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Efficacy and tolerance of oral versus parenteral cyanocobalamin supplement in normalising cobalamin status in dogs with hypocobalaminaemia and chronic enteropathy – a randomised trial

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Thesis prepared for the degree of Masters in Veterinary Medicine

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ABSTRACT

Hypocobalaminaemia is a common finding in dogs diagnosed with chronic enteropathies (CE). Currently, veterinary textbooks recommend parenteral or oral supplementation, although is it hypothesised that parenteral supplementation might be more effective as it by-passes impaired intestinal absorption. In dogs, two studies have reported equal efficacy of oral and parenteral cobalamin administration of cobalamin. The objectives of this study were to prospectively evaluate whether oral cobalamin supplementation can restore normocobalaminemia in dogs with CIE and hypocobalaminaemia, as effectively as parenteral supplementation. In addition to determine whether oral supplementation is well-tolerated by owners and dogs and to determine whether oral supplementation is effective even in dogs with protein-losing enteropathy, severe hypocobalaminaemia or severe gastrointestinal signs at inclusion.

Thirty-seven client-owned dogs with various signs of CE and hypocobalaminaemia were recruited in three UK referral centres. Dogs were randomly allocated to continuous oral or weekly parenteral cobalamin supplementation for 13 weeks. Serum cobalamin concentration, CIBDAI score and body weight were assessed at inclusion, week 7 and week 13. Methylmalonic acid was evaluated at inclusion and at week 13. Quality-of-life, palatability and tolerance questionnaires were fulfilled by owners at the completion of the study.

In total, nineteen dogs completed the study. All dogs became normocobalaminemic with oral cobalamin supplementation. There was no statistical difference in cobalamin concentration at week 13 in dogs treated with oral versus parenteral cobalamin, regardless of presence of PLE, severity of initial hypocobalaminaemia and/or CIBDAI score at inclusion. Both treatments were well tolerated by dogs and owners. There was no statistical difference in treatment tolerance between dogs receiving oral versus parenteral cobalamin supplementation.

In conclusion, oral supplementation is as effective and well-tolerated as parenteral cobalamin. Oral cobalamin should be considered in hypocobalaminaemia dogs secondary to CIE, regardless of the severity of the clinical presentation and/or measurable variables.

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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

I. A literature review of cobalamin metabolism, hypocobalaminaemia and cobalamin supplementation in dogs

a. Cobalamin metabolism in dogs

i. Sources or cobalamin

Cobalamin is a water-soluble vitamin that is of both diagnostic and therapeutic importance, also referred to as vitamin B12 (Kather *et al.* 2020). Cobalamin is unique because of its protein bound transport mechanism in plasma and because it can be stored in considerable amounts in body tissue (McDowell *et al.* 2000).

Cobalamin intake is mainly supplied by food of animal origin. In particular, the meat and milk of herbivorous ruminant animals (e.g. cattle and sheep), raw livers of beef, pork, and chicken, fish and jellyfish are excellent sources of vitamin B12 (Watanabe and Bito 2018).

To a lesser extent, vitamin B12 can also be produced by intestinal microbiota (Berghoff and Steiner 2011). As mostly produced by anaerobes which are mainly present in the colon, bacterial cobalamin synthesis is uncommon in the upper gastrointestinal tract (David and Alpers 2015).

The National Research Council recommendation for cobalamin is 35 µg/kg dry matter for canine diets, regardless of the canine life stage (NRC 2006). Although the dietary cobalamin content varies between foods (dry diets: 0.05-0.25 mg/kg dry matter basis; canned diets: 0.03-0.59 mg/kg dry matter basis), cobalamin is abundant in most commercial pet foods (Davenport *et al.* 1994, Tran *et al.* 2006). Moreover, diets restricted- or free of animal products, such as commercial vegan or vegetarian diets, are supplemented with cobalamin and therefore provide adequate cobalamin supply to dogs and cats (Hand *et al.* 2010, Semp 2014). Hence, a dietary cobalamin deficiency is uncommon in dogs (Berghoff and Steiner 2011, Kather *et al.* 2020, Steiner 2016).

However, a recent article assessing the risk of nutritional deficiencies for dogs on a weight loss plan published by Gaylord *et al.* (2018), showed that cobalamin was found at risk of deficiency in both non-therapeutic adult maintenance diets and non-therapeutic weight management diets. In healthy dogs, cobalamin concentration increases with higher dietary intakes (Tran *et al.* 2006).

ii. Cobalamin digestion and absorption

Dietary cobalamin is bound to dietary protein. Following ingestion, gastric pepsin and hydrochloric acid degrade the dietary protein, releasing cobalamin in the stomach (Figure 1 - A). Free cobalamin is then bound to haptocorrin, also called “R-protein” or “transcobalamin I”) which is produced by the gastric mucosa. This binding is essential to protect cobalamin from bacterial utilization in the proximal gastrointestinal tract (Kather *et al.* 2020, Steiner 2016).

In the duodenum, pancreatic proteinases digest the R-protein, releasing cobalamin. Free cobalamin in the duodenum is bound by intrinsic factor (Figure 1 - B). Intrinsic factor (IF) is a secreted glycoprotein, produced by parietal cells in the gastric mucosa and exocrine pancreas (Alpers and Russell-Jones 2013). In dogs and cats, intrinsic factor (IF) is mostly produced by the exocrine pancreas. Cobalamin remains bound to intrinsic factor during passage through the cranial small intestine (Figure 1 - C) (Kather *et al.* 2020, Marcoullis and Rothenberg 1981, Steiner 2016).

In the enterocytes of the ileum, the cobalamin/intrinsic factor complexes bind to specific receptors (Figure 1 - D), and the enterocytes process the intrinsic factor/cobalamin complex by receptor-mediated endocytosis.

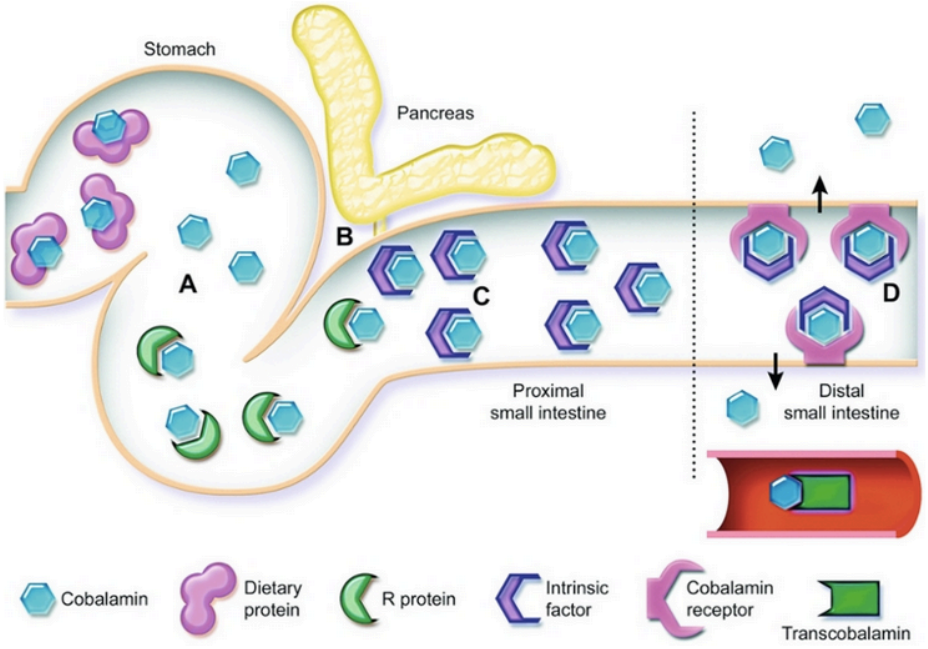

Figure 1: Intestinal cobalamin absorption in dogs and cats (Steiner 2016).

Ninety-nine percent of ingested cobalamin reaching the small intestine is absorbed at the ileal brush border by receptors called "cubam" (Figure 2). These receptor complexes are comprised of 2 subunits, the proteins amnionless (AMN) and cubilin (CUBN). In addition, approximately 1% of dietary cobalamin is absorbed via passive diffusion across the intestinal mucosal epithelium, most likely along the entire length of the gastrointestinal tract (Fyfe *et al.* 2004).

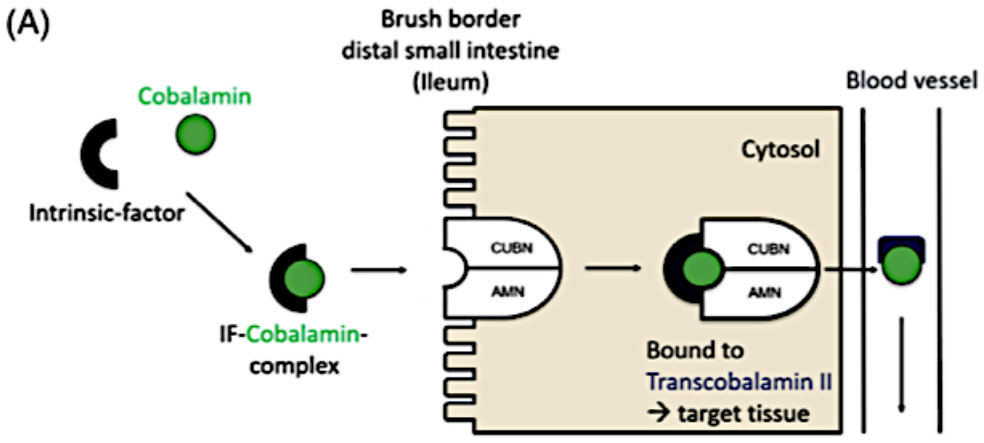

Figure 2: Schematic of the absorption of cobalamin by enterocytes in the distal small intestine (ileum) (Kather *et al.* 2020).

Cobalamin is separated from IF and the receptor within the lysosomes of the enterocytes by lysosomal proteolysis and pH effects, with subsequent binding to transcobalamin proteins (TCP) (Alpers and Russell-Jones 2013). Bound to transcobalamin proteins, cobalamin is then transported within the bloodstream to its target tissues (Kather *et al.* 2020, Steiner 2016). There are two types of transcobalamin proteins: transcobalamin I and transcobalamin II. Cobalamin bound to transcobalamin I is rendered unavailable for cellular uptake. However, if bound to transcobalamin II, cobalamin can be released to peripheral tissues. Fortunately, transcobalamin II appears to be much more abundant in dogs than in humans (Hall 1975).

Bound to transcobalamin I, cobalamin undergoes biliary excretion via enterohepatic circulation (EHC), improving the time for maintaining body stores, but also increasing gastrointestinal loss with concurrent intestinal malabsorption (David and Alpers 2015).

iii. Biochemical roles and intracellular function

Cobalamin is an essential cofactor for the intracellular enzymes, methionine synthase and methylmalonyl-CoA mutase (Allen *et al.* 1993, Kather *et al.* 2020).

- Methionine synthase

In the cytosol of most cells, methionine synthase and cobalamin catalyse the regeneration of methionine from homocysteine, and the transformation of folate into its biologically active form (tetrahydrofolate). Therefore, intracellular cobalamin deficiency is expected to cause a decrease in methionine synthase activity which would result in accumulation of homocysteine and functional hypofolataemia due to decreased synthesis of tetrahydrofolate (Figure 3).

- Methylmalonyl-CoA mutase

In the mitochondrion of most cells, cobalamin also acts as a co-factor for another enzyme, called “methylmalonyl-CoA mutase”. Methylmalonic CoA mutase catalyses the reaction from methylmalonyl CoA to succinyl CoA which is a key molecule in the tricarboxylic acid cycle (Krebs cycle) (Figure 3). Therefore, intracellular cobalamin deficiency leads to a reduced enzyme

activity and an accumulation of methylmalonic acid (MMA). MMA can also inhibit the activity of carbamoyl phosphate synthetase I. Carbamoyl phosphate synthetase I is an enzyme of the urea cycle which normally metabolizes ammonia to carbamoyl phosphate. When this metabolic process is impaired, plasma ammonia concentration increases.

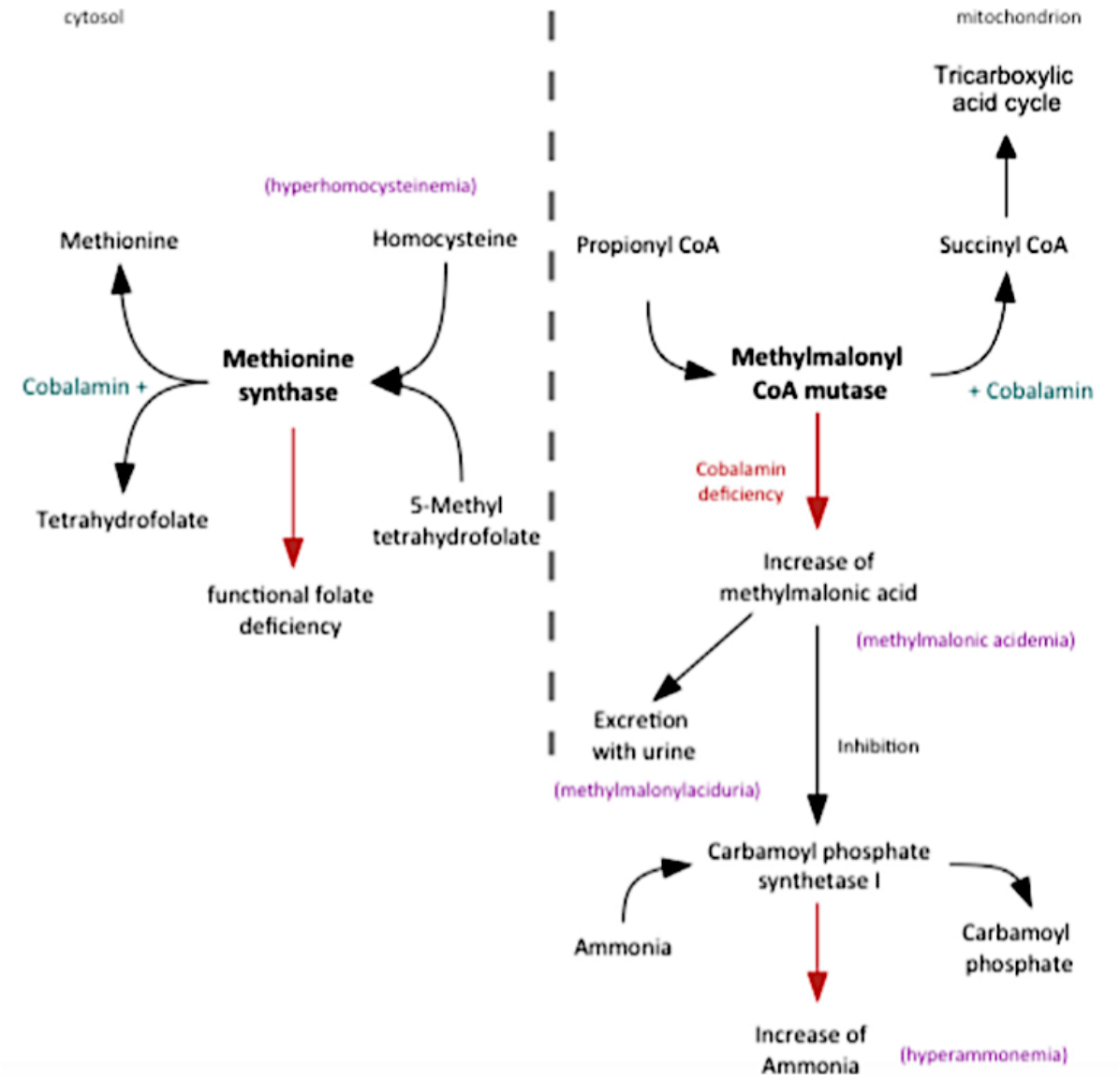

Figure 3: Intracellular pathways of cobalamin metabolism (Kather *et al.* 2020).

In summary, decreased intracellular cobalamin concentration is expected to result in hyperhomocysteinaemia, functional folate deficiency, methylmalonic acidaemia and hyperammonaemia.

iv. Cobalamin excretion and consumption by the intestinal microbiota

Cobalamin is mainly excreted in faeces following a lack of intestinal reabsorption and enterohepatic cycle. Due to a unique reabsorption process within the renal proximal tubules, urinary excretion is only minimal.

Although free cobalamin is excreted in the urine, cubam and megalin receptors are involved in phagocytic reabsorption of transcobalamin II-cobalamin complex in renal tubules (mainly the proximal tubules). The cubam receptor is also involved in the renal reabsorption of several proteins (e.g., albumin, transferrin, and vitamin D-binding protein) (Birn 2006).
In a prospective study published by Galler *et al.* (2012), serum and plasma cobalamin levels were not significantly different between healthy controls and nineteen dogs with chronic kidney disease (CKD).

Gut microbiota can also influence the available amount of cobalamin due to bacterial utilization of cobalamin (Dossin 2011, LeBlanc *et al.* 2011, Ruaux 2013, Steiner 2014). Changes in the small intestinal microbiota, including small intestinal bacterial overgrowth (SIBO) or intestinal dysbiosis, lead to increased bacterial utilization of cobalamin, resulting in decreased absorption of cobalamin by the ileum (Suchodolski 2016). Short-term probiotic administration of *Enterococcus faecium* SF68 has been shown to cause a significant reduction of mean cobalamin concentration and moderate hypocobalaminaemia in 45% of healthy dogs (Lucena *et al.* 2018). Nevertheless, measurement of serum cobalamin concentrations is only moderately sensitive and specific for canine SIBO (German *et al.* 2003, Rutgers *et al.* 1995).
In a study conducted by Rutgers et *al.* (1995), including 41 dogs with SIBO, high serum folate concentrations and low serum cobalamin concentrations had good specificity (79% and 87%, respectively) but low sensitivity (51% and 24%, respectively). The combination of high folate and low cobalamin had a sensitivity of 5% and specificity of 100% for SIBO. SIBO was diagnosed by duodenal juice culture and total bacterial counts above 10^5 cfu/mL or anaerobic bacterial counts above 10^4 cfu/mL.

b. Laboratory tests

i. Serum cobalamin concentration

1. Cobalamin concentration assessment

In veterinary medicine, several immunoassays for the measurement of serum cobalamin concentrations are commercially available. These laboratory tests include an automated chemiluminescence assay and the automated immunoassay provided by the analyser AIA-900 (Tosoh Bioscience, Tokyo, Japan) (McLeish *et al.* 2019). In North America and Europe, the chemiluminescence assay is routinely used and the reference intervals are similar between the different veterinary diagnostic laboratories (Kather *et al.* 2020). McLeish *et al.* (2019) have recently shown that the analyser AIA-900 yields different results than the chemiluminescent assay. This conclusion means that the results obtained by the two analysers cannot be used interchangeably and should be interpreted using instrument-specific reference intervals. However, both immunoassays demonstrated good analytical performance.

Serum cobalamin measurements are not affected by haemolysis, lipemia, or hyperbilirubinemia (Suchodolski and Steiner 2003).
Because cobalamin is abundant in commercial diets, some authors (Dossin 2011) recommend measuring serum cobalamin concentration in fasted animals. However, this precaution seems controversial as not advised in another publication (Suchodolski and Steiner 2003).

Storage has only very little effect on cobalamin concentration. Serum cobalamin concentration has shown to remain stable for 5 days when serum samples of dogs and cats are refrigerated at 6°C. The effect of light and room temperature is significant but quite small for samples stored with these exposures for a 5-day period (Kempf *et al.* 2018).

2. Indications for serum cobalamin assessment

Indications for serum cobalamin concentration assessment mainly aims at diagnosing low serum concentrations (hypocobalaminaemia), given the fact that this finding may lead to

cobalamin supplementation for the patient (oral or parenteral as discussed later in the presentation). Although high serum cobalamin concentration (hypercobalaminaemia) does not require further therapeutic actions, an association with hepatic and neoplastic diseases has been demonstrated in cats (Trehy et al 2014).

In human patients, hypocobalaminaemia has been reported with gastrointestinal diseases, reduced dietary intake, genetic disorders, pregnancy and lactation and medication side effect (Table 1) (Devalia *et al.* 2014). Therefore, if any of these diseases is suspected based on signalment, clinical signs, personal and family history, and medication history, serum cobalamin concentration should be assessed.

Population sector	Type of disease/mechanism causing hypocobalaminaemia	Disease causing of hypocobalaminaemia
All ages	Infections	H.pylori infection, Giardia lamblia infection, Fish tapeworm infection (*Diphyllobothrium latum*)
	Malabsorption	Pernicious anaemia
		Gastric resection for obesity or cancer Coeliac disease, Crohn disease Tropical sprue
	Inadequate dietary intake	Low intake of cobalamin-rich foods
Infants and children	Genetic disorders	Transcobalamin deficiency Imerslund Gräsbeck syndrome Other cobalamin mutations
	Inadequate dietary intake	Maternal strict vegetarian (vegan) diet in pregnancy, adherence to a vegan diet post-weaning
Women of child-bearing age	Pregnancy and lactation	Adherence to a low cobalamin diet during pregnancy may lead to metabolic signs of deficiency by the third trimester
Older persons	Malabsorption	Achlorhydria due to atrophic gastritis and proton pump inhibitors (malabsorption of food-bound cobalamin). Symptoms may develop slowly.

Table 1: Summary of the causes of cobalamin deficiency in human patients (Devalia *et al.* 2014).

In veterinary patients, hypocobalaminemia has been reported in many diseases of the gastrointestinal tract, liver and pancreas (Ruaux 2013). In particular, exocrine pancreas insufficiency (EPI), small intestinal bacterial overgrowth (SIBO), small intestinal bacterial dysbiosis, distal small intestinal disease, protein losing enteropathy, lymphangiectasia, congenital cobalamin malabsorption and ileal resection are well-documented causes of cobalamin deficiency in dogs (Dossin 2011). Other factors such as proton-pump inhibitor use, or administration of oral antibiotics, may result in serum hypocobalaminaemia or suboptimal cobalamin concentration (Berghoff *et al.* 2012, Manchester et *al.* 2019).

Albeit hypocobalaminemia is a highly specific indicator of gastrointestinal, pancreatic or hepatic dysfunction in an adult dog, it is not a specific diagnostic test for any of these diseases (Ruaux 2013).

ii. Whole-body cobalamin status assessment

1. General principles

According to the human literature, the interpretation of test results assessing cobalamin status remains challenging because there is no « gold standard » test to define deficiency (Devalia *et al.* 2014).

As cobalamin metabolic functions mainly occur in the cytosol of the cells, serum cobalamin concentration does not necessarily reflect the whole-body cobalamin status. Although serum cobalamin assessment is a useful tool to screen hypocobalaminaemic dogs, functional cobalamin deficiency can be identified by measuring methylmalonic acid, homocysteine and holotranscobalamin concentrations. These markers reflect more closely the intracellular availability of cobalamin.

Assessment of homocysteine, methylmalonic acid and holotranscobalamin concentrations is part of the gold standard approach for standard cobalamin status assessment in human medicine (Hvas and Nexo 2005).

In dogs, a holotranscobalamin assay is not available. Although not widely available, serum homocysteine and methylmalonic acid concentration measurements are recommended as part of the cobalamin status assessment (Kather *et al.* 2020).

Increased serum homocysteine and methylmalonic acid concentrations are expected in a patient with decreased intracellular cobalamin availability (Kather *et al.* 2020), even in patients with normal or low normal serum cobalamin concentration.

2. Serum methylmalonic acid concentration

Methylmalonyl-CoA mutase is an enzyme responsible for the synthesis of succinylCoA from propionylCoA. This enzymatic transformation required cobalamin as an important co-factor. In the case of cobalamin deficiency, this enzymatic reaction cannot occur, and methylmalonic acid (MMA) accumulates instead. MMA concentrations can be measured in serum (Berghoff *et al*. 2012, Ruaux *et al*. 2013) or urine (Lutz *et al.* 2012, Ruaux *et al*. 2013).

MMA is considered to be an accurate tool to assess cobalamin availability intracellularly. High MMA concentration is therefore an indirect marker of cobalamin deficiency at the cellular level. However, other potential causes of increased MMA concentration should be considered and include renal disease, plasma volume contraction and primary abnormalities in hepatic methyl-malonyl CoA mutase activity (Carmel *et al.* 2003, Ruaux *et al*. 2013).

- <u>Relationship between cobalamin and MMA concentration</u>

Quite logically, MMA concentration tends to be higher in hypocobalaminaemic dogs or dogs with a serum cobalamin concentration within the lower range of the reference interval.

⇨ A study undertaken by Berghoff *et al.* (2012) revealed that dogs with hypocobalaminaemia had significantly higher MMA concentrations. The authors also showed a trend for increasing serum MMA concentrations with decreasing serum cobalamin concentrations. High MMA concentration was identified in 63% of dogs with undetectable serum cobalamin concentrations. This finding was also demonstrated in another study where there was a significant negative correlation between serum

cobalamin and MMA concentrations in dogs with gastrointestinal disease (Berghoff *et al.* 2013).

- ⇨ In dogs with serum cobalamin concentration within the lower end of the reference interval, increased serum MMA concentration was present in 22% of cases (Toresson *et al.* 2019).
- ⇨ In humans and dogs with suboptimal cobalamin status, the response to cobalamin supplementation is a marked reduction in serum MMA concentration (Bolan *et al.* 2000, Toresson *et al.* 2019).

The opposite scenario has also been demonstrated. Sixty-nine percent of dogs with hypocobalaminaemia have normal MMA concentration (Berghoff *et al.* 2012). Kather *et al.* (2020) hypothesised that hypocobalaminemia in these dogs did not reflect a true whole-body cobalamin deficiency.

3. Serum homocysteine concentration

Homocysteine is an endogenous amino acid containing sulphur. This amino acid is synthetized from the systemic conversion of methionine, an exogenous amino acid present in animal proteins. Conversely, homocysteine can be transformed into methionine via the methionine-synthetase enzyme for which cobalamin, folic acid, and pyridoxine are essential cofactors (Heiman *et al.* 2018, Kather *et al.* 2020, Rossi *et al*. 2008).

Lutz *et al.* (2012) have demonstrated that serum homocysteine concentration increases early in the course of cobalamin deficiency in dogs. However, increased serum or plasma homocysteine concentration is less specific for intracellular lack of cobalamin than is increased serum methylmalonic acid concentration (Hermann and Obeid 2012).

In dogs, increased homocysteine concentration has been identified in patients with renal insufficiency (Amin *et al.* 2016), mitral valve disease (Lee and Hyun 2012) or hypothyroidism (Gołynski *et al.* 2017). Additionally, Rossi *et al.* (2008) suggested the use of homocysteine as a biomarker of heart and kidney diseases. In people, hyperhomocysteinaemia has also been

associated with thrombotic, neurogenerative and cardiovascular diseases (Grützner *et al.* 2013).

As circulating homocysteine is bound to albumin in the bloodstream, any disease resulting in hypoalbuminaemia could contribute to an underestimation of serum homocysteine concentration (Grützner *et al.* 2014). Breed may also play a role in homocysteine concentration variations as reported in Greyhounds (Heilmann *et al.* 2017).

4. Serum holotranscobalamin concentration

In plasma, cobalamin is bound to haptocorrin (also called « transcobalamin I ») and transcobalamin (also called « transcobalamin II »). The complex formed by cobalamin attached to transcobalamin is called holotranscobalamin. It represents the biologically active fraction that can be delivered into all cells (Hvas and Nexo 2005).
In human medicine, holotranscobalamin concentration is suggested as a suitable assay for assessment of cobalamin status in a routine diagnostic laboratory (Devalia *et al.* 2014). Unfortunately, such assay is currently not available in veterinary medicine.

In conclusion, serum cobalamin currently remains the first-line test to assess cobalamin status. Additionally, second-line plasma methylmalonic acid is sometimes measured to help clarify uncertainties of underlying biochemical/functional deficiencies.

c. Deficiency states

i. General principles

Because cobalamin is abundant in most commercial pet foods, a dietary deficiency remains overall uncommon (Berghoff and Steiner 2011, Kather *et al.* 2020, Steiner 2016). Hypocobalaminaemia results more likely from a disturbance within the absorptive mechanism of cobalamin (Berghoff and Steiner 2011) and/or intestinal bacterial dysbiosis.

ii. Clinicopathological findings associated with hypocobalaminaemia

1. Haematological and biochemical consequences of hypocobalaminaemia

- Haematological abnormalities

Tissues or cells with a high turnover rate such as enterocytes or blood cells are primarily affected by hypocobalaminaemia. In people, nonregenerative anaemia with megaloblastosis (desynchrony between cytoplasmic and nuclear maturation in erythroblasts and myeloblasts resulting in the presence of circulating megaloblasts in the blood film and megaloblastic changes in the bone marrow), neutropenia, and hypersegmented neutrophils are the typical features of clinical cobalamin deficiency (Devalia *et al.* 2014, Kather *et al.* 2020).

Stanley *et al.* 2019 have recently demonstrated that the association between cobalamin deficiency and macrocytic, nonregenerative anaemia established in humans was not routinely present in dogs. Although the prevalence of hypocobalaminaemia is high in anaemic dogs (64% in dogs with non-regenerative anaemia and 57% in dogs with regenerative anaemia), the prevalence of anaemia was not significantly different between normocobalaminaemic and hypocobalaminaemic dogs. Additionally, and in contrast to humans, the anaemia of cobalamin deficiency in dogs is rarely macrocytic (Fyfe *et al.* 1991, Stanley *et al.* 2019).

However, neutropenia is commonly reported in dogs with hereditary cobalamin malabsorption (Fyfe *et al.* 1991, Kook *et al*. 2014, Gold *et al.* 2015, Fyfe *et al.* 2018). In these dogs, megaloblastosis has also been reported in the erythrocytic and myelocytic series in bone marrow in these dogs (Fyfe *et al.* 1991).

- Biochemical abnormalities

Aside from hyperhomocysteinaemia and high methylmalonic acid concentration, dogs affected by hypocobalaminaemia secondary to hereditary cobalamin malabsorption can also display persistent hypoglycaemia and hyperammonaemia, as reported in several reports (Fyfe *et al.*

1991, Lutz *et al.* 2003, Battersby *et al.* 2005). These biochemical abnormalities resolved with cobalamin therapy.

An increase in liver enzymes due to hepatic degeneration was reported in four Beagles with hereditary cobalamin malabsorption (Kook *et al*. 2014, Murtagh *et al.* 2015). In these dogs, histopathologic examination of liver biopsy samples revealed multifocal areas of single-cell necrosis and mild periportal lymphoplasmacytic infiltration. The hypothetic cause of hepatic damage remains hyperhomocysteinaemia, as suggested from experimental studies in rodents (Ji *et al.* 2004, Robert *et al.* 2005, Matté *et al.* 2009).

- Proteinuria

Proteinuria has been commonly reported in dogs with hereditary cobalamin malabsorption (Fyfe *et al.* 2014, Fyfe *et al.* 2018).

The cubam receptor is expressed by the enterocytes of the ileal mucosa, but also by renal proximal tubular epithelial cells. This receptor allows renal epithelial cells to reabsorb a variety of protein ligands, such as albumin, apo AI (involved in lipid metabolism), haptoglobin, and vitamin D-binding protein from the glomerular filtrate. Hence, cubam dysfunction reported in dogs with hereditary cobalamin malabsorption not only causes intestinal cobalamin malabsorption but also a mild proteinuria of mid- to low-molecular weight proteins that are cubam ligands. Although this ligand-specific proteinuria is clinically benign, it is a useful diagnostic marker of cubam dysfunction (Lutz *et al.* 2013, Fyfe *et al.* 2018, Kook *et al.* 2018).

2. Clinical signs of hypocobalaminaemia

Clinical signs of hypocobalaminaemia vary between species. It also depends on the underlying condition responsible for cobalamin deficiency.

a. Gastrointestinal signs

Typically, dogs with primary cobalamin malabsorption, also called « Imerslund–Gräsbeck syndrome » (IGS), exhibit clinical signs at an early age, usually during the first year of life. Failure to thrive, inappetence, poor body condition, lethargy, vomiting and diarrhoea are the most common signs (Fordyce *et al.* 2000, Gold *et al.* 2015, Murtagh *et al.* 2015).
The cause of vomiting and diarrhoea seen in dogs with Imerslund–Gräsbeck syndrome is incompletely understood. One of the suggested underlying mechanism could be that impaired DNA synthesis associated with hypocobalaminaemia affects rapidly diving cells, including intestinal epithelial cells, and thus contribute to the development of gastrointestinal signs. Another possible explanation could be that there might be concurrent pathological change within the gastrointestinal tract.

Other gastrointestinal signs such as intermittent impaired swallowing (Lutz *et al*. 2013) or mucosal ulcerations (Murtagh *et al. 2015*) have been reported. Interestingly, oral mucosal ulceration resolved after cobalamin supplementation (Murtagh *et al.* 2015). In people with IGS, glossitis, glossodynia, and stomatodynia are described.

b. Neurological signs

Neurological disorders can be a complication of cobalamin deficiency states in dogs. MMA has a direct neurotoxic effect. As discussed above, MMA also inhibits carbamoyl phosphate synthase-1, an enzyme involved in the hepatic urea cycle, which in turn leads to hyperammonaemia. Together, MMA and ammonia can cause clinical signs of encephalopathy including lethargy and seizures (McLauchlan *et al.* 2015). To reinforce the presumptive role of methylmalonic acidaemia in the onset of signs of encephalopathy, a case report of epileptic seizures as the unique neurological manifestation in a dog with methylmalonic aciduria and hyperammonaemia without hypocobalaminaemia has been reported (Douralidou *et al.* 2020).

Metabolic changes caused by hypocobalaminaemia also include hypoglycaemia which might contribute to signs of encephalopathy.

In people, cobalamin deficiency has been associated with a variety of neurological manifestations, including peripheral demyelination and dementia (Reynolds, 2006). In hypocobalaminaemic cats, one case of encephalopathy (Simpson *et al.* 2012) and degenerative myelopathy (Salvadori *et al.*, 2003) have been described. Even magnetic resonance imaging abnormalities of the brain associated with hypocobalaminaemia have been documented in a cat (Simpson *et al.,* 2012) but not in dogs.

c. Concurrent infections

Hepatic and systemic fungal infections have been reported in two young dogs with congenital cobalamin deficiency (Kook *et al.* 2015, Erles *et al.* 2017). Neutrophil function defects are suspected to be responsible for the concurrent infections, as it has been identified in children with Imerslund–Gräsbeck syndrome (Broides *et al.* 2006). In combination with neutropenia, these factors may be playing a significant role in the onset of opportunistic infections.

d. Other signs associated with hypocobalaminaemia

- Pain and pyrexia

In puppies with Imerslund–Gräsbeck syndrome (IGS), pyrexia, joint pain, cervical pain and intra-abdominal neutrophilic lymphadenitis are reported in some cases. Interestingly, cervical pain resolved after cobalamin supplementation (Murtagh *et al.* 2015). The underlying pathophysiology for these symptoms remains unknown.

- Cardiovascular system

Bradyarrhythmia is a possible clinical sign in dogs (Lutz *et al.* 2013) with IGS. One could hypothesise that parasympathetic stimulation caused by intestinal inflammation might be the reason. In people, plasma MMA but not vitamin B_{12} is significantly associated with heart rate variability indices (Sucharita *et al.* 2013).

- Dermatological abnormalities

Reversible cutaneous hyperpigmentation occurs in children with IGS (Shivbalan and Srinath, 2013).

i. Causes of hypocobalaminaemia

1. Breed predisposition for hypocobalaminaemia

Breed predisposition for hypocobalaminaemia slightly differs between continents.

- ⇨ In the United-Kingdom, breeds of dogs with an increased frequency of hypocobalaminemia were the Chinese Shar-Pei, Staffordshire Bull Terrier, German Shepherd Dog, and mixed breed dogs (Dandrieux *et al.* 2013). Golden Retrievers had a decreased odds ratio for being diagnosed with hypocobalaminemia.
- ⇨ In North America, there is an increased frequency of hypocobalaminemia in Greyhounds, Chinese Shar-Peis and German Shepherd Dogs (Grützner *et al.* 2012, Heilmann *et al.* 2017).

It remains unclear whether hypocobalaminaemia in Greyhound is related to a breed-specific lower reference interval or whether Greyhounds are truly predisposed to hypocobalaminaemia. The high percentage of Greyhounds with hyperhomocysteinemia suggests that cobalamin deficiency is common in this breed (Heilmann *et al.* 2017).

2. Chronic inflammatory enteropathies

Hypocobalaminaemia is frequently reported in dogs with chronic inflammatory enteropathies (CIE). The prevalence of hypocobalaminaemia in these patients ranges from 19% to 38% (Heilmann, Parnell *et al.* 2016, Heilmann, Volkmann *et al.* 2016, Volkmann *et al.* 2017, Heilmann *et al.* 2018). However, the underlying mechanism has never been proven. Most authors assume that chronic mucosal ileal disease reduces the epithelial expression and/or function of the cubam receptor leading to a reduced mucosal uptake of cobalamin.

Prevalence of hypocobalaminemia is higher in dogs with non-neoplastic, noninfectious causes of protein-losing enteropathies (PLE), ranging from 43 to 75% (Allenspach *et al.* 2007, Equilino *et al.* 2015, Heilmann *et al.* 2016).

As cobalamin is essential for many cell functions and mucosal regeneration, cobalamin deficiency can contribute to mucosal inflammatory infiltration and villous atrophy (Berghoff and Steiner 2011).

An association between ileal mucosal inflammatory changes and hypocobalaminemia in dogs with CIE has been shown. Hypocobalaminemia correlates with an increased number of intra-epithelial lymphocytes in the ileal mucosa (Procoli *et al.* 2013).

3. Small intestinal dysbiosis

Small intestinal dysbiosis is characterised by an altered richness or composition or both of the intestinal microbiota (Suchodolski 2016). Cobalamin deficiency can result from competition between intestinal microbiota and intestinal cells for cobalamin absorption.
Most bacteria bind to free cobalamin. Bacteroides spp. has the ability to bind and utilise the cobalamin-IF complexes (Abrams 1977). As the diagnosis of SI dysbiosis remains challenging, the true prevalence of hypocobalaminaemia in dogs with SI dysbiosis remains unknown.

4. Hereditary selective cobalamin malabsorption

The cubam receptor expressed on the ileal brush border is required for the uptake of cobalamin from the small intestine. The cubam receptor is composed of two protein subunits, amnionless and cubilin, which are encoded by the AMN and CUBN genes respectively. Mutations in either the AMN or the CUBN gene, causing a loss of function, lead to hereditary selective cobalamin malabsorption also called Imerslund–Gräsbeck syndrome (IGS) (Drögemüller *et al.* 2014).

Border Collies, Beagles, Giant Schnauzers, Australian Shepherds and Komondors, are predisposed breeds to selective cobalamin malabsorption (Drögemüller *et al.* 2014, Fyfe *et al.*

2013, Fyfe *et al.* 2014, Fyfe *et al.* 2018, Kather et al. 2020, Kook *et al.* 2014, Murtagh *et al*. 2015).

The cubam receptor expression is affected by two independent mutations in the AMN gene in Australian Shepherd dogs and Giant Schnauzers. In Beagles, Border Collies, and Komondors, three independent mutations in the CUBN gene are involved in cobalamin deficiency.

5. Exocrine pancreatic insufficiency

Exocrine pancreatic insufficiency (EPI) results from inadequate production of digestive enzymes from pancreatic acinar cells. In dogs, lack of digestive enzymes secretion results from immune-mediated destruction of the exocrine pancreatic acinae and leads to the typical clinical signs of weight loss, polyphagia and increased faecal volume (Westermarck and Wiberg 2012).

There is a clear breed predisposition as German Shepherd Dogs, Rough Coated Collies, Chow-Chows, Cavalier King Charles Spaniels, and West Highland White Terriers, with German Shepherd Dogs represent about 60% of all cases of EPI (Batchelor *et al.* 2007).

Hypocobalaminaemia is a common finding in dogs diagnosed with exocrine pancreatic insufficiency, documented in 82% of cases. Thirty-six percent of them display severe hypocobalaminaemia (<100ng/L) (Batchelor *et al.* 2007). Hypothetical mechanisms causing failure to absorb cobalamin in dogs with EPI are as follows:

- Pancreatic secretion of intrinsic factor (IF) is reduced or absent,
- Impaired release of cobalamin from haptocorrin (due to a lack of digestive enzymes) and thus no binding of cobalamin to IF,
- Secondary small intestine dysbiosis compromising the endogenous production of cobalamin,
- Presence of toxic metabolites due to small intestine dysbiosis compromising intestinal absorption (Kather *et al.* 2020).

6. Neoplasia

a. Intestinal neoplasia

Hypocobalaminaemia has been documented in 40 to 71% of dogs with low-grade (lymphocytic) gastrointestinal lymphoma (Couto *et al.* 2018, Lane *et al.* 2018). It is presumed that the hypocobalaminaemia in dogs with lymphoma is a consequence of the ileal infiltration with neoplastic lymphocytes hypothetically resulting in a disruption of the receptor-mediated GI uptake of cobalamin.

b. Extra-intestinal neoplasia

Hypocobalaminaemia has been documented in 15.5% of dogs with multicentric lymphoma (Cook *et al.* 2009). According to Cook *et al.*, neoplastic infiltration of the ileum by tumoral lymphocytes seems to be the most likely hypothesis. However, the authors also speculated that cobalamin was consumed by the tumoral cells as a vital water-soluble vitamin needed for cell growth. Hence, another reasonable assumption to explain the poorer prognosis in dogs with multicentric lymphoma and hypocobalaminaemia might be that the hypocobalaminaemia develops as the tumoral burden increases due to peripheral cobalamin consumption by the tumoral cells.

ii. Prognostic implications of hypocobalaminaemia

Hypocobalaminaemia is a negative prognostic factor in dogs with chronic inflammatory enteropathies (Allenspach *et al.* 2007, Grützner *et al.* 2014), exocrine pancreatic insufficiency, as documented in two studies (Batchelor *et al.* 2017, Soetart *et al.* 2019), and lymphoma (Cook *et al.* 2009).

Hypocobalaminemia (<100 ng/L) has also been associated with shorter survival in dogs with exocrine pancreatic insufficiency (Batchelor et al., 2007).

Hypocobalaminaemia in dogs with CIE was shown to be associated with treatment refractoriness (Procoli *et al.* 2013). Dogs with chronic enteropathy and severe hypocobalaminaemia (serum cobalamin <200 ng/L) showed an odds ratio of 9.5 (95% CI 2.5–39.4) for a negative outcome (Allenspach *et al.*, 2007). In dogs with chronic enteropathy from several breeds including SharPeis (Grützner *et al.*, 2014) and Yorkshire Terriers (Grützner *et al.*, 2013), serum concentrations of albumin and alpha-1 proteinase inhibitor were significantly correlated with cobalamin concentration (Grützner *et al.* 2014).

d. Treatment of hypocobalaminaemia

iii. When is cobalamin supplementation required?

In veterinary medicine, appreciation of the prevalence and potential clinical impact of cobalamin deficiency in companion animals is growing. Cyanocobalamin has traditionally been used for supplementation, as it is widely available and inexpensive (Kather *et al.* 2020).

Most reference laboratories quote the lower limit of the normal canine reference interval for serum cobalamin concentration at 250 ng/L. However, Berghoff et *al.* (2012) showed evidence of cobalamin deficiency at a cellular level in 35 percent of dogs with serum cobalamin concentrations in the low normal range of the reference interval (251–450 ng/L). Hence, most clinicians tend to use 400ng/L as the lower limit for cobalamin supplementation. Kather *et al.* (2020), *Steiner* (2016) and *Toresson et al.* (2016) recommend that cobalamin should be supplemented whenever serum cobalamin concentration is subnormal, which means less than approximately 400ng/L, in dogs with chronic inflammatory enteropathies and/or EPI. Cyanocobalamin has traditionally been used for supplementation, as it is widely available and inexpensive.

Furthermore, cobalamin supplementation is vital in dogs diagnosed with selective cobalamin malabsorption. If left untreated, dogs with IGS will die as a result of metabolic derangements and immunodeficiency (Erles *et al*. 2018, Fyfe *et al.* 2014, Kook *et al.* 2014, Murtagh *et al*. 2015).

iv. Oral versus parenteral cobalamin supplementation

In human beings with cobalamin deficiency, all studies comparing oral and parenteral cobalamin supplementation have shown equal efficacy of oral compared to parenteral supplementation (Kuzminski *et al.,* 1998; Bolaman *et al.,* 2003; Castelli *et al.,* 2011; Kim *et al.,* 2011).

In dogs with chronic enteropathies, similar recommendations have been drawn. In 2016, Toresson *et al.* showed that oral cobalamin supplementation was effective in addressing hypocobalaminaemia in dogs with chronic enteropathies. Two years later, the same team showed equal efficacy of oral compared to parenteral supplementation to address hypocobalaminaemia in a similar canine population (Toresson *et al.* 2018).

Although oral and parenteral cobalamin supplementation seem to be both effective at maintaining an adequate cobalamin status in dogs with IGS, there is currently no comparative studies assessing the efficacy of oral versus parenteral cobalamin supplementation in dogs diagnosed with IGS or EPI.

v. Therapeutic recommendations: Doses and monitoring schedules

1. Oral and parenteral supplementation protocols

In dogs with CIE and suboptimal cobalamin status, cobalamin is recommended to be given at a dose of 50 µg/kg subcutaneously every 7 days or 50 µg/kg orally q24h (Toresson *et al.* 2018, Toresson *et al.* 2019).

The parenteral supplementation protocol involves weekly subcutaneous injections of 50 µg/kg SC cyanocobalamin over a period of 6 weeks, then an additional dose one month later, followed by measurement of serum cobalamin concentration one month after the last dose (Toresson *et al.* 2019).

The parenteral supplementation protocol involves daily cobalamin administration at 50 µg/kg over a period of 12 weeks, followed by a recheck of serum cobalamin concentration after

discontinuation of supplementation, typically one week after the last cobalamin tablet (Toresson *et al.* 2019).

In 2016, Kook *et al.* showed that monthly to bi-monthly intramuscular supplementation of 1mg of hydroxycobalamin was effective to maintain normal health status and MMA concentrations in Beagles with IGS. In 2019, Kook and Hersberger demonstrated that daily oral administration of 1 mg of cyanocobalamin represented an efficacious regimen for maintaining normal health status as well as unremarkable cellular cobalamin status, based on urine and serum MMA concentrations, in Beagles with IGS.

In some dogs, serum cobalamin concentrations are not adequately restored with cyanocobalamin supplementation. In these cases, hydroxocobalamin might be more effective (Kather et *al.* 2020). Hydroxycobalamin is likely more effective than cyanocobalamin because this compound presents the natural form of cobalamin (Farquharson and Adam, 1976).

2. Monitoring

Monitoring serum MMA concentrations alone or in combination with serum cobalamin concentrations might be a more appropriate way to reevaluate the canine cobalamin status after treatment (Kook *et al.* 2018).

vi. Treatment tolerance

There is no comparative study assessing treatment tolerance between injectable and oral cobalamin supplementation. In children with hypocobalaminaemia, intramuscular injections of hydroxocobalamin appear to cause less pain than cyanocobalamin (Gräsbeck, 2010). No such study has been conducted in dogs.

e. Aims of the study

The first objective of our study was to compare the efficacy of oral and parenteral cyanocobalamin supplementation in normalising serum cobalamin levels in dogs with

hypocobalaminaemia secondary to chronic enteropathy, in order to establish whether oral supplementation is non-inferior to parenteral supplementation. As proved in people and in one study in dogs with CIE and secondary hypocobalaminaemia (Toresson *et al.* 2018), oral cobalamin supplementation seems to be as effective as parenteral cobalamin supplementation to normalise the cobalamin status.

The second goal of the study was to compare treatment tolerance, for the patient and for the owner, in order to optimise treatment compliance in the future. We hypothesised that oral supplementation is less painful, less stressful and less expensive compared to parenteral supplementation which involves multiple visits to the vet and multiple subcutaneous injections.

The third goal of the study was to compare the efficiency of oral to parenteral cobalamin supplementation in dogs with specific subtypes such as protein-losing enteropathy, severe hypocobalaminaemia or severe gastrointestinal signs at inclusion. We hypothesised that oral cobalamin supplementation would be as effective as its parenteral counterpart in those dogs.

II. Materials and methods

a. Elaboration of the study protocol

i. Cases enrolment and owner's consent

A controlled, randomized, multicentre, non-inferiority study was designed to evaluate efficacy and tolerance of oral versus parenteral cyanocobalamin supplement in normalising cobalamin status in dogs with hypocobalaminaemia and chronic enteropathy.

Many veterinary teaching hospitals and private referral centres in the United-Kingdom were contacted by email. Two small animal referral centres and one out-of-hour emergency centre in the United-Kingdom agreed to participate to the study including:

- The University of Glasgow;
- Davies Veterinary Specialists;
- Vetsnow Glasgow.

Internal Medicine or Emergency and Critical Care clinicians from these centres were asked to recruit dogs with clinical signs of chronic enteropathy and hypocobalaminaemia (serum cobalamin <250 ng/mL) in a prospective manner. Cases were enrolled from August 2018 to April 2020.

As part of the inclusion criteria, the owner or authorised agent of each hypocobalaminaemic dog recruited in the study had given their consent for his/her animal to participate in the study (see appendix 2). Information to owner was delivered in order to optimise their understanding of the study (see appendix 3). This document included:

- A general approach to diagnosis and treatment of hypocobalaminaemia in dogs,
- An explanation to why we are looking for an alternative to cobalamin injections;
- What is a clinical trial;
- Why this study would be beneficial for themselves and their pet;
- The risks associated with the study;

- The obligations involved by the enrolment of their pet in the study.

ii. Inclusion and ethical approval

Animals which met the following inclusion criteria were enrolled in the study:

- Dogs with clinical signs of chronic enteropathy and hypocobalaminaemia;
    - Dogs with chronic enteropathy were characterised by chronic persistent or recurrent clinical signs of gastrointestinal disease (such as vomiting, diarrhoea, weight loss, or a combination of those) for longer than 3 weeks;
    - Hypocobalaminaemia was defined by serum cobalamin <250 ng/mL;
- Dogs must be suitable for the study when examined physically on Day 0;
- The owner or authorised agent had given the consent for his/her animal to participate in the study;

Animals which met one of the following exclusion criteria were excluded from the study:

- Animals that received cobalamin supplementation less than 12 weeks before Day 0;
- Animals with hypocobalaminaemia secondary to a genetic defect in cobalamin processing;
- Animals with known hypersensitivity to the active ingredients and/or excipients of the feed supplement or active control;
- Animals with severe systemic disease. Severe systemic disease was defined as a disease preventing the animal to take oral medication or a life-threatening disease (other than chronic enteropathy) with an estimated survival of less than 6 months.

Animals were excluded from the study subsequent to their inclusion for the following reasons:

- Removal due to worsening or non-improvement related to the condition under investigation;
- Removals due to concomitant disease, not allowing the animal to stay on study (e.g. other treatments needed not compliant with protocol);
- Animal's death;
- Animal owner's refusal to continue study participation;

- Incorrect administration of the cobalamin supplement (e.g. serious dosing error). A serious dosing error was defined as a dose at least ten times higher or lower than the recommended dose).

The study was submitted approved by the ethics committee of the School of Veterinary Medicine and Science, University of Nottingham in March 2018.

iii. Study design, randomisation and data collection

Each hypocobalaminaemic dog recruited underwent standard evaluation for intestinal disease and cobalamin status. The mandatory investigation workup included a full serum biochemistry, complete blood count, serum folate, cobalamin, canine TLI concentration and serum MMA concentration, following a 12 hour fast.
The need for additional investigation procedures was left at the discretion of the clinician in charge of the case. Hence, some dogs also had a faecal analysis, abdominal imaging (abdominal ultrasound or CT-scan) and/or intestinal biopsies (full thickness or endoscopic intestinal biopsies).

Physical examination, blood tests (serum biochemistry, complete blood count, serum folate, cobalamin, canine TLI concentration and serum MMA), and completion of questionnaires (quality of life questionnaire, tolerance & palatability) were undertaken in all dogs at week 0.

Cobalamin supplementation was initiated. Hypocobalaminaemic dogs with chronic enteropathy enrolled in the study were allocated into two groups:

- Group 1: Oral cyanocobalamin supplement administered once daily for twelve weeks;
- Group 2: Parenteral (subcutaneous) cyanocobalamin treatment comprising six doses administered once weekly for six weeks and a seventh dose four weeks later.

Dogs were randomly assigned (block randomisation, Excel, Microsoft Office 2016) to one of two groups (oral or parenteral cobalamin supplementation).

Following initiation of cobalamin supplementation, follow-up included:

- Further physical examination, body weight and history reporting, CIBDAI score, serum cobalamin and folate concentration assessment at week 7,
- Further physical examination, body weight and history reporting, CIBDAI score, serum cobalamin, folate and MMA concentration assessment, and completion of questionnaires (quality of life, tolerance & palatability) at week 13.

Based on a statistical power calculation, 50 dogs per group were required to achieve statistical significance between the two groups.

The number of dogs included in each of the two groups was calculated as follows:

1. According to Toresson et al.'s prospective study (2018), efficacy of parenteral cobalamin at 90 days is 95% (21/22 dogs became normocobalaminaemic at 90 days following parenteral cobalamin supplementation).
2. Based on these results, the expected success rate in the oral group was 95%.
3. We decided to take a significance level of 2.5% (one-sided t-test).
4. The sample size estimates for hypothesis testing was based on achieving 90% power.
5. The non-inferiority limit was established at 15%.

Based on this data, we calculated a sample size of 45 dogs per group. We decided to increase the sample size to 50 dogs per group to factor in losses.

Group	Name of Supplement	Content and additives	Route of administration	Dosage schedule	Days on study	Number of animals
T1	Cobalaplex® (Protexin, UK)	PXN-B12®, Preplex® prebiotic, folic acid, chicken flavour	per os	See Appendix 1: Dose selection	up to day 98	50 dogs
T2	Vitbee 250 (Dechra, UK)	0.025 % w/v cyanocobalamin, 0.500 % w/v phenol	Subcutaneous injection	See Appendix 1: Dose selection	up to day 98	50 dogs

Table 2: Study Design

Data were collected in April 2020 from the different clinicians who recruited hypocobalaminaemic cases.

iv. Blood sample handling, cobalamin and MMA assessment

All serum samples were refrigerated within 2h of collection. Blood samples for CBC, biochemistry, folate, cobalamin and TLI assessment: clinicians were asked to refrigerate 2mL of whole blood and 2mL of serum within 2h of collection. The stored serum was sent refrigerated with cold packs to the VPG Leeds laboratory within 48h of collection using priority delivery.

Blood samples for MMA assessment: clinicians were asked to freeze 1mL of serum at -20 degrees centigrade within 1–3 days until analysis. Frozen serum samples were sent for MMA analysis to the Synlab laboratory in Augsburg, Germany, in March 2021 on dry ice using express delivery. The conditions of the serum samples after shipment were not reported.

Cobalamin concentration was assessed by a chemiluminescent assay on the "Immulite 2000" automated analyser. The laboratory did not perform a dilution when the results exceeded the upper limit of the reference interval (>2000ng/L) for several reasons:

- Such values provide evidence of adequate supplementation.
- Such values do not raise concerns of a pathological state.
- Spike-and-recovery test and linearity-of-dilution test had not been undertaken for the cobalamin assay with the analyzer Immulite 2000.
    - A spike-and-recovery experiment is used to determine whether analyte detection is affected by a difference between the diluent used to prepare the standard curve and the biological sample matrix.
    - A linearity-of-dilution experiment provides information about the precision of assay results for samples tested at different levels of dilution in the chosen sample diluent.

MMA was analysed at the Synlab laboratory in Augsburg, Germany, using a liquid chromatography–mass spectrometry method.

v. Cobalamin supplementation protocols

1. Oral supplementation

Fifty percent of dogs included in the study received daily oral cyanocobalamin, based on their weight (see appendix 1).

The name of the oral supplement was "Cobalaplex®" (Protexin, UK). The list of additives reported by the manufacturer included PXN-B12®, Preplex® prebiotic, folic acid and chicken flavour (see Table 3).

Name	Cobalaplex
Qualitative and quantitative composition:	Technological additives: Preplex® prebiotic - acacia (gum arabic) Nutritional additives: Cyanocobalamin (vitamin B12) 0.5 mg per capsule Folic acid 0.2 mg per capsule
Formulation:	Sprinkle capsule
Nature and composition of immediate packaging:	Blister pack
Sponsor (Marketing authorization holder):	Probiotics International Ltd
Manufacturing company:	Probiotics International Ltd
Special precautions for storage:	Please store in cool, dry conditions (< 25°C).
Batch number(s):	Not recorded
Expiry date(s):	Not recorded
Special precautions for use:	For use in animals: For persons administering the test product to animals: Please see Material Safety Data Sheet

Table 3: Characteristics of the oral cobalamin supplement.

Each capsule contains 0.5mg of cyanocobalamin.

- Dogs weighing less than 10kgs received 0.5 capsule once daily (= 0.25mg). This dose represents 25 to 250ug/kg of cyanocobalamin once daily;
- Dogs weighing between 10 and 20kgs received 1 capsule once daily (= 0.5mg). This dose represents 25 to 50ug/kg of cyanocobalamin once daily;
- Dogs weighing more than 20kgs received 2 capsules once daily (= 1mg). This dose represents ≤ 50ug/kg of cyanocobalamin once daily.

2. Parenteral supplementation

Fifty percent of dogs included in the study received weekly subcutaneous injections of cyanocobalamin, for six weeks and then one more injection four weeks later. The total volume of cyanobalamin was calculated based on their weight (see appendix 1).

The name of the injectable supplement was "Vitbee 250" (Dechra, UK). The list of additives reported by the manufacturer included 0.500 % w/v phenol.

Name	Vitbee 250 Solution for Injection
Qualitative and quantitative composition:	Active substance: Cyanocobalamin 0.025% w/v Excipient: Phenol 0.500% w/v
Formulation:	Solution for injection
Nature and composition of immediate packaging:	50 ml type I amber glass vial, fitted with a red type I bung and aluminium overseal
Marketing authorization holder:	Dechra Limited
Manufacturing company:	Dechra Limited
Special precautions for storage:	Do not store above 25°C. Protect from light. Following withdrawal of the first dose, use the product within 28 days.
Batch number(s):	Not recorded
Expiry date(s):	Not recorded
Special precautions for use:	Observe normal aseptic precautions. Care should be taken to avoid accidental self-injection or ingestion. In the case of accidental self-

	injection or ingestion, seek medical advice as a precautionary measure. Following skin/eye contamination, wash/irrigate area thoroughly with cold water. Seek medical attention if irritation persists. Wash hands after use.

Table 4: Characteristics of the injectable subcutaneous cobalamin supplement.

Each millilitre of injectable cyanocobalamin contains 250ug of cyanocobalamin.

- Dogs weighing less than 10kgs received 1mL once weekly (= 0.25mg). This dose represents 25 to 250ug/kg of cyanocobalamin once weekly;
- Dogs weighing between 10 and 20kgs received 2mL once weekly (= 0.5mg). This dose represents 25 to 50ug/kg of cyanocobalamin once weekly;
- Dogs weighing more than 20kgs received 4mL once weekly (= 1mg). This dose represents ≤ 50ug/kg of cyanocobalamin once weekly.

vi. Assessment of treatment efficacy

1. Clinical signs and CIBDAI score monitoring

Following patient inclusion in the study, the veterinarian recorded a comprehensive history and performed a full clinical examination during the initial consultation (week 0). A Canine Inflammatory Bowel Disease Activity Index Score (CIBDAI) was also filled with the owner at week 0 (see appendix 4). Thereafter, physical re-examinations and CIBDAI score reassessments were undertaken at weeks 7, 9 (optional) and 13.

As discussed above, taking part in the trial required the owner to either administer an oral cobalamin supplement once daily for 12 weeks, or to bring the patient to the veterinary practice for a subcutaneous injection of cobalamin on seven separate occasions (week 1, 2, 3, 4, 5, 6 and 10).

2. Cobalamin status monitoring

The cobalamin status was monitored by assessing:

- Serum cobalamin concentration at week 0, 7 and 13, following a 12 hour fast;
- Serum MMA concentration at week 0 and 13, following a 12 hour fast.

vii. Assessment of tolerance to oral versus parenteral cobalamin supplementation

Treatment tolerance was assessed by two separate questionnaires filled after completion of the study at week 13. The first questionnaire looked at owners' quality of life during the trial, regardless of the type of cobalamin supplementation (oral or injectable) (see appendix 5). The second questionnaire was different depending on the type of cobalamin supplementation their dog received. Owners of dogs receiving cobalamin injections filled a "Tolerance Questionnaire for dogs receiving parenteral cobalamin supplementation" (Appendix 6). Owners of dogs receiving oral cobalamin supplements filled a "Tolerance and palatability questionnaire for dogs receiving oral cobalamin supplementation" (Appendix 7).

1. Owner Quality of Life Questionnaire

This first questionnaire entitled "Owner quality of life questionnaire following completion of the cobalamin study" (Appendix 5), was filled by every owner of dogs receiving oral cobalamin supplements. It included eight questions divided into three categories aiming at assessing the ease of treatment administration (administration, treatment planning, observance), the stress caused by treatment administration (to the owner and to the dog), and the owner's satisfaction. The questions have been proposed as follows:

- Ease of treatment administration (*sensu lato*)
    - Ease of administration: How easy or difficult is it to administer the cobalamin supplement in its current form?
    - Ease of treatment planning: How easy or difficult is it to plan when you will use the cobalamin supplement each time?

- Ease of observance (x2): How easy or difficult is it to adhere to the treatment regime as instructed? How easy did you find it to comply with the instructions the veterinary surgeon provided you with at the start of the study (i.e. manage to give the capsules each day or attend the veterinary appointments for the injections to be administered)?

- Stress related to treatment administration:
  - For the owner: How stressful (e.g. nervous/anxious) did you find it to administer the course of treatment as instructed?
  - For the dog: How stressful did your dog find the course of treatment?
- Owner's satisfaction:
  - Related to treatment administration: Thinking only about the administration of the treatment (i.e. not whether you think it worked or not), how satisfied or dissatisfied are you with how the cobalamin supplement is administered?
  - Related to the treatment modality: Taking all things into account, how satisfied or dissatisfied are you with the cobalamin supplement overall?

The owner was asked to answer each question as follows, with a score from 0 to 4 allocated to each answer:

Owner QOL questionnaire scoring system					
Very dissatisfied	0	Very difficult	0	Extremely stressful	0
Quite dissatisfied	1	Quite difficult	1	Moderately stressful	1
Neither dissatisfied nor satisfied	2	Neither difficult nor easy	2	Slightly stressful	2
Quite satisfied	3	Quite easy	3	Neither stressful nor easy	3
Very satisfied	4	Very easy	4	Easy	4

The last question of the quality-of-life questionnaire asked the owner if they would choose the same treatment that your dog received this time, or would you try the other treatment, if another cobalamin supplementation was needed (see appendix 5).

2. Tolerance Questionnaire for dogs receiving parenteral cobalamin supplementation

This second questionnaire entitled "Tolerance Questionnaire for dogs receiving parenteral cobalamin supplementation" (Appendix 6), is a validated questionnaire filled by the owners of dogs receiving cobalamin injections, included 23 questions divided into three categories:

- Dog's response to visiting the vets before this trial (11 questions). This part of the questionnaire is aiming at assessing the dog's tolerance of visiting the vet before the study;
- Dog's response to visiting the vets for the cobalamin injections (12 questions). This part of the questionnaire is aiming at assessing the dog's tolerance of visiting the vet during the study. It includes:
    - Eleven questions about the dog's behaviour at the vet before the injection;
    - One question about the dog' response to the cobalamin injections.

The owner was asked to answer each question as follows, with a score from 0 to 4 allocated to each answer:

Dog's response to visiting the vets before this trial

My dog…	Almost every time	A lot of the time	Sometimes	Rarely	Never
Has to be dragged or carried into the vets	0	1	2	3	4
Walks in hesitantly or hides behind you	0	1	2	3	4
Walks in pulling the lead	4	3	2	1	0
Walks in without pulling the lead or hiding behind you	0	1	2	3	4
Trembles when in the consulting room	0	1	2	3	4
Whimpers/whines in the consulting room	0	1	2	3	4
Pants when in the consulting room	0	1	2	3	4
Licks its lips regularly when in the consulting room	0	1	2	3	4

Yawns repeatedly when in the consulting room	0	1	2	3	4
Walks up to and greets the vet	4	3	2	1	0
Stays close to the door of the consulting room	0	1	2	3	4
Total score out of 44					

Dog's response to visiting the vets for the cobalamin injections

My dog…	Almost every time	A lot of the time	Sometimes	Rarely	Never
Had to be dragged or carried into the vets	0	1	2	3	4
Walked in hesitantly or hid behind you	0	1	2	3	4
Walked in pulling the lead	4	3	2	1	0
Walked in without pulling the lead or hiding behind you	0	1	2	3	4
Trembled when in the consulting room	0	1	2	3	4
Whimpered/whined in the consulting room	0	1	2	3	4
Panted when in the consulting room	0	1	2	3	4
Licked its lips regularly when in the consulting room	0	1	2	3	4
Yawned repeatedly when in the consulting room	0	1	2	3	4
Walked up to and greeted the vet	4	3	2	1	0
Stayed close to the door of the consulting room	0	1	2	3	4
Total score out of 44					
Pressed itself against you or tried to hide behind you when the injection was administered	0	1	2	3	4
Score out of 4					

3. Tolerance and palatability questionnaire for dogs receiving oral cobalamin supplementation (Appendix 7)

This third questionnaire entitled "Tolerance Questionnaire for dogs receiving oral cobalamin supplementation" (Appendix 7) was filled by the owners of dogs receiving oral cobalamin supplements. This questionnaire included 12 questions divided into four groups:

- Tolerance of taking capsules or tablets before the study (5 questions);

- Tolerance of taking cobalamin capsules during the study (5 questions);
- Behaviour changes while taking the cobalamin capsules during the study (11 questions);
- Modalities of Cobalaplex capsules administration (1 question).

The owner was asked to answer each question as follows, with a score ranging from 0 to 4 or from 0 to 8, allocated to each answer:

Dog's response to taking capsules or tablets before this trial

My dog…	Almost every time	A lot of the time	Sometimes	Rarely	Never
Eats tablets voluntarily as though they are food treats	8	6	4	2	0
Will readily eat a tablet when its hidden in food (i.e. wet food, chicken, cheese, peanut butter etc.)	4	3	2	1	0
Spits out or sifts out tablets that were hidden in food	0	1	2	3	4
Refuses to eat food if it thinks there's a tablet in it	0	1	2	3	4
Refuses to take a tablet no matter what, so I have to drop it down their throat by hand or use a pill pusher	0	2	4	6	8
Total score out of 28					

Dog's response to taking cobalamin capsules

My dog…	Every time (or almost every)	A lot of the time	Sometimes	Rarely	Never
Ate the supplement voluntarily as though it was a food treat	8	6	4	2	0
Ate the supplement when it was hidden in food (i.e. wet food, chicken, cheese, peanut butter etc.)	4	3	2	1	0
Spat out or sifted out the supplement that was hidden in food	0	1	2	3	4
Refused to eat food if it thought the supplement was in it	0	1	2	3	4

Refused to take the supplement no matter what, so I had to drop it down their throat by hand or use a pill pusher	0	2	4	6	8
Total score out of 28					

Behavioural changes when being given the cobalamin supplement

	My dog did this every time	My dog did this most of the time	My dog did this sometimes	My dog did this rarely (once or twice)	No, my dog never did this
Tried to hide or get away when it knew I was getting/had them	0	1	2	3	4
Tried to move/pull away from me	0	1	2	3	4
Approached me voluntarily when it knew I was getting/had them	4	3	2	1	0
Trembled or shook	0	1	2	3	4
Wagged its tail	4	3	2	1	0
Held its tail low between its legs	0	1	2	3	4
Growled	0	1	2	3	4
Whined	0	1	2	3	4
Barked	0	1	2	3	4
Began to pant	0	1	2	3	4
Appeared to want the supplement as though it was food	4	3	2	1	0
Total score out of 44					

Modalities of “Cobalaplex” capsules administration

- Capsule opened and contents sprinkled on top of the dog’s regular food, left unmixed
- Capsule opened and contents mixed with the dog’s regular food
- Capsule unopened (entire) hidden in the dog’s regular food
- Capsule unopened (entire) given with a treat or other palatable foodstuff
- Capsule unopened (entire) given alone
- Capsule given with another technique

For each administration modality, a score out of 4 is assigned (0 = never, 4 = every time)

viii. Summary of the study protocol

Procedure \ Study week		0	1	2-6	7	9	10-12	13
Obtain owner informed consent		X						
Animal details and history		X						
Inclusion/Exclusion criteria		X						
Body weight		X			X	[X]		X
Clinical examination		X			X	[X]		X
CIBDAI score		X			X	[X]		X
Blood sample	Haematology	X	[X]					X
	Serum biochemistry	X	[X]					X
	Serum cobalamin and folate	X			X			X
	TLI	X						
	Serum MMA	X						X
Randomization		X						
Oral supplementation		(X)						
Parenteral supplementation		(X)						
Study completion								X
Owner quality of life survey								X
Tablet tolerance and palatability survey or injection tolerance survey								X
Unexpected events		1	1		1	1		1
Concomitant treatment		1	1		1	1		1

X	These procedures have to be filled in the appropriate forms for every animal enrolled in the study.
(X)	Brackets indicate that the procedure may not need to be performed on that study day or on all animals in this week.
[X]	Facultative test or examination.
1	To be recorded in case unexpected/undesirable effect occurs or a concomitant treatment is given.

Table 4: Summary of the study protocol.

b. Data analysis

Data were initially inputted into a spreadsheet (Microsoft Excel). Data are presented descriptively. Continuous data were assessed for normality using the Shapiro Wilk test and presented with a mean ± sd if normally distributed or median (+ inter-quartile range) if not. Data were processed through the online statistics calculator "Statistics Kingdom": https://www.statskingdom.com/.

Age, body weight at week 7 and week 13, CIBDAI score at inclusion, and cobalamin at inclusion data sets, were normally distributed.
Body weight at inclusion, CIBDAI score at week 7 and 13, cobalamin at week 7 and 13 data sets, were not normally distributed.

The Mann–Whitney U test was used for body weight, CIBDAI score and serum cobalamin concentration comparisons between dogs receiving oral cobalamin or parenteral cobalamin at inclusion. This test was also used for body weight, CIBDAI score and serum cobalamin comparisons of improvement between groups.

Statistical significance for all tests was set at $P < 0.05$.

III. Results

a. Patients recruitment and randomisation

Initially, 37 dogs were initially enrolled in the study. Following initial recruitment, dogs were randomly assigned into one of the two study groups:

- Oral cobalamin supplementation (18 dogs)
- Parenteral cobalamin supplementation (19 dogs)

Nineteen dogs eventually completed the study. Among these nineteen dogs, eleven dogs received oral cobalamin supplementation and 8 dogs received parenteral cobalamin supplementation. One dog was recruited twice. This patient was initially enrolled in the parenteral group. Six months after the last cobalamin injection, gastrointestinal signs were persistent. Reassessment of serum cobalamin concentration revealed recurrent hypocobalaminaemia. A second cobalamin supplementation protocol was undertaken, and at the owner's request, the dog was included in the oral cobalamin group.

Eighteen dogs were excluded from the study for various reasons:

- Loss of clinical and/or blood follow-up (12 dogs)
- Euthanasia during the cobalamin supplementation protocol (six dogs)
    - Due to clinical deterioration of the clinical signs related to chronic enteropathy (five dogs)
    - Due to concurrent disease (one dog with concurrent splenic hemangiosarcoma)

Among the 18 dogs excluded from the study:

- Seven dogs received oral cobalamin supplementation:
    - Four dogs were excluded due to a lack of follow-up,
    - Three dogs were euthanised due to clinical deterioration.
- Eleven dogs received parenteral cobalamin supplementation:
    - Eight of these dogs were excluded due to a lack of follow-up,
    - Two dogs were euthanised due to clinical deterioration,
    - One dog was euthanised to due concurrent life-threatening disease (splenic hemangiosarcoma).

Among the five dogs excluded from the study due to clinical deterioration, two dogs belonged to the oral treatment group and three dogs belonged to the parenteral group. Therefore, it seems unlikely that the treatment modality had been a reason for poor treatment response. Quality of life and/or palatability and tolerance questionnaires were not available in two dogs, including one dog who was recruited twice.

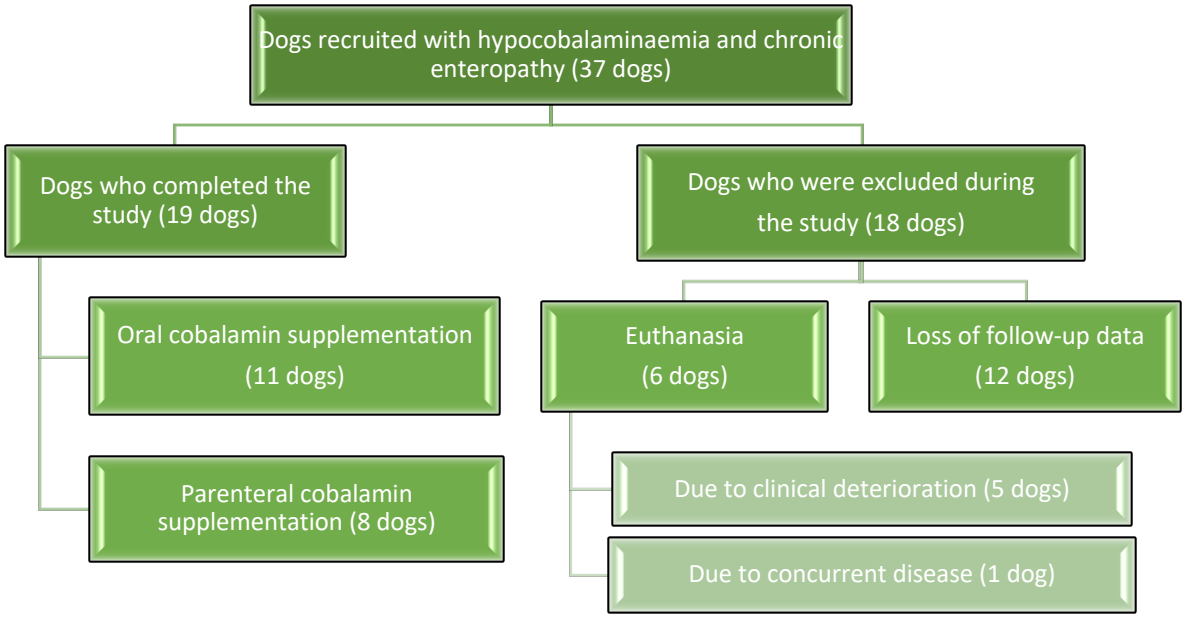

Figure 4: Diagram summarising the recruitment, inclusion and exclusion of 37 dogs with hypocobalaminaemia and chronic enteropathy during the study period.

b. Baseline data and clinical signs

Among the nineteen dogs who completed the study, thirteen different breeds were included, of which the most common were Labrador retrievers (n=5), Cairn Terriers (n=2), and mixed breed dogs (n=2). Other breeds were represented such as Beagle (n=1), Bichon Frise (n=1), Boxer (n=1), Cairn Terrier (n=1), Chihuahua (n=1), Dalmatian (n=1), Jack Russell Terrier (n=1), Miniature Schnauzer (n=1), Mixed breed (n=1), Norwich Terrier (n=1), Rottweiler (n=1), Staffordshire Bull Terrier (n=1), West Highland White Terrier (n=1), Miniature Schnauzer (n=1), Norwich Terrier (n=1), Rottweiler (n=1), Staffordshire Bull Terrier (n=1), West Highland White Terrier (n=1) (Table 5).

Females and males were equally represented, with 9 female neutered dogs (47.4%), 2 entire male dogs (10.5%) and 8 neutered male dogs (42.1%) (Table 5).

At the time of inclusion:

- the mean (+/-SD) age was 6 years 7 months (+/-3.55),
- the median (range) body weight in all dogs was 16.9 kgs (6.1-44),
- body weight was not statistically different between the dogs assigned to receive oral cobalamin supplementation and the dogs assigned to receive parenteral cobalamin supplementation (p-value = 0.103).

At the first follow-up at week 7:

- the median (range) body weight in all dogs was 16.3 kgs (6.7-44.6),
- At week 7, body weight was not significantly different in dogs receiving oral cobalamin supplementation compared to week 0 (p-value = 1),
- At week 7, body weight was not significantly different in dogs receiving parenteral cobalamin supplementation compared to week 0 (p-value = 0.66).

At the last follow-up at week 13:

- the median (range) body weight in all dogs was 15.9kg (7.1-47)
- At week 13, there was no statistical difference in body weight between dogs receiving oral cobalamin supplementation and dogs receiving parenteral cobalamin supplementation (p-value = 0.367).
- At week 13, body weight was not significantly different in dogs receiving oral cobalamin supplementation compared to week 0 (p-value = 0.97).
- At week 13, body weight was not significantly different in dogs receiving parenteral cobalamin supplementation compared to week 0 (p-value = 0.6).

The most common clinical signs were diarrhoea (n = 18), vomiting (n = 15), inappetence/anorexia (n = 13), weight loss (n = 11) and lethargy (n=11) (Table 5). Other clinical signs included abdominal distension (n = 8), borborygmi (n = 3), prayer stance (n = 2), constipation (n = 2) and pica (n = 1).

Most dogs (11/19, 57.9%) had shown clinical signs of gastrointestinal disease between one month and one year. A large proportion of dogs presented signs for less than one month (7/19, 36.8%).

Parameters at inclusion	Variable	Median (range) or number of dogs (%)
Age (years)	-	
Gender	Female entire Female neutered Male entire Male neutered	0 (0%) 9 (47.4%) 2 (10.5%) 8 (42.1%)
Body weight at inclusion (kgs)	-	16.9 (6.1-44)
Breed	Labrador Cairn Terrier Mixed breed Beagle Bichon Frise Boxer Chihuahua Dalmatian Jack Russell Terrier Miniature Schnauzer Norwich Terrier Rottweiler Staffordshire Bull Terrier West Highland White Terrier	5 2 2 1 1 1 1 1 1 1 1 1 1 1
Major clinical signs	Diarrhoea Vomiting Inappetence/Anorexia Weight loss Lethargy Abdominal distension Borborygmi Prayer stance Constipation Pica	18 (94.7%) 15 (78.9%) 13 (68.4%) 11 (57.9%) 8 (42.1%) 3 (15.8%) 2 (10.5%) 2 (10.5%) 1 (5.3%)
Duration of clinical signs	Up to 1 month 1 month to 1 year > 1 year	7 (36.8%) 11 (57.9%) 1 (5.3%)

Table 5: Baseline data at inclusion in the 19 dogs with hypocobalaminaemia and chronic enteropathy who completed the study.

c. Haematology, serum biochemistry, ancillary laboratory data

The most common haematological changes were neutrophilia (n = 7, median 14.44 x10^9 /L (9.97-38.87) and leucocytosis (n = 4, mean 28.64 x10^9 /L +/-12.79). Anaemia (n = 2), eosinopenia (n = 2), monocytosis (n = 2), eosinophilia (n =1), thrombocytopenia (n =1) and thrombocytosis (n = 1) were also identified (Table 6).

Parameter	Reference interval	Number of dogs affected by the abnormality	Median (range) or mean+/-SD
Increased total leucocyte count	5.9-14.5x10^9 /L	4	Mean 28.64 x10^9 /L (+/- 12.79)
Increased total neutrophil count	3.1-9.4x10^9 /L	7	Median 14.44 x10^9 /L (9.97-38.87)
Increased total eosinophil count	0.1-1.4x10^9 /L	1	1.71 x10^9 /L
Increased total monocyte count	0-1.6x10^9 /L	2	1.77-1.85 x10^9 /L
Decreased haematocrit	43-60 %	2	23.8-39.1 x10^9 /L
Thrombocytopenia	150-450 x10^9 /L	1	75x10^9 /L
Thrombocytosis	150-450 x10^9 /L	1	668 x10^9 /L
Decreased total protein	54-77 g/L	7	Mean 37.9g/L +/- 9.15
Decreased albumin	25-40 g/L	7	Mean 13.8g/L +/- 3.8
Decreased TLI	5-40 ng/mL	2	3.2-4ng/mL
Increased folate	7.2-23.8 mcg/L	1	24.6mcg/L
Decreased folate	7.2-23.8 mcg/L	7	Mean 4.4mcg/L +/- 1.7
Increased ALT	5-66 U/L	6	Mean 130U/L +/-63
Increased ALP	0.1-150 U/L	3	Mean 558U/L +/-338
Decreased cholesterol	3.8-7 mmol/L	6	Mean 2.65mmol/L +/-0.39
Increased creatinine	40-150 umol/L	2	157-260 umol/L

Table 6: Selected haematology and serum biochemistry data at inclusion in the 19 dogs with hypocobalaminaemia and chronic enteropathy who completed the study.

The most common biochemical changes were hypoproteinaemia (n = 7, mean 37.9 +/-9.15 g/L), hypoalbuminaemia (n = 7, mean 13.8 +/- 3.8 g/L), hypocholesterolaemia (n = 6, mean 2.65 +/- 0.39 mmol/L), and increased ALT (n = 6, mean 130 +/- 63 U/L). Increased ALP (n = 3, mean 558 +/- 338 U/L) and azotaemia (n = 2) were also documented (Table 6).

Two dogs had subnormal serum TLI concentrations of 3.2 and 4 ng/mL (reference interval, 5-40 ng/mL) making them diagnosed with subclinical exocrine pancreas insufficiency (EPI). Hypofolataemia, suggestive of proximal small intestinal malabsorption, was more frequent (n = 7, mean 4.4 +/-1.7 mcg/L) than hyperfolataemia (n = 1).

Basal cortisol and/or ACTH stimulation test was undertaken in six dogs. This(ese) test(s) allowed to rule out hypoadrenocorticism as a cause of ongoing gastrointestinal signs. Median basal cortisol was 59nmol/l (range: 32-114).

d. Faecal parasitology, abdominal imaging, histopathology of gastrointestinal biopsies

Results of parasitology, ultrasonography and histopathology of gastrointestinal biopsies are available as supplementary material were available in 13 dogs. The details of the diagnostic procedures were not provided in six dogs.

- Faecal parasitology was documented in two dogs,
- Abdominal ultrasound was performed in 11 dogs,
- Abdominal CT-scan was undertaken in one dog,
- Intestinal endoscopy associated with endoscopy-guided intestinal biopsies was undertaken in nine dogs.

Urinalysis was only available in three dogs and a bile acid stimulation test (BAST) was undertaken in one dog only.

e. Clinical diagnosis

A definitive diagnosis of chronic inflammatory enteropathy was achieved with endoscopy-guided intestinal biopsies in nine dogs. The details of the diagnostic procedures were not provided in six dogs.

Based on history, clinical findings, haematological and biochemical blood workup, and abdominal ultrasound results, presumptive chronic enteropathy was suspected in four dogs.

Among the 19 dogs recruited in the study, seven were diagnosed with protein-losing enteropathy (PLE) (= 7). Two dogs were diagnosed with subclinical EPI (n =2).

Among the 19 dogs who completed the study, concurrent diseases included:

- Chronic kidney disease (CKD) (n = 3),
- Pulmonary carcinoma (n =1),
- Primary hyperadrenocorticism (n = 1),
- Immune-mediated haemolytic anaemia (IMHA) (n = 1),
- Stump pyometra (n = 1),
- Idiopathic hyperlipidaemia (n = 1).

f. Concurrent therapies and diet during the trial

The details of the drugs provided during the trial was available in 17 dogs (17/19) (Table 7). In addition to cobalamin supplementation, prednisolone (n = 10), no additional treatment (n =5), anti-emetic/anti-nausea/appetite stimulant drugs (maropitant, metoclopramide, mirtazapine) (n = 5), anti-platelet drugs (clopidogrel) (n = 3), oral probiotics (n = 3), oral antibiotics (metronidazole, tylosin, amoxicillin/clavulanic acid) (n = 2), gastroprotectants (omeprazole, ranitidine) (n = 2), mycophenolate (n = 1), pancreatic enzymes (n = 1) and fenbendazole (n = 1) were also administered throughout the trial.

The details of the diet administered during the trial was available in 15 dogs (15/19) (Table 7). Among these 15 dogs, eleven dogs received a hydrolysed protein diet, two dogs received a low-fat diet, two dogs received a grain free diet and one dog received a novel protein diet.

Drugs administered	Number of dogs treated with the drug/diet in total	…within the oral cobalamin supplementation group	…within the parenteral cobalamin supplementation group
Prednisolone	10 (10/19)	6 (6/11)	4 (4/8)
Mycophenolate	1 (1/19)	1 (1/11)	0 (0/8)
Antibiotics (metronidazole, tylosin, amoxicillin/clavulanic acid)	2 (2/19)	1 (1/11)	1 (1/8)
Probiotics	3 (3/19)	1 (1/11)	2 (2/8)
Anti-emetic/anti-nausea drugs (maropitant, metoclopramide, mirtazapine)	5 (5/19)	2 (2/11)	3 (3/8)
Anthelmintic drugs (fenbendazole)	1 (1/19)	0 (0/11)	1 (1/8)
Gastroprotectants (omeprazole, ranitidine)	2 (2/19)	1 (1/11)	1 (1/8)
Anti-platelet drugs (clopidogrel)	3 (3/19)	2 (2/11)	1 (1/8)
Pancreatic enzymes	1 (1/19)	1 (1/11)	0 (0/8)
None	5 (5/19)	2 (2/11)	3 (3/8)
Unknown	2 (2/19)	1 (1/11)	1 (1/8)
Diets administered	**Number of dogs treated with the drug/diet in total**	**…within the oral cobalamin supplementation group**	**…within the parenteral cobalamin supplementation group**
Novel protein diet	1 (1/19)	0 (0/11)	1 (1/8)
Grain free diet	2 (2/19)	1 (1/11)	1 (1/8)
Hydrolysed protein diet	10 (10/19)	7 (7/11)	3 (3/8)
Low fat diet	2 (2/19)	1 (1/11)	1 (1/8)
Unknown	4 (4/19)	2 (2/11)	2 (2/8)

Table 7: Summary of the drugs and diets provided during the trial the 19 dogs with hypocobalaminaemia and chronic enteropathy who completed the study.

g. Canine inflammatory bowel disease activity index (CIBDAI)

At inclusion

At inclusion, the median CIBDAI was 8 (range 3–17) in the oral group and 10 (range 5–17) in the parenteral group at the time of inclusion, representing mild to severe gastrointestinal signs. The CIBDAI score was not statistically different between the dogs assigned to receive oral cobalamin supplementation and the dogs assigned to receive parenteral cobalamin supplementation (p-value = 0.77) (Figure 5).

At week 7

At week 7, the median CIBDAI was 2 (range 0-5) in the oral supplementation group and 2 (range 0-11) in the parenteral supplementation group at the time of inclusion.

The CIBDAI score was not statistically different between dogs receiving oral cobalamin supplementation and dogs receiving parenteral cobalamin supplementation (p-value = 0.932) (Figure 5).

At week 7, CIBDAI scores were statistically significantly lower in dogs receiving oral cobalamin supplementation compared to week 0 (p-value = 0.0001), and also lower in dogs receiving oral cobalamin supplementation compared to week 0 (p-value = 0.0004).

At week 13

At week 13, the median CIBDAI was 1 (range 0-6) in the oral supplementation group and 1 (range 0-5) in the parenteral supplementation group at the time of inclusion.

The CIBDAI score was not statistically different between dogs receiving oral cobalamin supplementation and dogs receiving parenteral cobalamin supplementation (p-value = 0.49) (Figure 5).

At week 13, CIBDAI scores were not statistically different in dogs receiving oral cobalamin supplementation compared to week 7 (p-value = 0.36). The CIBDAI scores were also not statistically different in dogs receiving parenteral cobalamin supplementation compared to week 7 (p-value = 0.10).

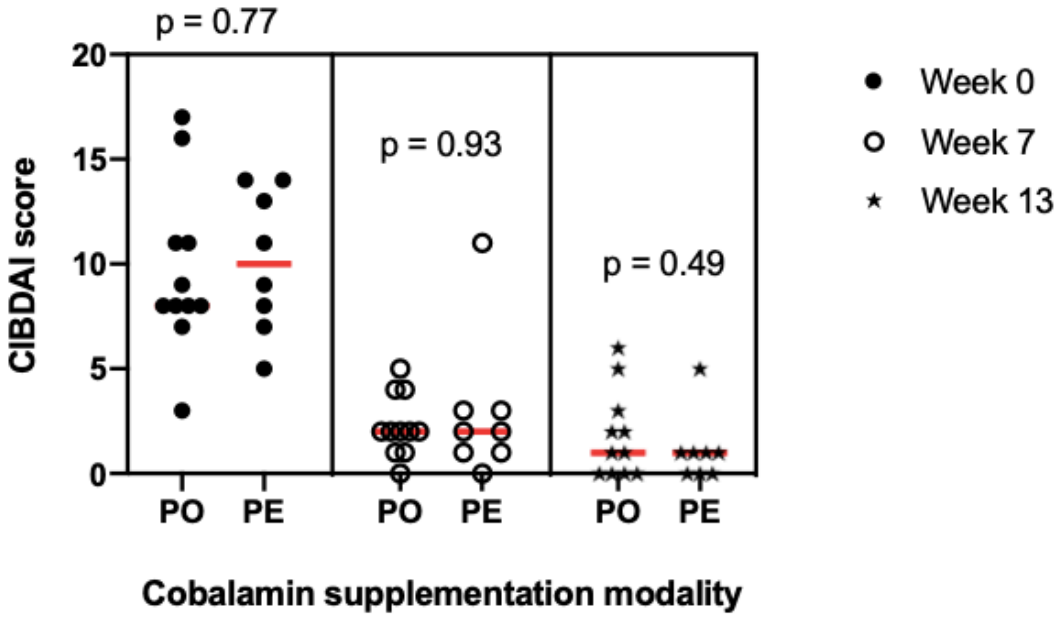

Figure 5: CIBDAI score at week 0, 7 and 13 in dogs receiving oral and parenteral cobalamin supplementation. Long horizontal lines represent medians. Data for the oral (peroral, PO) group are shown on the left in each panel (n = 11 at each time point) and data for the parenteral (PE) group are shown to the right (n = 8 at each time point).

h. Serum cobalamin concentration, MMA concentration

At inclusion

At inclusion, the median cobalamin concentration was 186ng/L (range 146-242) in the oral supplementation group and 199ng/L (range 150-252) in the parenteral supplementation group.

Serum cobalamin concentrations were not statistically different between the dogs assigned to receive oral cobalamin supplementation and the dogs assigned to receive parenteral cobalamin supplementation (p-value = 0.247) (Figure 6).

At week 7

At week 7, the median cobalamin concentration was 2000ng/L (range 1468-2000) in the oral supplementation group and 806ng/L (range 415-1775) in the parenteral supplementation group.

The cobalamin concentration was significantly higher in dogs receiving oral cobalamin supplementation compared dogs receiving parenteral supplementation (p-value = 0.0002) (Figure 6).

At week 7, the cobalamin concentration was significantly higher compared to week 0 (p-value = 0.00005) in dogs receiving oral cobalamin supplementation.

At week 7, the cobalamin concentration was significantly higher compared to week 0 (p-value = 0.0009) in dogs receiving parenteral cobalamin supplementation.

At week 13

At week 13, the median cobalamin concentration was 2000ng/L (range 412-2000) in the oral supplementation group and 453ng/L (range 231-805) in the parenteral supplementation group. The cobalamin concentration was significantly lower in the parenteral supplementation group (p-value = 0.0007) (Figure 6).

At week 13, there was no statistical difference in cobalamin concentration compared to week 7 (p-value = 0.8) in dogs receiving oral cobalamin supplementation.

At week 13, the cobalamin concentration was significantly lower in dogs receiving parenteral cobalamin supplementation compared to week 7 (p-value = 0.028), despite following strictly the supplementation protocol.

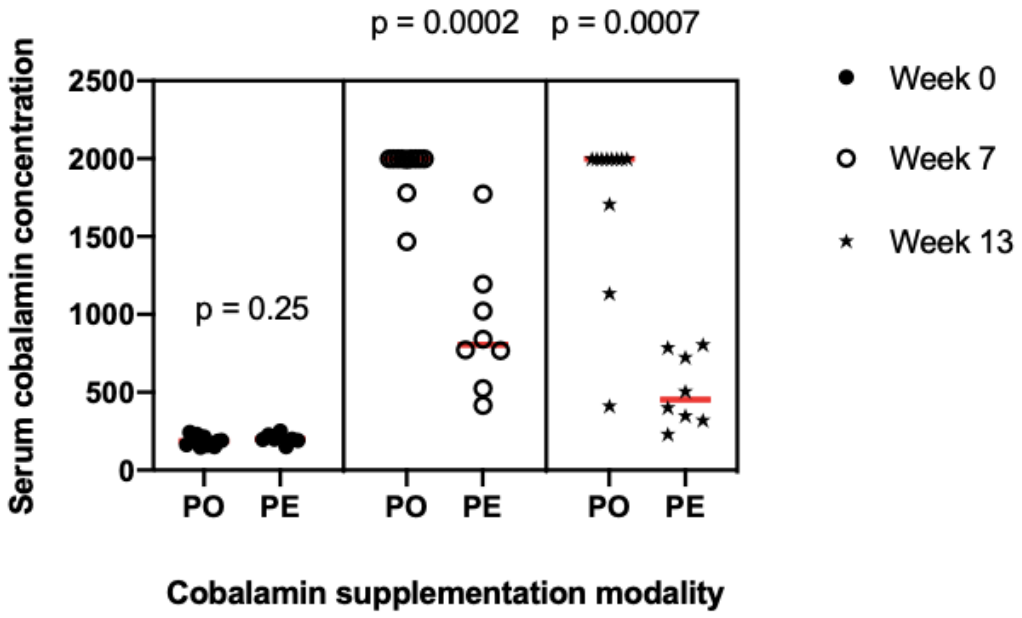

Figure 6: Serum cobalamin concentration at week 0, 7 and 13 in dogs receiving oral and parenteral cobalamin supplementation. Long horizontal lines represent medians. Data for the oral (peroral, PO) group are shown on the left in each panel (n = 11 at each time point) and data for the parenteral (PE) group are shown to the right (n = 8 at each time point).

Treatment failure defined by recurrence of hypocobalaminaemia despite cobalamin supplementation for 13 weeks, was identified in one dog. This dog was treated with parenteral supplementation (Table 8).

Persistent hypocobalaminaemia (cobalamin concentration < 250ng/mL)	At week 7		At week 13	
	Oral group	Parenteral group	Oral group	Parenteral group
Number of dogs affected	0	0	0	1

Table 8: Prevalence of persistent hypocobalaminaemia in 19 dogs with chronic inflammatory enteropathy despite oral or parenteral supplementation.

At the time of writing, the MMA concentration results were not available for interpretation.

i. Effects of the cobalamin supplementation modality in different subgroups of dogs

o Definition of subgroups of dogs

We decided to study the response to cobalamin supplementation and the effects of the cobalamin supplementation modality chosen in specific subtypes of dogs, including dogs with severe CIBDAI score at inclusion, dogs with PLE, and dogs with marked to severe hypocobalaminaemia at inclusion.

These subtypes of dogs were defined as follows:

- Presence or absence of PLE
    - We divided our dog population into two subgroups:
        - Dogs with PLE (n = 7)
            - 3 dogs received oral cobalamin supplement
            - 4 dogs received parenteral cobalamin supplement
        - Dogs without PLE (n = 12)
            - 8 dogs received oral cobalamin supplement
            - 4 dogs received parenteral cobalamin supplement
- Severity of hypocobalaminaemia at inclusion
    - We divided our dog population into two subgroups:
        - Dogs with mild hypocobalaminaemia (between 200 and 250ng/mL) (n = 7)
            - 4 dogs received oral cobalamin supplement
            - 3 dogs received parenteral cobalamin supplement
        - Dogs with moderate to severe hypocobalaminaemia (⩽200ng/mL) (n = 12)
            - 7 dogs received oral cobalamin supplement
            - 5 dogs received parenteral cobalamin supplement
- Severity of CIBDAI score at inclusion
    - We divided our dog population into two subgroups:
        - Dogs with mild to moderate CIBDAI score (⩽ 9) (n = 11)
            - 7 dogs received oral cobalamin supplementation
            - 4 dogs received parenteral cobalamin supplement
        - Dogs with severe CIBDAI score (⩾ 10) (n = 8)
            - 4 dogs received oral cobalamin supplementation
            - 4 dogs received parenteral cobalamin supplement

    - Dogs with protein-losing enteropathy (PLE)

Serum cobalamin concentration was assessed in dogs with PLE who received oral cobalamin supplementation, and compared between week 0, week 7 and week 13.

At inclusion

At inclusion, the median cobalamin concentration in dogs with PLE was 170ng/L (range 150-242) in the oral supplementation group and 199ng/L (range 197-252) in the parenteral supplementation group.

Serum cobalamin concentrations were not statistically different between the dogs assigned to receive oral cobalamin supplementation and the dogs assigned to receive parenteral cobalamin supplementation (p-value = 0.372).

At week 7

At week 7, the median cobalamin concentration was 2000ng/L (range 1468-2000) in the oral supplementation group and 931ng/L (range 768-1775) in the parenteral supplementation group.

The cobalamin concentration was significantly higher in dogs receiving oral cobalamin supplementation compared dogs receiving parenteral supplementation (p-value = 0.108).

We strongly suspect that the low number of cases has contributed to the lack of statistically significant difference between the two groups (n=3 in the oral group and n=4 in the parenteral group).

At week 7, the cobalamin concentration was not significantly higher compared to week 0 in dogs receiving oral cobalamin supplementation, but a significant statistical difference was nearly reached (p-value = 0.076). We strongly suspect that the low number of cases has contributed to the lack of statistically significant difference between the two groups.

At week 7, the cobalamin concentration was significantly higher compared to week 0 (p-value = 0.029) in dogs receiving parenteral cobalamin supplementation.

At week 13

At week 13, in dogs with PLE who received oral cobalamin supplementation, the median cobalamin concentration was 2000ng/L (range 2000-2000), compared to 170ng/L (range 150-242) at week 0. Nevertheless, the cobalamin concentration was not statistically different between week 0 and week 13 (p-value = 0.063). This might be due to the small number of cases within the subgroup of dogs (n = 3).

Serum cobalamin concentration was assessed in dogs with PLE who received oral cobalamin supplementation, and compared between week 0 and week 13.
At week 13, in dogs with PLE who received parenteral cobalamin supplementation, the median cobalamin concentration was 614ng/L (range 505-786), compared to 199ng/L (range 197-252) at week 0. The cobalamin concentration was significantly higher at week 13 compared to week 0 (p-value = 0.029).

Serum cobalamin concentration was compared in dogs with PLE who received oral cobalamin supplementation and dogs who received parenteral cobalamin supplementation at week 13.
At week 13, in the subgroup of dogs with PLE, the median cobalamin concentration was 614ng/L (range 505-786) in dogs who received parenteral cobalamin supplementation and 2000ng/L (range 2000-2000) in dogs who received oral cobalamin supplementation. The cobalamin concentration was significantly higher at week 13 in dogs who received oral cobalamin supplementation (p-value = 0.043).

Hence, we conclude that despite the small number of dogs with PLE (7 dogs in total, 3 dogs treated orally and 4 dogs treated parenterally), oral cobalamin supplementation might be at least non-inferior to parenteral cobalamin supplementation at increasing serum cobalamin levels at week 13.

- Dogs with moderate to severe hypocobalaminaemia at inclusion

Serum cobalamin concentration was assessed in dogs with moderate to severe hypocobalaminaemia at inclusion (⩽200ng/mL) who received oral cobalamin supplementation, and compared between week 0, week 7 and week 13.

At inclusion

At inclusion, the median cobalamin concentration in dogs with moderate to severe hypocobalaminaemia was 164ng/L (range 150-190) in the oral supplementation group and 197ng/L (range 150-199) in the parenteral supplementation group.

Serum cobalamin concentrations were not statistically different between the dogs assigned to receive oral cobalamin supplementation and the dogs assigned to receive parenteral cobalamin supplementation (p-value = 0.06).

At week 7

At week 7, dogs with moderate to severe hypocobalaminaemia at inclusion treated with oral cobalamin supplementation had a median cobalamin concentration of 2000ng/L (range 1468-2000) in the oral supplementation group and 772ng/L (range 415-1775) in the parenteral supplementation group.

The cobalamin concentration was significantly higher in dogs receiving oral cobalamin supplementation compared dogs receiving parenteral supplementation (p-value = 0.007).

At week 7, the cobalamin concentration was significantly higher compared to week 0 in dogs receiving oral cobalamin supplementation (p-value = 0.0017).

At week 7, the cobalamin concentration was significantly higher compared to week 0 (p-value = 0.011) in dogs receiving parenteral cobalamin supplementation.

At week 13

At week 13, dogs with moderate to severe hypocobalaminaemia at inclusion treated with oral cobalamin supplementation had a median cobalamin concentration of 2000ng/L (range 412-2000), compared to 164ng/L (range 146-190) at week 0. In this population of dogs, the cobalamin concentration was significantly higher at week 13 compared to week 0 (p-value = 0.0014).

Serum cobalamin concentration was assessed in dogs with moderate to severe hypocobalaminaemia at inclusion who received parenteral cobalamin supplementation, and compared between week 0 and week 13.

At week 13, dogs with moderate to severe hypocobalaminaemia at inclusion treated with parenteral cobalamin supplementation had a median cobalamin concentration of 402ng/L (range 320-786), compared to 197ng/L (range 150-199) at week 0. In this population of dogs, the cobalamin concentration was significantly higher at week 13 compared to week 0 (p-value = 0.011).

Serum cobalamin concentration was compared in dogs with moderate to severe hypocobalaminaemia at inclusion who received oral cobalamin supplementation and dogs who received parenteral cobalamin supplementation at week 13.

At week 13, in the subgroup of dogs with moderate to severe hypocobalaminaemia at inclusion, the median cobalamin concentration was 402ng/L (range 320-786) in dogs who received parenteral cobalamin supplementation and 2000ng/L (range 412-2000) in dogs who received oral cobalamin supplementation. Additionally, in dogs with moderate to severe hypocobalaminaemia at inclusion, the cobalamin concentration was significantly higher at week 13 in dogs who received oral cobalamin supplementation (p-value = 0.009).

Hence, we conclude that despite the small number of dogs with moderate/severe hypocobalaminaemia at inclusion (12 dogs in total, including 7 treated orally and 5 treated parenterally), oral cobalamin supplementation is at least non-inferior to parenteral cobalamin supplementation at increasing serum cobalamin levels at week 13.

- Dogs with high CIBDAI scores at inclusion

Serum cobalamin concentration was assessed in dogs with a high CIBDAI score (CIBDAI score $\geqslant$ 10) at inclusion who received oral cobalamin supplementation, and compared between week 0, week 7 and week 13.

At inclusion

At inclusion, the median cobalamin concentration in dogs with a high CIBDAI score was 201.5ng/L (range 159-242) in the oral supplementation group and 212ng/L (range 150-252) in the parenteral supplementation group.

Serum cobalamin concentrations were not statistically different between the dogs assigned to receive oral cobalamin supplementation and the dogs assigned to receive parenteral cobalamin supplementation (p-value = 0.885).

At week 7

At week 7, the median cobalamin concentration in dogs with a high CIBDAI score at inclusion was 2000ng/L (range 2000-2000) in the oral supplementation group and 806ng/L (768-1194) in the parenteral supplementation group.

At week 7, serum cobalamin concentrations were significantly higher in dogs assigned to receive oral cobalamin supplementation compared to dogs assigned to receive parenteral cobalamin supplementation (p-value = 0.021).

At week 7, the cobalamin concentration was significantly higher compared to week 0 in dogs receiving oral cobalamin supplementation (p-value = 0.021).

At week 7, the cobalamin concentration was significantly higher compared to week 0 (p-value = 0.028) in dogs receiving parenteral cobalamin supplementation.

At week 13

At week 13, dogs with a high CIBDAI score at inclusion treated with oral cobalamin supplementation had a median cobalamin concentration of 2000ng/L (range 2000-2000), compared to 201.5ng/L (range 159-242) at week 0. In this population of dogs, the cobalamin concentration was significantly higher at week 13 compared to week 0 (p-value = 0.021).

Serum cobalamin concentration was assessed in dogs with a high CIBDAI score at inclusion who received parenteral cobalamin supplementation, and compared between week 0 and week 13.

At week 13, dogs with a high CIBDAI score at inclusion treated with parenteral cobalamin supplementation had a median cobalamin concentration of 562ng/L (range 348-805), compared to 212ng/L (range 150-252) at week 0. In this population of dogs, the cobalamin concentration was significantly higher at week 13 compared to week 0 (p-value = 0.028).

Serum cobalamin concentration was compared in dogs with high CIBDAI score at inclusion who received oral cobalamin supplementation and dogs who received parenteral cobalamin supplementation at week 13.

At week 13, in the subgroup of dogs with moderate to severe hypocobalaminaemia at inclusion, the median cobalamin concentration was 562ng/L (range 348-805) in dogs who received parenteral cobalamin supplementation and 2000ng/L (range 2000-2000) in dogs who

received oral cobalamin supplementation. Additionally, in dogs with moderate to severe hypocobalaminaemia at inclusion, the cobalamin concentration was significantly higher at week 13 in dogs who received oral cobalamin supplementation (p-value = 0.021).

j. Treatment tolerance

Questionnaires were fulfilled at inclusion and week 13 in 16 dogs (16/19). Data from questionnaires were available in nine dogs treated with oral cobalamin supplementation (n = 9) and seven dogs with parenteral supplementation (n = 7).

o Quality of life: oral versus parenteral cobalamin supplementation

We decided to determine whether there was a difference in the "quality of life score" between the dog population who received oral cobalamin supplementation and the dog population who received parenteral cobalamin supplementation. As described in the "materiel & methods" chapter, the "quality of life score" summarises the quality of life of the treated dog and his/her owner during the study, as reported by the dog's owner via a thorough questionnaire. The scoring system provides a number out of 32 which was recalculated to obtain a final number out of 10.

Dogs treated with oral cobalamin had a median "quality of life score" of 10/10 (range: 8.4-10) compared to a median of 9.7/10 (range: 7.8-10) in dogs treated with parenteral cobalamin. According to the Whitney-Mann U test, there was no statistical difference of the "quality of life score" between dogs receiving oral and dogs receiving parenteral cobalamin supplementation (p = 0.097).

o Owners' satisfaction: oral versus parenteral cobalamin supplementation

The "owner's satisfaction score" used for dogs receiving oral or parenteral cobalamin corresponded to a subgroup of questions within the quality-of-life questionnaire. This score

was initially expressed out of 8 and then calculation allowed to obtain a final number out of 10.

There was no statistical difference of the "owner's satisfaction score" between dogs receiving oral and dogs receiving parenteral cobalamin supplementation (p = 0.55). Only one owner notified that he would have preferred the "other treatment modality", should another cobalamin supplementation protocol be necessary. His dog belonged to the parenteral cobalamin supplementation group.

- Tolerance to oral cobalamin capsules compared to cobalamin injections

In order to answer the question to whether one treatment modality was better tolerated to the other, we compared the "tolerance scores" between dogs receiving oral cobalamin capsules and dogs receiving cobalamin injections.

The "tolerance score" used for dogs receiving oral cobalamin was a mean of "the dog's response to taking cobalamin capsules" and "the behavioural changes when being given the cobalamin supplement" calculated out of 72.

The "tolerance score" used for dogs receiving parenteral cobalamin was "the dog's response to visiting the vets for the cobalamin injections" calculated out of 48. Both scores were expressed in a final number out of 10.

Dogs treated with oral cobalamin had a median "tolerance score" of 8.6/10 (range: 4.4-10) compared to a median of 7.7/10 (range: 5-9.2) in dogs treated with parenteral cobalamin. According to the Whitney-Mann U test, there was no statistical difference in terms of treatment tolerance between dogs receiving oral versus parenteral cobalamin supplementation (p = 0.22).

- Tolerance to oral cobalamin capsules administration compared to other tablets

In order to determine whether the "Cobalaplex" capsules were better tolerated than other types of capsules/tablets, we compared the "tolerance score" of dogs when they received

other oral treatments in the past ("Dog's response to taking capsules or tablets before this trial"), to their current "tolerance score" while receiving the "Cobalaplex" capsules ("Dog's response to taking cobalamin capsules"). Both scores were calculated to obtain a number out of 10. The nature of the tablets or capsules administered prior to this trial was not documented.

Dogs treated with oral tablets before this trial (other than cobalamin tablets), had a median "tolerance score" of 6.4/10 (range: 0-10). The same dogs given Cobalaplex capsules had a median "tolerance score" of 8.6/10 (range: 0-10). According to the Whitney-Mann U test, there was no statistical difference between the two sets of "tolerance score" ($p = 0.44$).

o "Cobalaplex" administration modalities

Owners were asked how they administered the "Cobalaplex" capsules to their dog, including:

- Capsule opened, sprinkled on the dog's food and left unmixed;
- Capsule opened and contents mixed with the dog's regular food;
- Capsule unopened (entire) hidden in the dog's regular food;
- Capsule unopened (entire) given with a treat or other palatable foodstuff;
- Capsule unopened (entire) given alone;
- Capsule given with another technique.

Two different administration modalities were reported in three dogs. In the remaining six dogs, only one capsule administration modality was used. The most commonly used administration modality was "capsule unopened (entire) hidden in the dog's regular food" which was reported in five dogs (5/9). The entire unopen capsule was given alone in three dogs (3/9) or wrapped in a treat in three other dogs (3/9). The capsule required to be opened and sprinkled on the dog's food in only one dog (1/9) (Table 9).

These data, along with high quality of life scores and high tolerance scores in dogs receiving oral "Cobalaplex" suggest that these capsules were easy to administer and well-tolerated by dogs and owners.

Oral cobalamin supplement administration modality	Number of dogs given this administration modality (n = 9)
Capsule opened, sprinkled on the dog's food and left unmixed	1 (1/9)
Capsule opened and contents mixed with the dog's regular food	0 (0/9)
Capsule unopened (entire) hidden in the dog's regular food	5 (5/9)
Capsule unopened (entire) given with a treat or other palatable foodstuff	3 (3/9)
Capsule unopened (entire) given alone	3 (3/9)
Capsule given with another technique	0 (0/9)

Table 9: Details of the "Cobalaplex" administration modality(ies) spontaneously chosen by dogs' owners.

IV. Discussion

This study compared oral and parenteral cobalamin supplementation in dogs with chronic inflammatory enteropathy and hypocobalaminaemia.

In this study, we have shown that oral cobalamin supplementation was non inferior to parenteral cobalamin supplementation in dogs with hypocobalaminaemia secondary to chronic inflammatory enteropathy. Similar conclusions had been demonstrated by other authors recently (Toresson et *al.* 2018).

- At inclusion, the two groups of dogs (oral and parenteral cobalamin supplementation) were homogeneous in terms of body weight, CIBDAI score and cobalamin concentration. Body weight, CIBDAI score, and cobalamin concentration were not statistically different between the dogs assigned to receive oral cobalamin supplementation and the dogs assigned to receive parenteral cobalamin supplementation.
- At week 7, both oral and parenteral supplementations were effective at increasing the serum cobalamin concentrations. We showed that cobalamin concentration was significantly higher at week 7 in dogs receiving oral and parenteral cobalamin supplementation compared to week 0.
- At week 13, compared to week 7, serum cobalamin levels were stable in the group of dogs receiving oral cobalamin. At week 13, the serum cobalamin concentration was significantly higher in the oral cobalamin supplementation group compared to the parenteral cobalamin supplementation group.

We also demonstrated that parenteral cobalamin supplementation resulted in a significant serum cobalamin concentration increase at week 7, followed by a significant drop at week 13. Additionally, the only treatment failure at week 13 identified, was in a dog belonging to the parenteral cobalamin supplementation group.

- We hypothesised several causes for this decrease in parenteral cobalamin supplementation efficiency at increasing serum cobalamin levels at week 13:
  1) The form of injectable cobalamin administered

     Our dog population treated with parenteral cobalamin supplementation received injectable cyanocobalamin. However, hydroxycobalamin is likely more effective than cyanocobalamin because this compound presents the natural form of cobalamin (Farquharson and Adam, 1976). Additionally, some veterinarian authors have already suspected that hydroxocobalamin might have been more effective in some dogs whose serum cobalamin concentrations had not been adequately restored following cyanocobalamin supplementation (Steiner J.M., personal communication).
  2) In our study, based on current guidelines, the oral supplementation protocol provided a much higher weekly amount of cobalamin compared to the parenteral supplementation protocol. Dogs treated with oral cyanocobalamin supplementation received 25-50ug/kg once daily compared to 25-50ug/kg once weekly in dogs with parenteral supplementation. This means that dogs treated with oral supplementation received seven times more cobalamin, on a weekly basis, compared to dogs supplemented parenterally.
- Nevertheless, this drop in cobalamin concentration may not be clinically relevant.
  1) First, the CIBDAI scores were significantly lower at week 13 compared to week 0 in dogs receiving parenteral cobalamin supplementation (p-value 0.009). However, CIBDAI scores were not statistically different at week 13 compared to week 7 in the same dog population (p-value = 0.10).
  2) Second, the dog who technically experienced a “treatment failure” had a low CIBDAI score of 1 which renders its clinical relevance questionable.
- Interestingly, a drop in serum cobalamin concentration was identified between day 28 and day 90, in dogs receiving parenteral cobalamin supplementation. The same trend was identified by Toresson et *al.* (2018) in dogs receiving parenteral cobalamin supplementation. In the parenteral group, the median serum cobalamin concentration was 228 ng/L (range 150–285 ng/L) at the time of inclusion, 2065 ng/L (range 725–10,009 ng/L; $P < 0.001$) after 28 days and 874 ng/L (range 188–1267 ng/L; $P < 0.001$) after 90 days. It would be interesting to follow these patients for a longer period to determine whether this trend continues to worsen.

It may be important to notice that dogs receiving parenteral cyanocobalamin supplementation received their weekly injections at day 0, 7, 14, 21, 28, 35 and day 63. This means that at day 90 (which represents the end of the study period), the last cobalamin injection has been administered 27 days before. Conversely, dogs with oral supplementation were still receiving daily cobalamin capsules at day 90. This may explain the drop in cobalamin between week 7 and week 13 in dogs receiving parenteral cobalamin.

One of the main limitations of the study was the small number of dogs enrolled. Despite the prospective nature of the study, numerous data were missing which reduced considerably the number of dogs able to be included in the final data analysis.

Another limitation of the study is the presumptive "inflammatory" nature of the underlying chronic enteropathy in a number of cases. In our study population, hypocobalaminaemia was assumed to be secondary to chronic inflammatory enteropathy without excluding:

- Common causes of hypocobalaminaemia such as intestinal lymphoma, infectious enteritis (intestinal parasitism, bacterial enteritis…),
- Less common causes of hypocobalaminaemia such as primary intestinal lymphangiectasia, hypoadrenocorticism,
- Uncommon aetiologies of hypocobalaminaemia such as IGS and dietary cobalamin deficiency.

IGS was not excluded via genetic tests available in some breeds, although this disease represented an exclusion criterion. There was only one dog, a 11-year-old Beagle dog, who belonged to a predisposed breed for IGS, and who was enrolled in the study. However, the age of this dog makes familial cobalamin deficiency less likely than chronic inflammatory enteropathy.

The diet provided before the start of the trial, including the exact amount of daily dietary cobalamin, was only documented in a few dogs. Therefore, dietary cobalamin deficiency could not be fully excluded as a cause of hypocobalaminaemia. Nevertheless, most commercial foods and non-vegetarian/non-vegan home-made foods are not restricted in cobalamin which makes dietary cobalamin deficiency relatively unlikely in our dog population.

Data related to investigative procedures to diagnose the underlying gastrointestinal disease remained unknown in six dogs (6/19). Moreover, intestinal endoscopy-guided biopsies were conducted in only nine dogs (9/19). No full thickness biopsies were performed. Faecal analysis was conducted in only two dogs. Therefore, conditions such as intestinal parasitism, primary lymphangiectasia or intestinal lymphoma could have been misdiagnosed.

In order to address these weaknesses, in addition to the initial serum cobalamin and MMA assessment, the initial diagnostic workup should include:

- Haematology, serum biochemistry, electrolytes, serum folate, TLI, basal cortisol (+ ACTH stimulation test if the basal cortisol is below or equal 55nmol/L),
- A full faecal analysis (faecal coproscopy, faecal culture, faecal Giardia and Cryptosporidium antigen detection),
- An abdominal ultrasound,
- Intestinal biopsies (full thickness, or endoscopic biopsies).

Ideally, hereditary cobalamin malabsorption and dietary cobalamin deficiency should be ruled out by:

- Performing a genetic test for IGS in every case recruited,
- Recording precisely the diet provided and its composition.

Among the seven dogs diagnosed with hypoproteinaemia and/or hypoalbuminaemia secondary to PLE, other differentials for these biochemical abnormalities such as protein-losing nephropathy (PLN) or hepatic insufficiency were not systematically ruled out.

We showed that oral and parenteral cobalamin supplementations were both well tolerated and provided similar scores in terms of “quality of life”, “owner’s satisfaction” or “treatment tolerance”.

Interestingly, oral cobalamin supplementation was shown to be at least non-inferior to parenteral cobalamin supplementation at treating hypocobalaminaemia associated with CIE in dogs presented with PLE or moderate/severe hypocobalaminaemia or severe CIBDAI scores at inclusion. This finding emphasises that the severity of the disease should not prevent clinicians to use an oral supplementation modality.

Two dogs were diagnosed with subclinical exocrine pancreas insufficiency. It would have been interesting to be able to determine whether these dogs demonstrated a different response to these two cobalamin supplementation modalities. A larger dog population would be required to evaluate this assumption which opens the possibility for future studies.

V. Future research

As mentioned in the discussion section above, the main limitation of our study remains the small number of dogs recruited. Therefore, a larger study population would be required to confirm our results. We are currently investigating the possibility to recruit a larger number of dogs for this purpose.

At the time of writing the MMA results were not available in our study population. Further work should include collection and interpretation of the MMA concentration pre- and post-cobalamin supplementation. It would be interesting to determine:

1. Whether hypocobalaminaemia correlates with an increase in MMA level (i.e. functional cobalamin deficiency)
2. Whether one treatment modality is more performant than the other to address functional cobalamin deficiency.

A drop tendency was identified in the cobalamin concentration of dogs supplemented parenterally between week 7 and week 13 without statistical significance. However, this trend was also documented in Toresson et al.'s study (2019). Hence, it may be of interest to reassess cobalamin and MMA concentration two months after week 13 in our study population, in order to determine:

1. Whether dogs have persistent eucobalaminaemia, and normal MMA concentration.
2. Whether one route of administration (oral versus parenteral) provides better results.

Several medications such as antibiotic, probiotics and proton pump inhibitors can affect intestinal cobalamin absorption by modifying the intestinal pH and/or the intestinal microbiota. It would be of great interest to assess:

1. The impact of those drugs on cobalamin and MMA concentration in our study population.
2. Whether one treatment modality is more performant than the other to address cobalamin deficiency in dogs with hypocobalaminaemia secondary to chronic enteropathy receiving these medications.

Finally, two dogs in our study population were diagnosed with EPI. We would like to recruit more of these patients to be able to compare the ability of oral versus parenteral cobalamin supplement to reach eucobalaminaemia in dogs with clinical and subclinical EPI.

Interestingly, the median quality of life and tolerance scores were slightly higher in the oral group without statistical difference. Part of the reason for comparing the two routes of cobalamin supplementation was to see whether one route was better tolerated in the absence of less efficacy. This trend suggests that the oral cobalamin supplement might be preferable from an owner and dog point of view. A larger study would be valuable in order to see whether a statistical difference would have been reached with a larger dog population.

VI. Conclusion

We have demonstrated that oral cobalamin supplementation was non inferior to parenteral cobalamin supplementation at increasing serum cobalamin levels at week 13 in dogs with hypocobalaminaemia secondary to chronic inflammatory enteropathy. Despite the small number of dogs allocated to the different subgroups, oral cobalamin supplementation was shown to be at least non-inferior to parenteral cobalamin supplementation at increasing serum cobalamin levels at week 13, in hypocobalaminaemic dogs presented with PLE or moderate/severe hypocobalaminaemia at inclusion or severe CIBDAI score at inclusion. This finding highlights the relevance of oral cobalamin supplementation, even in patients with a severe clinical picture at presentation. Regardless the clinical presentation, clinicians should consider equally oral or parenteral supplementation modalities. Both supplementation modalities were well tolerated and provided comparable "quality of life", "owner's satisfaction" or "treatment tolerance" scores.

In conclusion, oral cobalamin supplementation is a suitable and well-tolerated treatment modality to address hypocobalaminaemia in dogs with chronic inflammatory enteropathy. Additionally, this treatment option might be found more convenient and less stressful for some owners compared to weekly parenteral injections.

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APPENDICES

Appendix 1: Dose selection

Animals will be supplemented as follows:

1. **Oral cobalamin supplementation:**

Cobalaplex capsules (0.5 mg cyanocobalamin/ capsule)

Body weight	Number of capsules	Frequency
<10 kg	0.5	Once daily
10 kg to 20 kg	1	Once daily
>20 kg	2	Once daily

2. **Parenteral cobalamin supplementation:**

Vitbee 250 solution for injection (0.025% w/v cyanocobalamin – 250ug/mL)

Body weight	Volume (ml) injected subcutaneously	Frequency
<10 kg	1	Once a week for 6 weeks and then once 4 weeks later
10 kg to 20 kg	2	Once a week for 6 weeks and then once 4 weeks later
>20 kg	4	Once a week for 6 weeks and then once 4 weeks later

Appendix 2: Consent form for owners

Protexin® veterinary

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Consent form

Title of the study: Efficacy of oral versus parenteral cyanocobalamin supplement in normalising serum cobalamin in dogs with hypocobalaminaemia and chronic enteropathy

Researchers:
Dr Mark Dunning – University of Nottingham, School of Veterinary Medicine and Science mark.dunning@nottingham.ac.uk or 01332548911

Collaborators:
Dr Sophie Nixon - Protexin Veterinary® Sophie.Nixon@protexin.com

Purpose of the study:
Vitamin B12 (cobalamin) deficiency in dogs can occur due to a variety of diseases, these are most commonly gut problems such as long-standing diarrhoea but, in some dogs, (and people) it can also result from a problem they are born with that makes absorbing cobalamin very difficult. Low cobalamin can make dogs very unwell as it is important for a number of processes to occur effectively in the body. These include blood cell production, making sure the immune system functions properly and making sure general health is appropriate. Cobalamin deficiency is currently treated by injections to bring the levels back to normal. These are required every month for 4-6 months and sometimes monthly for longer periods of time. The injections can sting and cause local irritation. A tablet version of cobalamin is available, and from initial studies it seems as effective as injections for bringing cobalamin levels back to normal. We are therefore asking owners of dogs who require cobalamin to help with a study to gather more evidence to show that the tablet form of cobalamin is as effective. Further information about the study is available on the accompanying study sheets.

Consent:
This consent form is a formal way of indicating that you agree to participate in this study and that you understand that any information collected by the researchers:

- will be used for a research study
- may be written in a report for publication
- may be presented at research conferences or meetings
- will be anonymised and treated confidentially
- will only be accessed by research colleagues
- that you can request to see a copy/summary of the completed study

- that you can request to see any information written down/kept during the process of data collection.

	please tick
I have read the Owner Information Sheet and the nature and purpose of the research project has been explained to me. I understand and agree to take part.	
I understand that participation in this study is voluntary and that I may choose to no longer be part of the study at any time (without needing to provide reasons for doing so). I understand this will not affect my status now or in the future.	
I agree that information I give during the study can be used in a report, a published paper or a conference or meeting presentation.	
I understand that the study is being conducted for the purposes of research.	
I understand that data will be stored for the purposes of this research project. This will be stored securely and will be accessed only by the researchers taking part in this study.	
I understand that I can request to see a summary of the findings, and I can also request to see any notes made during the process of my data collection.	
I understand that I may contact the researcher if I require further information about the research.	

If you have any queries regarding this study, please speak to the researcher directly or contact them via e-mail or phone (details above).

Please tick here if you would like to receive a summary of the study results, and indicate how you would like to be contacted

.....

Participant

Name:..... Signature:.....

Date:.....

Researcher

Name: Dr Mark Dunning Signature: 

Thank you very much for participating in this study we very much appreciate your involvement.

Appendix 3: Owner background information sheet

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School of Veterinary Medicine and Science

Owner background information sheet

We would like to introduce our project which is looking at investigating whether a different method to supplementing vitamin B_{12} can be as effective as the current method which uses periodic injections. Your dog has been diagnosed with a vitamin B_{12} (cobalamin) deficiency. Cobalamin is a vitamin that is present in food and is absorbed from the gut when dogs (and people for that matter) eat. When a dog has damaged intestines, for example from a longstanding problem such as inflammatory bowel disease (similar to Crohn's disease in humans), the wall of the intestines which absorbs cobalamin, doesn't work properly. In addition, if the normal bacteria in the gut are out of balance they can use the cobalamin. In both of these situations the body becomes deficient in cobalamin and this can lead to serious illness. Rarely low cobalamin can occur because dogs have been born with an inability to absorb appropriate levels of the vitamin from their food.

Cobalamin is important in various processes in the body, including how blood cells and the immune system work, it is also important in keeping the body generally healthy as animals that have low cobalamin levels are generally poorly thrifty. Dogs with low cobalamin can show various signs of being unwell including poor appetite, weight loss, ongoing problems with their intestines, susceptibilities to infections, anaemia and problems with their nervous systems. It is important therefore that treating a low cobalamin level is effective as this has a significant impact on animals' quality of life. Unfortunately, when treating the underlying problem in dogs with low cobalamin (whether this is intestinal problems or abnormal bacterial levels in the gut), the levels don't always improve. As a result of this it is usually necessary to supplement cobalamin in addition to the treatment for the underlying problem. Cobalamin has been treated for many years by regular injections. This is a very effective treatment, with dogs receiving injections every week for 6 weeks and then monthly until the blood levels are normal. Sometimes dogs need to remain on these injections indefinitely.

So, why would we be looking for an alternative?

Cobalamin injections require your dog to go to your vet weekly or monthly, and the bottle containing the injection only lasts for 28 days. The injection can sting when it is given and can cause some local reaction under the skin.

Tests in humans have recognised that using cobalamin tablets or capsules can be a very effective alternative to injections to treat low cobalamin. The use of tablets instead of the injection would mean fewer visits to the vet and avoid the injections and discomfort this may cause. In addition, tablets should be cheaper and reduce the need to visit the vets.

What is a clinical trial?

A clinical trial is a piece of research to test if a particular treatment is effective. This study is examining whether the tablets are an easy and equally effective method by which cobalamin can be supplemented in dogs when compared to the current standard treatment which is the injection. There is already some evidence that cobalamin tablets work in dogs and so taking part in this study will not put your dog at a disadvantage to those having injections. Some dogs in this trial will be given cobalamin capsules and some will be given cobalamin injections. By comparing the response in these two groups we will establish if tablets are equally or more effective than the injectable version in treating dogs with cobalamin deficiency.

What is the benefit to me and my pet?

Your dog will be closely monitored throughout the trial by your vet. Your dog will not need to have any additional blood samples taken compared to a patient being treated normally for cobalamin deficiency outside of the study. The cost of the cobalamin supplementation and the blood tests to measure the concentration in the blood will be covered for the length of the study.

Are there any risks?

All the ingredients in both the oral (capsules) and the injectable cobalamin supplement are safe and already used to treat cobalamin deficiency in dogs. If your dog's condition worsens during the trial and they need further medication they will immediately be removed from the trial and treated appropriately. If a blood test 7 weeks after starting the trial shows that your dog still has a low blood cobalamin level they will be withdrawn from the study. You can however remove your dog from the trial at any time should you so wish.

What do I need to do?

During the initial consult with your vet at the start of week 0, the vet will discuss the trial with you and ask you questions to ensure that your pet is eligible for the trial. They will also give your dog a Canine Inflammatory Bowel Disease Activity Index (CIBDAI) score following discussion with you and give you instructions to complete this score yourself during the study. Your dog may require a blood sample at this appointment.

To determine whether the cobalamin treatment is working your dog will need to see your vet for a re-examination and repeat blood tests at week 7 and week 13 of the study. If your dog is receiving the injectable version of cobalamin, they will need to be taken to your vet once a week for 6 weeks and then every month until the levels are normal. If your dog is receiving cobalamin tablets, you will need to give these once daily for the entire duration of the study (this is a powder that can be mixed with food to help with administration).

If you have any further questions, please do not hesitate to discuss them with your vet in the first instance. If they have any further questions they will be able to discuss them with the organisers of the study.

We would like to thank you in advance for participating in this study, it is extremely important. The information it provides will help vets to make better choices for their patients and improve the long-term quality of life for dogs with low levels of cobalamin in their blood.

Dr Mark Dunning
(on behalf of the collaborators and collaborating institutions)

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RCVS and EBVS® European Veterinary Specialist in Small Animal Internal Medicine
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Appendix 4: Canine Inflammatory Bowel Disease Activity Index Form (CIBDAI)

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Canine Inflammatory Bowel Activity Index Form

Name	
Age	
Sex	
Date	

Parameter	Score	Scoring	Comments
Attitude/Activity		0 - normal 1 – slightly decreased 2 – moderately decreased 3 – severely decreased	
Appetite		0 – normal 1 – slightly decreased 2 – moderately decreased 3 – severely decreased	
Vomiting		0 - none 1 – mild (1x/week) 2 – moderate (2-3x/wk) 3 – severe (>3x/wk)	
Stool consistency		0 – normal 1 – slightly soft or blood or mucus or both 2 – very soft faeces 3 – watery diarrhoea	
Stool frequency		0 – normal 1 – slightly increased (2-3x/d) 2 – moderately increased (4-5x/d) 3 – severely increased (>5x/d)	
Weight loss		0 – none 1 – mild (<5%) 2 – moderately (5-10%) 3 – severe (>10%)	
Total			

Results

0-3	clinically insignificant disease
4-5	mild IBD
6-8	moderate IBD
9 or >	severe IBD

Appendix 5: Owner quality of life questionnaire following completion of the cobalamin study

Owner quality of life questionnaire following completion of the cobalamin study

We would like to thank you for being involved in this study investigating the benefits of different methods of vitamin B12 (cobalamin) in dogs. We hope very much you enjoyed being part of this study which will help us improve our approach to treating this condition. As you will therefore be aware, we can treat low blood levels of cobalamin either by the administration of a series of injections given at your veterinary practice a twelve-week period, or by the administration of a capsule that is opened and sprinkled on food once daily for twelve weeks. An important part of understanding how effective a particular treatment can be for dogs, is understanding how easy an owner feels it is to administration or adhering to the instructions provided by the veterinary surgeon and the time of the initial prescription. We would therefore as one final task, ask you to complete this short questionnaire. This will enable us to understand more about each treatment type and whether in the end using capsules instead of injections seems like a feasible alternative. During this study your dog has received either the injections or capsules. If your dog received treatment with cobalamin by injection, for the purpose of these questions 'administration' of treatment includes making and attending appointments for your dog's injections. For dogs in both treatment groups these questions refer only to treatment for your dog's cobalamin deficiency, and not to other treatment that your dog may have received over the same time period. Thinking about the treatment that your dog received, please answer the following questions:

- How easy or difficult is it to administer the cobalamin supplement in its current form?

very difficult	quite difficult	neither difficult nor easy	quite easy	very easy

- How easy or difficult is it to plan when you will use the cobalamin supplement each time?

very difficult	quite difficult	neither difficult nor easy	quite easy	very easy

- How easy or difficult is it to adhere to the treatment regime as instructed?

very difficult	quite difficult	neither difficult nor easy	quite easy	very easy

- How stressful (e.g. nervous/anxious) did you find it to administer the course of treatment as instructed

extremely stressful	moderately stressful	slightly stressful	neither stressful nor easy	easy

- How stressful did your dog find the course of treatment (not stressful at all… very stressful)

extremely stressful	moderately stressful	slightly stressful	neither stressful nor easy	easy

- Thinking only about the administration of the treatment (i.e. not whether you think it worked or not), how satisfied or dissatisfied are you with how the cobalamin supplement is administered?

very dissatisfied	quite dissatisfied	neither dissatisfied nor satisfied	quite satisfied	very satisfied

- How easy did you find it to comply with the instructions the veterinary surgeon provided you with at the start of the study (i.e. manage to give the capsules each day or attend the veterinary appointments for the injections to be administered)

very difficult	quite difficult	neither difficult nor easy	quite easy	very easy

- Taking all things into account, how satisfied or dissatisfied are you with the cobalamin supplement overall?

very dissatisfied	quite dissatisfied	neither dissatisfied nor satisfied	quite satisfied	very satisfied

- Finally, if your dog required treatment for cobalamin deficiency in the future, would you choose the same treatment that your dog received this time, or would you try the other treatment?

Same treatment	The other treatment

- Please indicate whether your dog was using the capsules or having the injections

Capsules	Injections

Thank you once again for taking part in this study, your involvement is invaluable in determining the most appropriate way in which to supplement dogs with cobalamin.

Dr Mark Dunning
(on behalf of the collaborators and collaborating institutions)

MA VetMB PhD CertSAM DipECVIM-CA MRCVS
RCVS and EBVS® European Veterinary Specialist in Small Animal Internal Medicine
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Appendix 6: Tolerance Questionnaire for dogs receiving Parenteral Cobalamin Supplementation

Tolerance Questionnaire for dogs receiving Parenteral Cobalamin Supplementation

1) When thinking about your dog's response to visiting the vets before this trial, please select the most appropriate descriptions:

My dog…	Almost every time	A lot of the time	Sometimes	Rarely	Never	N/A
Has to be dragged or carried into the vets						
Walks in hesitantly or hides behind you						
Walks in pulling the lead						
Walks in without pulling the lead or hiding behind you						
Trembles when in the consulting room						
Whimpers/whines in the consulting room						
Pants when in the consulting room						
Licks its lips regularly when in the consulting room						
Yawns repeatedly when in the consulting room						
Walks up to and greets the vet						
Stays close to the door of the consulting room						

2) When thinking about your dog's response to visiting the vets for the cobalamin injections, please select the most appropriate descriptions:

My dog…	Almost every time	A lot of the time	Sometimes	Rarely	Never
Had to be dragged or carried into the vets					

Walked in hesitantly or hid behind you					
Walked in pulling the lead					
Walked in without pulling the lead or hiding behind you					
Trembled when in the consulting room					
Whimpered/whined in the consulting room					
Panted when in the consulting room					
Licked its lips regularly when in the consulting room					
Yawned repeatedly when in the consulting room					
Walked up to and greeted the vet					
Stayed close to the door of the consulting room					
Pressed itself against you or tried to hide behind you when the injection was administered					

Appendix 7: Tolerance and Palatability Questionnaire for dogs receiving Oral Cobalamin Supplementation

Tolerance & Palatability Questionnaire for dogs receiving Oral Cobalamin Supplementation

1) When thinking about **your dog's response to taking capsules or tablets before this trial**, please select the most appropriate descriptions:

My dog…	Almost every time	A lot of the time	Sometimes	Rarely	Never	N/A
Eats tablets voluntarily as though they are food treats						
Will readily eat a tablet when its hidden in food (i.e. wet food, chicken, cheese, peanut butter etc.)						
Spits out or sifts out tablets that were hidden in food						
Refuses to eat food if it thinks there's a tablet in it						
Refuses to take a tablet no matter what, so I have to drop it down their throat by hand or use a pill pusher						

2) Thinking about your dog's response to taking **these cobalamin supplements**, please select the most appropriate descriptions:

My dog…	Every time (or almost every)	A lot of the time	Sometimes	Rarely	Never	N/A
Ate the supplement voluntarily as though it was a food treat						
Ate the supplement when it was hidden in food (i.e. wet food, chicken, cheese, peanut butter etc.)						

Spat out or sifted out the supplement that was hidden in food						
Refused to eat food if it thought the supplement was in it						
Refused to take the supplement no matter what, so I had to drop it down their throat by hand or use a pill pusher						

3) Please select whether your dog ever showed any of the following behaviour **when being given the cobalamin supplement:**

	My dog did this every time	My dog did this most of the time	My dog did this sometimes	My dog did this rarely (once or twice)	No, my dog never did this
Tried to hide or get away when it knew I was getting/had them					
Tried to move/pull away from me					
Approached me voluntarily when it knew I was getting/had them					
Trembled or shook					
Wagged its tail					
Held its tail low between its legs					
Growled					
Whined					
Barked					
Began to pant					
Appeared to want the supplement as though it was food					

Please answer the following additional question regarding administration of the cobalapex product to help us interpret the results from the above survey:

How did you administer the supplement (please check every box that applies):

- ☐ I opened the capsule and sprinkled the contents on top of my dog's food and left unmixed
- ☐ I opened the capsule and mixed the contents with my dog's regular food
- ☐ I hid the entire unopened capsule in my dog's regular food
- ☐ I gave the entire unopened capsule with a treat or other foodstuff
- ☐ I gave the entire unopened capsule alone
- ☐ I used another technique (please indicate this to us below):

Please indicate for each of the methods selected above how successful each was:

My dog…	Every time (or almost every)	A lot of the time	Sometimes	Rarely	Never	N/A
I opened the capsule and sprinkled the contents on top of my dog's food and left unmixed						
I opened the capsule and mixed the contents with my dog's regular food						
I hid the entire unopened capsule in my dog's regular food						
I gave the entire unopened capsule with a treat or other foodstuff						
I gave the entire unopened capsule alone						
I used another technique (please indicate this to us below)						

Many thanks for taking the time to complete this short survey on the potential impact on your dog's quality of life whilst they were receiving their medication.