

Original Article
Internal Medicine



Serum homocysteine concentration in dogs with immunosuppressant-responsive enteropathy

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Received: Aug 6, 2019

Revised: Mar 10, 2020

Accepted: Mar 27, 2020

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ABSTRACT

Background: Homocysteine (HCY) was evaluated in healthy and chronic enteropathic dogs, however no studies on dogs with immunosuppressant-responsive enteropathy are available.

Objectives: The aim was to evaluate serum HCY concentrations and its prognostic role in dogs with immunosuppressant-responsive enteropathy compared to healthy dogs.

Methods: Serum HCY concentration was statistically compared between 24 healthy dogs and 29 dogs with immunosuppressant-responsive enteropathy. Correlation analyses between serum total protein, albumin (ALB), C-reactive protein (CRP), folate and cobalamin, and serum HCY concentration were performed in immunosuppressant-responsive enteropathic dogs.

Results: The associations between serum HCY concentration and clinical, histological, endoscopic scores and follow-up were evaluated. Mean serum HCY concentration was higher in immunosuppressant-responsive enteropathic dogs compared to control dogs (30.22 ± 8.67 $\mu\text{mol/L}$ vs. 5.26 ± 2.78 $\mu\text{mol/L}$; $p < 0.0001$). No association between serum HCY concentration and total protein, ALB, CRP, folate concentration as well as, clinical score, histological and endoscopic scores was found. A negative correlation between serum HCY concentration and cobalamin was noted ($p = 0.0025$, $r = -0.54$). No significant difference in HCY was found between responsive and non-responsive dogs or between survivors and non-survivors.

Conclusions: Although, serum HCY concentration was higher in immunosuppressant-responsive enteropathy, its prognostic value remains unclear. However, further prospective, large-scale studies are warranted to better investigate the possible prognostic role of HCY in immunosuppressant-responsive enteropathic dogs.

Keywords: Cobalamin; folate; CCECAI; canine; HPLC

INTRODUCTION

Homocysteine (HCY) is an endogenous sulphur-containing amino acid, produced by the systemic conversion of methionine, which is an exogenous amino acid present in animal proteins [1,2]. HCY is a pro-inflammatory molecule and its hematic concentration is influenced by nutritional determinants, including folate (vitamin B9), cobalamin (vitamin B12), pyridoxin (vitamin B6), as well as the genetic polymorphism of enzymes

Conflict of Interest

The authors declare no conflicts of interest. No third-party funding or support was received in association with the present study or the writing or publication of the manuscript.

Authors Contributions

Conceptualization: Benvenuti E, Marchetti V; Data curation: Benvenuti E, Pierini A, Pietra M, Meucci V; Formal analysis: Pierini A; Investigation: Benvenuti E; Methodology: Benvenuti E; Project administration: Marchetti V; Supervision: Marchetti V; Writing - original draft: Benvenuti E; Writing - review & editing: Benvenuti E, Pierini A, Gori E, Bottero E, Pietra M, Lippi I, Meucci V, Marchetti V.

involved in its metabolic pathway [1,3]. In human medicine, methionine synthase requires methylcobalamin for the re-methylation of methionine from HCY. Methionine synthase dysfunction results in suboptimal cobalamin availability and a consequent increase in HCY serum [4]. Hyperhomocysteinemia is commonly reported in humans with inflammatory bowel disease (IBD), and it seems to be linked to cobalamin and/or folate deficiency [5] and, HCY is therefore a sensitive marker for vitamin B deficiency [6-8]. In people affected by IBD, serum HCY is significantly higher compared to healthy people, and is associated with low serum folate and cobalamin concentration [9].

In veterinary medicine, altered serum HCY concentrations have been reported in healthy dogs and dogs with various diseases. For instance, HCY has been reported to increase in dogs with heart and kidney disease [10] and in hypothyroid dogs [2]. The association between elevated serum HCY concentration and hypocobalaminemia has been demonstrated in Shar-Pei dogs with chronic diarrhoea [11].

In a study by Rossi et al. [12], serum HCY concentration has also been investigated in 17 dogs with chronic enteropathies showing a significant negative correlation with serum cobalamin. Afterwards, Heilmann et al. [13] showed that serum cobalamin and folate are negatively correlated with serum HCY in Greyhounds with chronic gastrointestinal disease and with thrombotic disease. Lastly, the serum HCY have been studied in four different breeds of healthy dogs establishing breed-related reference ranges [14].

In dogs with chronic enteropathy, many studies have shown that hypoalbuminemia is associated with a poor outcome [15-17]. Moreover, serum cobalamin and folate can be measured to determine if supplementation is required and low serum cobalamin concentration has also been associated with a negative prognosis [15,18]. Serum C-reactive protein (CRP) has been shown to correlate with clinical disease activity (Canine Inflammatory Bowel Disease Activity Index [CIBDAI]) and implies that severe clinical disease may be accompanied by a systemic inflammatory process [19]. Clinical disease activity or CRP can also serve as a baseline for determining the response to treatment [20]. Chronic enteropathies are a group of diseases which includes food-responsive enteropathy (FRE), antibiotic-responsive enteropathy (ARE), immunosuppressant-responsive enteropathy (IRE) and non-responsive enteropathy (NRE) [16]. The IRE describes the intestinal idiopathic inflammation in dogs that typically implies failed treatment trials with diet and subsequently antibiotics, intestinal inflammation demonstrated by histological examination and necessity of immunosuppressant treatment [16,21,22]. In veterinary literature, HCY was evaluated in healthy dogs [14] and in dogs with chronic enteropathy [12].

However, as far as we know, no studies have investigated serum HCY in dogs with IRE and its prognostic role.

The aims of our study were: 1) to evaluate serum HCY concentration in dogs with IRE compared to healthy dogs, and 2) to investigate association between serum HCY and laboratory examinations, clinical, endoscopic and histologic scores, response to therapy and mortality.

MATERIALS AND METHODS

Clinical data

For this retrospective study, serum HCY concentration was evaluated on exceeding serum samples of IRE dogs included in a larger prospective study, conducted at two veterinary teaching hospitals (University of Pisa and University of Bologna) between March 2017 and September 2018, for which ethical approval was obtained (Approval No. 31834/2017).

The twenty-nine dogs included had IRE. Other extra-intestinal diseases (such as hepatic, pancreatic or renal disease, hypoadrenocorticism, hypothyroidism and hypercalcaemia), infectious or parasitic diseases and intestinal diseases of other aetiologies (e.g. mechanical obstruction from intussusception, foreign bodies, or intestinal tumours) were excluded. Additionally, dogs with kidney disease (history, clinical signs, biochemical profile and urinalysis) and heart disease (history, clinical signs, electrocardiography, thoracic radiography and echocardiography) were also excluded. FRE, improvement when fed with hydrolysed or restricted antigen diets, and dogs with ARE, improvement while on tylosin (15 mg/kg, PO q12 h). All dogs included in the IRE group were fed with a hydrolyzed diet in and in all dogs an immunomodulatory therapy was set using prednisolone (from 0.5 to 1 mg/kg every 12 h) or budesonide (3 mg/m² every 24 h) association or not with cyclosporine to 5 mg/kg every 24 h or chlorambucil 2–4 mg/m² at discretion of the clinician.

The clinical severity of the disease was evaluated at diagnosis (T0), and 6 months (T6) and 12 months (T12) by using the previously published CCECAI score [15]. The CCECAI score includes clinical and laboratory signs: attitude/activity, appetite, vomiting, stool consistency, stool frequency, and weight loss, serum albumin (ALB) concentration, peripheral oedema or ascites and severity of pruritus [15]. The result of total CCECAI score classifies patients according to the degree of illness: insignificant disease (0–3), mild disease (4–5), moderate disease (6–8), severe disease (9–11), and very severe disease (12 or higher) [15].

A biochemical profile was performed in all dogs at T0. Hypoproteinaemia and hypoalbuminemia were defined as total protein < 5.8 g/dL (reference interval 5.8–7.8 g/dL) and ALB < 2.7 g/dL (reference interval 2.7–4.1 g/dL), respectively [17,23]. The presence of protein losing enteropathy (PLE) was defined if serum ALB concentration was lower than 2.7 g/dL [17].

The CRP was evaluated in all patients (laboratory reference interval 0–0.3 mg/dL) at the presentation. Serum cobalamin was also evaluated in all dogs using a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulate 1000, Diagnostic Products Corporation, DPC, USA). Folate concentration was measured by means of a validated automated solid-phase chemiluminescence assay (Immulate 1000, Siemens Healthcare Diagnostic Inc., USA). The lower laboratory reference values of cobalamin and folate was 251 ng/L and 7.7 µg/L, respectively.

Endoscopy and histology of the duodenum were scored by according to the standard of the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group [20,23,24].

Dogs were divided into two groups (responders and non-responders) on the basis of the response to treatment at T6 and T12. At T6-T12, responders had to have ALB ≥2.7 g/dL and CCECAI ≤ 3 [17]. The non-survivors and survivors were also assessed during follow-up.

Control dogs

Client consent was obtained for the client-owned dog of various breeds and ages. The control dogs (CD) were healthy dogs ($n = 24$) that were recruited during annual routine checks, prior to the annual prophylaxis for heartworms and/or for vaccination. All CD were clinically healthy at physical examination, with no historical complaints and normal blood works. Some of the serum from their blood work was used for HCY determination.

HCY evaluation

The serum HCY concentration was assessed through the High-Performance Liquid Chromatography (HPLC) system, using a HCY in serum (HPLC) Chromsystem[®]-Diagnostic Kit (Germany). The HPLC system consisted of a Series 200 Perkin Elmer gradient pump (USA) coupled to a Jasco FT-1520 fluorimetric detector (Jasco, Inc., Japan). Excitation wavelength was set at 385 nm and the emission wavelength was set at 515 nm. Turbochrome Navigator software (Perkin Elmer) was used for data processing. The HPLC method was internally validated. The mean HCY recoveries in spiked samples ranged from 87.1% to 90.4%. The LOD and LOQ calculated were 0.1 and 0.5 $\mu\text{mol/L}$, respectively. Intra-day and inter-day coefficients of variations were $< 7.9\%$ and $< 9.5\%$, respectively. The HCY serum evaluation was performed in all dogs at T0.

Statistical analysis

To perform the statistical analyses, GraphPad Prism 6 (GraphPad Software, USA) was used. A D'Agostino-Pearson test was used to assess the normality of data distribution of all continuous variables. Normally and non-normally distributed continuous variables were reported as mean \pm SD and as median and range, respectively. Age and sex distribution were compared between healthy and IRE groups with unpaired t -test and Fisher's exact test, respectively. Serum HCY was compared between the healthy and IRE groups using the unpaired t -test. In IRE dogs, serum total protein, ALB, CRP, folate and cobalamin concentrations were correlated to serum HCY concentrations using Pearson or nonparametric Spearman correlation tests. IRE dogs were divided into two groups (normal and low cobalamin/folate) based on serum cobalamin and folate. They were then compared to serum HCY concentrations using the unpaired t -test. Associations between serum HCY and CCECAI, histologic and endoscopic scores were evaluated using the t -test or ANOVA one-way test. A p value of < 0.05 was considered significant.

RESULTS

Study population

Twenty-nine dogs with a final diagnosis of IRE were enrolled. Median age was 4.79 years (ranging between 1–11 years). Twenty-two dogs were male (1 neutered) and 7 were female (4 spayed). Median Body Condition Score (BCS) was 3.5 (range 2–6). Seven dogs were mixed breed and the rest belonged to 13 different breeds: German Shepherd (6), Maltese (4), Dachshund (2), Pinscher (2), Basenji (1), Irish Setter (1), Weimaraner (1), English Cocker Spaniel (1), Jack Russel Terrier (1), Dobermann (1), Pug (1), and Beagle (1).

The CD included 24 dogs of different breeds: Jack Russel Terrier (4), English setter (3), English bulldog (2), Dachshund (2), Beagle (1), and Cocker Spaniel (1). Eleven dogs were mixed breed. Eight dogs were female and 16 were male. Median age was 4.5 years (1–8 years) and median BCS was 4.5 (range 4–6). No difference in age and sex between the study groups (IRE and CD) was found.

HCY in IRE dogs and CD

Mean serum HCY concentration was $30.22 \pm 8.67 \mu\text{mol/L}$ and $5.26 \pm 2.78 \mu\text{mol/L}$ in IRE and CD group, respectively, statistically higher in the IRE group than in CD ($p < 0.0001$; **Fig. 1**).

At T0, 14 out of 29 IRE-dogs (48.3%) showed hypoproteinaemia ($4.43 \pm 1.05 \text{ g/dL}$), while hypoalbuminemia ($2.01 \pm 0.48 \text{ g/dL}$) occurred in 12 (41.3%) out of 29 dogs and sixteen dogs showed an increase in CRP ($0.53 \pm 0.50 \text{ mg/dL}$).

Median serum HCY concentration in dogs with hypoproteinaemia and hypoalbuminemia was $27.91 \mu\text{mol/L}$ (range $14.11\text{--}51.07 \mu\text{mol/L}$) and $26.9 \mu\text{mol/L}$ (range $19.86\text{--}39.92 \mu\text{mol/L}$), respectively. No association between serum HCY and ALB ($p = 0.097$), total protein ($p = 0.061$), CRP ($p = 0.705$) and folate ($p = 0.440$) concentration was found.

Eighteen (62%) out of 29 dogs had hypocobalaminemia (mean $184.35 \pm 41.24 \text{ ng/L}$), and nine dogs showed hypofolatemia (mean $4.42 \pm 1.91 \mu\text{g/L}$) (31%). Median HCY was significantly different between dogs with (35.48 , range $19.90\text{--}51.07 \mu\text{mol/L}$) or without hypocobalaminemia (23.36 , range $14.11\text{--}33.98 \mu\text{mol/L}$) ($p = 0.001$). Serum cobalamin concentration showed a moderate negative correlation with serum HCY concentration ($p = 0.002$, $r = -0.54$; **Fig. 2**).

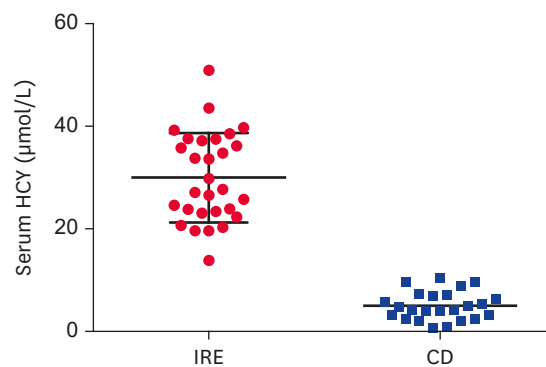


Fig. 1. Serum HCY evaluation in CD and in IRE dogs. HCY, homocysteine; CD, control dogs; IRE, immunosuppressant-responsive enteropathy.

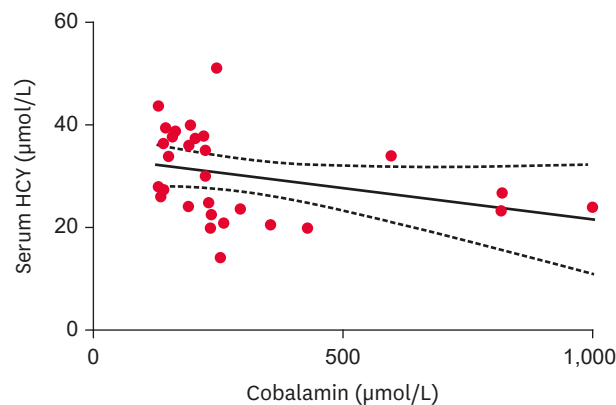


Fig. 2. Correlation between serum cobalamin and serum HCY concentration in immunosuppressant-responsive enteropathy dogs ($p = 0.002$). HCY, homocysteine.

Table 1. IRE dogs clinical scores at admission (T0) and after 6 months (T6) and 12 months (T12) and endoscopic and histological score

No.	CCECAI (T0)	CCECAI (T6)	CCECAI (T12)	Endoscopic score	Histological score
1	15	0*	0*	3	3
2	7	0*	0*	2	2
3	5	5 [†]	0*	2	2
4	5	0*	0*	3	2
5	7	0*	0*	2	3
6	13	4 [†]	0*	2	2
7	7	Non survivor		3	3
8	4	0*	4 [†]	2	2
9	5	5 [†]	6 [†]	3	2
10	5	Lost follow-up		2	2
11	8	Lost follow-up		2	2
12	13	0*	0*	3	2
13	12	0*	2*	3	3
14	8	1*	0*	3	2
15	3	5 [†]	0*	3	2
16	4	1*	2*	3	3
17	6	4 [†]	0*	3	2
18	9	Non survivor		3	3
19	7	0*	7 [†]	3	2
20	7	3*	2*	1	2
21	6	2*	2*	3	2
22	8	0*	0*	3	3
23	5	0*	2*	2	1
24	6	Non survivor		3	2
25	9	0*	0*	3	2
26	2	0*	3*	3	2
27	4	0*	2*	2	1
28	5	Lost follow-up		2	2
29	6	0*	0*	3	2

IRE, immunosuppressant-responsive enteropathy; CCECAI, Canine Chronic Enteropathy activity index. *, responders; †, non-responders; T0, admission; T6, after 6 months; T12, after 12 months.

Evaluation of prognostic significance of HCY in association with clinical, endoscopic, and histological scores, and response to therapy and mortality

At admission, the median CCECAI score was 6 (range 2–15) in IRE group. No significant correlation between serum HCY concentration and clinical scores at admission was found. At T6, three dogs were lost at follow-up and three dogs died. At T12 twenty-three dogs were evaluated. Serum HCY concentration was similar between non-survivors and survivors (median 24.74 and 30.85 µmol/L, respectively).

No statistical difference in serum HCY concentrations at T6 and T12 was found between responsive (T6: 18 dogs, T12: 20) and non-responsive dogs (T6: 5 dogs, T12: 3 dogs) ($p > 0.05$; **Table 1**).

Median endoscopic score was 2 (range 0–2) and median histological score was 2 (range 1–3; **Table 1**). Serum HCY did not correlate with duodenal endoscopic ($p = 0.083$) and histological scores ($p = 0.400$).

DISCUSSION

In veterinary medicine, there are no studies on serum HCY concentration in dogs with IRE, and only a few reports on serum HCY in dogs with gastrointestinal disease [11-13].

In our study, dogs affected by IRE showed a higher serum HCY concentration than healthy dogs. The two groups (CD and IRE) were similar in terms of age and sex and this particular data allowed us to compare HCY between groups avoiding age and sex interpreting bias.

In CD, serum HCY ranged from 1 and 10.55 $\mu\text{mol/L}$ (median 4.75 $\mu\text{mol/L}$, mean 5.26 ± 2.77). In Grützner, the reference interval for serum HCY concentration was calculated as 5.2–25.9 $\mu\text{mol/L}$ and a recent study reported a reference interval of 5.9–31.9 $\mu\text{mol/L}$ [11,18]. In the present study the median value was lower than the previously reported intervals [11,18].

In the study by Çayir and Kozat [14], the HCY ranged from 8.88 $\mu\text{mol/L}$ to 11.43 $\mu\text{mol/L}$ in German Shepherd, Golden Retriever, Labrador Retriever and Terrier and no differences were found between these four breeds [14]. In our data, the serum HCY concentration in healthy dogs was similar to Çayir and Kozat's [14] study, despite the higher number of breeds involved in the present study. However, elevated HCY concentrations were also found in 12 healthy Greyhounds, in which the median serum HCY was 30.8 $\mu\text{mol/L}$ (range 15–56 $\mu\text{mol/L}$) and the potential cause remained unknown [13]. In Greyhounds, elevated serum HCY has been hypothesized as being related to hereditary hypocobalaminemia [13]. In our study healthy Greyhounds were not included.

In our study, serum HCY concentration was measured with HPLC, contrarily to previous studies which used other analytical methods (ELISA and Gas Chromatography Mass Spectrometry [GC/MS]) making comparisons hard [11,12,14,18]. Consequently, in our opinion, the discrepancy between our data and those reported in healthy dogs [11,18] may be due to the different analytical method.

In the literature, serum HCY concentrations have been reported to be higher in dogs with hypocobalaminemia [11,12] and hypofolatemia [13], as well as in hypoalbuminemic dogs [13,26].

The IRE dogs showed an increase of HCY with a mean value of $30.22 \pm 8.67 \mu\text{mol/L}$ (min 20.85–max 52.07 $\mu\text{mol/L}$). In veterinary medicine, there are few studies on HCY evaluation and chronic enteropathy. In Berghoff, in 20 dogs the median of serum HCY concentration was 9.5 $\mu\text{mol/L}$ (min 2.7–max 71.4) [5]. Toresson and colleagues evaluated the HCY concentration in hypocobalaminemic dogs and the reference interval of serum HCY concentration was 3.3–62.2 $\mu\text{mol/L}$ [18]. Moreover, in Rossi et al. [12], median serum HCY concentration in dogs with hypocobalaminemia was 8.70 $\mu\text{mol/L}$ (range 7.30–13 $\mu\text{mol/L}$) and the median HCY in dogs without hypocobalaminemia was 4.25 (range 1.30–11.30 $\mu\text{mol/L}$). Our IRE dogs had higher values of HCY compared to the results reported by these papers. We evaluated the serum HCY concentration in dogs with IRE. The IRE group included dogs that underwent trials (diet and antibiotics) in order to exclude FRE and ARE [16]. To the best of our knowledge, serum HCY concentration has not been investigated for this specific disease in dogs. The IRE is defined as intestinal inflammatory diseases, requiring immunosuppressant therapy which may sometimes be severe [9,15,16]. In humans, malnutrition is a common condition in patients with IBD and malabsorption and hypoalbuminemia have been reported also in dogs [9,15,16].

In both human and veterinary medicine, the multifactorial condition of increased serum HCY is still not completely understood. In human medicine, a negative correlation has been reported between increased serum HCY and decreased serum folate or cobalamin in IBD patients [6-8]. Deficiencies in cobalamin and the cobalamin component linked to the

transcobalamin genes, are characterized by an increase in the serum concentration of HCY > of 20 $\mu\text{mol/L}$ [4]. Our results confirm the increase in HCY in hypocobalaminemic dogs and agree with the results of Rossi et al. [12] who found a negative correlation between the serum concentrations of HCY and cobalamin serum concentrations.

In our study, serum HCY concentration did not correlate with folate concentrations. Hyperhomocysteinaemia was also present in dogs without cobalamin and folate deficiencies. HCY has a complex metabolism, it involves methionine, nutrition determinants, including cobalamin, folate, and pyridoxin, and various enzymes [3]. Our findings may indicate that HCY metabolism could also be altered in the absence of overt cobalamin and folate deficiencies. In that half of human patients with IBD, a micronutrient deficiency is reported. Most common deficiencies involve iron, cobalamin, pyridoxin, thiamine (vitamin B1), cholecalciferol (vitamin D), vitamin K and folic acid, selenium and zinc [27]. According to Weisshof and Chermesh [27], hyperhomocysteinaemia in dogs without cobalamin and folate deficiencies may be secondary to malnutrition and micronutrient deficiency and it could be a possible early marker of malabsorption. In fact, although cobalamin and folate concentrations were in the reference range, the increase in HCY could be caused by the lack of other micronutrients, such as pyridoxin [27].

All dogs were fed using only hydrolyzed veterinary diets. In human medicine, variations in the dietary routine have been demonstrated to be the cause of various degrees of a reduction in folate absorption and an increase in HCY concentrations [3,9]. In our study, the use of a commercial diet in all dogs likely reduced the bias due to possible nutrient deficiencies.

An increase in serum HCY concentration did not correlate with serum ALB and total protein. In a study on hypocobalaminemic dogs of different breed [26], an association between an increase in HCY and methylmalonic acid was found along with a negative correlation between serum HCY and serum ALB. In our study, no correlation between serum HCY and serum ALB was found for hypocobalaminemic dogs.

The discrepancy with Grützner et al. [26] may be due to factors such as the enrolment of only hypocobalaminemic dogs, different breeds, and the lack of characterization of the clinical presentation of the included dogs. Moreover, in our study, we included dogs of different breeds that had IRE, with or without hypocobalaminemia.

Our data seem to suggest that a high concentration of serum HCY concentration is not associated only with malabsorption. Indeed, in dogs with IRE, high intestinal inflammation can occur in both dogs with or without hypoalbuminemia and can increase HCY blood concentrations. HCY acts as a pro-oxidant agent and has been implicated in the decrease in antioxidant capacity in patients with IBD [28,29]. In addition, HCY is considered as a pro-inflammatory molecule [2], which in human medicine has been associated with an increased CRP concentration [9].

In human IBD, CRP is used as an activity marker, as it correlates with clinical disease activity and histologic inflammation [30]. In dogs with IRE, CRP has been considered as a sensitive biomarker of inflammation, which at the time of diagnosis is significantly elevated, compared to healthy dogs, and compared to post treatment time in dogs with IRE [31]. In our study, sixteen dogs showed CRP elevation, with no association between HCY and CRP concentration. Based on our results, it is not clear whether HCY acts as a pro-inflammatory molecule in dogs with IRE.

One aim of the present study was to evaluate the hypothetical prognostic significance of HCY in association with clinical, endoscopic, and histological scores. We found that serum HCY concentration was not associated with the clinical score at the time of diagnosis and the clinical score after therapy (T6-T12). In addition, HCY was not associated with the endoscopic and histological score. There was no difference in HCY serum concentrations between responsive and non-responsive dogs, nor between survivors and non-survivors. Despite cases in human medicine [32,33], serum HCY did not appear to be a risk factor for mortality. Therefore, serum HCY should not be considered as a prognostic marker in dogs with IRE.

In humans, increased serum HCY concentration has been proposed as a risk factor and sensitive marker for venous and arterial thrombosis, cardiovascular, neurodegenerative disorders, and folic acid deficiency [32,33]. Although, HCY has been considered as prothrombotic due to its ability to increase the production of several inflammatory cytokines [34], many studies have failed to find an association between increased serum HCY and thrombosis in human IBD patients [9,35]. In IRE dogs, hypoalbuminemia was frequently present and hypercoagulability may be a common finding in PLE dogs [16,20]. Evaluation of haemostatic function is recommended to determine if hypo- or hyper-coagulability developed as a consequence of enteric protein loss [20]. PLE dogs with hypercoagulability had a death rate of 66% within 5 months [36]. In the present study, higher levels of serum HCY were not associated neither with ALB level nor with mortality. It would be interesting also to evaluate systemic thrombotic risk in IRE dogs and hyperhomocysteinaemia.

This study has some limitations. Firstly, the relatively low number of dogs enrolled, and the lack of HCY evaluation during follow-up at T6 and T12. Despite HCY at the time of the diagnosis was not associated neither with CCECAI nor with the mortality rate, it would be interesting to evaluate HCY during the follow-up and potential fluctuation in responders and non-responders and in non-survivors. Moreover, in Toresson, the HCY was evaluated at diagnosis and after 28 days and 90 days in hypocobalaminemic dogs before and after cobalamin supplementation. In this study no significant difference showed between the two groups at any time points [18].

In a recent study [37], increased HCY concentration was associated with the severity of the mitral valve cardiac disease and it was evaluated in comparison with clinical signs. In our study, although HCY was not evaluated with single clinical sign, it was evaluated in relation to the CCECAI score, since this scoring system includes various clinical signs of enteropathic dogs.

Another interesting research topic would be the evaluation of any possible variation in HCY of IRE dogs before and after immuno-suppressant therapy. In addition, the evaluation of HCY concentration between IRE and other types of chronic enteropathies, as FRE, may be another interesting research topic.

The lacking of HCY reference intervals in healthy dogs is another study limitation. Considering the low number of enrolled dogs, it was possible only to insert and consider an HCY cut-off. It would be interesting to evaluate the HCY reference range using the HPLC method in healthy and IRE dogs.

A decreased glomerular filtration rate has been associated with increased serum HCY in hypothyroid dogs [2]. Although, dogs with overt chronic kidney disease (CKD) were not included in the study, glomerular filtration rate was not measured. As a consequence, we

cannot rule out that the enrolled patients might have had early stage CKD (International Renal Interest Society, stage 1).

In conclusion, dogs with IRE have a markedly increased serum HCY concentration compared to healthy dogs, and also show a negative correlation between serum HCY concentration and cobalamin regardless of folate concentrations. Serum HCY did not predict outcome and was not associated with clinical, endoscopic or histological scores. However, further prospective, large-scale studies are warranted to better understand the real role of HCY in dogs with immunosuppressant-responsive enteropathy.

ACKNOWLEDGEMENTS

We would like to thank Pietro Ruggiero and Giuseppe De Cata for their contribution to this paper.

REFERENCES

1. Kather S, Grützner N, Kook PH, Dengler F, Heilmann RM. Review of cobalamin status and disorders of cobalamin metabolism in dogs. *J Vet Intern Med.* 2020;34(1):13-28.
[PUBMED](#) | [CROSSREF](#)
2. Golyński M, Lutnicki K, Krumrych W, Szczepanik M, Golyńska M, Wilkołek P, Adamek Ł, Sitkowski Ł, Kurek Ł. Relationship between total homocysteine, folic acid, and thyroid hormones in hypothyroid dogs. *J Vet Intern Med.* 2017;31(5):1403-1405.
[PUBMED](#) | [CROSSREF](#)
3. Zhu S, Li J, Bing Y, Yan W, Zhu Y, Xia B, Chen M. Diet-induced hyperhomocysteinaemia increases intestinal inflammation in an animal model of colitis. *J Crohns Colitis.* 2015;9(9):708-719.
[PUBMED](#) | [CROSSREF](#)
4. Harrington DJ. Laboratory assessment of vitamin B12 status. *J Clin Pathol.* 2017;70(2):168-173.
[PUBMED](#) | [CROSSREF](#)
5. Berghoff N, Suchodolski JS, Steiner JM. Serum homocysteine concentrations in dogs with chronic enteropathies. *J Vet Intern Med.* 2013;27:699-700.
6. Lippi G, Plebani M. Hyperhomocysteinemia in health and disease: where we are now, and where do we go from here? *Clin Chem Lab Med.* 2012;50(12):2075-2080.
[PUBMED](#) | [CROSSREF](#)
7. Joseph J, Loscalzo J. Methoxistasis: integrating the roles of homocysteine and folic acid in cardiovascular pathobiology. *Nutrients.* 2013;5(8):3235-3256.
[PUBMED](#) | [CROSSREF](#)
8. Saibeni S, Cattaneo M, Vecchi M, Zighetti ML, Lecchi A, Lombardi R, Meucci G, Spina L, de Franchis R. Low vitamin B(6) plasma levels, a risk factor for thrombosis, in inflammatory bowel disease: role of inflammation and correlation with acute phase reactants. *Am J Gastroenterol.* 2003;98(1):112-117.
[PUBMED](#) | [CROSSREF](#)
9. Oussalah A, Guéant JL, Peyrin-Biroulet L. Meta-analysis: hyperhomocysteinaemia in inflammatory bowel diseases. *Aliment Pharmacol Ther.* 2011;34(10):1173-1184.
[PUBMED](#) | [CROSSREF](#)
10. Rossi S, Rossi G, Giordano A, Paltrinieri S. Homocysteine measurement by an enzymatic method and potential role of homocysteine as a biomarker in dogs. *J Vet Diagn Invest.* 2008;20(5):644-649.
[PUBMED](#) | [CROSSREF](#)
11. Grützner N, Heilmann RM, Stupka KC, Rangachari VR, Weber K, Holzenburg A, Suchodolski JS, Steiner JM. Serum homocysteine and methylmalonic acid concentrations in Chinese Shar-Pei dogs with cobalamin deficiency. *Vet J.* 2013;197(2):420-426.
[PUBMED](#) | [CROSSREF](#)
12. Rossi G, Breda S, Giordano A, Pengo G, Dall'Ara P, Rossi G, Bo S, Paltrinieri S. Association between hypocobalaminaemia and hyperhomocysteinaemia in dogs. *Vet Rec.* 2013;172(14):365.
[PUBMED](#) | [CROSSREF](#)

13. Heilmann RM, Grützner N, Iazbik MC, Lopes R, Bridges CS, Suchodolski JS, Couto CG, Steiner JM. Hyperhomocysteinemia in Greyhounds and its association with hypofolateemia and other clinicopathologic variables. *J Vet Intern Med.* 2017;31(1):109-116.
[PUBMED](#) | [CROSSREF](#)
14. Çayır C, Kozat S. Investigation of Homocysteine levels in healthy dogs. *JVSAH.* 2016;4:305.
15. Dandrieux JR. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? *J Small Anim Pract.* 2016;57(11):589-599.
[PUBMED](#) | [CROSSREF](#)
16. Nakashima K, Hiyoshi S, Ohno K, Uchida K, Goto-Koshino Y, Maeda S, Mizutani N, Takeuchi A, Tsujimoto H. Prognostic factors in dogs with protein-losing enteropathy. *Vet J.* 2015;205(1):28-32.
[PUBMED](#) | [CROSSREF](#)
17. Allenspach K, Wieland B, Gröne A, Gaschen F. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. *J Vet Intern Med.* 2007;21(4):700-708.
[PUBMED](#) | [CROSSREF](#)
18. Toresson L, Steiner JM, Spodsberg E, Olmedal G, Suchodolski JS, Lidbury JA, Spillmann T. Effects of oral versus parenteral cobalamin supplementation on methylmalonic acid and homocysteine concentrations in dogs with chronic enteropathies and low cobalamin concentrations. *Vet J.* 2019;243:8-14.
[PUBMED](#) | [CROSSREF](#)
19. Jergens AE, Schreiner CA, Frank DE, Niyo Y, Ahrens FE, Eckersall PD, Benson TJ, Evans R. A scoring index for disease activity in canine inflammatory bowel disease. *J Vet Intern Med.* 2003;17(3):291-297.
[PUBMED](#) | [CROSSREF](#)
20. Simpson KW. Managing chronic enteropathies in dogs Part 1 and 2. *Pacific Veterinary Conference 2017*; June 29, 2017 - July 2, 2017; Long Beach, CA, USA. Sacramento: California Veterinary Medical Association; 2017, 10-17.
21. Washabau RJ, Day MJ, Willard MD, Hall EJ, Jergens AE, Mansell J, Minami T, Bilzer TW; WSAVA International Gastrointestinal Standardization Group. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. *J Vet Intern Med.* 2010;24(1):10-26.
[PUBMED](#) | [CROSSREF](#)
22. Allenspach KA, Mochel JP, Du Y, Priestnall SL, Moore F, Slayter M, Rodrigues A, Ackermann M, Krockenberger M, Mansell J. Correlating gastrointestinal histopathologic changes to clinical disease activity in dogs with idiopathic inflammatory bowel disease. *Vet Pathol.* 2019;56:435-443.
[PUBMED](#) | [CROSSREF](#)
23. Moser K, Mitze S, Teske E, von Bomhard W, Stockhaus C. Correlation of clinical, diagnostic and histopathological parameters in dogs with chronic lymphocytic-plasmacytic enteropathy. *Tierarztl Prax Ausg K Klientiere Heimtiere.* 2018;46(1):15-20.
[PUBMED](#) | [CROSSREF](#)
24. Day MJ, Bilzer T, Mansell J, Wilcock B, Hall EJ, Jergens A, Minami T, Willard M, Washabau R; World Small Animal Veterinary Association Gastrointestinal Standardization Group. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. *J Comp Pathol.* 2008;138 Suppl 1:S1-S43.
[PUBMED](#) | [CROSSREF](#)
25. Slovak JE, Wang C, Sun Y, Otoni C, Morrison J, Deitz K, LeVine D, Jergens AE. Development and validation of an endoscopic activity score for canine inflammatory bowel disease. *Vet J.* 2015;203(3):290-295.
[PUBMED](#) | [CROSSREF](#)
26. Grützner N, Suchodolski JS, Steiner JM. Relationship between cobalamin-dependent metabolites and both serum albumin and alpha1-proteinase inhibitor concentrations in hypcobalaminemic dogs of 7 different breeds. *Vet Clin Pathol.* 2014;43(4):561-566.
[PUBMED](#) | [CROSSREF](#)
27. Weissshof R, Chermesh I. Micronutrient deficiencies in inflammatory bowel disease. *Curr Opin Clin Nutr Metab Care.* 2015;18(6):576-581.
[PUBMED](#) | [CROSSREF](#)
28. Koutroubakis IE, Malliaraki N, Dimoulios PD, Karmiris K, Castanas E, Kouroumalis EA. Decreased total and corrected antioxidant capacity in patients with inflammatory bowel disease. *Dig Dis Sci.* 2004;49(9):1433-1437.
[PUBMED](#) | [CROSSREF](#)
29. Mansoor MA, Bergmark C, Svardal AM, Lønning PE, Ueland PM. Redox status and protein binding of plasma homocysteine and other aminothiols in patients with early-onset peripheral vascular disease. Homocysteine and peripheral vascular disease. *Arterioscler Thromb Vasc Biol.* 1995;15(2):232-240.
[PUBMED](#) | [CROSSREF](#)

30. Solem CA, Loftus EV Jr, Tremaine WJ, Harmsen WS, Zinsmeister AR, Sandborn WJ. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. *Inflamm Bowel Dis*. 2005;11(8):707-712.
[PUBMED](#) | [CROSSREF](#)
31. Otoni CC, Heilmann RM, García-Sancho M, Sainz A, Ackermann MR, Suchodolski JS, Steiner JM, Jergens AE. Serologic and fecal markers to predict response to induction therapy in dogs with idiopathic inflammatory bowel disease. *J Vet Intern Med*. 2018;32(3):999-1008.
[PUBMED](#) | [CROSSREF](#)
32. Finch JM, Joseph J. Homocysteine, cardiovascular inflammation, and myocardial remodeling. *Cardiovasc Hematol Disord Drug Targets*. 2010;10(4):241-245.
[PUBMED](#) | [CROSSREF](#)
33. Nilsson K, Gustafson L, Hultberg B. Elevated plasma homocysteine level in vascular dementia reflects the vascular disease process. *Dement Geriatr Cogn Dis Extra*. 2013;3(1):16-24.
[PUBMED](#) | [CROSSREF](#)
34. Danese S, Sgambato A, Papa A, Scaldaferrì F, Pola R, Sans M, Lovecchio M, Gasbarrini G, Cittadini A, Gasbarrini A. Homocysteine triggers mucosal microvascular activation in inflammatory bowel disease. *Am J Gastroenterol*. 2005;100(4):886-895.
[PUBMED](#) | [CROSSREF](#)
35. Twig G, Zandman-Goddard G, Szyper-Kravitz M, Shoenfeld Y. Systemic thromboembolism in inflammatory bowel disease: mechanisms and clinical applications. *Ann N Y Acad Sci*. 2005;1051(1):166-173.
[PUBMED](#) | [CROSSREF](#)
36. Goodwin LV, Goggs R, Chan DL, Allenspach K. Hypercoagulability in dogs with protein-losing enteropathy. *J Vet Intern Med*. 2011;25(2):273-277.
[PUBMED](#) | [CROSSREF](#)
37. Lee CM, Jeong DM, Kang MH, Kim SG, Han JI, Park HM. Correlation between serum homocysteine concentration and severity of mitral valve disease in dogs. *Am J Vet Res*. 2017;78(4):440-446.
[PUBMED](#) | [CROSSREF](#)