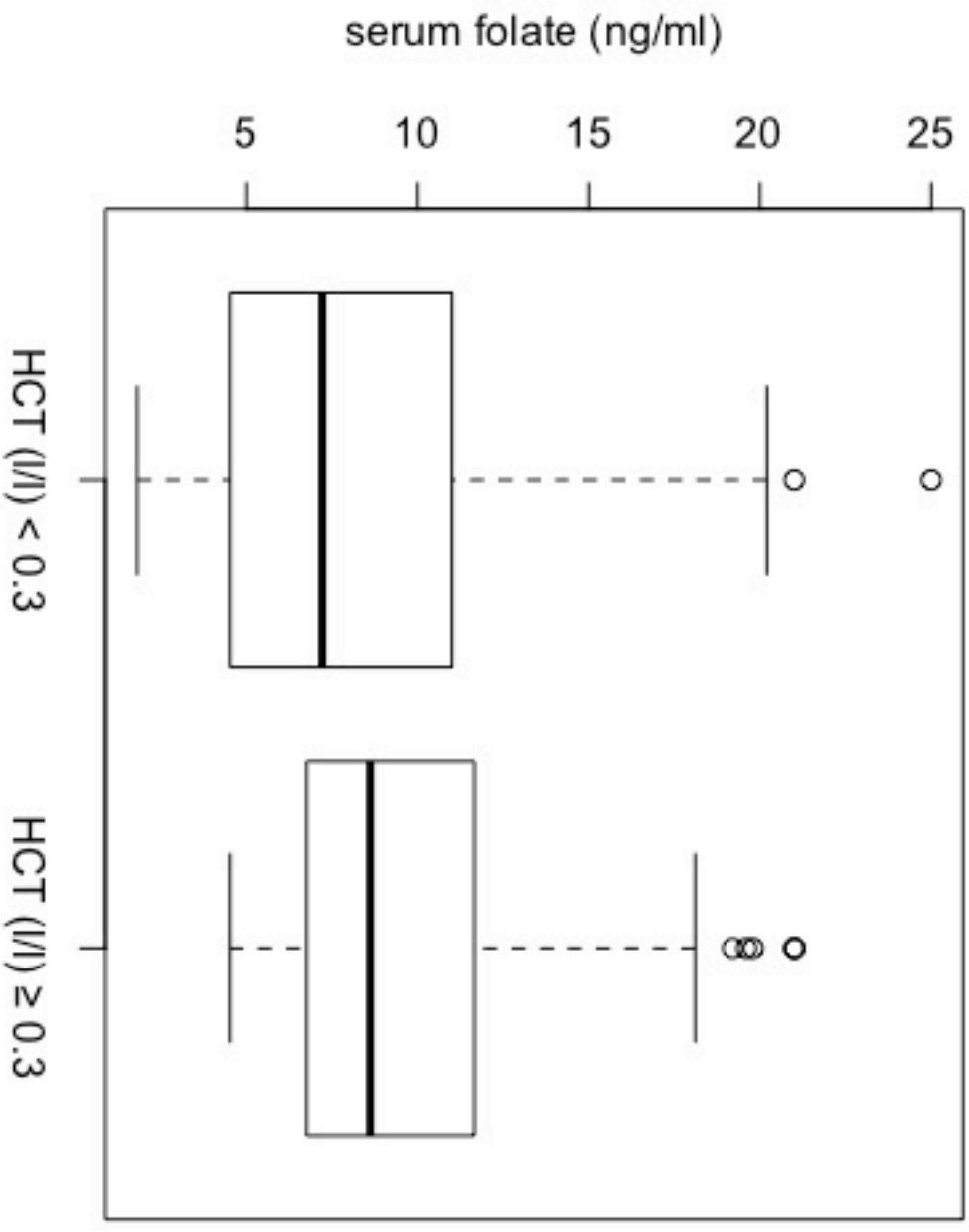
539 FE, female entire; FN, female neutered; ME, male entire; MN, male neutered
540

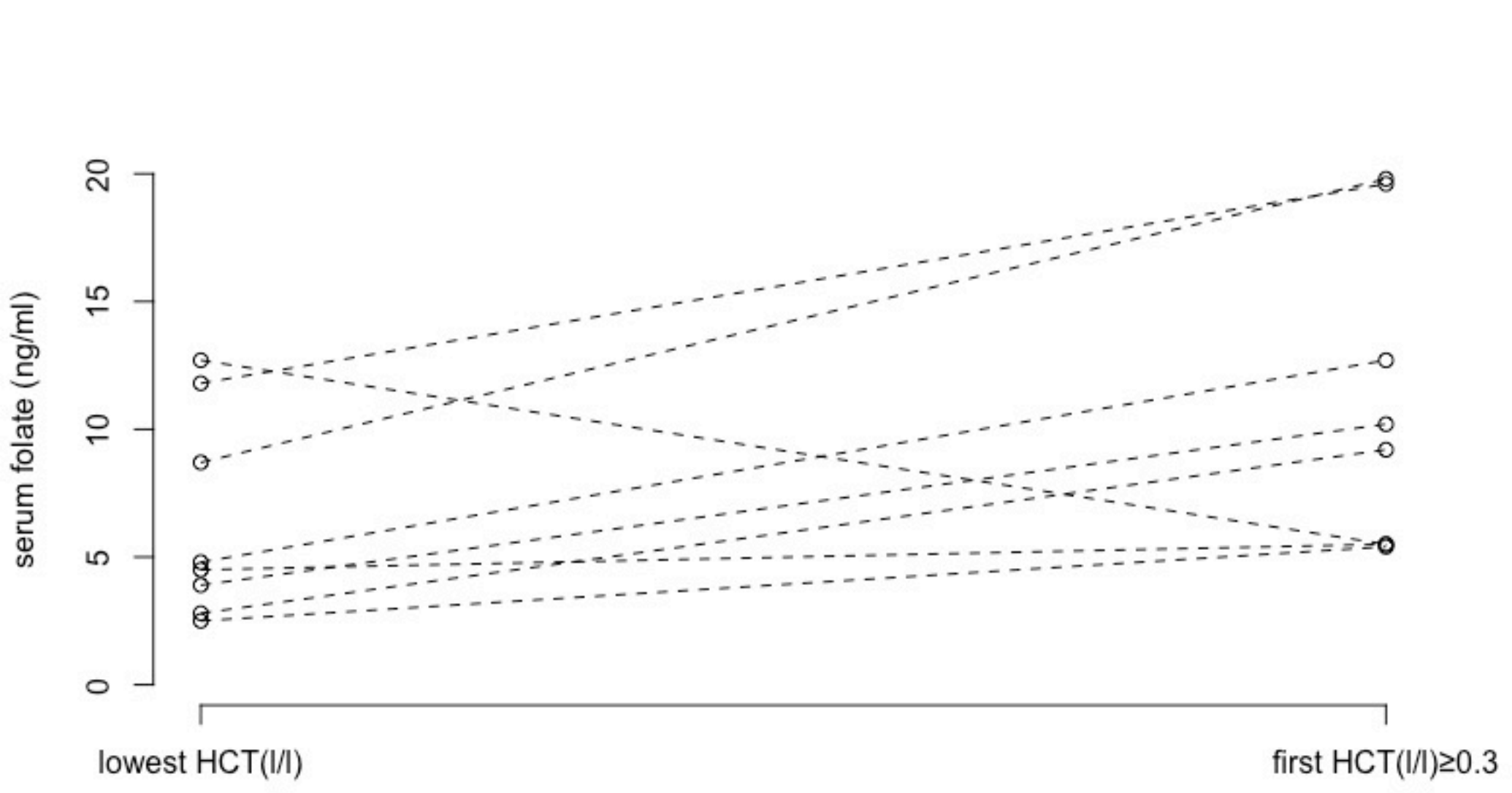
Table 2: Estimates of the regression coefficients from the linear mixed effects model including serum folate concentrations, HCT, sex, body weight, age, and laboratory used.

Fixed effects	Estimate of regression coefficient ± SE	t-value	*P*-value
Intercept	2.92 ± 5.26	0.55	0.580
HCT	7.78 ± 3.93	1.98	0.050
Sex	2.84 ± 2.51	1.13	0.281
Body weight	0.11 ± 0.18	0.59	0.565
Age	0.01 ± 0.03	0.34	0.739
Laboratory used	0.81 ± 1.76	0.46	0.644

HCT, haematocrit; SE, standard error


serum folate (ng/ml)
5
10
15
20
25
0.1
0.2
0.3
0.4
0.5
HCT (l/l)


serum folate (ng/ml)
5
10
15
20
25
HCT (I/I) < 0.3
HCT (I/I) ≥ 0.3


serum folate (ng/ml)
0
5
10
15
20
lowest HCT(l/l)
first HCT(l/l)≥0.3