# COBALAMIN (VITAMIN $B_{12}$) DECICIENCY IN THE CHINESE SHAR PEI – EVALUATION OF A POTENTIAL HEREDITARY ETIOLOGY

A Dissertation

by

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## ABSTRACT

In recent history, no other dog breed has grown in popularity and/or population size in such a short period of time as is the case for the Chinese Shar Pei in North America. After being introduced to North America in the 1970s, the breed suffered from rushed breeding carried out by inexperienced breeders. This resulted not only in a dramatically different look for the Chinese Shar Pei breed, but also in a large number of health problems. A report from 1991 revealed that Chinese Shar Pei have a predisposition for cobalamin deficiency. In this context, a comparison of serum cobalamin concentrations between dogs of different breeds would help to better understand this condition in the Chinese Shar Pei. Cobalamin-deficient Chinese Shar Peis show several clinical signs, which can be characterized by inflammatory markers, markers for chronic intestinal disease, and immunological markers. Other serum markers of cobalamin-related cellular biochemistry include homocysteine and methylmalonic acid, which are a reflection of intracellular cobalamin availability and thus might provide insights in the intracellular cobalamin metabolism in Chinese Shar Peis with cobalamin deficiency. The Chinese Shar Pei phenotype changed over the last few decades and a survey would identify which of the two types (i.e., traditional type vs. meatmouth type) is more commonly affected with cobalamin deficiency. Genetically speaking, genome-wide scans can be used to identify potential regions on the canine chromosome that are linked to cobalamin deficiency in Chinese Shar Peis. Further sequencing may identify the actual mutation responsible for the condition in this breed.

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# 1. INTRODUCTION

## 1.1 Cobalamin (vitamin $B_{12}$)

Cobalamin is a water-soluble organometallic molecule and represents the most chemically complex vitamin. In 1947, Karl Folkers and co-workers first crystallized cobalamin (Rickes et al., 1948). The three-dimensional structure of cobalamin was then ascertained in 1956 by Hodgkin and coworkers (Hodgkin et al., 1956). Cobalamin contains cobalt as a centrally located metal ion, for which there are six coordination sites. Four of these are provided by the corrin ring, whereas the fifth is supplied by a dimethylbenzimidazole group. The sixth coordination site is variable, and the specific functional groups attached to this site determine the type of cobalamin.

### Cobalamin sources

Humans, animals, and plants do not have the capability to synthesize cobalamin, but cobalamin can be absorbed from certain dietary sources. In general, dietary sources high in cobalamin include meat, dairy products, and fish (especially shellfish), whereas vegetables do not contain any significant amounts of dietary cobalamin (Herbert, 1988).

Studies investigating the cobalamin concentration in various mammalian tissues revealed high concentrations of cobalamin in the liver and kidney, with a higher concentration in the liver compared to the kidney (Retey, 1982), indicating that the main storage site for cobalamin is the liver (Glass, 1959; Beedholm-Ebsen et al., 2010).

**Absorption and transport of cobalamin**

The absorption of dietary cobalamin and its conversion to an intracellularly active co-enzyme is complex and involves many physiological processes, including gastric release of protein-bound cobalamin, intestinal uptake by a carrier-mediated transport process, intravascular transport, cellular uptake, intracellular release, and intracellular compartmentalization (Hall, 1979). The entire process of cobalamin absorption and transport depends on numerous proteins, including R-binder, intrinsic factor, transcobalamin, cell membrane receptors, and intracellular binding proteins. Hansen characterized three main classes of cobalamin transport proteins (Hansen, 1990).

R-binders (also called haptocorrins and transcobalamin I) are glycoproteins with a high affinity for cobalamin, and have been isolated from plasma, tissue extracts, secretions (e.g., saliva and bile), and the cytoplasm of erythrocytes, granulocytes, and platelets (Fenton & Rosenberg, 1989; Cooper & Rosenblatt, 1987). Following its ingestion, cobalamin is removed from dietary components and thought to bind to salivary and gastric R-binders (Fenton & Rosenberg, 1989). Within the proximal duodenal lumen cobalamin is released from these R-binders by pancreatic proteolytic enzymes.

Intrinsic factor is also a glycoprotein that mediates the uptake of cobalamin in the gastrointestinal lumen. In humans, intrinsic factor is exclusively synthesized by gastric parietal cells (Fenton & Rosenberg, 1989), whereas in dogs, intrinsic factor is mainly synthesized and secreted by pancreatic acinar cells and only to a much smaller degree from the gastric mucosa (Batt et al., 1989; Simpson et al., 1993). Upon the binding of

cobalamin, intrinsic factor molecules decrease in size, resulting in an increased affinity of the intrinsic factor-cobalamin complex for specific receptors located within the brush border membrane of ileal enterocytes (Levine et al., 1985; Gueant et al., 2001). Within the brush border membrane of the ileum, a specific receptor recognizes the intrinsic factor-cobalamin complex and allows for receptor-mediated endocytosis (Fyfe et al., 2004). Thus, the intrinsic factor-cobalamin complex is crucial for the absorption of cobalamin via receptor-mediated endocytosis into the ileal mucosa (Fyfe et al., 2004).

The absorption of cobalamin from the enterocyte into the circulation is accomplished by passage across the basal membrane of enterocytes, after which cobalamin is bound to transcobalamin II and enters the portal circulation. Transcobalamin II represents the third class of molecular cobalamin binders and is expressed by many additional tissues and body fluids (Fenton & Rosenberg, 1989). In humans, it has been reported that transcobalamin II is synthesized by intestinal villi in areas where the vascular endothelium is abundant (Quadros et al., 1999). Transcobalamin II binds only a small percentage (about 20 %) of the total circulating cobalamin, but only transcobalamin II-cobalamin complexes can be internalized by peripheral tissue cells (Seetharam & Alpers, 1985; Allen, 1975). After the binding of the transcobalamin II-cobalamin complex to its cellular membrane receptor, the complex is absorbed by way of endocytosis. The transcobalamin II-cobalamin complex is degraded in the lysosome to yield free cobalamin, which is converted to methylcobalamin within the cytosol and/or to adenosylcobalamin inside the mitochondria. Cobalamin within the cell is bound to proteins, which are mainly cobalamin-dependent enzymes, such as methionine synthase

and methylmalonyl-CoA mutase and both bind more than 95 % of the intracellular cobalamin (Mellman et al., 1977; Kolhouse & Allen, 1977). Neither transcobalamin I nor II have been specifically reported in dogs so far.

Methionine synthase – Intracellular cobalamin undergoes a methylation to methylcobalamin, which represents the active form of cobalamin that is required for the conversion of homocysteine to methionine that is catalyzed by methionine synthase (Fenton & Rosenberg, 1989). Methionine synthase requires the presence of the co-factor cobalamin as well as a methyl group. The methyl group is provided through the conversion of 5-methyltetrahydrofolate to tetrahydrofolate, which is the biologically active form of vitamin $B_9$. 5-tetrahydrofolate is required for purine and pyrimidine biosynthesis and is thus important for the *de novo* synthesis of DNA and RNA.

Methylmalonyl-CoA mutase – Methylmalonyl-CoA mutase is the second enzyme that requires cobalamin. It catalyzes the final isomerization step in the metabolism of propionyl-CoA to succinyl-CoA in mammalian cells (Cannata et al., 1965; Chandler et al., 2006). Propionyl-CoA results from the catabolism of certain amino acids, such as valine, isoleucine, methionine, and threonine. In the pathway from propionyl-CoA to succinyl-CoA the conversion of L-methylmalonyl-CoA to succinyl-CoA can only occur when sufficient amounts of cobalamin are available within the cell. In turn, succinyl-CoA is incorporated into the tricarboxylic acid cycle (Krebs' cycle), which fuels cellular energy production. In summary, cobalamin serves as an essential co-factor for both methylmalonyl-CoA mutase and methionine synthase in mammalian cells.

## 1.2 Disorders of cobalamin absorption, transport, and intracellular metabolism

### Disorders of cobalamin absorption and transport

The absorption of dietary cobalamin is complex and involves many steps, including gastric release of protein-bound cobalamin, intestinal uptake by a carrier-mediated transport process, intravascular transport, and cellular uptake. This process of cobalamin absorption and transport depends on numerous proteins including R-binder, intrinsic factor, transcobalamin, and cell membrane receptors. Disorders affecting the aforementioned proteins have been described in both the human and veterinary literature.

R-binder deficiency – Only a few case reports of human patients with R-binder deficiency have been described (Carmel et al., 2003). Affected individuals usually have decreased serum cobalamin concentrations but no clinical signs of cobalamin deficiency.

Intrinsic factor deficiency – Humans with an inherited intrinsic factor deficiency show megaloblastic pernicious anemia, and have decreased serum cobalamin concentrations (Shevell & Rosenblatt 1992). However, clinical signs of cobalamin deficiency are absent for the first few months of life, which might be due to an alternative pinocytic absorption mechanism for cobalamin, leading to an overall sufficient cobalamin absorption (Fenton & Rosenblatt, 1989).

Cobalamin malabsorption – Imerslund-Gräsbeck syndrome in humans is a selective cobalamin malabsorption by enterocytes, and is caused by a defect of the cobalamin-intrinsic factor complex receptor, the internalization of cobalamin-receptor complexes,

or the transfer of cobalamin to transcobalamin II (Burman et al., 1985; Fyfe et al., 1991). In humans, the clinical findings in affected patients include megaloblastic anemia, low serum cobalamin concentrations, and proteinuria (Gräsbeck, 1972 and Gräsbeck, 2006). A similar syndrome of cobalamin malabsorption with an absence of ileal intrinsic factor-cobalamin complex receptors has also been described in a family of Giant Schnauzers (Fyfe et al., 1991a). It has been suggested that the receptor for intrinsic factor-cobalamin complexes is not being expressed in the brush border membrane of these dogs.

Transcobalamin II deficiency – Transcobalamin II deficiency in humans is generally characterized by megaloblastic anemia, vomiting, failure to thrive, pancytopenia, and eventually the development of immunologic and neurologic abnormalities (Shevell & Rosenblatt, 1992). Usually, clinical symptoms can be observed within the first two months of life. Transport of cobalamin into the cells is hindered in the absence of transcobalamin II. However, due to the presence of R-binder proteins, the majority of which are in the vascular space (about 80%), patients with transcobalamin II deficiency may have a normal serum cobalamin concentration (Frisbie & Chance, 1993). However, the absorption of cobalamin is usually affected in these patients, suggesting that, at least in humans, transcobalamin II to be essential for cobalamin entering cells (Cooper & Rosenblatt, 1987; Seetharam & Yammani, 2003).

**Disorders of intracellular cobalamin metabolism**

In general, methylmalonic acidemia/aciduria is a metabolic consequence of cobalamin deficiency and is characterized by an accumulation of large amounts of methylmalonic

acid (MMA) in serum, and as a result, excretion of MMA in the urine of affected individuals (Cooper & Rosenblatt, 1987; Fenton & Rosenblatt, 1978). This is caused by an intracellular failure of the conversion of methylmalonyl-CoA to succinyl-CoA and the accumulation of the metabolite MMA in the vascular space, which in turn is excreted in the urine and can be used as a marker for cobalamin deficiency at the cellular level. In healthy animals, only small amounts of MMA are detectable in blood, urine, or cerebrospinal fluid.

In humans and dogs with methylmalonic acidemia the characteristic laboratory findings include secondary hyperammonemia, increased concentrations of ketones, and MMA in the urine (Cooper & Rosenblatt, 1987; Fyfe et al., 1991). In human patients, response to parenteral administration of cobalamin is variable. These differences in response to therapy suggest that different intracellular forms of cobalamin deficiency can lead to methylmalonic acidemia/aciduria.

**Metabolic consequences of intracellular cobalamin deficiency – Cobalamin A to G diseases**

Biochemical studies have described human patients with selective or combined deficiencies of adenosylcobalamin and methylcobalamin. Patients with such selective or combined deficiencies have been grouped into 7 groups: cobalamin A to cobalamin G disease, which provide insights into the pathways of intracellular cobalamin transport (Gravel et al., 1975; Willard et al., 1978; Shevell & Rosenblatt, 1992).

Cobalamin A and cobalamin B disease are characterized by a deficient activity of L-methylmalonyl-CoA mutase, which leads to methylmalonic aciduria (Cooper & Rosenblatt, 1987). Human patients with cobalamin A disease have a selective deficiency of adenosylcobalamin, but have normal concentrations of methylcobalamin intracellularly and no homocysteinuria (Cooper & Rosenblatt, 1987). In contrast, patients with cobalamin B disease were shown to lack adenosylcobalamin, indicating normal cobalamin reduction but a deficiency in cobalamin I ATP-adenosyltransferase (Fenton & Rosenblatt, 1981). Neither cobalamin A or B disease have been specifically reported in dogs so far.

Cobalamin C and cobalamin D disease are associated with a decreased synthesis of adenosylcobalamin and methylcobalamin, resulting in both methylmalonic aciduria and homocystinuria (Rosenblatt & Cooper, 1987). However, serum concentrations of cobalamin are usually within the reference interval because affected patients have normal intestinal absorption and vascular transport of cobalamin, leading to a normal serum cobalamin concentration (Rosenblatt & Fenton, 1989). Symptoms of cobalamin C disease become evident within the first year of life or during adolescence (Shinnar & Singer, 1984). The clinical findings in human patients usually are a failure to thrive, lethargy, inappetence, developmental delay, microcephaly, seizures, hypotonia, and hypomethioninemia (Cooper & Rosenblatt, 1987). In general, the ability to exchange the cyanide group for a hydroxyl group can be used to distinguish patients with cobalamin C disease from those with cobalamin D disease (Mellman et al., 1978). Furthermore, different studies have shown that cobalamin D disease is similar to cobalamin C disease,

but it is associated with less severe abnormalities compared to those found in patients with cobalamin C disease. Neither cobalamin C or D disease have been specifically reported in dogs so far.

Cobalamin F disease has been described only in a few human patients. One of the patients reported was an infant with developmental delay, cobalamin responsive methylmalonic aciduria, but no indication of megaloblastic anemia or homocysteinuria (Rosenblatt et al., 1986; Rosenblatt et al., 1985). The defect in patients with cobalamin F disease has been suggested to affect the release of lysosomal cobalamin into the cytoplasm (Rosenblatt, 1992). Cobalamin F disease has not been specifically reported in dogs so far.

Cobalamin E and cobalamin G diseases are characterized by a failure of methionine biosynthesis and the accumulation of homocysteine in the urine. In patients with cobalamin E or cobalamin G disease, serum cobalamin concentrations are usually within the reference interval (Fenton & Rosenberg, 1989). In most patients, the disease becomes apparent within the first year of life, but it has also been diagnosed in a 21 year old patient (Carmel et al., 1988). Patients usually present with homocysteinemia and hypomethioninemia, but not with methylmalonic aciduria (Cooper & Rosenblatt, 1987; Rosenblatt & Cooper, 1989; Watkins & Rosenblatt, 1989). Cobalamin E and cobalamin G disease both represent a heterogeneous group of patients characterized by a functional methionine synthase deficiency due to a decreased methionine biosynthesis, leading to decreased intracellular concentrations of methylcobalamin (Rosenblatt, 1992; Rosenblatt

et al., 1984). Neither cobalamin E or G disease have been specifically reported in dogs so far.

### 1.3 Canine genetics

During the last decade, the domestic dog, *Canis lupus familiaris*, has frequently been used as a model for the study of hereditary diseases and gene expression in humans. The dog is believed to be the oldest domesticated animal species. Selective breeding over the last centuries has created more than 300 different dog breeds with a certain number of genes that have not been characterized for each breed. These dog breeds represent isolated, inbred populations as most of them have developed more than 250 years ago (Ostrander & Giniger, 1999). Therefore, these breeds demonstrate genotypic and phenotypic homogeneity, giving rise to founder effects (e.g., color and height) and population bottlenecks. One effect of such breeding practices appears to be the large number of genetic diseases observed in dogs. Approximately 450 hereditary diseases have been described in dogs in the Online Mendelian Inheritance in Animals database (OMIA 2003). Many of these diseases resemble clinical syndromes of similar hereditary diseases in humans, and some even share the mutation of a gene that is responsible for the disease (Ostrander & Giniger, 1997; Ostrander et al., 2000). These spontaneous mutations in the dog offer the possibility to study canine spontaneous diseases as a model of human diseases (Kijas et al., 2002).

## Linkage analysis

The unique population structure of the dog lends itself to the study of hereditary diseases that appear to be similar to human hereditary diseases, but it should be pointed out that there are many diseases unique to the dog. It has been suggested that about two-thirds of hereditary diseases in dogs are transmitted by autosomal recessive traits (Ostrander & Kruglyak, 2000). Therefore, elimination of the respective alleles represents a challenge for breeder clubs and breeders.

With hereditary diseases the clinical symptoms can occur in either young or older dogs, which should be considered for planning association studies. Linkage analysis is aimed at the identification of markers that associate with a particular disease by identity-by-descent, which allows for subsequent development of a PCR-based test to identify potential carriers and affected animals prior to the onset of clinical signs.

## Linkage analysis strategies

Causative disease factors may be investigated using two different approaches. Many diseases in dogs have a significant genetic basis, and one commonly used technique to identify genetic risk factors for complex disorders is the candidate gene approach, which directly tests the effects of genetic variants of a potentially contributing gene in an association study. The candidate gene approach is used where genes are known to control the physiologic function that is affected in diseased patients. These candidate genes are usually chosen based on known mutations in similar syndromes in different species or genes that code for proteins that might play an important role in the disease

process of interest. However, there are diseases where the underlying disease process does not allow for the selection of a suitable candidate gene and a genome wide scan is needed. For instance, cobalamin deficiency in the Shar Pei has not shown any similarities to the various forms of hereditary cobalamin deficiency in humans or similar conditions in veterinary patients (Fyfe et al., 1991a; Carmel, 2000; Fordyce et al., 2000). This may suggest that the genetic basis for the disease in Shar Peis is different from the one identified in humans with cobalamin deficiency. Thus, a genome-wide scan would hold more promise for the evaluation of cobalamin deficiency in the Shar Pei than a candidate gene approach. Regardless, both a candidate gene approach as well as genome-wide scan requires the construction of pedigrees.

**MSS-2 and SNPs**

For studies where a specific condition, such as cobalamin deficiency, is suspected to be hereditary and where the aim of that study is to identify a locus or loci that co-segregate with the disease, performing a genome wide scan using the canine minimal screening set-2 (cMSS-2) and/or a SNP map are both considered suitable approaches. Microsatellite markers have been used successfully to locate mutations in humans with genetic disorders (Holmes, 1994). More recently, the 327 microsatellite markers contained in the cMSS-2 set have been used to identify genetic regions of interest in various canine hereditary diseases (Lowe et al., 2003; Clark et al., 2006; Lippmann et al., 2007). On the other hand, SNP arrays have been used to identify a locus or loci that co-segregate with a disease in humans (e.g., carcinogenesis) and also in veterinary

studies (multiple diseases of the German Shepherd Dog) (Liang et al. 2012; Tsai et al. 2012). Furthermore, SNP arrays would cover the dog genome in more detail and thus might provide a better likelihood of detecting associated effects (Fukuda et al. 2009).

Genome-wide association studies using the cMSS-2 and SNP array would help to further identify regions on the canine chromosome that co-segregate with cobalamin deficiency in Shar Peis. After identifying those regions a targeted resequencing approach, which has been successfully applied and described by Seabury et al., (2011) or Olsson et al., (2011), would be useful to pinpoint the locus or loci that co-segregate with the phenotype (cobalamin deficiency) in Shar Peis and may ultimately aid in deciphering the likely defect(s) causing and/or resulting in cobalamin deficiency in Shar Peis.

### 1.4 Chinese Shar Peis

The Shar Pei is a dog breed that comes originally from the Guangdong province of China. For many years, the Shar Pei was used for hunting, protecting and herding livestock, and guarding the home and family in the Chinese countryside. During that time, the Shar Pei was bred for intelligence, strength, and a scowling face, but not for the amount of wrinkles.

In the 1970s, the Shar Pei was considered by *The Guinness Book of Records* to be the rarest dog in the world. The Shar Pei population dwindled dramatically because of the communist revolution in China. Matgo Law, a Hong Kong businessman, rescued several Shar Peis and brought them to North America. In an attempt to save the breed, he smuggled an estimated 200 Shar Peis into North America. The current American Shar

Pei population stems mainly from these Shar Peis, which, genetically speaking, is a classic example of the bottleneck phenomenon. In other words, this small group of Shar Peis represents the limited source of genetic material in the current American Shar Pei population.

After being introduced to North America in the 1970s, the breed suffered from rushed breeding by inexperienced breeders. This resulted not only in a dramatically different look for the Shar Pei (as its most characteristic features, including its wrinkles and rounded snout, were greatly exaggerated), but also in a large number of health problems.

As of August 1992, the breed began competing in the nonsporting group at AKC shows. Back then the registration figures showed that in the Shar Pei Club of America a total of 75,000 individual Shar Peis and 47,000 litters were registered. In the same year the Shar Pei breed became the 134$^{th}$ recognized AKC dog breed. Interestingly, over the last 5 years, the Shar Pei has been ranked around position 50 in the list of most common registered dog breeds by the AKC. In recent history, no other dog breed has grown in popularity and/or population size in such a short period of time as has the Shar Pei.

**Cobalamin deficiency in Chinese Shar Peis**

In Shar Peis subnormal serum cobalamin concentrations were first reported in 1991 (Williams, 1991). In a small group of Shar Peis (n=26) evaluated, 21 dogs were reported to have subnormal serum cobalamin concentrations and in 19 of these dogs serum

cobalamin concentration was undetectable (Williams, 1991). This study led to the hypothesis that Shar Peis have a predisposition for cobalamin deficiency.

Often times, cobalamin deficiency in the Shar Pei is associated with clinical signs of chronic small intestinal disease (small bowel diarrhea and weight loss), and can also be associated with gastrointestinal protein loss (Williams 1991; Peterson & Willard 2003).

In 2007, another study confirmed a high prevalence of cobalamin deficiency in the Shar Pei (Bishop et al., 2007). In that study, about 64% (n=89) of serum samples from Shar Peis submitted to the Gastrointestinal Laboratory at Texas A&M University (2002 to 2006) had a cobalamin concentration below the lower limit of the reference interval and 38.1% of those dogs had serum cobalamin concentrations below the detection limit of the assay (Bishop et al., 2007). Compared to dogs of other breeds, Shar Peis were 7.6 times more likely to have a serum cobalamin concentration below the lower limit of the reference interval (i.e., < 249 ng/L) and were 55.6 times more likely to have an undetectable serum cobalamin concentration (i.e., < 100 ng/L). However, there was no statistically significant difference between serum cobalamin concentrations in healthy Shar Peis and healthy dogs of other breeds (Bishop et al., 2007). These findings suggest that cobalamin deficiency occurs frequently in Shar Peis, but it also suggests that not all individuals within this breed are affected.

Based on a genome-wide scan using the cMSS-2, cobalamin deficiency in the Shar Pei has recently been linked to a genomic locus in close proximity to two microsatellite markers (DTR13.6 and REN13N11) on canine chromosome 13 (Grützner et al. 2010). However, the previous study does not conclusively narrow down the region on

chromosome 13 as the major locus for a gene or genes responsible for cobalamin deficiency in the Shar Pei. In this context, a cSNP array would cover the dog genome in more detail and thus might provide a better likelihood of detecting associated effects (Fukuda et al. 2009).

In both, human medical (Stabler et al. 1986) and veterinary studies (Ruaux et al. 2009; Berghoff et al. 2011), an increased serum MMA concentration has been suggested to reflect cobalamin deficiency at the cellular level. A combination of decreased serum cobalamin and increased serum MMA concentrations might therefore represent stronger evidence for cobalamin deficiency at the cellular level than a decreased serum cobalamin concentration alone. Thus, a phenotypic re-classification based on serum cobalamin and MMA concentrations may lead to identification of a different region on chromosome 13 or even a location on a different chromosome.

**Other diseases commonly seen in Chinese Shar Peis**

Two other conditions are frequently reported in Shar Peis, Shar Pei fever and cutaneous mucinosis, both of which are also suspected to be hereditary. Shar Pei fever describes an autoimmune syndrome that causes periodic flare-ups associated with joint pain and fever. Cutaneous mucinosis, is a disorder characterized by the deposition of excessive amounts of mucin in the dermis of the skin, a condition that primarily occurs in Shar Peis. To our knowledge, serum cobalamin concentrations have not been reported in studies investigating Shar Pei fever and/or cutaneous mucinosis. Thus, further studies are

necessary to test the hypothesis of a potential association between cobalamin deficiency and these other two common diseases in the Shar Pei.

## 1.5 Hypotheses and research objectives

### Hypotheses

The hypotheses of this project are that: a) Chinese Shar Peis (Shar Peis) have a higher prevalence of decreased serum cobalamin concentrations and a higher occurrence of increased methylmalonic acid concentrations than other breeds; b) due to longstanding gastrointestinal disease, serum concentrations of inflammatory markers, markers for chronic intestinal disease, and immunological markers are altered in cobalamin-deficient Shar Peis; c) serum homocysteine and methylmalonic acid concentrations, which reflect intracellular cobalamin availability, differ between cobalamin-deficient Shar Peis and cobalamin-deficient dogs of other breeds; and d) genome scans using canine single nucleotide polymorphism array and canine minimal screening set-2 will provide potential regions on the canine chromosome that are linked with cobalamin deficiency in Shar Peis.

### Research objectives

The objectives of this study are: 1) to compare proportions of dogs with a decreased serum cobalamin concentration and to compare the number of submissions (to the Gastrointestinal Laboratory) for serum cobalamin analysis by breed to the American

Kennel Club (AKC) breed ranking list of 2009, 2) to evaluate serum concentrations of inflammatory markers, markers for chronic intestinal disease, and immunological markers in Shar Peis with and without cobalamin deficiency, 3) to assess serum homocysteine (HCY) and methylmalonic acid (MMA) concentrations in cobalamin-deficient Shar Peis and cobalamin-deficient dogs of 6 other breeds, 4) to determine if cobalamin deficiency predominates in one of the two types of Shar Peis (i.e., traditional type and meatmouth type), 5) to quantify serum cobalamin and MMA concentrations in cobalamin-deficient Shar Peis at initial testing and after parenteral cobalamin supplementation, 6) to analyze the MYC_CANFA gene, which is the closest known gene to the microsatellite marker DTR13.6 on canine chromosome 13 that has been linked to cobalamin deficiency in Shar Peis by using the canine minimal screening set-2 (cMSS-2), and 7) to perform genome-wide scans using canine single nucleotide polymorphism (cSNP) array and cMSS-2 to identify potential regions of the canine genome that are linked with cobalamin deficiency in Shar Peis.

# 2. EVALUATION OF SERUM COBALAMIN CONCENTRATIONS IN DOGS OF 164 DOG BREEDS (2006-2010)*

## 2.1 Overview

Altered serum cobalamin concentrations have been observed in dogs with gastro-intestinal disorders such as exocrine pancreatic insufficiency (EPI) or gastrointestinal inflammation. The aims of the current study were 1) to identify breeds with a higher proportion of dogs with a decreased serum cobalamin concentration, 2) to determine whether dogs with such decreased concentrations tend to have serum canine trypsin-like immunoreactivity (cTLI) concentrations diagnostic for EPI, and 3) to compare the number of submissions for serum cobalamin analysis by breed to the American Kennel Club (AKC) breed ranking list of 2009. In this retrospective study, results of 28,675 cobalamin tests were reviewed. Akitas, Chinese Shar Peis, German Shepherd Dogs, Greyhounds, and Labrador Retrievers had increased proportions of serum cobalamin concentrations below the lower limit of the reference interval (<251 ng/L; all $p < 0.0001$). Akitas, Chinese Shar Peis, German Shepherd Dogs, and Border Collies had increased proportions of serum cobalamin concentrations below the detection limit of the assay (<150 ng/L; all $p < 0.0001$). Akitas, Border Collies, and German Shepherd Dogs with serum cobalamin concentrations <150 ng/l were more likely to have a serum cTLI concentration considered diagnostic for EPI (≤2.5 µg/L; all $p \leq 0.001$).

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The breed with the highest proportion of samples submitted for serum cobalamin analysis in comparison with the AKC ranking list was the Greyhound (odds ratio: 84.6; $p < 0.0001$). In Akitas and Border Collies, further investigations are warranted to clarify if a potentially breed-specific gastrointestinal disorder is responsible for the increased frequency of decreased serum cobalamin and cTLI concentrations.

## 2.2 Introduction

Cobalamin (vitamin $B_{12}$) is essential for a wide variety of metabolic processes in many tissues and organs. Immunoassays for the measurement of cobalamin concentrations in serum from human beings, cats, and dogs are routinely used to diagnose cobalamin deficiency.

In dogs, cobalamin deficiency can be caused by exocrine pancreatic insufficiency (EPI; Simpson et al., 1989), severe and longstanding ileal disease, small intestinal dysbiosis, or an inherited condition (Fyfe et al., 1991). Cobalamin deficiency can also be associated with systemic metabolic complications such as central and peripheral neuropathies (Battersby et al., 2005) and immunodeficiencies (Cook et al., 2009), and is also associated with intestinal changes, such as villous atrophy (Rutger et al., 1995) or malabsorption of vitamins and other nutrients.

In cases of longstanding ileal disease, low serum cobalamin concentrations have been documented in both human and canine patients with chronic enteropathies such as inflammatory bowel disease (Yakut et al., 2010; Allenspach et al., 2007). Chronic enteropathies have been commonly described in canine patients of different breeds such

as the Basenji (MacLachlan et al., 1988), Boxer (German et al., 2000a), German Shepherd Dog (German et al., 2000b), Irish Setter (Batt, 1985; Garden et al., 2000), and Soft Coated Wheaten Terrier (Littman et al., 2000). A comparison with data from the American Kennel Club (AKC), which shows the number of dogs of various breeds that are registered based on popularity, could help identify additional breeds with disproportionately high numbers of serum submissions (e.g., to the Gastrointestinal Laboratory at Texas A&M University [GI Lab], College Station, TX) for serum cobalamin analysis.

Low serum cobalamin concentrations have been observed in dogs with EPI, which is recognized as a potential cause of cobalamin deficiency (Simpson et al., 1989). The measurement of serum canine trypsin-like immunoreactivity (cTLI) is considered the gold standard test for the diagnosis of canine EPI (Batt, 1993). An investigation of serum cTLI concentrations in dogs with low serum cobalamin concentrations could help to identify breeds where EPI is associated with cobalamin deficiency.

In the past decade, cases of cobalamin deficiency have been reported in several dog breeds. For instance, a family of Giant Schnauzers (Fyfe et al., 1991), a Beagle (Fordyce et al., 2000), 2 juvenile Border Collies (Battersby et al., 2005; Morgan & McConnell, 1999), juvenile Australian Shepherds (Morgan & McConnell, 1999), and Chinese Shar Peis [Shar Peis] (Williams, 1991; Bishop et al., 2012) have been described with selective malabsorption of cobalamin and deficiency of this vitamin. A breed predisposition for cobalamin deficiency has been described for Shar Peis in North America (Bishop et al., 2012). In the United Kingdom, cobalamin deficiency has been described for the Shar

Pei, Staffordshire Bull Terrier, as well as a group of mixed-breed dogs (Dandrieux et al., 2010).

Due to the variety of breeds that were represented at GI Lab, serum cobalamin concentrations of 164 breeds (based on the AKC breed ranking list of 2009) were investigated. The first aim of the study was to identify breeds with higher proportions of decreased serum cobalamin concentrations. The second aim was to look for serum cTLI concentrations that were diagnostic for EPI in the dogs with decreased serum cobalamin concentrations to identify breeds in which EPI is associated with cobalamin deficiency. Finally, the study compared the number of serum submissions for cobalamin analysis by breed with the AKC breed ranking list of 2009 to identify breeds with disproportionately high numbers of serum submissions for serum cobalamin analysis. A trend or discovery of a high number of serum submissions for serum cobalamin analysis in a certain dog breed could help to identify a clinical problem in a specific breed perceived by veterinarians and may help to direct future investigations.

## 2.3 Materials and methods

### Selection of serum cobalamin data

The current retrospective study covered a period of 4 years (from March 1, 2006 through February 28, 2010). Information on canine serum samples in the database of the GI Lab was reviewed. Serum samples that had been submitted for evaluation of serum cobalamin concentration were selected, but the clinical history and disease status of the dogs were not provided by the referring veterinarian. A total of 28,675 canine

submissions (belonging to 164 breeds, represented by the AKC ranking list of 2009) for analysis of serum cobalamin concentration were reviewed, and sex and age were identified where reported on the submission form. Resubmissions and duplicates were excluded. The concentrations of serum cobalamin had been measured using an automated chemiluminescence assay (Immulite 2000, Vitamin $B_{12}$; Siemens Healthcare Diagnostics Inc., Deerfield, IL). The reference interval for canine serum cobalamin concentration had previously been established as 251-908 ng/L (Gastrointestinal Laboratory at Texas A&M University, College Station, TX; http://vetmed.tamu.edu/gilab/service/assays/b12folate; accessed May 1, 2012). The frequency of decreased (<251 ng/L) and undetectable (<150 ng/L) serum cobalamin concentrations recorded in the GI Lab database were compared between breeds by calculating the odds ratio (OR) and the 95% confidence interval (CI) for 164 breeds that were listed in the AKC breed ranking list of 2009 (American Kennel Club breed ranking list of 2009; http://www.akc.org/reg/dogreg_stats.cfm; accessed November 1, 2010). Serum cobalamin concentrations between 251 ng/L and 150 ng/L were excluded for the proportion analyses of dogs with serum cobalamin concentration <150 ng/L. Only breeds with at least 30 submitted samples were included in the calculation. However, the 5 breeds, Giant Schnauzer, Beagle, Border Collie, Australian Shepherd, and Shar Pei, that had been mentioned in case reports of cobalamin deficiency over the past two decades (1990-2010) were reported regardless of the OR.

Breeds with a significantly higher odds of having samples with serum cobalamin concentrations <150 ng/L were subsequently investigated for proportions of dogs with a

serum cTLI concentration that is considered diagnostic for EPI (≤2.5 μg/L as measured at roughly the same time as serum cobalamin concentration; i.e., serum cTLI may have been measured up to 48 hr before or after serum cobalamin concentration due to logistical reasons, but would have been measured on the same serum sample). The concentration of serum cTLI was measured using a commercially available radioimmunoassay (Canine TLI Double Antibody Radioimmunoassay, Siemens Healthcare Diagnostics Inc., Deerfield, IL), and the reference interval has previously been established as 5.7-45.2 μg/L (Gastrointestinal Laboratory at Texas A&M University, College Station, TX; http://vetmed.tamu.edu/gilab/service/assays/tli; accessed May 1, 2012). Breeds that were identified as having a significant OR for a serum cTLI concentration ≤2.5 μg/L were considered to have an association of undetectable serum cobalamin concentration and EPI. Only breeds that showed a significant OR were reported for all analyses. However, data for the 5 breeds that had been mentioned in case reports of cobalamin deficiency over the past two decades were reported regardless of the OR.

**Comparison of submissions for serum cobalamin measurement with the 2009 AKC breed ranking list**

Due to possible annual variation of submissions, the average number of serum samples submitted for cobalamin analysis to GI Lab over a 4-year period was calculated for each breed. Thus, a total of 7,203 canine submissions, the calculated average number of serum samples submitted for cobalamin analysis to GI Lab for 1 year, were compared by

calculating the OR and the 95% CI to the AKC breed ranking list of 2009 to identify breeds with higher proportions of submissions for serum cobalamin analysis. Again, only breeds with at least 30 sample submissions were included in the calculation. The AKC ranking list of 2009 contained a total of 649,677 registered dogs. Subsequently, for all breeds with a higher proportion of serum sample submissions for serum cobalamin analysis, the serum cobalamin concentrations and age were compared among the breeds (averaged across the 4 years). Also, serum cTLI concentrations were subsequently investigated in breeds with significantly higher proportions of serum samples submitted for cobalamin analysis.

**Statistical analyses**

A commercially available software (JMP version 8, SAS Institute Inc., Cary, NC) was used to perform statistical analyses. All variables, the breed proportion of dogs with a serum cobalamin concentration of <251 ng/L, those with a serum cobalamin concentration <150 ng/L, and those with a cobalamin concentration within the reference interval, were compared by using a Fisher's exact test. Breeds for which the 95% CI of breed distribution of submissions to the GI Lab database population and those in the AKC ranking list of 2009 differed were considered potentially overrepresented or underrepresented in the population of dogs for the respective group. Because of multiple comparisons between 164 dog breeds of the AKC ranking, statistical significance level for a difference was adjusted from $p < 0.05$ to $p < 0.0003$ using a Bonferroni correction for multiple statistical comparisons (Bonferroni correction for multiple statistical

comparisons; http://www.quantitativeskills.com/sisa/calculations/bonfer.htm; accessed May 1, 2012). Breeds with significantly higher proportions of samples with undetectable serum cobalamin concentrations and those with higher proportions of serum sample submissions for cobalamin analysis were subsequently investigated for proportions of dogs with a serum cTLI diagnostic for EPI using a Fisher's exact test; statistical significance was set at $p < 0.05$. A Kruskal–Wallis test with a Dunn post test was used to compare serum cobalamin concentrations and age in breeds with a higher proportion of submissions for serum cobalamin analysis over the 4-year period ($p < 0.05$).

**2.4. Results**

Data from the GI Lab database showed that the Akita, Shar Pei, German Shepherd Dog, Greyhound, and Labrador Retriever had significantly higher proportions of dogs with serum cobalamin concentrations <251 ng/L (OR > 1; all $p < 0.0001$; Table 1). In contrast, the Belgian Malinois, Boxer, Golden Retriever, Great Dane, Miniature Schnauzer, and Standard Poodle had significantly lower proportions of dogs with serum cobalamin concentrations <251 ng/L (OR < 1; all $p < 0.0001$; Table 1).

**Table 1.** Over- and underrepresented dog breeds with regard to serum cobalamin concentration below the lower limit of the reference interval (<251 ng/L) using data from the GI Lab (Texas A&M University, College Station, Texas) database.

| Breed* | 2009 AKC ranking position | Age† | Cobalamin‡ | | Odds ratio§ |
|---|---|---|---|---|---|
| | | | <251 ng/L | 251-908 ng/L | |
| **1. Odds ratio > 1** | | | | | |
| Chinese Shar Pei | 47 | 6.0 (8.5) | 82/5,646 | 71/21,428 | 4.4 (3.2–6.0)¦ |
| Akita | 50 | 7.0 (3.3) | 43/5,685 | 59/21,440 | 2.8 (1.9–4.1)¦ |
| Greyhound | 140 | 9.0 (5.1) | 174/5,554 | 262/21,237 | 2.5 (2.1–3.1)¦ |
| German Shepherd Dog | 2 | 5.0 (8.8) | 1,095/4,633 | 2,767/18,732 | 1.6 (1.5–1.7)¦ |
| Labrador Retriever | 1 | 7.0 (6.9) | 738/4,990 | 2,098/19,400 | 1.4 (1.3–1.5)¦ |
| **2. Odds ratio < 1** | | | | | |
| Golden Retriever | 4 | 8.0 (8.5) | 224/5,504 | 1,152/20,347 | 0.7 (0.6–0.8)¦ |
| Boxer | 6 | 6.0 (5.6) | 126/5,602 | 930/20,569 | 0.5 (0.4–0.6)¦ |
| Great Dane | 21 | 5.0 (10.0) | 40/5,688 | 310/21,189 | 0.5 (0.4–0.7)¦ |
| Standard Poodle | 9 | 7.0 (1.8) | 56/5,672 | 469/21,030 | 0.4 (0.3–0.6)¦ |
| Miniature Schnauzer | 11 | 8.0 (11.3) | 62/5,666 | 597/20,902 | 0.4 (0.3–0.5)¦ |
| Belgian Malinois | 81 | 9.0 (0.0) | 3/5,725 | 73/21,426 | 0.2 (0.1–0.5)¦ |
| **3. Case reports** | | | | | |
| Australian Shepherd | 28 | 8.0 (6.3) | 64/5,664 | 187/21,312 | 1.3 (1.0–1.7)¶ |
| Beagle | 5 | 8.0 (9.0) | 89/5,639 | 357/21,142 | 0.9 (0.7–1.2)¶ |
| Giant Schnauzer | 89 | 8.0 (0.0) | 7/5,721 | 9/21,490 | 2.9 (1.1–7.9)¶ |
| Border Collie | 52 | 5.0 (4.8) | 104/5,624 | 295/21,204 | 1.3 (1.1–1.7)# |
| Chinese Shar Pei | 47 | See above | | | |

* Table shows the dog breeds with a higher (1.) or lower (2.) proportion of decreased serum cobalamin concentrations (<251 ng/L). Also shown are data for 5 breeds that had previously been reported in case reports describing cobalamin deficiency in a group of dogs of a single breed (3. Case reports).
† Median age (in years) for all dogs of each breed. Dogs where age was not reported is shown in parentheses. Both values are in percentages.
‡ Number of dogs of a particular breed/number of dogs of the remaining dog breeds in which decreased serum cobalamin concentrations (<251 ng/L) and normal serum cobalamin concentrations (251-908 ng/L) were identified.
§ Calculated odds ratio, 95% confidence interval (in parentheses) for each breed, and the corresponding *p* values (¦ = < 0.0003, # = < 0.05, ¶ = > 0.05).

Furthermore, the Akita, Border Collie, Shar Pei, and German Shepherd Dog had significantly higher proportions of dogs with serum cobalamin concentrations <150 ng/L (OR > 1; all $p < 0.0001$; Table 2). In contrast, the Boxer, Golden Retriever, Miniature Schnauzer, and Standard Poodle had significantly lower proportions of dogs with serum cobalamin concentrations <150 ng/L (OR < 1; all $p < 0.0001$; Table 2). Also, for the Akita, Border Collie, and German Shepherd Dog, but not for the Shar Pei, submissions with undetectable serum cobalamin concentrations were more likely associated with a serum cTLI concentration considered diagnostic for EPI than those submissions with a normal serum cobalamin concentration (all $p \leq 0.001$; Table 3).

A total of 19 breeds were found to have disproportionately higher proportions of serum samples submitted for serum cobalamin analysis (all $p < 0.0001$, Table 4) relative to the AKC breed ranking list of 2009. The breed with the highest proportion of serum samples submitted for serum cobalamin analysis was the Greyhound (Table 4). In contrast, 7 breeds were found to have disproportionately lower proportions of serum samples submitted for serum cobalamin analysis (all $p < 0.0001$, Table 4). For the Cairn Terrier, Cardigan Welsh Corgi, Cocker Spaniel, Dalmatian, Wire Fox Terrier, West Highland White Terrier, and Australian Shepherd (1 of the 5 breeds previously reported in a case series with cobalamin deficiency), submissions with undetectable serum cobalamin concentrations were more likely to be associated with serum cTLI concentrations considered diagnostic for EPI than those with normal cobalamin concentrations (all $p < 0.05$; Table 3).

**Table 2.** Over- and underrepresented dog breeds with regard to undetectable serum cobalamin concentrations (<150 ng/L) using data from the Gastrointestinal Laboratory (Texas A&M University, College Station, Texas) database.*

| Breed* | 2009 AKC ranking position | Age† | Cobalamin‡ | | Odds ratio§ |
|---|---|---|---|---|---|
| | | | <150 ng/L | 251-908 ng/L | |
| **1. Odds ratio > 1** | | | | | |
| Chinese Shar Pei | 47 | 5.0 (1.6) | 63/1,670 | 71/21,428 | 11.4 (8.1–16.0)¦ |
| Akita | 50 | 6.8 (22.2) | 18/1,715 | 59/21,440 | 3.8 (2.2–6.5)¦ |
| Border Collie | 52 | 4.5 (4.0) | 50/1,683 | 295/21,204 | 2.1 (1.6–2.9)¦ |
| German Shepherd Dog | 2 | 5.0 (7.3) | 354/1,379 | 2,767/18,732 | 1.7 (1.5–2.0)¦ |
| **2. Odds ratio < 1** | | | | | |
| Golden Retriever | 4 | 8.0 (6.8) | 44/1,689 | 1,152/20,347 | 0.5 (0.3–0.6)¦ |
| Boxer | 6 | 7.0 (12.1) | 33/1,700 | 930/20,569 | 0.4 (0.3–0.6)¦ |
| Miniature Schnauzer | 11 | 8.0 (20.0) | 20/1,713 | 597/20,902 | 0.4 (0.3–0.6)¦ |
| Standard Poodle | 9 | 7.0 (0.0) | 10/1,723 | 469/21,030 | 0.3 (0.1–0.5)¦ |
| **3. Case reports** | | | | | |
| Australian Shepherd | 28 | 7.0 (0.0) | 19/1,714 | 187/21,312 | 1.3 (0.8–2.0)¶ |
| Beagle | 5 | 8.5 (7.7) | 26/1,707 | 357/21,142 | 0.9 (0.6–1.3)¶ |
| Giant Schnauzer | 89 | 6.5 (0.0) | 1/1,732 | 9/21,490 | 1.4 (0.2–11.0)¶ |
| Border Collie | 52 | See above | | | |
| Chinese Shar Pei | 47 | See above | | | |

* Table shows the dog breeds with a higher (1.) or lower (2.) proportion of undetectable serum cobalamin concentrations (<150 ng/L). Also shown are data for 5 breeds that had previously been reported in case reports describing cobalamin deficiency in a group of dogs of a single breed (3. Case reports).
† Median age (in years) for all dogs of each breed. Dogs where age was not reported is shown in parentheses. Both values are in percentages.
‡ Number of dogs of a particular breed/number of dogs of the remaining dog breeds with undetectable serum cobalamin concentrations (<150 ng/L) and normal serum cobalamin concentrations (251-908 ng/L) were identified.
§ Calculated odds ratio, 95% confidence interval (in parentheses) for each breed, and the corresponding *p* values (¦ = < 0.0003, # = < 0.05, ¶ = > 0.05).

**Table 3.** Breeds with proportions of serum samples submitted to the Gastrointestinal Laboratory (GI Lab; Texas A&M University, College Station, Texas) for serum cobalamin analysis when compared with the American Kennel Club (AKC) ranking list of 2009.

| Breed* | AKC ranking position | GI Lab† (*n*) | AKC† (*n*) | Odds ratio‡ |
|---|---|---|---|---|
| **1. Odds ratio > 1** | | | | |
| Greyhound | 140 | 110/7,093 | 119/649,358 | 84.6 (65.2–110.0) |
| Parson Russell Terrier | 87 | 121/7,082 | 691/648,786 | 16.0 (13.2–19.5) |
| Standard Schnauzer | 99 | 58/7,145 | 559/648,918 | 9.4 (7.2–12.4) |
| American Eskimo Dog | 118 | 23/7,180 | 318/649,159 | 6.5 (4.3–10.0) |
| Cardigan Welsh Corgi | 83 | 43/7,160 | 818/648,659 | 4.8 (3.5–6.5) |
| Border Collie | 52 | 102/7,101 | 2,009/647,468 | 4.6 (3.8–5.7) |
| Wire Fox Terrier | 94 | 28/7,175 | 622/648,855 | 4.1 (2.8–6.0) |
| Soft Coated Wheaten Terrier | 62 | 58/7,145 | 1,367/648,110 | 3.8 (3.0–5.0) |
| Keeshond | 102 | 20/7,182 | 542/648,935 | 3.3 (2.1–5.2) |
| Irish Setter | 73 | 39/7,164 | 1,044/648,433 | 3.4 (2.5–4.7) |
| English Setter | 95 | 22/7,181 | 622/648,855 | 3.2 (2.1–4.9) |
| Dalmatian | 75 | 35/7,168 | 1,001/648,476 | 3.2 (2.3–4.4) |
| Cairn Terrier | 56 | 54/7,149 | 1,791/647,686 | 2.7 (2.1–3.6) |
| Bichon Frise | 35 | 113/7,090 | 4,161/645,316 | 2.5 (2.1–3.0) |
| Australian Shepherd | 67 | 34/7,169 | 1,271/648,206 | 2.4 (1.7–3.4) |
| German Shepherd Dog | 2 | 976/6,227 | 40,938/608,539 | 2.3 (2.2–2.5) |
| Lhasa Apso | 54 | 49/7,154 | 1,932/647,545 | 2.3 (1.7–3.1) |
| West Highland White Terrier | 36 | 98/7,105 | 4,096/645,381 | 2.2 (1.8–2.7) |
| Cocker Spaniel | 23 | 171/7,032 | 8,282/641,195 | 1.9 (1.6–2.2) |
| **2. Odds ratio < 1** | | | | |
| Labrador Retriever | 1 | 722/6,481 | 89,599/559,878 | 0.7 (0.6–0.8) |
| Standard Poodle | 9 | 138/7,065 | 18,601/630,876 | 0.7 (0.6–0.8) |
| Pomeranian | 14 | 76/7,127 | 11,415/638,062 | 0.6 (0.5–0.8) |
| French Bulldog | 24 | 36/7,167 | 7,381/642,096 | 0.4 (0.3–0.6) |
| Beagle | 5 | 117/7,086 | 30,672/618,805 | 0.3 (0.3–0.4) |
| English Springer Spaniel | 29 | 21/7,182 | 5,896/643,581 | 0.3 (0.2–0.5) |
| Bulldog | 7 | 54/7,149 | 23,248/626,229 | 0.2 (0.2–0.3) |

* Shown are 19 dog breeds with a higher proportion of samples submitted for serum cobalamin analyses that were considered overrepresented (1.), and 7 breeds with a lower proportion of samples submitted for serum cobalamin analysis that were considered underrepresented (2.).

† Number of dogs of a particular breed/number of dogs of the remaining dog breeds that had been identified by the GI Lab database and in the AKC ranking list of 2009.

‡ Calculated odds ratio and 95% confidence interval (in parentheses) for each breed (*p* values for all < 0.0003).

**Table 4.** Comparison of dog breeds with a higher proportion of dogs with decreased serum canine trypsin-like immunoreactivity (cTLI; ≤2.5 µg/L) concentrations and undetectable serum cobalamin (COB) concentrations (<150 ng/L) and those with a higher proportion of decreased serum cTLI (≤2.5 µg/L) concentrations but a normal serum cobalamin concentration (251–908 ng/L).

| Breed* | 2009 AKC ranking position | cTLI ≤2.5 µg/L and COB <150 ng/L† | cTLI ≤2.5 µg/L and COB 251-908 ng/L† | Odds ratio‡ | *p* value‡ |
|---|---|---|---|---|---|
| **A. Cobalamin < 150 ng/l** | | | | | |
| Chinese Shar Pei | 47 | 0/53 | 3/49 | NA | 1.0 |
| Akita | 50 | 10/16 | 7/50 | 8.6 (2.3–31.3) | **0.001** |
| Border Collie | 52 | 9/37 | 8/198 | 7.6 (2.7–21.4) | **0.0002** |
| German Shepherd Dog | 2 | 131/316 | 335/2,024 | 3.6 (2.8–4.6) | **<0.0001** |
| **B. Cobalamin submissions** | | | | | |
| Greyhound | 140 | 0/1 | 29/209 | NA | 1.0 |
| Parson Russell Terrier | 87 | 2/14 | 17/214 | 1.9 (0.4–9.3) | >0.05 |
| Standard Schnauzer | 99 | 1/7 | 1/78 | 12.8 (0.7–231.8) | >0.05 |
| American Eskimo Dog | 118 | ¼ | 1/31 | 10.0 (0.5–204.1) | >0.05 |
| Cardigan Welsh Corgi | 83 | 4/7 | 9/76 | 9.9 (1.9–51.7) | **<0.05** |
| Border Collie | 52 | See above | See above | | |
| Wire Fox Terrier | 94 | 2/4 | 2/55 | 26.5 (2.4–296.7) | **<0.05** |
| Soft Coated Wheaten Terrier | 62 | 0/5 | 0/138 | NA | 1.0 |
| Keeshond | 102 | 0/1 | 0/42 | NA | 1.0 |
| Irish Setter | 73 | 0/6 | 1/91 | NA | 1.0 |
| English Setter | 95 | 0/3 | 0/55 | NA | 1.0 |
| Dalmatian | 75 | 2/7 | 1/77 | 30.4 (2.3–395.4) | **<0.05** |
| Cairn Terrier | 56 | 12/20 | 24/91 | 4.2 (1.5–11.5) | **<0.01** |
| Bichon Frise | 35 | 0/16 | 0/180 | NA | 1.0 |
| Australian Cattle Dog | 67 | 3/8 | 7/57 | 4.2 (0.8–22.0) | >0.05 |
| German Shepherd Dog | 2 | See above | See above | | |
| Lhasa Apso | 54 | 1/10 | 6/88 | 1.5 (0.2–14.1) | >0.05 |
| West Highland White Terrier | 36 | 8/22 | 30/200 | 3.2 (1.3–8.4) | **<0.05** |
| Cocker Spaniel | 23 | 4/34 | 8/331 | 5.4 (1.5–18.9) | **<0.05** |
| **C. Case reports** | | | | | |
| Australian Shepherd | 28 | 4/18 | 4/109 | 7.5 (1.7–33.4) | **<0.05** |
| Beagle | 5 | 0/22 | 3/235 | NA | 1.0 |
| Giant Schnauzer | 89 | NA | NA | NA | NA |
| Border Collie | 52 | See above | See above | | |
| Chinese Shar Pei | 47 | See above | See above | | |

* Shown are dog breeds (A) from Table 2: with a higher proportion of undetectable serum cobalamin concentrations (<150 ng/L), (B) from Table 4: with a higher proportion of samples submitted for serum cobalamin analysis, and (C) dog breeds reported in case reports of cobalamin deficiency and their calculated proportion of low serum cTLI concentrations (≤2.5 µg/L) diagnostic for exocrine pancreatic insufficiency when compared to normal cobalamin concentrations (251-908 ng/L).

† Number of dogs that had a serum cTLI concentration ≤2.5 µg/L and serum cobalamin concentrations <150 ng/L/total number of dogs or a serum cTLI concentration ≤2.5 µg/L and serum cobalamin concentrations 251-908 ng/L/total number of dogs. NA = not applicable.

‡ Calculated odds ratio, 95% confidence interval (in parentheses) for each breed, and the corresponding *p* values. NA = not applicable.

Among the 19 breeds with higher proportion of serum sample submissions, serum cobalamin concentrations as well as ages were significantly different (both: $p < 0.0001$; Figure 1 and 2, respectively; Table 5). Dunn post test showed that serum cobalamin concentrations in the Greyhound were significantly lower than those in the other 18 breeds (all $p \leq 0.01$; Figure. 1). Also, the ages differed significantly among the 19 breeds ($p < 0.0001$; Figure 2). Post test revealed that German Shepherd Dogs were significantly younger than those other 17 breeds, but not the Irish Setter (all $p \leq 0.05$; Figure 2). Furthermore, Dunn post test showed that the age in the Irish Setters differed significantly from those in 16 other breeds, but not the German Shepherd Dog or Soft Coated Wheaten Terrier (all $p \leq 0.05$; Figure 2). The American Eskimo Dog, Dalmatian, and Keeshond had a median age of 10 years, which differed significantly from the Border Collie, Cairn Terrier, Cardigan Welch Corgi, German Shepherd Dog, Irish Setter, and Soft Coated Wheaten Terrier (all $p \leq 0.05$; Figure 2).

**Table 5.** Gastrointestinal Laboratory (GI Lab; Texas A&M University, College Station, Texas) data set for a period of 4 years (2006-2010) for 19 breeds with their corresponding sex, median age, and median serum cobalamin concentration.*

| Breed* | 2009 AKC ranking position | GI Lab† (*n*) | Age‡ | Cobalamin§ (ng/L) |
|---|---|---|---|---|
| **1. Odds ratio > 1** | | | | |
| Greyhound | 140 | 441 | 8.0 (4.5) | 286 |
| Parson Russell Terrier | 87 | 484 | 8.8 (7.8) | 518 |
| Standard Schnauzer | 99 | 231 | 8.0 (6.0) | 575 |
| American Eskimo | 118 | 91 | 10.0 (11.0) | 422 |
| Cardigan Welsh Corgi | 83 | 171 | 6.0 (4.1) | 416 |
| Border Collie | 52 | 409 | 6.0 (8.3) | 368 |
| Wire Fox Terrier | 94 | 111 | 8.0 (9.9) | 515 |
| Soft Coated Wheaten Terrier | 62 | 230 | 5.6 (9.6) | 409 |
| Keeshond | 102 | 80 | 10.0 (7.5) | 392 |
| Irish Setter | 73 | 154 | 4.0 (6.5) | 391 |
| English Setter | 95 | 86 | 7.0 (3.5) | 520 |
| Dalmatian | 75 | 141 | 10.0 (3.5) | 346 |
| Cairn Terrier | 56 | 214 | 6.0 (5.1) | 411 |
| Bichon Frise | 35 | 452 | 8.0 (7.3) | 526 |
| Australian Cattle Dog | 67 | 136 | 7.0 (7.4) | 379 |
| German Shepherd Dog | 2 | 3,905 | 3.0 (8.9) | 342 |
| Lhasa Apso | 54 | 195 | 8.0 (9.2) | 443 |
| West Highland White Terrier | 36 | 393 | 7.0 (5.3) | 458 |
| Cocker Spaniel | 23 | 684 | 9.0 (7.9) | 472 |

* Table shows the dog breeds with a higher (1.) proportion of serum samples submitted to GI Lab.
† Number of dogs per breed in which higher proportion of serum samples were submitted to GI Lab.
‡ Median age (in years) for all dogs of each breed. Dogs where age was not reported is shown in parentheses. Both values are in percentages.
§ Median serum cobalamin concentrations (in ng/l) for all breeds.

**Figure 1.** Serum cobalamin concentrations in 19 dog breeds. Serum cobalamin concentrations differed significantly among these 19 breeds ($p < 0.0001$). Furthermore, serum cobalamin concentrations in Greyhounds differed significantly from those in the other 18 dog breeds ($p \leq 0.01$). Order of listed breeds in the figure is the same as in Tables 3 and 4.

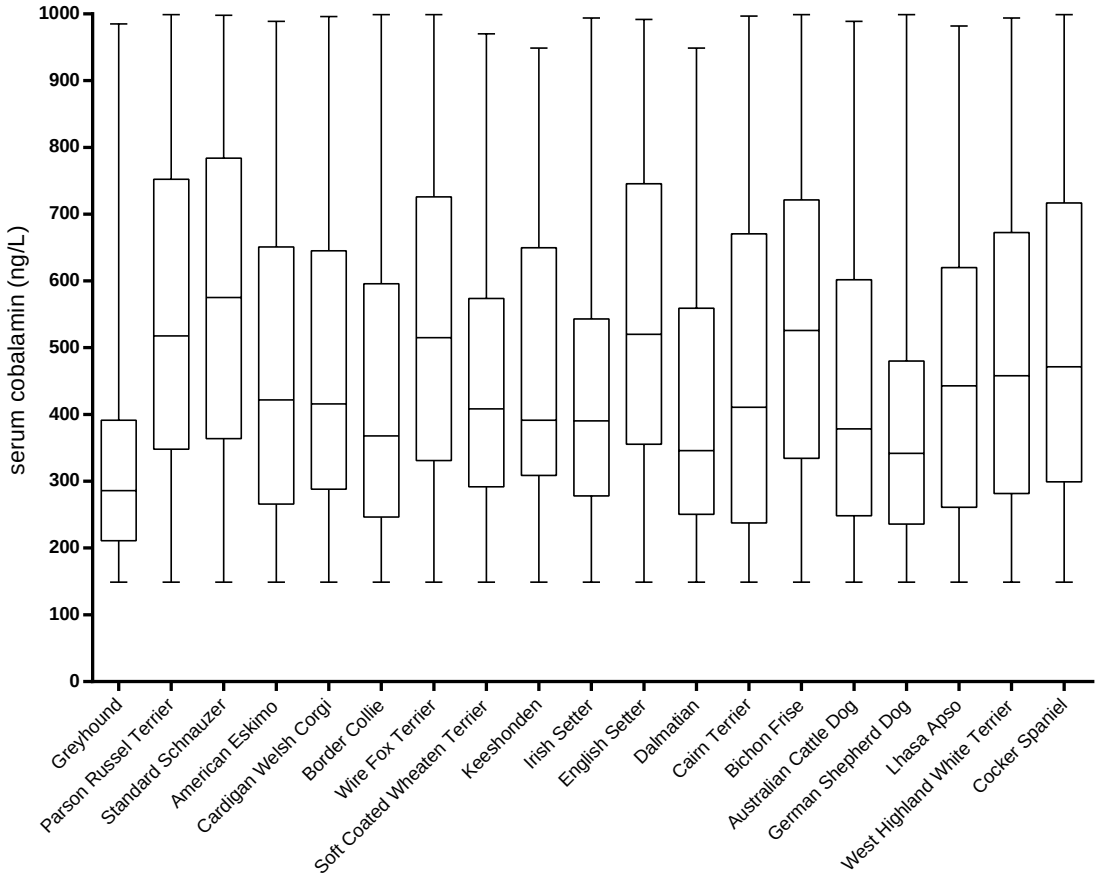
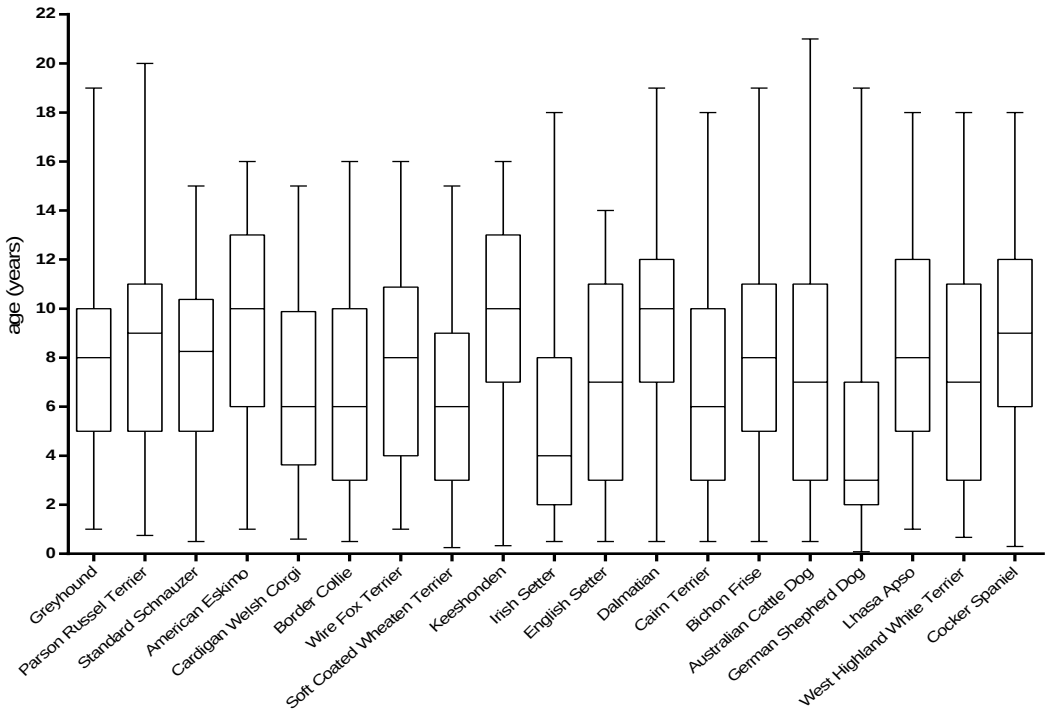


**Figure 2.** Ages of dogs of 19 dog breeds. The ages among the 19 breeds differed significantly ($p < 0.0001$). Also, the ages of German Shepherd Dogs differed significantly from those of the 17 dog breeds other than the Irish Setter ($p \leq 0.05$). The American Eskimo Dog, Keeshond, and Dalmatian (median age: 10 years) were significantly older than the Cardigan Welsh Corgi, Border Collie, Soft Coated Wheaten Terrier, Cairn Terrier, Irish Setter, and German Shepherd Dog ($p \leq 0.05$). Order of the listed breeds in the figure is the same as in Figure 1 and Tables 3 and 4.



## 2.5 Discussion

In the present retrospective study, 5 breeds (the Akita, Shar Pei, German Shepherd Dog, Greyhound, and Labrador Retriever) were observed to be overrepresented, and 6 breeds (Belgian Malinois, Boxer, Golden Retriever, Great Dane, Miniature Schnauzer, and Standard Poodle) were underrepresented with regard to a serum cobalamin concentration below the lower limit of the reference interval. Furthermore, 4 breeds (Akita, Border Collie, Shar Pei, and German Shepherd Dog) were overrepresented with regard to undetectable serum cobalamin concentrations, and 4 breeds (Boxer, Golden Retriever, Miniature Schnauzer, and Standard Poodle) were underrepresented in this regard.

The GI Lab database results also revealed that the Akita, Border Collie, and German Shepherd Dog, but not the Shar Pei, with undetectable serum cobalamin concentrations, were more likely to also have a serum cTLI concentration considered diagnostic for EPI than dogs with a normal cobalamin concentration. Of the 5 breeds that were mentioned in previous reports regarding cobalamin deficiency during the past two decades, the Border Collie and the Shar Pei were the only breeds in the current study that showed an association with undetectable serum cobalamin concentrations. Of these 5 breeds, the Border Collie and Australian Shepherd, with undetectable serum cobalamin concentrations, revealed an association with serum cTLI concentrations considered diagnostic for EPI. In contrast, the Beagle showed no such association in the present study, which could indicate that the case report of cobalamin deficiency was an isolated case and not a reflection of a breed predilection. Because there were less than 30 serum

submissions for the Giant Schnauzer (1 dog with undetectable serum cobalamin concentration), that breed was excluded from the analysis.

The findings of the current study suggest that the Akita, Australian Shepherd, Border Collie, and German Shepherd Dog, but not the Shar Pei, may have a higher prevalence of cobalamin deficiency due to exocrine pancreatic insufficiency. Pancreatic secretions play an important role in the intestinal absorption of cobalamin in the dog (Simpson et al., 1989). Intrinsic factor, which is essential for cobalamin absorption, is secreted mainly from pancreatic acinar cells in dogs (Batt et al., 1985). Therefore, in the Akita, Australian Shepherd, Border Collie, and German Shepherd Dog with decreased serum cobalamin concentration and serum cTLI concentration considered diagnostic for EPI, further investigations of the findings are warranted.

Nineteen breeds had disproportionately high numbers of serum samples submitted for cobalamin analysis relative to the AKC breed ranking list of 2009. For some breeds, such as the Cairn Terrier, Cardigan Welsh Corgi, Cocker Spaniel, Dalmatian, West Highland White Terrier, and Wire Fox Terrier, submissions with undetectable serum cobalamin concentrations were associated with a serum cTLI concentration considered diagnostic for EPI, suggesting that in these breeds cobalamin deficiency is due to EPI. In contrast, 10 breeds with undetectable serum cobalamin concentrations showed no association with a serum cTLI concentration considered diagnostic for EPI. Consequently, this suggests that cobalamin deficiency in these breeds was most likely independent of EPI.

The ages of dogs for which serum was submitted for cobalamin analysis differed significantly among the 19 breeds for which disproportionate numbers of samples were submitted. Veterinarians requested serum cobalamin analysis more frequently in younger German Shepherd Dogs and Irish Setters, which suggests that early in life both breeds are susceptible to gastrointestinal disease. It has been shown in North America and in Europe that EPI in German Shepherd Dogs (Batchelor et al., 2007) is a disease that occurs early in life and is suspected to be hereditary. The same applies for the gluten-sensitive enteropathy in Irish Setters, but this condition has been reported only in the United Kingdom (Batt, 1985; Garden et al., 2000). In contrast, American Eskimo Dogs, Dalmatians, and Keeshonds were significantly older than Border Collies, Cairn Terriers, Cardigan Welsh Corgis, German Shepherd Dogs, Irish Setters, and Soft Coated Wheaten Terriers, which could suggest that the former breeds have a predilection to late-onset gastrointestinal disease that is associated with cobalamin malabsorption.

The Greyhound, which was 1 of 19 breeds with a higher proportion of serum sample submissions for cobalamin measurement, had by far the highest proportion of serum samples submitted for serum cobalamin analysis and the lowest serum cobalamin concentration, suggesting that cobalamin deficiency is frequently suspected in this breed. It also suggests that cobalamin deficiency is common in this breed or that serum cobalamin concentrations in Greyhounds are lower than those in other breeds and that a breed-specific reference interval should be investigated.

It should be noted that there were several limitations of the current study. For instance, mixed-breed dogs might have been included if a dog owner reported the dog to

be pure bred. Also, it is possible that animal hospitals did not correctly report the dog breeds. In addition, veterinarians might have submitted samples from certain breeds more frequently because a disease is associated with a particular breed. Also, it might be possible that dog-breeding clubs are aware of certain gastrointestinal diseases in their breed of interest and due to breed-club initiatives submissions rates by breeds can be influenced.

Serum cobalamin concentrations below the lower limit of the reference interval have previously been described in the German Shepherd Dog (Rutger et al., 1995) and Shar Pei (Bishop et al., 2012) but not in the Akita, Greyhound, or Labrador Retriever. Undetectable serum cobalamin concentrations have been reported in the Border Collie (Morgan & McConnell, 1999), Shar Pei (Bishop et al., 2012), and German Shepherd Dog (Batt, 1993), but not in the Akita. An association of undetectable serum cobalamin concentration and a serum cTLI concentration considered diagnostic for EPI has been previously identified in the German Shepherd Dog (Batt, 1993), but not in the Akita or Border Collie. In contrast, the Shar Pei did not show an association with a serum cTLI concentration considered diagnostic for EPI. Therefore, it appears that only some breeds with undetectable serum cobalamin concentration have an association with EPI. In the Shar Pei, for which a high prevalence of cobalamin deficiency has previously been described in North America (Bishop et al., 2012) and the United Kingdom (Dandrieux et al., 2010), it appears that the cobalamin deficiency is not associated with EPI but rather with a defect in cobalamin metabolism (Bishop et al., 2012; Grützner et al., 2013).

Nineteen breeds had higher proportions of samples submitted for serum cobalamin analysis. Breeds such as the American Eskimo, Keeshond, and Standard Schnauzer with undetectable serum cobalamin concentrations did not show an association with EPI. Those breeds might have been overrepresented in the present study because they have been identified in another study as having abnormal findings on SpecCPL testing (Bishop et al., 2010). SpecCPL is a test used to diagnose pancreatitis in dogs (Huth et al., 2010), and therefore veterinarians might have submitted serum samples from those breeds more frequently to GI Lab for concurrent serum cobalamin analysis.

The Chinese Shar Pei, Staffordshire Bull Terrier, and a mixed-breed dog have been described in the United Kingdom as having a higher risk of low serum cobalamin concentration, while the Boxer, Bullmastiff, English Setter, Flat-Coated Retriever, Golden Retriever, Old English Sheepdog, and Weimaraner have a low risk for low serum cobalamin concentration (Dandrieux et al., 2010). In the current retrospective study, the Staffordshire Bull Terrier did not show a higher risk of low serum cobalamin concentration. On the other hand, breeds such as the Boxer and Golden Retriever were underrepresented in both North America and the United Kingdom with regard to decreased cobalamin concentration, which suggests that neither breed is predisposed to cobalamin deficiency.

In conclusion, results of the present retrospective study indicate that the Akita, Shar Pei, German Shepherd Dog, Greyhound, and Labrador Retriever had an increased proportion with regard to a serum cobalamin concentration below the lower limit of the reference interval. Akitas, Shar Peis, German Shepherd Dogs, and Border Collies had an

increased proportion of serum cobalamin concentrations below the detection limit of the assay. Furthermore, undetectable serum cobalamin concentrations were associated with a serum cTLI concentration considered diagnostic for EPI in the Akita, Australian Shepherd, Border Collie, German Shepherd Dog, Cairn Terrier, Cardigan Welsh Corgi, Cocker Spaniel, Dalmatian, West Highland White Terrier, and Wire Fox Terrier. However, in the Shar Pei, undetectable serum cobalamin concentrations were not associated with serum cTLI concentrations suggestive of EPI. Greyhounds had the highest proportion of serum samples submitted for serum cobalamin analysis. Further investigations are warranted in the breeds identified in this study to clarify if any breed-specific gastrointestinal disorders may exist.

# 3. INFLAMMATORY, IMMUNOLOGICAL, AND OTHER BIOMARKERS IN COBALAMIN-DEFICIENT CHINESE SHAR PEIS

## 3.1 Overview

Chinese Shar Peis (Shar Peis) have a high prevalence of cobalamin deficiency and commonly present with clinical signs suggestive of severe and longstanding gastrointestinal disease. Cutaneous mucinosis and Shar Pei fever are also prevalent in this breed, and their potential association with cobalamin deficiency has not been investigated. Therefore, the goal of this study was to evaluate serum concentrations of inflammatory markers, immunological markers, and markers for intestinal disease in Shar Peis with and without cobalamin deficiency. Serum concentrations of inflammatory markers (i.e., C-reactive protein [CRP], calprotectin [CP], and S100A12), hyaluronic acid, parameters commonly altered in chronic intestinal diseases (i.e., albumin, zinc, canine alpha$_1$-proteinease inhibitor [c$\alpha_1$PI], IgA, and IgM), and creatinine were compared between Shar Peis with and without cobalamin deficiency. Serum concentrations of CRP, CP, S100A12, hyaluronic acid, zinc, and c$\alpha_1$-PI concentrations did not differ between the groups of Shar Peis ($p > 0.05$). Concentrations of serum albumin and creatinine were significantly lower in cobalamin-deficient Shar Peis than in normocobalaminemic Shar Peis (both $p < 0.0001$). Higher serum IgA concentrations and lower serum IgM concentrations were observed in cobalamin-deficient Shar Peis than in normocobalaminemic Shar Peis (both $p < 0.0001$). In conclusion, between the two groups of Shar Peis differences were only found in parameters that may be altered in

patients with chronic enteropathies (e.g., albumin and IgA). The findings of this study might suggest that cobalamin deficiency in Shar Peis is not associated with other highly prevalent diseases in Shar Peis; however, further studies are needed before definitive conclusions can be drawn.

### 3.2 Introduction

In both North America and the United Kingdom the Chinese Shar Pei (Shar Pei) has been described as having a high prevalence of cobalamin (vitamin $B_{12}$) deficiency (Bishop et al, 2012; Dandrieux et al., 2013). Cutaneous mucinosis and Shar Pei fever are also prevalent in this breed, and their potential association with cobalamin deficiency has not been investigated.

Shar Peis with cobalamin deficiency commonly present with clinical signs suggestive of severe and longstanding gastrointestinal disease such as diarrhea, vomiting, and/or weight loss (Bishop et al., 2012). It has been shown that cobalamin deficiency in Shar Peis is associated with hyperhomocysteinemia, which suggests that the function of the intracellular cobalamin-dependent enzyme (i.e., methionine synthase) is impaired in cobalamin-deficient Shar Peis (Grützner et al., 2013). In this context, hyperhomocysteinemia has been described in humans with chronic inflammatory diseases such as rheumatic disease (Szekanecz & Koch, 2008), cardiovascular and end-stage renal disease (van Guldener et al., 2007), and inflammatory bowel disease (IBD) (Romagnulo et al., 2001).

Serum concentrations of C-reactive protein (CRP), a recognized acute phase reactant (Baykal et al., 1993); calprotectin (CP), a marker of granulocytic inflammation (Heilmann et al., 2012; Lügering et al., 1995); and S100A12, a sensitive marker of neutrophilic inflammation (Kallinich et al., 2010), have recently been described in dogs with chronic gastrointestinal diseases and were observed to be altered in dogs with chronic enteropathies (e.g., IBD) (Jergens et al., 2003; Heilmann et al., 2012; Heilmann et al., 2011). However, to the authors' knowledge such inflammatory markers (i.e., CRP, CP, and S100A12) have not yet been reported in Shar Peis with cobalamin deficiency.

Hyaluronan (also called hyaluronic acid [HA]) has been suggested as another potential marker for inflammation by Hascall et al. (2004). Interestingly, increased serum HA concentrations have been described in Shar Peis with cutaneous mucinosis when compared to healthy controls and it has been proposed that this condition is a consequence of a genetic defect involving HA (Hascall et al., 2004; Muller, 1990; Zanna et al., 2008). Therefore, it appears reasonable to measure serum HA concentrations in Shar Peis with cobalamin deficiency to evaluate if a potential association between cutaneous mucinosis and cobalamin deficiency in Shar Peis might exists.

A higher production of cell surface hyaluronan has been documented on mucosal endothelial cells in human patients with IBD when compared to healthy controls (Kessler et al., 2008). Along those lines, low serum cobalamin concentrations have also been documented in both human and canine patients with chronic enteropathies such as IBD (Allenspach et al., 2007; Yakut et al., 2010). We hypothesize that certain serum parameters (e.g., albumin [Allenspach et al., 2007], zinc [Gingerich et al., 2008], and

canine alpha$_1$-proteinease inhibitor [Heilmann et al., 2013; Grützner et al., 2013]), which have been reported to be affected in dogs with severe and longstanding intestinal disease might be altered in Shar Peis with cobalamin deficiency.

In humans, immunoglobulin A (IgA) has been suggested to play a role in the pathogenesis of chronic intestinal diseases leading to cobalamin deficiency (Baz et al., 2004). In dogs, decreased concentrations of IgA and IgM in serum have been observed in Shar Peis with a suspected primary immunodeficiency syndrome (Moroff et al., 1986; Rivas et al., 1995). However, to the authors knowledge those two immunoglobulins have not been documented in Shar Peis with cobalamin deficiency.

Shar Pei Fever is an autoimmune disorder causing periodic flare-ups, which predisposes Shar Peis to systemic reactive amyloidosis causing renal failure (DiBartola et al., 1989; Segev et al., 2012). A study that investigated renal amyloidosis in dogs demonstrated that serum creatinine concentrations were higher in Shar Peis with renal amyloidosis when compared to non-Shar Peis with renal amyloidosis (Segev et al., 2012). Also, dogs affected with Shar Pei fever frequently show clinical signs such as anorexia and weight loss, which have also been reported in cobalamin-deficient Shar Peis. However, to the best of our knowledge neither serum cobalamin concentrations have been reported in Shar Peis with Shar Pei Fever nor serum creatinine concentrations have been evaluated in Shar Peis with cobalamin deficiency.

Several conditions have frequently been reported in Shar Peis but a potential association between those conditions has not been investigated. Therefore, the aims of this study were to compare serum concentrations of inflammatory markers, markers for

chronic intestinal disease, and immunological markers in Shar Peis with and without cobalamin deficiency, and to evaluate if an inflammatory phenotype exists in Shar Peis with cobalamin deficiency.

## 3.3 Materials and methods

### Sample Collection

For the purpose of this study, serum samples from Shar Peis were collected between March 1st of 2006 and December 1st of 2009. The protocol for collection of serum samples from healthy dogs was reviewed and approved by the Clinical Research Review Committee at Texas A&M University (CRRC#2003-51, CRRC#2007-30). These serum samples had been collected from Shar Peis from various parts of the United States, and the owner of each dog completed a questionnaire, which included questions concerning the signalment and the current health status of the dog. Some of the samples were collected from dogs that also had been used for a genome-wide association study of cobalamin deficiency in the Shar Pei as reported elsewhere (Grützner et al., 2010). Not all parameters were evaluated in all samples (Table 6).

### Concentrations of serum cobalamin

Serum cobalamin concentrations in Shar Peis were measured using an automated chemiluminescence assay (Immulite®2000; Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) with a reference interval of 251-908 ng/L. Only dogs were included that either were normocobalaminemic or that had an undetectable serum cobalamin

concentration (<150 ng/L; these dogs were considered to be cobalamin-deficient; Table 6). Shar Peis with cobalamin deficiency had clinical signs suggestive of severe and longstanding gastrointestinal disease such as diarrhea, vomiting, and/or weight loss. Whereas Shar Peis with normal cobalamin concentrations were apparently health based on the study questionnaires which were filled out by primary care veterinarian and pet owners.

**Concentrations of serum inflammatory markers**

Concentrations of serum CRP were quantified in Shar Peis with and without cobalamin deficiency (Table1) using a commercial ELISA kit (Tridelta, Maynooth, Ireland) with a reference interval of 0-7.6 mg/L (Gastrointestinal Laboratory at Texas A&M University; College Station, TX, USA; http://vetmed.tamu.edu/gilab/service/assays/canine-c-reactive-protein.; Accessed August 5, 2013). Serum calgranulin concentrations were measured using an in-house ELISA for canine CP (reference interval: 0.9-11.9 mg/L; [Heilmann et al., 2011]) and an in-house radioimmunoassay for canine S100A12 (reference interval: 33.0-233.0 µg/L; [Heilmann et al., 2010]).

**Concentrations of serum HA**

Serum HA concentrations were measured in samples from Shar Peis (Table 1) with and without cobalamin deficiency by use of a commercially available ELISA kit (Echelon Biosciences, Salt Lake City, USA). Due to the lack of an in-house control interval for canine serum HA concentrations, serum from 7 healthy German Shepherd Dogs was

used to provide an approximate control range. Because of the small sample size, serum HA concentrations of healthy control dogs were compared to measurements of healthy control dogs of other studies (Zanna et al., 2008; Seki et al., 2008). The protocol for collection of serum samples from healthy German Shepherd Dogs was reviewed and approved by the Clinical Research Review Committee at Texas A&M University (CRRC#2005-35).

**Concentrations of serum chronic intestinal diseases markers**

Concentrations of serum albumin (reference interval: 2.4-4.5 g/dL) were measured for Shar Peis with and without cobalamin deficiency using an automated clinical chemistry analyzer (Stanbio Laboratory, Boerne, TX, USA). An external laboratory (Texas A&M Veterinary Medical Diagnostic Laboratory) was used to measure serum zinc concentrations (reference interval: 0.7-2.0 ppm; Texas Veterinary Medical Diagnostic Laboratory; College Station, TX, USA; http://tvmdl.tamu.edu.; Accessed August 5, 2013) in Shar Peis with and without cobalamin deficiency. Finally, serum $c\alpha_1$PI concentrations were measured using an in-house radioimmunoassay (reference interval: 732-1,802 mg/L; [Heilmann et al., 2013]).

**Table 6.** Number (n) and proportion of cobalamin-deficient (COB deficient) Shar Peis and normocobalaminemic (normal COB) Shar Peis that were included in this study are listed for each test performed (hyaluronic acid [HA], canine C-reactive protein [CRP], canine calprotectin [CP], canine S100A12 [A12], albumin, creatinine, zinc, canine alpha$_1$-proteinase inhibitor [c$\alpha_1$PI], canine IgA [IgA], and canine IgM [IgM] concentration). The remaining columns show the number of female and male dogs and the median age (in years) for all dogs of each group.

| | | Chinese Shar Peis | | | | | |
|---|---|---|---|---|---|---|---|
| **Tests** | **n** | **# COB deficient** | **♀ /age** | **♂ /age** | **# normal COB** | **♀ /age** | **♂/age** |
| **HA** | 46 | 16 | 9 / 6.0 | 7 / 6.0 | 30 | 17 / 3.0 | 13 / 4.0 |
| **CRP** | 68 | 24 | 13 / 7.0 | 11 / 6.0 | 44 | 24 / 5.0 | 20 / 5.0 |
| **CP** | 39 | 14 | 8 / 6.5 | 6 / 6.0 | 25 | 13 / 4.0 | 12 / 4.0 |
| **A12** | 39 | 14 | 8 / 6.5 | 6 / 6.0 | 25 | 13 / 4.0 | 12 / 4.0 |
| **Albumin** | 66 | 22 | 11 / 7.0 | 11 / 6.0 | 44 | 26 / 5.0 | 18 / 4.0 |
| **Creatinine** | 66 | 22 | 11 / 7.0 | 11 / 6.0 | 44 | 26 / 5.0 | 18 / 4.0 |
| **Zinc** | 40 | 15 | 7 / 6.0 | 8 / 5.0 | 25 | 10 / 9.0 | 15 / 4.0 |
| **c$\alpha_1$PI** | 50 | 18 | 9 / 7.0 | 9 / 5.0 | 32 | 18 / 5.0 | 14 / 4.0 |
| **IgA** | 71 | 23 | 12 / 7.5 | 11 / 5.0 | 48 | 30 / 5.0 | 18 / 4.0 |
| **IgM** | 71 | 23 | 12 / 7.5 | 11 / 5.0 | 48 | 30 / 5.0 | 18 / 4.0 |

**Concentrations of serum Immunoglobulins markers**

Serum IgA and immunoglobulin M (IgM) concentrations were quantified by ELISAs using commercial kits (Bethyl Laboratories, Montgomery, TX, USA).

**Concentrations of serum creatinine**

Concentrations of serum creatinine (reference interval: 0.5-1.4 mg/dL) were measured in Shar Peis with and without cobalamin deficiency using an automated clinical chemistry analyzer (Stanbio Laboratory, Boerne, TX, USA).

**Data analysis**

To conduct statistical analyses, a commercial software package (GraphPad Prism5, GraphPad Software, La Jolla, CA, USA) was used. A Mann-Whitney *U* test for non-parametric data was used to compare serum HA, CRP, CP, S100A12, albumin, creatinine, zinc, c$\alpha_1$PI, IgA, and IgM concentrations between cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis. In addition, a Kruskal-Wallis test with a Dunn's post test for non-parametric data was used to compare serum HA concentrations between cobalamin-deficient Shar Peis, normocobalaminemic Shar Peis, and healthy controls. A Fisher's exact test was used to evaluate if cobalamin deficiency in Shar Peis is associated with decreased serum albumin concentrations and the odds ratio (OR) and the 95% confidence interval (CI) were calculated. Significance for all tests was set at $p < 0.05$.

## 3.4 Results

### Serum inflammatory markers concentrations

Concentrations of serum CRP, CP, and S100A12 were not significantly different between cobalamin-deficient Shar Peis (medians: 6.3 mg/L, 13.6 mg/L, and 196.3 μg/L, respectively) and normocobalaminemic Shar Peis (medians: 2.8 mg/L [$p = 0.3011$], 10.8 mg/L [$p = 0.5581$], and 144.5 μg/L [$p = 0.4914$], respectively; Figure 3, Table 7). Fifty percent of cobalamin-deficient Shar Peis had serum CP concentrations above the upper limit of the reference interval, 43% had serum S100A12 concentrations above the suggested upper limit of the reference interval, and 29% of cobalamin-deficient Shar Peis had a serum CRP concentration above the upper reference limit.

### Serum HA concentrations

No significant difference of serum HA concentrations was identified between cobalamin-deficient Shar Peis (medians: 597 ng/ml) and normocobalaminemic Shar Peis (medians: 672 ng/ml; $p = 0.8087$). However, serum HA concentrations were significantly higher in both cobalamin-deficient Shar Peis (median: 597 ng/ml) and normocobalaminemic Shar Peis (median: 672 ng/ml) when compared to healthy controls (median: 227 ng/ml; $p = 0.0156$; Figure 4; Table 7).

**Table 7.** This table shows the medians, ranges, and the *p* values for comparison of the different parameters between cobalamin-deficient (COB deficient) and normocobalaminemic (normal COB) Shar Peis. Parameters are listed in the same order as in table 1.

| | Chinese Shar Peis | | |
|---|---|---|---|
| **Tests** | **COB deficient** | **normal COB** | ***p* value** |
| **HA** (ng/ml) | 597 (195-3086) | 672 (100-2759) | 0.8087 |
| **CRP** (mg/L) | 6.3 (0.0-55.6) | 2.8 (0.0-86.4) | 0.3011 |
| **CP** (mg/L) | 13.6 (3.8-41.0) | 10.8 (2.8-41.0) | 0.5581 |
| **A12** (µg/L) | 196.3 (49.3-1315.0) | 144.5 (51.6-628.7) | 0.4914 |
| **Albumin** (g/dL) | 2.5 (1.3-3.2) | 2.9 (1.9-3.7) | **<0.0001** |
| **Creatinine** (mg/dL) | 1.0 (0.5-1.8) | 1.2 (0.6-2.9) | **0.0095** |
| **Zinc** (ppm) | 0.9 (0.5-1.2) | 1.0 (0.6-2.7) | 0.0963 |
| **c$\alpha_1$PI** (mg/L) | 1706 (1033-3210) | 1494 (904-3258) | 0.1270 |
| **IgA** (g/L) | 1.7 (0.4-4.0) | 0.6 (0.2-2.5) | **<0.0001** |
| **IgM** (g/L) | 0.9 (0.3-2.5) | 2.0 (0.4-5.0) | **<0.0001** |

**Figure 3.** Comparison of serum **A)** C-reactive protein (CRP), **B)** calprotectin (CP), and **C)** S100A12 (A12) concentrations between cobalamin-deficient (COB deficient) and normocobalaminemic (normal COB) Shar Peis (median: [CRP: 6.3 mg/L and 2.8 mg/L, $p$ = 0.3011; CP: 13.6 mg/L and 10.8 mg/L, $p$ = 0.5581; A12: 196.3 µg/L and 144.5 µg/L, $p$ = 0.4914; respectively). The reference interval for CRP (0.0-7.6 mg/L), CP (0.9-11.9 mg/L), and A12 (33.0-233.0 µg/L) are indicated by the dashed horizontal lines.

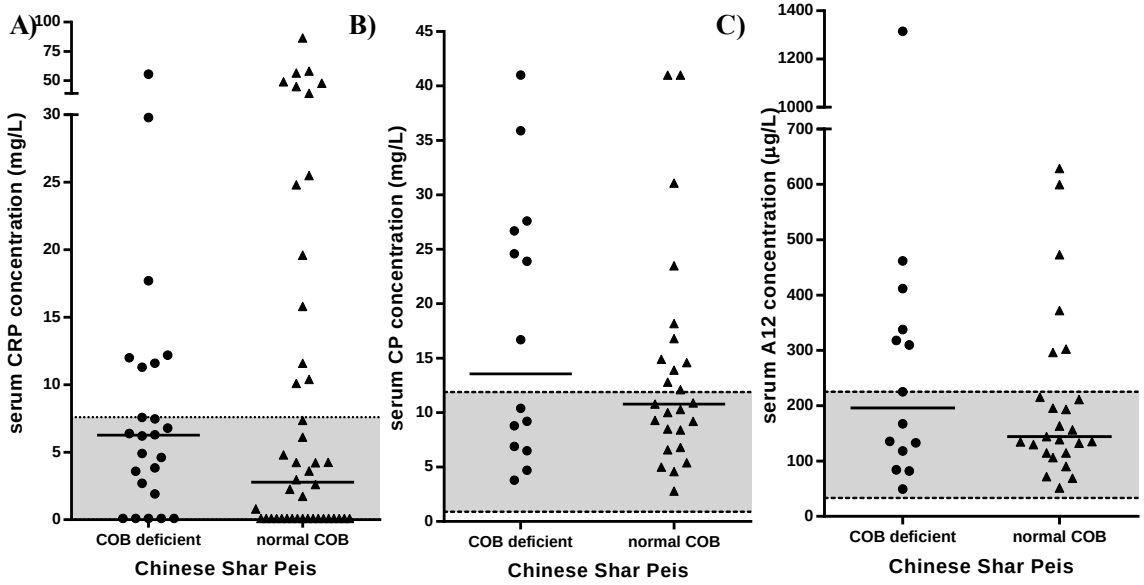
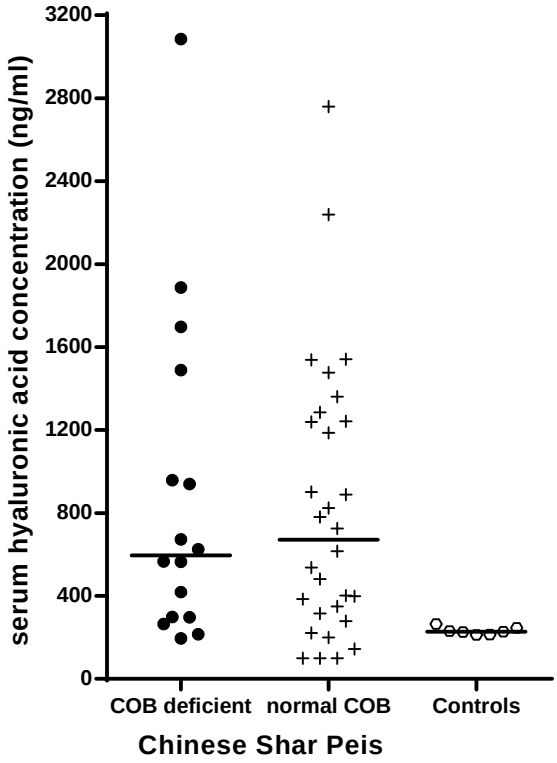


**Figure 4.** Comparison of serum hyaluronic acid (HA) concentrations between cobalamin-deficient (COB deficient) Shar Peis, normocobalaminemic (normal COB) Shar Peis, and healthy control dogs of other breeds (median: 597 ng/ml, 672 ng/L, and 227 ng/mL, respectively; $p = 0.0156$).



**Serum markers of chronic intestinal disease**

Concentrations of serum albumin were significantly lower in cobalamin-deficient Shar Peis (median: 2.5 g/dL) compared to normocobalaminemic Shar Peis (median: 2.9 g/dL; $p < 0.0001$; Figure 5). Approximately 57% (n=8) cobalamin-deficient Shar Peis had a serum albumin concentration below the lower limit of the reference interval (<2.4 g/dL), and cobalamin deficiency was significantly associated with hypoalbuminemia (OR: 12.0, CI: 2.8-63.4, $p = 0.0015$). Cobalamin-deficient Shar Peis had lower median serum zinc concentrations (median: 0.9 ppm) than normocobalaminemic Shar Peis (median: 1.0 ppm), but this difference was not significant ($p = 0.0963$; Figure 6; Table 7). Serum concentrations of $c\alpha_1PI$ were not different between cobalamin-deficient Shar Peis (median: 1,706 mg/L) and normocobalaminemic Shar Peis (median: 1,494 mg/L; $p = 0.1270$).

**Serum Immunoglobulins concentrations**

Serum IgA concentrations were significantly higher in cobalamin-deficient Shar Peis (median: 1.705 g/L) than in normocobalaminemic Shar Peis (median: 0.6339 g/L; $p < 0.0001$; Figure 7). In contrast, serum IgM concentrations were significantly lower in cobalamin-deficient Shar Peis (median: 0.9217 g/L) than in normocobalaminemic Shar Peis (median: 1.958 g/L; $p < 0.0001$; Figure 7; Table 7). The IgA-to-IgM ratio was significantly higher in cobalamin-deficient Shar Peis (median: 1.329) than in normocobalaminemic Shar Peis (median: 0.238; $p < 0.0001$).

**Serum creatinine concentrations**

Concentrations of serum creatinine were significantly lower in cobalamin-deficient Shar Peis (median: 1.0 mg/dL) compared to normocobalaminemic Shar Peis (median: 1.2 mg/dL; $p$ = 0.0095; Figure 6; Table 7). Approximately 5% (n=1) cobalamin-deficient Shar Peis had a serum creatinine concentration above the upper limit of the reference interval (>1.4 g/dL), and cobalamin deficiency was not significantly associated with increased creatinine concentrations (data not shown). Furthermore, both groups of Shar Peis had a median serum creatinine concentration within the reference interval.

**Figure 5.** Comparison of serum **A)** albumin and **B)** creatinine concentrations between cobalamin-deficient (COB deficient) and normocobalaminemic (normal COB) Shar Peis (median: [albumin: 2.5 g/dL and 2.9 g/dL, $p < 0.0001$; creatinine: 1.0 mg/dL and 1.2 mg/dL, $p = 0.0095$, respectively]). The reference interval for albumin (2.4–4.5 g/dL) and creatinine (0.5-1.4 mg/dL) are indicated by the dashed horizontal lines.

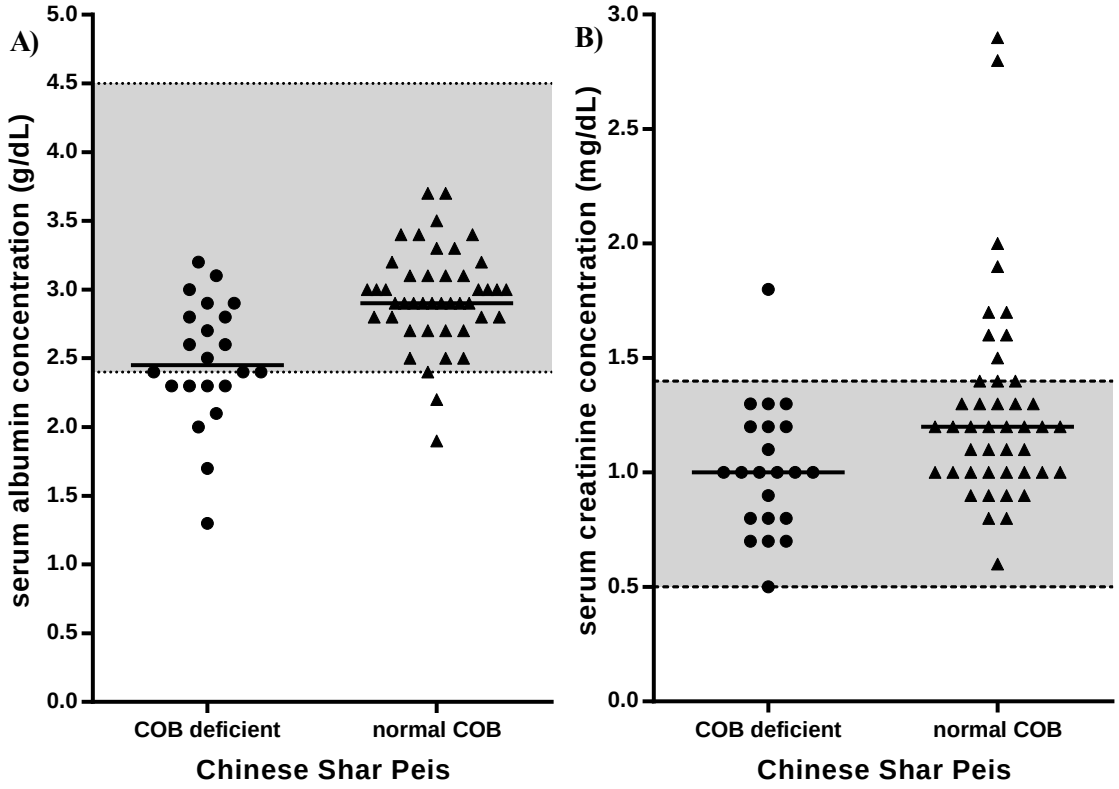


**Figure 6.** Comparison of serum zinc concentrations between cobalamin-deficient (COB deficient) and normocobalaminemic (normal COB) Shar Peis (median: 0.9 ppm and 1.0 ppm, respectively; $p = 0.0963$). The dashed horizontal lines indicate the reference interval (0.7-2.0 ppm) for serum zinc concentrations in dogs.

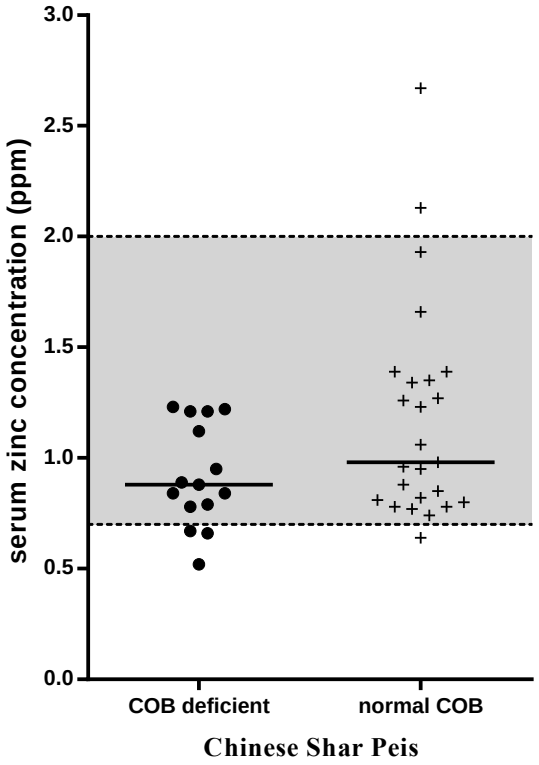
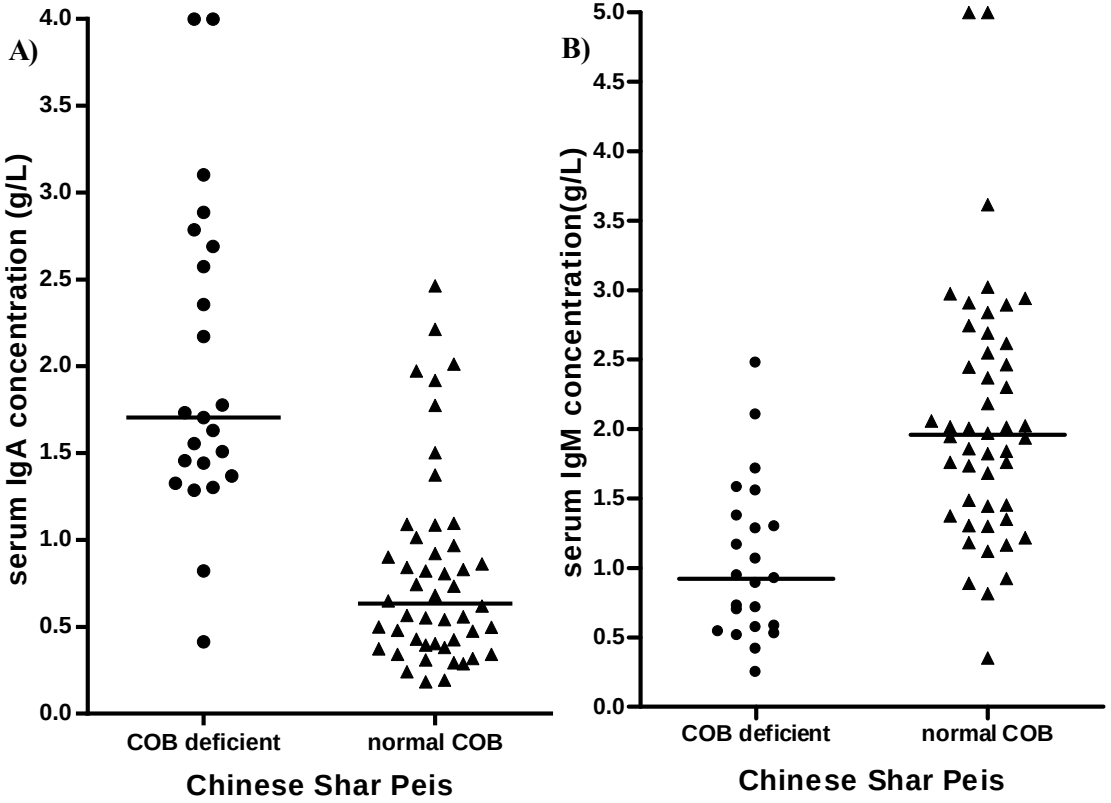


**Figure 7.** Comparison of serum **A)** IgA and **B)** IgM concentrations between cobalamin-deficient (COB deficient) and normocobalaminemic (normal COB) Shar Peis (median: [IgA: 1.7 g/L and 0.6 g/L; IgM: 0.9 g/L and 2.0 g/L, respectively; both $p < 0.0001$).



### 3.5 Discussion

The Shar Pei has been described as having a high prevalence of cobalamin (vitamin $B_{12}$) deficiency and clinical signs of cobalamin-deficient Shar Peis are suggestive of severe and longstanding gastrointestinal disease such as diarrhea, vomiting, and/or weight loss. The current study assessed serum concentrations of inflammatory markers, markers for chronic intestinal disease, and immunological markers in Shar Peis with and without cobalamin deficiency and if an inflammatory phenotype exists in Shar Peis with cobalamin deficiency.

Serum concentrations of the inflammatory markers CRP, the calgranulins (i.e., CP and S100A12), and HA, did not differ significantly between cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis. This finding might suggest that cobalamin deficiency in Shar Peis is not associated with other potential disease in Shar Peis (e.g., cutaneous mucinosis) because increased serum HA concentrations have been described in Shar Peis with cutaneous mucinosis. However, further studies are needed to determine serum cobalamin concentrations in Shar Peis with confirmed cutaneous mucinosis.

It is interesting to note that all three inflammatory markers (CRP, and the calgranulins [i.e., CP and S100A12]) were increased in both cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis. Serum CRP concentrations were increased above the upper limit of the reference interval in 29% and 31%, respectively, whereas 50% and 44% of the cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis, respectively, had serum CP concentrations above the suggested upper reference limit. In contrast, we found serum S100A12 concentrations to be more frequently increased

above the suggested reference limit in cobalamin-deficient Shar Peis (43%) compared to normocobalaminemic Shar Peis (24%), although this difference did not reach significance ($p > 0.05$; data not shown). The increase in inflammatory markers in this study in both cobalamin-deficient and normocobalaminemic Shar Peis would suggest that an inflammatory phenotype exists in both groups of Shar Peis and is not associated with cobalamin deficiency in this breed. The high proportion of dogs with a serum CP concentration above the reference interval in the groups of Shar Peis with and without cobalamin deficiency is interesting because increased serum CP concentrations have also been observed in dogs with chronic enteropathies (Heilmann et al., 2012). However, further studies to investigate serum CRP and calgranulins in dogs (e.g., Shar Peis) with chronic intestinal diseases are needed and are currently underway.

Hyperhyaluronic academia was not associated with cobalamin deficiency in Shar Peis in this study. It is possible that the increased HA concentrations in both Shar Peis with and without cobalamin deficiency reflect a high production of cell surface hyaluronan on mucosal endothelial cells as has been documented in human patients with IBD (Kessler et al., 2008). Although only a few control dogs were used in this study, serum HA concentrations in these dogs were comparable to historical controls (median: 244.12 μg/L) and dogs with hepatic diseases (median: 59.17 ng/ml), while Shar Peis with and without cobalamin deficiency had much higher serum HA concentrations similar to those reported by others (Zanna et al., 2008; Seki et al., 2008). Elevated serum HA concentrations together with increased concentrations of the calgranulins, and especially CP, in serum from both Shar Peis with and without cobalamin deficiency

might reflect compromised gastrointestinal health. However, further studies are needed to confirm this hypothesis.

Cobalamin-deficient Shar Peis had lower serum albumin concentrations and hypoalbuminemia was more frequently detected in cobalamin-deficient compared to normocobalaminemic Shar Peis. This result appears of significance given that hypo-cobalaminemia in dogs with chronic enteropathies has been shown to be associated with hypoalbuminemia (Allenspach et al., 2007; Heilmann et al., 2012). Hypoalbuminemia could also occur due to renal protein-loss (Cook & Cowgill, 1996), and chronic kidney disease is frequently associated with an increased serum creatinine concentration. Chronic kidney disease as a cause of hypoalbuminemia cannot be definitively ruled out but seems rather unlikely given that in 21 (95%) cobalamin-deficient Shar Peis the serum creatinine concentration was within the reference interval. In humans, hypoalbuminemia has also been associated with the inflammatory process or amyloidosis (Shin et al., 2013) and an increased concentration of protease inhibitors (e.g., $\alpha_1$PI) has been hypothesized to contribute to the pathogenesis of amyloidosis (Vaden, 2010). Along these lines, we have shown that hypoalbuminemia is associated with a decreased serum c$\alpha_1$PI concentration in cobalamin-deficient Yorkshire Terriers and hypothesized that serum c$\alpha_1$PI concentrations might have prognostic implications in dogs with chronic small intestinal disease (Grützner et al., 2013). However, in the present study, serum concentrations of c$\alpha_1$PI were not significantly different between cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis suggesting that cobalamin deficiency in Shar Peis is not associated with altered serum c$\alpha_1$PI

concentrations. Because albumin and $\alpha_1$PI are lost at the same rate, the altered availability of albumin and c$\alpha_1$PI in serum raises the question whether another source of c$\alpha_1$PI (such as inflammatory cells) might have an impact on the serum c$\alpha_1$PI concentration. Therefore, further studies are needed to investigate the relationship of serum albumin and serum/fecal c$\alpha_1$PI in Shar Peis and in dogs with chronic gastrointestinal disease and gastrointestinal protein loss.

Serum concentrations of creatinine were significantly lower, albeit numerically only slightly different, in cobalamin-deficient Shar Peis compared to normocobalaminemic Shar Peis. However, the median serum creatinine concentrations were within the reference interval in both groups. Increased serum creatinine concentrations have been observed in 1 (5 %) cobalamin-deficient Shar Peis and in 9 (20%) normocobalaminemic Shar Peis. A study that investigated renal amyloidosis in dogs showed that serum creatinine concentrations were 3-fold higher in Shar Peis with renal amyloidosis compared to non-Shar Peis with renal amyloidosis (Segev et al., 2012). Furthermore, this study revealed that hypoalbuminemia occurs more often in non-Shar Peis with renal amyloidosis than in Shar Peis with renal amyloidosis (Segev et al., 2012). Based on the results of the present study it might be speculated that cobalamin deficiency in Shar Peis is likely not associated with renal amyloidosis taking into account the findings by Segev et al. (2012). However, given the lack of urine samples and/or renal biopsies for analysis and further phenotypic characterization of the Shar Peis enrolled in this study, the results of the present study do not allow any definitive conclusions.

Amyloid deposition has been reported in Shar Peis and non-Shar Peis with renal amyloidosis (Segev et al., 2012). Because extra-renal amyloid depositions have been shown to more commonly occur in Shar Peis compared to non-Shar Peis with renal amyloidosis it is possible that amyloid deposition in the gastrointestinal tract, pancreas, and central nervous system (Segev et al., 2012) affects cobalamin metabolism and/or malabsorption of nutrients such as cobalamin and zinc. In line with this, serum zinc concentrations were also numerically lower in cobalamin-deficient Shar Peis compared to normocobalaminemic Shar Peis, although this difference was not significant. Thus, investigation of the effect of intramural amyloid and HA deposition within the gastrointestinal tract on malabsorption of certain nutrients in Shar Peis with chronic enteropathies warrants further research.

Cobalamin-deficient Shar Peis had higher serum IgA concentrations and, in contrast, lower serum IgM concentrations than in normocobalaminemic Shar Peis. The results of this study are in contrast to lower serum IgA concentrations in Shar Peis as reported by Rivas et al. (1995) and Moroff et al. (1986). This finding supports that IgA deficiency (Moroff et al., 1986) and the primary immunodeficiency syndrome in Shar Peis (Rivas et al., 1995) are not associated with cobalamin deficiency in Shar Peis. Interestingly, in human patients with plasma cell dyscrasias, a link between serum IgA and the pathogenesis of cobalamin deficiency has been suggested in that IgA may have an anti-intrinsic factor-like activity or be involved in other mechanisms that have an impact on normal cobalamin absorption (Baz et al., 2004). In dogs, high serum IgA concentrations have been observed in patients with inflammatory diseases such as meningitis-arteritis

(Schwartz et al., 2011). Although speculative, an increased serum IgA concentration may argue for an inflammatory phenotype in cobalamin-deficient Shar Peis. Along these lines, increased serum IgA and decreased IgM concentrations have been described in humans with type 2 diabetes mellitus when compared to healthy controls, and this finding was considered to be due to low-grade systemic inflammation (Cai et al., 2013). Similar trends for these two immunoglobulins, best reflected by the IgA-to-IgM ratio, were seen in cobalamin-deficient Shar Peis in this study. However, these results warrant further investigation of the gastrointestinal immunoglobulin secretion in Shar Peis with cobalamin deficiency.

In conclusion, Shar Peis have a high prevalence of cobalamin (vitamin $B_{12}$) deficiency and clinical signs are suggestive of severe and longstanding gastrointestinal disease such as diarrhea, vomiting, and/or weight loss. No difference was found in serum concentrations of CRP, the calgranulins (CP and S100A12), and HA, between Shar Peis with and without cobalamin deficiency. However, the results of this study suggest that an inflammatory phenotype exists in both Shar Peis with and without cobalamin deficiency. In contrast, cobalamin-deficient Shar Peis had higher serum IgA concentrations and lower serum IgM, albumin, and creatinine concentrations when compared to normocobalaminemic Shar Peis. These findings might suggest that cobalamin deficiency in Shar Peis is not associated with other highly prevalent diseases in Shar Peis (such as cutaneous mucinosis and Shar Pei Fever) although all three diseases in Shar Peis have been suspected to be hereditary in a dog breed classified as being rare. However, further studies are needed to determine serum cobalamin

concentrations in Shar Peis with confirmed cutaneous mucinosis and/or Shar Pei Fever and to investigate gastrointestinal immunoglobulin secretion in Shar Peis with cobalamin deficiency.

# 4. SERUM HOMOCYSTEINE AND METHYLMALONIC ACID CONCENTRATIONS IN CHINESE SHAR PEIS WITH COBALAMIN DEFICIENCY*

## 4.1 Overview

Cobalamin deficiency is suspected to be hereditary in Chinese Shar Peis (Shar Peis), and inherited causes of cobalamin deficiency may affect the cellular processing of cobalamin. In humans, a defect of the two main cobalamin-dependent intracellular enzymes (i.e., methionine synthase and methylmalonyl-CoA mutase) may lead to hyperhomocysteinemia and hypermethylmalonic acidemia. The aim of this retrospective study was to evaluate serum homocysteine (HCY) and methylmalonic acid (MMA) concentrations in cobalamin-deficient Shar-Peis and dogs of six other breeds. Serum samples (n = 297) from cobalamin-deficient dogs (Shar Peis, German Shepherd Dogs, Labrador Retrievers, Yorkshire Terriers, Boxers, Cocker Spaniels, and Beagles) were analyzed for serum HCY and MMA concentrations. A Fisher's exact test was used to evaluate if cobalamin deficiency in Shar Peis is associated with hyperhomocysteinemia.

Serum HCY and MMA concentrations were higher in cobalamin-deficient Shar Peis compared to cobalamin-deficient dogs of the six other breeds ($p < 0.0001$). Hyper-homocysteinemia was associated with cobalamin deficiency in Shar Peis ($p = 0.009$).

*Reprinted with permission from Grützner N, Heilmann RM, Stupka KC, Rangachari VR, Weber K, Holzenburg A, Suchodolski JS, Steiner JM. 2013. "Serum methylmalonic acid and homocysteine concentrations in Chinese Shar Peis with cobalamin deficiency" *Vet J* 197, 420-426, Copyright (2013) by Elsevier.

In addition, serum HCY and MMA concentrations did not differ between cobalamin-deficient German Shepherd Dogs with and without exocrine pancreatic insufficiency (EPI), a potential cause of secondary cobalamin deficiency. These findings suggest that the function of the two intracellular cobalamin-dependent enzymes is impaired in Shar Peis with cobalamin deficiency.

## 4.2 Introduction

A breed disposition for cobalamin deficiency has been described for the Chinese Shar Pei (Shar Pei) in North America and in the United Kingdom, and this condition is speculated to be hereditary (Bishop et al., 2011; Dandrieux et al., 2010). Inherited causes of cobalamin deficiency have been reported in humans and may affect the absorption, transport, or cellular processing of cobalamin.

In humans, various defects of the intracellular cobalamin metabolism have been reported and summarized by Froese and Gravel (2010). The net result of these defects is a deficient function of methionine synthase or methylmalonyl-CoA mutase, the two main cobalamin-dependent enzymes, which may lead to hyperhomocysteinemia and hypermethylmalonic acidemia, respectively. In humans, hyperhomocysteinemia can occur as a result of deficiencies of folic acid (vitamin $B_9$), pyridoxine (vitamin $B_6$), or cobalamin (vitamin $B_{12}$) (Iqbal et al., 2009; Acharya et al., 2008). It has also been shown in humans that increased serum homocysteine (HCY) concentrations are associated with cardiovascular, thrombotic, and neurodegenerative diseases (Refsum et al., 2004; Stanger et al., 2003). Rossi et al. (2008) measured serum HCY concentrations in dogs

with various diseases, such as heart disease, gastrointestinal disease, or renal failure. This study showed that dogs with gastrointestinal diseases have low serum HCY concentrations; however, to the authors' knowledge, serum HCY concentrations have not been reported in dogs with cobalamin deficiency.

Hypermethylmalonic acidemia has been described in humans and dogs (Bjørke Monsen and Ueland, 2003; Berghoff et al., 2012). Based on human and veterinary studies, an increased serum methylmalonic acid (MMA) concentration can occur due to cobalamin deficiency and has been suggested to reflect cobalamin deficiency at the cellular level (Stabler et al., 1986; Ruaux et al., 2009; Berghoff et al., 2012). In this context, it has been shown in humans that MMA concentrations are higher in patients with genetic disorders affecting intracellular processing than in patients with genetic defects affecting the gastrointestinal processing and the extracellular transport of cobalamin (Fowler et al., 2008).

The aim of this study was to evaluate serum HCY and MMA concentrations in Shar-Peis and dogs of other breeds with cobalamin deficiency. We hypothesized that serum HCY and/or MMA concentrations differ between cobalamin-deficient Shar Peis and cobalamin-deficient dogs of other breeds suggesting a defect of intracellular cobalamin metabolism in Shar Peis. This might provide further insight whether cobalamin deficiency in Shar Peis represents an inherited disorder.

## 4.3 Materials and methods

### Sample collection

For the purpose of this retrospective study, which covered a 3-year period (2008-2011), we reviewed information on canine serum samples in the database of the Gastrointestinal Laboratory, Texas A&M University. Serum samples (n = 297, belonging to Shar Peis and dogs of six other breeds) with an undetectable serum cobalamin concentration (<150 ng/L; i.e., below the minimum detection limit of the assay) were identified. Sex and age of the dogs from which the samples had been collected from were identified (Table 8). The serum samples had been submitted by the primary care veterinarian to the Gastrointestinal Laboratory for evaluation of gastrointestinal function; however, the clinical history and disease status of the dogs were not provided.

To ensure an adequate number of samples per group, the six non-Shar Pei dog breeds, namely, German Shepherd Dogs (GSDs), Labrador Retrievers (Labradors), Yorkshire Terriers, Boxers, Cocker Spaniels, and Beagles, were selected based on the popularity of the breed according to the American Kennel Club ranking list of 2009 (all among the first 23 breeds) and the estimated number of samples that had been submitted to the Gastrointestinal Laboratory over a 2-year period (2006–2008). A study in humans showed that the compounds measured for the purpose of this study, including cobalamin, HCY, MMA, and creatinine, are stable in serum samples for several years (Hustad et al., 2012). While this does not definitively prove that these stability data translate to dogs, it is reasonable to assume that they do. Only serum samples from dogs were included into

this study where a sufficient amount of serum (>500 μL) was available for the measurement of serum concentrations of HCY and MMA.

**HCY assay**

Serum HCY concentrations were measured in samples from the seven different dog breeds using a gas chromatography–mass spectrometry method (GC/MS) as described by Stabler et al. (1987) (Table 8). Due to the lack of a published reference interval for canine serum HCY concentrations, a reference interval was established from HCY concentrations in 35 healthy pet dogs using the robust method with a Box–Cox transformation (Geffré et al., 2011). This non-parametric robust method was chosen because the number of reference individuals in this study did not reach 120, which is generally accepted as a bar for a parametric calculation of the reference interval (Geffré et al., 2011).

**Table 8.** Breed, age (median, in years), and sex distribution of all dogs that were included in this study. The last column shows the number of dogs for which information about sex or age were not available.

| Breed | n | female/age | male/age | sex/age unknown |
|---|---|---|---|---|
| **Chinese Shar Pei** | 30 | 17 / 7.5 | 13 / 5.0 | -/1 |
| **German Shepherd Dog** | 95 | 50 / 4.8 | 45 / 4.0 | -/1 |
| **Labrador Retriever** | 76 | 39 / 6.5 | 36 / 7.0 | 1/2 |
| **Yorkshire Terrier** | 41 | 18 / 10.0 | 23 / 8.0 | -/1 |
| **Boxer** | 20 | 12 / 7.0 | 8 / 7.0 | -/1 |
| **Cocker Spaniel** | 20 | 9 / 10.0 | 10 / 11.0 | 1/1 |
| **Beagle** | 15 | 8 / 5.5 | 7 / 11.0 | -/- |

The healthy pet dogs had a median age (range) of 5.2 (1.3–13.5) years. The sex distribution of the healthy pet dogs was 17 males and 18 females. The healthy pet dog group included the following dog breeds: mixed-breeds (n = 10), Labradors (n = 4), GSDs (n = 4), Border Collies (n = 4), Boxers (n = 3), Yorkshire Terriers (n = 3), Dachshunds (n = 2), and one each of Pug, Beagle, Australian Heeler, Brittany Spaniel, and Miniature Schnauzer. The protocol for collection of serum samples from healthy dogs was reviewed and approved by the Clinical Research Review Committee at Texas A&M University (CRRC 2010-07).

In addition, left-over serum specimens from a previous association study of cobalamin deficiency in the Shar Pei (Grützner et al., 2010) were used to compare serum HCY concentrations between cobalamin-deficient Shar Peis (n = 10; median age [range]: 4.5 [1.5–11.0] years; sex: 5 females and 5 males) and normocobalaminemic Shar Peis (n = 28; median age [range]: 4.0 [2–12] years; sex: 15 females and 13 males). These serum samples had been collected from Shar Peis from various parts of the United States, and the owner of each dog completed a questionnaire, which included information about the signalment and the current health status of the dog.

**MMA assay**

Serum MMA concentrations (reference interval: 415–1193 nmol/L; Berghoff et al., 2012) were measured in samples from dogs belonging to one of the seven different dog breeds using a GC/MS method described by Ruaux et al. (2001) (Table 8).

**Creatinine assay**

Serum creatinine (CRE) concentrations (reference interval: 0.5–1.4 mg/dL) were measured using an automated clinical chemistry analyzer (Sirrus Clinical Chemistry Analyzer, Stanbio Laboratory) and were used to normalize serum HCY and MMA concentrations for evaluation of HCY/CRE and MMA/CRE ratios, respectively. In humans, both serum HCY and MMA concentrations are affected by chronic kidney disease like serum CRE concentrations (Rasmussen et al., 1990). Therefore, serum MMA and HCY concentrations were normalized to serum CRE concentrations for the purpose of this study to account for changes in renal excretion expected to be reflected by an increase in serum CRE concentration (Hyas et al., 2000; Rasmussen et al., 1990).

**Trypsin-like immunoreactivity (TLI) assay**

Low serum cobalamin concentrations are frequently detected in dogs with exocrine pancreatic insufficiency (EPI), making EPI an important cause of cobalamin deficiency in the dog (Simpson et al., 1989). If available, the concentration of canine trypsin-like immunoreactivity (cTLI) in serum, which is considered the gold standard test for the diagnosis of EPI (Batt, 1993), was used for identifying dogs with EPI. The reason was to compare serum HCY and MMA concentrations between cobalamin-deficient GSDs and Labradors with a serum cTLI concentration diagnostic for EPI (≤2.5 μg/L) and with a cTLI concentration within the reference interval (5.7–45.2 μg/L). The remaining five dog breeds were not investigated for a potential association with EPI due to the small

number of dogs (n ≤ 2 dogs per breed) with a serum cTLI concentration that was diagnostic for EPI.

**Data analysis**

To perform statistical analyses, a commercially available software package (GraphPad Prism5, GraphPad Software) was used. A Kruskal–Wallis test with a Dunn's post-test for non-parametric data was used to compare age, HCY, MMA, HCY/CRE, and MMA/CRE between the seven dog breeds. A Mann–Whitney U test for non-parametric data served to compare age, HCY, MMA, HCY/CRE, and MMA/ CRE between male and female dogs of each breed. A Fisher's exact test was used to evaluate the relation between cobalamin deficiency and hyperhomocysteinemia in Shar Peis. A Mann–Whitney U test served to compare serum HCY concentrations between cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis and to compare serum HCY, MMA, HCY/CRE, and MMA/CRE in cobalamin-deficient GSDs and Labradors with EPI and those without EPI. Significance for all tests was set at $p < 0.05$.

**4.4 Results**

There were no sex-specific differences ($p > 0.05$; Table 8) among Shar Peis, GSDs, Labradors, Yorkshire Terriers, Boxers, Cocker Spaniels, and Beagles with an undetectable serum cobalamin concentration. Ages were different among the seven breeds ($p < 0.0001$); the post-test showed that GSDs were younger than Yorkshire

Terriers or Cocker Spaniels ($p < 0.001$) and that Shar Peis were younger than Cocker Spaniels ($p < 0.05$).

Serum HCY concentrations were higher in cobalamin-deficient Shar Peis than in cobalamin-deficient dogs of the six other breeds, except for the Boxer ($p < 0.0001$; Figure 8; Table 9). GSDs, Labradors, and Boxers had higher serum HCY concentrations compared to both the group of Yorkshire Terriers and Cocker Spaniels ($p < 0.05$). For all seven breeds, serum HCY concentrations were not different between males and females. Serum HCY concentrations ranged from 5.2 to 25.9 μmol/L (median: 10.3 μmol/L) in healthy pet dogs and the reference interval for serum HCY was established as 5.0-22.1 μmol/L (Figure 9). Only Shar Peis had a median serum HCY concentration above the reference interval, with about two-thirds of the dogs showing hyperhomocysteinemia.

Serum HCY concentrations in cobalamin-deficient Shar Peis (median [range]: 25.4 [4.6-241.1] μmol/L; n = 40) were higher than in normocobalaminemic Shar Peis (median [range]: 13.7 [8.6-51.9] μmol/L; n = 28; $p < 0.05$; Figure 10), and cobalamin deficiency in Shar Peis was associated with hyperhomocysteinemia ($p < 0.001$). The majority of normocobalaminemic Shar Peis (83%) had a serum HCY concentration within the reference interval (Figure 10). More than half of the cobalamin-deficient Shar Peis were hyperhomocysteinemic, whereas only a small proportion (0-20%) of dogs of the remaining six breeds had a serum HCY concentration above the upper limit of the reference interval (Figure 8).

**Table 9.** Medians (ranges) for serum homocysteine (HCY; μmol/L), methylmalonic acid (MMA; nmol/L), and creatinine (CRE; mg/dL) concentrations and the HCY/CRE (μmol/g) and MMA/CRE ratios (μmol/g) for dogs included in this study.

| Breed | HCY | MMA | CRE | HCY / CRE | MMA / CRE |
|---|---|---|---|---|---|
| **Chinese Shar Pei** | 33.6 (4.6-241.1) | 29,145 (2,330-420,709) | 1.1 (0.6-1.6) | 34.9 (5.2-160.8) | 28,546 (1,664-280,473) |
| **German Shepherd Dog** | 10.0 (2.1-31.8) | 2,341 (465-31,322) | 1.0 (0.3-3.5) | 9.8 (1.9-86.2) | 2,614 (179-50,425) |
| **Labrador Retriever** | 10.4 (2.5-53.7) | 1,733 (500-21,076) | 1.1 (0.6-4.3) | 9.6 (2.3-171.9) | 1,737 (147-35,361) |
| **Yorkshire Terrier** | 3.8 (1.0-42.3) | 1,229 (528-11,252) | 0.6 (0.2-1.6) | 5.5 (2.4-26.4) | 2,028 (836-22,503) |
| **Boxer** | 12.0 (3.7-108.1) | 2,275 (530-7,217) | 0.9 (0.2-1.4) | 12.1 (5.3-108.1) | 2,243 (663-8,136) |
| **Cocker Spaniel** | 5.0 (2.0-20.2) | 1,073 (383-2,244) | 0.6 (0.3-1.3) | 8.0 (1.8-40.3) | 1,622 (519-6,698) |
| **Beagle** | 8.4 (2.5-27.0) | 1,115 (329-14,279) | 0.6 (0.3-0.8) | 15.0 (5.0-64.0) | 1,891 (657-28,559) |

**Figure 8.** Serum homocysteine (HCY) concentrations (left) and HCY/CRE ratios (right) were higher in cobalamin-deficient Shar Peis than in cobalamin-deficient dogs of 6 other breeds ($p < 0.0001$; y-axis in a log 2 scale). The solid black lines indicate the median for each breed. The area between the two dotted lines represents the reference interval (5.0−22.1 µmol/L).

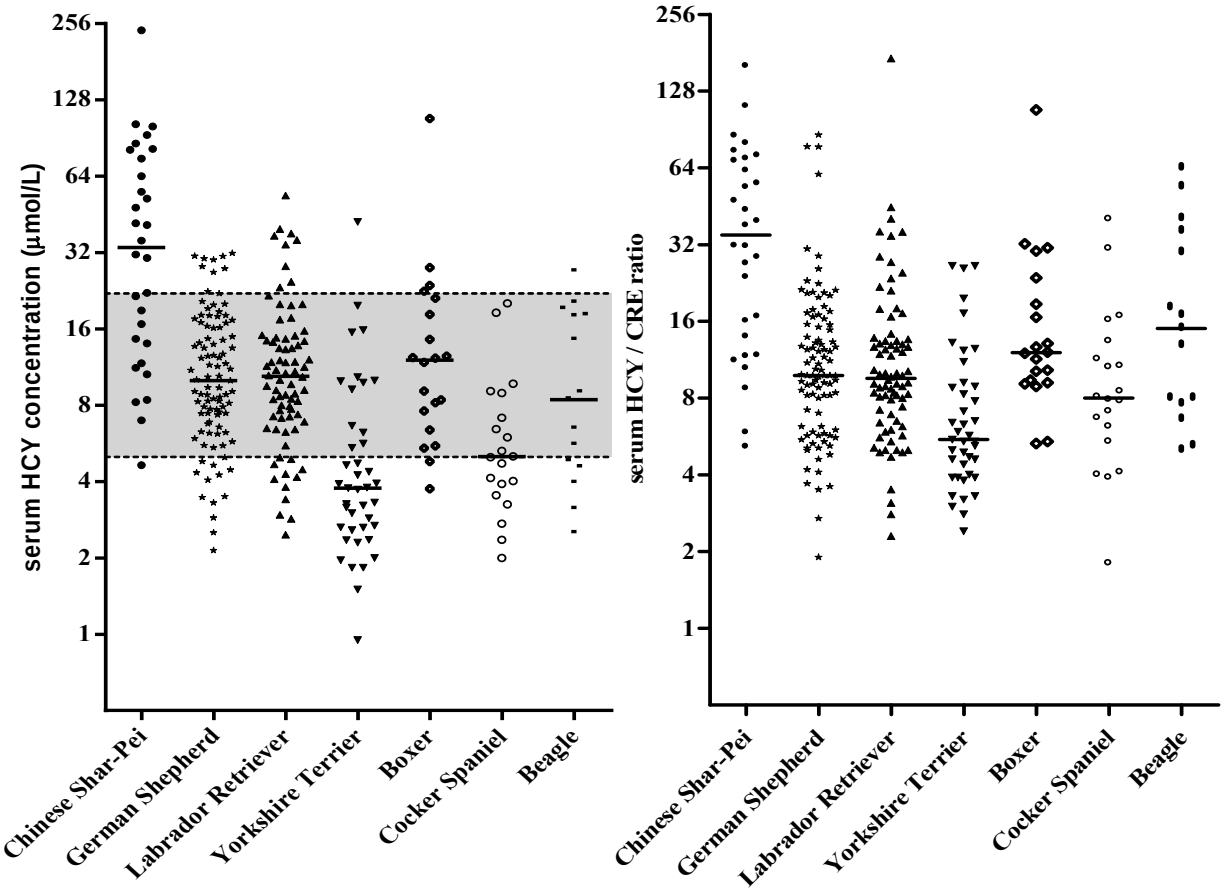


**Figure 9.** Serum homocysteine (HCY) concentrations ranged from 5.2-25.9 µmol/L (median: 10.3 µmol/L; solid line) in 35 healthy pet dogs; the reference interval for serum HCY concentration was calculated as 5.0-22.1 µmol/L (area between the two dotted lines).

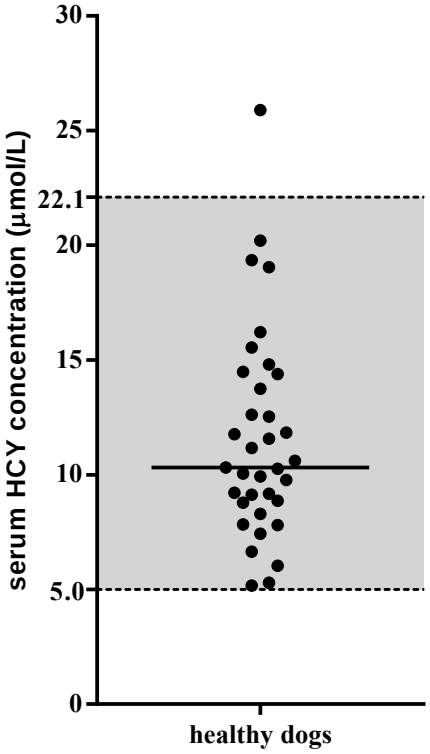


**Table 10.** Medians (ranges) of serum homocysteine (HCY; µmol/L) and methylmalonic acid (MMA; nmol/L) concentrations and the HCY/CRE (µmol/g) and MMA/CRE ratios (µmol/g) for German Shepherd Dogs and Labrador Retrievers with a cTLI concentration that is considered diagnostic for EPI (≤2.5 µg/L) or a cTLI concentration within the reference interval (RI: 5.7–45.2 µg/L).

| Breeds | HCY | MMA | HCY / CRE | MMA / CRE | *p* value |
|---|---|---|---|---|---|
| **German Shepherd Dog** | | | | | **>0.05** |
| cTLI ≤ 2.5 µg/L | 12.4 (2.9-0.3) | 1,711 (559-24,382) | 12.8 (4.8-30.8) | 1,988 (430-30,478) | |
| cTLI in RI | 9.5 (3.3-31.8) | 2,533 (465-31,322) | 9.8 (1.9-86.2) | 2,789 (179-50,424) | |
| **Labrador Retriever** | | | | | **>0.05** |
| cTLI ≤ 2.5 µg/L | 12.1 (2.5-38.0) | 1,674 (500-3,152) | 11.7 (6.2-40.4) | 1,907 (786-6,624) | |
| cTLI in RI | 10.5 (3.8-53.7) | 1,944 (514-21,076) | 9.1 (2.3-44.8) | 1,739 (147-17,563) | |

Serum MMA concentrations were higher (~10 times) in cobalamin-deficient Shar Peis when compared to the other investigated breeds ($p < 0.0001$; Figure 11; Table 9). All cobalamin-deficient Shar Peis, but only a proportion (33-88%) of cobalamin-deficient dogs of the other six breeds had a serum MMA concentration above the upper limit of the reference interval. In addition, MMA concentrations in serum samples from both Yorkshire Terriers and Cocker Spaniels were lower when compared to GSDs ($p < 0.01$). Serum MMA concentrations showed sex-specific differences in both Beagles (medians: males 3714 nmol/L, females 881 nmol/L; $p = 0.02$) and Boxers (medians: males 3041 nmol/L, females 1688 nmol/L; $p = 0.02$), but not for the remaining five breeds.

Serum CRE concentrations (median and ranges) from all seven breeds are summarized in Table 9. HCY/CRE ratios were higher in cobalamin-deficient Shar Peis than in cobalamin-deficient dogs of the six other breeds, except for the Boxer and the Beagle ($p < 0.0001$; Figure 8 and Table 9). In addition, GSDs, Labradors, Boxers, and Beagles had higher serum HCY/CRE ratios than Yorkshire Terriers ($p < 0.05$).

MMA/CRE ratios were substantially higher in cobalamin-deficient Shar Peis when compared to the other investigated breeds ($p < 0.0001$; Figure 11 and Table 9), which was similar to serum MMA concentrations not adjusted for serum CRE concentrations. In addition, MMA/CRE ratios in serum samples from GSDs were higher than those of Labradors ($p < 0.05$).

Serum HCY and MMA concentrations as well as HCY/CRE and MMA/CRE ratios did not differ between GSDs with a cTLI concentration diagnostic for EPI (n = 24) and GSDs with a cTLI concentration within the reference interval (n = 54; $p > 0.05$; Table 10). No differences were observed for Labradors with a cTLI concentration diagnostic for EPI (n = 10) and Labradors with a cTLI concentration within the reference interval when comparing serum HCY and MMA concentrations, as well as HCY/CRE and MMA/CRE ratios (n = 53; $p > 0.05$; Table 10). Serum MMA concentrations in cobalamin-deficient GSDs and Labradors with low and normal cTLI concentrations were above the reference interval in more than 80% and more than 70% of dogs, respectively.

**Figure 10.** Serum homocysteine (HCY) concentrations in 40 cobalamin-deficient Shar Peis (COB deficient; median: 25.4 μmol/L, solid line) and 28 normocobalaminemic Shar Peis (normal COB; median: 13.7 μmol/L, solid line) were significantly different ($p < 0.05$).

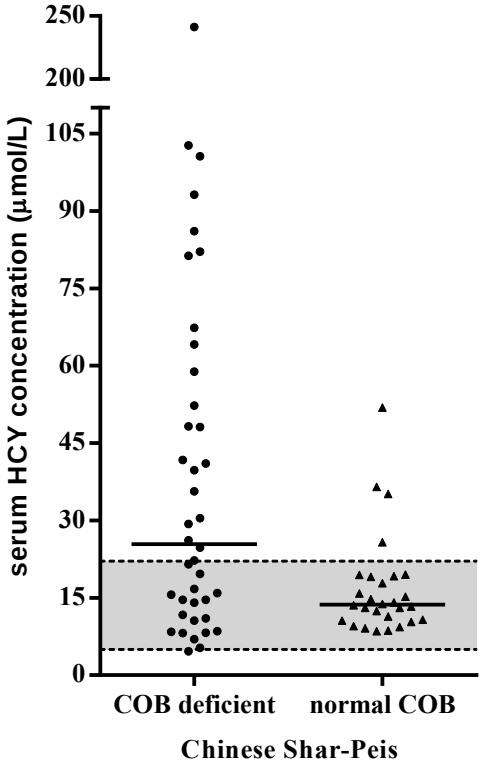


**Figure 11.** Serum methylmalonic acid (MMA) concentrations (left) and MMA/CRE ratios (right) were higher in cobalamin-deficient Shar Peis than in cobalamin-deficient dogs of 6 other breeds ($p < 0.0001$; y-axis on a log 10 scale). Solid lines indicate the median for each breed. The area between the two dotted lines represents the reference interval (415-1,193 nmol/L).

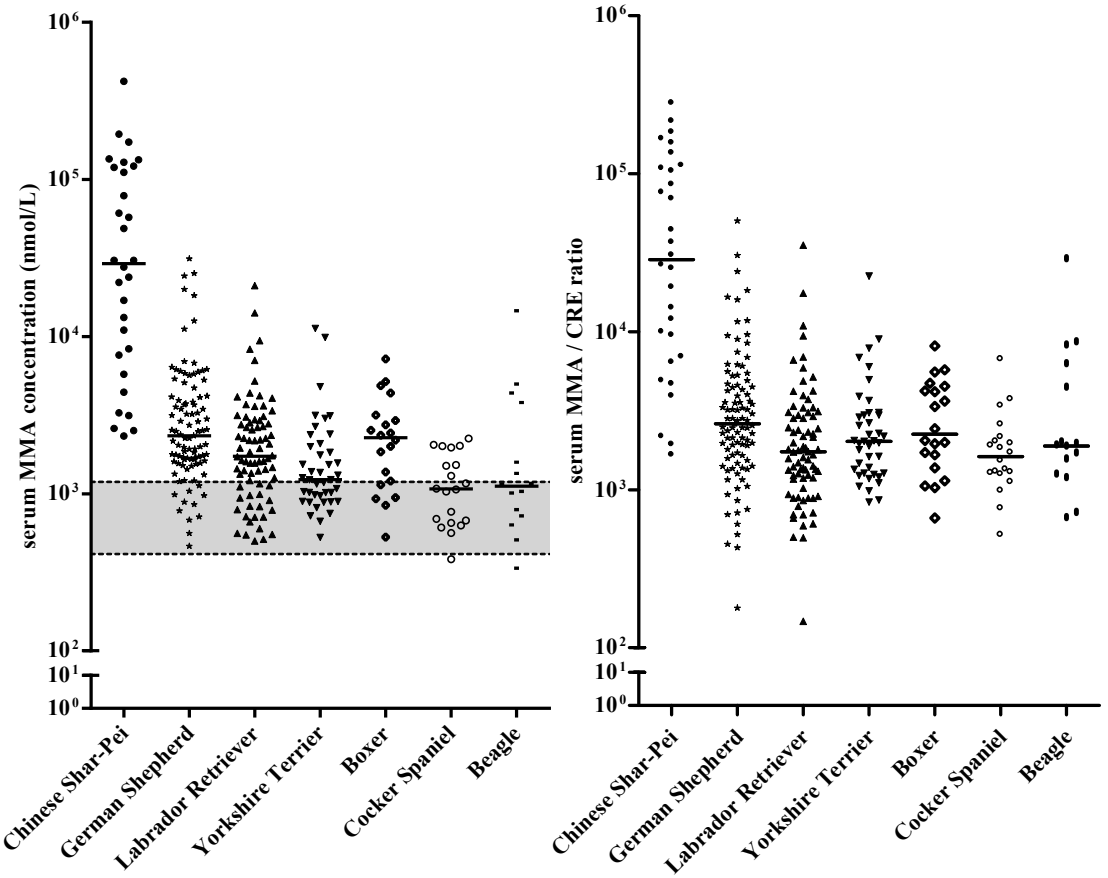
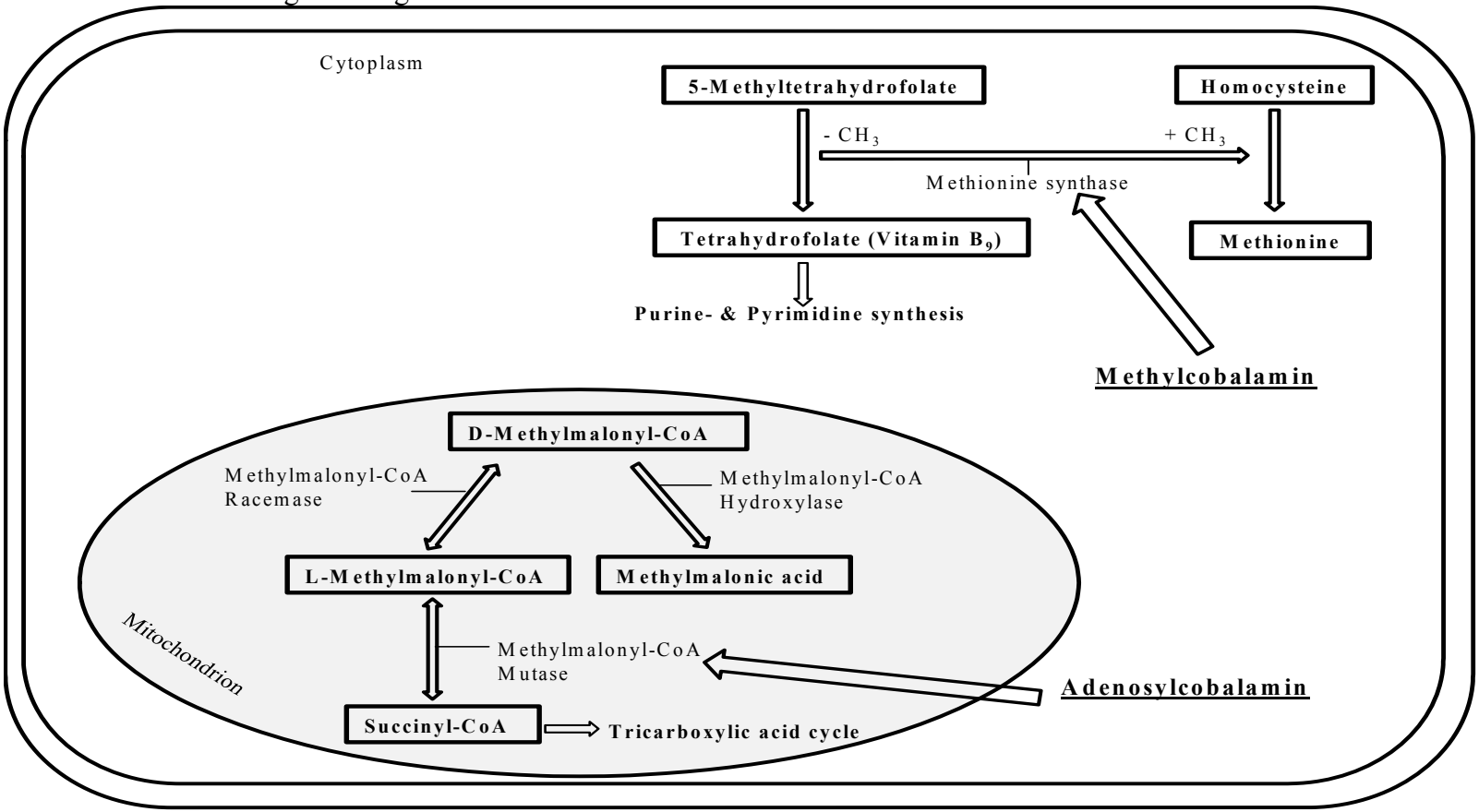


**Figure 12.** Function of the two main intracellular cobalamin-dependent enzymes, methionine synthase and methylmalonyl-CoA mutase. (1) The methionine synthase reaction, which is required for the conversion of homocysteine to methionine (Fenton et al. 1989), occurs in the cytoplasm and requires methylcobalamin. For this active form of cobalamin to be available, cobalamin has to undergo a methylation to methylcobalamin. (2) The reaction catalyzed by methylmalonyl-CoA mutase takes place in the mitochondrion and requires adenosylcobalamin. Within the mitochondrion, a 5'-deoxyadenosyl group is transferred to cobalamin generating this active form of cobalamin.



## 4.5 Discussion

In this retrospective study, 297 previously archived serum samples from Shar Peis, GSDs, Labradors, Yorkshire Terriers, Boxers, Cocker Spaniels, and Beagles with an undetectable serum cobalamin concentration were used to evaluate differences of serum HCY and MMA concentrations in cobalamin-deficient dogs between these breeds. In general, cobalamin-deficient dog breeds were found to have a high frequency of hypermethylmalonic acidemia, and especially Shar Peis showed a high frequency of hyperhomocysteinemia. Both conditions may be the net results of the malfunction of the two main intracellular cobalamin-dependent enzymes, methionine synthase in the cytosol and mitochondrial methylmalonyl-CoA mutase. The availability of cobalamin as a cofactor is essential for the reactions catalyzed by these two enzymes and depends on cobalamin in complex with transcobalamin entering the cell via receptor-mediated endocytosis, followed by its release from this complex and, in the case of methylmalonyl-CoA mutase subsequent transport into the mitochondrion (Figure 12).

In dogs, the measurement of serum cobalamin concentrations is routinely used to diagnose cobalamin deficiency, and archives of laboratories that offer this test can be helpful to collect left-over serum samples from a variety of dog breeds with a certain condition, such as cobalamin deficiency. The present study showed that serum HCY concentrations were higher in cobalamin-deficient Shar Peis than in cobalamin-deficient dogs from other breeds. Considering the established reference interval for healthy pet dogs, only Shar Peis had a median serum HCY concentration above the reference interval. Although the HCY/CRE ratios showed differences between the seven

cobalamin-deficient dog breeds compared to the serum HCY concentrations, the overall picture of the comparison did not change. Taking both serum HCY concentrations and HCY/CRE ratios into account, the results of this study showed that cobalamin-deficient Shar Peis have higher HCY values than cobalamin-deficient dogs of the six other breeds studied. This suggests that adjusting serum HCY concentrations for serum creatinine concentrations would not have had a considerable impact on the results of this study.

The comparison of serum HCY concentrations between cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis indicated that cobalamin deficiency in Shar Peis is associated with hyperhomocysteinemia. The normocobalaminemic Shar Peis had serum HCY concentrations within the reference interval, which suggests that not all Shar Peis are equally affected, if at all, by a putative genetic cause of cobalamin deficiency. The same was demonstrated by Bishop et al. (2011) for serum MMA concentrations in Shar Peis with and without cobalamin deficiency. In addition, our study (data not shown) and Bishop et al. (2011) described that Shar Peis with and without cobalamin deficiency had no differences in serum vitamin B9 concentrations, which suggests that hyperhomocysteinemia in cobalamin-deficient Shar Peis results mainly from the lack of cobalamin. However, to rule out completely that only the lack of cobalamin is responsible for hyperhomocysteinemia in cobalamin-deficient Shar Peis, the impact of vitamin B6 should be investigated, because hyperhomocysteinemia can also be due to vitamin B6 deficiency (Iqbal et al., 2009).

Interestingly, serum MMA concentrations were approximately 10 times higher in cobalamin-deficient Shar Peis than in cobalamin-deficient dogs of the six other breeds.

The MMA/CRE ratios showed a similar pattern compared to the serum MMA results. Comparing the HCY and MMA results of this study, it seems that serum MMA concentrations in cobalamin-deficient Shar Peis compared to the cobalamin-deficient dogs of the six other breeds have a bigger difference compared to serum HCY concentrations. We hypothesize that this bigger difference between hypocobalaminemic Shar Peis and cobalamin-deficient dogs of the six other breeds for serum MMA concentrations is due to the fact that methylmalonyl-CoA mutase is only dependent on cobalamin, while methionine synthase is cobalamin, vitamin B9, and vitamin B6 dependent. Comparing the non-normalized serum HCY and MMA concentrations, the HCY/CRE and MMA/CRE ratios showed differences between cobalamin-deficient dogs of the six other breeds, which may indicate that serum creatinine concentration should be considered when evaluating serum MMA and HCY concentrations in dogs.

The results of this study showed that cobalamin-deficient Shar Peis have higher serum HCY and MMA concentrations than cobalamin-deficient dogs of six other breeds. Thus, the current study provides further evidence that cobalamin deficiency in Shar Peis is considerably different compared to cobalamin-deficient dogs of other breeds. In addition, the current results help to further pinpoint the intracellular compartment and the pathways affected by the lack of their cofactor and may ultimately facilitate the detection of the defect(s) causing and/or resulting in cobalamin deficiency in Shar Peis.

It appears that the two main intracellular cobalamin-dependent enzymes (methionine synthase and methylmalonyl-CoA mutase) do not have a sufficient amount of cobalamin available for both enzymatic reactions. However, it is possible that the defect occurs in

the mitochondrial pathway, because the serum MMA concentrations in cobalamin-deficient Shar Peis compared to the cobalamin-deficient dogs from other breeds showed more severe alterations than the serum HCY concentrations. Consistent with higher serum MMA concentrations in humans with genetic disorders affecting the intracellular processing of cobalamin (Fowler et al., 2008), this leads us to speculate that a defect may be located intracellularly and may affect the mitochondrial pathway. However, further studies are needed to investigate the intracellular processing of cobalamin in Shar Peis with cobalamin deficiency.

Serum HCY concentrations have been measured previously in dogs by use of an enzymatic method (Rossi et al., 2008). In that study, dogs with gastrointestinal disorders (i.e., pyloric stenosis, intestinal obstruction, or gastric stenosis) had decreased serum HCY concentrations, whereas the highest serum HCY concentrations were reported in dogs with heart or kidney disease. Interestingly, Galler et al. (2011) showed that serum cobalamin concentrations were not different between dogs with and without chronic kidney disease, whereas no serum HCY and MMA concentrations were investigated. Therefore, further studies are needed to investigate serum HCY and MMA concentrations in dogs with gastrointestinal diseases and concurrent disease of the heart and/or kidneys.

Variations of serum HCY and MMA concentrations have been observed between breeds (e.g., GSDs and Cocker Spaniels). These differences may be due to the minimum detection limit of the cobalamin assay (i.e. 150 ng/L), such that dogs categorized as cobalamin-deficient in the current study could have different serum cobalamin

concentrations. However, to address this possibility, an assay more sensitive than the automated chemiluminescence assay (which is routinely used in North America and in Europe) would be needed. Furthermore, cobalamin-deficient Shar Peis had a median serum HCY concentration above the upper limit of the reference interval, whereas the remaining six breeds evaluated (except the cobalamin-deficient Yorkshire Terriers) had median serum HCY concentrations within the reference interval. Interestingly, cobalamin-deficient Yorkshire Terriers had a median HCY concentration below the reference interval, which indicates that the cytoplasmic cobalamin pathway is less affected in cobalamin-deficient Yorkshire Terriers compared to the six other dog breeds. In this present study, five dog breeds (except for the Cocker Spaniel and the Beagle) with undetectable serum cobalamin concentrations had a median serum MMA concentration above the upper limit of the reference interval, which suggests a lack of cobalamin at the level of the mitochondrial pathway (Stabler et al., 1986; Ruaux et al., 2009).

We only identified sex-differences for serum MMA concentrations in Beagles and Boxers. Male cobalamin-deficient Beagles were older (median, 11 years) than their female counterparts (median, 5.5 years), but this difference was not significant. To investigate whether male Beagles might have an impaired absorption of cobalamin later in life, it would be necessary to compare a greater number of cobalamin-deficient Beagle dogs. As a breed, the Boxer has been suggested to have a higher risk of developing inflammatory bowel disease (IBD), which can be accompanied by low serum cobalamin concentrations, but no sex differences were reported in that particular study (Kathrani et

al., 2011). Further investigation regarding serum cobalamin and MMA as well as HCY concentrations are warranted in a larger group of cobalamin-deficient Beagles and Boxers, since in humans some studies have shown age and sex differences in biomarkers related to cobalamin (Carmel et al., 2012).

EPI (Batt et al., 1993) and IBD (Kathrani et al., 2011) in GSDs have been shown to be associated with low serum cobalamin concentrations. Intrinsic factor, which is almost exclusively secreted in pancreatic juice, plays an important role in the intestinal absorption of cobalamin in the dog (Batt et al., 1993) and cobalamin absorption is often dramatically decreased in dogs with EPI. Interestingly the comparison of serum HCY and MMA concentrations in both cobalamin-deficient GSDs and Labradors, with and without cTLI concentrations diagnostic for EPI, showed no significant difference. Nevertheless, the majority of cobalamin-deficient GSDs and Labradors with low or normal cTLI concentrations had a serum MMA concentration above the reference interval. In contrast, only a small proportion of both cobalamin-deficient GSDs and Labradors with low or normal cTLI concentrations had a serum HCY concentration above the upper limit of the reference interval. These results suggest that cobalamin-deficient GSDs and Labradors with EPI or other gastrointestinal diseases such as IBD have a similar lack of cobalamin at the cellular level. However, further investigations are needed to confirm these findings.

In conclusion, cobalamin-deficient Shar Peis have higher serum HCY concentrations compared to cobalamin-deficient dogs from six other breeds, and also have a higher frequency of hyperhomocysteinemia than normocobalaminemic Shar Peis. Cobalamin-

deficient Shar Peis had 10-fold higher median serum MMA concentration compared to cobalamin-deficient dogs from other dog breeds. In addition, serum HCY and MMA concentrations did not differ between cobalamin-deficient GSDs with and without EPI, a potential cause of secondary cobalamin deficiency. These findings suggest that the function of the two main cobalamin-dependent enzymes (i.e., methionine synthase and methylmalonyl-CoA mutase) is impaired in cobalamin-deficient Shar Peis.

# 5. ASSOCIATION OF SKIN PHENOTYPE AND COBALAMIN DEFICIENCY IN CHINESE SHAR PEIS

## 5.1 Overview

Three conditions have frequently been reported in Chinese Shar Peis (Shar Peis): Shar Pei fever, cutaneous mucinosis, and cobalamin deficiency, all of which are suspected to be hereditary. Recently, two genome wide association studies (GWAS) have been conducted for these conditions in Shar Peis and all three conditions have been linked to canine chromosome 13 in an area that is also associated with skin thickness. Therefore, a survey was conducted to evaluate if cobalamin deficiency predominates in one of these two types of Shar Peis (i.e., traditional type versus meatmouth type). Normo-cobalaminemic Shar Peis with normal serum methylmalonic acid (MMA) concentrations (Shar Peis considered to have a normal cobalamin status) and cobalamin-deficient Shar Peis were surveyed using a standardized questionnaire showing illustrations of meatmouth and traditional type Shar Peis to ensure consistent responses from the owners of Shar Peis enrolled. To test for an association between cobalamin deficiency and the type of Shar Pei (i.e., meatmouth versus traditional type), a Fisher's exact test was used, and the odds ratio (OR) and the 95% confidence interval (CI) were calculated. A $p < 0.05$ was considered significant. Responses to the survey were obtained for 16 cobalamin-deficient Shar Peis and 33 Shar Peis with a normal cobalamin status. Cobalamin-deficient Shar Peis were 20 times (95%CI: 3.5-113.3; $p = 0.0002$) more likely to belong to the traditional type than the meatmouth type Shar Pei. Cobalamin

deficiency in Shar Peis was found to occur more frequently in the traditional type (i.e., the original Shar Pei, which originated from China) than in the meatmouth type of this breed. However, overlaps between both types existed.

## 5.2 Introduction

Three conditions have frequently been reported in Chinese Shar Peis (Shar Peis): Shar Pei fever, cutaneous mucinosis, and cobalamin deficiency, all of which are suspected to be hereditary. Two genome wide association studies (GWAS) for these conditions have recently been conducted in the Shar Pei:

The first GWAS was performed in Shar Peis with Shar Pei fever and cutaneous mucinosis and revealed that both conditions are linked to the hyaluronic acid synthase 2 (*HAS2*) gene on canine chromosome 13 (Olsson et al., 2011). This gene encodes hyaluronan, the main component of mucin, which accumulates in the thickened skin of affected Shar Peis. A high copy number of a 16.1 kb duplication close to the *HAS2* gene was found to be associated with the thickened skin in meatmouth type Shar Peis, whereas a high copy number of a 14.3 kb duplication close to the *HAS2* gene was found to be associated with the traditional type Shar Pei (Figure 13; Olsson et al., 2011).

The second GWAS showed that cobalamin deficiency in Shar Peis is also linked to the area of the *HAS2* gene on canine chromosome 13 (Figure 14; Grützner et al., 2010). *HAS2* has been reported to be located on canine chromosome 13 in the region of location 23,348,773-23,364,912 bp, with a distance of approximately 0.42 Mb and 0.47 Mb to the

canine single nucleotide polymorphisms (cSNP) and cMSS-2 marker that were associated with cobalamin deficiency in this breed in previous studies, respectively.

Both human and veterinary studies have suggested that an increased serum methylmalonic acid (MMA) concentration reflects cobalamin deficiency at the cellular level (Stabler et al., 1986; Ruaux et al., 2009; Berghoff et al., 2012). A combination of the measurement of serum cobalamin and serum MMA concentration might therefore be considered to be stronger evidence of cobalamin deficiency at the cellular level than a decreased serum cobalamin concentration alone. Thus, a phenotypic re-classification based on serum cobalamin and MMA concentrations may lead to identification of a stronger phenotype in Shar Peis with cobalamin deficiency.

Therefore, the aim of this study was to conduct a survey to evaluate 1) if cobalamin deficiency, based on undetectable serum cobalamin concentrations, and 2) if cobalamin deficiency based on undetectable serum cobalamin and increased MMA concentrations, predominates in one of these two types of Shar Peis (i.e., traditional type versus meatmouth type).

**Figure 13.** Meatmouth and traditional type Shar Peis. The picture (Olsson et al., 2011) shows the thickened skin of various degrees of the meatmouth type Shar Pei (**A, B, and C**) as opposed to the traditional type Shar Pei (**D**).

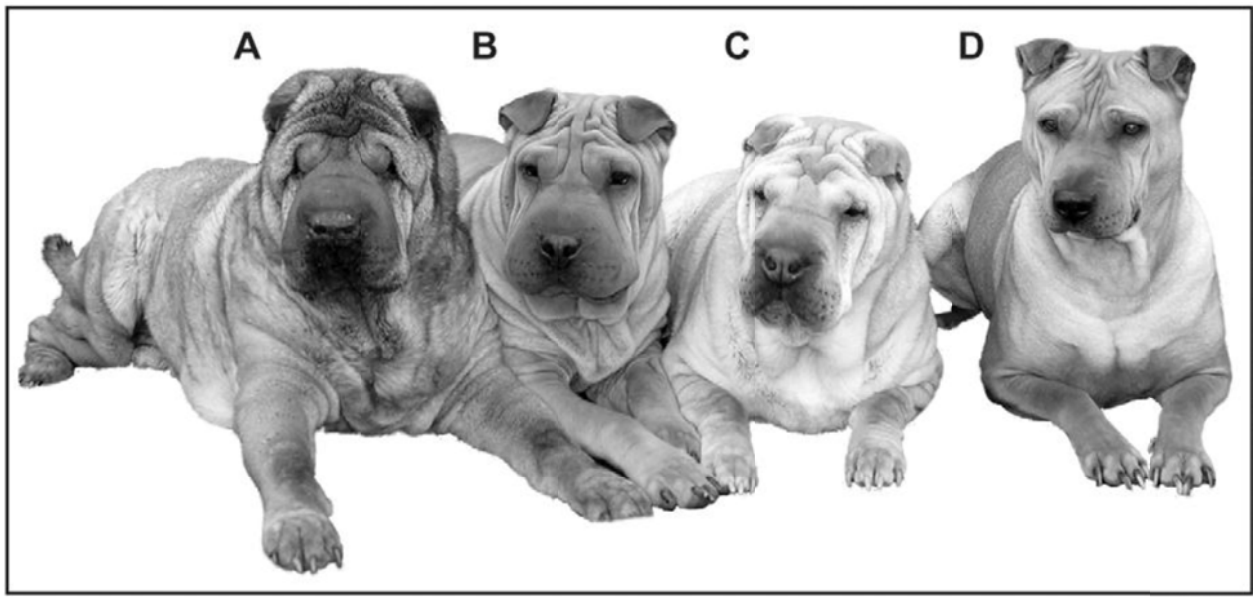
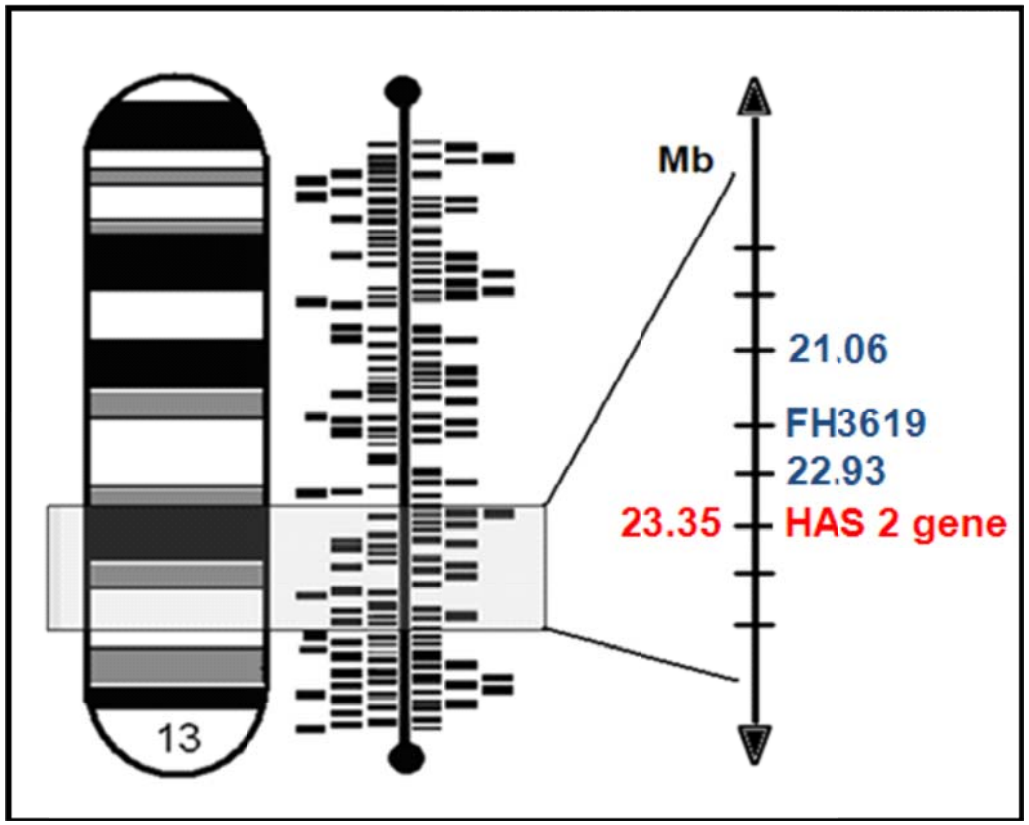


**Figure 14.** Ideogram of canine chromosome 13. The microsatellite marker FH3619, the two cSNP array markers, and the *HAS2* gene are shown at their respective locations on canine chromosome 13. Note that the *HAS2* gene is located in close proximity to the microsatellite and cSNP markers associated with cobalamin deficiency.



## 5.3 Materials and methods

### Sample population

Unrelated pure-bred Shar Peis, previously enrolled in the GWAS, were surveyed using a standardized questionnaire. Both Shar Peis with a normal cobalamin status (both serum cobalamin and MMA concentrations within the reference intervals; cobalamin: 251-908 ng/L; Gastrointestinal Laboratory at Texas A&M University; http://vetmed.tamu.edu /gilab/service/assays/b12folate; accessed December 19, 2011); MMA: 415-1,193 nmol/L (Berghoff et al., 2011) and cobalamin-deficient Shar Peis (cobalamin <150 ng/L, the detection limit of the assay and MMA >1,193 nmol/L) were enrolled.

### Survey analysis

The questionnaire included illustrations of the meatmouth and traditional type Shar Pei (Figure 13) to ensure consistent responses from the Shar Pei owners. Associations between cobalamin-deficiency based on 1) undetectable serum cobalamin concentrations and 2) undetectable serum cobalamin and increased serum MMA concentration) and the type of Shar Pei (i.e., meatmouth vs. traditional type) were tested. However, it was not possible to measure serum MMA concentrations for all Shar Peis so that less numbers of dogs were used for second analysis (Table 11). Fisher's exact test was used and the odds ratio (OR) and the 95% confidence interval (CI) were calculated and a $p < 0.05$ was considered significant.

**Table 11.** Number of dogs, sex distribution, and age (median, in years) of the cobalamin-deficient Shar Pei (A) and the normocobalaminemic Shar Pei (B) that were included in the two parts of this study. The last two columns show the medians (ranges) for serum cobalamin (COB; ng/L) and methylmalonic acid (MMA; nmol/L) concentrations for included Shar Pei dogs.

| | n | female/age | male/age | COB (ng/L) | MMA (µmol/L) |
|---|---|---|---|---|---|
| **Part 1** | | | | | |
| **A** | 20 | 7 / 3.5 | 13 / 5.0 | <150 | N/A |
| **B** | 41 | 24 / 4.8 | 17 / 3.0 | 529 (251-908) | N/A |
| **Part 2** | | | | | |
| **A** | 16 | 7 / 3.5 | 9 / 5.0 | <150 | 14,742 (1,533-262,969) |
| **B** | 33 | 21 / 4.0 | 12 / 2.5 | 529 (251-908) | 777 (372-1,177) |

## 5.4 Results

**Part 1** – Responses to the survey were obtained for 20 cobalamin-deficient Shar Peis and 41 normocobalaminemic Shar Peis. Cobalamin-deficient Shar Peis were 19 times (95%CI: 4.4-83.2; $p < 0.0001$) more likely to be of the traditional type than the meatmouth type (Table 11, Figure 15).

**Part 2** – Responses to the survey were obtained for 16 cobalamin-deficient Shar Peis with increased serum MMA concentrations and 33 normocobalaminemic Shar Peis with a normal serum MMA concentration. Cobalamin-deficient Shar Peis with an increased serum MMA concentration were 20 times (95%CI: 3.5-113.3; $p = 0.0002$) more likely to belong to the traditional type than the meatmouth type (Table 11, Figure 16).

**Figure 15.** This bar graph shows the proportions of cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis that are of either the traditional or the meatmouth type. The traditional type Shar Peis was more frequently observed in cobalamin-deficient (COB deficient) Shar Peis (60.0 %) compared to normocobalaminemic (normal COB) Shar Peis (7.3 %; $p < 0.0001$).

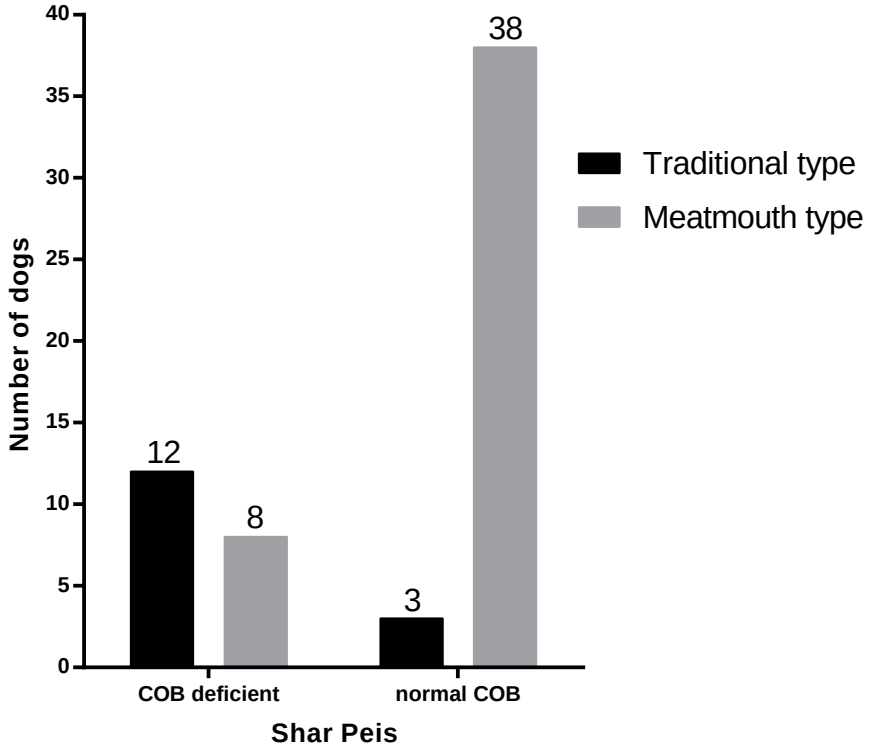
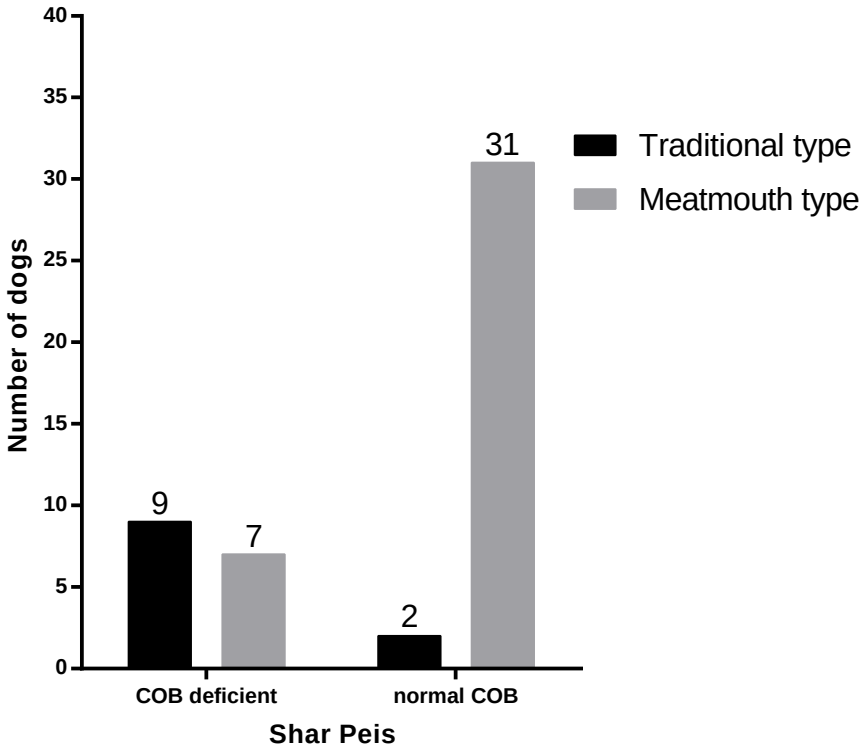


**Figure 16.** Bar graph showing the distribution of cobalamin-deficient Shar Peis with increased serum MMA concentrations and normocobalaminemic Shar Peis with normal serum MMA concentrations regarding the traditional versus meatmouth type. Traditional type Shar Peis were more frequently observed in cobalamin-deficient (COB deficient) Shar Peis with increased serum MMA concentrations (56.3 %) compared to normocobalaminemic (normal COB) Shar Peis with normal serum MMA concentrations (6.1 %; $p = 0.0002$).



## 5.5 Discussion

In this study we evaluated Shar Peis with an undetectable serum cobalamin concentrations and normocobalaminemic Shar Peis to determine if any specific type of Shar Pei (i.e., traditional type versus meatmouth type) predominates in regards to cobalamin deficiency. Cobalamin deficiency in Shar Peis was found to occur more frequently in the traditional type than in the meatmouth type of this breed. However, there were overlaps for both types.

Three conditions have frequently been reported in Shar Peis (i.e., Shar Pei fever, cutaneous mucinosis, and cobalamin deficiency), all of which are suspected to be hereditary. GWAS for these conditions have shown linkage to the *HAS2* gene on canine chromosome 13. A high copy number of a 16.1 kb duplication close proximity to the *HAS2* gene was found to be associated with the thickened skin in meatmouth type Shar Peis, whereas a high copy number of a 14.3 kb duplication close proximity to the *HAS2* gene was found to be associated with the traditional type Shar Pei (Figure 13; Olsson et al., 2011). Based on the results of the present study it would be interesting to determine whether cobalamin-deficient Shar Peis have a low copy number of a 16.1 kb duplication and a high copy number of a 14.3 kb duplication close proximity to the *HAS2* gene (Olsson et al. (2011).

A small number of Shar Peis have been introduced to North America, which reflects a classical example for a bottleneck phenomenon. Due to the breeding of the Shar Pei in North America, which was potentially aimed at increasing the wrinkles, resulted not only in a dramatically different look for the Shar Pei (as its most characteristic features,

including its wrinkles and rounded snout, were greatly exaggerated), but also in a large number of health problems. Therefore, it is possible that the three aforementioned conditions in Shar Peis (i.e., Shar Pei fever, cutaneous mucinosis, and cobalamin deficiency) have arisen due to the same limited source of genetic material and a differentiation could potentially be made based on the copy numbers of a 16.1 kb duplication and a 14.3 kb duplication close to the *HAS2* gene (Olsson et al., 2011). However, the present study showed an overlap between both types (traditional and meatmouth type), which could be explained by the objective description of the Shar Pei by the dog owners based on the pictures provided (Figure 13).

Several studies have suggested that an increased serum MMA concentration reflects cobalamin deficiency at the cellular level and a combination of the measurement of serum cobalamin and serum MMA concentrations might therefore be considered to be stronger evidence for cobalamin deficiency at the cellular level than a decreased serum cobalamin concentration alone (Stabler et al., 1986; Ruaux et al., 2009; Berghoff et al., 2012). Based on our study, both phenotypes (cobalamin deficiency with [part 2] and without [part 1] serum MMA concentrations) showed a similar occurrence of cobalamin deficiency in the traditional type of Shar Pei. However, one limitation of this study was that the number of Shar Peis in the second part of the study was lower than that in the first part, which may have led to a larger difference for the part of the study with a higher number of Shar Peis enrolled.

In conclusion, cobalamin-deficient Shar Peis more commonly belonged to the traditional type Shar Pei, which originated from China, than the meatmouth “thickened

skin" type of this breed. Further studies are needed to determine the copy numbers of the two duplications that were shown close to the *HAS2* gene by Olsson et al. (2011) for Shar Peis with cobalamin deficiency.

# 6. SERUM COBALAMIN AND METHYLMALONIC ACID CONCENTRATIONS IN COBALAMIN-DEFICIENT CHINESE SHAR PEIS FOLLOWING COBALAMIN SUPPLEMENTATION

## 6.1 Overview

Chinese Shar Peis (Shar Peis) have a high prevalence of cobalamin deficiency. Following supplementation with cobalamin, a complete resolution of methylmalonic acidemia coupled with an increase in serum cobalamin concentration has been shown in both human and veterinary patients with selective enterocyte cobalamin malabsorption, but has not previously been shown in Shar Peis with cobalamin deficiency. Therefore, this study was aimed at comparing serum cobalamin and methylmalonic acid (MMA) concentrations in cobalamin-deficient Shar Peis at initial testing and after parenteral cobalamin supplementation. Serum samples were collected from 8 cobalamin-deficient Shar Peis. All 8 Shar Peis repeatedly received cobalamin subcutaneously and a follow-up serum sample was obtained from each dog 22 to 66 days after the initial testing. Serum cobalamin and MMA concentrations were compared to baseline values using a Wilcoxon matched pairs test. Following parenteral cobalamin supplementation, serum cobalamin concentrations were significantly higher (median: 243 ng/L) compared to baseline values (median: 149 ng/L; $p = 0.0156$). In 3 of these Shar Peis, serum cobalamin was within the reference interval or higher after cobalamin supplementation. Serum MMA concentrations were found to be significantly decreased after cobalamin supplementation (median: 2,085 nmol/L vs. 21,602 nmol/L; $p = 0.0078$). In 5 of these

Shar Peis, serum MMA was within the reference interval after cobalamin supplementation. Cobalamin-deficient Shar Peis showed an increase of serum cobalamin concentrations and a decrease of serum MMA concentrations after supplementation with cobalamin. These data suggest that parenteral cobalamin supplementation in Shar Peis with cobalamin deficiency reaches the cellular level.

## 6.2 Introduction

A high prevalence of cobalamin (vitamin $B_{12}$) deficiency in Shar Peis has previously been reported (Williams, 1991; Bishop et al., 2011). Recently, cobalamin-deficient Shar Peis were also shown to have a significantly higher serum methylmalonic acid (MMA) concentration compared to cobalamin-deficient dogs of six other dog breeds (Grützner et al., 2011 and 2012), suggesting cobalamin deficiency on a cellular level. A complete resolution of methylmalonic aciduria/methylmalonic acidemia, coupled with an increase in serum cobalamin concentration has been shown following supplementation of cobalamin in veterinary patients with selective enterocyte cobalamin malabsorption (Battersby et al., 2005). Variable responses to cobalamin supplementation have been reported in human patients that are deficient in intracellular adenosyl- and/or methylcobalamin due to disturbances of cobalamin metabolism at the cellular level (Matsui et al., 1983).

Undetectable and decreased serum cobalamin concentrations have been documented in dogs with chronic gastrointestinal diseases (Allenspach et al., 2007; Berghoff et al., 2013). Cobalamin measurements are recommended in dogs with gastrointestinal diseases

to assess the need for cobalamin supplementation. However, no data have been reported about the effectiveness of parenteral cobalamin supplementation in Shar Peis with cobalamin deficiency. Therefore, this study was aimed at comparing serum cobalamin and MMA concentrations in cobalamin-deficient Shar Peis at initial testing and after parenteral cobalamin supplementation and to evaluate if parenterally supplemented cobalamin reaches the cellular level.

## 6.3 Materials and methods

### Sampling population

Pure-bred Shar Peis were investigated (n=8; 3 males and 5 females [age: 2–10 years]). All 8 Shar Peis were considered cobalamin-deficient based on an undetectable serum cobalamin concentration (<150 ng/L) and an increased serum MMA concentration (>1,193 nmol/L; Table 12).

### Data Collection

Serum samples were collected from each dog and study questionnaires were completed for each dog. The clinical signs for cobalamin-deficient Shar Peis consisted of diarrhea (8/8), weight loss (8/8), and vomiting (5/8). Serum cobalamin and MMA concentrations were measured in all dogs, respectively. Cobalamin-deficient Shar Peis received cobalamin subcutaneously - one dose weekly for 6 weeks (dosage was roughly dependent on body weight; Figure 17; Table 13). A follow-up serum sample was

obtained from each dog 22 to 66 days after the initial testing and 5 to 7 days after the last serum cobalamin injection (Table 12).

**Sample analysis**

Serum cobalamin concentration was measured in all 8 dogs using an automated chemiluminescence assay (Immulite®2000; Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) with a reference interval of 251-908 ng/L. Serum MMA concentration was measured in all 8 dogs using a stable isotope dilution gas chromatography-mass spectrometry assay with a reference interval of 415-1,193 nmol/L (Berghoff et al., 2011; Stabler et al., 1986). Serum cobalamin and MMA concentrations were compared between time points using a Wilcoxon matched pairs test and significant difference set at $p < 0.05$.

**Table 12.** The table shows the cobalamin-deficient Shar Peis (n=8) that were included in this study and the tests (cobalamin and methylmalonic acid measurements) that were performed before and after cobalamin supplementation. The remaining columns show the days between testing, sex (F: female and M: male), and age (in years) for all cobalamin-deficient Shar Peis.

| | **Cobalamin (ng/L)** | | **MMA (nmol/L)** | | | | |
|---|---|---|---|---|---|---|---|
| | Cobalamin supplementation | | | | | | |
| **Dogs** | **Before** | **After** | **Before** | **After** | **Days between testing** | **Age** | **Sex** |
| **1** | <150 | 313 | 24,613 | 3,090 | 48 | 3 | F |
| **2** | <150 | 158 | 16,066 | 4,408 | 66 | 2 | F |
| **3** | <150 | 149 | 74,479 | 24,627 | 35 | 10 | F |
| **4** | <150 | 968 | 29,212 | 705 | 38 | 6 | M |
| **5** | <150 | 292 | 18,591 | 1,067 | 55 | 6 | M |
| **6** | <150 | 200 | 6,680 | 989 | 22 | 5 | F |
| **7** | <150 | 193 | 29,896 | 1,000 | 41 | 3 | M |
| **8** | <150 | 185 | 2,775 | 1,162 | 32 | 4 | F |

**Table 13.** The table shows the current recommendations for subcutaneous cobalamin injections in dogs (http://vetmed.tamu.edu/gilab/research/cobalamin-information).

| **Body weight of dogs** | **< 5 kg** | **5-10 kg** | **10-20 kg** | **20-30 kg** | **30-40 kg** | **40-50 kg** | **> 50 kg** |
|---|---|---|---|---|---|---|---|
| **Dose of cobalamin** | 250 µg | 400 µg | 600 µg | 800 µg | 1000 µg | 1200 µg | 1500 µg |

**Figure 17.** Cyanocobalamin. This figure **s**hows a bottle of cyanocobalamin, which is commonly used for supplementation in dogs and is both widely available and inexpensive.

## 6.4 Results

In the 8 Shar Peis evaluated in this study serum cobalamin concentrations were significantly higher following parenteral cobalamin supplementation (median: 197 ng/L [range: 149-968 ng/L]) compared to baseline values (median: 149 ng/L [ for all 8 Shar Peis]; $p = 0.0156$; Figure 18). In 3 of these 8 Shar Peis serum cobalamin was within the reference interval or higher after cobalamin supplementation.

Serum MMA concentrations were measured in 8 Shar Peis and were found to be significantly lower after cobalamin supplementation (median: 1,115 nmol/L [range: 705-24,627]) than at baseline (median: 21,602 nmol/L [range: 2,775-74,480]; $p = 0.0078$; Figure 19). In 5 of these 8 Shar Peis serum MMA concentrations were within the reference interval after cobalamin supplementation.

**Figure 18.** Comparison of serum cobalamin concentrations of 8 cobalamin-deficient Shar Peis before and after cobalamin supplementation. Serum cobalamin concentrations were significantly higher after cobalamin supplementation (median: 197 ng/L) compared to baseline values (median: 149 ng/L; $p = 0.0156$). The reference interval for serum cobalamin concentrations (251-908 ng/L) is indicated by the dashed horizontal lines.

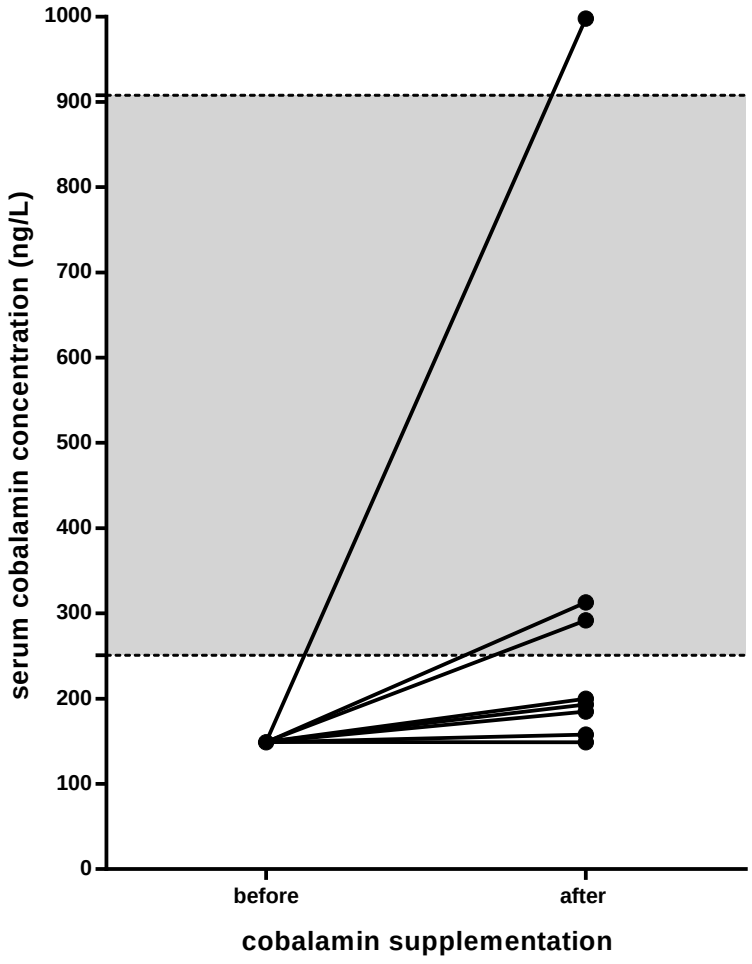
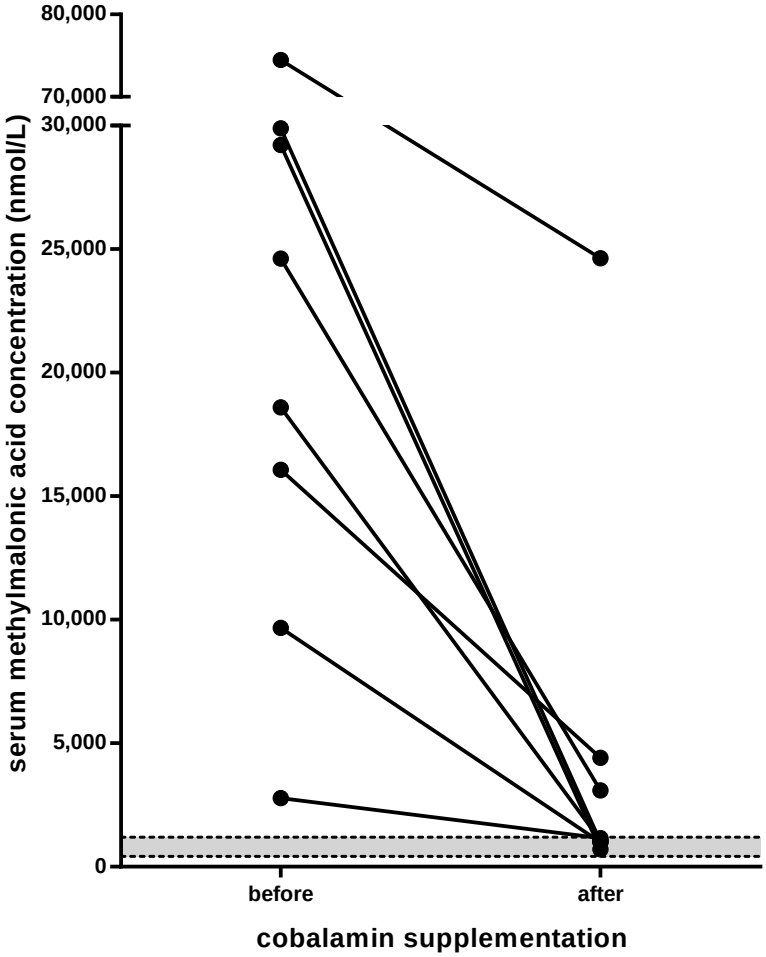


**Figure 19.** Comparison of serum MMA concentrations in 8 cobalamin-deficient Shar Peis before and after cobalamin supplementation. Serum methylmalonic acid (MMA) concentrations were significantly lower (median: 1,115 nmol/L) after cobalamin supplementation compared to baseline values (median: 21,602 nmol/L; $p = 0.0078$). The reference interval for serum MMA concentrations (415-1193 µmol/L) is indicated by the dashed horizontal lines.



## 6.5 Discussion

This study was conducted to determine if parenterally supplemented cobalamin has an impact on cobalamin metabolism on the cellular level in cobalamin-deficient Shar Peis by comparing serum cobalamin and MMA concentrations at initial testing and after parenteral cobalamin supplementation. Cobalamin-deficient Shar Peis showed an increase in serum cobalamin concentrations and a decrease in serum MMA concentrations after parenteral cobalamin supplementation. Based on human and veterinary studies, serum MMA concentrations have been suggested to reflect cobalamin status at the cellular level (Berghoff et al., 2011; Stabler et al., 1986). Therefore, these data suggest that in Shar Peis with cobalamin deficiency, parenterally supplemented cobalamin reaches the cellular level.

This study had several limitations. For instance, it would have been ideal to measure serum cobalamin and MMA concentrations in all cobalamin-deficient Shar Peis weekly to evaluate when the cells are saturated with cobalamin (vitamin $B_{12}$) and when the circulating cobalamin reaches the reference interval. Also, it was not possible to include cobalamin-deficient dogs of other breeds to compare their response to parenteral cobalamin supplementation. In addition, it would have been optimal to evaluate parenteral cobalamin supplementation in normo-cobalaminemic Shar Peis and normocobalaminemic dogs of other breeds. Regardless, this is the first study that investigated parenteral cobalamin supplementation in Shar Peis with cobalamin deficiency. Further studies to investigate pharmacokinetics of cobalamin (vitamin $B_{12}$) in healthy dogs are needed and are currently underway.

It should be noted that it is possible that some of the owners and breeders may have independently supplemented their Shar Pei with cobalamin in an effort to improve their dog's general health, which may have resulted in an effect on serum cobalamin and MMA concentrations at the time of retesting of these dogs. For instance, oral supplementation of foods high in cobalamin, such as meat, fish [especially shellfish], or a cobalamin supplement) could have had an effect on serum cobalamin and MMA concentrations in the enrolled cobalamin-deficient Shar Peis. However, based on the veterinary literature no studies have been reported that would suggest that oral cobalamin supplementation has an impact on serum cobalamin or MMA concentrations in dogs with cobalamin deficiency.

In conclusion, Shar Peis with cobalamin deficiency showed an increase in serum cobalamin concentrations and a decrease in serum MMA concentrations after cobalamin supplementation. These data suggest that in Shar Peis with cobalamin deficiency, parenterally supplemented cobalamin reaches the cellular level.

# 7. EVALUATION OF THE MYC_CANFA GENE IN SHAR PEIS WITH COBALAMIN DEFICIENCY*

## 7.1 Overview

A recent genome wide scan using the canine minimal screening set 2 (MSS-2) showed that cobalamin deficiency appears to be hereditary in Chinese Shar Peis (Shar Peis) and is linked to the microsatellite markers DTR13.6 and REN13N11 on canine chromosome 13. The goal of this study was to evaluate the MYC_CANFA gene, which is the closest known gene with a distance of approximately 0.06 Mega bases (Mb) to the microsatellite marker DTR13.6, for any mutations in this breed. Microsatellite markers (Myc and G15987) for genotyping and primers for sequencing were used to evaluate the MYC_CANFA gene. The genotype and gene sequence were compared between cobalamin-deficient Shar Peis, Shar Peis with normal serum cobalamin concentrations, and the DNA sequences published as part of the Ensemble Genomic map. Neither the microsatellite markers (Myc and G15987) nor the sequences of the MYC_CANFA gene showed a significant difference among both groups of Shar Peis and the published canine DNA sequence. The data presented here suggest that cobalamin deficiency in Shar Peis is not related to any mutations of the MYC_CANFA gene according to the genotyping and sequencing results in this study.

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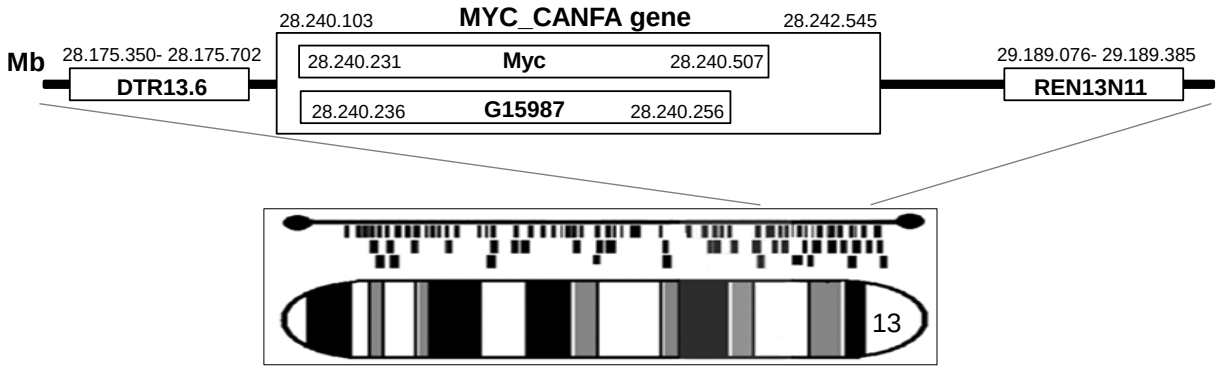
Further investigations are warranted to find a potential genomic locus in proximity to DTR13.6 and REN13N11 that shows mutations in cobalamin-deficient Shar Peis.

## 7.2 Introduction

It has been reported in both North America and the United Kingdom that Chinese Shar Peis (Shar Peis) have a high prevalence of cobalamin deficiency (Bishop et al., 2011; Dandieux et al., 2010). A genome wide scan using the canine minimal screening set 2 (MSS-2) has previously shown that cobalamin deficiency appears to be hereditary in Shar Peis and is linked to the microsatellite markers DTR13.6 and REN13N11 on canine chromosome 13 (Grützner et al., 2010). This study provided the first evidence of an association between cobalamin deficiency and a region located on canine chromosome 13. In this region, there are no previously identified genes reported to be associated with cobalamin deficiency in dogs or any other species. Interestingly and according to the Ensemble Genome Browser, the MYC_CANFA gene is located between the two microsatellite markers, approximately 0.06 Mega base (Mb) from microsatellite marker DTR13.6 and 1.01 Mb from REN12N11 (Figure 20).

**Figure 20.** Ideogram of canine chromosome 13. The microsatellite markers DTR13.6, REN13N11, G15987, and Myc are all located in proximity to the MYC_CANFA gene (distances in bp are illustrated).



The MYC gene family encodes a group of transcription factors that control cell proliferation and differentiation and are conserved on certain chromosomes in both clinically normal and diseased animals, but are associated with different binding motifs (Grandori & Eisenman, 1997; Atchley & Fitch, 1995; Miyoshi et al., 1991). The physiologic control of cell proliferation has been shown to be altered in cancer patients due to a dysregulation of the MYC gene as a result of retroviral transduction and insertional mutagenesis, chromosomal translocation, or gene amplification (Cowley et al., 1987). Thus, dysregulation of the MYC gene may be associated with a variety of malignant neoplasms (Cowley et al., 1987). A study in people showed that the transcobalamin II gene has at least one binding site (motif) for the *myc* protein, the product of the MYC gene (Regec et al., 1995). This may explain why human patients with abnormal transcobalamin II concentration had a variety of different malignant disorders, but especially multiple myeloma and lymphoproliferative disease (Areekul et al., 1995; Vreugdenhil et al., 1992; Kaikov et al., 1991). In this context, human studies have shown that patients with transcobalamin II deficiency have a normal total circulating serum cobalamin concentration (Kaikov et al., 1991; Sacher et al., 1983). However, one human case report describes a congenital transcobalamin II deficiency that was associated with a low serum cobalamin concentration (Carmel & Ravindranath, 1984). Low or undetectable serum cobalamin concentration in people and Shar Peis could be due to abnormalities of cobalamin-binding proteins such as transcobalamin II in the serum because of an altered binding reaction of the *myc* protein on the transcobalamin II gene.

Thus, the goal of this study was to evaluate the MYC_CANFA gene, the closest known gene on chromosome 13 in the region of the microsatellite markers DTR13.6 and REN12N11, for any mutations in the Shar Pei breed.

### 7.3 Materials and methods

Samples were used from the association study of cobalamin-deficient and normocobalaminemic Shar Peis (Grützner et al., 2010). All owners signed informed client consent before enrolling the dogs into this study. Briefly, whole blood and serum samples had been collected from 42 Shar Peis from various parts of the United States. The owners of the Shar Peis completed a questionnaire about their dog (including sex, age, and health status).

The canine genomic map was used to identify microsatellite markers, which are specific for the MYC_CANFA gene. Two stable microsatellite markers (Myc and G15987; NCBI map database: *Canis familiaris* (CanFam 2.0 [2005]). Available at: http://www.ncbi.nlm.nih.gov/map view/map_search.cgi?taxid=9615. Accessed October 2011) were identified (Figure 20) to investigate whether certain alleles of both markers are associated with cobalamin deficiency in Shar Peis, supporting the hypothesis that the MYC_CANFA gene might be a candidate gene for cobalamin deficiency. To the author's knowledge, both markers have not been evaluated in dogs and therefore, the informativeness of each marker by using the polymorphic information content (PIC) value is not known. However, Myc and G15987 were amplified and genotyped in 14 cobalamin-deficient Shar Peis and in 28 Shar Peis with serum cobalamin concentrations

within the reference interval. The most frequent allele of microsatellite markers Myc and G15987 in Shar Peis with cobalamin deficiency was identified. A Fisher's exact test was used to determine a possible association of this allele with cobalamin deficiency in Shar Peis. Evidence of genetic association was defined as $p < 0.05$.

In addition, primers for the MYC_CANFA gene were chosen to amplify exons I and II. Two primer pairs (I-a and I-b) were used for sequencing exon I, which is 757 base pairs (bp) long, and one primer pair (II-a) for sequencing exon II with a size of 563 bp (Table 14). Additional primer pairs (I-c and II-b, respectively) were designed to reach approximately 25 bp into the intron/exon boundary area of both borders of exon I and approximately 50 bp into the intron/exon boundary area of both borders of exon II (Table 14). DNA samples from three cobalamin-deficient Shar Peis and three normocobalaminemic Shar Peis were used for sequencing of the MYC_CANFA gene.

**Table 14.** Primer sequences used for genotyping microsatellite markers Myc and G15987, and PCR amplification of both exons I and II of the MYC_CANFA gene (5'end- to-3'end).

| Primer | Forward | Reverse |
|---|---|---|
| **Myc** | CGCGCCCAGTGAGGATATC | CCACATACAGTCCTGGATGAT |
| **G15987** | TCTTCCAGATATCCTCGCTG | TATGACCTCGACTACGACTCG |
| | | |
| **Exon I-a** | CCCGTAACTCAAGATCGCCC | TCCAGACCTAACGTTTCCCTTCCT |
| **Exon I-b** | TCCAGGACTGCATGTGGAGCGGCT | AGCCGCTCCACATGCAGTCCTGGA |
| **Exon I-c** | TACCCGCTCAATGACAGCAGCTCG | ATCCTCGCTGGGCGCCGGCGGCTG |
| **Exon II-a** | TCATCTGGTCACTGGTGGCTTGAA | TTCCAGTTCCTCCCTCCAATAGGT |
| **Exon II-b** | CGTGATCAGATCCCGGAGTTGGAA | TGGGTGGACACATGGCATCTCTTA |

The PCR reaction was performed in a Mastercycler (Eppendorf North America, Westbury, NY). For exon I the following cycling program was utilized: 3 min at 94°C followed by 35 cycles of 30s at 94°C, 30s at 59°C, and 30s at 72°C. The same cycling conditions as practiced for the multiplex PCR for markers of the cMSS-2 set were also used for amplification of exon II (Clark et al., 2004). The PCR products were purified (Purification, DNA Clean & Concentrator – 5™, Zymo Research Corporation, Orange, CA) and visualized on a 1% agarose electrophoresis gel (Gel, Fisher BioReagents, Pittsburgh, PA) using a horizontal gel electrophoresis system (Gel electrophoresis system, Horizon® 58, Whatman Inc., Florham Park, NJ). The identity of the product was then further verified by direct sequencing on a Genetic Analyzer (ABI 3130x/ Genetic Analyzer, Applied Biosystem, Foster City, CA). The sequencing results were compared between the cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis, the published canine sequence, and the cDNA sequence (Ensemble Genome Browser Web-site. *Canis Familiaris* (CanFam 2.0 [2005]). Available at: http://useast.ensemble.org/canis_familiaris/Gene/Summary?g=ENSCAFG00000001086;r=13:2824010328242545;t=ENSCAFT00000001656. Accessed October 2011).

**7.4 Results**

Allele 275 of microsatellite marker Myc and allele 199 of microsatellite marker G15987 occurred most often in both cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis (Table 15). Both markers (Myc and G15987) have probably a low PIC value because the allele distribution within the Shar Peis population is limited to one or four

alleles, respectively. Using Fisher's exact test for linkage analysis of alleles 275 and 199 in cobalamin-deficient Shar Peis revealed a *p*-value of 1.0 and 0.2, respectively. No statistically significant difference was reached, revealing no association of allele 275 and 199 with cobalamin deficiency in Shar Peis.

The MYC_CANFA gene was analyzed with primer pairs designed for exon I and II, and an agarose gel electrophoresis of the purified PCR products showed a single clear band with the same product size for both exons. This gel was performed with two cobalamin-deficient Shar Peis and two normocobalaminemic Shar Peis. Subsequently, DNA samples from three cobalamin-deficient Shar Peis and three normocobalaminemic Shar Peis were sequenced. No difference was found in the entire exonic and intron boundaries of the DNA sequence of the MYC_CANFA gene for any of the dogs in this study or in the published canine sequences.

**Table 15.** Observations for alleles 275 bp and 199 bp for microsatellite markers Myc and G15987, respectively, in cobalamin-deficient (CD) Shar Peis and control Shar Peis (*p*-values of 1.0 and 0.2, respectively). Note that the allele distribution within the Shar Peis population is limited to one or four alleles, respectively.

| | **CD Shar Peis** | **Control Shar Peis** | **Σ** |
|---|---|---|---|
| **Myc** | | | |
| **Allele 275** | 28 (100%) | 56 (100%) | 84 (100%) |
| **other Alleles** | 0 | 0 | 0 |
| **Σ** | 28 | 56 | 84 |
| **G15987** | | | |
| **Allele 199** | 28 (100%) | 50 (89.3%) | 78 (92.9%) |
| **other Alleles** | 0 | 6 (10.7%) | 6 (7.1%) |
| **Σ** | 28 | 56 | 84 |

## 7.5 Discussion

A previous genome wide scan showed that cobalamin deficiency appears to be hereditary in Shar Peis and linked to the microsatellite markers DTR13.6 and REN13N11 on canine chromosome 13 (Grützner et al., 2010). The only known gene in the region between these two microsatellite markers on canine chromosome 13 is the MYC_CANFA gene. In this study, both microsatellite markers Myc and G15987, specific for the MYC_CANFA gene, revealed no association of allele 275 or 199 with cobalamin deficiency in Shar Peis. In addition, the sequenced MYC_CANFA gene showed no differences when compared to the published canine sequence, the cDNA sequence, cobalamin-deficient Shar Peis, and Shar Peis with normal serum cobalamin concentrations. The data presented here would suggest that cobalamin deficiency in Shar Peis is not related to any mutations of the MYC_CANFA gene. Further investigations are warranted to find a potential genomic locus in proximity to DTR13.6 and REN13N11 that shows mutations in cobalamin-deficient Shar Peis.

In mammalian species, cell proliferation and differentiation are fundamental to growth, development, and also evolution. The MYC gene family encodes a group of transcription factors that control cell proliferation and differentiation (Grandori & Eisenman, 1997; Atchley & Fitch, 1995). The identification of at least one binding site for the *myc* protein, a product of the MYC gene, on the human transcobalamin II gene (Regec et al., 1995), showed a possible role of these two gene products in patients with abnormal transcobalamin II concentration and various malignant disorders (Areelkul et al., 1995; Vreugdenhil et al., 1992; Kaikov et al., 1991). Nevertheless, the data presented

here suggest that cobalamin deficiency in Shar Peis is not related to any mutations of the MYC_CANFA gene.

The success of a genetic association study is dependent on the correct assignment of the phenotypes. The exclusion criteria in a genome wide association study were that cobalamin-deficient Shar Peis had undetectable serum cobalamin concentrations (Grützner et al., 2010). However, cobalamin has been shown to be involved in many enzymatic reactions in mammalian cells. Human and veterinary studies have suggested that an increased serum methylmalonic acid concentration reflects cobalamin deficiency at the cellular level (Stabler et al., 1986; Ruaux et al., 2009; Berghoff et al., 2012). A combined evaluation of serum cobalamin and serum methylmalonic acid concentrations might therefore provide stronger evidence of cobalamin deficiency at the cellular level than a decreased serum cobalamin concentration alone. Thus, a phenotypic re-classification based on serum cobalamin and methylmalonic acid concentrations may lead to the identification of a different region on chromosome 13 or even on a different chromosome.

Both, this present report and the previous genome wide association study could be inconclusive because the cMSS-2 set contains only 327 microsatellite markers with an average marker spacing of 9 Mb, leaving large gaps and a chance of missing potential mutations. To narrow down the potential mutated regions on chromosome 13 as the major locus or primary gene responsible for cobalamin deficiency in the Shar Pei, refined mapping by single nucleotide polymorphism (SNP) determination is currently under way. Hopefully this will allow both, verifying and providing a refined map of the

region associated with cobalamin deficiency in the Shar Pei and lead to the identification of additional candidate genes for further investigation.

At the time this study was conducted no other genes had been identified in the region approximately 1 Mb up- or down-stream of the microsatellite markers DTR13.6 and REN13N11. However, the *HAS2* gene, which is located approximately 5 Mb downstream of the region from the microsatellite markers (DTR13.6 and REN13N11) and the MYC gene which could be a potential candidate gene. An increased *HAS2* expression has been shown in Shar Peis with cutaneous mucinosis, a highly prevalent and suspect hereditary condition in Shar Peis (Zanna et al., 2009). So far, serum cobalamin concentrations have not been reported in studies that investigated Shar Peis with cutaneous mucinosis.

In people and cats it has been reported that the MYC gene consists of three exons, whereas in dogs no third exon has been identified yet according to the Ensemble Genome Browser Web-site. The third exon of the MYC gene in cats consists of three nucleobases. A general investigation with regard to the MYC_CANFA gene in the canine genome is required to determine whether there is an additional exon of the MYC_CANFA gene in dogs, and whether it is a candidate for clinically relevant mutations. The present study focused on and used sequences of the published exons of the canine genome sequence.

A limitation of the study was that only a small section was sequenced while it might have been helpful to sequence a larger proportion (approximately 2 Mb) of the microsatellite marker DTR13.6 and REN13N11 region. An association can be dependent

upon the respective size of a population, for instance a linkage disequilibrium can occur in the range of kb in popular breeds (e.g., Labrador Retriever, Yorkshire Terrier, and German Shepherd Dog), whereas in a rare breed like the Shar Pei (ranked 50th by the American Kennel Club in 2011; http://www.akc.org/reg/dogreg_stats.cfm; accessed March 1st, 2012) it can occur in the range of Mb (Sutter et al., 2004). As mentioned above, a canine SNP would be useful to both verify and fine map this region that is associated with cobalamin deficiency in Shar Peis.

In conclusion, the DNA sequence of the MYC_CANFA gene determined in this present study revealed no differences when compared to the published canine sequence, the cDNA sequence, and Shar Peis of both groups. Consequently, cobalamin deficiency in Shar Peis does not appear to be related to a mutation of the MYC_CANFA gene according to the genotyping and sequencing results in this study. Further investigations are warranted to find a potential genomic locus in proximity to DTR13.6 and REN13N11 microsatellite markers that shows mutations in cobalamin-deficient Shar Peis.

# 8. GENOME-WIDE SCANS IN COBALAMIN-DEFICIENT CHINESE SHAR PEIS

## 8.1 Overview

Cobalamin deficiency in Chinese Shar Peis (Shar Peis) has been linked to canine chromosome 13 using the canine minimal screening set-2 (cMSS-2), but because of an average marker spacing of 9 megabases, this genome scan might be inconclusive. Therefore, study aims were 1) to corroborate previous results from genome scans using the canine single nucleotide polymorphism (cSNP) array in cobalamin-deficient Shar Peis, 2) to evaluate the candidate gene, *HAS2*, located on canine chromosome 13, for mutations in Shar Peis with cobalamin deficiency, and 3) to ascertain whether cobalamin-deficient Shar Peis have a low or high copy number of the duplications close to the *HAS2* gene. First, the cSNP analysis of cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis revealed 2 markers (21,600,902 bp and 22,937,592 bp; $p < 1.0\times10^{-6}$). Allele 283 of the cMSS-2 marker FH3619 was found significantly more frequently in cobalamin-deficient Shar Peis than normocobalaminemic Shar Peis ($p = 1.8\times10^{-6}$). Second, the sequencing of the 3 exons of *HAS2* gene revealed no differences between Shar Peis with and without cobalamin deficiency. Third, cobalamin-deficient Shar Peis had a higher copy number of a 14.3 kb duplication and a lower copy number of a 16.1 kb duplication that are close proximity to the *HAS2* gene than normocobalaminemic Shar Peis, and cobalamin deficiency was associated with higher copy number of a 14.3 kb duplication and with lower copy number of a 16.1 kb

duplication ($p \leq 0.0001$). In conclusion, cSNP and cMSS-2 analyses revealed a cluster of cSNP markers on canine chromosome 13 that co-segregate with cobalamin deficiency in Shar Peis. The copy number assay analysis suggests that cobalamin deficiency in Shar Peis is associated with the traditional type Shar Pei (i.e., the original Shar Peis that originated from China). The findings of this study provide further evidence that a region of chromosome 13 contains one or more genes responsible for this condition in the Shar Pei.

## 8.2 Introduction

Cobalamin (vitamin $B_{12}$) deficiency is a common disorder in the Chinese Shar Pei (Shar Pei) and is suspected to be hereditary (Bishop et al. 2011). Based on a genome-wide scan using the canine minimal screening set-2 (cMSS-2), cobalamin deficiency in the Shar Pei has recently been linked to a genomic locus in close proximity to two microsatellite markers (DTR13.6 and REN13N11) on canine chromosome 13 (Grützner et al. 2010). The cMSS-2 contains 327 microsatellite markers with an average marker spacing of 9 megabases (Mb), but no gaps larger than 17.1 Mb (Clark et al. 2004). Thus, the previous study does not conclusively narrow down the region on chromosome 13 as the major locus for a gene or genes responsible for cobalamin deficiency in this breed. Also, not all genes that have been associated with cobalamin deficiency in humans or genes encoding for cobalamin binding proteins have been identified in the dog (Tanner et al. 2005; Hauck et al. 2008). In this context, a single nucleotide polymorphism (SNP) array would cover the dog genome in more detail and thus might be more informative for such an

association study (Fukuda et al. 2009). Therefore, the first aim of the current study was to scan the whole genome using canine SNPs (cSNP) to identify genes or regions that may be associated with cobalamin deficiency in the Shar Pei.

In human (Stabler et al. 1986) and veterinary studies (Ruaux et al. 2009; Berghoff et al. 2011), an increased serum methylmalonic acid (MMA) concentration has been suggested to reflect cobalamin deficiency at the cellular level. A combination of decreased serum cobalamin and increased serum methylmalonic acid concentrations might therefore be stronger evidence of cobalamin deficiency at the cellular level than a decreased serum cobalamin concentration alone. Thus, a phenotypic re-classification based on serum cobalamin and methylmalonic acid concentrations may lead to identification of a different region on chromosome 13 or even on a different chromosome. Therefore, the second aim of this study was to re-evaluate results from the previous cSNP array and the cMSS-2 results based on a comparison of Shar Peis with decreased serum cobalamin and increased serum MMA concentrations to those with serum cobalamin and MMA concentrations within the respective reference intervals.

In addition, it has previously been shown that Shar Peis have a high prevalence of cutaneous mucinosis, which is also suspected to be hereditary. Cutaneous mucinosis in Shar Peis is a condition that has been shown to be associated with an increased hyaluronan synthase gene (*HAS2*) expression, a gene that is located within the genomic region of canine chromosome 13 (Olsson et al., 2011). *HAS2* gene has been reported to be located on canine chromosome 13, at location 23,348,773-23,364,912 bp, with a distance of approximately 0.42 Mb and 0.47 Mb to the cSNP and cMSS-2 markers that

have previously been reported to be associated with cobalamin deficiency in this breed (Figure 21; Grützner et al., 2010). The aim of this part of the study was to evaluate the candidate gene *HAS2* located on canine chromosome 13 for any mutations in Shar Pei with cobalamin deficiency based on a decreased serum cobalamin and an increased serum MMA concentrations.

Olsson et al., has reported a link of Shar Pei fever and cutaneous mucinosis with the *HAS2* gene on canine chromosome 13 (Olsson et al., 2011). A high copy number of a 16.1 kb duplication close proximity to the *HAS2* gene was found to be associated with the thickened skin in the meatmouth type Shar Pei, while a high copy number of a 14.3 kb duplication close proximity to the *HAS2* gene was suggested to be associated with the traditional type Shar Pei (Olsson et al., 2011). Therefore, the copy number assay analysis was conducted to ascertain whether cobalamin-deficient Shar Peis have a low or high copy numbers of the two duplications close proximity to the *HAS2* gene (Olsson et al. 2011).

## 8.3 Materials and methods

### First study aim

**Part A** – 42 pure-bred Shar Peis that had previously been used in an association study of cobalamin deficiency in Shar Peis by using the cMSS-2 set were investigated (Grützner et al. 2010; Table 16).

**Part B** – The Shar Peis from part A of the study and an additional 19 pure-bred Shar Peis were investigated (Table 16).

For both parts of the study, the protocol for collection of blood samples from Shar Peis was reviewed and approved by the Clinical Research Review Committee at Texas A&M University (CRRC# 2007-30). Owners were asked to complete a questionnaire requesting information concerning their dogs, including age, sex, sexual status, health status (including current medications and vaccination status), and history of any prior supplementation with cobalamin alone or as a vitamin B complex preparation. Food was withheld from the dogs for at least 12 hours before collection of blood samples.

For both parts of the study, serum and blood samples were collected from each dog. Serum cobalamin concentration and serum cobalamin and MMA concentrations, respectively, were measured in each serum sample. Whole blood was used for subsequent DNA extraction and analysis.

**Table 16.** The table lists the number (n) and proportion of cobalamin-deficient (COB deficient) Shar Peis and normocobalaminemic (normal COB) Shar Peis that were included for each part of the study (1. Part A: cSNP and microsatellite marker FH3619; Part B: cSNP and microsatellite marker FH3619; 2. sequencing of the *HAS2* gene and C/T deletion; 3. copy number assays: CNV-Eastern and CNV_23.759; with and without increased MMA concentration). The remaining columns show the number of female and male dogs and the median age (in years) for all dogs of each group.

| Study aims | n | # COB Deficient | ♀/age | ♂/age | # normal COB | ♀/age | ♂/age |
|---|---|---|---|---|---|---|---|
| **1. Genome scans** | | | | | | | |
| **Part A** | | | | | | | |
| **cSNP** | 42 | 14 | 9 / 6.0 | 5 / 6.0 | 28 | 17 / 3.0 | 13 / 4.0 |
| **FH3619** | 69 | 28 | 16 / 6.5 | 12 / 6.0 | 41 | 21 / 4.0 | 20 / 4.0 |
| **Part B** | | **with increased MMA** | | | | | |
| **cSNP** | 34 | 10 | 5 / 6.5 | 5 / 6.0 | 24 | 13 / 4.0 | 11 / 4.0 |
| **FH3619** | 56 | 20 | 12 / 6.5 | 8 / 6.0 | 36 | 16 / 4.0 | 19 / 4.0 |
| **2. Sequencing** | | | | | | | |
| ***HAS2* gene** | 8 | 4 | 2 / 7.0 | 2 / 6.0 | 4 | 2 / 5.0 | 2 / 4.0 |
| **C/T deletion** | 26 | 12 | 7 / 6.0 | 5 / 5.0 | 14 | 6 / 9.0 | 8 / 4.0 |
| **3. Copy number assays** | | | | | | | |
| **CNV-Eastern** | 69 | 28 | 16 / 7.5 | 12 / 5.0 | 41 | 21 / 5.0 | 20 / 4.0 |
| **CNV_23.759** | 69 | 28 | 16 / 7.5 | 12 / 5.0 | 41 | 23 / 5.0 | 18 / 4.0 |
| | | **with increased MMA** | | | | | |
| **CNV-Eastern** | 56 | 20 | 12 / 7.5 | 8 / 5.0 | 36 | 16 / 5.0 | 19 / 4.0 |
| **CNV_23.759** | 56 | 20 | 12 / 7.5 | 8 / 5.0 | 36 | 16 / 5.0 | 19 / 4.0 |

**Part A** – Serum cobalamin concentration was measured in each dog using an automated chemiluminescence assay (Immulite®2000; Siemens Healthcare Diagnostics), with a reference interval of 252-908 ng/L (Gastrointestinal Laboratory at Texas A&M University, College Station, TX; http://vetmed.tamu.edu/gilab/service/assays/b12folate; accessed May 1, 2012). DNA was extracted from whole blood using a commercial DNA extraction kit (Gentra Systems). Spectrophotometry was used to evaluate purity and quantity of the DNA extracted prior to further analysis. A total of 49,633 SNPs were genotyped using the Affymetrix v2 Platinum cSNP array. The analysis of the genotype data was conducted using a whole genome association analysis toolset (Plink v1.05) and compared to the CanFam 2.0 (2005). Bonferroni correction for multiple statistical comparisons was used to evaluate the significance of any potential association (alpha level for each individual test adjusted to 0.05). Significance was set at $p$ value <0.000001 ($P < 1.0\times10^{-6}$).

**Part B** – Serum cobalamin concentration was measured as for part A. Serum MMA concentration was measured in each dog by using a stable isotope dilution gas chromatography-mass spectrometry method, with a reference interval of 415-1,193 nmol/L (Berghoff et al. 2012). DNA was handled as described above. cSNP results were re-analyzed with the combined measurements of cobalamin and MMA concentration and the analysis was conducted as for the first part of the study (comparison to CanFam 2.0 [2005]). In addition, 313 microsatellite markers from the cMSS-2 were re-analyzed and

genotyped with both serum parameters using an ABI 3130 series capillary electrophoresis-based Genotyper (Applied Biosystems) and Gene-Mapper® software (version 3.7; Applied Biosystems). Fisher's exact test was used for statistical analysis to evaluate for a potential association. Significance was set at $p$ value of $< 1.0\times10^{-4}$.

**Second aim of the study**

For sequencing of the *HAS2* gene, 8 pure-bred Shar Peis were investigated (4 normocobalaminemic Shar Peis with a serum methylmalonic acid (MMA) concentration within the reference interval and 4 cobalamin-deficient Shar Peis with an increased serum MMA concentration [Table 16]). Serum and blood samples were collected from each dog. Serum cobalamin concentrations using an automated chemiluminescence assay (Immulite®2000; Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA), with a reference interval of 252-908 ng/L and MMA concentrations using a gas chromatography-mass spectrometry assay, with a reference interval of 415-1,193 nmol/L (Berghoff et al., 2012) were measured in each serum sample.

Whole blood was used for subsequent DNA extraction and analysis. Primers for amplification for all three exons of the *HAS2* gene were chosen (Table 17). DNA was extracted from whole blood using a commercial DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN, USA). PCR reaction was performed in a Mastercycler (Eppendorf North America, Westbury, NY, USA). For all exons the same cycling program was used: 3 min at 94°C followed by 35 cycles of 30s at 94°C, 30s at 59°C, and 30s at 72°C. PCR products were purified (Purification, DNA Clean & Concentrator-5™,

Zymo Research Corporation, Orange, CA, USA), and visualized on a 1% agarose electrophoresis gel (Gel, Fisher BioReagents, Pittsburgh, PA) using a horizontal gel electrophoresis system (Horizon® 58, Whatman Inc., Florham Park, NJ, USA). The identity of the product was then further verified by direct sequencing on a genetic analyzer (ABI 3130x/ Genetic Analyzer, Applied Biosystem, Foster City, CA, USA). Sequencing results were compared between cobalamin-deficient Shar Peis and Shar Peis with normal serum cobalamin and methylmalonic acid concentrations and published DNA sequences for this gene as published in the Ensemble genomic map.

Further evaluation of the intronic region following exon 2 of the *HAS2* gene was evaluated in DNA samples from 26 unrelated Shar Peis (Table 17). Genomic DNA was extracted from whole blood from each dog and a primer pair was chosen to amplify the intron region following exon 2 of the *HAS2* gene (Forward: GGATGCTCAATGTTGA CTGC and / Reverse: TCAGCCAAAACAGACAAGAA and the PCR products were analyzed by direct sequencing. Occurrence of the C/T deletion within the intron following exon 2 of the *HAS2* gene was compared between Shar Peis with and without cobalamin deficiency (phenotype) and based on allele 283 of microsatellite marker FH3619. Fisher's exact test was used and the odds ratio (OR) and the 95% confidence interval (CI) were determined.

**Table 17.** This table shows the chosen forward and reverse primers for the amplification of exon 1 (1a and 1b), exon 2 (2a), and exon 3 (3a, 3b, and 3c) of the canine *HAS2* gene.

| Exon | Forward | Reverse |
|---|---|---|
| **1a** | CCAAGTGCTTCTCGTCCAAT | CCCAGGGTAGGTTAGCCTCT |
| **1b** | TGCAAATGAGCAAACCTGAG | ATAGGCAGCGATGCAAAGAG |
| **2a** | GCACATCACTTCAGCTGGTC | GTGGGTCCAAGGCACATACT |
| **3a** | CGCATGCACACAATTTATCA | CATCGCATTGTACAGCCACT |
| **3b** | TTCCTGGATCTCCTTCCTCA | AGCTGGCAAAGGAGGATTTT |
| **3c** | GGGGTAAAATTTGGAACATCC | GTTCAAGTCCCAGCAGCAGT |

**Third aim of the study**

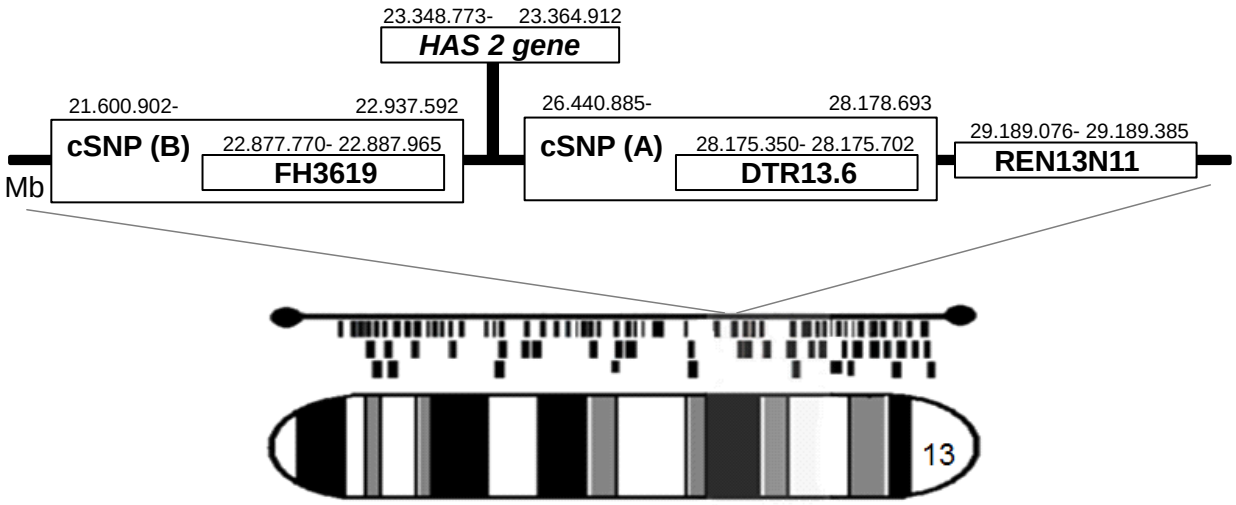
Previously, researcher from Uppsala University conducted the duplication genotyping by using two assay sets: 1) CNV-Eastern assay estimates the copy numbers of a 14.3 kb duplication 0.4 Mb away from the *HAS2* gene (related to the traditional type of Shar Peis) and 2) CNV_23.759 assay estimates the copy number of a 16.1 kb duplication 0.3 Mb away from the *HAS2* gene (related to the meatmouth type of Shar Peis). Both assay sets were used for the comparison of cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis, and cobalamin-deficient Shar Peis with an increased serum MMA concentration and normocobalaminemic Shar Peis with a normal serum MMA concentration using a Mann-Whitney *U* test (Table 16). A Fisher's exact test was used to evaluate whether cobalamin deficiency in Shar Peis is associated with a low or high copy numbers of the two duplications close proximity to the *HAS2* gene exist (Table 16). For all tests statistical significance was set at a *p* value of 0.05.

## 8.4 Results

### First study aim

**Part A** – Serum cobalamin concentrations were undetectable (< 150 ng/L) in 14 of 42 (33.3%) Shar Peis enrolled. Serum cobalamin concentrations were within the reference interval in the other 28 dogs (66.7%). The cSNP analysis revealed 4 markers with a *p*-value of $< 1.0\times10^{-6}$. These four markers were located between 26,440,885-28,178,693 bp on chromosome 13 (Figure 21).

**Figure 21.** Ideogram of canine chromosome 13. The microsatellite markers DTR13.6, REN13N11, and FH3619 are shown at their respective locations in mega bases (Mb) and the closely located *HAS2* gene is also shown. The cSNP array analysis for the first part of the study revealed four markers that are located between 26,440,885-28,178,693 bp on chromosome 13 and illustrated as **cSNP (A)** ($p$ = $<1.0\times10^{-6}$). The analysis of the cSNP array for the second part of the study revealed two significant markers located in close proximity to microsatellite marker FH3619 on chromosome 13 and illustrated as **cSNP (B)** ($p = 4.1\times10^{-7}$ and $4.5\times10^{-7}$, respectively).



**Part B** – 61 Shar Peis were included in this part of the study, with 19 Shar Peis (32.8%) having an undetectable serum cobalamin concentration (<150 ng/L) and an increased serum MMA concentration (>1,193 nmol/L). Data from the cSNP array (part A) were available for 34 of these dogs, with 10 (29.4%) having an undetectable serum cobalamin concentration and an increased serum MMA concentration. The other 24 (70.6%) dogs had serum cobalamin and MMA concentrations within the reference intervals. The analysis of the cSNP array revealed two significant markers (21,600,902 bp; $p = 4.1\times10^{-7}$ and 22,937,592 bp; $p = 4.5\times10^{-7}$, respectively) located in close proximity to microsatellite marker FH3619 (from cMSS-2) on chromosome 13 (Figure 21).

cMSS-2 data from the previously conducted association study of cobalamin deficiency in the Shar Pei (Grützner et al., 2010) plus additional pure-bred Shar Peis were investigated for cMSS-2 marker FH3619, which is located between the significant cSNP array markers. Allele 283 of the cMSS-2 marker FH3619, located on chromosome 13 (22,877,770 bp), was found significantly more frequently in the 28 cobalamin-deficient Shar Peis (30 of 56 alleles, 53.6%) than in the 41 normocobalaminemic Shar Peis (12 of 82 alleles, 14.6%; $p = 1.1\times10^{-6}$; Table 18; Figure 21). Allele 283 of the cMSS-2 marker FH3619 was found significantly more frequently in the 20 cobalamin-deficient Shar Peis with increased MMA concentrations (25 of 40 alleles, 62.5%) than in the 36 normocobalaminemic Shar Peis with normal serum MMA concentrations (12 of 72 alleles, 16.7%; $p = 1.8\times10^{-6}$; Table 18; Figure 21).

**Table 18.** Frequencies for allele 283 bp for microsatellite marker FH3619 in **A)** cobalamin-deficient (COB deficient) Shar Peis and normocobalaminemic (Control) Shar Peis ($p$ value = $1.2\times10^{-6}$) and **B)** cobalamin-deficient (COB deficient) Shar Peis with increased serum MMA concentrations and normocobalaminemic (Controls) Shar Peis with normal serum MMA concentrations ($p$ value = $1.8\times10^{-6}$).

| | **COB deficient Shar Peis** | **Control Shar Peis** | **Σ** |
|---|---|---|---|
| **A) FH3619** | | | |
| **Allele 283** | 30 (53.6%) | 12 (14.6%) | 42 (30.4%) |
| **other Alleles** | 26 | 70 | 96 |
| **Σ** | 56 | 82 | 138 |
| **B) FH3619** | | | |
| **Allele 283** | 25 (62.5%) | 12 (16.7%) | 37 (33.0%) |
| **other Alleles** | 15 | 60 | 75 |
| **Σ** | 40 | 72 | 112 |

**Second aim of the study**

The sequencing of the 3 exons of the *HAS2* gene revealed no differences between any of the dogs belonging to either of the two groups of Shar Peis as well as the published sequence for this gene in the dog. Interestingly, the intron region following exon 2 showed a deletion of two nucleobases (cytosine and thymine) located at intron positions 87 bp and 88 bp, respectively, in all four Shar Peis with undetectable serum cobalamin and increased serum MMA concentrations, but none of the healthy control dogs (Figure 22).

Furthermore, the sequencing results of the intron region following exon 2 of the *HAS2* gene were compared between 12 cobalamin-deficient Shar Peis with normal serum MMA concentrations and 14 normocobalaminemic Shar Peis with normal serum MMA concentrations. Five of 12 (41.7%) cobalamin-deficient, but only 3 of 14 (21.4%) normocobalaminemic Shar Peis had the C/T deletion within the intron following exon 2 of the *HAS2* gene. However, this numerical difference was not statistically significant ($p$ = 0.4004; OR=1.8 [95%CI: 0.6-5.5]; Figure 23). In contrast, a significant association was found between allele 283 of microsatellite marker FH3619 and the C/T deletion in both groups of Shar Peis ($p$ = 0.0147; OR=7.8 [95%CI: 1.5-41.0]; Figure 23).

**Figure 22.** This figure shows the alignment (ChromasPro software) between *HAS2* sequences from two healthy control Shar Peis (**A and C**) and two cobalamin-deficient Shar Peis (**B and D**). The bases cytosine and thymine (CT) are missing in Shar Peis B and D compared to healthy Shar Peis A and C.

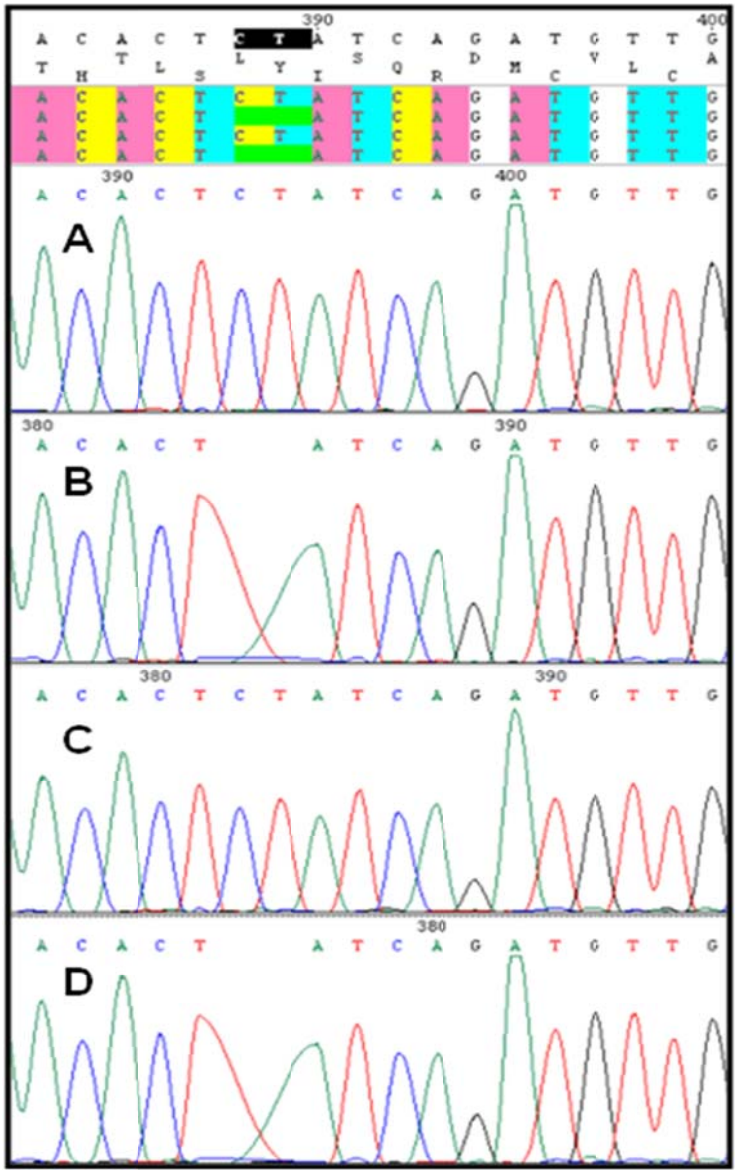
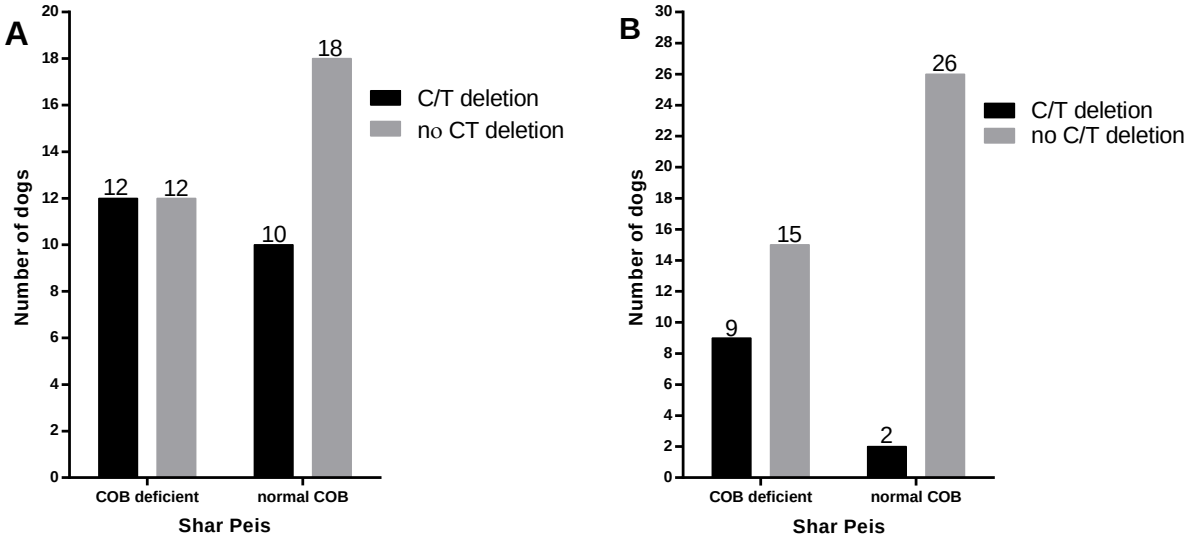


**Figure 23.** Bar graph showing the proportions of cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis with and without C/T deletion within the intron following exon 2 of the *HAS2* gene. **A)** C/T deletions were no more frequently observed in cobalamin-deficient (COB deficient) Shar Peis (50.0%) than in normocobalaminemic (normal COB) Shar Peis (35.7%; $p$ = 0.4004). **B)** C/T deletions were associated with allele 283 of microsatellite marker FH3619 in both groups of dogs: cobalamin-deficient (COB deficient) Shar Peis (37.5%) and normocobalaminemic (normal COB) Shar Peis (7.1%; $p$ = 0.0147).



**Third study aim**

Higher copy numbers of the CNV-Eastern assay (traditional type of Shar Peis) and lower copy numbers of the CNV_23.759 assay (meatmouth type of Shar Peis) were observed in cobalamin-deficient Shar Peis than in normocobalaminemic Shar Peis (for both: $p<0.0001$; Figure 24). The CNV-Eastern assay showed that approximately 57% (n=16) of the cobalamin-deficient Shar Peis had higher copy numbers (≥4), and cobalamin deficiency was associated with a higher copy number (OR: 7.8 [CI: 2.5-24.4]; $p = 0.0004$; Figure 25). Whereas, for the CNV_23.759 assay approximately 71 % (n=20) of the cobalamin-deficient Shar Peis had lower copy numbers (<4) and cobalamin deficiency was associated with a lower copy number (OR: 14.5 [CI: 4.4-48.1]; $p < 0.0001$; Figure 25).

Similarly, higher copy numbers of the CNV-Eastern assays and lower copy numbers of the CNV_23.759 assay were observed in cobalamin-deficient Shar Peis with increased serum MMA concentrations than in normocobalaminemic Shar Peis with serum MMA concentrations within the reference intervals (for both: $p<0.0001$; Figure 26). The CNV-Eastern assay revealed that approximately 70% (n=14) of the cobalamin-deficient Shar Peis with increased serum MMA concentrations had higher copy numbers (≥4) and cobalamin deficiency was associated with a higher copy number (OR: 11.7 [CI: 3.2-42.7]; $p = 0.0001$; Figure 27). Whereas, for the CNV_23.759 assay revealed that approximately 75% (n=15) of the cobalamin-deficient Shar Peis with increased serum MMA concentrations had lower copy numbers (<4) and cobalamin deficiency was associated with a lower copy number (OR: 15.0 [CI: 3.9-57.2]; $p < 0.0001$; Figure 27).

**Figure 24.** Copy number assay (CNV-Eastern and CNV_23.759) results for 28 cobalamin-deficient Shar Peis and 41 normocobalaminemic Shar Peis. **A)** CNV-Eastern assay showed that cobalamin-deficient (COB deficient) Shar Peis (median: 4.5; range: 1.8-7.0) had higher copy numbers than normocobalaminemic (normal COB) Shar Peis (median: 2.5; range: 1.7-7.1; $p < 0.0001$). **B)** The CNV_23.759 assay showed that cobalamin-deficient (COB deficient) Shar Peis (median: 2.5; range: 1.0-9.7) had lower copy numbers than normocobalaminemic (normal COB) Shar Peis (median: 5.4; range: 2.4-11.6; $p < 0.0001$).

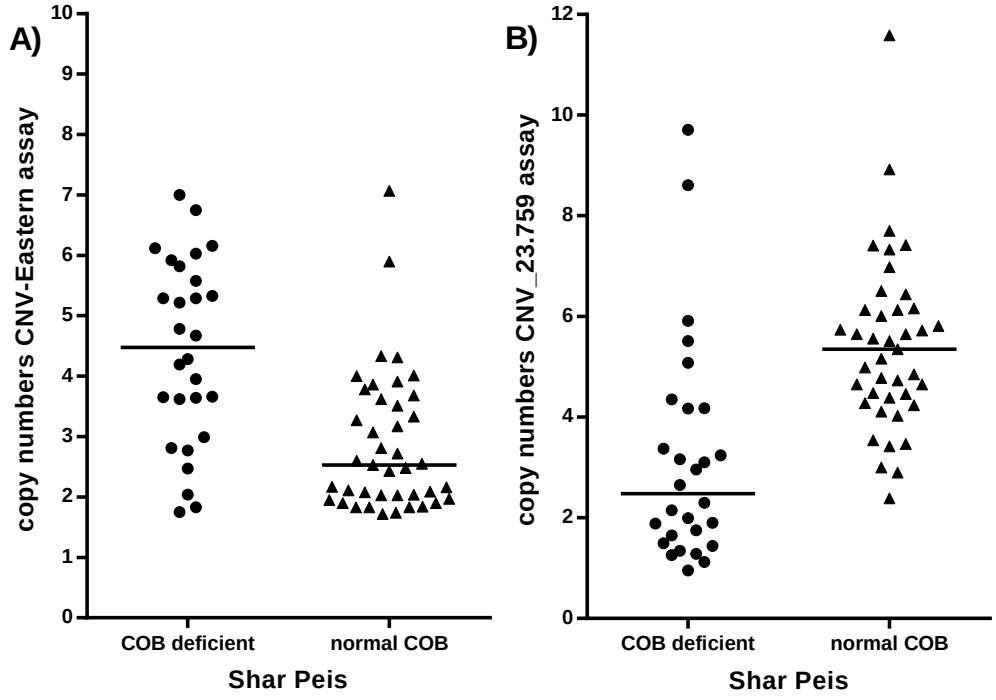


**Figure 25.** Bar graph showing the proportions of cobalamin-deficient Shar Peis and normocobalaminemic Shar Peis with low and high copy numbers using both assays (CNV-Eastern and CNV_23.759). **A)** The CNV-E assay revealed that high copy numbers (≥4) were more frequently observed in cobalamin-deficient (COB deficient) Shar Peis (57.0%) than in normocobalaminemic (normal COB) Shar Peis (14.6%; $p = 0.0004$). **B)** In contrast, the CNV_23.759 assay showed that low copy numbers (<4) were found more frequently in cobalamin-deficient (COB deficient) Shar Peis (71.4%) than in normocobalaminemic (normal COB) Shar Peis (14.6%; $p < 0.0001$).

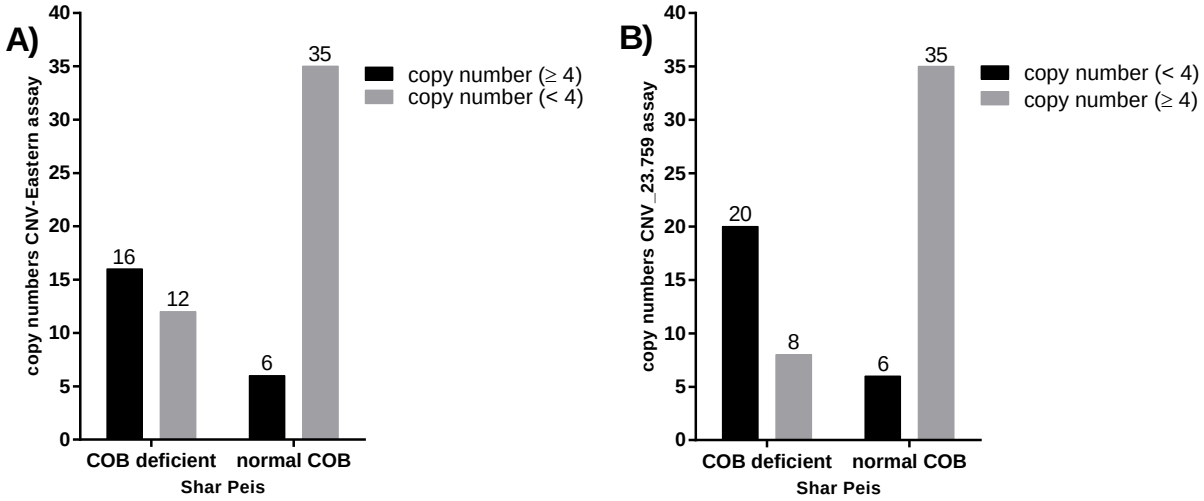


**Figure 26.** Copy number assay (CNV-Eastern and CNV_23.759) results for 20 cobalamin-deficient Shar Peis with an increased serum MMA concentration and 36 normocobalaminemic Shar Peis with normal serum MMA concentrations. **A)** The CNV-Eastern assay showed that cobalamin-deficient (COB deficient) Shar Peis with increased serum MMA concentrations had higher copy numbers (median: 5.0; range: 2.0-7.0) than normocobalaminemic (normal COB) Shar Peis with normal serum MMA concentrations (median: 2.5; range: 1.7-7.1; $p = 0.0001$). **B)** In contrast, the CNV_23.759 assay showed that cobalamin-deficient (COB deficient) Shar Peis with increased serum MMA concentrations had lower copy numbers (median: 2.1; range: 1.0-8.6) than normocobalaminemic (normal COB) Shar Peis with normal serum MMA concentrations (median: 5.3; range: 2.4-11.6; $p < 0.0001$).

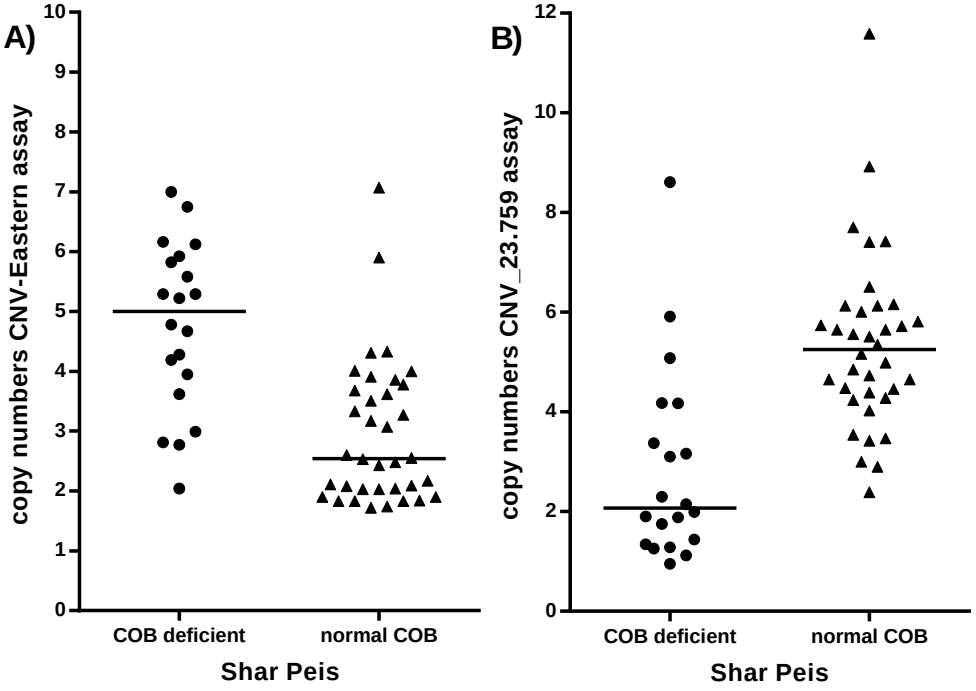
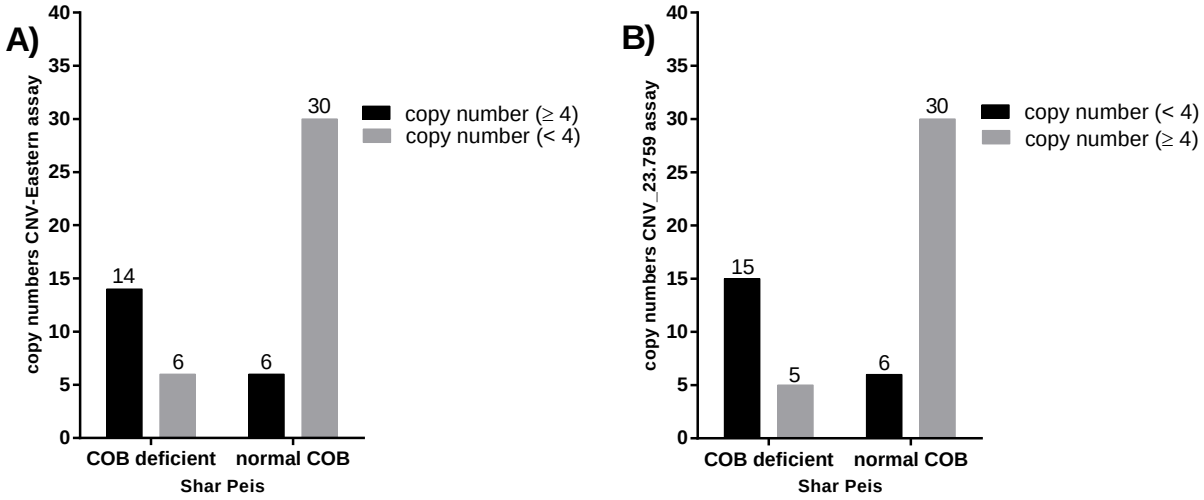


**Figure 27.** Bar graph showing the proportions of cobalamin-deficient Shar Peis with increased serum MMA concentration and normocobalaminemic Shar Peis with normal serum MMA concentrations with low and high copy numbers using both assays (CNV-Eastern and CNV_23.759). **A)** The CNV-Eastern assay revealed that high copy numbers (≥4) were more frequently observed in cobalamin deficient (COB deficient) Shar Peis with an increased serum MMA concentration (70.0%) than in normocobalaminemic (normal COB) Shar Peis with normal serum MMA concentrations (16.7%; $p = 0.0001$). **B)** In contrast, the CNV_23.759 assay showed that low copy numbers (<4) were found more frequently in cobalamin-deficient (COB deficient) Shar Peis with increased serum MMA concentrations (75.0%) than in normocobalaminemic (normal COB) Shar Peis with normal serum MMA concentrations (16.7%; $p < 0.0001$).



## 8.5 Discussion

This genome wide association study was conducted using cSNP arrays and the cMSS-2 to corroborate previous results and to analyze Shar Peis with cobalamin deficiency based on two different phenotypes. First, the cSNP analysis revealed a cluster of cSNP markers on chromosome 13, which were significantly associated with undetectable serum cobalamin concentrations in this group of Shar Peis. Interestingly, previous results from the association study using the cMSS-2 had pointed to the same region of chromosome 13 (Grützner et al. 2010). Second, the analysis of the cSNP array and cMSS-2 revealed one region on chromosome 13 that is significantly associated with cobalamin deficiency (based on a combination of an undetectable serum cobalamin concentration and an increased serum MMA concentration). The cSNP and cMSS-2 markers are approximately 5 Mb downstream from those identified in the first part of this study and the previously conducted association study by using the cMSS-2 (Grützner et al., 2010). Thus, the findings of this study provide further evidence that this region of chromosome 13 contains the causative gene or genes for this condition.

However, the phenotypic re-classification based on serum cobalamin and methylmalonic acid concentrations led to the identification of a slightly different region on chromosome 13, but no other region on any other canine chromosome. Since measurement of a combination of serum cobalamin and methylmalonic acid concentrations may provide a stronger phenotype than a decreased serum cobalamin concentration alone, this newly identified region maybe more accurately reflect the region of interest. The identified region on chromosome 13 approximately 5 Mb

downstream from a previously identified region warrants further investigation with regard to candidate genes for this condition.

Other conditions that are reported to occur frequently in Shar Peis could help to identify a candidate gene. For instance, two other conditions have been described extensively in Shar Peis, cutaneous mucinosis and Shar Pei fever. Cutaneous mucinosis is a skin disorder that has been reported frequently in Shar Peis and is also suspected to be hereditary in this breed (Muller, 1990; von Bomhard & Kraft, 1998, Zanna et al. 2008). This condition in Shar Peis is associated with an increased *HAS2* gene expression (Zanna et al. 2009). Interestingly, the *HAS2* gene is located on canine chromosome 13, at location 23,348,773-23,364,912 bp, which is in close proximity to the cSNP and cMSS-2 markers that have been identified in this study as a region of interest for cobalamin deficiency.

Shar Pei fever has been shown to be associated with renal amyloidosis and renal failure in several studies. In affected Shar Peis, amyloid deposits have been found in tissues such as the liver, spleen, stomach, small intestine, lymph nodes, and the pancreas (DiBartola et al. 1990). The clinical signs of Shar Peis fever, include vomiting, anorexia, fever, and weight loss (DiBartola et al. 1990; May et al. 1992; Rivas et al. 1992; Clements et al. 1995), which have also been reported in cobalamin-deficient Shar Peis. Interestingly, another research group found an unstable duplication close to the *HAS2* gene with a preferred skin and a fever syndrome phenotype in Shar Peis (Olsson et al. 2011). However, to our knowledge, serum cobalamin concentrations have never been reported in dogs with Shar Pei fever.

Higher production of cell surface hyaluronan (also called hyaluronic acid) has been reported on mucosal endothelial cells in human patients with inflammatory bowel disease than in healthy controls (Kessler et al. 2008). Additionally, low serum cobalamin concentration has been documented in both human and canine patients with chronic enteropathies such as inflammatory bowel disease (Yakut et al. 2010; Allenspach et al. 2007). It is thus reasonable to hypothesize that cutaneous mucinosis in Shar Peis may be related to cobalamin deficiency in this breed. Further investigation of the gene is warranted, for any mutations in this breed because to our knowledge, there are no previously published reports regarding any mutation of the *HAS2* gene and the regions around the *HAS2* gene in Shar Peis with cobalamin deficiency.

The results of the second part of the study suggest that undetectable serum cobalamin and increased serum MMA concentrations in Shar Peis do not appear to be due to a mutation within the three exonic regions of the *HAS2* gene. However, the deletion of the two nucleotides in the intron region following exon 2 of the *HAS2* gene were associated with allele 283 of the microsatellite marker FH3619, which has been linked to Shar Peis with cobalamin deficiency. This genetic variation within the region following exon 2 of the *HAS2* gene was also recognized by Olsson et al. (2011). However, further studies would be needed to ascertain whether the intron region following exon 2 of the *HAS2* gene plays a role in cobalamin-deficient Shar Peis and/or in Shar Peis with cutaneous mucinosis.

The third part of the study revealed high copy numbers of a 14.3 kb duplication and low copy numbers of a 16.1 kb duplication close proximity to the *HAS2* gene in

cobalamin deficient (based on a decreased serum cobalamin concentration and an increased serum MMA concentration) Shar Peis. Olsson et al. described that a high copy number of the of a 14.3 kb duplication close proximity to the *HAS2* gene was found to be associated with the traditional type Shar Pei. On the other hand a high copy number of a 16.1 kb duplication close proximity to the *HAS2* gene was found to be associated with the thickened skin in the meatmouth type Shar Pei and with Shar Pei fever and cutaneous mucinosis (Olsson et al., 2011). In this current study the copy number assay analysis suggests that cobalamin deficiency in Shar Peis is associated with the traditional type Shar Pei as described by Olsson et al. (2011).

To date, possible associations between cobalamin deficiency and either cutaneous mucinosis or Shar Pei fever in Shar Peis have not been investigated. However, a link between these diseases in a breed classified as being rare (the Shar Pei has been ranked 50$^{th}$ by the American Kennel Club in 2010) cannot be ruled out (http://www.akc.org/reg /dogreg_stats.cfm; accessed November 10$^{th}$, 2011), and therefore, further investigations are warranted.

In conclusion, cSNP analysis revealed a cluster of cSNP markers on canine chromosome 13 that co-segregate with cobalamin deficiency in Shar Peis. Previous results from an association study using the cMSS-2 have pointed to the same region of chromosome 13. The cSNP array analysis and cMSS-2 revealed a region of chromosome 13 that is significantly associated with the combination of an undetectable serum cobalamin concentration and an increased serum MMA concentration. The region identified in the second part of study is approximately 5 Mb downstream of the

previously identified region associated with an undetectable serum cobalamin concentration alone. The findings of this study provide further evidence that a region of chromosome 13 contains one or more genes responsible for this condition in the Shar Pei. Undetectable serum cobalamin and increased serum MMA concentrations in Shar Peis do not appear to be due to a mutation within the three exonic regions of the *HAS2* gene. However, the deletion of the two nucleotides in the intron region following exon 2 of the *HAS2* gene was associated with allele 283 of the microsatellite marker FH3619. Lastly, the copy number assay analysis suggests that cobalamin deficiency in Shar Peis is associated with the traditional type Shar Pei (i.e., the original Shar Peis that originated from China).

# 9. CONCLUSIONS OF RESEARCH OBJECTIVES

In recent history, no other dog breed has grown in popularity and/or population size in such a short period of time as is the case for the Chinese Shar Pei in North America. After being introduced to North America in the 1970s, the breed suffered from rushed breeding carried out by inexperienced breeders. This resulted not only in a dramatically different look for the Shar Pei breed, but also in a large number of health problems. A report from 1991 revealed that Shar Peis have a predisposition for hypocobalaminemia, indicating a likely increased predisposition for cobalamin deficiency.

A comparison of serum cobalamin concentrations between dogs of different breeds indicated that most significantly the Shar Pei, but also the Akita, German Shepherd Dog, and Border Collie had an increased proportion of serum cobalamin concentrations below the detection limit of the assay. Furthermore, undetectable serum cobalamin concentrations were associated with a serum cTLI concentration considered diagnostic for EPI in the Akita, Australian Shepherd, Border Collie, German Shepherd Dog, Cairn Terrier, Cardigan Welsh Corgi, Cocker Spaniel, Dalmatian, West Highland White Terrier, and Wire Fox Terrier. However, in the Shar-Pei, undetectable serum cobalamin concentrations were not associated with serum cTLI concentrations suggestive of EPI.

The Shar Pei has been described as having a high prevalence of cobalamin deficiency and clinical signs are suggestive of severe and longstanding gastrointestinal disease such as diarrhea, vomiting, and/or weight loss. No difference was found in serum concentrations of HA, CRP, and the calgranulins (i.e., calprotectin and S100A12)

between Shar Peis with and without cobalamin deficiency. However, this study also showed that an inflammatory phenotype does exist in some Shar Peis with and some without cobalamin deficiency. In contrast, cobalamin-deficient Shar Peis showed higher serum IgA concentrations and lower serum IgM, albumin, and creatinine concentrations when compared to normocobalaminemic Shar Peis. The findings of this study might suggest that Shar Peis with and without cobalamin deficiency are not associated with other potential diseases in Shar Peis such as cutaneous mucinosis and Shar Pei Fever. However, further studies are needed to determine if serum cobalamin concentrations are affected in Shar Peis with confirmed cutaneous mucinosis or Shar Pei Fever.

Other serum markers of cobalamin-related cellular biochemistry include homocysteine and methylmalonic acid, which can be used to assess intracellular cobalamin availability. A comparison showed that cobalamin-deficient Shar-Peis had a higher serum HCY concentration compared to cobalamin-deficient dogs from six other breeds, and also had a higher frequency of hyperhomocysteinemia than normocobalaminemic Shar-Peis. Cobalamin-deficient Shar-Peis had a 10-fold higher median serum MMA concentration compared to cobalamin-deficient dogs from other dog breeds. In addition, serum HCY and MMA concentrations did not differ between cobalamin-deficient German Shepherd Dogs with and without EPI, a potential cause of secondary cobalamin deficiency. These findings suggest that the functions of the two main cobalamin-dependent enzymes (i.e., methionine synthase and methylmalonyl-CoA mutase) are impaired in cobalamin-deficient Shar-Peis.

The Shar Pei phenotype changed over the last few decades and a survey showed that cobalamin deficiency in Shar Peis was found to occur more frequently in the traditional type (i.e., the original Shar Pei that originated from China) than in the meatmouth type of this breed. However, overlaps between both types existed. Due to the breeding of the Shar Pei in North America, which was aimed at increasing the wrinkles, it is understandable that an overlap between both types (traditional and meatmouth type) existed.

Variable responses to cobalamin supplementation have been reported in human patients that are deficient in intracellular adenosyl- and/or methylcobalamin due to disturbances of cobalamin metabolism at the cellular level. In this part of the study, cobalamin-deficient Shar Peis showed an increase in serum cobalamin concentrations and a decrease in serum MMA concentrations after cobalamin supplementation. Based on human and veterinary studies, an increased serum MMA concentration has been suggested to reflect cobalamin deficiency at the cellular level. Therefore, these data suggest that in Shar Peis with cobalamin deficiency, parenteral cobalamin supplementation does successfully reach the cellular level.

The initial genome scan by Grützner et al. (2010) showed that cobalamin deficiency appears to be hereditary in Shar Peis and is linked to the microsatellite markers DTR13.6 and REN13N11 on canine chromosome 13. The DNA sequence of the MYC_CANFA gene, which represents the closest known gene with a distance of approximately 0.06 Mega bases (Mb) to the microsatellite marker DTR13.6, determined in this present study revealed no differences when compared to the published canine sequence, the cDNA

sequence, and Shar Peis of both groups. Consequently, cobalamin deficiency in Shar Peis does not appear to be related to a mutation of the MYC_CANFA gene according to the genotyping and sequencing results in this study.

Genome-wide scans can be used to identify potential regions on the canine chromosome that are linked with cobalamin deficiency in the Shar Pei. Further sequencing may identify the actual mutation responsible for the condition in this breed. The cSNP analysis revealed a cluster of cSNP markers on canine chromosome 13 that co-segregate with cobalamin deficiency in Shar Peis. As mentioned above, previous results from an association study using the cMSS-2 had pointed to the same region on chromosome 13. The cSNP array analysis and cMSS-2 revealed a region of chromosome 13 that is significantly associated with the combination of an undetectable serum cobalamin concentration and an increased serum MMA concentration. The region identified in this part of study is approximately 5 Mb downstream of the previously identified region associated with undetectable serum cobalamin concentration alone. Thus, the findings of this study provide further evidence that a region of chromosome 13 contains one or more genes responsible for this condition in the Shar Pei.

The *HAS2* gene, is a gene that is located within the genomic region of interest of canine chromosome 13, and has been linked to Shar Peis with Shar Pei fever and cutaneous mucinosis. However, no studies had previously been conducted in Shar Peis with cobalamin deficiency. In our study the undetectable serum cobalamin and increased serum MMA concentrations in Shar Peis do not appear to be due to a mutation within the three exonic regions of the *HAS2* gene. However, the deletion of the two nucleotides

in the intron region following exon 2 of the *HAS2* gene was associated with allele 283 of the microsatellite marker FH3619.

Olsson et al. (2011) had previously linked Shar Pei fever and cutaneous mucinosis to the *HAS2* gene on canine chromosome 13. A high copy number of a 16.1 kb duplication close to the *HAS2* gene was found to be associated with the thickened skin in meatmouth type Shar Peis, while a high copy number of a 14.3 kb duplication close to the *HAS2* gene was suggested to be associated with the traditional type Shar Peis (Olsson et al., 2011). Our copy number assay analysis suggest that cobalamin deficiency in the Shar Pei with or without increased serum MMA concentrations is associated with the traditional type Shar Pei (i.e., the original Shar Peis that originated from China). This might suggest that the markers identified on chromosome 13 are simply markers of coat and skin type in Chinese Shar Peis, rather than markers for cobalamin deficiency. Thus, further investigations of traditional type Shar Peis with cobalamin deficiency are warranted.

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