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ORAL COBALAMIN SUPPLEMENTATION IN DOGS WITH CHRONIC ENTEROPATHIES AND LOW SERUM COBALAMIN CONCENTRATIONS

COMPARATIVE STUDIES AND EFFECTS ON BIOCHEMICAL MARKERS OF INTRACELLULAR COBALAMIN DEFICIENCY

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ACADEMIC DISSERTATION

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ABSTRACT

Cobalamin (cbl) deficiency is a common sequela to chronic enteropathies (CE) in dogs. Numerous metabolic and clinical consequences have been reported in association with cbl deficiency, as has a poorer prognosis of the underlying disease. The recommended treatment for dogs is multiple parenteral cbl injections. This is based on the theoretical reasoning that the diseased intestine in CE is incapable of absorbing cbl and on case reports of successful parenteral cbl supplementation. However, to the best of our knowledge, no studies have been performed on dogs with CE and low cbl concentrations to evaluate the effect of oral cbl supplementation, nor has the currently recommended parenteral cbl protocol been systematically validated. This is in contrast to human medicine, where comparative studies have shown equal efficacy of parenteral and high-dose oral cbl supplementation. Oral supplementation is a cost-effective and very simple treatment in human patients since it can be performed at home, as opposed to parenteral treatment.

This thesis aimed to evaluate the effects of oral cbl supplementation on serum cbl concentrations in dogs with CE and low serum cbl concentrations. Further, a comparison of oral and parenteral supplementation was performed and intracellular markers of cbl deficiency, such as homocysteine (HCY) and methylmalonic acid (MMA), were analyzed and compared during the treatment period.

First, a retrospective study was performed on dogs with CE and low serum cbl concentrations that had received oral cbl supplementation in clinical practice by the author. A significant difference was found when comparing serum cbl concentrations before and after supplementation. All dogs had serum concentrations within or above the cbl reference interval after supplementation. The increase in serum cbl concentrations did not differ between dogs with low or high Canine Inflammatory Bowel Disease Activity Index (CIBDAI) at inclusion. Neither did the response to oral cbl differ between dogs with a subnormal serum cbl at inclusion and dogs with a low-normal serum cbl concentration (i.e. within the lowest 5% of the normal reference interval). Further, the increase in serum cbl concentration did not differ between dogs that had no change in diet or treatment apart from the addition of cbl versus dogs that were treated with cbl plus a change of diet and/or change of medical treatment during supplementation.

Second, a prospective block-randomized study was performed, comparing serum cbl concentrations in dogs treated with peroral (PO) versus parenteral (PE) cbl supplementation. All dogs of both groups were within the upper half of the reference interval or above the reference interval limit after 28 days of cbl supplementation. The PO group had significantly lower serum cbl concentrations at this time-point than the the PE group. Ninety days after starting supplementation, the PO group had a significantly higher serum cbl concentration than the PE group, in which one dog was again hypocobalaminemic. In the PO group, a significant continuous increase in

serum cbl concentration was registered when comparing baseline and 28 days of supplementation, as well as when comparing 28 days and 90 days of treatment. In the PE group, a significant increase in serum cbl concentration was evident when comparing baseline and 28 days of treatment, but a significant decrease was seen after 90 days relative to 28 days. At this time-point, the last cbl injection had been given 3-4 weeks earlier.

Methylmalonic acid (MMA) is the intracellular marker of cbl deficiency that has been most studied in dogs with CE and hypocobalaminemia. MMA decreased significantly in both groups between baseline and 28 days of treatment, with no further reduction after 90 days compared to 28 days in either group. No significant differences in MMA concentrations emerged between the groups at any time-point.

Homocysteine is another intracellular marker of cbl deficiency. It appears very valuable in congenital cbl malabsorption in dogs, but less valuable in cbl deficiency in canine CE. Serum HCY concentrations did not differ between baseline and 28 days after initiation of cbl supplementation in any group. Ninety days after cbl supplementation was started, a small increase in serum HCY concentration compared to 28 days was noted in both groups. However, the increase was only significant in the PE group.

In parallel with the MMA results, there were no significant differences in HCY concentrations between the groups at any time point. The studies on intracellular markers of cbl deficiency suggest that both treatment protocols are equally effective on a cellular level.

In conclusion, this thesis provides evidence-based data that oral cbl supplementation can be used as an alternative treatment to the traditional parenteral protocol. Further, the parenteral supplementation protocol has been validated. Our results suggest that, similar to humans, an alternative intestinal absorptive pathway of cbl may exist in dogs.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Toresson, L., Steiner, J.M., Suchodolski, J. S., Spillmann, T., 2015. Oral cobalamin supplementation in dogs with chronic enteropathy and hypocobalaminemia. Journal of Veterinary Internal Medicine 30, 101-7.

II Toresson, L., Steiner, J.M., Razdan, P., Spodsberg, E., Olmedahl. G., Suchodolski, J. S., Spillmann, T., 2018. Comparison of efficacy of oral and parenteral cobalamin supplementation in normalising low cobalamin concentrations in dogs: A randomised controlled study. The Veterinary Journal 232, 27–32

III Toresson, L., Steiner, J.M., Razdan, P., Spodsberg, E., Olmedahl. G., Suchodolski, J. S., Lidbury, J. A., Spillmann, T., 2018. Effects of oral versus parenteral cobalamin supplementation on methylmalonic acid and homocysteine concentrations in dogs with chronic enteropathies and low cobalamin concentrations. Manuscript under revision for the Veterinary Journal.

These publications are referred to in the text by their Roman numerals and have been reprinted with the permission of their copyright holders. In addition, some unpublished material is presented.

AUTHOR'S CONTRIBUTIONS

I	LT recruited the majority of the patients, performed the clinical examinations and designed the plan for further work-up, prescribed the medication and established the oral cobalamin dose, collected and analyzed the data, and prepared and finalized the manuscript.
II	LT was responsible for the study design and was the primary investigator. She recruited the majority of the patients, performed the clinical examinations and devised the plan for further work-up, prescribed the medication, maintained contact with clients, examined the majority of the patients at follow-up consultations, collected and analyzed the data, and prepared and revised the manuscript.
III	LT was responsible for the study design and was the primary investigator. She recruited the majority of the patients, performed the clinical examinations and designed the plan for further work-up, prescribed the medication, maintained contact with the clients, examined the majority of the patients at follow-up consultations, collected and analyzed the data, and prepared the manuscript.

ABBREVIATIONS

BCS	Body condition score
BW	Body weight
Cbl	Cobalamin
CE	Chronic enteropathy
CIBDAI	Canine IBD activity index
EPI	Exocrine pancreatic insufficiency
ESAHHS	Evidensia Specialist Animal Hospital, Helsingborg, Sweden
Fig.	Figure
GC-MS	Gas chromatography-mass spectrometry
HCY	Homocysteine
HoloTC	Holotranscobalamin
IBD	Inflammatory bowel disease
IF	Intrinsic factor
IGS	Imerslund-Gräsbeck syndrome
MMA	Methylmalonic acid
PE	Parenteral
PLE	Protein-losing enteropathy
PO	Peroral (oral)
SD	Standard deviation
SNAP cPL	SNAP canine pancreatic lipase
Spec cPL	Specific canine pancreatic lipase immunoreactivity
TLI	Trypsin-like immunoreactivity

1. INTRODUCTION

Cobalamin (cbl), also known as vitamin B12, is a water-soluble vitamin available in dietary protein of animal origin. All cells in the mammalian body require cbl since it is a vital cofactor in two enzymatic processes necessary for protein synthesis and cell metabolism (Banerjee, 2006).

The gastrointestinal transport and absorption of cbl are complex processes. Cobalamin is ingested bound to food particles, released from these in the stomach, and then bound to haptocorrin (also called R-protein). In the duodenum, cbl is cleaved from haptocorrin and bound to intrinsic factor (IF), which is mainly produced by the exocrine pancreas in dogs (Batt et al, 1989; Simpson et al., 1989; Simpson et al., 1993). This complex is transported to the specific cubam receptor in the ileum, where cobalamin is absorbed via endocytosis.

The reported prevalence of cbl deficiency in chronic enteropathy (CE) is 6-73% (German et al., 2003; Craven et al. 2004; Allenspach et al, 2007; Kathrani et al, 2009; Berghoff et al., 2013). Once cbl deficiency occurs, it can be associated with numerous clinical and metabolic consequences. The clinical signs related to cbl deficiency in dogs are best characterized in congenital cbl malabsorption since it is difficult to distinguish between clinical signs related to CE and signs related to cbl deficiency in dogs affected by both of these conditions. Thus, clinical signs reported in congenital cobalamin malabsorption in dogs are anorexia, lethargy, weight loss, failure to thrive, central and/or peripheral neuropathies, anemia, and leukopenia (Fyfe et al., 1991; Morgan and McConnell, 1999; Fordyce at al., 2000; Battersby et al., 2005; Lutz et al., 2013; Gold et al., 2015). Further, immunodeficiency has been reported in rodent models of cbl deficiency, and intestinal changes, such as villous atrophy and malabsorption of other vitamins and nutrients, have been described in humans affected with cbl deficiency (Arvanitakis, 1978; Funada et al., 2001). It has also been suggested that dogs and cats with cbl deficiency have a poorer response to medical treatment for CE if the deficiency is not corrected (Ruaux, 2013). Lastly, cbl deficiency has been associated with a negative prognosis and increased risk of euthanasia in chronic enteropathy, chronic diarrhea, and exocrine pancreatic insufficiency (EPI) in dogs (Allenspach et al., 2007; Batchelor et al., 2007; Volkmann et al., 2017).

Suggested mechanisms behind cbl deficiency in dogs with CE are damage to the ileal mucosal receptors and/or dysbiosis (Ruaux, 2013). Certain anaerobic bacteria (mainly some *Clostridia* and *Bacteroides*) can adsorb and utilize cobalamin (Gianella et al., 1972). Consequently, bacterial competition for nutrients could lead to less cobalamin available for absorption in the ileum. However, to the best of our knowledge, no direct evidence linking dysbiosis with an increased amount of the previously mentioned bacterial groups to cbl deficiency in dogs has been published. Thus, decreased expression of the cubam receptor due to mucosal disease is the most likely mechanism behind cbl deficiency in dogs with CE.

To diagnose cbl deficiency in humans, decreased serum cbl concentrations combined with increased serum concentrations of intracellular

markers of cbl deficiency are usually required (Allen 2012). Serum cbl concentrations may not correctly diagnose intracellular cbl deficiency (Herrmann and Obeid, 2012). Whether this approach to diagnose cbl deficiency should be used routinely in veterinary medicine as well requires further studies. However, several studies and case reports have demonstrated a correlation between low serum cbl concentrations and increased serum or urinary concentrations of serum methylmalonic acid (MMA) and/or increased serum concentrations of homocysteine (HCY) (Fyfe et al., 1991; Morgan and McConnell, 1999; Fordyce et al., 2000; Battersby et al., 2005; Berghoff et al 2012; Lutz et al., 2012; Berghoff et al., 2013; Grützner et al., 2013; Lutz et al., 2013; Gold et al., 2015; McLauchlan et al., 2015).

The recommended supplementation protocol for cbl deficiency in dogs comprises multiple parenteral injections (Allenspach et al., 2007; Ruaux, 2013; Gold et al., 2015; McLauchlan et al., 2015; Volkmann et al., 2017). This recommendation is based on pathophysiologic justification, clinical empiric experience, and specialist opinion (Allenspach et al., 2007; Lutz et al., 2013; Ruauax, 2013; Gold et al., 2015; McLauchlan et al., 2015). However, no parenteral protocol for dogs has thus far been validated.

In humans, several comparative studies have shown equal efficacy of oral versus parenteral cbl supplementation (Kuzminski et al., 1998; Bolaman et al., 2003; Castelli et al., 2011; Kim et al., 2011). A Cochrane review from 2005 stated that oral cbl supplementation "may be as effective as parenteral", although only 2/4 comparative studies had been published at the time (Vidal-Alaball et al., 2005).

This thesis aims to evaluate the efficacy of a novel oral cbl supplementation protocol for dogs with CE and cbl deficiency, to investigate potential factors that could influence the response to oral supplementation, and to compare the oral protocol with a current parenteral protocol regarding the effects on serum cbl, MMA, and HCY concentrations.

2. REVIEW OF THE LITERATURE

Historical background

The effects of cbl were first identified by George Minot and William Murphy after intense studies on pernicious anemia (see Section 2.2.1) in the 1920s. The researchers found that they could cure this fatal condition by treating the patient with a daily diet containing a large amount of whole liver. At the time, they were unaware of which substance in the liver diet that exerted the anti-anemic effect (Chanarin, 2000). Minot and Murphy were, together with the physician George Whipple, awarded the Nobel Prize in 1934 "for their discoveries concerning liver therapy in cases of anaemia". Twenty-two years after Minot and Murphy's liver diet studies, cbl was finally isolated and the chemical structure determined by almost simultaneous publications from a British and an American group (Chanarin, 2000).

2.1 Cobalamin in health

2.1.1 Cobalamin and its metabolism

Cobalamin (vitamin B12) is a water-soluble vitamin found in protein of animal origin. It is the most complex molecule of all vitamins. The chemical structure is a corrinoid molecule with a cobalt atom in the center, hence, the name cobalamin (Fig. 1). The terms vitamin B12 and cbl are often used interchangeably. However, according to some authors, cbl is the correct term to describe the coronoid compounds that are biologically available to mammals since the term vitamin B12 refers to all corrinoid compounds with similar biological activity to cbl (Ruaux, 2013). Some of these compounds are not biologically active in eukaryotes.

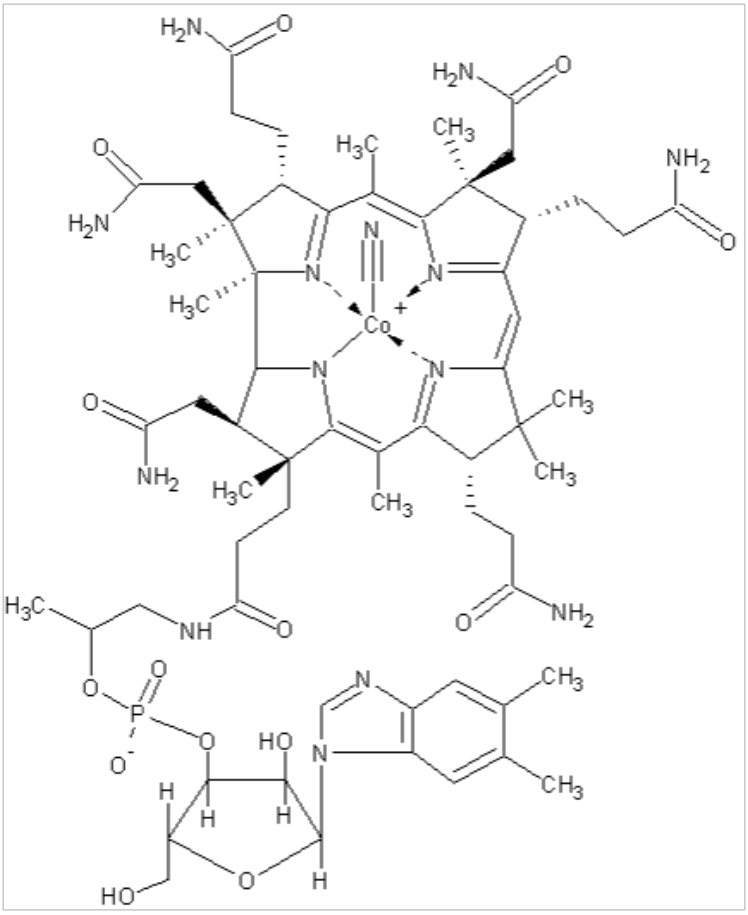

Fig. 1. Chemical structure of cobalamin (cyanocobalamin) (from Wikipedia, https://sv.wikipedia.org/wiki/Kobalamin).

Two biologically active forms of cbl exist in mammals – adenosylcobalamin and methylcobalamin (Herrmann and Obeid, 2012). Both of these are essential cofactors for two enzymatic processes.

Methylcobalamin is active in the cytoplasm and serves as a cofactor for methionine synthase, which converts HCY to methionine (Fig. 2). Methionine is an important start codon for protein synthesis. Adenosylcobalamin is a cofactor for methylmalonyl-CoA mutase, located in the mitochondrion. This enzyme catalyzes the reaction of methylmalonyl-CoA being isomerized to succinyl-CoA. Succinyl-CoA then enters the tricarbocyclic acid (Kreb's) cycle (Fig. 2).

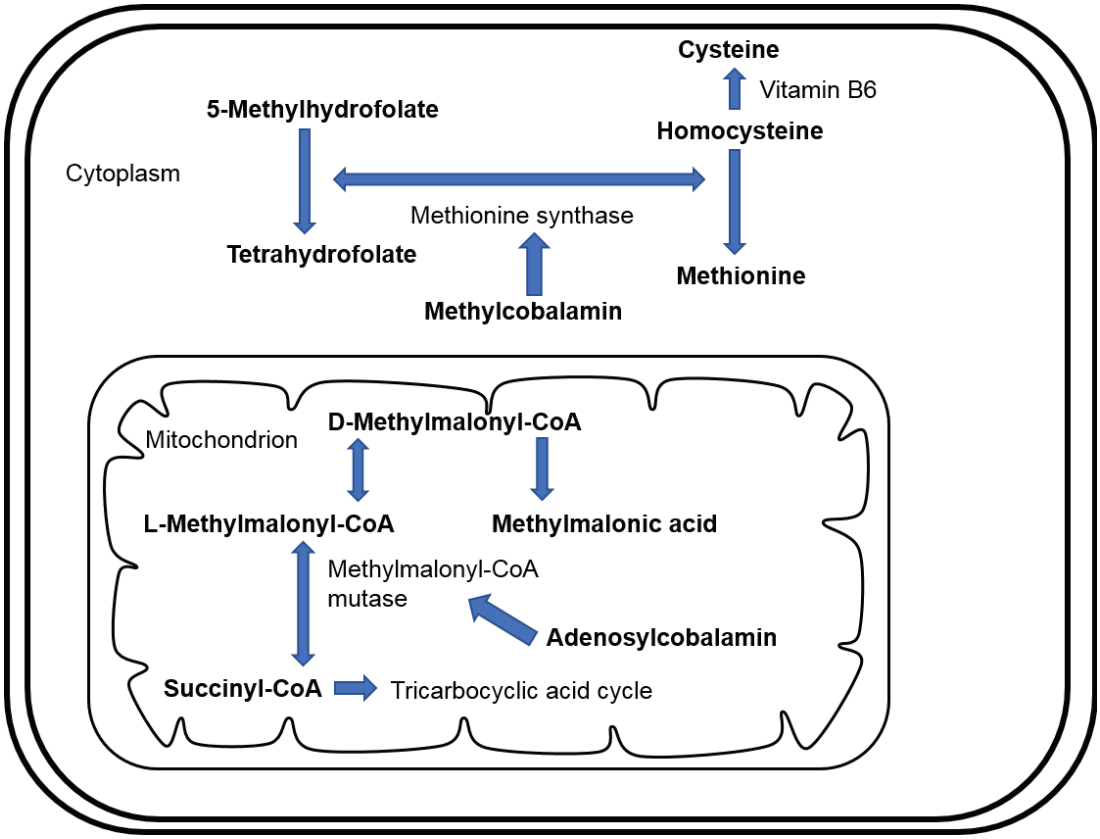

Fig. 2. A schematic view of the intracellular utilization of cobalamin. Within the mitochondrion, adenosylcobalamin is required as a cofactor for methylmalonyl-CoA-mutase, converting L-methylmalonyl-CoA to succinyl-CoA. In the cytoplasm, methylcobalamin is required as a cofactor for methionine synthase, converting homocysteine to methionine.

In short, cbl is required for normal maturation and development of all DNA-synthesizing cells, regeneration of methionine for protein synthesis, methylation, and fat synthesis (Allen, 2012).

2.1.2 Sources of cobalamin

Despite the fact that cbl is produced by certain bacteria, protein of animal origin is the only unfortified source of cbl available to humans and dogs. Cobalamin-producing bacteria can be ingested when herbivores eat plants and roots, particularly when contaminated with feces (Greibe, 2017). Cobalamin is stored in body tissues, with the highest concentration occurring in the liver. Meat from older animals contains higher cbl content than that from younger animals. Further, meat from ruminants contains higher concentrations of cbl than meat from monogastric animals (Greibe, 2017). With the information available at present, ruminants are the only mammals capable of absorbing cbl produced by intestinal microbiota.

Some plants and algae can produce cobalamin-like substances. These are called cbl analogs and have no nutritional value to humans (Dagnelie et al., 1991). Until proven otherwise, we should assume that the same is true for dogs.

Three major forms of cbl are available in food: adenosylcobalamin, methylcobalamin, and hydroxocobalamin. Adenosylcobalamin and methylcobalamin are light-sensitive. When exposed to light, a conversion to hydroxocobalamin can occur (Greibe, 2017). However, when food-cbl enters cells, conversion to adenosylcobalamin and methylcobalamin occurs.

Cyanocobalamin, a very stable, synthetic form of cbl, is the most common form used for clinical supplementation and fortification of food. At present, it is unknown whether the bioavailability of cyanocobalamin differs from that of naturally occurring hydroxocobalamin. Recent studies in rats suggest that hydroxocobalamin and cyanocobalamin are absorbed to the same extent, but hydroxocobalamin has a higher affinity for the liver while cyanocobalamin has a higher affinity for the kidneys (Kornerup et al., 2015).

2.1.3 Normal transport and absorption

Transport and absorption of cbl are components in a complex process. Cobalamin is ingested bound to protein and released from food particles in the stomach by pepsinogen and gastric acid. Thereafter, cbl is bound to haptocorrin (also named R-protein). Cobalamin is then complexed with Intrinsic Factor (IF), which is mainly produced by the exocrine pancreas in dogs, with a minor part produced by the gastric mucosa (Batt et al., 1989). In humans, all production of IF occurs in the gastric mucosa. The cbl-IF complex is further transported to the ileum, where the cubam receptors are located. These receptors are constructed of the proteins cubilin and amnionless (Fyfe et al., 2004). Receptor recognition leads to cbl absorption via endocytosis. After absorption by the ileal enterocytes, cbl is transported in the circulation bound to transcobalamin I (often referred to as haptocorrin) and transcobalamin II. Only cbl bound to transcobalamin II is available for receptor-mediated endocytosis by body tissues. In dogs, the major storage site of cbl is the liver (Glass and Mersheimer, 1958).

2.2 Cobalamin deficiency – causes and consequences

2.2.1 Diseases associated with cbl deficiency in humans

Cobalamin deficiencies in humans are most often acquired, caused by nutritional deficiencies or malabsorption (Greibe, 2017). Cobalamin deficiency due to poverty and a poor diet is endemic in countries such as Mexico, India, Guatemala, and Kenya, among others (Stabler and Allen, 2004). The recommended daily dietary intake of cbl is 2.4 μg/day for adults, but this dietary need is not met in many parts of the world. Nutritional deficiencies are further commonly diagnosed in strict vegetarians (vegans).

Lacto-ovo vegetarians have a lower risk of developing cbl deficiency than vegans, but a higher risk than omnivores (Herrmann et al., 2003).

In Western countries, cbl deficiency is particularly common in the elderly. The most common reasons are atrophic gastritis type A, an autoimmune disease resulting in autoantibodies against IF, and atrophic gastritis type B (Herrmann and Obeid, 2012). The former results in a very low amount of IF available in the small intestine, and subsequently, less cbl absorbed. The latter is caused by *Helicobacter pylori* infection. The consequence of the gastritis type B is decreased production of hypochloric acid, resulting in hypochlorhydria or achlorhydria and increased pH of the stomach. This results in impaired release of cbl bound to dietary protein. The same effect can occur in patients with prolonged treatment with H2-blockers or proton-pump inhibitors.

Cobalamin deficiency can also occur in other gastrointestinal diseases associated with malabsorption such as Morbus Crohn, colitis ulcerosa, pancreatic insufficiency, tropical sprue, and celiac disease. Further, it is a common sequela to ileal or gastric resection. Reports of cobalamin malabsorption related to infection with *Diphyllobothrium latum, Giardia intestinalis,* and Taenia infestation also exist, although a case-control study showed no significant difference in serum cobalamin concentrations between Giardia-positive individuals and healthy controls (Nyberg et al 1961; Springer and Key, 1997; Vuylsteke et al., 2004; Askari et al., 2007; Zarebavani et al, 2012). At present, 12 different inborn defects of cobalamin transport, absorption, or metabolism have been reported (Watkins and Rosenblatt, 2011). These are rare conditions that usually present in early childhood. Each condition is inherited as an autosomal recessive trait. The most common is Imerslund-Gräsbeck syndrome (IGS) characterized by defect expression of the cubilin, amnionless, or both parts of the cubam receptor and associated with impaired absorption of cbl and persistent proteinuria (Gräsbeck, 2006). The second most common is inherited deficiency of intrinsic factor (Watkins and Rosenblatt, 2011). Further, transcobalamin deficiency, haptocorrin deficiency, transcobalamin receptor deficiency, methylmalonyl CoA mutase deficiency, and errors of intracellular cobalamin metabolism have been reported.

2.2.2 Diseases associated with cbl deficiency in dogs

The most common diseases associated with cbl deficiency in dogs are CE and EPI. Cobalamin deficiency has been reported in 6-73% of dogs diagnosed with CE (German et al., 2003; Craven et al., 2004; Allenspach et al., 2007; Kathrani et al., 2009; Berghoff et al., 2013). The suggested mechanism behind the cbl deficiency in CE is decreased expression of the cubam receptor, causing impaired cbl absorption (Ruauax, 2013). Another suggested mechanism is dysbiosis, which is a common sequela to CE (Honneffer et al., 2014). Certain anaerobic bacteria (mainly some *Clostridia* and *Bacteroides*) can adsorb and utilize cbl (Giannella et al, 1972). Thus, bacterial competition for nutrients could theoretically lead to less cbl available for absorption in the ileum. However, no direct evidence, such as identification of causative organisms clearly linking dysbiosis to canine cbl malabsorption, has been published in dogs to date.

The prevalence of cbl deficiency in EPI is 74-83% (Hall et al., 1991; Batchelor et al, 2007). Decreased production and secretion of IF are well-described phenomena in this disease, resulting in reduced amounts of cbl-IF complexes available for absorption (Batt et al 1989; Simpson et al., 1989; Simpson et al., 1993).

Cobalamin deficiency has also been reported in 16% of dogs with multicentric lymphoma (n=58) in one study (Cook et al., 2009). Although the authors were not able to establish the exact mechanism underlying the cbl deficiency, they speculated that the most likely was neoplastic infiltration of the ileum, damaging the cubam receptors.

Familial cbl deficiency has been described in the Border Collie, Giant Schnauzer, Beagle, Australian Shepherd, and Shar Pei (Fyfe et al., 1989; Fyfe et al, 1991; Morgan and McConnell, 1999; Fordyce et al., 2000; Battersby et al., 2005; He et al, 2005; Grützner et al 2010; Lutz et al., 2012; Lutz et al., 2013; Gold et al., 2015). In Australian Shepherds and Giant Schnauzers, a genetic defect of chromosome 8, containing the amnionless gene, has been proven (Fyfe et al., 1989; He at al., 2005; Gold et al., 2015). In Beagles, mutations of the genes coding for cubilin have been described (Fyfe et al., 2014), which have also been described in Border Collies (Owczarek-Lipska et al., 2013; Fyfe et al, 2013), although the location of the mutation differs between the breeds. The familial cbl deficiency described in Giant Schnauzers, Australian Shepherd, Beagles, and Border Collies represent the canine version of Imerslund-Gräsbeck syndrome (IGS). One case report of a very young Yorkshire terrier with cbl deficiency and increased urinary MMA also exists, but further characterization of the suspected underlying receptor abnormality was not performed (McLauchlan et al, 2015).

In Shar Peis, the genetic defect has been localized to chromosome 13, but the exact genetic basis has not been identified (Grützner et al., 2010; Grützner et al. 2013). No reports of early onset disease and typical clinical signs of IGS exist in Shar Peis at present. Until proven otherwise, the familial cbl deficiency in Shar Peis likely represent another type of congenital cbl malabsorption than IGS.

Cobalamin deficiency has also been reported in some dogs with *Giardia intestinalis* (Volkmann et al, 2017), and in anecdotal reports of short bowel syndrome (Allenspach and Gaschen, 2008). To the best of our knowledge, there are no canine reports of cbl deficiency due to a poor diet in the peer-reviewed literature, but a significant correlation between cbl content in the diet and serum cbl concentrations has been shown (Davenport et al., 1994).

2.2.3 Biochemical, functional, and immunological effects of cbl deficiency

Adenosylcobalamin is a cofactor for methylmalonyl-CoA mutase required for the conversion of methylmalonyl-CoA to succinyl-CoA (Fig. 2). In cbl deficiency, this pathway will not function properly, and methylmalonyl-CoA will accumulate, which will be converted to methylmalonic acid (MMA). This will result in increased serum concentrations of MMA and methylmalonic aciduria. Among other consequences, accumulated methylmalonyl-CoA

causes decreased myelin synthesis and incorporation of abnormal fatty acids into neuronal lipids (Briani et al., 2013).

Cobalamin in the form of methylcobalamin is further required as a cofactor when HCY is converted to methionine (Fig. 2). Subsequently, HCY will accumulate in the absence of cbl. Elevated HCY concentrations have been linked to vascular and thrombotic disease (Carmel et al., 2003). Furthermore, methionine is the start codon of all protein synthesis. Cbl deficiency can thus lead to lack of methionine and impaired protein synthesis.

In humans, malabsorption has been documented in pernicious anemia, associated with impaired intestinal permeability and absorptive function as well as with pathological intestinal histopathology (Arvanitakis, 1978). These changes were normalized after parenteral cbl supplementation. Studies in mice fed a cbl-deficient diet for 90 days showed that an immunologic shift occurred, with significantly increased serum concentrations of IgE, but significantly decreased concentrations of IgG and IgM compared with healthy controls (Funada et al., 2001).

Leukopoiesis is affected in humans with cbl deficiency of various etiologies and in dogs with IGS. In humans, hypersegmented polymorphonuclear neutrophils are a hallmark in a peripheral blood smear, and children with IGS are prone to infections (Briani et al, 2013; Gräsbeck, 2006). Likewise, dogs with IGS often exhibit neutropenia (Fyfe et al., 1991; Fordyce et al., 2000; Lutz et al., 2013; Gold et al., 2015).

2.2.4 Clinical effects of cbl deficiency in humans

Clinical manifestation of cbl deficiency in humans is typically anemia and/or neurological symptoms. Pernicious anemia (megaloblastic anemia) is a major consequence of cbl deficiency in humans (Allen, 2012). Cobalamin is needed for all DNA synthesis. Subsequently, in relatively severe cbl deficiency, sustained DNA synthesis in rapidly dividing blood cells can occur. The effect is large red cells with increased mean corpuscular volume (MCV). However, a diagnosis of megaloblastic anemia is not a sensitive or specific tool for diagnosing cbl deficiency (Herrmann and Obeid, 2012).

Neurological manifestations of cbl deficiency are frequently encountered. These can occur in patients with megaloblastic anemia, but are also seen in cbl-deficient patients without anemia. Cobalamin deficiency exerts a multitude of pathological effects on the nervous system, including decreased myelin synthesis, incorporation of abnormal fatty acids into neuronal lipids, and diminished synthesis and levels of some growth factors and cytokines in the CNS (Scalabrino, 2009). Hence, cbl deficiency can result in myelopathy, neuropathy, and neuropsychiatric abnormalities such as memory loss, psychosis, dementia, depression, and mania. Peripheral neuropathies are seen in 25-40% of all patients with cbl deficiencies (Shorvon et al., 1980; Briani et al., 2013). Neurological signs can be irreversible, especially if cbl deficiency has lasted longer than one year (Allen, 2012). Infants with cbl deficiency can have marked developmental regression, impaired growth of the brain, poor intellectual outcome, impaired communicative reactions, and defective fine and gross motor functions (Herrmann and Obeid, 2012).

Other reported clinical effects of cbl deficiency are sterility due to the effects on the gonads and impaired bactericidal activity (Briani et al., 2013).

2.2.5 Clinical effects of cbl deficiency in companion animals

Dogs with IGS typically present at an early age with lethargy, failure to thrive, anorexia, poor body condition score, and vomiting and diarrhea (Fyfe et al. 1989; Fyfe et al, 1991; Fordyce et al., 2000; Fyfe et al., 2013; Lutz et al., 2013; Fyfe et al., 2014; Gold et al., 2015). Seizures and encephalopathy have also been described (Fyfe et al., 1989; Fordyce et al., 2000; Battersby et al., 2005). Common clinicopathological findings are normocytic non-regenerative anemia, neutropenia, hypoglycemia, hyperammonemia, hypoproteinemia, methylmalonic aciduria, and mild proteinuria (Fyfe et al 1991; Morgan and McConnell, 1999; Battersby et al., 2005; Fyfe et al., 2013; Lutz et al., 2013; Fyfe et al., 2014; Gold et al., 2015). All of these changes (except proteinuria) are reversible with treatment.

In adult dogs with cbl deficiency, the clinical signs are usually dominated by the underlying gastrointestinal disorder. For this reason, it is difficult to assess which signs are related to the primary disease and which signs to the cbl deficiency (Ruaux 2013). However, cbl deficiency has been reported as a negative prognostic factor in dogs with CE, EPI, and chronic diarrhea (Allenspach et at., 2007; Batchelor et al., 2007; Volkmann et al., 2017). It has also been suggested that dogs and cats with gastrointestinal diseases do not respond as well to medical treatment of the underlying condition if the cbl deficiency is not corrected (Ruaux 2013). Cobalamin supplementation in cbl-deficient cats caused significant weight gain and reduction of clinical signs in one study (Ruaux et al., 2005). No change of diet or concurrent treatment occurred during the study. In another study in cats with gastrointestinal diseases and hypocobalaminemia, cbl supplementation improved clinical disease activity score (Kempf el al., 2017). After discontinuation of cbl, but no other change in treatment, disease activity again increased. In one study of cats with EPI, significantly improved clinical response to medical treatment was shown in cats supplemented with cbl, regardless of whether or not the cats were hypocobalaminemic (Xenoulis et al, 2016). Similar studies have not been performed in dogs.

2.3 Diagnosing cobalamin deficiency

In human medicine, it is generally considered that there is no single perfect test to diagnose cbl deficiency (Carmel et al., 2003; Herrmann and Obeid, 2012; Devalia et al., 2014). For this reason, a diagnosis of cbl deficiency usually requires analysis of serum cbl concentration and one additional biomarker. In veterinary medicine, a few studies have been published regarding intracellular markers of cbl deficiency in dogs. However, it is very likely that, just as in human medicine, no single perfect test to diagnose cbl deficiency exists in veterinary medicine.

2.3.1 Serum cbl concentrations in humans and dogs

Serum cbl concentration is usually the standard initial routine diagnostic test to diagnose cbl deficiency in humans, but it is not without limitations (Devalia et al., 2014). The major advantages of measuring serum cbl concentrations to detect cbl deficiency are the low cost and the wide availability. The method is automated, based on IF-binding of cbl and immunochemiluminescence-based assays (Devalia et al., 2014).

When serum cbl concentration is measured, the total amount of serum cbl is measured. However, the majority of serum cbl is bound to haptocorrin (also named holohaptocorrin or transcobalamin 1), which is biologically inactive. The first stage of cbl deficiency is a decreased amount of holotranscobalamin (holoTC), the biologically active form of cbl (Herrmann and Obeid, 2012). During this stage, total serum cbl may still be within the reference range. The sensitivity and specificity for cbl deficiency are reported to be especially low in the lowest range of the cbl reference interval (150 to 350 pmol/L, 203-474 ng/L) (Herrmann and Obeid, 2012). It has been estimated that up to 10% of humans with true cbl deficiency have serum concentrations within the reference range (Green, 1995). The opposite also occurs, with subnormal serum cbl concentrations, but normal biomarkers of cbl deficiency and no clinical signs of cbl deficiency.

In dogs, as in humans, analysis of serum cbl concentration is the first-line test. Parallel to human medicine, it is likely that the test can be false-positive or false-negative. In a study by Berghoff and co-workers (2012), 12% of dogs with normal serum cbl concentrations had increased MMA concentrations. This suggests that these dogs may have intracellular cbl deficiency despite normal cbl concentrations. When only dogs in the lowest end of the normal cbl reference range (185-258 pmol/L, 251–350 ng/L) were studied, 19% had supranormal serum MMA concentrations.

2.3.2 Methylmalonic acid concentrations in humans and dogs

In the absence of cbl, methylmalonyl-CoA will accumulate, which will be converted to MMA (see Section 2.2.3 and Fig. 2). Elevated serum or urinary MMA is considered a more specific test than serum cbl or HCY concentrations for cbl deficiency in humans (Allen, 2012). However, MMA concentrations may be falsely increased in patients with renal disease, intestinal dysbiosis, or hemoconcentration (Devalia et al., 2014). Intestinal dysbiosis may result in production of propionic acid, which is a precursor of MMA (Herrmann and Obeid, 2012). Analysis of serum creatinine is required for correct assessment of MMA concentrations in humans since even subclinical renal disease can result in increased MMA concentrations. MMA must be analyzed with gas chromatography-mass spectrometry (GC-MS), which makes the test more technically demanding and expensive. These are major drawbacks.

Elevated serum MMA concentrations have been reported in 25-46% of dogs with subnormal serum cbl concentrations (Berghoff et al., 2012, Berghoff et al., 2013). When only serum samples from dogs with undetectable cbl concentration were selected, 63% of dogs had increased serum MMA

concentrations (Berghoff et al., 2012). In that study, significantly higher serum MMA concentrations were demonstrated in dogs with serum cbl concentrations below 251 ng/L (185 pmol/L) than in dogs with higher serum cbl concentrations. Further, some dogs with normal serum cbl concentrations had elevated serum MMA concentrations, which may indicate a functional cbl deficiency (in parallel with subtle cbl deficiency in humans).

In another study, serum MMA concentrations were analyzed in dogs of seven different breeds with undetectable serum cbl concentrations (Grützner et al., 2013). Marked breed variation in serum MMA concentrations was demonstrated. All Shar Peis had highly elevated serum MMA concentrations, whereas the median serum MMA concentrations of Beagles and Cocker Spaniels were just within the reference interval.

Increased urinary MMA has also been shown in case series and case reports of dogs with familial cbl deficiency (Fyfe et al., 1991; Morgan and McConnell, 1999; Fordyce at al., 2000; Battersby et al., 2005; Lutz et al., 2012; Fyfe et al., 2013; Lutz et al., 2013; Gold et al., 2015; McLauchlan et al., 2015).

2.3.3 Homocysteine concentrations in humans and dogs

Total plasma HCY concentration increases in cbl deficiency, as cbl is required as a cofactor for methionine synthase, which catalyzes the conversion of HCY to methionine (see Section 2.2.3 and Fig. 2). Homocysteine can further be metabolized to cysteine via a transsulfuration pathway (Fig. 3). Elevated HCY concentrations have been linked to vascular and thrombotic disease in humans (Carmel et al., 2003).

Increased plasma HCY concentrations are less specific for cbl deficiency in humans than MMA (Hermann 2012). Plasma HCY concentrations also increase in folate, riboflavin, and vitamin B6 deficiencies (Devalia et al., 2014). Furthermore, conditions such as hypothyroidism and renal disease can increase plasma HCY concentrations in humans. Folate deficiency causes a more pronounced HCY increase than cbl deficiency (Herrmann and Obeid, 2012).

Cobalamin-deficient vegetarians often do not have increased HCY concentrations, in contrast to increased MMA concentrations. A high dietary intake of folate compensates for low cbl concentrations on HCY concentrations (Herrmann and Obeid, 2012).

Cobalamin deficiency can further cause a secondary folate deficiency (Herrmann and Obeid, 2012). Since methionine synthase require cobalamin, cbl deficiency can lead to a retention of 5-methylhydrofolate (Fig. 3). The transfer of the methyl group required to convert 5-methylhydrofolate to tetrahydrofolate (the activated form of folate) is inhibited, which results in “the folate trap”. Although serum concentrations of folate may be normal or high, this is mostly 5-methylhydrofolate and not active tetrahydrofolate.

Plasma HCY can be measured with a variety of techniques, such as gas chromatography (GC), high-performance liquid chromatography, fluorescent probes based on cyclization mechanisms, colorimetric methods, or redox-sensitive fluorometric detection (Peng et al, 2014)

In dogs, increased serum HCY concentrations have been reported in Giant Schnauzers, Shar Peis, and Border Collies with familial cbl deficiency

(Fyfe et al., 1991, Lutz et al 2012; Grützner et al., 2013; Lutz et al 2013). However, in dogs of six other breeds with undetectable serum cbl concentration, median HCY concentration was normal (Grützner et al., 2013). Further, no significant difference in HCY concentrations was detected between healthy Greyhounds and Greyhounds with diarrhea and/or cbl deficiency in a recent study (Heilmann et al., 2017).

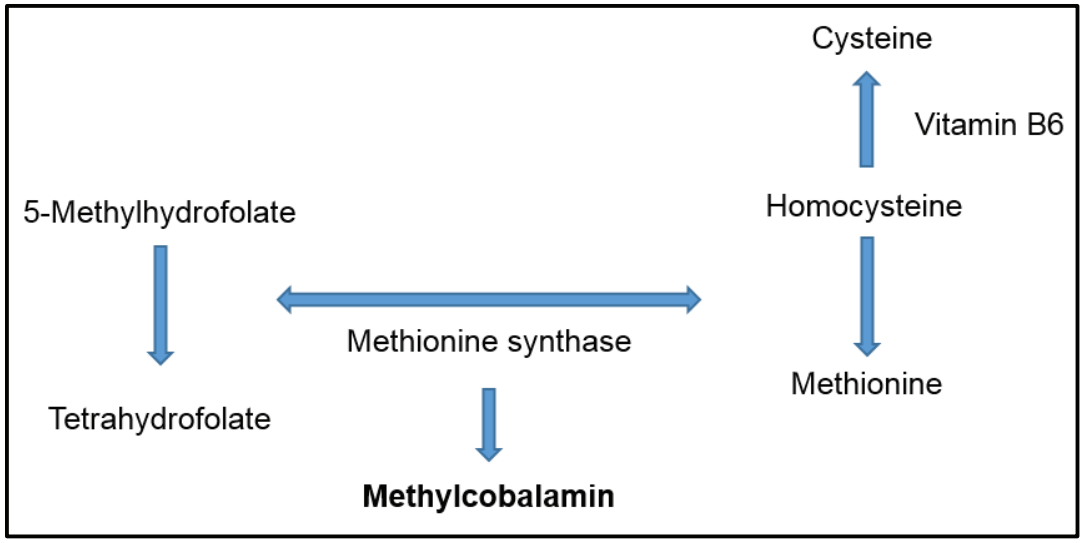

Fig. 3. Homocysteine metabolism. The conversion of homocysteine to methionine requires folate and cobalamin as enzyme cofactors. Vitamin B6 is required for the transsulfuration pathway, which converts homocysteine to cysteine.

2.3.4 Holotranscobalamin concentrations in humans and dogs

The majority of cbl transported in the circulation of humans is bound to transcobalamin I, often referred to as haptocorrin. Only 6-20% of cbl detected by serum cbl concentration is bound to transcobalamin II. This fraction is referred to as holotranscobalamin (holoTC) (Hermann and Obeid, 2012). Thus, in people, holoTC is the biologically active form of cbl available in the circulation. Holotranscobalamin is regarded as a more sensitive and specific marker of cbl deficiency than regular serum cbl concentration (Lindgren et al., 1999; Hermann and Obeid, 2012; Devalia et al., 2014). Further, holoTC appears to decrease earlier in the disease process than serum cbl concentration (Bor et al., 2004). Sample handling is easy and the samples can be stored for later batch analysis (Hermann and Obeid, 2012). Commercial immunoassays for analysis of holoTC are now available. If the analysis becomes more cost-effective in the future, holoTC has the potential of being a better routine first-line assessment of cbl deficiency than regular serum cbl concentration (Devalia et al., 2014). However, the clinical utility of the test needs to be evaluated further. Just as with serum MMA concentrations, renal disease can affect test results, causing increased holoTC concentrations (Carmel et al.,

2001). Further, oral contraceptives can result in a 25% reduction of holoTC despite normal MMA and HCY concentrations (Riedel et al. 2005).

As far as we are aware, no studies regarding holoTC in dogs have been published.

2.4 Cobalamin supplementation

2.4.1 Cobalamin supplementation in humans

The most utilized way of supplementation worldwide is PE, usually intramuscular injections of cyanocobalamin (Andres et al., 2008). Hydroxocobalamin and methylcobalamin are also used, but not as commonly. Many different protocols have been applied, and different indications require different doses (Hermann and Obeid, 2012). The supplementation protocols vary markedly between countries (Andres et al., 2008). The Swedish PE protocol is 1000 μg daily over 1-2 weeks (until clinical remission), then maintenance therapy of 1000 μg from once a month to every third month.[1] The higher doses are used in patients with neurological manifestation of cbl deficiency.

In a study published in 1968, effective treatment of oral cbl supplementation was demonstrated in a systematic evaluation. The supplementation was effective even in patients with atrophic gastritis, i.e. lacking IF. By using radioactively labeled cbl, it was demonstrated that approximately 1% of a given oral dose was absorbed along the entire small intestine. This absorptive capacity was of the same magnitude in individuals with normal absorption as in patients with atrophic gastritis, patients with malabsorption due to ileitis, or patients who had undergone major gastrointestinal resections (Berlin et al., 1968).

At present, four studies comparing the effects of oral versus parenteral cbl supplementation have been published in humans with cbl deficiency (Kuzminski et al., 1998, Bolaman et al., 2003; Castelli et al., 2011; Kim et al., 2011). The patients were suffering from cbl deficiency for various reasons, such as atrophic gastritis, Crohn's disease, celiac disease, dietary deficiencies (in vegetarians or vegans or due to poverty), prolonged use (>3 months) of H2-blockers or proton-pump inhibitors, and patients with a history of ileal or gastric surgery. In all four studies, oral supplementation was statistically as effective as parenteral, except at two of three time-points (2 and 4 months after start of supplementation) in one study (Kuzminski et al., 1998). At these time-points, patients in the PO supplementation group had significantly higher serum cbl concentrations than patients in the PE group. The PE group further had a significantly higher serum MMA concentration after 4 months than the PO group, but mean HCY concentration was the same at all time-points in both groups. In the study by Castelli and co-workers (2011), no significant difference in serum cbl or HCY concentrations were noted at any time-point, but the decrease in serum MMA concentration was significantly higher in the PO group than in the PE group on day 91.

[1] http://www.fass.se/LIF/product?userType=0&nplId=19640101000042

A commonly used oral protocol is 1000 – 2000 μg of cyanocobalamin daily for 1-2 months and then 250-1000 μg daily as maintenance therapy, depending on diagnosis (the lower dose for food-cbl malabsorption, the higher dose in pernicious anemia, Andres et al., 2009). Individual absorption varies considerably between patients. Daily oral doses of 500 μg of cbl are sufficient for some but not all patients. However, daily oral cbl doses of 1000-2000 μg are considered to produce successful long-term results (Elia, 1998).

A Cochrane review from 2005 states that oral supplementation "may be as effective as intramuscular administration" (Vidal-Alaball et al., 2005). At this time, two of the four comparative studies had not yet been published (Castelli et al, 2011; Kim et al., 2011).

The effect of oral cbl has been specifically addressed in patients suffering from Crohn's disease in a recent retrospective study (Gomollon et al., 2017). Similar to other oral cbl supplementation studies, significant increases in serum cbl concentrations were achieved.

When calculating the cost benefits for the healthcare budget, PO supplementation is superior to PE, mainly due to no costs incurred for healthcare nurses to book appointments and give injections (Nyholm et al., 2003; Masucci et al 2013). Despite compelling evidence of the effectiveness of PO supplementation, oral cbl is still regarded as "Medicine's best-kept secret" (Lederle et al., 1991; Graham et al., 2007).

2.4.2 Cobalamin supplementation in dogs

Parenteral cbl supplementation has been the only recommended treatment for dogs prior to our studies on oral cbl supplementation (Fyfe et al., 1989; Fordyce et al., 2000; Allenspach et al., 2007; Batchelor et al., 2007; Lutz et al., 2013; Ruauax, 2013; Gold et al., 2015). Several supplementation protocols exist, based on weekly injections for 6-10 weeks in CE. In canine IGS, more frequent injections have been given as start-up treatment, followed by once monthly to bimonthly injections (Fyfe et al., 1989; Fordyce et al., 2000; Lutz et al., 2013; Gold et al., 2015, Kook et al., 2018). In case reports and cases series of IGS, follow-up serum cbl concentrations are available, demonstrating various efficacy of the treatment. However, to the best of our knowledge, there have been no studies systematically evaluating the efficacy of the recommended parenteral protocol in dogs with CE, nor have there been studies evaluating the efficacy of daily oral cbl supplementation in dogs.

There are two previous reports on the use of oral cbl supplementation in dogs with cbl deficiency (Fyfe et al., 1989; Fyfe et al., 2013). In a case series of Giant Schnauzer puppies with familial cbl malabsorption, one puppy was supplemented orally with cyanocobalamin at a dose of 10 μg/day for seven days (Fyfe et al., 1989). This treatment did not improve clinical signs or laboratory parameters. However, the cbl dose was very low, representing only 1/25 of the lowest amount used by our group for supplementation (Toresson et al., 2016; Toresson et al., 2018). Further, in a case series of Border Collies with IGS, one dog had been treated with PO cbl for 3 weeks without effect. The dose is not mentioned in the text (Fyfe et al., 2013). However, a very recent case report in a young Border Collie with IGS demonstrated efficacy of daily oral cbl supplementation on serum cobalamin concentrations (McCallum and

Watson, 2018). Further, oral cbl supplementation was associated with resolution of clinical signs and normalization of hematological parameters.

3. AIMS OF THE THESIS

The objective of this thesis was to evaluate oral cbl supplementation in dogs with CE and low serum cbl concentrations. Detailed aims were as follows:

1. To evaluate whether oral cbl supplementation results in a significant increase in serum cbl concentrations in dogs with CE and low serum cbl concentrations.

2. To evaluate whether any of the following parameters at inclusion affected serum cbl concentrations after oral cbl supplementation: Canine Inflammatory Bowel Disease Activity Index (CIBDAI), medical or dietary intervention during supplementation, and serum cbl concentration at baseline (hypocobalaminemia versus serum cbl concentration in the lowest end of the reference interval).

3. To validate the oral protocol used in Study I and also to validate a recommended, previously unvalidated, parenteral protocol.

4. To compare the effects of oral versus parenteral cobalamin supplementation on serum cbl concentrations in a longitudinal, prospective, randomized study.

5. To investigate the prevalence of increased serum MMA and HCY concentrations in dogs with CE and low normal serum cbl concentrations.

6. To compare the effects of oral versus parenteral cobalamin supplementation on intracellular markers of cbl deficiency.

4. MATERIALS AND METHODS

4.1 Study design and study protocol

4.1.1 Study I

This study was retrospective, based on the review of medical records of dogs with CE and serum cbl concentrations ≤270 ng/L (≤199 pmol/L) (reference interval: 234-811 ng/L; 173-599 pmol/L), treated with oral cbl at Evidensia Specialist Animal Hospital, Helsingborg, Sweden (ESAHHS). Serum cbl concentrations ≤ 270 ng/L (≤ 199 pmol/L) will be referred to as "low cbl concentrations" in this text with regard to Study I. All patients were supplemented between January 2012 and March 2014.

4.1.2 Studies II and III

These studies were multicenter studies performed in an open, prospective, randomized manner from March 2014 to July 2016.

Dogs with serum cbl concentrations ≤ 285 ng/L (≤ 210 pmol/L, referred to as "low cbl concentrations" in Studies II and III) (reference interval 244-959 ng/L; 180-708 pmol/L) and CE were enrolled after informed owner consent. All dogs were enrolled from two Swedish centers: ESAHHS and Hälsinge Small Animal Clinic, Ljusdal. A schematic overview of the study design is presented in Fig. 4.

Owners of dogs that met the inclusion criteria were contacted by phone or invited to participate during a consultation when test results were reported. At baseline, a detailed medical history was taken, a clinical investigation was performed, and the Canine Inflammatory Bowel Disease Activity Index (CIBDAI) was calculated (Jergens, 2004). The consultation when the blood sample demonstrating a low serum cbl concentration was collected was considered the baseline consultation. If too little information was available in medical records, additional questions were asked during the consultation or over the phone at the time of inclusion.

A blood sample for serum concentrations of cbl, MMA, and HCY was collected at baseline and at 28 ± 5 days and 90 ± 15 days after cbl supplementation was initiated (Fig. 4).

At the follow-up visits, a detailed medical history was again obtained, a clinical evaluation performed, and the CIBDAI calculated. For dogs in the PO group, the owners were instructed to bring the pill container for tablet counting at the two follow-up visits. They were further instructed not to give the cbl tablet on the same day as the follow-up visit prior to the visit. All owners were instructed to keep their dogs fasted for a minimum of 8 hours before the follow-up visit. For legislative reasons, all cbl injections were given at the participating centers or by referring veterinarians.

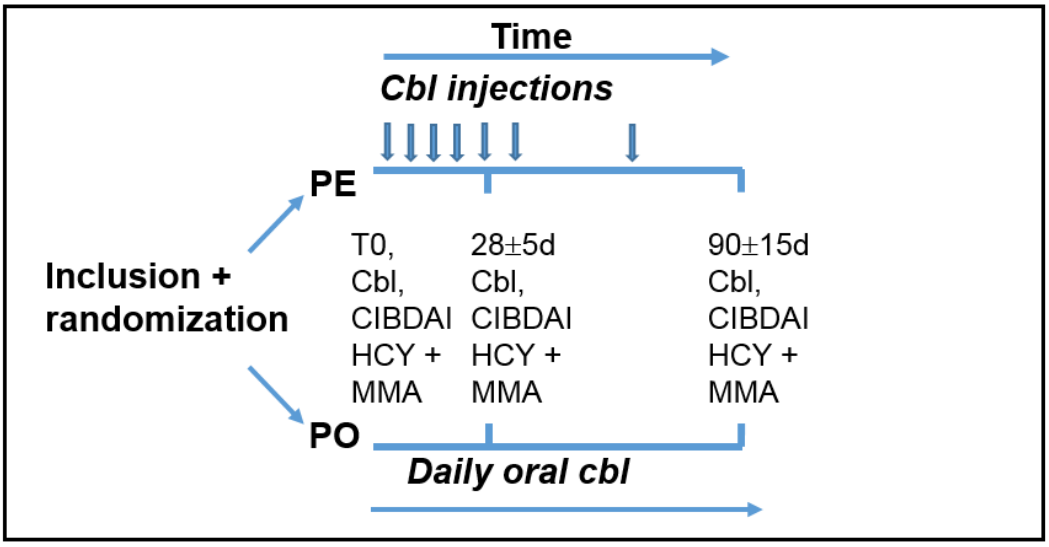

Fig. 4. Schematic design of Studies II and III. Cbl – cobalamin, CIBDAI – canine inflammatory bowel disease activity index, HCY – homocysteine, MMA – methylmalonic acid, PO – oral supplementation PE – parenteral supplementation

4.2 Ethical approval (I – III)

Since Study I was a retrospective study, no ethical approval was necessary. All owners were informed that the recommended treatment for cbl deficiency in dogs was PE supplementation, but that PO supplementation was effective in humans. They were further informed that previous patients with cbl deficiency treated at ESAHHS had responded very well to PO supplementation.

Studies II and III were approved by the Local Animal Ethics Committee in Uppsala, Sweden. The date of approval was 27 September 2013; approval number C109/13.

4.3 Study population (I – III)

The study population in Study I consisted of client-owned dogs with signs of CE and an initial serum cbl concentration ≤ 270 ng/L (≤ 199 pmol/L) (reference interval: 234-811 ng/L; 173-599 pmol/L) supplemented with PO cbl at ESAHHS. No dogs with protein-losing enteropathy (PLE) and serum albumin concentrations at or below 20 g/L were included in the study since PO cbl was not prescribed to that patient group at the time of the study.

The dogs were treated according to a standardized cbl protocol. All dogs included were identified by an electronic database search of the hospital electronic patient record. The brand name of cbl used at the hospital during the last 5 years was searched in the database fields for treatment and prescription but also as free text. Oral cbl tablets were administered daily by the owners, but withheld on the day of the follow-up visit, during which a

serum sample for cbl determination was collected. The owners were further instructed to withhold food from their dogs for a minimum of 8 hours prior to sample collection. In a few patients, the follow-up sample had been collected on several occasions. For these patients, the first sample collected was chosen.

The study population for Studies II and III consisted of client-owned dogs with signs of CE and serum cbl concentrations ≤ 285 ng/L (≤ 210 pmol/L) (reference interval 244-959 ng/L; 180-708 pmol/L). Dogs were included after informed owner consent. All dogs were patients at ESAHHS or Hälsinge Small Animal Clinic, Ljusdal, Sweden.

4.4 Inclusion and exclusion criteria (I – III)

Inclusion criteria for Study I comprised a documented physical examination at inclusion and follow-up, a dietary and medical history at both time-points, current clinical signs compatible with CE or previously documented CE verified with intestinal biopsies, an initial serum cbl concentration ≤ 270 ng/L (reference interval 234-811 ng/L), a second serum sample for cbl analyses collected after institution of oral cbl supplementation, and treatment following a set protocol (see Section 4.5). Dogs that had previously been treated with cbl were included if the last cbl treatment was administered at the latest 30 days before a serum cbl of ≤ 270 ng/L was determined.

Exclusion criteria were dogs receiving any form of cbl supplementation when a low serum cbl concentration was documented, those receiving oral and parenteral cbl supplementation in parallel, EPI without a histologically verified concurrent CE, documented failure to comply with the cbl supplementation protocol, too little data for retrospective calculation of the canine inflammatory bowel disease activity index (CIBDAI), or incomplete medical records.

Inclusion criteria in Studies II and III were dogs with signs of CE and serum cbl concentrations ≤ 285 ng/L (≤ 210 pmol/L) (reference interval 244-959 ng/L; 180-708 pmol/L). This represented the lower end of the reference interval or below. Exclusion criteria were ongoing cbl supplementation at the time of diagnosis of cbl deficiency, gastrointestinal neoplasia, or EPI without concurrent CE, verified with small intestinal biopsies.

For Study III, an additional exclusion criterion was dogs lacking a baseline serum MMA or HCY concentration due to insufficient serum remaining after cbl analysis or transport errors.

4.5 Cobalamin supplementation (I – III)

The oral supplementation protocol used in Studies I, II, and III was designed by the primary investigator and extrapolated from human data (Table 1). All dogs were treated with daily cyanocobalamin tablets (Behepan, 1 mg, Pfizer). The owners were allowed to give the tablet with food.

Table 1. Oral cbl supplementation protocol

BW (kg):	<10	10 ≤ 20	20 ≤ 50	>50
Cbl dose (µg):	250	500	1000	1500

BW = body weight; Cbl = Cobalamin

The parenteral protocol used in Studies II and III was described by Ruaux in 2013. Dogs received one cbl injection per week for 6 weeks. Four weeks later, an additional injection was given. All dogs were supplemented with hydroxocobalamin (Behepan 1 mg/ml, Pfizer). Hydroxocobalamin was the only available form of injectable cbl in Sweden at the time of the study.

Table 2. Parenteral cbl supplementation protocol

BW (kg):	<5	5≤10	10≤20	20≤30	30≤40	40≤50	>50
Cbl dose (µg):	250	400	600	800	1000	1200	1500

BW = body weight; Cbl = Cobalamin

4.6 Randomization (II, III)

Study I was not randomized. Studies II and III were block-randomized. The randomization was performed by an external statistician (Linda Palm Simonsson, I-Mind Consulting, Lund, Sweden) prior to study start-up.

Each patient entering the study was assigned a number from a list prepared by the statistician. This patient data list, which also served as a template for the data needed during the study, was available at the local network at ESAHHS. The number corresponded to an envelope, also prepared by the statistician, containing information about the treatment that the patient should receive. Each envelope was opened by the clinicians at ESAHHS after a new patient was included. A separate patient data list with a higher number series and the corresponding envelopes were available to Pia Razdan, DVM, at Hälsinge Small Animal Clinic. A master list of the randomization existed, but the information was only available to the primary investigator.

4.7 CIBDAI (I – III)

In Study I, CIBDAI at study inclusion was calculated retrospectively using information available from the patient data record. For patients already treated with immunomodulatory therapy, CIBDAI was also calculated for the time-point before endoscopy and prior to commencement of immunomodulatory treatment. CIBDAI at inclusion was compared between dogs receiving immunosuppressive therapy and those not receiving immunosuppressive therapy. Further, CIBDAI in dogs receiving immunosuppressive therapy at study inclusion was compared with the

CIBDAI that each dog in this group previously had when immunosuppressive therapy had started.

In Studies II and III, CIBDAI was calculated for each visit. An Excel file was used to calculate the CIBDAI for each patient. This Excel file was available on the local network at ESAHHS. A similar Excel file was available to Pia Razdan at Hälsinge Small Animal Clinic. During busy clinic days it was sometimes not possible to take the extra time to fill in the Excel file on the same day. Under such circumstances, the CIBDAI was calculated at the latest within 14 days of the patient visit by the clinician in charge of the case or by the primary investigator, using information available in the patient data record. The template for medical history at ESAHHS specifically asks for details regarding each dog's gastrointestinal signs, general condition, weight changes, etc. The program further asks for BCS at each visit. CIBDAI was compared between the PO and PE group at each time-point as well as within each group at inclusion, at 28 ± 5 days, and at 90 ± 15 days.

4.8 Ancillary diagnostic tests

4.8.1 Selected hematology and serum biochemistry (I, II)

Blood samples were collected from the cephalic or jugular vein in all dogs. The blood was placed in EDTA tubes for hematology analysis and in regular plain serum tubes for serum biochemistry analysis. Hematology was analyzed in-house at ESAHHS using ProCyte Dx (IDEXX Laboratories, Ludwigsburg, Germany). Serum biochemistry was analyzed in-house using a Konelab Prime 30i (Diamond Diagnostics, Holliston, Massachusetts, USA) except for cbl, folate, trypsin-like immunoreactivity (TLI), and specific canine pancreatic lipase (Spec cPL, see Section 4.8.2). All in-house laboratory analyses were performed without delay.

Hematology from the patients at Hälsinge Small Animal Clinic was further analyzed in-house at the local clinic using ProCyte Dx (IDEXX Laboratories), and serum biochemistry was analyzed using Catalyst DX (IDEXX Laboratories), except for cbl, folate, trypsin-like immunoreactivity (TLI), and specific canine pancreatic lipase (Spec cPL).

4.8.2 Folate and pancreatic tests (I, II)

For the retrospective study (I), serum folate and TLI concentrations were analyzed at IDEXX Laboratories, Ludwigsburg, Germany, with an ADVIA centaur (Siemens Healthcare). Canine pancreatic lipase (cPL) was also analyzed at IDEXX Laboratories, Ludwigsburg, Germany, using the Spec cPL test kit from IDEXX, which is a sandwich ELISA. Serum samples were sent by refrigerated transport twice weekly. Samples that were transported within 48 hours were kept in a fridge, whereas serum samples that would be transported later were stored at -20°C until transport.

For the prospective comparative study (II), serum samples for folate and TLI analysis were refrigerated within 2 h of collection. Thereafter, samples

were frozen at -20°C for 1-3 days and sent to the Laboratory Department at Evidensia Specialist Animal Hospital, Strömsholm, Sweden, with cold packs using priority delivery. The samples were analyzed using Immulite 2000 (Siemens Healthcare Diagnostics, Erlangen, Germany). Spec cPL was analyzed at IDEXX Laboratories as for Study I.

4.8.3 Parasitology (I, II)

Fecal samples from dogs included at ESAHHS were collected on three consecutive days and submitted for fecal parasitology. The samples were analyzed in-house with the sedimentation-flotation method and the IDEXX SNAP Giardia test. Positive SNAP Giardia tests were sent for confirmation using direct immunofluorescence assay to the National Veterinary Institute, Uppsala, Sweden.

Fecal samples from Hälsinge Small Animal Clinic were collected on three consecutive days and submitted for fecal parasitology to the National Veterinary Institute, Uppsala, Sweden. Samples were analyzed with the sedimentation-flotation method and direct immunofluorescence assay for detection of *Giardia intestinalis*.

4.8.4 Abdominal ultrasound (I, II)

Abdominal ultrasound examinations were performed by veterinary clinicians from the Diagnostic Imaging Department at ESAHHS (Mia Nilsson, Anna Djupsjöbacka, Ditte Ljungqvist, Anna Frennesson, Sofia Honkavaara, and Stine Hoelgaard). The scans were performed using a Philips HD11XE or a GE logiq E9 scanner. All participating veterinarians had a minimum of 5 years' experience with ultrasound imaging.

Dogs included at the Hälsinge Small Animal Clinic had abdominal ultrasound scans performed by Eva Eriksson, DVM, using a Zonare Z one scanner.

4.8.5 Endoscopy and histopathology (I, II)

In most dogs, endoscopic biopsies were collected under general anesthesia using a Fujinon EG-250WR endoscope. Biopsies of the stomach and small and large intestine were processed by routine histopathology. On average, eight biopsies were collected from each site in each dog. One dog in Study I had biopsies collected surgically via laparotomy by a referring veterinarian, and three dogs in Study II had full-thickness biopsies collected during surgery for a small intestinal foreign body. For these three dogs, both surgical and endocsopic biopsies were collected.

The samples were submitted for histopathology to the veterinary pathology laboratory Biovet in Sollentuna, Sweden, and analyzed by board-certified pathologists. However, biopsies were not graded according to the World Small Animal Veterinary Association gastrointestinal standardization template

(Washabau et al., 2010). No Swedish laboratory at the time of the study used these guidelines.

4.9 Diet and medical treatment

4.9.1 Study I

Information on diet and medical treatment at inclusion was retrieved from the electronic database search of the electronic patient record of ESAHHS. The template for medical history specifically asks for details on diet and current treatment. Information on possible previous cbl supplementation was specifically searched for in all records of dogs included. Changes of diet and/or medical treatment were common during the study period. These changes were based on clinical judgment and noted in the medical database.

4.9.2 Studies II and III

Information on diet and medical treatment at inclusion was available in the medical records from the baseline visit. On rare occasions, this information was lacking and the owners were specifically asked for these details at inclusion. To determine the cbl content of all commercial diets that the dogs were fed at inclusion, all pet food manufacturers were contacted by email or phone. Changes of diet and medical treatment during the study were based on clinical judgment and noted in the medical database.

4.10 Serum cobalamin – sample handling and analysis

4.10.1 Study I

Serum samples were sent by refrigerated transport twice weekly. Samples that were transported within 48 hours were kept in a fridge, whereas serum samples that would be transported later were stored at -20°C until transport. Serum cbl was analyzed at IDEXX Laboratories, Ludwigsburg, Germany, using an automated chemiluminescence immunoassay (ADVIA Centaur, Siemens Healthcare). Stable serum cbl concentrations have previously been demonstrated under similar storage conditions (Drammeh et al., 2008).

4.10.2 Study II

Serum samples for cbl analysis were refrigerated within 2 h of collection, frozen at -20°C for 1-3 days, and sent to the Laboratory Department at Evidensia Specialist Animal Hospital, Strömsholm, Sweden, with cold packs using priority delivery. The samples were analyzed using an automated chemiluminescence immunoassay (Immulite 2000, Siemens Healthcare Diagnostics). As in Study I, serum cbl concentrations were considered very

stable under such handling and storage conditions (Drammeh et al., 2008). Dose-response curves were calculated in a routine fashion for both groups. The increase in serum cbl concentration (Δ) after initiation of cbl supplementation was calculated at 28 days relative to day 0 and at 90 days relative to 28 days in both groups. Furthermore, the cbl dose in mg/kg was calculated based on body weight at inclusion for both groups.

4.11 Serum MMA and HCY – sample handling and analysis

4.11.1 Serum sample handling (III)

Serum samples collected for cbl analysis at ESAHHS or Hälsinge Small Animal Clinic were refrigerated within 2 hours of collection, frozen at -20°C within 1-3 days, and sent to the Laboratory Department of Evidensia Specialist Animal Hospital, Strömsholm, with cold packs using priority delivery.

The laboratory staff at Evidensia Specialist Animal Hospital in Strömsholm was instructed to save all remaining serum from patients with a serum cbl concentration ≤ 285 ng/L and store it in the freezer during the study period, as well as any remaining serum from the two follow-up blood samples at 28±5 days and 90±15 days. Extra serum samples were further collected and stored in the freezer at -20°C at ESAHHS at the follow-up visits. The stored, frozen serum samples at Strömsholm were returned to Helsingborg with cold packs every 12 months using priority delivery. Frozen serum samples were then sent from Helsingborg on dry ice every 12-18 months to the Gastrointestinal Laboratory at Texas A&M University, College Station, Texas, using express delivery. The condition of the serum samples was recorded after every transatlantic shipment. If insufficient serum volumes were available for both MMA and HCY analyses, MMA analysis was prioritized.

4.11.2 MMA analysis (III)

MMA was analyzed at the Gastrointestinal Laboratory at Texas A&M University, College Station, Texas, with stable isotope dilution gas chromatography–mass spectrometry (GC-MS) methods, as previously reported (Ruaux et al., 2001; Berghoff et al., 2012). Prior to analysis of the serum samples, a set of nine standards of known concentrations, ranging from 62.5 to 16 000 nmol/L (reference interval 415.0 – 1193.0 nmol/L), was run. When the serum samples were run, each batch also contained several stored serum samples of known MMA concentrations and a blank for accuracy.

4.11.3 HCY analysis (III)

Homocysteine was also analyzed at the Gastrointestinal Laboratory at Texas A&M University using a stable isotope dilution gas GC-MS method, as previously described (Stabler et al., 1986; Grützner et al., 2013). A set of seven

standards of known concentrations was run prior to analysis of the serum samples. The concentrations of the standards ranged from 3.13 to 200 µmol/L (reference interval 5.9 – 31.9 µmol/L). Additionally, each batch of serum samples analyzed also contained a blank and a known control for accuracy.

4.12 Outcome measures

4.12.1 Study I

In retrospective Study I, the primary outcome measure was serum cbl concentrations before and after cbl supplementation. Also investigated was whether the response in serum cbl concentration was affected by such factors as clinical disease activity at inclusion or degree of cbl deficiency or whether the response differed between dogs only supplemented with cbl and dogs that had a concurrent change in medical treatment and/or diet. Thus, the dogs were stratified into three different groups based on CIBDAI at inclusion. They were also stratified in two different groups based on serum cbl concentration at inclusion (hypocobalaminemia versus serum cbl concentration at the lowest end of the reference interval). Lastly, the dogs were stratified in two different groups based on concurrent treatment during the study period (unaltered treatment apart from cbl ± folate versus dogs with a change in diet and/or medical treatment besides supplementation with cbl ± folate). Serum cbl concentrations were compared between the groups.

4.12.2 Study II

Outcome measures in Study II were comparisons of serum cbl concentrations within the PO and PE group at baseline, 28 ± 5 days, and 90 ± 15 days. The increases in serum cbl concentrations (cbl Δ) were compared between the groups at 28 ± 5 days and 90 ± 15 days. Further, CIBDAI was calculated and compared between the groups at three different time-points.

4.12.3 Study III

In Study III, outcome measures were comparisons of serum MMA and HCY concentrations within the PO and PE group at baseline and at 28 ± 5 days and 90 ± 15 days after initiation of cbl supplementation. Serum MMA and HCY concentrations were compared between the groups at baseline and at 28 ± 5 days and 90 ± 15 days after initiation of cbl supplementation. Serum MMA concentrations were also compared within and between the groups at day 0 and day 28 after removal of the dogs with supranormal MMA at inclusion.

4.13 Statistical methods (I – III)

A commercially available software package (GraphPad Prism 6.0, GraphPad Software) was used for all data analyses. The D'Agostino & Pearson omnibus normality test was used for normality testing. The significance threshold was set at 0.05. In the retrospective study (I), serum cbl concentrations before and after cbl supplementation were normally distributed and were thus analyzed with a paired t-test. An unpaired t-test was used to compare increases in serum cbl concentrations stratified after initial serum cbl concentrations (hypocobalaminemia versus serum cbl concentrations within the lowest end of the reference interval).

Additionally, dogs were stratified into three groups based on CIBDAI at inclusion. Increases in serum cbl concentration between these groups were compared using a one-way ANOVA. Lastly, increases in serum cbl concentration in dogs that underwent changes in medical treatment or diet during supplementation versus dogs that had an unaltered treatment plan were compared using an unpaired t-test.

Neither of the CIBDAI data sets in Study I followed a Gaussian distribution. For that reason, a Mann-Whitney U-test was used for comparing CIBDAI in dogs receiving immunosuppressive therapy with CIBDAI in dogs not receiving immunosuppressive therapy. CIBDAI was also compared between the time of initiation of immunosuppressive therapy and the time of study inclusion using a Wilcoxon matched-paired signed-rank test.

In Study II, the dose-response curves were calculated using the same software package. Furthermore, none of the data sets (i.e. BCS, weight, CIBDAI, serum cbl concentrations) were normally distributed. Consequently, the Mann-Whitney U-test was used for comparisons of CIBDAI between the PO and PE group. This test was also used for comparisons of serum cbl concentrations between the groups, as well as comparisons of serum cbl concentration (Δ) improvement between the groups. Comparisons of BW and BCS before and after cbl supplementation, and serum cbl concentration within each group at baseline, 28 ± 5 days, and 90 ± 15 days, were performed using the Wilcoxon matched-pairs signed-rank test.

Neither MMA nor HCY data (Study III) were normally distributed in the PO or PE group. When the dogs with supranormal MMA were removed from both groups, the data from both these new groups were normally distributed. Comparisons between the total PO and PE groups at baseline, 28 ± 5 days, and 90 ± 15 days were performed using the Mann-Whitney U- test and comparisons within the groups using Wilcoxon matched-pairs signed-rank test. The Wilcoxon matched-pairs signed-rank test was used for comparison between 10 serum samples stored under different conditions. The serum MMA concentrations of the PO and PE groups, containing only dogs with MMA concentrations within the reference range, were compared with an un-paired t-test at day 0 and 28. Comparisons within these groups were performed with a paired t-test.

5. RESULTS

5.1 Baseline data and clinical signs

5.1.1 Study I

Fifty-one dogs aged 1.3 to 12.8 years (median 4.9 years) met the inclusion criteria. The majority of these dogs were males (n=34, 67%; 26 intact and 8 neutered). Of the remaining 17 female dogs (33%), 15 were intact and two were spayed. The body condition score ranged from 2 to 7/9 (median 5/9) at inclusion, and the BW ranged from 2.6 to 52.0 kg (median 14.0 kg).

In total, 30 different breeds were represented. The most common was mixed breed dogs (7/51; 14%), followed by the German Shepherd (n=3, 6%), Labrador Retriever (n=3, 6%), Soft Coated Wheaten Terrier (n=3, 6%), Cavalier King Charles Spaniel (n=3, 6%), and Miniature Poodle (n=3, 6%). Of the remaining 29 dogs of 24 different breeds, two were Giant Schnauzers (Table 3). No other dogs of breeds known for familial cbl deficiency were present.

The most commonly reported clinical signs at inclusion were anorexia (n=21, 41%), diarrhea (n=17, 33%), lethargy (n=16, 31%), weight loss (n=14, 27%), and vomiting (n=13, 25%) (Table 3).

Table 3. Selected data at inclusion in 51 dogs with low cbl concentrations (Study I)

Parameter at inclusion	Variable	Range (median) /amount (%)
Age (years)		1.3 - 12.8 (4.9)
BW (kg)	-	2.6 -52.0 (14.0)
BCS	-	2/9 - 7/9 (5/9)
Breed	Mixed breed	7 (14)
	Labrador Retriever	3 (6)
	German Shepherd	3 (6)
	Soft Coated Wheaten Terrier	3 (6)
	Cavalier King Charles	3 (6)
	Spaniel	3 (6)
	Miniature Poodle	
	Jack Russell Terrier	2 (4)
	Danish-Swedish Farmdog	2 (4)
	Papillon	2 (4)
	Giant Schnauzer	2 (4)
	Miniature Schnauzer	2 (4)
	Miscellaneous[a]	18 (35)
Presenting clinical signs	Anorexia	21(41)
	Diarrhea	17(33)
	Lethargy	16 (31)
	Weight loss	14 (27)
	Vomiting	13 (25)
	↑ frequency of defecation	9 (18)
	Signs of abdominal pain	6 (12)
	Pica	3 (6)
	Borborygmus	3 (6)
	↑ licking (mouth, paws, floor)	3 (6)
	Miscellaneous[b]	11 (22)
CIBDAI	All dogs	1 - 13 (5)
	No IS at inclusion	2 - 13 (6)
	IS at inclusion	1 - 13 (3)
	Before IS started (pre-inclusion)	2 - 10 (6)

[a]19 additional breeds, represented by 1 individual [b] halitosis (2), retching (2), melena (2), bad hair coat (1), syncope (1), seizures (1), and polyuria and polydipsia (1) BW – body weight; BCS – body condition score; CIBDAI – canine inflammatory bowel disease index; IS – immunosuppressive treatment

5.1.2 Study II

All dogs enrolled in the study were presented to ESAHHS or Hälsinge Small Animal Clinic with clinical signs of CE. In total, 55 dogs were included, but two dogs were euthanized prior to the first follow-up due to severe deterioration of protein-losing enteropathy (1) or concurrent chronic bronchitis (1). Of the remaining 53 dogs, 4 were lost after the second follow-up. Reasons for drop-out were non-compliance to the treatment protocol (3) or euthanasia due to deterioration of the CE in combination with an acute pyometra (1).

The 53 dogs were aged 1.5-13.1 (median 6.2) years at inclusion (Table 4). Thirty-three dogs were males, 22 of which were intact. Of the 20 females, 9 were spayed and the rest intact. Twenty-eight different breeds were included (Table 4). The most common breeds were Labrador Retrievers (n=8), mixed breed dogs (n=7), German Shepherds (n=6), and Bernese Mountain Dogs (n=3). One 9.5-year-old Border Collie was included, but no other dogs of breeds for which familial cbl deficiency has been reported were included.

The median (range) weight at inclusion was 14.1 (3.1-49.0) kg, which had increased significantly to 15.2 kg (4.2-50.7 kg; $p<0.003$) at the last follow-up. The BCS also increased significantly, from 4/9 (2/9-7/9) at inclusion to 5/9 (1/9-7/9; $p<0.001$) at the last follow-up.

At inclusion, the majority of dogs presented with clinical signs of CE such as diarrhea (n=31, 58%), anorexia (n=24, 45%), vomiting (n=23, 43%), and lethargy (n=18, 24%) (Table 4).

Table 4. Baseline data at inclusion in 53 dogs with CE and low cbl concentrations (Study II)

Parameter at inclusion or after 90 days	Variable	Range (median) or Number of dogs (%)
Age at inclusion (years)	-	1.5-13.1 (6.2)
BW at inclusion (kg)	-	3.1-49.0 (14.1)
BCS at inclusion	-	2/9-7/9 (4/9)
BW at 90 days (kg)	-	4.2-50.7 (15.2)
BCS after 90 days	-	1/9-7/9 (5/9)
Breed	Labrador Retriever	8 (15)
	Mixed breed	7 (13)
	German Shepherd	6 (11)
	Bernese Mountain Dog	3 (6)
	Cavalier King Charles Spaniel	2 (4)
	Miniature Schnauzer	2 (4)
	Shetland Sheepdog	2 (4)
	Golden Retriever	2 (4)
	Border Terrier	2 (4)
	Miscellaneous [b]	19 (36)
Major clinical signs	Diarrhea	31 (58)
	Anorexia	24 (45)
	Vomiting	23 (43)
	Lethargy	18 (34)
	Weight loss	12 (23)
	↑ frequency of defecation	6 (11)
	Pica	5 (9)
Duration of clinical signs	Up to 1 month	1 (2)
	1 month to 1 year	14 (26)
	> 1 year	38 (72)

[b] Nineteen additional breeds, each represented by one individual; BW – body weight; BCS – body condition score

5.1.3 Study III

Thirty-six of the 53 dogs participating in Study II participated in Study III. Reasons for exclusion of the 17 dogs were no baseline serum available (7 dogs) and serum lost in a shipment from Strömsholm to Helsingborg (10 dogs). When the samples from these dogs were found after 13 days of room storage temperature, serum concentrations of MMA and HCY differed to such an extent that it was decided to exclude the affected samples from those dogs (see Section 5.7.1).

5.2 CIBDAI

5.2.1 Study I

The CIBDAI range in all dogs at study inclusion was 1-13 (median 5). The dogs that were receiving immunosuppressive treatment at inclusion had a significantly lower CIBDAI (n=22; range 1-13, median 3, p=0.0005) than those not receiving immunosuppressive treatment (n=29; range 2-13, median 6). However, the dogs receiving immunosuppressive treatment at inclusion had a significantly higher CIBDAI (range 2-10, median 6, p=0.006) prior to medical intervention.

5.2.2 Study II

Median (range) CIBDAI was 6.5 (2-13) in the PO group and 8 (2-14) in the PE group at inclusion, which decreased in both groups over time (Fig. 5). No statistically significant differences between the PE and PO group were present at baseline or after 28 days (p=0.45 and p=0.12, respectively). After 90 days of treatment, the PO group had a significantly lower CIBDAI than the PE group (p=0.036).

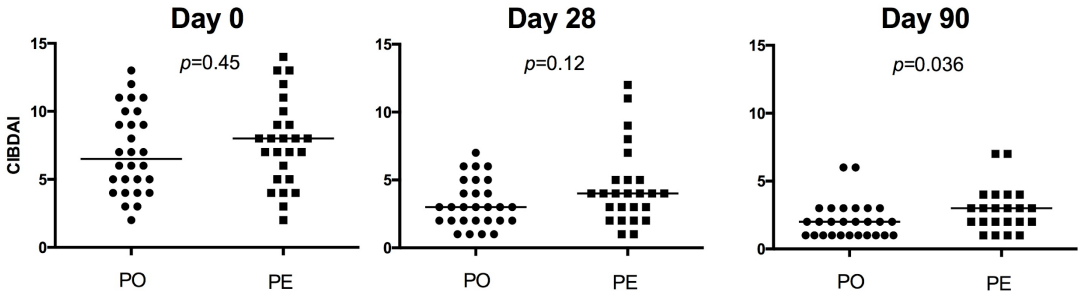

Fig. 5. Canine Inflammatory Bowel Disease Activity Index at day 0, day 28, and day 90. Long horizontal lines represent the median. Data from the PO group are displayed to the left in each panel (n=27 at each time-point) and data from the PE group to the right (n=26 at day 0 and day 28 and n=22 at day 90).

5.3 Clinical diagnosis

5.3.1 Study I

Thirty of the 51 dogs that had been diagnosed with or suspected of having CE responded to immunosuppressive therapy to a various extent. Of the 30 dogs treated with immunosuppressive therapy, two dogs (both Cavalier King Charles Spaniels) were diagnosed with chronic pancreatitis based on a consistently increased serum Spec cPL concentration (> 400 µg/L; reference interval: 0-200 µg/L). Concurrent CE was suspected, but the owners declined further work-up. Hence, corticosteroid treatment was started.

One dog with confirmed CE had concurrent EPI. The remaining 21 dogs had food-responsive enteropathy, one of which had concurrent *Giardia intestinalis* infection. The two other dogs with gastrointestinal parasites had small intestinal biopsies confirming lymphocytic-plasmacytic inflammation of the gastrointestinal tract, which was believed to be the major clinical diagnosis. No dogs with protein-losing enteropathy (PLE) and a serum albumin concentration below 20 g/L were included.

5.3.2 Study II

The most common diagnosis was CE without PLE or simultaneous EPI. Thirty-six of these dogs responded to immunosuppressive treatment to various extents. One dog had biopsy-confirmed CE and chronic pancreatitis with a consistently supranormal Spec cPL concentration (> 400 µg/L) during and after the study. The remaining 9 dogs with increased serum Spec cPL concentrations at inclusion regained a normal Spec cPL concentration during the study. Food-responsive diarrhoea (FRD) was present in 6 dogs. One additional dog had both FRD and severe *Toxocara* sp. infestation. In the five other dogs diagnosed with intestinal parasites (see Section 5.4.3), parasite infection was not considered the main diagnosis. The two dogs with Isospora infection did not have diarrhea, the dog diagnosed with Giardiasis was asymptomatic on corticosteroid treatment when parasitology results were available, and the dog with hookworm infection did not respond clinically to deworming. Finally, the dog with cryptosporidiosis had previously been diagnosed with CE and had a negative fecal parasite samples at inclusion, but later tested positive for cryptosporidiosis.

Ten dogs had a serum albumin concentration below 20 g/L at some time-point during the study and were classified as suffering from CE with PLE. Two of these dogs with histologically verified CE and PLE had concurrent EPI.

5.4 Ancillary diagnostic tests

5.4.1 Selected serum biochemistry and hematology (I, II)

In Study I, a retrospective evaluation of serum biochemistry and hematology results was performed to exclude dogs with diseases having clinical signs similar to CE. Consequently, dogs with renal disease IRIS stage 2 or higher were excluded.

Regarding Study II, the most common hematological change identified was leukocytosis, observed in 44% of the dogs (Table 5). The total leukocyte count in these dogs was 11.5-31.9 x 10^9/L, with a median of 15.0 (reference range 6.2-11.4 x 10^9/L). The majority of these dogs had a mild leukocytosis (75^{th} percentile: 16.9). Furthermore, the most common change of the leukogram was neutrophilia, which was mild in most patients (12.1-22.0 x 10^9/L (median 13.6, reference interval 3.0-11.0 x 10^9/L). A decreased hematocrit, ranging from 25% to 42% (median 39; reference interval 43-52%) was seen in 29% of the dogs, although the decrease was mild for most patients (75^{th} percentile 39.5).

Hypoproteinemia was very common, seen in 81% of the dogs. Median serum total protein was 57.5 g/L in affected dogs (range 26-64 g/L; reference interval 65-75 g/L). Decreased serum albumin concentration was seen in 42% of the dogs, with a range from 14-28 g/L (median 23 g/L, reference interval 29-39 g/L). Seven dogs had a serum albumin concentration < 20 g/L at inclusion. Serum albumin decreased below 20 g/L in three additional dogs throughout the study.

A mild increase in serum alanine aminotransferase was noted in 9% of the patients, and 4% (n=2) had an increased serum creatinine concentration. Both of these dogs had normal creatinine and blood urea nitrogen concentrations at the next follow-up and a normal urine specific gravity.

Table 5. Selected serum biochemistry and hematology data at inclusion (Study II)

Parameter	Reference interval	Range (median) and number of dogs (%)
Increased total leukocyte count [a]	6.2-11.4 x 10^9/L	11.5-31.9 x 10^9/L (15.0) 23 (44)
Increased total neutrophil count [a]	3.0-11.0 x 10^9/L	12.1-22.0 x 10^9/L (13.6) 13 (30)
Increased total lymphocyte count [a]	1.0-4.8 x 10^9/L	5.0-5.3 x 10^9/L (5.2) 2 (5)
Increased total eosinophil count [a]	0-1.25 x 10^9/L	1.44-6.48 x 10^9/L (3.96) 2 (5)
Increased total monocyte count [a]	0-1.35 x 10^9/L	1.36-3.43 x 10^9/L (1.61) 10 (23)
Decreased hematocrit [a]	43-52%	25-42% (39) 15 (29)
Increased hematocrit [a]	43-52%	53-60% (53.5) 4 (8)
Decreased total protein [a]	65-75 g/L	26-64 g/L (57.5) 42 (81)
Decreased albumin [a]	29-39 g/L	14-28 g/L (23) 22 (42)
Increased spec cPL[b]	0-200 mg/L	213-795 mg/L (582) 10 (19)
Negative Snap cPL[c]	Negative	Negative 19 (36)
Decreased TLI	5.5-35 mg/L	1-2.7 mg/L (1.9) 2 (4)
Decreased folate	7-20 ng/mL	2-6 ng/mL (5) 15 (28)
Increased folate	7-20 ng/mL	21->24 ng/mL (22) 6 (11)
Increased ALT	0-80 U/L	108-301 U/L (176) 5 (9)
Increased creatinine [d]	0.7-1.2 mg/dL	1.3-1.5 mg/dL (1.4) 2 (4)

[a]n=52 (for all other data, n=53 if not specified). [b]Spec cPL was measured in 34/53 dogs; six dogs had Spec cPL concentrations > 400 µg/L and four dogs had Spec cPL concentrations of 200-400 µg/L.[c]SNAP cPL was negative in all dogs tested. [d]Both dogs had normal creatinine, blood urea nitrogen, and urine specific gravity at the next follow-up. ALT – alanine aminotransferase

5.4.2 Serum folate and pancreatic test

5.4.2.1 Study I

Results from serum folate analysis were available in 49/51 dogs. Two of these dogs had previously been diagnosed with folate deficiency and were under folate supplementation when tested. Of the remaining dogs, serum folate concentrations below the lower limit of the reference interval were detected in 20/47 dogs (43%) and increased folate concentrations in 7/47 dogs (15%).

Serum trypsin-like immunoreactivity (TLI) results were available for 31/53 dogs. One dog with histologically verified CE had a serum TLI concentration consistent with EPI (1.6 g/L, reference interval 8.5-35 g/L). An increased serum TLI was seen in 2/31 dogs (6%). These dogs were further tested with serum Spec cPL concentration or SNAP cPL test, with normal/negative results.

In total, 35/51 dogs were tested for pancreatitis with serum Spec cPL concentrations or SNAP cPL tests. Four dogs had increased Spec cPL concentrations, of which one had a mildly increased concentration of 251 μg/L and the other 3 had concentrations above 400 μg/L, consistent with pancreatitis. The SNAP cPL was negative in all dogs tested, but none of the dogs with an increased serum Spec cPL were tested with SNAP cPL.

5.4.2.2 Study II

Increased serum folate concentrations were noted in 6 dogs (11%) and subnormal serum folate concentrations in 15 dogs (28%) (Table 5).

Two of 53 dogs had markedly subnormal serum TLI concentrations (1.0 and 2.7 g/L, reference interval 5.5-35 g/L, Laboratory Department of Evidendisa Specialist Animal Hospital, Strömsholm). Besides EPI, both of these dogs had concurrent histologically verified CE.

An increased serum TLI concentration was seen in 3/53 dogs (6%). These dogs were further tested for pancreatitis by measurement of serum canine pancreatic lipase immunoreactivity (Spec cPL), which was within the reference interval in two dogs. The remaining dog had a Spec cPL of 651 μg/L, consistent with pancreatitis (reference interval 0-200 μg/L)

Results for Spec cPL or SNAP cPL were available for all dogs in Study II. An increased serum Spec cPL concentration was detected in 10 dogs. Of these dogs, four had Spec cPL concentrations in the “gray zone” between 200 and 400 μg/L. Serum Spec cPL concentration > 400 μg/L, suggestive of pancreatitis, was detected in six dogs. SNAP cPL was negative in all dogs tested, but none of the dogs with an increased serum Spec cPL were tested with SNAP cPL

5.4.3 Parasitology (I, II)

Intestinal parasites were detected in three dogs in Study I. Two dogs were diagnosed with *Giardia intestinalis* and one with coccidiosis (*Isospora canis*).

In Study II, intestinal parasites were found in six dogs. *Isospora canis* was the most common parasite detected, found in two dogs. Of the remaining dogs, one dog each was diagnosed with *Giardia intestinalis, Cryptosporidium canis, Uncinaria stenocephala,* and *Toxocara canis*.

5.4.4 Diagnostic imaging (I, II)

No evidence of intestinal neoplasia was present on X-rays or abdominal ultrasound in any of the dogs enrolled in Study I.
In Study II, 51/53 dogs had an abdominal ultrasound scan, which was unremarkable in 13 dogs (25%). The most common change was a mild to moderate thickening of the small intestinal wall or thickening of the muscularis layer, noted in 17 dogs (33%), followed by enlargement of the mesenteric, ileocecal, or colonic lymph nodes in 11 dogs (22%), striations or "speckles" of the small intestine in 6 dogs (12%) and a fluid-filled stomach, small intestine, or colon in 5 dogs (10%).

5.4.5 Histopathology

5.4.5.1 Study I

Histopathological analysis of biopsies from the stomach and small and/or large intestine were available for 33/51 dogs (65%) in Study I. For all dogs, except one, biopsies were retrieved endoscopically. Biopsies from the remaining dog were collected surgically via laparotomy.

In 28 dogs, biopsies were collected from the gastric mucosa, duodenum, and colon. The remaining four dogs had biopsies collected from the gastric and small intestinal mucosa. Only the dog that underwent laparotomy had ileal biopsies retrieved.

No dog had evidence of neoplasia in any biopsies. The majority of dogs (28/33, 85%) had lymphocytic-plasmacytic inflammation of the gastrointestinal tract. A predominantly eosinophilic inflammation was found in 5/33 dogs (15%).

5.4.5.2 Study II

Results from gastrointestinal mucosal biopsies were available for 37/53 dogs. Nine dogs had biopsies collected 11 days to 9.3 years prior to study inclusion, and 28 dogs had biopsies collected during the study period. Of the remaining 16 dogs, three were considered unsuitable for general anesthesia, eight responded to dietary changes and five had owners who declined further work-up. Lymphocytic-plasmacytic inflammation of mild to moderate severity was the most common type of small intestinal inflammation, identified in 23/36 dogs (Table 6). Ileal biopsies were not available from any dog, and none of the dogs had changes consistent with neoplasia. Nine dogs had lymphangiectasia combined with chronic inflammation.

Table 6. Gastrointestinal histopathological biopsy reports (Study II)

Histopathological findings	Severity	n (%)
Gastric changes (n=37)		
Lymphocytic-plasmacytic gastritis	Mild	12 (32)
	Moderate	11 (30)
	Severe	2 (5)
Eosinophilic gastritis	Mild	1 (3)
	Moderate	5 (14)
Mixed cell type inflammation	Moderate	1 (3)
Fibrosis without inflammation	Moderate	1 (3)
Ulcerative gastritis	Moderate	2 (5)
Normal stomach		2 (5)
Small intestinal changes (n=36)		
Lymphangiectasia	Mild-moderate	9 (25)
Lymphocytic-plasmacytic enteritis	Mild	10 (28)
	Moderate	13 (36)
Eosinophilic enteritis	Mild	1 (3)
	Moderate	1 (3)
	Severe	1 (3)
Mixed cell type inflammation	Moderate	2 (6)
Erosive/ulcerative enteritis	Moderate	3 (8)
Normal duodenum		5 (14)
Large intestinal changes (n=29)		
Lymphocytic-plasmacytic colitis	Mild	7 (24)
	Moderate	13 (45)
Eosinophilic colitis	Moderate	2 (7)
	Severe	1 (3)
Mixed cell type inflammation	Moderate	2 (6)
Erosive/ulcerative colitis	Mild	1 (3)
	Moderate	2 (6)
Normal colon		1 (3)

5.5 Diet and medical treatment

5.5.1 Study I

At inclusion, 40/51 dogs were fed a commercial kibble diet from a major pet food company. Thirty of these dogs were fed diets with a single protein source, a hydrolyzed protein diet, or a diet labeled “intestinal” (Table 7). Of 51 dogs, 5 were being fed a commercial raw food diet, 4 were fed a commercial kibble diet combined with a home-cooked diet, and 2 were fed a home-cooked diet. At the time of collection of the follow-up blood sample, 19/51 dogs had switched to a new diet.

When the study started, 22/51 dogs were already receiving immunomodulatory treatment (Table 7). This treatment had lasted 9-2811 days (median 566 days) prior to study inclusion. Some of these dogs also received olsalazine (6), metoclopramide (3), folate (2), or metronidazole (1).

Of the remaining 29 dogs, four were treated with miscellaneous drugs for gastrointestinal disorders, i.e. folate (2), sucralfate (1), or pancreatic enzymes (1). Eight additional dogs were started on immunosuppressive therapy during the study period, six of which had biopsies confirming CE. The other two dogs had concurrent chronic pancreatitis and strongly suspected CE, but the owners did not want to proceed with endoscopy and further work-up. Hence, immunosuppressive treatment was started without a histopathological diagnosis, resulting in clinical improvement in both dogs.

Twelve dogs (24%) had previously been supplemented with cbl (Table 7). This treatment had ended 37-1788 days before study enrollment. After the previous cbl supplementation had ended, a low serum cbl concentration was demonstrated in all of these dogs, prior to study inclusion. Eight of these dogs had been treated with PE cbl weekly for 4-6 weeks, and four dogs had received a combination of PO and PE supplementation. This typically consisted of 1-4 injections in parallel with PO treatment for 100-200 days.

In summary, 25/51 dogs underwent a diet change or an altered medical treatment during the study period. The remaining 26 dogs continued on the same diet and medical treatment as at inclusion during the study, apart from the addition of oral cyanocobalamin (20/26) or cyanocobalamin and folate (6/26).

Table 7. Diet and medical treatment in 51 dogs with low serum cbl concentrations (Study I)

Parameter at inclusion or follow-up	Variable	Number of dogs (%)
Treatment at inclusion	Corticosteroids [a]	21 (41)
	Cyclosporine [b]	2 (4)
	Azathioprine [c]	3 (6)
	Other [d]	9 (17)
Diet at inclusion	Kibble diet (KD)	40 (78)
	KD: 'Intestinal'	9 (18)
	KD; single protein	15 (29)
	KD; hydrolyzed	6 (12)
	Home-cooked [e]	6 (12)
	Raw food (commercial)	5 (10)
Dietary change during study	-	19 (37)
Treatment at follow-up	Corticosteroids [f]	29 (57)
	Cyclosporine [g]	4 (8)
	Azathioprine [c]	3 (6)
	Other [h]	27 (53)
Diet at follow-up	Kibble diet (KD)	43 (84)
	KD: 'Intestinal'	8 (16)
	KD; single protein	17 (34)
	KD, hydrolyzed	12 (24)
	Home-cooked	5 (10)
	Raw food (commercial)	3 (6)

[a]Prednisolone (10), methylprednisolone (10), budesonide (1) [b]One dog was treated with cyclosporine+corticosteroids [c]Combined with corticosteroids [d]Olsalazine (6), folate (4), metoclopramide (3), sucralfate (1), metronidazole (1), pancreatic enzymes (1) [e]Home-cooked mixed with kibbles in 4 dogs, only home-cooked in 2 [f]Prednisolone (12), methylprednisolone (15), budesonide (2) [g]3 dogs treated with cyclosporine+ corticosteroids [h]Folate (22), olsalazine (5), metoclopramide (2), metronidazole (2), tylosine (1), pancreatic enzymes (1)

5.5.2 Studies II and III

At inclusion, the majority of the dogs (39/53) received commercial pet food kibbles from major pet food companies (Table 8). Two additional dogs were fed kibbles, but the owners could not specify the brand. Eight dogs were fed primarily or exclusively a home-cooked meat-based diet and four dogs received commercial raw food diets. Sixteen of the 39 dogs being fed a commercial kibble diet were on a diet labeled "intestinal" at inclusion, and 13/39 were on a single protein or hydrolyzed protein diet.

Upon contacting the different manufacturers, the cbl content of 38/39 known kibble diets was disclosed. The cbl range was 0.046-0.35 mg/kg (median 0.13 mg/kg) on a dry matter (DM) basis. No diet contained a lower

amount of cbl than the American Association of Feed Control Officials' minimum recommendation of 0.028 mg/kg DM.[2]

None of the manufacturers of the raw food diets could specify the cbl content. Furthermore, the cbl content of the home-made diets was not available. During the study 20/53 dogs were fed the same diet as at inclusion, whereas the remaining 33 dogs were switched to another type of diet (Table 8).

Twenty-four dogs were being treated for gastrointestinal disorders when the study commenced. The most common drug was corticosteroids in various forms (i.e. prednisolone, methylprednisolone, or budesonide) (n=11, Table 8), followed by olsalazine (n=5), cyclosporine (n=4), and metronidazole (n=4). At the end of the study, 41 of the remaining 49 dogs were receiving immunomodulatory treatment (Table 8).

Three dogs had previously been supplemented with oral cobalamin. Supplementation had ended 30-385 days prior to the blood sample, demonstrating recurrence of low serum cbl concentrations at study inclusion.

[2]See:http://www.merckvetmanual.com/management-and-nutrition/nutrition-small-animals/nutritional-requirements-and-related-diseases-of-small-animals

Table 8. Medication and diet in 53 dogs with chronic enteropathy and low serum cobalamin concentrations at inclusion; 49 dogs remaining after 90 days (Study II)

Parameter at inclusion or after 90 days	Variable	Number of dogs (%)
Treatment at inclusion	Corticosteroids [a]	11 (23)
	Cyclosporine [b]	4 (8)
	Antibiotics [c]	6 (11)
	Other [d]	9 (17)
Diet at inclusion	Kibble diet (KD)	41 (77)
	KD: 'Intestinal'	16 (30)
	KD; single protein	11 (21)
	KD; hydrolyzed	2 (4)
	Home-cooked	8 (15)
	Raw food (commercial)	4 (8)
Dietary change during study	-	33 (67)
Treatment after 90 days	Corticosteroids in total[e]	43 (88)
	Cyclosporine	4 (8)
	Chlorambucil [f]	9 (18)
	Antibiotics [g]	2 (49)
	Cortiocosteroids + miscellaneous [h]	21 (43)
	Miscellaneous [i]	3 (6)
Diet after 90 days	Kibble diet (KD)	45 (85)
	KD: 'Intestinal'	14 (26)
	KD; single protein	18 (34)
	KD, hydrolyzed	12 (23)
	Home-cooked	2 (4)
	Raw food (commercial)	2 (4)

[a]Corticosteroids (prednisolone, methylprednisolone or budesonide) (11), alone or in combination with olsalazine (5) [b]Three dogs were treated with cyclosporine+ corticosteroids [c]Metronidazole (4), amoxicillin (1), unknown antibiotics (1) [d]Omeprazole (3), sucralfate (2), metoclopramide (1), chaolin clay (1), chaolin clay + probiotics (1), pancreatic enzymes (1), folate (1), clomipramine (1) [e]Prednisolone/methylprednisolone (31), budesonide (11), prednisolone+ budesonide (1) [f]All dogs were treated with corticosteroids + chlorambucil [g]Metronidazole.[h]Folate (10), psyllium (7), olsalazine (7), pancreatic enzymes (2), sucralfate (2) [i]Folate (1), psyllium (1), metoclopramide (1).

5.5.3 Cobalamin tablet count (II)

Twenty-five of 27 dog owners remembered to bring the remaining cbl tablets for counting at the first follow-up appointment (day 28). At the second follow-up appointment (day 90), 22/27 dog owners brought the tablet container. The owners of the remaining dogs had forgotten to bring the tablets. The number of remaining pills differed by one tablet or less from the calculated consumption in 13/25 dogs at the first follow-up and in 11/22 dogs at the

second follow-up (Fig. 6). One dog had eight less remaining tablets than expected; the owner of that dog had dropped the container on the floor and believed that they had not found all of the tablets.

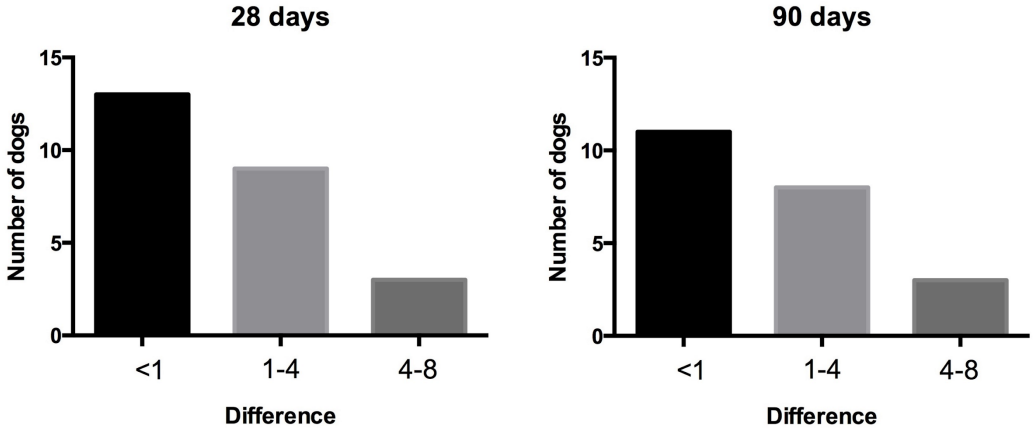

Fig. 6. Difference between expected number of remaining cobalamin tablets and tablet count at 28 (n=25 (of 27)) and 90 days (n=22 (of 27)).

5.6 Serum cobalamin concentrations

5.6.1 Study I

At study enrollment, mean ± SD serum cbl concentration was 223±33 ng/L (reference interval 234-811 ng/L), which had increased significantly to 1017±460 ng/L at follow-up ($p<0.0001$; Fig. 7). The follow-up blood sample was collected 20-202 (median 72) days after initiation of supplementation. Mean increase in serum cbl concentration (Δ) relative to baseline was 794±462 ng/L.

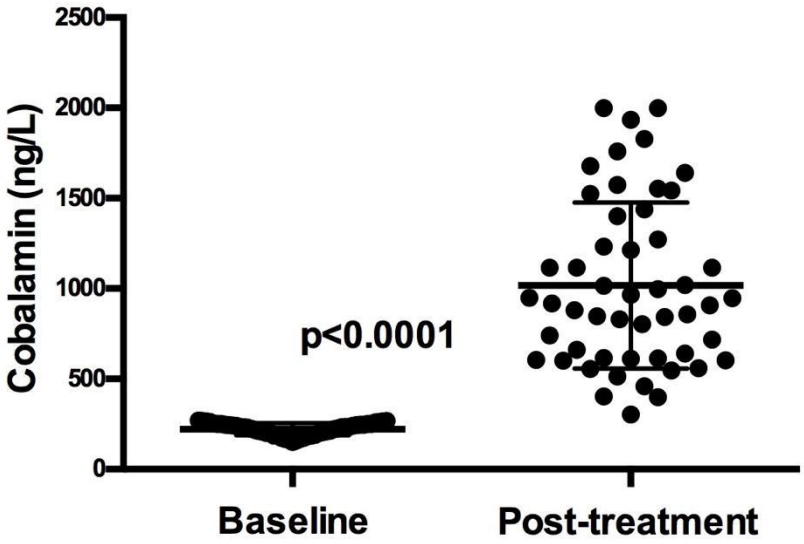

Fig. 7. Serum cobalamin concentrations at baseline and post-treatment in 51 dogs with hypocobalaminemia treated with oral cobalamin supplementation. Long horizontal line represents mean, short horizontal line standard deviation.

The three dogs with the poorest response to oral supplementation had a serum cbl Δ of 73, 152, and 183 ng/L after 54, 64, and 76 days of supplementation, respectively. The dog with the lowest response to oral cbl of all dogs in total was a German Shepherd (increase in serum cbl concentration 73 ng/L after 54 days of supplementation) that previously had been treated with parenteral cbl supplementation, achieving an equally poor response. Unfortunately, this dog was lost to follow-up. The two other dogs with the smallest response in Study I continued on oral cbl supplementation. At the next follow-up, serum cbl Δ concentrations had continued to increase, reaching 346 and 406 ng/L, respectively, compared with baseline. No correlation between the three dogs with the lowest response to treatment regarding breed, degree of inflammation, dose in mg/kg, duration of disease, or other characteristics could be identified.

The dogs were further stratified into two groups based on serum cbl concentrations (low-normal or subnormal) at inclusion and the increase in serum cbl concentration (cbl Δ) was compared. No significant difference was found between the groups (p=0.98, Fig. 8).

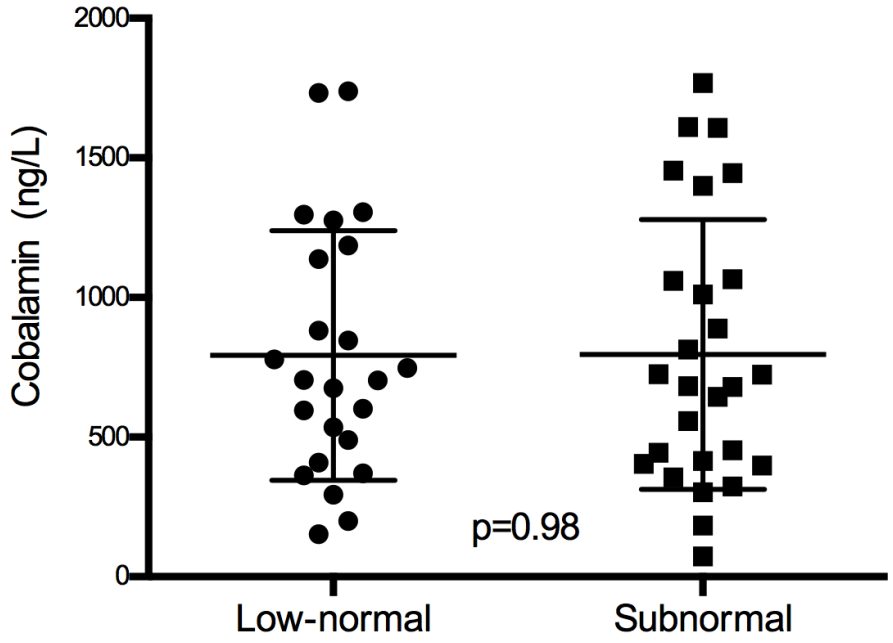

Fig. 8. Increase in serum cobalamin concentrations (cobalamin Δ) post-supplementation in dogs with low-normal (n=24) or subnormal (n=27) serum cbl concentration at inclusion. Long horizontal line represents mean, short horizontal line standard deviation.

Further, cbl Δ was stratified based on CIBDAI at time of study inclusion, with no significant difference between the groups (p=0.83, Fig. 9).

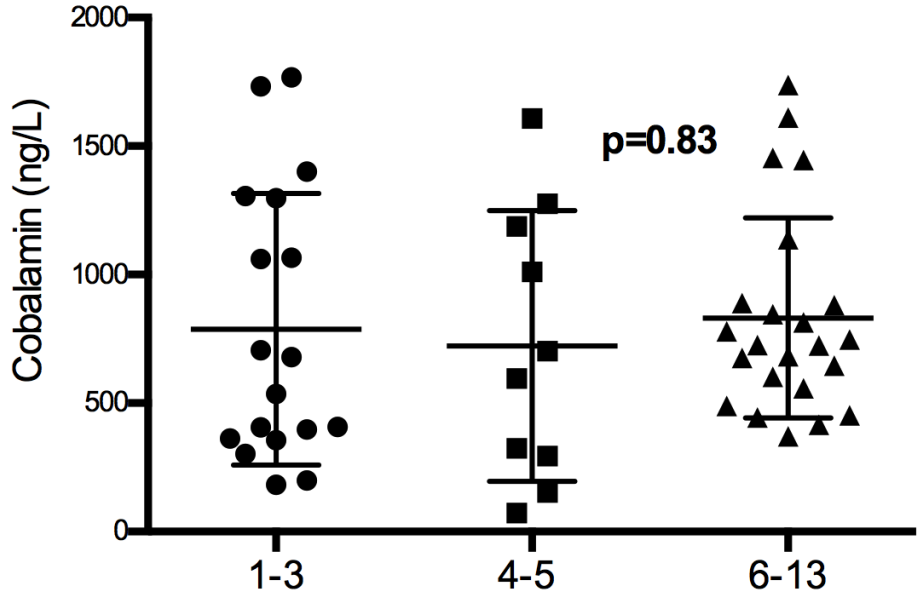

Fig. 9. Increase in serum cobalamin concentrations (cobalamin Δ) post-supplementation in dogs based on canine inflammatory bowel disease activity index (CIBDAI) at inclusion (CIBDAI 1–3; n=18, CIBDAI 4–5; n=10, CIBDAI > 6; n=23). Long horizontal line represents mean, short horizontal line standard deviation.

Finally, cbl Δ was compared between dogs that had a change in diet or medication during the study and dogs that had unaltered diet and medical treatment. No significant difference was found (p=0.27, Fig. 10).

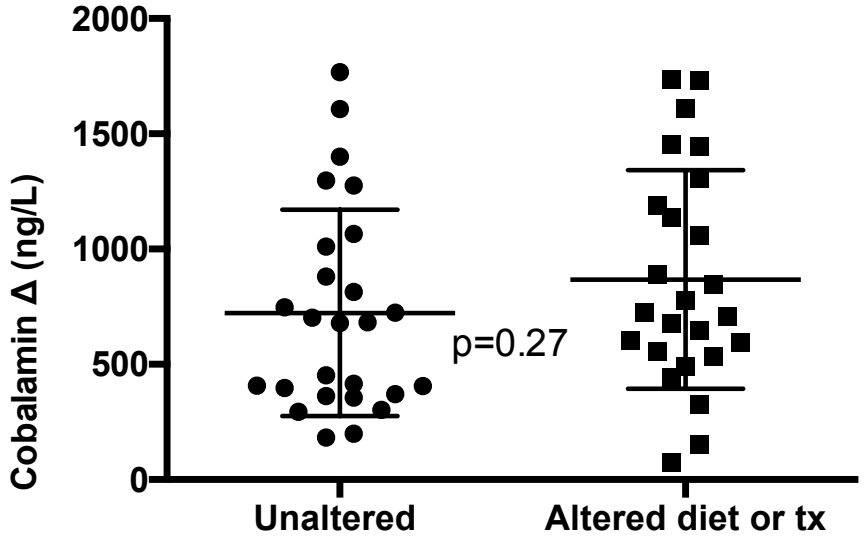

Fig. 10. Increase in serum cobalamin concentrations (cobalamin Δ) post-supplementation in dogs with unaltered diet and medical treatment during supplementation (n = 26) compared with dogs that had a change in diet or treatment (n = 25). Long horizontal line represents mean, short horizontal line standard deviation.

5.6.2 Study II

Serum cbl concentrations at inclusion were 150-285 ng/L (median 249) in the PO group and 150-285 ng/L (median 228) in the PE group (reference interval 244-959 ng/L). At this time-point, 3 dogs in the PO group and 3 dogs in the PE group had undetectable serum cbl concentrations (≤ 150 ng/L), which were truncated to 150 ng/L. After initiating supplementation, serum cbl increased significantly over time in the PO group. After 28 days, median (range) had increased to 955 ng/L (564-2385; p<0.0001), which had further increased to 1244 ng/L (768-4999; p<0.0001 compared to 28 days; Fig. 11) after 90 days.

In the PE group, serum cbl concentrations had increased significantly after 28 days of treatment, but decreased significantly at 90 days compared to 28 days. After 28 days, the median (range) serum cbl concentration was 2065 ng/L (725-10009; p<0.0001), which had decreased to 874 ng/L (188-1267; p<0.0001 compared to 28 days) after 90 days.

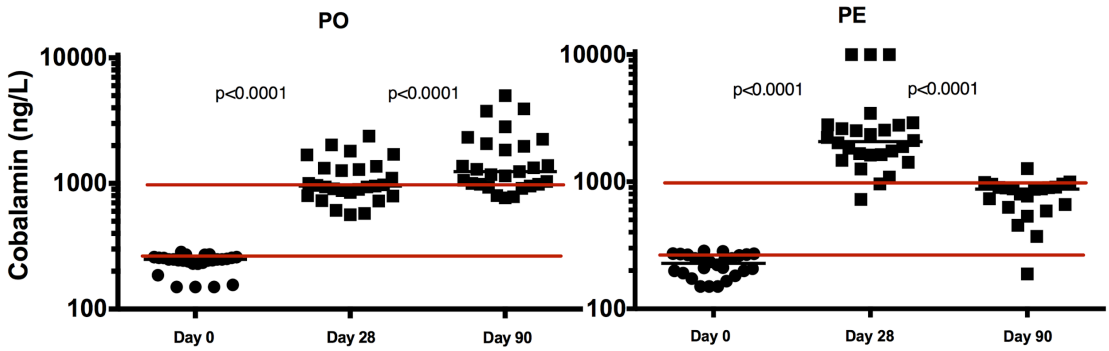

Fig. 11. Serum cbl concentrations over time in the PO (left panel, n=27) and PE (right panel, n=26 on days 0 and 28; n=22 on day 90) groups. Long horizontal lines represent reference interval and short horizontal lines represent median, Log10 scale.

The increase in serum cbl concentration (Δ cbl) was compared between the two groups. The median (range) Δ cbl was significantly higher in the PE group than in the PO group after 4 weeks (1799 ng/L (575-9827) and 696 ng/L (316-2235), respectively, $p<0.0001$). Ninety days after initiation of supplementation, the median (range) Δ cbl was significantly higher in the PO group (1045 ng/L (516-4849) than in the PE group (600 ng/L (38-997): $p<0.0001$, Fig. 12).

Looking specifically at the dogs with the lowest response to supplementation, the lowest serum Δ cbl concentration in the PO group was 316, 362, and 391 ng/L, respectively, after 28 days. After 90 days of treatment, these three dogs had a Δ cbl of 516, 535, and 582 ng/L compared with baseline. In the PE group, the lowest serum Δ cbl concentrations were 575, 762, and 814 ng/L, respectively, after 28 days. At the 90-day follow-up, serum Δ cbl had decreased to 38, 180, and 183 ng/L compared with baseline for the lowest responders in the PE group.

In the PO group, four dogs with PLE and serum albumin <20 g/L were included. After 28 days of supplementation, the Δ cbl for these dogs was 362, 690, 850, and 2235 ng/L, respectively, which had increased to 683, 1045, 1026, and 4849 ng/L (median 1036), respectively, after 90 days. Consequently, one of the three dogs with the lowest response to oral cbl after 28 days was diagnosed with PLE. However, the dog with the best response of all dogs in the PO group, at both follow-up visits, was also suffering from histologically verified CE with concurrent PLE and EPI.

In the PE group, six dogs with PLE were included. The Δ cbl range after 4 weeks of cbl supplementation was 575-2378 ng/L (median 1327). Ninety days after study initiation of cobalamin supplementation, Δ cbl was 38-786 ng/L (median 569) in this group of dogs.

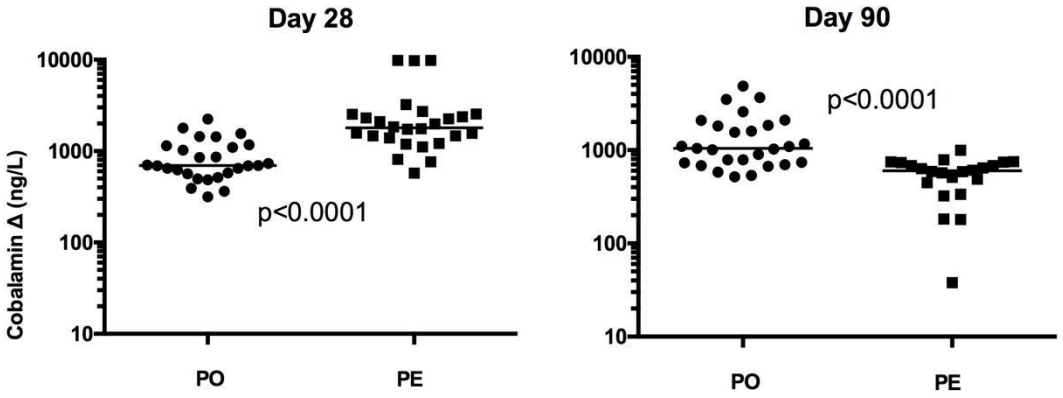

Fig. 12. Difference in serum cbl concentrations (cobalamin Δ) compared to baseline over time after cbl supplementation in the PO (n = 27) and PE (n = 26 after 28 days, n = 22 after 90 days) groups. Log10 scale, long horizontal lines represent median.

5.6.3 Dose-response curves (II)

The dose-response curves are shown in Fig. 13, with r^2 being 0.28 for the PO group and 0.01 for the PE group.

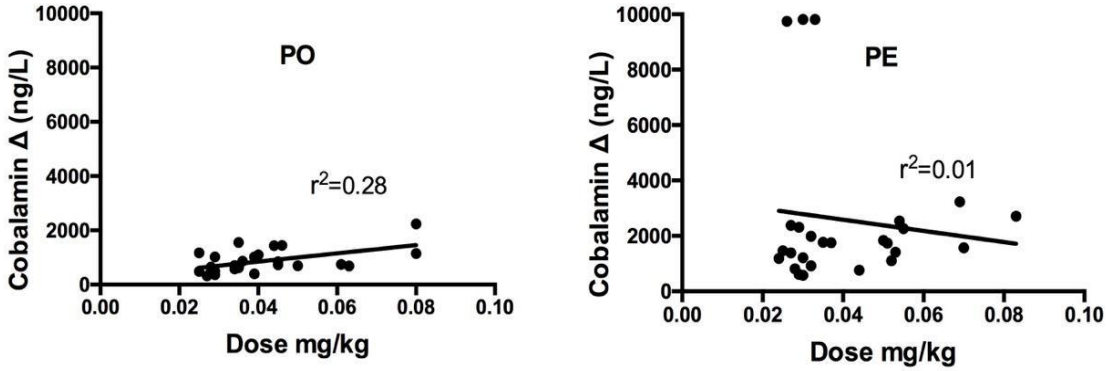

Fig. 13. Dose-response curves after 28 days of cbl supplementation based on serum cbl Δ in the PO (left panel, n=27l) and PE (right panel, n=26) groups.

5.7 Serum MMA and HCY concentrations (III)

5.7.1 Transport failure, accidental room-temperature storage, and stability (III)

Baseline serum samples from 10 dogs and follow-up serum samples from 11 dogs were lost during transport from the laboratory department at Evidensia Specialist Animal Hospital in Strömsholm to ESAHHS. When the samples were found after 13 days of room-temperature storage, they were immediately frozen again at -20°C. Paired samples from one occasion were available from

10 dogs; i.e. the samples had been drawn on the same occasion and split into two aliquots. One sample had been stored in the freezer at -20°C at ESAHHS the whole time and the corresponding sample had been sent to Strömsholm for cbl analysis.

The serum MMA concentrations of these corresponding samples were compared. All of these samples were analyzed in the same batch. Serum MMA concentration had decreased in 8 samples and increased in 2 samples (Fig. 14). The difference between the samples was compared and almost reached statistical significance (p=0.084). This difference, expressed as the percentage of serum MMA concentration of the sample stored at room temperature, compared with the corresponding sample stored in the freezer, was 35-228% (median 74%). The wide range of difference and the fact that MMA concentrations could either increase or decrease with prolonged storage at room temperature led to the decision to exclude all of the samples that had been stored at room temperature for 13 days. Homocysteine concentrations were not compared since the serum volume was too small.

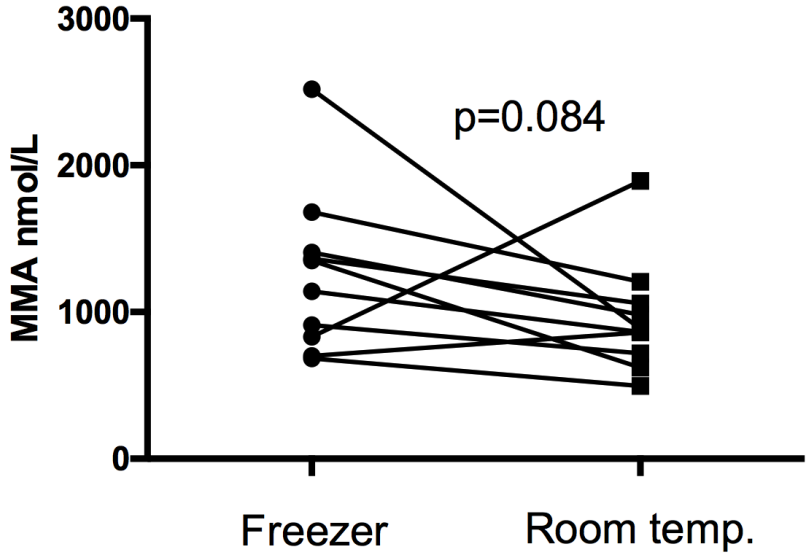

Fig. 14. Comparison of serum methylmalonic acid (MMA) concentrations in samples accidently stored at room temperature for 13 days and paired samples from the same dogs collected at the same time-point stored at -20°C. The samples stored in the freezer appear on the left, and those stored at room temperature on the right.

5.7.2 Serum MMA concentrations (III)

Serum samples from 36 dogs included in Study II were available for MMA analysis. Seventeen samples from Study II could not be used in Study III due to insufficient volume of baseline serum sample (n=7) or serum samples lost in transport (n=10). Nine of 36 dogs (25%) had an increased serum MMA concentration at inclusion (reference interval 415-1193 nmol/L; Fig. 15), of which 5 belonged to the PO group and 4 to the PE group. Serum MMA concentrations decreased significantly in both groups after 28 days of treatment (p<0.0001 in both groups), with no further reduction after 90 days

of treatment compared to 28 days (p=0.58 in PO group and p=0.76 in PE group).

In the PO group, median (range) serum MMA concentrations were 932 (566-2468) nmol/L (566-2468, n=18) at baseline, 705 nmol/L (386-1465, p<0.0001, n=18) after 28 days, and 739 nmol/L (450-1221, p=0.58 compared to 28 days, n=17) after 90 days (Fig. 12). Median (range) serum MMA concentrations in the PE group were 943 nmol/L (508-1900, n=18) at baseline, 696 nmol/L (377-932, p<0.0001, n=18) after 28 days, of supplementation and 690 nmol/L (349-1145, p=0.76 compared to 28 days, n=13) after 90 days (Fig. 15). When comparing the serum MMA concentration between the PO and PE groups over time, no statistical differences emerged at the three different time-points (p=0.99 at inclusion, p=0.28 after 28 days, and p=0.59 after 90 days).

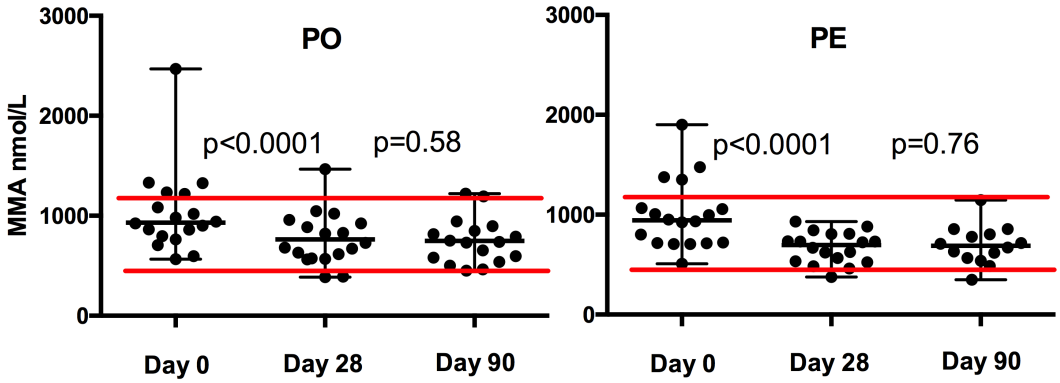

Fig. 15. Serum MMA concentrations over time in the PO (left) and PE (right) groups. Long horizontal lines represent reference interval, medium horizontal lines median, and short horizontal lines range.

In total, serum MMA concentrations had decreased at day 28 compared to day 0 in 32/36 dogs. In two dogs in each group, serum MMA concentrations had increased with 1-23 nmol/L (median 13.5) compared to baseline at day 28. However, at day 90, serum MMA concentrations had decreased compared to baseline and day 28 in three of those four dogs. Thus, serum MMA concentrations decreased compared to day 0 in 35/36 dogs during the study.

In the 27 dogs with serum MMA concentrations within the reference range at inclusion, significant decreases in serum MMA concentrations were seen in both groups (Fig. 16). Serum MMA concentrations in the PO group (mean (range)) were 846 (566 – 1083) nmol/L at day 0 and 681 nmol/L (386-959; p=0.0001) at day 28. In the PE group, mean serum MMA concentrations were 843 (508 – 1067) nmol/L at day 0 and 653 nmol/L (377-932 ; p=0.0004) at day 28. There was no significant difference between the PO and PE group at day 0 (p=0.97) or day 28 (p=0.68).

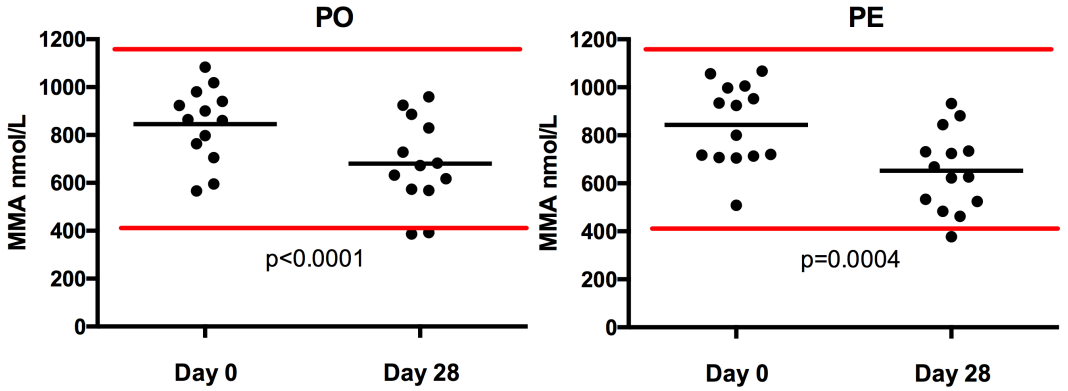

Fig. 16. Serum MMA concentrations at day 0 and day 28 in the PO (left) and PE (right) groups after removal of dogs with supranormal serum MMA concentrations at inclusion. Long horizontal lines represent reference interval and short horizontal lines mean.

5.7.3 Serum HCY concentrations (III)

Homocysteine concentrations at inclusion were supranormal in 4/35 dogs (11%) (reference interval 5.9 - 31.9 µmol/L), of which three dogs were in the PO group and one dog in the PE group (Fig. 17). No significant difference was present in serum HCY concentration after 28 days compared to baseline in either group (p=0.61 in PO group and p=0.28 in PE group). After 90 days of treatment, a small increase of HCY concentration was seen in both groups compared to 28 days. This increase was only significant in the PE group (p=0.07 in PO group and p=0.0007 in PE group).

The median (range) serum HCY concentration in the PO group was 12.2 µmol/L (3.3-62.2, n=18) at inclusion, 12.5 µmol/L (5.0-45.0, p=0.44 compared to baseline, n=19) after 28 days, and 17.7 µmol/L (7.3-60.0, p=0.038 compared to 28 days, n=16) after 90 days of supplementation. In the PE group, the median (range) serum cbl concentration at the time of inclusion was 8.4 µmol/L (3.7-34.8, n=17), 8.0 µmol/L (3.8-18.3, p=0.43 compared to baseline, n=17) after 28 days, and 12.4 µmol/L (6.3-33.1, p=0.0007 compared to 28 days) after 90 days (n =13) of supplementation.

No significant differences were detected between the groups at any time-points (p=0.35 at baseline, p=0.12 after 28 days, and p=0.11 after 90 days). Of the four dogs with increased HCY concentrations at inclusion, the HCY concentrations had decreased to normal concentrations after 28 days in two dogs, one belonging to the PO and the other to the PE group. The dog in the PE group was lost to follow-up due to euthanasia shortly thereafter. The dog in the PO group with normalized serum HCY concentration at day 28 had serum HCY concentrations within the reference range at 90 days as well. The remaining two dogs with increased HCY concentrations at inclusion, both in the PO group, had consistently supranormal HCY concentrations over time. In the PE group, one dog had HCY concentration very close to the upper limit of the reference interval at inclusion and supranormal HCY concentration at 90 days (33.1 µmol/L).

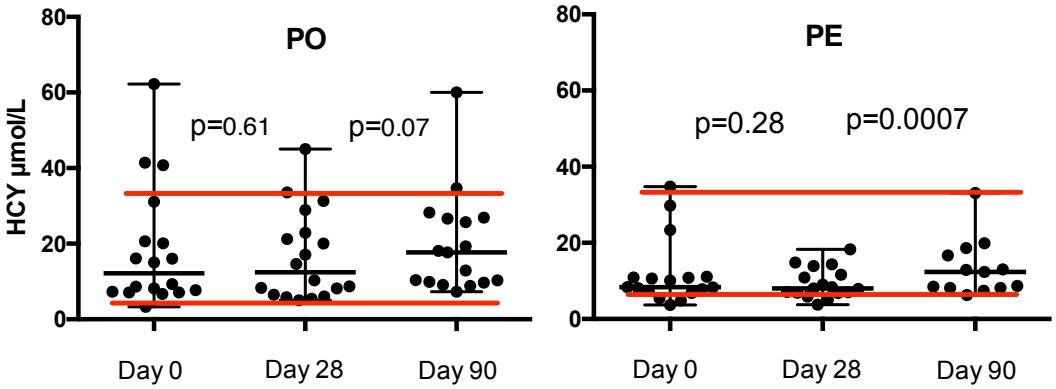

Fig. 17. Serum HCY concentrations over time in the PO (left) and PE (right) groups. Long horizontal lines represent reference interval, medium horizontal lines median, and short horizontal lines range.

6. DISCUSSION

6.1 Serum cbl inclusion range (I – III)

In all three studies, dogs with hypocobalaminemia (below the lower limit of the reference interval) and dogs within the lowest end of the reference interval were included. In Study I, dogs with serum cbl concentrations within the lowest 5% of the reference interval were included (serum cbl concentration 270 ng/L [199 pmol/L], reference interval: 234-811 ng/L [173-599 pmol/L]). In Studies II and III, dogs up to 30 units (based on SI units) above the low end of the reference interval were included (serum cbl concentration ≤ 285 ng/L [210 pmol/], reference interval 244-959 ng/L [180-708 pmol/L]). These ranges are referred to as "low cbl concentrations" throughout the text.

It could be argued that it is questionable to include dogs at the low end of the cbl reference interval. However, data from a previous study on serum MMA concentrations in dogs with various serum cbl concentrations, based on left-over serum samples at a laboratory, demonstrated that 19% of dogs with a serum cbl concentration of 251–350 ng/L (low end of the reference interval) had increased serum MMA concentrations (Berghoff et al, 2012). This may indicate intracellular cbl deficiency, similar to subtle cbl deficiency in humans.

Furthermore, in human patients where cbl deficiency can neither be excluded, nor confirmed, a significant decrease in serum MMA concentration after cobalamin supplementation indicates pre-clinical deficiency (Herrmann and Obeid 2012). Even in the dogs with serum MMA concentrations within the reference range at inclusion (study III), a significant decrease in serum MMA concentrations was seen after cobalamin supplementation, possibly indicating an early stage of cbl deficiency in parallel with human medicine.

The negative effects of cbl deficiency combined with the high safety profile of cbl supplementation has led some authors and centers[3] to recommend rapid identification and early intervention (Dossin, 2011).

As mentioned previously, cbl supplementation has a very high safety profile. In human medicine, no toxic effects of cbl supplementation have been reported, even when administered PE at 300–3000 times the recommended daily dietary amount (Allen, 2012).

It is a well-known fact in human medicine that reference intervals regarding cbl, MMA, HCY, and holoTC differ substantially between labs using different laboratory methods (Aparicio-Ugarriza et al., 2015). The age of the referenced population also differs. Further, the reference interval differs between countries where regular dietary staple items are fortified with B vitamins and countries where food fortification is not common (Aparicio-Ugarriza et al., 2015). Hence, there is no gold standard or exact cut-off value for when to start cbl supplementation. In Western countries, additional biomarkers of cbl deficiency are often analyzed if a subnormal or low-normal

[3] http://vetmed.tamu.edu/gilab/research/cobalamin-information

serum cbl concentration is found. Supplementation is started despite a serum cbl within the reference interval if MMA and/or HCY is supranormal or if holoTC is subnormal (Herrmann and Obeid, 2012).

The analysis of MMA and HCY is technically complex and not widely available in veterinary medicine, and holoTC has not been studied in dogs to our knowledge. Given the high safety profile of cbl, results from studies on MMA in dogs with low cbl concentrations, and supplementation recommendations in humans, it appears to be a reasonable clinical approach to supplement dogs with serum cbl concentration at the lowest end of the normal reference range that exhibit signs of chronic gastrointestinal disease. These arguments were behind the decision to include dogs with clinical signs of CE and serum cbl concentration at the lowest end of the normal reference range.

6.2 Effect of oral supplementation on serum cbl concentrations (I, II)

Oral cbl supplementation was associated with a significant increase in serum cbl concentrations in both Study I ($p<0.0001$) and Study II after 28 and 90 days ($p<0.0001$ after 28 days compared to baseline, $p<0.0001$ at 90 days compared to 28 days). Similarly to studies in human patients, serum cbl concentrations increased significantly with PO treatment (Berlin et al., 1968; Kuzminski et al., 1998, Bolaman et al.,2003; Eussen et al., 2005; Vanderbrink et al., 2010; Castelli et al., 2011; Kim et al., 2011; Gomollón et al., 2017).

A few dogs in the retrospective study (I) had a worse response to supplementation than any dogs in the prospective study (II). The three dogs with the lowest response to oral supplementation in Study I had a cbl Δ of 73, 152, and 183 ng/L after 54, 64, and 76 days of supplementation, respectively. These results can be compared with cbl Δ of 316, 362, and 391 ng/L for the three dogs with the lowest cbl Δ after 28 days of PO supplementation in Study II. After 90 days of treatment, the three dogs with the smallest increase of serum cbl concentrations had a cbl Δ of 516, 535, and 582 ng/L compared with baseline (Study II).

A possible explanation for why the dogs in Study II responded better to supplementation than those in Study I is that the owners of the dogs in the prospective study could have been more meticulous about cbl supplementation. They were fully aware of the fact that their dog participated in a study and that the remaining tablets would be counted at every re-check. Although pill count has been shown to overestimate compliance, a positive correlation exists between the number of clinician pill counts and adherence to treatment in humans (Pullar et al., 1989, Berg and Arnsten, 2006, Achieng et al., 2013).

Varied responses of serum cbl concentrations to supplementation could also be explained with dose differences. Using the current supplementation protocol, the oral dose range for dogs participating in Study II was 0.025-0.08 mg/kg. However, a dose-response curve showed a very weak linear correlation ($r^2=0.28$), making individual factors a more likely reason for a variable response to supplementation.

Indeed, the response to both PO and PE supplementation varied substantially between individuals in Study II. Potential individual factors that could influence response to treatment are degree of malabsorption associated with mucosal damage, composition of the microbiota, shortened half-life of exogenous cbl in small intestinal disease (at present only proven in cats, Simpson et al., 2001), concurrent pancreatic or liver disease, and possibly extra-intestinal malfunction of the cbl transport system such as decreased amount of functional transcobalamin II (Watkins and Rosenblatt, 2011). The latter has been shown to play a role in the intestinal absorption of cobalamin in humans, but has not been studied in dogs, as far as we are aware.

6.3 Factors affecting response to oral supplementation

In human medicine, dose and duration of supplementation affect serum cbl concentration, hematological deviations, and neurological signs (Berlin et al., 1968, Kuzminski et al., 1998; Eussen et al., 2005; Vanderbrink et al., 2010; Castelli et al., 2011; Kim et al., 2011). A study in cbl-deficient humans demonstrated that 0.5 mg oral cbl daily produced a satisfactory response in most patients, but caused borderline circulating serum cbl concentrations in some patients (Hathcock et al., 1991). A daily dose of 1.0 mg was associated with successful long-term results (Elia, 1998, Nyholm et al., 2003). In one study, a dose of 2.0 mg of oral daily cbl supplementation was used, which was associated with a higher serum cbl concentration than that produced by the standard parenteral protocol (Kuzminski et al., 1998).

As mentioned in Section 6.2, the dose-response curve calculated for the dogs in the PO group in Study II revealed a very weak correlation. Possibly, a wider dose range and a larger case number are needed for a better correlation between dose and response. The dose range used in Studies I and II was 0.25 – 1.0 mg in dogs with a BW of 2.6-52 kg. This can be compared with a dose-finding trial in cbl-deficient human patients older than 70 years, where daily cyanocobalamin doses at 0.0025, 0.1, 0.25, 0.5, and 1.0 mg were used (Eussen et al., 2005). The mean reductions in plasma MMA concentrations for the different groups were 16%, 16%, 23%, 33%, and 33%, respectively. A clear correlation between dose and response was shown in this study.

A longer duration of treatment in Study II was significantly associated with increased serum cbl concentration ($p<0.0001$ comparing serum cbl concentrations at 28 and 90 days), which also has been shown in human studies (Kuzminski et al., 1998, Vanderbrink et al., 2010, Kim et al., 2011, Castelli et al., 2011). A similar conclusion could not be drawn in Study I since serum cbl concentrations were only measured at one time-point.

Clinical disease activity (CIBDAI) at inclusion was not associated with response in serum cbl concentrations in Study I ($p=0.83$). This is supported by the results from dogs with PLE in the PO group in Study II, where serum cbl concentrations after supplementation were scattered from among the lowest responses to the highest response of all in the PO group. In human medicine, the non-IF-dependent absorption of oral cbl (see Section 6.4) was of the same magnitude in individuals with normal absorptive capacity as in patients with pernicious anemia, in patients with idiopathic malabsorption, or

in patients who had undergone extensive gastric, small intestinal, or ileal surgery (Berlin et al., 1968).

It could be speculated that cbl absorption would be facilitated by reducing the inflammation of the small intestine with a dietary change or adding immunomodulatory treatment. However, there was no significant difference in response to oral cbl if diet and/or concurrent medication was changed, compared with dogs with unaltered diet or medication apart from the addition of cbl or cbl + folate (Study I). This result is further in line with the study by Berlin and co-workers, where the non-IF-dependent absorption of oral cbl was similar in healthy individuals and in patients affected by malabsorption.

6.4 Possible mechanisms behind response to oral supplementation

A direct uptake of cbl, independent of IF, has been proven in humans by using radioactively labeled cbl (Berlin et al., 1968). The absorption was roughly proportional to the oral dose. Approximately 1.2% of the oral dose administered was absorbed via this direct route. This absorption was not affected by concurrent malabsorption, lack of IF, or extensive surgery of the gastrointestinal canal. The exact mechanism behind the uptake is not known, but a passive diffusion process was suggested, based on the correlation between dose and uptake. A constant ratio between dose and absorption was proven in a wide dose range from 1 pg to 100 000 pg (=100 mg) of cbl.

One dog each in Studies I and II had EPI and CE. Both dogs, however, had a very good response to oral cbl supplementation. Recently, excellent response to oral cbl has been proven in 10 dogs with EPI (Toresson et al., 2017, abstract). The response to oral cbl in dogs with EPI is especially interesting as it can be assumed that these dogs lack IF. The pancreatic enzymes available in Sweden should not contain any IF, although negligible traces, due to contamination, could exist (personal communication, Malin Lindgren, Mylan). However, even if traces of hog IF were present, it is not known whether dogs can utilize hog IF. It has been shown that dogs cannot use IF of bovine origin (Simpson et al., 1989). The finding that dogs with EPI can absorb oral cbl may support the theory that dogs too have an alternative absorptive pathway of cbl, independent of IF. Additionally, successful oral cbl supplementation in a dog with IGS further suggests the presence of an alternative absorptive pathway beyond the ileal cubam receptor (McCallum and Watson, 2018).

Intestinal microbial cbl saturation is another potential mechanism behind successful PO cbl supplementation. Dysbiosis is a common sequela or an initiating factor of CE. Certain anaerobes, such as some *Clostridia* and *Bacteroides,* are capable of binding to the cbl-IF complex, and thus, utilizing cbl (Giannella et al., 1972, Honneffer et al., 2014). An increased amount of these bacteria in the small intestine could theoretically induce competition for cbl with the host, resulting in less cbl available for absorption in the ileum. Oral cbl supplementation will lead to a large amount of cbl available for the intestinal microbes. This could potentially saturate the cbl binding bacteria.

As a consequence, more cbl-IF complexes may be available for ileal absorption. However, to the author's knowledge, no direct evidence has been published linking dogs with hypocobalaminemia to dysbiosis with increased amounts of *Clostridia* and *Bacteroides*. While dysbiosis may be the reason for low cbl concentrations in some canine patients, it seems less likely that microbial cbl saturation is the sole mechanism behind successful oral cbl supplementation in all dogs with low cbl concentrations, especially considering the efficacious oral cbl supplementation in dogs with EPI, and the case report of successful oral cbl supplementation in a dog with IGS (Toresson et al., 2017, McCallum and Watson, 2018).

6.5 Comparison of PO and PE cbl supplementation (II)

In humans, the major benefits of PO cbl supplementation is the cost-effectiveness and, for the individual patient, avoiding the pain with which injection may be associated and having a simple supplementation protocol that is easy to follow at home. In one study, 83% of cbl-deficient human patients preferred PO treatment over PE (Nyholm et al., 2003). Parenteral cbl is administered intramuscularly or subcutaneously in humans, and both routes may be associated with pain, especially in thin patients (Elia, 1998). On rare occasions, cbl injections have caused adverse reactions, such as tissue necrosis or localized scleroderma, but these reactions are mainly believed to be caused by the carrier substance, not by cbl itself (Hovding et al., 1968, Ho et al., 2004). Dogs, by contrast, rarely show pain when subcutaneous injections are administered.

Regarding compliance, 8/19 patients suffering from Crohn's disease in a recent report were non-compliant with a PE protocol, but complied with a PO protocol (Plener et al., 2014). The PO protocol restored normocobalminemia in these patients. Eleven percent of human cbl-deficient patients were non-compliant with a PE protocol in another study (Elia, 1998). This was especially common when PE cbl was administered by patients or friends instead of healthcare professionals. In Study II, all dogs that did not complete the study (3/3) due to non-compliance with the study protocol belonged to the PE group. It is likely that it was more difficult for the dog owners to remember veterinary appointments at irregular intervals than to give a daily tablet.

Naturally, non-compliance also occurs in humans on a PO cbl supplementation protocol, especially in young males or patients suffering from dementia (Elia, 1998; Gomollón et al., 2017). Although tablet count is by no means a perfect method to assess compliance, tablet counting in Study II suggests that many dog owners missed a few doses (Achieng et al., 2013, Berg and Arnsten, 2006). However, missing a daily oral dose on a few occasions should have a much smaller impact on serum cbl concentrations than missing one or several weekly cobalamin injections. The possibility to choose between a PE and PO supplementation protocol increases the chance to find a protocol that suits the individual client's circumstances and may increase compliance overall.

It may be questioned whether it is appropriate to compare daily PO

supplementation with a PE protocol entailing initially weekly then monthly injections. However, the study design compares two different absorptive mechanisms. Since it can be assumed that the dogs in the study had a malfunctioning intestine, resulting in low serum cbl concentration, this reduced absorptive capacity had to be overridden. This could either be achieved by bypassing the diseased intestine with PE supplementation or by using high daily PO doses. Human studies have shown that if sufficiently high oral cbl doses are given, the absorption of cbl does not differ between healthy individuals and those lacking IF, those suffering from malabsorption, or those having had extensive surgical resections of the stomach, small intestine, or ileum (Berlin et al., 1968). Furthermore, a study design similar to the one used in Study II has been applied in several human studies (Kuzminski et al., 1998; Castelli et al., 2011; Kim et al., 2011).

The PO group had a significantly lower serum cbl Δ concentration than the PE group after 28 days (Study II). However, this situation was reversed after 90 days, when the PO group had a significantly higher serum Δ cbl concentration than the dogs of the PE group. At this time-point, dogs in the PE group had had the last cbl injection 3-4 weeks earlier. Studies using radioactively labeled cbl in dogs showed that higher doses of cbl given parenterally were associated with a smaller fractional uptake of cbl by the liver and a larger fraction lost in urine, whereas the proportional uptake of cbl by the liver increased and the fraction lost in urine was diminished using smaller doses of cbl (Glass and Mersheimer, 1958).

It is possible that low doses of cbl given frequently are more effective in loading depleted body stores of cbl in dogs and cats than high doses given less frequently. A study in cats on a cbl-deficient diet showed that very low doses of PE cbl administered daily (10 μg) normalized urinary MMA concentrations and resulted in complete clinical remission (Morris et al., 1977). This dose corresponds to 70 μg/week, which is less than one-third of the currently recommended weekly dose of 250 μg/cat, but was still effective in normalizing urinary MMA and reversing severe weight loss and other clinical signs of cbl deficiency. Although the daily oral doses given in Studies I and II/III appear to be high, only about 1% of the PO dose administered is absorbed, if results from human studies can be extrapolated to dogs (Berlin et al., 1968). Thus, after intestinal absorption, the oral cbl supplementation protocol in Studies I and II may be equivalent to a low dose given frequently.

Regarding the efficacy of cbl supplementation in dogs with PLE, the median (range) Δ cbl after 28 days was 770 (362-2235) ng/L in the PO group (n=4) and 1327 (575-2378) ng/L in the PE group (n=6). After 90 days, the median (range) Δ cbl was 1035 (683-4849) ng/L in the PO group (n=4) and 569 (38-786) ng/L in the PE group (n=6). It should be noted that, of all dogs in the PO group (n = 27), 1/3 dogs with the lowest Δ cbl after 28 days was a dog with PLE. However, the dog with the highest Δ cbl at 28 and 90 days in the PO group was also a dog with PLE. Of all dogs in the PE group (n=26 at day 28, n=22 at day 90), the dog with the lowest Δ cbl after 28 and 90 days was a dog with PLE. It is difficult to draw any major conclusions based on the results from such a small number of dogs. Further studies are needed to evaluate the efficacy of oral cbl supplementation in dogs with PLE.

The dose-response curves calculated show a very weak linear correlation for both groups (r^2=0.28 for PO group and r^2=0.01 for PE group).

A wider dose range and a larger study group may be needed for a better dose-response correlation. A logarithmic correlation is also possible. It can further be speculated that individual factors regarding transport and absorption of cbl are the major driver of the weak correlation.

The low Δ cbl seen in a few dogs in the PE group 3-4 weeks after the last cbl injections stresses the importance of measuring serum cbl concentration after discontinuation of cbl supplementation. Until proven otherwise, this recommendation refers to both oral and parenteral supplementation. Further studies are needed to evaluate recurrence or persistence of low serum cbl concentrations after PO versus PE supplementation.

In summary, Study II demonstrates that both the PE and the PO supplementation protocols were effective in treating dogs with low serum cbl concentrations. The choice of treatment can be based on client preferences. In Finland and Sweden, dog owners are not allowed to give their dogs injections at home, with the exception of insulin treatment and allergen-specific immunotherapies. The oral supplementation protocol thus represents a very cost-effective treatment alternative. When comparing the costs of PO versus PE supplementation in Sweden and addressing only the cost of the supplement, the PO treatment is associated with less than one-third of the cost of the PE supplementation protocol during the treatment period used in this study.

6.6 Relationship between intestinal parasites and serum cobalamin concentrations (I, II)

In humans, an association between tapeworm infection and decreased serum cbl concentrations has been shown (Nyberg et al 1961.; Vuylsteke et al., 2004). Furthermore, some studies have demonstrated a relationship between low serum cbl concentrations and *Giardia intestinalis* infection, whereas other studies have failed to show such a relationship (Springer and Key, 1997; Askare et al., 2007; Zarebavani et al, 2012.) It has thus been suggested that the association between *Giardia intestinalis* infection and low serum cbl concentrations only occurs if other co-founding factors, such as malnutrition, are present (Stabler et al., 2004).

Giardia intestinalis was associated with subnormal serum cbl concentrations in a recent study in dogs with chronic diarrhea (Volkmann et al., 2017). Of the 9 dogs diagnosed with intestinal parasites in Studies I and II, 3 were diagnosed with *Giardia intestinalis*. Whether this was the primary cause of cbl deficiency, a contributing factor, or an incidental finding is difficult to assess. Infection with *Giardia intestinalis* can be asymptomatic, but may also result in mild to severe diarrhea. In human patients, persistent gastrointestinal signs can often be present long after eradication of the parasite (Robertson et al., 2010). This is very likely true for dogs as well. A long-standing parasite infection could further possibly result in an exaggerated intestinal immune response, potentially triggering chronic inflammation in susceptible dogs.

Of the nine dogs diagnosed with intestinal parasites, the clinical impression, based on response to deworming, was that only 1/3 dogs with *Giardia intestinalis* infection and the one dog with *Toxocara canis* infection had parasite infection as the primary problem, possibly resulting in cbl deficiency. However, long-standing parasite infection in some of the other dogs could potentially have contributed to the development of CE. The association between low serum cbl concentrations and intestinal parasite infections, with and without CE, warrants further studies.

6.7 Effects on markers of cellular cbl deficiency (III)

6.7.1 Serum MMA concentrations (III)

At inclusion, 9/36 dogs (25%) in Study III had increased serum MMA concentrations. This proportion is consistent with another study in dogs with CE, in which 25% of the dogs with cbl deficiency had increased serum MMA concentrations (Berghoff et al., 2013). It has previously been shown that supranormal serum MMA concentrations are more prevalent in dogs with undetectable serum cbl concentrations (63%) than in dogs with subnormal but detectable serum cbl concentrations (31%) or dogs with serum cbl concentrations at the lowest end of the reference interval (19%) (Berghoff et al., 2012). In another study, serum MMA concentrations were supranormal in 33-100% of dogs of seven different breeds with undetectable serum cbl concentrations (Grützner at al., 2013). Since dogs with both subnormal and low-normal serum cbl concentrations were included in Study III, the expected prevalence of increased serum MMA concentrations, based on previously published studies, is roughly 19-31%. When stratifying the data into two groups according to the serum cbl concentration, 5/18 dogs (28%) with subnormal and 4/18 dogs (22%) with low-normal concentrations had supranormal serum MMA concentration. Thus, the difference between the subnormal and low-normal cbl groups was smaller than expected based on a previous study (Berghoff et al., 2012). However, in the study by Berghoff and coworkers, serum samples from 555 dogs were included. If our study populations had been larger, the difference between the subnormal and low-normal group regarding prevalence of increased serum MMA concentrations may have been larger.

Compared with most human studies on metabolic markers of cbl deficiency, the correlation between increased serum MMA concentrations and decreased serum cbl concentrations is much stronger in humans (Stabler et al., 1986, Herrmann et al., 2000, Carmel, 2011). In a study investigating serum MMA concentrations in human patients with episodes of clinical cbl deficiency (anemia and/or neurological symptoms), 98% of patients with subnormal serum cbl concentrations had supranormal MMA concentrations (Stabler et al., 1986). In a review paper, >95% of patients with pernicious anemia had increased serum MMA concentrations (Carmel, 2011). However, in a very recent study on cbl deficiency in humans with IBD, only 38% of the patients with subnormal serum cbl concentrations had supranormal serum MMA

concentrations (Battat et al., 2017).

Cobalamin deficiency in humans has by some authors been described to progress from low serum cbl concentrations to decreased concentrations of holoTC combined with decreased cellular stores of cbl, followed by biochemical cbl deficiency, and finally clinical signs of overt cbl deficiency (Herrmann et al., 2001). It is possible that the human patients with IBD and subnormal serum cbl concentrations in the study by Battat and co-workers (2017) had not yet reached the state of biochemical cbl deficiency, which could explain the discrepancy between serum cbl and MMA concentrations. This explanation could apply to dogs as well. Potentially, several dogs with CE might have been diagnosed with cbl deficiency while the deficiency was still subclinical, prior to the stage of increased markers of cbl deficiency. Although the majority of the dogs in Studies II and III had showed clinical signs for more than a year prior to study inclusion, the time period may not have been sufficient to deplete body stores of cbl. To the best of our knowledge, there are no studies in dogs with malabsorption describing the half-life of cbl or the amount of time required to deplete cbl body stores due to malabsorption. One study demonstrated that the half-life of cbl stored in the liver was 16 weeks in healthy dogs, but studies in dogs with gastrointestinal disease are lacking (Glass and Mersheimer, 1958). Cats have a very short half-life for cbl in both healthy animals (12.75 days) and cats with CE (5 days) (Simpson et al., 2001). This could cause serum MMA concentrations to increase earlier during the course of gastrointestinal disease in cats than in dogs. In a study of 20 cats with CE and subnormal serum cbl concentrations, 19/20 had supranormal MMA concentrations (Kempf et al., 2017). This is in contrast to the previously cited study in dogs with CE, in which only 5/20 dogs with cbl deficiency had increased serum MMA concentrations (Berghoff et al., 2013).

Despite the low number of dogs with increased serum MMA concentrations at inclusion, serum MMA decreased significantly between baseline and 4 weeks of treatment in both treatment groups, with no statistical difference between the groups. Furthermore, the decreases in serum MMA concentrations were not limited to dogs with supranormal serum MMA concentrations at inclusion, instead being a general occurrence. In humans with border-line serum cbl concentrations and intracellular markers of cbl deficiency within the reference range, a significant decrease in serum MMA concentration after cbl supplementation implies pre-clinical cbl deficiency (Herrmann and Obeid 2012). Possibly, the dogs with serum MMA concentrations within the reference range at baseline, but a significant decrease after cbl supplementation, had early stage cbl deficiency.

The lack of statistical difference between the PO and PE treatment groups after 28 day implies that both treatment protocols had equal effects on the intracellular cobalamin status. This is consistent with human comparative studies, where PO and PE supplementation had similar effects on serum MMA concentration at the first follow-up visits (Kuzminski et al., 1998, Castelli et al., 2011). However, at the last follow-up visit in the same human studies, serum MMA concentrations had decreased the most in the PO group, which was not the case in our study.

After 28 days of treatment, median serum MMA concentrations were in the lower half of the reference interval in both groups. There was no further reduction of serum MMA concentrations between 28 and 90 days in either

group. One possible explanation for the lack of further decrease may be that the cellular demand and uptake of cobalamin had already stabilized after 28 days. The finding that serum MMA concentrations were affected to the same extent over time in both groups supports the recommendation that both oral and parenteral cbl supplementation can be used in dogs with CE and cbl deficiency.

6.7.2 Serum HCY concentrations (III)

Only 4 of 35 dogs had increased serum HCY concentrations at inclusion. Previous studies in dogs have shown mixed results regarding the usefulness of serum HCY concentrations as a diagnostic marker for cbl deficiency. In dogs of breeds affected with familial or congenital cbl malabsorption, the correlation between subnormal serum cbl and supranormal serum HCY was very strong (Fyfe et al., 1991, Lutz et al 2012; Lutz et al 2013; Grützner et al., 2013). However, despite undetectable serum cbl concentrations, median serum HCY concentrations were within the reference interval in dogs of six breeds not known for congenital cbl malabsorption (Grützner et al., 2013). A recent study in Greyhounds detected no significant differences in HCY concentrations in healthy Greyhounds compared to Greyhounds with diarrhea and/or cbl deficiency (Heilmann et al., 2017). Furthermore, in a study of dogs with systemic inflammatory response syndrome, no correlation was found between serum cbl concentrations and HCY concentrations. The only dog in Study II belonging to a breed known for congenital cbl malabsorption was a 9.5-year-old Border collie, but serum from this dog was not available for HCY and MMA analyses. Thus, no dogs of breeds known for congenital or familial cbl malabsorption were included in Study III.

A possible explanation for why only 4/35 dogs had increased HCY concentrations is that dogs may utilize the transsulfuration step to convert HCY to cysteine more efficiently than humans with cbl deficiency, subsequently accumulating less HCY. In humans, 50% of the intracellular HCY undergoes transsulfuration to form cysteine (Schindler et al., 2000). Another reason may be that cbl deficiency was still subclinical and had not lasted long enough to induce biochemical cbl deficiency in the majority of patients.

In humans, HCY concentrations are more sensitive to folate levels than cbl concentrations (Herrmann and Obeid, 2012). This was confirmed in a study in vegans, in which hypocobalaminemia and supranormal serum MMA concentrations were detected, but HCY levels were normal (Herrmann et al., 2013). Vegans usually have a high intake of dietary folate, which can compensate for the effects of cbl deficiency when evaluating serum HCY concentrations. In Study III, one dog had increased serum folate concentrations at inclusion and one dog was under supplementation with folate. However, even if HCY concentrations were affected by folate status in these two dogs, this does not fully explain why only 4/35 dogs with low serum cbl concentrations had supranormal HCY concentrations.

Hyperhomocysteinemia is reversed with cbl supplementation in oral or parenteral form in humans (Kuzminski et al., 1998, Castelli et al., 2011, Kim et al., 2011). Very few dogs had hyperhomocysteinemia at inclusion in Study III, but the hyperhomocysteinemia was not reversed in 2/4 dogs after 28 and 90

days. The lack of normalization of HCY concentrations after cobalamin supplementation in these dogs may be due to deviant biochemical pathways and altered HCY metabolism.

No significant difference in serum HCY concentrations relative to baseline was detected after 28 days of cbl supplementation in the PO or PE group. After 90 days, a small increase in serum HCY concentrations was seen in both groups, but this increase was only significant in the PE group ($p=0.07$ in the PO group and $p=0.007$ in the PE group, compared with 28 days). This could be due to inter-assay variability; results on day-to-day variations in canine HCY have not been published as far as we know. Furthermore, as previously mentioned, HCY is more sensitive to changes in folate concentrations than cbl concentrations in humans (Hermann and Obeid, 2012). Ten of the dogs in Study III were supplemented with folate during the study, which may have resulted in increased HCY concentrations. However, median HCY concentrations were still in the lower half of the reference interval at 90 days in the PE group, where a significant difference in HCY concentration between 28 and 90 days was found. The low median HCY concentration at 90 days reflects a clinically insignificant change.

Despite the low number of dogs with hyperhomocysteinemia at inclusion and the lack of effect on serum HCY concentrations after cbl supplementation, we concluded that there was no significant difference between PO and PE cbl supplementation on HCY concentrations at baseline, day 28, and day 90.

6.7.3 Conclusion of the results on cbl cellular deficiency markers (III)

Oral cobalamin supplementation affected serum MMA and HCY concentrations to the same extent as parenteral supplementation, suggesting that both treatment protocols were equally effective on a cellular level in dogs with CE and low serum cbl concentrations. Serum MMA concentrations decreased significantly and to the same extent after 28 days of treatment compared with baseline in both groups. Even in dogs with serum MMA concentrations within the reference range at inclusion, a significant decrease was seen in both groups after 28 days of supplementation. Homocysteine did not appear to be a useful marker of cbl deficiency in this group of dogs.

The results support the use of oral cobalamin as an alternative to the currently recommended parenteral supplementation protocol. Utilization of either the parenteral or the oral cobalamin supplementation protocol can thus be considered evidence-based medicine.

7. LIMITATIONS

The major limitation of Study I is its retrospective nature. This was associated with a very wide treatment range from 20 to 202 days, which might have affected the results. Several studies in human medicine have demonstrated a correlation between duration of treatment and serum cbl concentrations (Kuzminski et al., 1998; Eussen et al., 2005; Vanderbrink et al., 2010; Castelli et al., 2011; Kim et al, 2011). Furthermore, serum TLI concentrations were not available for 20/51 dogs. Consequently, some dogs may have had undiagnosed EPI. This might have affected the results and caused inclusion of dogs into the study with an incorrect phenotype, having low serum cbl due to EPI and not CE. However, in a later study we were able to demonstrate that dogs with EPI also respond to PO treatment (Toresson et al., 2017, abstract). Another limitation is the retrospective calculation of CIBDAI values, possibly leading to discrepancies compared with CIBDAI calculated in close proximity to the baseline clinical examination.

In Studies I, II, and III, the dog owners were instructed not to give cbl PO on the day of the follow-up blood sample collection. However, only in Studies II and III were the owners specifically asked at each follow-up visit when they had given the last cbl tablet. In Study I, this information was not available for all dogs. Thus, blood sampling could potentially have occurred within a few hours of oral cbl supplementation in some dogs. It is, however, unlikely that such mistakes in single animals would have had a major influence on the overall outcome since all dogs had an increase in serum cbl concentration after PO medication. Furthermore, the overall response to PO supplementation was better in Study II than in Study I. In Study II, no dogs had oral supplementation on the day of blood sampling.

In Study I, intracellular biomarkers of cbl deficiency, such as MMA and HCY, had not been analyzed since these parameters had not previously been routinely used for the diagnosis of cbl deficiency. This should, however, not have had an influence on the actual results of Study I that showed that cbl PO causes an increase in serum cbl of dogs with low serum cbl concentrations, regardless of whether or not they were cbl-deficient at the cellular level.

For the histological assessment of intestinal mucosal biopsies, it could be considered a limitation that the grading was not performed according to the World Small Animal Veterinary Association gastrointestinal standardization template in any of the studies (Washabau et al., 2010). Swedish histopathology laboratories did not use these guidelines at the time of the studies. This may have affected the histopathology reports by increasing the risk of inter-observer variability (Willard et al., 2002).

Another limitation was that not all dogs had a full diagnostic work-up in any of the studies. Although intestinal biopsies were available for the majority of the patients (65% in Study I and 70% in Study II), the remaining dogs may have had another reason for cbl deficiency than CE, especially the dogs lacking TLI results in Study I. In any case, all dogs of Studies I and II had

an increase in serum cbl concentrations after oral administration, showing that PO cbl supplementation is a viable alternative to PE administration.

Regarding Study II, a double-blind design would have resulted in the highest level of evidence-based medicine. However, this would have made the study much more complicated to perform since all dogs would have had to be treated with both injections (cbl versus placebo) and tablets (placebo versus cbl). A double-blind design would also have been more expensive to perform. Either all dog owners would have had to be charged for injections, which would have been hard to justify with a 50% risk that the owners were paying for placebo injections, or the project budget would have had to have been significantly increased, delaying the project in order to achieve sufficient funding.

The laboratory running the cbl samples were blinded to which supplementation group the dogs belonged to, but the owners and clinicians were aware of the treatment. Consequently, this may have affected the owner's perception of clinical signs, and thus, the repeated CIBDAI recordings of the clinician.

Another limitation is sample size. The size of the study populations in peer-reviewed human comparative cbl studies has ranged from 33 to 60 patients (median 55) (Kuzminski et al., 1998; Bolaman et al., 2003; Castelli et al., 2011; Kim et al., 2011). Compared with these numbers, the sizes of the study populations in Studies I and II appear reasonable, although the study population in Study III was smaller than desired. However, it is still larger than the human study with the smallest study population of 33 patients (Kuzminski et al., 1998).

Insufficient communication and transport errors ended in a large proportion of dogs being excluded from Study III due to inadequate volumes of the baseline serum samples being saved and, mainly, transport errors leading to the spoilage of serum samples stored for an extended time at room temperature. The importance of fact that one can never be too abundantly clear regarding communication was a very expensive lesson to learn.

Additional limitations are the methods used for HCY and MMA analysis. These methods have previously been described for use in canine studies, but no laboratory validation has been published in peer-reviewed journals (Ruaux et al., 2001; Berghoff et al., 2012; Grützner et al., 2013). Since both methods are technically demanding and involve extensive manual work, a substantial risk for intra- and inter-assay variability exists. Consequently, this may have affected the results. Further, stability of MMA and HCY concentrations in frozen canine samples has not yet been reported, although excellent stability has been demonstrated for both MMA and HCY in human serum and plasma stored for up to 25 years at -20°C (Hustad et al, 2012). The serum samples for HCY and MMA analyses further underwent two freeze-thaw cycles, when the serum was shipped from Helsingborg to the laboratory in Strömsholm and then later returned to Helsingborg. This could have affected the stability of the samples and the study results. The shipment to the Gastrointestinal Laboratory at Texas A&M University, however, was the least problematic transport since all serum samples arrived frozen.

Another limitation is the poor correlation between the intracellular markers of cbl deficiency and serum cbl concentrations. HCY was only

increased in 4/35 dogs and MMA in 9/36 dogs. These results are similar to some other studies in dogs, and probably more so reflect the sensitivity and specificity of these tests in dogs with CE and low serum cbl concentrations than inadequate quality of the study (Berghoff et al., 2013; Patterson et al., 2013; Grützner et al, 2013, Heilmann et al., 2017).

Holotranscobalamin has, to the best of our knowledge, not been investigated in dogs, but this marker could potentially add valuable information to the definition of cbl deficiency in dogs. As in human medicine, it is very likely that no single marker can be used to correctly diagnose cbl deficiency in dogs.

8. FUTURE PROSPECTS

The studies of this dissertation highlight the need for additional research to better characterize and understand cbl deficiency in dogs. The aims of such studies should include the following:

1. To evaluate persistence or recurrence of low serum cbl concentration after cbl supplementation in dogs with CE.

2. To investigate whether dogs also have an alternative uptake of cbl, independent of IF or an intact ileum. This could be achieved using radioactive cbl, labeled with a stable isotope, in dogs with EPI and cbl deficiency.

3. To compare the microbiota of dogs with CE and cbl deficiency versus dogs with CE but without cbl deficiency, with a special focus on the previously mentioned *Clostridia* and *Bacteroides* spp.

4. To evaluate the effects of PO supplementation in dogs with EPI in a larger group of dogs.

5. To investigate whether cbl supplementation can result in weight gain and reduction of CIBDAI, similar to previous work in cats (Morris et al., 1977, Ruaux et al., 2005, Kempf et al., 2017).

6. To describe the prevalence of hypocobalaminemia in dogs with idiopathic epilepsy in a case-control study.

9. CONCLUSION

1. Oral cbl supplementation resulted in a significant increase in serum cbl concentrations in dogs with CE and low serum cbl concentrations in both Study I and Study II.

2. The increase in serum cbl concentration after supplementation was not affected by any of the following parameters: CIBDAI at inclusion, medical or dietary intervention during supplementation, or serum cbl concentration at baseline (hypocobalaminemia versus serum cbl concentration in the lowest normal reference range).

3. Both the PO and the PE protocols were effective in increasing serum cbl concentrations in dogs with low serum cbl concentrations. Thus, both protocols have been validated.

4. The effects of PO versus PE cbl supplementation on serum cbl concentration were compared. The PE protocol resulted in a larger increase in serum cbl Δ than the PO protocol after 28 days, but continuous PO supplementation resulted in a higher serum cbl Δ after 90 days. At this time-point, the last cbl injection had been given in the PE group 3-4 weeks earlier.

5. Serum MMA concentrations were supranormal in 25% of dogs with low serum cbl concentrations and HCY concentrations were increased in 11% of the dogs.

6. No statistical difference between the PO and PE group regarding serum MMA or HCY concentrations occurred at baseline, day 28 or day 90. Consequently, both protocols affected serum MMA and HCY concentrations to the same extent.

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