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Genetische Studie zum Cobalamin-Mangel beim Chinesischen Shar-Pei

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This thesis is dedicated to:

my parents Bernd and Ulrike, my sister Anke, my brother Lars, and my wife Romy

List of abbreviations

Shar Pei	Chinese Shar Pei
µl	Microliter
µg	Microgram
L	Liter
ng	Nanogram
p	*p*-value
F	Forward
R	Reverse
bp	Base pairs
OH-	Hydroxy-group
CANFA	*Canis familiaris*
CN	Cyanide
Mb	Mega base
MSS-2	Minimal Screening Set-2
PCR	Polymerase chain reaction
PIC	Polymorphism information content
CoA	Coenzyme A
MYC gene	v-myc, myelocytomatosis viral oncogene
cM	centi-Morgan
Co	Cobalt
CH_3-	Methyl-group

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I Introduction

Cobalamin (vitamin B_{12}) is a biologically active co-enzyme that is characterized as a water soluble, organometallic molecule with a planar corrin ring, surrounding a central cobalt atom (KOBAYASHI and SHIMIZU, 1999). Cobalamin can only be synthesized by certain fungi, actinomycetes, and a few species of bacteria (QURESHI et al., 1994). Cobalamin affects a wide variety of metabolic processes in many tissues and organs, including cell growth (HALL, 1979) and peripheral and central nervous system function (VOLKOV et al., 2006; SCALABRINO et al., 2000). Furthermore, cobalamin affects both the bone marrow (FYFE et al., 1991) and bone formation (TUCKER et al., 2005). Cobalamin deficiency has been reported to lead to hyperpigmentation and other effects in the skin (VOLKOV et al., 2006; BARTHELEMY et al., 1986) and the oral mucosa (FIELD et al., 1995). In humans, cobalamin deficiency can also lead to cardiovascular and thrombotic diseases, such as portal vein thrombosis (KANBAY et al., 2005; STANGER et al., 2003).

Transport and metabolism of cobalamin are dependent on a number of different classes of proteins, which all can bind the cobalt atom. Intrinsic factor, transcobalamin I and II, membrane receptors, and intracellular binding proteins, such as methionine synthase or methylmalonyl-CoA mutase are examples of these proteins (QURESHI et al., 1994). There are two different cobalamin binding proteins in plasma, transcobalamin I and transcobalamin II, the former of which is present at higher concentrations (FOWLER, 1998). The latter is responsible for transport of cobalamin into cells by pinocytosis. Transcobalamin II is synthesized by amniocytes, fibroblasts, endothelial cells, and enterocytes, and is also present in cerebrospinal fluid, semen, and extracellular fluid (COOPER and ROSENBLATT, 1987). By virtue of the complex interaction of all these cobalamin binding proteins, many physiological functions of the body are affected by cobalamin. With cobalamin malabsorption, depletion of the cobalamin stores may therefore cause malfunction of multiple organ systems (VOLKOV et al., 2006). Inherited disorders of cobalamin homeostasis are commonly reported in humans and may affect absorption, transport, or cellular uptake of cobalamin (FOWLER, 1998).

It has been shown that some humans with juvenile cobalamin deficiency have a mutation of the gene encoding intrinsic factor (TANNER et al., 2005). Another group of patients characterized by an abnormally low serum cobalamin concentration and presenting signs suggestive of neurologic dysfunction, were found to have congenital transcobalamin II deficiency (CARMEL and RAVINDRANATH, 1984). In yet another group of patients, mutations of the cubilin and amnionless genes lead to cobalamin deficiency. These patients have Imerslund-Gräsbeck syndrome, which has been described in both humans and dogs (HE et al., 2003; FYFE et al., 1991a). Cubilin is part of the intestinal cobalamin receptor, which is located on the brush border membrane of ileal epithelial cells, and is responsible for endocytosis of intrinsic factor-cobalamin complexes (KOZYRAKI et al., 1999). Various defects of intracellular cobalamin deficiency have been reported in several different studies and have been designated as cobalamin deficiency type A to G. All these defects result in a deficient function of the two main cobalamin-dependant enzymes, methylmalonyl-CoA mutase and methionine synthase (FOWLER, 1998).

In dogs, selective intestinal cobalamin malabsorption has been reported in a family of Giant Schnauzers (FYFE et al., 1991a). Affected puppies started to show clinical signs of chronic inappetence and failure to thrive between 6 and 12 weeks of age, and were found to have neutropenia with hypersegmentation, anemia with anisocytosis and poikilocytosis, and megaloblastic changes of the bone marrow (FYFE et al., 1991a). There has also been a case report of anemia due to cobalamin deficiency in a Border collie (MORGAN and MCCONNELL, 1999). The dog was found to have erythroblastic anemia, methylmalonic aciduria and low serum cobalamin concentration. Interestingly, these findings in these dogs of Giant Schnauzers were similar to the megaloblastic anemia, proteinuria, and low serum cobalamin concentrations seen in human patients with Imerslund-Gräsbeck syndrome (GRÄSBECK, 2006). Moreover, the so-called canine Imerslund-Gräsbeck syndrome has also been mapped to a region which is homologue to the human form (HE et al., 2003). This syndrome in humans is associated with a selective cobalamin malabsorption by enterocytes, and is caused by a defect of the cobalamin receptor, the process of internalization of cobalamin-receptor complexes, or the transfer of cobalamin to transcobalamin II (HE et al., 2003; BURMAN et al., 1985).

Subnormal serum cobalamin concentrations in Shar Peis were first reported in a conference proceeding (WILLIAMS, 1991). In this report, 21 of 26 Shar Peis evaluated (80.8 %) had subnormal serum cobalamin concentrations. Nineteen of these 21 dogs had serum cobalamin concentrations below the detection limit of the assay (WILLIAMS, 1991). These data suggested that disorders affecting the absorption of cobalamin are overrepresented in this breed (WILLIAMS, 1991). In addition, in another report, 89 of 139 (64.0 %) serum samples from Shar Peis that were submitted to the Gastrointestinal Laboratory at Texas A&M University between 2002 and 2006 had serum cobalamin concentrations below the lower limit of the reference interval (252-908 ng/L) (BISHOP et al., 2007) and 53 of these 139 (38.1 %) serum samples had a serum cobalamin concentration below the detection limit of the assay (BISHOP et al., 2007). This study reported a significant difference between the serum cobalamin concentration in serum samples submitted from Shar Peis, when compared to samples from dogs of other breeds, including German Shepherd dogs, Labrador Retrievers, Boston Terriers, Yorkshire Terriers, Cocker Spaniels, and Beagles (p - value < 0.0001; Figure 1) (BISHOP et al., 2007). However, in the same study there was no statistically significant difference between the mean serum cobalamin concentration of Shar Peis and dogs of the above mentioned breeds, when samples that had results outside the reference interval were excluded from comparison (529.6 ng/L and 500.7 ng/L, respectively; $p = 0.9145$) (BISHOP et al., 2007), suggesting that not all Shar Peis are affected by a putative genetic cause of cobalamin deficiency.

Because of the increased incidence of cobalamin deficiency and the clinical signs previously described in Shar Peis with cobalamin deficiency (WILLIAMS 1991; PETERSON and WILLARD 2003), the objective of this study was to conduct a whole genome scan using the canine minimal screening set-2 (MSS-2) (CLARK et al., 2004) to identify linkage of a microsatellite marker with cobalamin deficiency in the Shar Pei. The MSS-2 is a set of microsatellite markers that has previously been used for whole genome scans in dogs with other diseases, including dermatomyositis in the Shetland Sheepdog (CLARK et al., 2005a), Collie Eye Anomaly (CEA) (LOWE et al., 2003), and pancreatic acinar atrophy (PAA) in the German Shepherd Dog (CLARK et al., 2005b). Except for the latter study, these whole genome scans pointed to a specific linkage of these diseases to a specific chromosomal region.

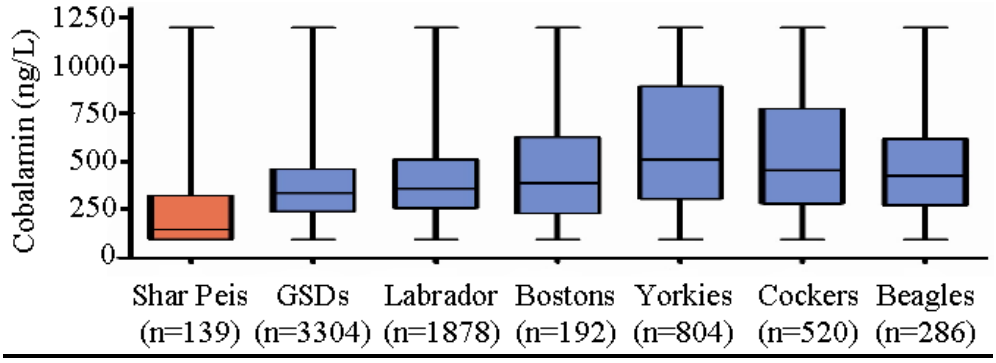

Figure 1: Comparison of the serum cobalamin concentration in dogs of different breeds. The serum cobalamin concentration in samples submitted from Shar Peis was significantly lower than in dogs of six other breeds (*p*-value < 0.0001) (BISHOP et al., 2007).

II Literature review

1. Cobalamin (vitamin B_{12})

1.1 Chemistry of cobalamin

Cobalamin (vitamin B_{12}) represents the most chemically complex vitamin. Karl Folkers and his coworkers first crystallized cobalamin in 1947 (RICKES et al., 1948). The three-dimensional structure was ascertained in 1956 by Hodgkin and coworkers (HODGKIN et al., 1956).

Cobalamin is a water-soluble organometallic molecule with a planar corrin ring surrounding a central cobalt atom. The corrin contains four pyrrole rings in a planar formation, and as such is closely related to the porphyrin ring found in heme and cytochrome. However, with corrin, one pair of adjacent pyrrole rings are linked directly, while all four interpyrroline linkages in porphyrins are achieved through a carbon bridge (Figure 2) (HOGENKAMP, 1975; HUENNEKENS et al., 1982).

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 and last coordination site is variable, and the specific functional groups attached to the cobalt determine the type of cobalamin. Two of the resulting cobalamins, methylcobalamin ($-CH_3$) and 5'-deoxyadenosylcobalamin (5'-deoxyadenosyl-group), have coenzyme activity, and as such are biologically active. Other common functional groups yield cyano- (-CN), aquo- ($-H_2O$), or hydroxocobalamin (-OH). The central cobalt atom exists in different oxidized forms. In cyano-, aquo-, and hydroxocobalamin the cobalt is in the oxidized 3+ state, but cobalamin also occurs intracellularly with cobalt in the 2+ state and 1+ state, called cobalamin (2) and cobalamin (1). To date, the activities of other cobalamins, such as the glutathionylcobalamin complex and sulfitocobalamin, have not been intensively investigated.

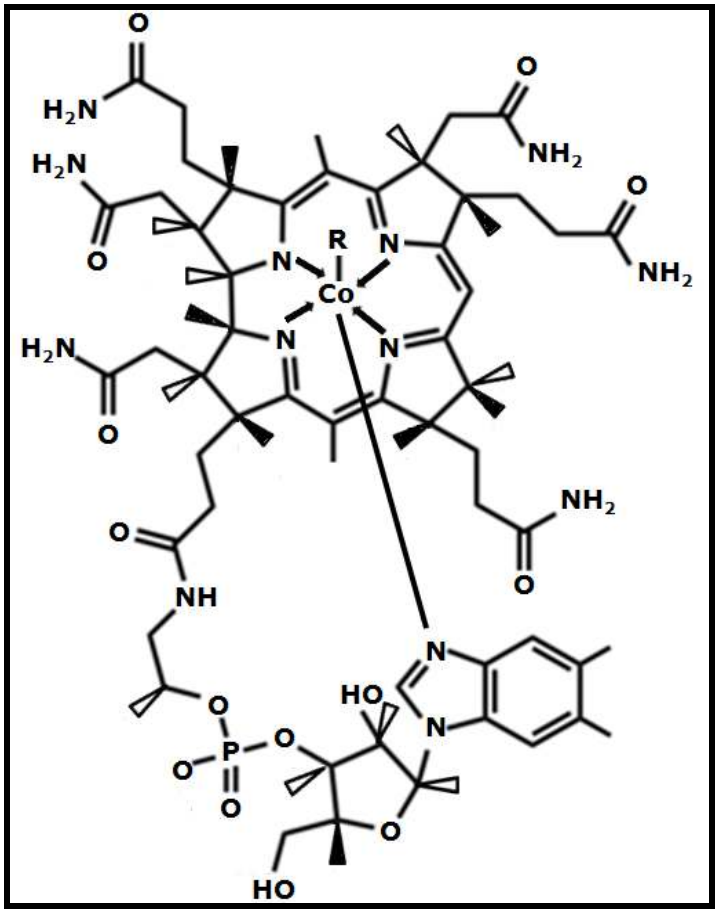

Figure 2. Chemical structure of cobalamin. This figure displays the chemical structure of cobalamin with a planar corrin ring and the central cobalt atom (R= -CN, -H_2O, -CH_3, -OH, -5'-deoxyadenosyl-residue). (Modified from chemical structure of vitamin B_{12} (Azazell0); article: Symptoms of vitamin B_{12} deficiency by Val Tobin (June 6th 2010)).

1.2. Cobalamin sources

Cobalamin cannot be synthesized by animals or plants, as only certain organisms have the enzymes required for the synthesis of cobalamin (BECK, 1982). The synthesis of cobalamin has first been described in detail by Woodward (WOODWARD, 1973) and Eschenmoser and colleagues (ESCHENMOSER and WINTNER, 1977).

Cobalamin can be synthesized by certain fungi (*Streptomyces griseus*), actinomycetes (*Streptomyces aureofaciens*), and bacteria (*Aerobacter* spp., *Agrobacterium* spp., Alcaligenes spp., *Azotobacter* spp., *Bacillus* spp., *Clostridium* spp., *Corynebacterium* spp., *Flavobacterium* spp., *Mycobacterium* spp., *Nocardia* spp., *Propionibacterium* spp., *Proteus* spp., *Pseudomonas* spp., *Rhizobium* spp., *Salmonella* spp., *Serratia* spp., *Streptococcus* spp., and *Xanthomonas* spp.) (MARTENS et al., 2002; ROTH et al., 1996). The industrial production of cobalamin is achieved through fermentation of selected microorganisms, especially *Pseudomonas denitrificans* and *Propionibacterium shermanii* (DE BAETS et al., 2000; WATANABE, 2007). However, other microorganisms, such as *Escherichia coli*, *Lactobacillus leichmanii*, and yeast do not have the repertoire of enzymes to synthesize the corrin ring (BECK, 1982; HERBERT, 1988).

Humans, animals, and plants do not have the ability to synthesize cobalamin, but cobalamin can be absorbed from 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 kidneys and the liver, with a higher concentration in the liver compared to the kidneys (RETEY, 1982), indicating that the main storage of cobalamin in the body occurs in the liver (GLASS, 1959; BEEDHOLM-EBSEN et al., 2010). In humans, the half-clearance time of cobalamin from the body is several months (MARKLE, 1996). In cats, however, it has been

estimated that the half-clearance time of cobalamin is 12-13 days (SIMPSON et al., 2001), whereas in dogs such data have not been reported.

1.3 Absorption and transport of cobalamin

The absorption of dietary vitamin B_{12} and its conversion to 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 intrinsic factor, R-binder, transcobalamin, cell membrane receptors, and intracellular binding proteins.

Hanson characterized three main classes of cobalamin transport proteins (HANSEN, 1990). Firstly, the large molecular size binder protein family, also known as R-binders, comprises transcobalamin I, transcobalamin III, haptocorrin, and cobalophilins. R-binders 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 and ROSENBERG, 1989; COOPER and ROSENBLATT, 1987). Following its ingestion, cobalamin is removed from dietary components and thought to bind to salivary and gastric R-binders (FENTON and ROSENBERG, 1989). Within the proximal duodenal lumen cobalamin is released from these R-binders by proteolytic enzymes from the pancreas. R-binders contain different amounts of carbohydrates and have different isoelectric points due to a variable sialic acid content. In humans different R-binders in the systemic circulation bind about 80 % of the circulating cobalamin, albeit with a slow turnover (FRISBIE and CHANCE, 1993).

Intrinsic factor, also known as S-binder, belongs to the second class of cobalamin transport proteins. Intrinsic factor is a glycoprotein that mediates the uptake of cobalamin from R-binders in the gastrointestinal tract. In humans intrinsic factor is produced exclusively by gastric parietal cells (FENTON and ROSENBERG, 1989),

whereas in dogs, intrinsic factor is mainly secreted from pancreatic acinar cells and to a smaller degree from the gastric mucosa (BATT et al., 1989; SIMPSON et al., 1993). While intrinsic factor binds cobalamin, it does not bind or mediate the uptake of cobalamin analogues, thereby minimizing their accumulation (SEETHARAM and ALPERS, 1985). 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., 1984; GUEANT et al., 2001). The process of cobalamin binding to intrinsic factor, the intrinsic factor-cobalamin receptor, as well as transcobalamin and R-binder, requires a neutral pH and the presence of Ca^{2+} ions (FEDOSOV et al., 2002; ANDERSEN et al., 2010).

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 in the ileum (FYFE et al., 2004). The intracellular release of cobalamin from intrinsic factor is presumed to be upon lysosomal digestion (ROTHENBERG and QUADROS, 1995). However, within the enterocytes cobalamin is never free and is bound either to intrinsic factor or to transcobalamin II (RAMANUJAN et al., 1991). The absorption of cobalamin from the enterocyte into the circulation is accomplished by passage of the basal membrane of the enterocytes, after which cobalamin is bound to transcobalamin II and enters the portal circulation. In humans, it has been reported that transcobalamin II is synthesized in the intestinal villi where the vascular endothelium is abundant (QUADROS et al., 1999). Transcobalamin II represents the third class of molecular cobalamin binders and is expressed by many additional tissues and body fluids (FENTON and ROSENBERG, 1989). More specifically, transcobalamin II is found in cerebrospinal fluid, plasma, semen, and extracellular fluids (COOPER and ROSENBLATT, 1987). It is also synthesized by amniocytes (ROSENBLATT et al., 1987), fibroblasts (BERLINER and ROSENBERG, 1981), endothelial cells, and enterocytes (CHANARIN et al., 1978), as well as by the myocardium, kidneys, spleen, and several other tissues (FERNANDEZ-COSTA and METZ, 1982; HALL et al., 1984). 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 tissues (SEETHARAM and ALPERS, 1985; ALLEN, 1975). After binding of cobalamin, transcobalamin II undergoes a transformation that results in an increased affinity for its cellular membrane receptor. Comparable with the binding to intrinsic factor, this process also requires the presence of Ca^{2+} (NEXO and OLESEN, 1982; YOUNGDAHL-TURNER et al., 1978). 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.

In addition, two alternative mechanisms of cobalamin absorption have been reported in the literature (QURESHI et al., 1994). It has been shown *in vitro* that fibroblasts support the internalization of free cobalamin by use of a saturable and Ca^{2+} independent transport system with an electron transport chain across the membrane (BERLINER and ROSENBERG, 1981). Also, in humans, asialoglycoprotein receptors have been found in hepatocytes and enterocytes (MU et al., 1997; SEETHARAM and ALPERS, 1985). These hepatocytes with asialoglycoprotein receptors are able to clear cobalamin bound to R-protein from plasma and excrete it into the bile (SEETHARAM and ALPERS, 1985).

Cobalamin is an essential co-factor for the cytoplasmic enzymes methionine synthase and methylmalonyl-CoA mutase, which bind more than 95 % of the intracellular cobalamin (MELLMAN et al., 1977; KOLHOUSE and ALLEN, 1977). The 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 and ROSENBERG, 1989). Methionine synthase requires the presents of the co-factor cobalamin as well as a methyl group. The methyl group is derived from the demethylation of 5-methyltetrahydrofolate that leads to its conversion to

tetrahydrofolate, which is the biologically active form of vitamin B_9. 5-Methyltetrahydrofolate, the monoglutamyl form of folate, is required for the re-methylation of cobalamin and binds to methionine synthase. 5-Methyltetrahydrofolate is required for the purine and pyrimidine biosynthesis and is thus important for the *de novo* synthesis of DNA and RNA (Figure 3). Methylcobalamin is the major circulating form of cobalamin that is bound to transcobalamin I, and accounts for 60-80 % of the total cobalamin in human plasma (FENTON and ROSENBERG, 1989).

Methylmalonyl-CoA mutase, the second enzyme requiring cobalamin, catalyzes the final isomerization step in the metabolism of propionyl-CoA to succinyl-CoA in mammalian tissues (CANNATA et al., 1965; CHANDLER et al., 2006). Propionyl-CoA results from beta oxidation of fatty acids. Propionyl-CoA also results from the catabolism of certain amino acids, such as valine, isoleucine, methionine, and threonine. Propionyl-CoA carboxylase and methylmalonyl-CoA racemase aid in the conversion of propionyl-CoA to D-methylmalonyl-CoA and further to L-methylmalonyl-CoA. The subsequent conversion of L-methylmalonyl-CoA to succinyl-CoA can only occur when sufficient amounts of cobalamin are available within the cell. Thereafter, succinyl-CoA is being incorporated in the tricarboxylic acid cycle (Krebs' cycle), which fuels the cellular energy (adenosine triphosphate (ATP)) production (Figure 4). Thus, in summary, cobalamin serves as an essential co-factor for both methyl-CoA mutase and methionine synthase in mammalian cells.

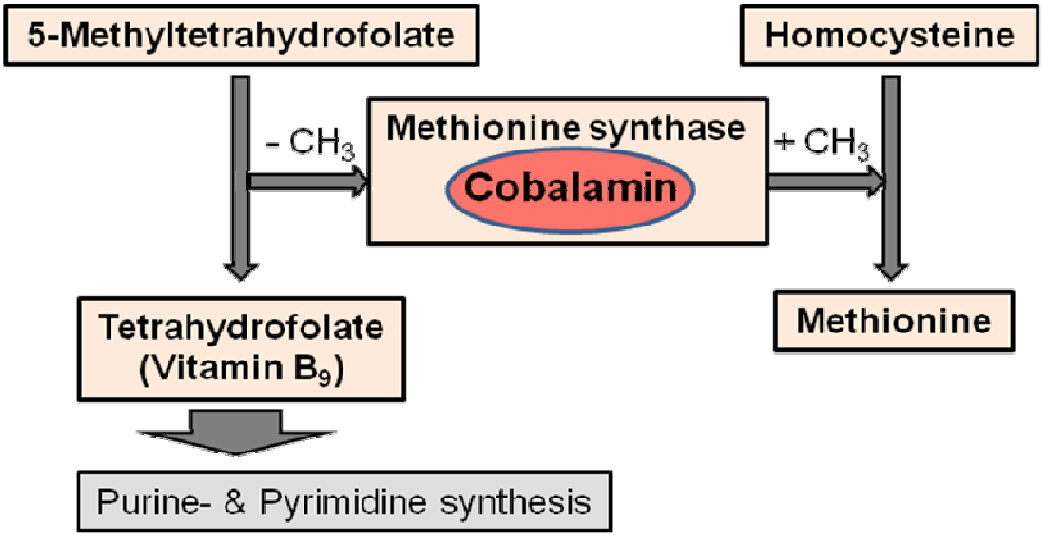

Figure 3. Cobalamin represents an essential co-factor for intracellular methionine synthase:

Methionine synthase is an enzyme that catalyzes the methylation of homocysteine to methionine. For this reaction to occur, methionine synthase requires the presence of the co-factor cobalamin as well as a methyl group, which is derived from the concurrent demethylation of 5-methyltetrahydrofolate to tetrahydrofolate, the biologically active form of vitamin B_9, which is important for purine- and pyrimidine synthesis, and thus for the *de novo* synthesis of DNA and RNA.

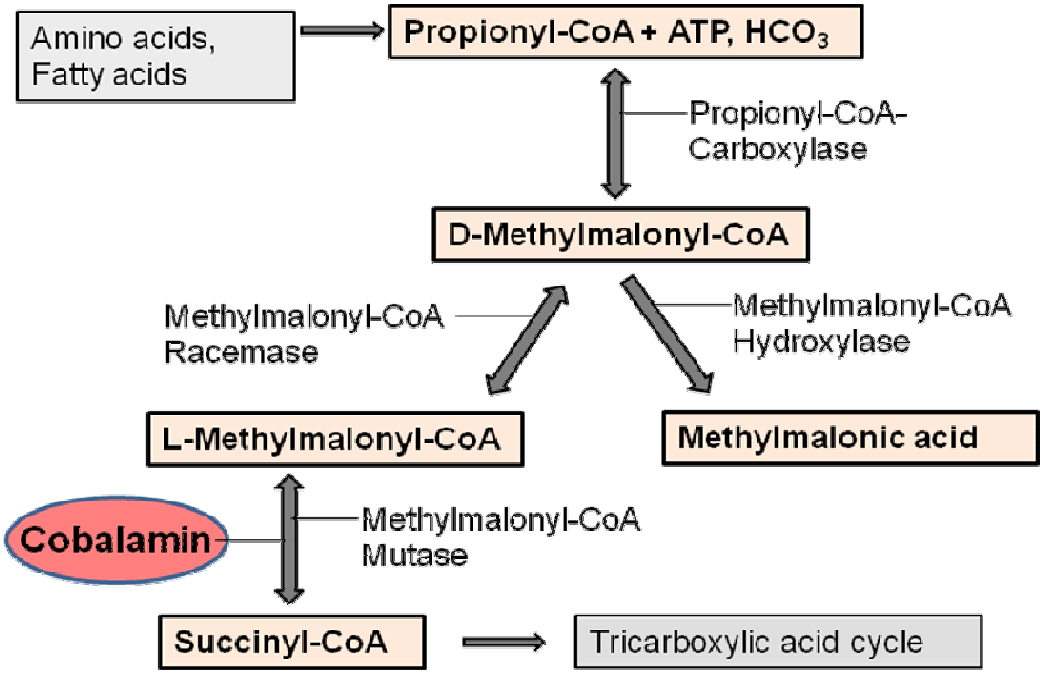

Figure 4. Cobalamin is an essential co-factor for intracellular methylmalonyl-CoA mutase:

Methylmalonyl-CoA mutase is an enzyme that catalyzes the conversion of L-methylmalonyl-CoA to succinyl-CoA, for which it requires cobalamin as a co-factor. L-Methylmalonyl-CoA is a product of propionyl-CoA, which in turn is a product of the beta oxidation of fatty acids and the catabolism of certain amino acids. The conversion of L-methylmalonyl-CoA to succinyl-CoA is an essential step for the incorporation of these catabolic products into the tricarboxylic acid cycle.

2. Disorders of cobalamin absorption, transport and intracellular metabolism

2.1 Disorders of cobalamin absorption and transport

The absorption of dietary vitamin B_{12} 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. However, disorders affecting the aforementioned proteins have been described in both the human and veterinary literature.

2.1.1 R-binder deficiency

There are only a few case reports of human patients with R-binder deficiency in plasma, saliva, and leukocytes (CARMEL et al., 2003). Affected individuals usually have a low serum cobalamin concentration, but no clinical signs of cobalamin deficiency. The reason for the lack of clinical signs of cobalamin deficiency in patients with R-binder deficiency is unclear. Also, serum concentrations of transcobalamin II have been reported to be within the reference interval in these patients (CARMEL, 1982).

2.1.2 Intrinsic factor deficiency

Humans with an inherited intrinsic factor deficiency show megaloblastic pernicious anemia, and have low serum cobalamin concentrations (SHEVELL and 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 transport (FENTON and ROSENBERG, 1989). The cause of the intrinsic factor deficiency has

been an area of intensive research. One suggested pathogenetic mechanism is an increased susceptibility of the intrinsic factor to proteolytic degradation (YANG et al., 1985).

2.1.3 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). About 60 cases of this disease have been reported in humans, and the clinical findings in these patients include megaloblastic anemia, low serum cobalamin concentrations, and proteinuria (GRÄSBECK, 1972 and GRÄSBECK, 2006). Individuals with this disease develop clinical signs of cobalamin deficiency between 1 and 15 years of age. Imerslund-Gräsbeck syndrome is characterized by a normal transcobalamin II concentration, a normal intrinsic factor secretion and function, and a normal gastrointestinal morphology (COOPER and ROSENBLATT, 1987). Following cobalamin injection, these findings such as methylmalonic aciduria and low serum cobalamin concentration completely resolve, with the exception of persistent proteinuria. 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.

2.1.4 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 and ROSENBLATT, 1992). Usually, the symptoms start within the first two months of life. Transport of

cobalamin into the cells is hindered in the absence of transcobalamin II, but due to the presence of R-binder, which can resume some of the function of the missing transcobalamin, and that leads to a normal total circulating cobalamin concentration. However, the absorption of cobalamin is usually affected in these patients, suggesting an essentiality of transcobalamin II to enter the cells (COOPER and ROSENBLATT, 1987; SEETHARAM and YAMMANI, 2003).

2.2 Disorders of intracellular cobalamin metabolism

2.2.1 Introduction

In general, methylmalonic 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 and ROSENBLATT, 1987; FENTON and ROSENBERG, 1978). This is caused by an intracellular failure of the conversion of methylmalonyl-CoA to succinyl-CoA and the accumulation of the metabolite methylmalonic acid in the blood, which in turn is excreted in the urine and can be used as a marker for cobalamin deficiency at the cellular level (Figure 5). In healthy animals, small amounts of methylmalonic acid are found in the blood, urine, and cerebrospinal fluid.

In humans and dogs with methylmalonic aciduria the characteristic laboratory findings include secondary hyperammonemia, which is caused by an accumulation of propionyl-CoA, hypoglycemia, which is due to an impaired mitochondrial transport of glucose phosphate, and increased concentrations of ketones and methylmalonic acid in the urine (COOPER and ROSENBLATT, 1987; FYFE et al., 1991). In human patients, response to parenteral administration of cobalamin is variable. Patients that respond to cobalamin supplementation have been shown to be affected with cobalamin deficiency that only affects the synthesis of adenosylcobalamin in fibroblasts, whereas in individuals that did not respond to cobalamin injections, the synthesis of methylmalonyl-CoA mutase has been demonstrated to be affected. This

difference suggests that different intracellular forms of cobalamin deficiency can lead to methylmalonic aciduria.

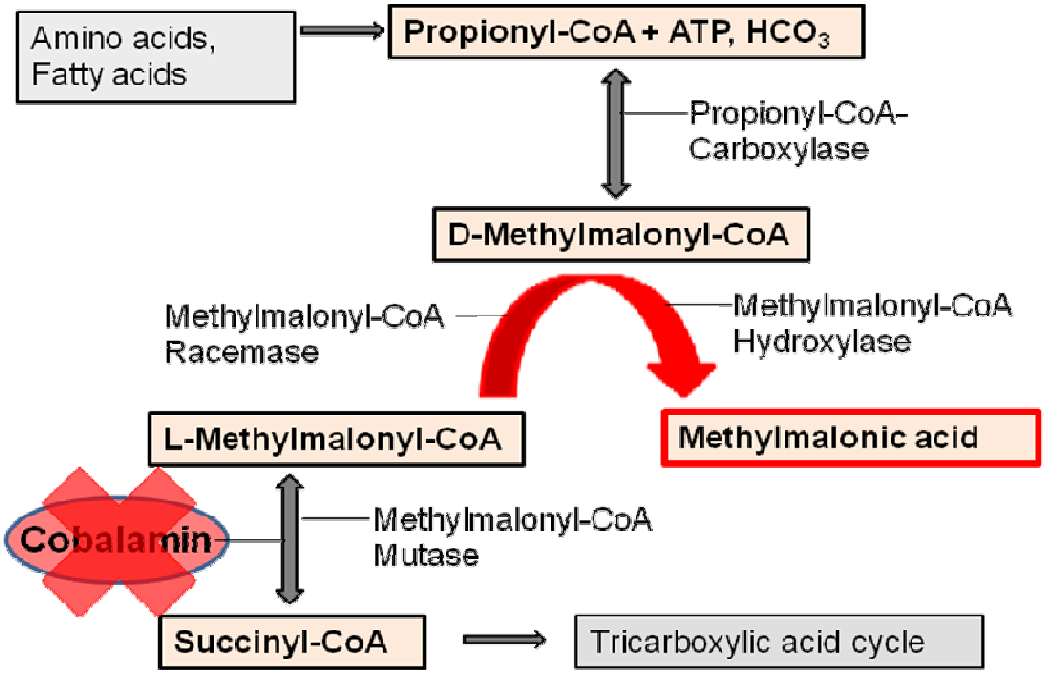

Figure 5. This figure shows the cause of methylmalonic acid accumulation in patients with cobalamin deficiency. A lack of cobalamin leads to a failure to convert L-methylmalonyl-CoA to succinyl-CoA. In turn some L-methylmalonyl-CoA is converted to D-methylmalonyl-CoA (enzyme: methylmalonyl-CoA mutase), which in turn is converted to methylmalonic acid (enzyme: methylmalonyl-CoA hydroxylase).

2.2.2 Metabolic consequences of cobalamin deficiency - Cobalamin A to G diseases

Biochemical studies have described human patients with selective or combined deficiencies of adenosylcobalamin and methylcobalamin, and those with selective or combined deficiencies have been characterized in cultured skin fibroblasts using different methods such as the conversion of propionate to succinate to evaluate the existence of the intermediary enzyme methylmalonyl CoA mutase and to assess the cellular uptake of cobalamin and its conversion to active coenzymes with the aid of a specific cobalamin adenosyltransferase assay (FENTON and ROSENBERG, 1978). Patients with such selective or combined deficiencies have been grouped into 7 independent groups: cobalamin A to cobalamin G disease, which provide insights into the pathway of intracellular cobalamin transport (GRAVEL et al., 1975; WILLARD et al., 1978; SHEVELL and ROSENBLATT, 1992).

Cobalamin A and cobalamin B disease are characterized by a deficient activity of L-methylmalonyl-CoA mutase, which is caused by the deficiency of that cobalamin-dependent enzyme and which leads to methylmalonic aciduria (COOPER and 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 and ROSENBLATT, 1987). In cell extracts from patients with cobalamin A disease usually have a normal adenosylcobalamin synthesis and a decreased accumulation of adenosylcobalamin in intact fibroblasts due to the non-enzymatic reduction of cobalamin (2) to cobalamin (1) followed by the synthesis of adenosylcobalamin (WILLARD et al., 1978; MAHONEY et al., 1975). In contrast, cell extracts and intact fibroblasts from patients with cobalamin B disease were shown to lack adenosylcobalamin, indicating normal cobalamin reduction but a deficiency in cobalamin I ATP-adenosyltransferase (FENTON and ROSENBERG, 1981). Approximately 90 % of patients with cobalamin A disease, but less than 40 % of patients with cobalamin B disease respond to supplementation with cobalamin, indicating a good prognosis for patients with cobalamin A disease compared to those with cobalamin B disease (MATSUI et al., 1983). Finally, a few years ago, the genes responsible for both cobalamin A and

cobalamin B disease in humans have been identified (DOBSON et al., 2002; DOBSON et al., 2002).

Cobalamin C and cobalamin D disease are associated with a decreased synthesis of adenosylcobalamin and methylcobalamin, resulting in both methylmalonic aciduria and homocystinuria (ROSENBLATT and COOPER, 1987). However, serum concentrations of cobalamin are usually within the respective reference intervals because affected patients have normal intestinal absorption and vascular transport of cobalamin leading to a normal total circulating cobalamin concentration (ROSENBERG and FENTON, 1989). Cobalamin C disease has been reported in more than 75 humans and has been suggested to be an inherited disorder (ASPLER et al., 1993; PLETCHER et al., 1991). Symptoms of cobalamin C disease become evident within the first year of life or during adolescence (SHINNAR und SINGER, 1984). The clinical findings in human patients usually are a failure to thrive, lethargy, inappetence, developmental delay, microcephaly, seizures, hypotonia, and hypomethioninemia (COOPER and ROSENBLATT, 1987). Furthermore, hemolytic uremic syndrome (GERAGHTY et al., 1992), hepatic dysfunction (CAOUETTE et al., 1992), and hydrocephalus (PLETCHER et al., 1991) have been documented in these patients. Cobalamin D disease has been demonstrated in two siblings with different clinical pictures (WILLARD et al., 1978). One of the patients was asymptomatic, whereas the older brother of that patient had mental and behavioral abnormalities (GOODMAN et al., 1970). Intracellular cobalamin in fibroblasts from patients with cobalamin D disease were unaffected by cyanocobalamin in the culture medium, indicating an inability to exchange the cyanide group for a hydroxyl group. 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). 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.

A few patients have been described as having cobalamin F disease. One of the cases reported represented 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). In this case a Schilling test revealed a decrease in cobalamin absorption (LAFRAMBOISE et al., 1992). A Schilling test can be used to evaluate the ability of a human patient to absorb cobalamin. This test measures the uptake of orally administered ^{57}Co- or ^{58}Co-labeled cobalamin following the saturation of the cobalamin receptors in the liver by an intramuscular injection of cobalamin. The latter ensures that the majority of orally given cobalamin circumvents the liver and is being cleared from the circulation by the kidneys. Thus, the radioactivity measured in a urine sample within 24 hours after oral cobalamin supplementation theoretically reflects the amount of cobalamin absorbed from the gastrointestinal lumen. This value should be at least 5 % in a human patient with normal cobalamin absorption.

Another patient, 11 years of age, presented with pancytopenia, an increased erythrocyte mean corpuscular volume, a decreased serum cobalamin concentration, an abnormal Schilling test, and methylmalonic aciduria. Intracellular methyltetrahydrofolate was decreased in patients with cobalamin F disease (ROSENBLATT et al., 1986). Incubation of fibroblasts obtained from patients with cobalamin F disease with cyanocobalamin yielded no synthesis of either adenosylcobalamin or methylcobalamin but an abnormal accumulation of free cobalamin within the lysosomes and no megaloblastic anemia or homocystinuria (VASSILIADIS et al., 1991). However, the internalization of cobalamin bound to transcobalamin II was unchanged in cells from patients with cobalamin F disease (ROSENBLATT et al., 1985). Therefore, the defect in patients with cobalamin F disease has been suggested to affect the release of lysosomal cobalamin into the cytoplasm (ROSENBLATT, 1992).

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 and folate concentrations are usually within the reference interval (FENTON and ROSENBERG, 1989). Patients usually present with homocysteinemia and hypomethioninemia, but not with methylmalonic aciduria (COOPER and ROSENBLATT, 1987; ROSENBLATT and COOPER, 1989; WATKINS and ROSENBLATT, 1989). Surprisingly, one patient with cobalamin E disease presented with transient methylmalonic aciduria

(TUCHMAN et al., 1988). Two types of cobalamin G disease have been reported, which are differentiated based on a normal or reduced activity of methionine synthase in cell extracts (WATKINS and ROSENBLATT, 1989). In most cases, the disease presents itself within the first year of life, but it has also been diagnosed in a 21 year old patient (CARMEL et al., 1988). 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).

2.2.3 Methylmalonyl-CoA disease

This disorder is characterized by a defect of the mitochondrial enzyme methylmalonyl-CoA mutase. This enzyme catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA and thus is crucial for the metabolism of odd-chain fatty acids, cholesterol, and branched-chain amino acids to enter the tricarboxylic acid cycle in mammals (BANERJEE, 2003). The defect of methylmalonyl-CoA mutase is characterized by the presence of methylmalonyl aciduria in the absence of homocysteinuria. Also, methylmalonyl aciduria does not resolve upon supplementation with cobalamin. Two different mutant cell lines have been described that lead to this disease (WILLARD and ROSENBERG, 1980). Patients with one of the mutant cell lines showed a detectable methylmalonyl-CoA mutase activity in cell extracts. This was demonstrated by an increased ability to utilize propionate after supplementation with hydroxocobalamin. This mutant cell line was termed mut^{-}. In contrast, the second mutant cell line showed an undetectable mutase activity in cell extracts after hydroxocobalamin supplementation and this mutant cell line was termed mut^{0}. A genetic test has been developed to identify patients with either the mut^{-} or mut^{0} genotypes, and to differentiate them from patients with other causes of cobalamin deficiency (WILKEMEYER et al., 1991).

3. Canine Genetics

3.1 Introduction

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. All the species related to the domestic dog such as coyotes, jackals, and various wolves, are phylogenetically closely related and are thus capable of interbreeding (WAYNE and OSTRANDER, 1999). It has been suggested that the large phenotypic diversity within the dog species represents the result of multiple founding species believed to have all diverged from the wolf (*Canis lupus*). Different studies revealed that the domestic dog is related to the Chinese Wolf (WAYNE and OSTRANDER, 1999) as well as to wolves of East Asian origin (SAVOLAINEN et al., 2002). Results of one study pointed to a single domestication event that would have occurred roughly 15,000 years ago (SAVOLAINEN et al., 2002). Selective breeding by humans over the last centuries has created more than 300 different dog breeds with a clear number of genes that are uniquely characteristic for each breed. These dog breeds represent isolated, inbred populations as most of them have developed more than 250 years ago (OSTRANDER and GINIGER, 1999). Therefore, these breeds demonstrate genotypic and phenotypic homogeneity, giving rise to founder effects and population bottlenecks. One effect of such breeding practices appears to be the large number of genetic diseases described 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 similar to hereditary diseases in humans, and some even share the mutation of a gene that is responsible for the disease (OSTRANDER and GINIGER, 1997; OMIA 2003). These spontaneous mutations in the dog offer the possibility to study canine spontaneous diseases as a model of human diseases (KIJAS et al., 2002).

It has previously been shown that the 1.5X coverage of the canine genome sequence (with 6.22 million sequence reads) indicate that the dog and human genome sequences are more similar to each other when compared to the mouse genome

sequence (KIRKNESS and BAFNA, 2003). The canine genome was sequenced *inter alia* by the National Human Genome Research Institute. One aim of this project was to understand the genetics of hereditary diseases in dogs. One further advantage of studying dogs is that the inter-individual variation in dogs is lower than that in humans, mice, or rats, but similar to that of other domesticated animals (ZAJC et al., 1997). Therefore, the dog was selected as a superior model for genome sequencing instead of some other species such as mice or rats.

In general, the term linkage disequilibrium is defined as a non-random association of alleles of two or more loci. Those alleles can be located on the same chromosome, but this is not always the case. Linkage disequilibrium depicts a situation in which allele combinations or genetic markers occur more or less frequently in a population. A genetic association is dependent upon the respective population, and linkage disequilibrium can extend from 400 to 700 kb in popular dog breeds (e.g., German Shepherd dogs, Labrador Retrievers), whereas it can range from 3 to 3.2 Mb in dog breeds with a smaller population size such as the Shar Pei (SUTTER et al., 2004; GUYON, 2003). In the United States, for instance, Shar Peis appear to be a rare breed, ranked 47th by the American Kennel Club in 2009 (compared for example to position 3 for the German Shepherd dog) (http://www.akc.org/reg/dogreg_stats.cfm; accessed June 29th, 2010).

3.2 Genomic Map

Over the last decade, various maps of the canine genome have been established. Mellersh and colleagues published the first linkage map for dogs in 1997, which at that time included 139 microsatellite markers and 30 linkage groups (MELLERSH and LANGSTON, 1997). Two years later, a specific canine (donor) -rodent (recipient) cell line was used to construct a canine radiation hybrid map (VIGNAUX et al., 1999). That panel comprised 218 markers for genes and 182 microsatellite markers (VIGNAUX et al., 1999). In 2001, Breen and colleagues constructed an integrated linkage-radiation hybrid map of the canine genome (BREEN et al., 2001). These maps and the identified microsatellite markers have been designed for whole genome

scan studies. Ideally, the microsatellite markers should be spread out evenly across all chromosomes. Also, polymorphic information content (PIC) values, which can range from 0.0 (not informative) to 1.0 (very informative), should be considered to determine how informative each of the markers are for an association study.

The first microsatellite marker set that provided coverage of the canine genome was published by Richman in 2001. This set, called the Minimal Screening Set-1 (MSS-1), comprised 172 microsatellite markers, of which 64 were di-, 3 were tri-, and 104 were tetrameric nucleotide markers with an average polymorphic information content (PIC) value of 0.74 (RICHMAN et al., 2001). The MSS-1 had an average spacing of the markers of 10 cM with gaps between markers no greater than 20 cM (RICHMAN et al., 2001). Two years later, an updated radiation hybrid map was published containing a data for 3,270 markers. The distance between these markers was reduced to about 1 Mb (GUYON et al., 2003). This panel represented a more comprehensive screening set than the Minimal Screening Set-2 (MSS-2), which was developed by Clark et al. also for conducting genome-wide scans (CLARK et al., 2004b). A total of 327 microsatellite markers were contained in the MSS-2, and the average spacing was 9 Mb with no gaps larger than 17.1 Mb (Clark et al., 2004b; GUYON, 2003). The nucleotide repeats were distributed as follows: 151 di-, 3 tri-, and 171 tetramers with an average polymorphic information content (PIC) value of 0.73 (Table 1.). It should be noted that one-sixth of the microsatellite markers of the MSS-1 were also contained in the MSS-2 (RICHMAN et al., 2001; CLARK et al., 2004b).

Table 1. Chromosome-specific microsatellite multiplex set (MSS-2)

Chromosome/ Multiplex (1)	Marker (2)	Primer volume (3)	Fluorescent label (4)
1.1	FH3413	0.8	P
	REN112I02	0.8	V
	C01.424	0.8	F
	C00901	0.8	P
	FH2793	0.6	P
	FH2326	1.0	N
1.2	FH3325	0.8	P
	FH3300	0.8	N
	C01.251	0.8	N
	FH2309	0.8	V
	REN143K19	0.6	V
1.3	FH2663	1.1	F
	FH3603	1.5	F
	FH3922	1.1	F
	FH2294	0.3	N
2.1	FH3210	0.8	P
	REN303H07	0.8	V
	REN70M14	0.8	V
	FH3965	0.8	F
2.2	FH2890	0.4	N
	C02.609	0.6	P
	FH2613	0.6	V
	FH2132	0.6	F
2.3	FH2274	0.8	N
	FH2608	0.8	P
	C02.342	0.8	F
3.1	REN161A12	0.6	F
	FH3252	0.6	P
	FH3464	0.6	V
	FH2316	0.8	N
	FH3377	0.6	N
3.2	FH3115	0.8	N
	C03.629	1.2	V
	FH2145	0.8	P
	REN260I04	0.6	F
3.3	FH3396	1.0	F
	FH2302	0.6	V
4.1	REN298N18	0.4	P
	REN303C04	0.4	V
	FH2732	0.8	F
	FH3310	0.8	F
	REN74B13	0.6	V
	AHT103	0.8	F
4.2	FH2776	1.0	P
	REN195B08	0.8	N
	FH2097	0.6	N
	G07704	0.6	V
5.1	FH3004	0.8	F
	DTR05.8	0.8	N
	FH3978	1.0	P
	REN175P10	0.6	V
	CPH14	0.8	N
5.2	FH3928	0.8	P
	FH3320	0.6	N
	FH3702	0.8	F
	FH3089	0.8	V
5.3	FH2140	0.8	P
	REN285I23	0.8	V
	FH3278	0.8	P
	C05.771	0.6	V

Chromosome/ Multiplex (1)	Marker (2)	Primer volume (3)	Fluorescent label (4)
6.1	FH2525	0.8	V
	FH2561	1.4	N
	FH2734	0.8	V
	FH2164	0.8	V
	FH3303	0.5	P
6.2	FH2576	0.6	F
	FH3933	0.8	P
	FH2370	0.8	N
6.3	REN285H12	0.8	F
	FH2119	0.8	N
	REN111L07	0.8	P
7.1	REN97M11	0.8	P
	FH3972	0.6	N
	REN162C04	0.8	V
	REN143L20	0.6	P
	FH2860	0.4	V
7.2	FH2226	0.8	N
	VIASD10	0.8	P
	FH2973	0.8	P
8.1	FH3241	0.8	P
	REN204K13	0.8	N
	FH3316	0.8	V
	C08.618	0.8	F
8.2	FH3425	0.8	N
	C08.410	0.8	F
	REN178J05	0.6	F
	FH2989	1.0	V
9.1	GALK1	0.6	V
	FH2263	0.8	N
	C09.173	0.4	N
	REN54L20	0.4	F
	G06401	0.6	P
	REN287G01	0.6	N
9.2	FH2186	1.4	V
	REN145P07	0.6	P
	FH3835	0.3	F
	REN73K24	0.3	V
	FH2885	0.4	N
10.1	FH2537	0.8	N
	FH4081	0.8	P
	C10.781	0.6	V
	ZUBECA1	0.4	N
	DTR10.5	0.8	F
	FH3381	0.8	P
10.2	REN06H21	0.8	P
	FH2293	0.8	V
	C10.16	0.8	F
	FH2422	0.8	N
11.1	FH3203	0.8	V
	REN242K04	0.8	F
	FH2004	0.6	F
	C11.868	0.8	P
	C11.873	0.8	V
	DGN13	0.8	V
11.2	AHT137	0.3	V
	FH4031	0.8	P
	FH2319	1.0	N
	FH2019	0.2	N

Table 1 – continued: Chromosome – specific microsatellite multiplex set (MSS-2)

Chromosome/ Multiplex (1)	Marker (2)	Primer volume (3)	Fluorescent label (4)
12.1	REN153O12	0.6	F
	FH2401	0.6	V
	FH3591	0.6	N
	G01811	0.6	P
	REN94K11	0.6	N
12.2	REN258L11	0.8	P
	REN213F01	0.8	F
	FH3711	0.8	N
	FH1040	0.6	V
	FH3748	0.8	P
13.1	C13.391	0.8	N
	REN120P21	0.6	F
	FH3619	0.6	P
	DTR13.6	0.6	F
	FH2348	1.2	V
	FH3800	0.6	N
	FH3494	0.8	V
	REN227M12	0.8	P
14.1	FH3951	1.0	F
	FH3725	0.6	P
	FH2658	1.0	P
	FH2763	0.4	N
14.2	C14.866	0.8	F
	FH3285	0.8	P
	PEZ10	0.8	V
15.1	FH4012	0.6	P
	FH3813	0.8	V
	FH2171	0.6	N
	CPH4	0.8	N
	REN230G12	0.6	F
15.2	FH3802	0.6	V
	REN06C11	0.6	F
	FH2360	0.8	P
16.1	REN214L11	0.4	V
	FH2670	0.6	F
	REN73O19	0.6	P
	REN85N14	0.8	P
	FH3592	0.8	V
16.2	FH2155	1.0	N
	REN275L19	0.4	N
	FH2175	1.2	F
17.1	REN240A05	0.6	F
	FH3369	0.6	P
	REN294E18	0.6	V
	FH3995	0.8	F
17.2	FH3047	0.8	P
	FH4023	0.8	P
	PEZ8	1.0	N
	FH2869	0.6	V
18.1	FH4060	1.0	N
	FH3944	0.8	P
	FH3824	0.4	V
	FH3815	0.4	F
	REN54P11	0.8	P
	FH2834	0.4	N
	REN47J11	0.8	V
	AHT130	0.6	F

Chromosome/ Multiplex (1)	Marker (2)	Primer volume (3)	Fluorescent label (4)
19.1	REN213G21	0.6	V
	FH3491	0.4	F
	FH3313	1.0	P
	FH2206	0.8	P
	FH2380	0.6	N
19.2	FH3299	0.6	V
	FH3834	0.6	F
	FH3969	0.6	N
20.1	PEZ19	0.8	N
	FH2951	0.6	F
	FH2158	1.0	P
	REN114M19	0.4	F
20.2	REN55P21	0.8	N
	REN100J13	0.8	P
	REN93E07	0.2	V
	AHTk209	1.0	N
21.1	FH3803	1.0	P
	FH2233	0.8	F
	REN118B15	0.3	V
	FH2441	0.8	N
	REN37A15	0.3	V
	FH3398	0.8	P
	FH2312	0.8	N
22.1	REN42F10	0.8	V
	FH3355	0.8	V
	FH3411	0.8	N
	FH3853	0.8	P
22.2	REN49F22	1.0	N
	REN128E21	1.0	P
	C22.279	0.4	V
	REN78I16	0.6	F
23.1	FH3078	0.8	P
	FH2508	1.0	P
	REN181K04	0.8	P
	REN113M13	0.6	V
	REN02P03	0.8	N
	FH2626	0.8	F
24.1	FH3023	0.8	P
	FH2261	0.8	F
	AHT125	0.8	P
	FH3287	0.8	F
	REN228J19	0.8	V
24.2	FH3750	0.8	P
	FH2159	0.8	N
	REN106I06	0.6	V
	REN272I16	0.4	F
25.1	REN54E19	0.8	F
	FH3245	0.8	P
	FH2324	0.3	N
	FH2141	1.0	N
	FH3627	0.3	V
	FH4027	0.7	F
26.1	REN62M06	0.4	N
	DTR26.9	0.4	V
	FH3426	0.4	V
	DGN10	0.8	P
	FH2130	1.0	N
	C26.733	0.6	F

Table 1 – continued: Chromosome – specific microsatellite multiplex set (MSS-2)

Chromosome/ Multiplex (1)	Marker (2)	Primer volume (3)	Fluorescent label (4)
27.1	FH3221	0.8	P
	PEZ6	0.6	F
	REN181L14	0.4	N
	REN72K15	0.4	V
27.2	FH2289	0.8	P
	PEZ16	0.3	N
	LEI002	0.3	F
	FH3924	0.6	V
28.1	C28.176	0.8	V
	FH3963	0.8	P
	FH2585	0.8	F
	REN146G17	0.8	V
	FH2208	0.8	N
29.1	FH2952	0.8	P
	FH2364	0.8	F
	REN52D08	0.4	P
	REN45F03	0.8	F
	FH2385	1.0	V
	FH1007	0.3	V
30.1	FH3489	0.4	F
	REN51C16	0.4	P
	REN248F14	0.8	V
	FH2290	1.0	N
	FH3632	0.8	P
	FH3053	0.4	F
31.1	FH2189	0.8	N
	RVC11	0.6	V
	REN43H24	0.6	N
	REN109B10	1.2	P
	REN110K04	0.8	P
	FH2712	0.4	F
32.1	REN244E04	0.3	F
	CPH2	0.4	V
	FH2875	0.6	N
	FH3635	1.0	F
	FH3236	0.8	N
	AHT127	0.3	V
	FH3294	0.8	P
33.1	FH2790	0.4	F
	FH3608	0.8	F
	FH2361	0.3	V
	REN186B12	0.6	V
	FH2165	0.8	N
34.1	FH3721	0.8	P
	REN174M24	0.6	F
	REN243O23	0.8	F
	REN314H10	0.4	V
34.2	REN109L16	0.8	N
	FH2377	0.8	V
	FH3836	0.8	N
35.1	FH3570	0.8	F
	REN282I22	0.4	V
	REN94K23	0.6	N
	REN112C08	0.6	P
36.1	REN106I07	0.8	V
	FH2611	0.8	P
	REN179H15	0.8	P
	FH3865	0.8	V
	DTR36.3	0.8	N
37.1	FH3272	0.8	F
	H10101	0.8	V
	REN67C18	0.8	P
	FH3449	0.8	F
	FH2532	0.8	N
38.1	FH2766	0.8	P
	REN02C20	0.8	F
	REN164E17	0.8	N
X.1	FH2916	0.8	F
	REN101G16	1.0	N
	D04614	0.8	F
	REN144O22	0.6	V
X.2	FH3027	0.8	N
	FH1020	0.8	F
	FH2985	0.6	N
	REN230I20	0.6	V
X.3	REN130F03	0.8	F
	FH2584	0.8	N
	REN75A05	0.8	P
Y.1	REN197E16	0.8	V
	REN44K10	0.6	F
	DTRY.13	0.4	F
	REN75H09	0.2	V
	REN173O16	0.2	P

Table 1. This table represents all microsatellite markers (chromosome 1 to Y) of the canine Minimal Screening Set-2 (MSS-2). Column (1) lists the chromosome and multiplex set number within the respective chromosome. Column (2) shows each microsatellite marker. Column (3) represents the volume (in µl) of each forward and reverse primer used for the multiplex PCR. Column (4) lists the fluorescent label (F = 6FAM, P = PET, V = VIC, N = NED) used for the respective marker.

3.3 Linkage analysis

The unique population structure of the dog lends itself to the study of human hereditary diseases, but it should be pointed out that there are many diseases unique to the dogs. However, due to the role of the dog in our society as a guardian and companion, it would also be worthwhile to eliminate specific hereditary diseases of the dog. It has been reported that about two-thirds of hereditary diseases in dogs are transmitted by autosomal recessive traits (OSTRANDER and 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, which allows for subsequent development of a PCR-based test to identify potential carriers and affected animals prior to the onset of clinical signs.

3.4 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 that 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 human patients with symptoms of hereditary cobalamin deficiency or similar conditions in veterinary patients (FYFE et al., 1991a; CARMEL,

2000; FORDYCE et al., 2000). The lack of the classical signs of cobalamin deficiency suggests that other unknown mechanisms may be responsible for this condition in Shar Peis. Thus, instead of a candidate gene approach, recombination mapping strategies, such as classical linkage analysis and linkage disequilibrium analysis, are warranted. Classical linkage studies are used to trace inheritance through the pedigree of a family. Linkage disequilibrium has been suggested for small numbers of unrelated and purebred dogs (HYUN et al., 2003). However, both the candidate gene approach and genome wide scan require the construction of pedigrees.

3.5 Multiplex-PCR based approach

Microsatellite markers have proven to be a valuable tool for linkage analysis, linkage disequilibrium, and forensic investigations in the dog (LEOPOLDINO and PENA, 2003). Microsatellite markers can also be used for the identification of individuals as well as the determination of parentage (such as relationship and haplotype estimation) of a specific breed as well as between breeds (SUTTON et al., 1998; ZAJC and SAMPSON, 1999; MULLER et al., 1999; CLARK et al., 2004a).

Microsatellite profile "genotyping" requires the availability of genomic DNA and an optimized PCR in order to allow for the accurate determination of genotypes. As it would be costly and time consuming to evaluate each single microsatellite marker contained in a microsatellite marker set through a separate PCR reaction, a multiplex PCR suitable for linkage studies has been developed for humans (BEEKMAN et al., 2001), but also for forensic investigations for several other mammals including the dog (KOSKINEN und BREDBACKA, 1999; ALTET et al., 2001; CLARK et al., 2004a). Thus, a multiplex PCR approach for a set of selected microsatellite markers reduces the cost and time to perform a genome scan.

3.6 Data collection methods

In general, the amount of DNA that can be extracted from buccal swabs from dogs is small whereas the amount of DNA that can be extracted from whole blood samples is much larger. It has also been reported that the quality of the extracted DNA depends largely on the method of sampling and the main difference between DNA extracted from whole blood and DNA extracted from buccal swabs is the amount of DNA that is not from the dog being sampled. It has been reported for humans that the proportion of bacterial DNA can exceed 90 % when buccal swabs are being used. Similarly, it has been estimated that most of the DNA comes from bacteria present in the dog's oral cavity or from food remnants when a buccal sample is taken too soon after the dog has eaten (NEUHAUS et al., 2004). This contamination with bacterial DNA can interfere with genetic methods (NEUHAUS et al., 2004). Also, in order to reduce time, effort, and material when performing whole genome scans, a multiplex approach (e.g., using the MSS-2) should be employed. Using this approach, an exact concentration of extracted DNA is required to ensure successful multiplex PCR performance (CLARK et al., 2004b).

More specifically, primer pairs of the MSS-2 were specifically designed to allow for multiplexing of microsatellite markers for each chromosome, and the primers were labeled with different fluorescent dyes (Table 1) based on their location and expected product size. The optimal amount of each primer for the multiplexing approach was determined by Clark et al. and yielded a total of 70 chromosome-specific panels comprising the 327 microsatellite marker of the MSS-2 (CLARK et al., 2004b). PCR products obtained by use of a regular PCR-cycler need to be analyzed by electrophoretic techniques (e.g., capillary gel electrophoresis on a genetic analyzer) against an internal size standard (CLARK et al., 2004b). Standard fragment analysis software programs (such as the Genemapper® software) represent a helpful tool for evaluating the data. To verify the accuracy of the results and define homozygous and heterozygous alleles, this software allows for comparison of genotyping results to the published repeats for the MSS-2 (CLARK et al., 2004b). If additional microsatellite markers are needed to more closely evaluate a specific region, it is important to evaluate these markers with regard to allele appearance and distribution. For example,

the genotyping profiles of the canine MSS-2 microsatellite marker DTR13.6 and of REN13N11, which is not contained in the MSS-2, in Shar Peis demonstrate different homozygous or heterozygous alleles as shown in figures 6 and 7, respectively. The calculation of the polymorphic information content (PIC), which provides information concerning the informativeness of a marker, is crucial for interpretation of the results.

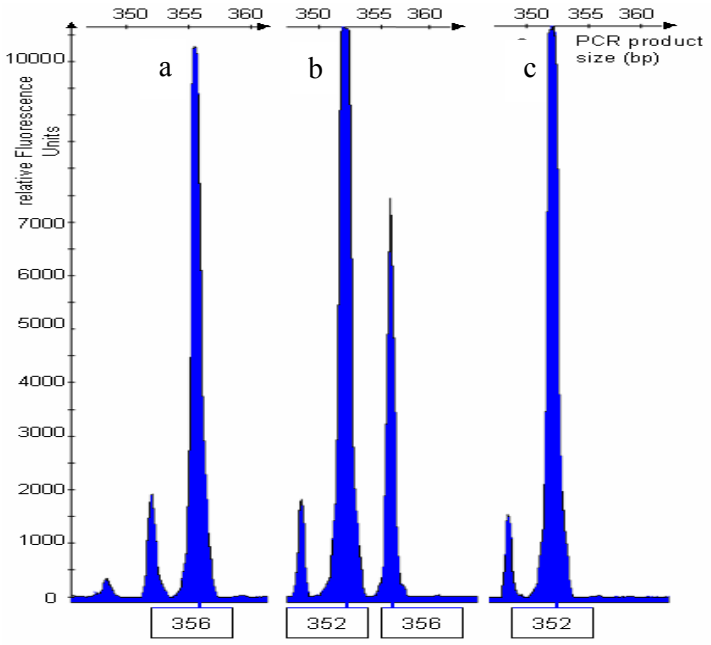

Figure 6: Genotyping peaks for the canine microsatellite marker DTR13.6 from the MSS-2. Results for 3 Shar Peis are shown. Panel **a)** shows a dog that is homozygous for DTR13.6 with the allele 356. Panel **b)** shows a dog that is heterozygous for DTR13.6 with alleles 352 and 356. Panel **c)** shows a dog that is homozygous for DTR13.6 with allele 352 (GRÜTZNER et al., 2008).

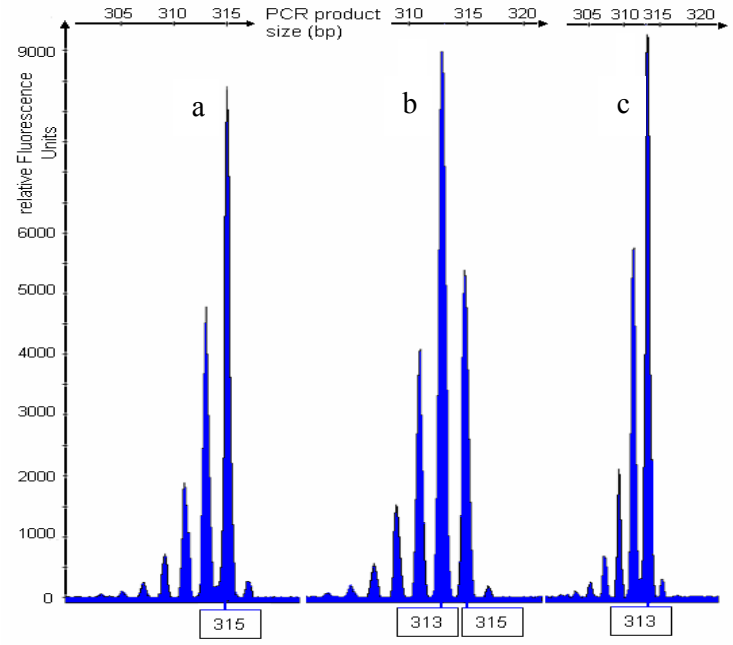

Figure 7: Genotyping peaks of the canine microsatellite marker REN13N11. This marker is not part of the MMS-2. Results for 3 Shar Peis are shown. Panel a) shows a dog that is homozygous for REN13N11 with allele 315. Panel b) shows a dog that is heterozygous for REN13N11 with alleles 313 and 315. Panel c) shows a dog that is homozygous for REN13N11 with allele 313 (GRÜTZNER et al., 2008).

3.7 Use of microsatellite markers

For studies where a specific condition such as cobalamin deficiency, is suspected to be hereditary and the aim of that study is to identify a locus or loci that cosegregate with the disease, performing a genome wide scan using the MSS-2 is considered a suitable approach. 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 MSS-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). Also, linkage disequilibrium analysis has been used to evaluate for linkage as this approach allows for the use of small numbers of unrelated affected as well as unaffected individuals (NORDBORG und TAVARE, 2002; NOLTE and TE MEERMAN, 2001; CLARK et al., 2005b). This method has also been shown to be effective for studying genetic diseases in purebred dog populations (HYUN et al., 2003; SUTTER et al., 2004; CLARK et al., 2005a; CLARK et al., 2006; AWANO et al., 2009).

A reasonable approach for linkage analysis is the evaluation of microsatellite markers located in close proximity to potential candidate genes. For instance, two microsatellite markers have been discovered to be linked to progressive retinal atrophy (PRA) in American Eskimo dogs (MOODY et al., 2005). However, that particular study focused on selected microsatellite markers located close to candidate genes. If the underlying disease process prevents the selection of suitable candidate genes, which is commonly the case, a whole genome scan is necessary. As an example, the linkage study for dermatomyositis in the Shetland sheepdog was performed using a whole genome scan using the MSS-2 (CLARK et al., 2005a). This study discovered one microsatellite marker on canine chromosome 35, which showed significant linkage to dermatomyositis in the Shetland sheepdog (CLARK et al., 2005a). A similar approach was utilized in the study of generalized progressive retinal atrophy (PRA) in Schapendoes dogs (LIPPMANN et al., 2007) and copper toxicosis in Bedlington Terriers (YUZBASIYAN-GURKAN, 1997).

4. MYC gene (v-myc myelocytomatosis viral oncogene)

The MYC gene family encodes a group of transcription factors that control cell proliferation and differentiation (GRANDORI and EISENMAN, 1997; ATCHLEY and FITCH, 1995). Cell proliferation and differentiation are fundamental to growth, development, and also evolution. The genes of the MYC gene family are conserved on certain chromosomes in both clinically normal and diseased animals, but are associated with different binding motifs (MIYOSHI et al., 1991). In cancer patients, the physiologic control of cell proliferation has been shown to be altered due to a dysregulation of the MYC gene as the result of retroviral transduction and insertional mutagenesis, chromosomal translocation, or gene amplification. In turn, such dysregulation leads to increased cell proliferation, impaired cell differentiation, and the potential induction of a preneoplastic state. Thus, dysregulation of the MYC gene may be associated with a variety of malignant neoplasms (COWLEY et al., 1987). Based on these findings, products of the MYC gene represent an important component of physiologic cellular activity as well as neoplastic transformation.

In one study, the human transcobalamin II gene was shown to contain at least one binding site (motif) for the myc protein a product of the MYC gene (REGEC et al., 1995). The identification of these motifs within the transcobalamin II gene in humans may explain the abnormalities of transcobalamin II concentration in patients with 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). Thus, the increased plasma concentration of transcobalamin II in some patients with multiple myeloma could be due to upregulation of transcription of this binding protein by the MYC ligand, a nuclear protein that is believed to regulate transcription and gene expression (REGEC et al., 1995).

However, this association could not be confirmed in two other publications, where transcobalamin II deficiency was seen in the face of a normal total circulating cobalamin concentration (SACHER et al., 1983; KAIKOV et al., 1991). Interestingly, only one case report in human patients described a congenital transcobalamin II deficiency with a low serum cobalamin concentration (CARMEL and

RAVINDRANATH, 1984). An explanation for this finding could be that low serum cobalamin concentrations were due to abnormalities of other cobalamin-binding proteins in the serum.

5. Cobalamin deficiency in Chinese Shar Peis and other breeds

5.1 Cobalamin deficiency in Chinese Shar Peis

Subnormal serum cobalamin concentrations in Shar Peis were first documented in 1991 (WILLIAMS, 1991). In a small group of 26 Shar Peis evaluated, 21 dogs were reported to have subnormal serum cobalamin concentrations and in 19 of these dogs cobalamin was undetectable in the serum (WILLIAMS, 1991). This study led to the hypothesis that Shar Peis have a predilection for cobalamin deficiency. Serum IgA concentrations were also measured in this population, but no association of cobalamin deficiency and IgA deficiency could be identified (WILLIAMS, 1991). In 2007, another study confirmed a high prevalence of cobalamin deficiency in the Shar Pei (BISHOP et al., 2007). In this study, about 64 % of serum samples from Shar Peis submitted to the Gastrointestinal Laboratory at Texas A&M University between 2002 and 2006 had a cobalamin concentration below the lower limit of the reference interval (i.e., < 249 ng/L) and 38.1 % of those dogs had serum cobalamin concentrations below 100 ng/L, the detection limit of the assay (BISHOP et al., 2007). Serum cobalamin concentrations were significantly lower in Shar Peis, with a median of 149 ng/L, compared to dogs of other breeds (median: 415 ng/L). Also, compared to dogs of other breeds, Shar Peis were 7.6 (95 % confidence interval [CI]: 5.4-10.8) 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 (95 % CI: 37.3-83.1) 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.

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 and WILLARD 2003). However, the absorption of dietary vitamin B_{12} (cobalamin) is complex and involves many steps, including gastric release of protein-bound cobalamin, intestinal binding to intrinsic factor, intestinal uptake by a carrier-mediated transport process, intravascular transport, and cellular uptake. It has been shown in the human and veterinary literature that cobalamin deficiency can be associated with R-binder deficiency (CARMEL et al., 2003), intrinsic factor deficiency (SHEVELL and ROSENBLATT 1992), cobalamin malabsorption (GRÄSBECK 2006; FYFE et al., 1991a), and disorders of the intracellular cobalamin processing. However, cobalamin absorption, transport, or intracellular processing have not been studied in detail in cobalamin deficient Shar Peis with clinical signs due to cobalamin deficiency. Hence, the diagnostic approach of measuring only serum cobalamin concentration may be inadequate in Shar Peis as it is not known whether sufficient amounts of cobalamin reach the tissues in these patients. However, measurement of the cobalamin concentration in serum is the most commonly used screening test for cobalamin deficiency in both human and veterinary patients. Evaluation of other biomarkers that are involved in cobalamin metabolism in addition to the serum cobalamin concentration may yield a better understanding of cobalamin deficiency in Shar Peis. Homocysteine and methylmalonic acid (MMA) concentrations in serum, for example, have both been shown to be increased in human patients with cobalamin deficiency (MILLER et al., 2009). Measurement of serum MMA has also been described in dogs (BERGHOFF et al., 2009) and in cats (RUAUX et al., 2009). In both species, an increased serum MMA concentration has been observed with a decreasing serum cobalamin concentration. Thus, the combined assessment of both serum cobalamin and serum MMA concentrations might yield stronger evidence for cobalamin deficiency at the cellular level than the measurement of serum cobalamin concentration alone.

5.2 Cobalamin deficiency in dogs of other breeds

During the last decade, cobalamin deficiency has also been identified in dogs of other breeds than Shar Peis and has been documented in six case reports. For instance, in two juvenile Border Collies, a mild anemia, neutropenia, proteinuria, hyperammonemia, and methylmalonic aciduria were associated with serum cobalamin deficiency (BATTERSBY et al., 2005; MORGAN and MCCONNELL, 1999). Also, a Beagle dog was found to have a low serum cobalamin concentration associated with anemia, leukopenia, and methylmalonic aciduria (FORDYCE et al., 2000). In another study, selective cobalamin malabsorption was described in a group of juvenile Australian Shepherd dogs and was associated with vomiting, diarrhea, and seizures (BATTERSBY et al., 2005). Also, a family of 17 Giant Schnauzers presenting with low serum cobalamin concentrations and methylmalonic aciduria was shown to be affected by a selective intestinal cobalamin malabsorption (FYFE et al., 1991a). In contrast, a Labrador retriever showing neurological signs was found to have methylmalonic aciduria, but this dog did neither have hypocobalaminemia nor hyperammonemia (PODELL et al., 1996). Finally, a Maltese with an increased urinary methylmalonic acid concentration but a normal concentration of serum methylmalonic acid (MMA) presented with hypoglycemia, acidosis, and ketonuria after a period of seizures and stupor. Following a subcutaneous cyanocobalamin injection and feeding of a low fat diet these clinicopathological findings normalized (O'BRIEN et al., 1999). Interestingly, cases of cobalamin deficiency in dogs of other breeds have only been reported from North America and the United Kingdom possibly due to the availability of an MMA assay in these two countries. Also, the clinical pathology findings seen in these breeds were different from those reported in Shar Peis and more closely resembled those in human patients with cobalamin deficiency.

The family of Giant Schnauzers with a selective intestinal cobalamin malabsorption has been shown to be able to serve as a model for cobalamin deficiency in humans (FYFE et al., 1991a). In this group of Giant Schnauzers, normal gastrointestinal function and ileal morphology suggests a selective defect of cobalamin absorption at the receptor level of the ileal enterocytes. Affected puppies showed neutropenia with

hypersegmentation, anemia with anisocytosis and poikilocytosis, and megaloblastic changes of the bone marrow, but no abnormality of intrinsic factor or transcobalamin II function (FYFE et al., 1991a). Interestingly, these findings were similar to the megaloblastic anemia, proteinuria, and low serum cobalamin concentrations seen in human patients with Imerslund-Gräsbeck syndrome (Gräsbeck, 2006). This syndrome in humans is caused by a selective cobalamin malabsorption by enterocytes, and was shown to be due to a mutation of the cubilin gene (called: CUBN) (HE et al., 2003; HAUCK et al., 2008). Cubilin is a membrane associated protein that is part of the receptor for the intrinsic factor-cobalamin complex (KRISTIANSEN et al., 1999). In the group of Giant Schnauzers with cobalamin deficiency evaluated, expression of the intrinsic factor-cobalamin complex receptors in the apical brush border membrane in both the ileal mucosa and the kidneys were demonstrated to be decreased (FYFE et al., 1991a). However, there clinicopathologic findings in cobalamin deficient Shar Peis and cobalamin deficient dogs of other breeds differ, suggesting that Shar Peis have a very unique disorder of cobalamin metabolism.

6. Other diseases commonly diagnosed in Chinese Shar Peis

Two other conditions are reported frequently in Shar Peis, Shar Pei fever and cutaneous mucinosis. Shar Pei fever, describes an autoimmune syndrome that causes periodic flare-ups and is suspected to be hereditary. The second, cutaneous mucinosis, is a disorder characterized by the deposition of excessive amounts of mucin in the dermis of the skin and occurs primarily in Shar Peis. To date, a possible association between Shar Pei fever and/or cutaneous mucinosis and cobalamin deficiency in Shar Peis has not been investigated. However, a link between these diseases in a breed classified as being rare (the Shar Pei has been ranked 47th by the American Kennel Club in 2009) cannot be ruled out (http://www.akc.org/reg/dogreg_stats.cfm; accessed June 29th, 2010).

Shar Pei fever has been shown to be associated with renal amyloidosis and failure in several studies. The clinical signs of affected Shar Peis included vomiting, anorexia, fever, and weight loss (DIBARTOLA et al., 1990; MAY et al., 1992; RIVAS et al.,

1992; CLEMENTS et al., 1995). Amyloid deposits have been found in different tissues such as the liver, spleen, stomach, small intestine, lymph nodes, and the pancreas (DIBARTOLA et al., 1990). In contrast, hepatic amyloidosis without any evidence of renal involvement was reported in another study (LOEVEN, 1994). A syndrome similar to Shar Pei fever has been described in humans and is called Familial Mediterranean Fever (RUPIEPER and HELLER 1991). Both diseases, Shar Pei fever and Familial Mediterranean Fever, are characterized by an increased serum concentration of interleukin 6 (IL-6), an important mediator of fever and the acute phase response (BAYKAL et al., 1993; RIVAS et al., 1992). In a study that investigated if Shar Pei fever and/or renal amyloidosis are inherited diseases similar to Familial Mediterranean Fever in humans, the proportion of affected dogs from different litters ranged between 25 % and 50 % when a phenotypically healthy parent was bred to an affected dog (RIVAS et al., 1993). Dogs affected with Shar Pei fever showed clinical signs such as anorexia and weight loss, which have also been reported in cobalamin deficient Shar Peis. However, serum cobalamin concentrations have never been reported in dogs with Shar Pei fever.

Cutaneous mucinosis is a skin disorder that is commonly seen in Shar Peis and is suspected to be hereditary in this breed (MULLER, 1990; VON BOMHARD and KRAFT, 1998). Clinically, cutaneous mucinosis is associated with signs, such as localized alopecia, hyperpigmentation and ulceration of the skin, and swollen hind limbs (which can extend from the hock to the hip). This condition is usually confirmed by skin biopsies (LOPEZ et al., 1999; ZANNA et al., 2008). Histopathologic evaluation of biopsies from Shar Peis with cutaneous mucinosis and healthy dogs of other breeds revealed an increased involvement of a certain mast cell subtype, characterized by an increased production of chymase, in the pathogenesis of cutaneous mucinosis (WELLE et al., 1999). Lopez et al. suggested a potential association between cutaneous mucinosis and the development of mast cell tumors in the Shar Pei (LOPEZ et al., 1999). A more recent study investigated whether the concentration of serum hyaluronic acid (also known as hyaluronan), the main component of mucin, and the expression of CD44, the major cell surface receptor for hyaluronic acid, are associated with cutaneous mucinosis in Shar Peis. Serum hyaluronic acid concentrations were shown to be significantly higher in Shar Peis

with cutaneous mucinosis than in healthy controls, which suggest that this condition is a consequence of a genetic defect involving hyaluronic acid (ZANNA et al., 2008). However, the fact that there was no correlation between hyaluronic acid concentration in serum and CD44 expression in this study (ZANNA et al., 2008) would indicate a defect in the metabolism of hyaluronic acid rather than an abnormality at the level of its receptor. About a year after this report, the same investigators reported that cutaneous mucinosis in Shar Peis is associated with an increased expression of the hyaluronan synthase gene (ZANNA et al., 2009). Interestingly, a higher production of cell surface hyaluronan has also been documented on mucosal endothelial cells in human patients with inflammatory bowel disease when compared to healthy controls (KESSLER et al., 2008). In this context, 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). Therefore, it is reasonable to hypothesize that cutaneous mucinosis in Shar Peis may be related to cobalamin deficiency in this breed.

The concentration of serum cobalamin concentration has not been reported in any of the above mentioned 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.

III Publication

1. Association Study of cobalamin deficiency in the Chinese Shar Pei

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1.1 Abstract

Cobalamin deficiency is a common disorder in Chinese Shar Peis (Shar Peis) and is thus suspected to be hereditary. The objective of this study was to identify a genomic region or locus with the phenotype of cobalamin deficiency in Shar Peis. Serum cobalamin concentration were measured, and blood for genomic DNA extraction was collected from 14 cobalamin-deficient Shar Peis and 28 Shar Peis with a serum cobalamin concentration in the reference range. The 327 microsatellite markers from the canine minimal screening set 2 and 4 additional markers were amplified by polymerase chain reaction and genotyped by automated capillary electrophoresis. Two microsatellite markers, DTR13.6 ($P = 1.4 \times 10^{-6}$) and REN13N11 ($P = 1.5 \times 10^{-5}$), both on canine chromosome 13, showed evidence of linkage disequilibrium. These findings indicate that the region of chromosome 13 near these markers should be mapped and closely examined for potential mutations associated with this disease in Shar Peis.

1.2 Key words

Chinese Shar Peis, cobalamin, linkage, microsatellite

1.3 Introduction

Cobalamin (vitamin B_{12}) is a water-soluble vitamin that is synthesized by microorganisms and essential for DNA, heme, and fatty acid synthesis and also for intermediate metabolism of sulfur-containing amino acids (Hall 1979; Carmel et al. 2003; Solomon 2007). Cobalamin is absorbed via receptor-mediated endocytosis in the brush border of the ileum (Fyfe et al. 2004). Cobalamin is then transcytosed through the intestinal epithelial cells and into the bloodstream and transported to various cells to act as a cofactor for the functions mentioned above (Fowler 1998; Kozyraki et al. 1999; Solomon 2007). Cobalamin is involved in a variety of metabolic processes in many tissues, including cell growth (Scalabrino et al. 2000) and

peripheral and central nervous system function (Volkov et al. 2006; Scalabrino et al. 2000). Bone marrow (Fyfe et al. 1991) as well as bones (Tucker et al. 2005) are also affected by cobalamin. A variety of gastrointestinal disorders can cause cobalamin malabsorption, which will lead to depletion of cellular cobalamin stores and may result in malfunction of multiple organ systems (Volkov et al. 2006).

Subnormal serum cobalamin concentrations have been reported anecdotally in Chinese Shar Peis (Shar Pei) (Williams 1991; Bishop 2007). Interestingly, over a 4 year period, 64.0 % of serum samples from Shar Peis that were submitted to the Gastrointestinal Laboratory at Texas A&M University for the measurement of serum cobalamin concentration had serum cobalamin concentrations below the lower limit of the reference range (252 - 908 ng/L) (Bishop et al. 2007). Furthermore, 53 of 139 (38.1 %) of all submitted serum samples from Shar Peis had serum cobalamin concentrations below the detection limit of the assay (Bishop et al. 2007).

The authors hypothesize that in the Shar Pei, cobalamin deficiency is a genetic condition. Therefore, the aim of this study was to perform a genome wide scan using the canine minimal screening set-2 (MSS-2) to identify a locus or loci that cosegregate with cobalamin deficiency. The MMS-2 set is a group of microsatellite markers that have been used previously to identify candidate gene regions of interest in canine hereditary diseases (Lowe et al. 2003; Clark et al. 2006; Lippmann et al. 2007). This set of 327 markers provides an average spacing of 9 mega bases (Mb) throughout the canine genome (Guyon et al. 2003; Clark et. al. 2004b). In order to evaluate linkage, linkage analysis was utilized as this approach allows for the use of small numbers of unrelated affected and unaffected individuals (Nordborg and Tavare, 2002; Nolte and Te Meerman, 2002; Clark et. al. 2005). This technique has previously been shown to be effective for studying genetic diseases in pure bred dog populations (Hyun et al. 2003; Sutter et al. 2004; Clark et al. 2005, 2006; Awano et al. 2008).

1.4 Materials and Methods

Sample Collection

Whole blood and serum samples were collected from 42 unrelated Shar Peis over a 4 year period, from various parts of the United States. The pedigree information was evaluated for a minimum of 3 generations whenever possible to ensure that dogs were not closely related. 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, collecting information concerning their dogs, including age, sex, sexual status, health status (including current medications and vaccination status), and the history of any prior supplementation with cobalamin, either in pure form or as part of a vitamin B complex preparation. Food was withheld from the dogs for at least 12 hours before collection of blood samples. Measurements of serum concentrations of cobalamin and canine trypsin-like immunoreactivity (cTLI) were performed at the Gastrointestinal Laboratory at Texas A&M University. The reference range for serum cobalamin and cTLI concentrations have previously been established as 252 - 908 ng/L and 5.7 - 45.2 µg/L, respectively (Gastrointestinal Laboratory at Texas A&M University; http:// www.cvm.tamu.edu /gilab/assays/index.shtml; accessed February 9th, 2009).

The concentrations of serum cobalamin were measured using an automated chemiluminescence assay (Immulite®2000, vitamin B_{12}, Siemens Healthcare Diagnostics Inc., Deerfield, IL) and serum cTLI was measured using a radioimmunoassay (Canine TLI Double Antibody Radioimmunoassay, Siemens Healthcare Diagnostics, Deerfield, IL). The cobalamin assay has a detection limit of 150 ng/L. Dogs with a serum cobalamin concentration < 150 ng/L were considered to be severely cobalamin deficient. In order to avoid including any dogs with mild hypocobalaminemia into the control group, Shar Peis with a serum cobalamin concentration within the reference range but ≤ 400 ng/L were excluded from the control group. A Mann-Whitney test was used to compare the median of age between the two groups of Shar Peis. A Fisher's exact test was used to evaluate whether gender status was associated with cobalamin deficiency. All data was tested for

normal distribution using the Kolmogorov-Smirnov test. Significance was set at $p < 0.05$.

Genotyping

Genomic DNA was isolated from whole blood samples using a commercial kit (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis, MN). Spectrophotometry was used to evaluate purity and quantity of the extracted DNA prior to further analysis. The 327 microsatellite markers of the MSS-2 were used for the genome scan. PCR primers for these markers and multiplex PCR conditions have previously been described (Clark et al. 2004b). PCR was performed using a Mastercycler (Eppendorf North America, Westbury, NY) and the PCR products were analyzed by capillary gel electrophoresis on a genetic analyzer (ABI Prism 3130x Sequencer, Applied Biosystem, Foster City, CA), with an internal size standard (GeneScan 500 LIZ, Applied Biosystem, Foster City, CA) as previously described (Clark et al. 2004b). Standard fragment analysis software (Genemapper 3.5 software, Applied Biosystem, Foster City, CA) was used to evaluate the data. The genotyping results were compared with published repeats (Clark et al. 2004b).

Four other stable microsatellite markers, not included in the canine MSS-2 (REN65A19, RH103480, RH12645, and REN13N11), were chosen to evaluate the region around microsatellite marker DTR13.6 on chromosome 13. These additional microsatellite markers had their forward primer labeled with a fluorescent dye (6FAM, Gene Technologies Lab, College Station, TX) and were amplified and genotyped in all 42 enrolled Shar Peis. Information regarding the canine genome was obtained from the CanFam2.0 assembly (http://www.ncbi.nlm.nih.gov/genome/guide/dog/index.html; accessed February 9th, 2009).

Linkage studies

For each microsatellite marker, the most frequent allele in the group of Shar Peis with an undetectable serum cobalamin concentration was identified, and was compared to the frequency in Shar Peis with a serum cobalamin concentration above 400 ng/L. A Fisher's exact test was used to evaluate the significance of the association between the two variables in a 2×2 contingency table format for single microsatellite markers. Evidence of genetic association was defined as a $p < 1.6\times10^{-4}$ using Bonferroni's correction for multiple comparisons. The PIC (polymorphism information content) value, which can range from 0.0 (not informative) to 1.0 (very informative) was used to determine how informative each of the markers was for the association studies (Clark et al. 2004a). For the microsatellite markers that were not part of the canine MSS-2, the PIC was calculated from the estimated allelic frequencies in different dog breeds (Clark et al. 2004a; Leopoldino and Pena 2003). The PIC value, which can range from 0 (not informative) to 1 (very informative) is a measure of heterozygosity and therefore, represents a parameter to describe the informativeness of a marker used for association studies (Clark et al. 2004a). After the initial genome scan was performed, samples from an additional seven Shar Peis were collected. The genotype data for these 7 dogs were analyzed only for the two microsatellite markers of interest (DTR13.6 and REN13N11).

1.5 Results

Serum cobalamin concentrations were measured in 42 unrelated Shar Peis. Undetectable serum cobalamin concentrations (< 150 ng/L) were found in 14 of these 42 dogs (33.3 %) and these 14 dogs were considered to be severely cobalamin deficient. Serum cobalamin concentrations in the remaining 28 Shar Peis ranged from 400-958 ng/L (median: 608 ng/L). The median age of cobalamin deficient Shar Peis was 7.0 years (range: 2 to 10 years). The median age of the enrolled Shar Peis with serum cobalamin concentrations in the reference range was 4.5 years (range: 2 to 12 years). There was a statistically significant difference between the median age of the two groups ($p = 0.0191$). The gender distribution of the cobalamin deficient Shar Peis

was 6 male and 8 female dogs. Thirteen of the enrolled Shar Peis with normal serum cobalamin concentrations were male and 15 were female. There was no significant association of sex and cobalamin deficiency (p = 1.000). Serum concentrations for canine trypsin-like immunoreactivity (cTLI) in cobalamin deficient Shar Peis enrolled were all within the reference range (5.7-45.2 µg/L). A cTLI concentration within the reference range rules out exocrine pancreatic insufficiency (EPI) in the dog. This was important as EPI has been documented to be a potential cause of cobalamin deficiency (Cooper and Rosenblatt 1987; and Simpson et al. 1989).

Three hundred thirteen microsatellite markers of the canine MSS-2 were genotyped across all 38 autosomes and sex chromosomes in all 42 dogs (14 Shar Peis with an undetectable serum cobalamin concentration and 28 Shar Peis of the control group with a serum cobalamin concentration within the reference range). Eight markers of the canine MSS-2 could not be amplified. Additionally, six markers of the canine MSS-2 could not be consistently genotyped. The amplicons of those 6 microsatellite markers showed a broad peak spectrum with non-distinct allele values.

Following the genome-wide scan with the canine MSS-2, only the microsatellite marker DTR13.6, located at position 28,175,350 - 28,175,702 on canine chromosome 13, yielded significant results (Figure 1). PCR product sizes of 340, 341, 352, and 356 base pairs (bp) were observed. Allele 356 (bp) of this microsatellite marker was seen significantly more frequently in Shar Peis with undetectable serum cobalamin concentrations (19 of 28 alleles, 67.9 %) than in the Shar Peis of the control group (8 of 56 alleles, 14.3 %; Table 1). The genotyped alleles of the microsatellite marker DTR13.6 are shown in Table 2. A Fisher's exact test as a single analysis method and the Bonferroni correction indicated an association for allele 356 (bp) of DTR13.6 with cobalamin deficiency in Shar Peis ($p = 1.4\times10^{-6}$). Four other stable microsatellite markers, that are not included in the canine MSS-2, were chosen to evaluate the region around microsatellite marker DTR13.6. The canine microsatellite markers REN65A19, RH103480, RH12645, and REN13N11 are all located in close proximity to microsatellite marker DTR13.6 on chromosome 13 (Figure 2). Of these, REN65A19, RH103480, and RH12645, revealed no association with cobalamin deficiency in Shar Peis ($p = 8.5\times10^{-2}$, 1.8×10^{-1}, and 1.5×10^{-1}, respectively) based on a

Fisher's exact test with a Bonferroni correction for multiple comparisons. However, allele 315 (bp) of microsatellite marker REN13N11, which is located at position 29,189,076 - 29,189,385 showed a significant association with cobalamin deficiency ($p = 1.5\times10^{-5}$) using a Fisher's exact test and Bonferroni's correction for multiple comparisons.

In fact, 18 of 28 (64.3 %) cobalamin deficient Shar Pei chromosomes carried this allele compared to only 9 of 56 Shar Peis of the control group (16.1 %; Table 1). The genotyped alleles of the microsatellite marker REN13N11 are shown in Table 2. In addition, the *p* - value for genetic disequilibrium of both microsatellite markers DTR13.6 and REN13N11 was computed. An adjusted *p* - value of 0.01 revealed that these two markers form a single haplotype.

To the author's knowledge, there are no previously published reports regarding microsatellite marker REN13N11 in different dog breeds. Consequently, the characteristics of this canine microsatellite marker and the frequency of occurrence of different alleles were evaluated in Shar Peis and in healthy dogs of other breeds (16 German Shepherd Dogs (GSD), 16 White Shepherd Dogs (WSD), and 16 Miniature Schnauzers). Genotyping of the PCR products of REN13N11 showed the same alleles in Shar Peis compared to GSD, WSD, and Miniature Schnauzers, and the alleles identified in all dogs were in the same size range (Table 3). The PIC value for the canine microsatellite marker REN13N11, calculated based on the alleles obtained for the different dog breeds, was 0.79 (Clark et al. 2004a; Leopoldino and Pena 2003). Being relatively close to a value of 1.0, this PIC value indicates a sufficient polymorphism of the microsatellite marker REN13N11 and thus the usefulness of its interpretation in this association study.

A pedigree of a Shar Pei family was investigated with their respective cobalamin concentrations and the contributions of the significant allele 356 of microsatellite marker DTR13.6 and the significant allele 315 of the microsatellite marker REN13N11. We could only obtain samples from the Sire, Dam, and two puppies from one of their litters. The male puppy was diagnosed with cobalamin deficiency and was homozygous for both significant alleles of the respective microsatellite markers,

whereas the female puppy had a normal serum cobalamin concentration and was homozygous for a different allele. The two unaffected Shar Pei parents were both heterozygous with one significant allele present (Figure 3). The result of this analysis would suggest that the cobalamin deficiency in those few Shar Peis may follow an autosomal recessive trait.

After completion of the genome scan with the 42 Shar Peis, seven additional Shar Peis (4 cobalamin deficient, 3 with a cobalamin concentration of the control group) were genotyped for the two microsatellite markers of interest, leading to a further decreased p - value for both DTR13.6 and REN13N11 (7.8×10^{-7} and 2.09×10^{-6}, respectively).

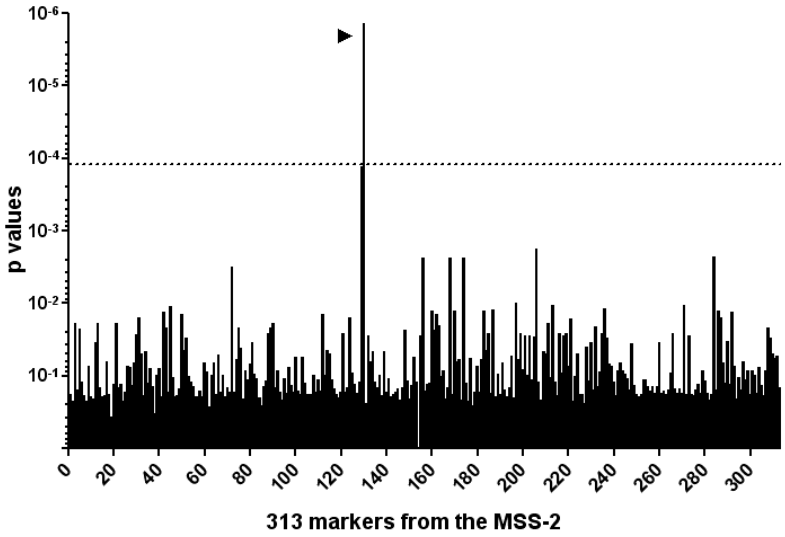

Figure 1: This figure represents the respective *p* - values for all 313 consecutive microsatellite markers computed for the genome wide scan. The arrow indicates the significant *p* - value of microsatellite marker DTR13.6 (1.4×10^{-6}), and the dashed line defines the level for this study using Bonferroni's correction.

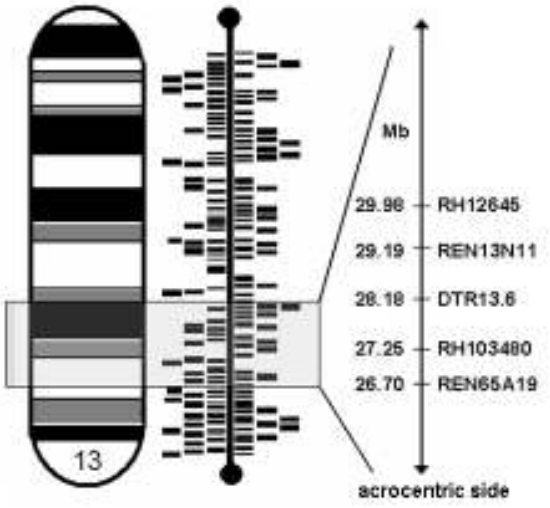

Figure 2: Ideogram of canine chromosome 13. The microsatellite markers REN65A19, RH103480, DTR13.6, REN13N11, and RH12645 are shown at their location on chromosome 13. Their approximate locations to the respective microsatellite marker DTR13.6 are illustrated in mega bases (Mb).

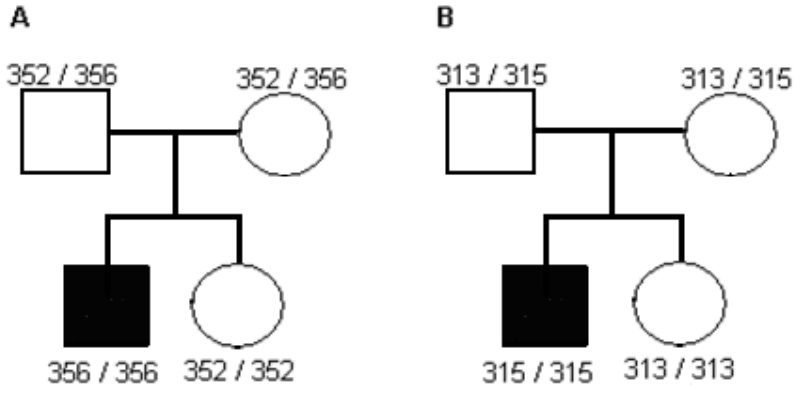

Figure 3: Pedigree of a Shar Pei family. This figure shows a pedigree of a sire and dam and two puppies from one of their litters, characterizing the contributions of the significant allele 356 of microsatellite marker DTR13.6 (**A**) and the significant allele 315 of microsatellite marker REN13N11 (**B**). The allele combination for both littermates being homozygous for both significant alleles is shown (female: normal serum cobalamin concentration and male: cobalamin deficient). The two unaffected Shar Pei parents present with only one of the significant alleles each.

DTR13.6	CD Shar Peis	Control Shar Peis	Σ
Allele 356	19	8	27
other Alleles	9	48	57
Σ	28	56	84
REN13N11			
Allele 315	18	9	27
other Alleles	10	47	57
Σ	28	56	84

Table 1. Observations for alleles 356 bp and 315 bp for microsatellite markers DTR13.6 and REN13N11, respectively, in cobalamin deficient (CD) Shar Peis and control Shar Peis (*p* values of 1.4×10^{-6} and 1.5×10^{-5}, respectively).

DTR13.6	Alleles	CD Shar Peis	Control Shar Peis
Homozygous	356 / 356	7	1
Heterozygous	352 / 356	5	5
Heterozygous	340 / 356		1
Homozygous	352 / 352	2	15
Heterozygous	340 / 352		5
Homozygous	340 / 340		1
	Σ	14	28

REN13N11	Alleles	CD Shar Peis	Control Shar Peis
Homozygous	315 / 315	6	1
Heterozygous	313 / 315	6	6
Heterozygous	315 / 317		1
Homozygous	313 / 313	2	18
Heterozygous	307 / 313		1
Heterozygous	305 / 313		1
	Σ	14	28

Table 2. Genotyped alleles of the two significant microsatellite markers (with the observations for all alleles of DTR13.6 and REN13N11, respectively) in cobalamin deficient (CD) Shar Peis and control Shar Peis.

	Observed Alleles (bp)	Allele Frequency
Miniature	311	43.8% (14/32)
Schnauzer	301	43.8% (14/32)
	307	9.3% (3/32)
	303	3.1% (1/32)
White	309	56.3% (18/32)
Shepherd Dog	307	25.0% (8/32)
	305	9.3% (3/32)
	303	6.3% (2/32)
	301	3.1% (1/32)
German	307	56.3% (18/32)
Shepherd Dog	309	40.7% (13/32)
	305	3.1% (1/32)
Chinese Shar	313	65.5% (55/84)
Pei	315	32.1% (27/84)
	317	1.2% (1/84)
	307	1.2% (1/84)

Table 3. This table shows the observed alleles for microsatellite marker REN13N11 and their respective frequencies in four different dog breeds.

1.6 Discussion

Inherited disorders of cobalamin metabolism in humans may affect absorption, transport, or cellular uptake of cobalamin (Fowler 1998). Humans with juvenile cobalamin deficiency were found to have a mutation of the gene encoding intrinsic factor (Tanner et al. 2005). Another group of human patients showed mutations of the cubilin and amnionless genes, leading to Imerslund-Gräsbeck syndrome (Hauck et al. 2008; He et al., 2005). These two genes code for proteins that form the cubam receptor, which is located on the brush border membrane of the ileal epithelial cells, and they are jointly responsible for endocytosis of the intrinsic factor-cobalamin complexes (Kozyraki et al. 1999; Fyfe et al. 2004). The various effects of cobalamin deficiency are ultimately due to deficient action of the two main cobalamin-requiring enzymes, methylmalonyl-CoA mutase and methionine synthase (Fowler 1998).

In dogs, a selective intestinal cobalamin malabsorption has been reported in a family of Giant Schnauzers (Fyfe et al. 1991). This syndrome is similar to Imerslund-Gräsbeck syndrome (He et al. 2003). Affected puppies show clinical signs between 6 and 12 weeks of age, including neutropenia with hypersegmentation, anemia with anisocytosis, poikilocytosis, and megaloblastic changes of the bone marrow (Fyfe et al. 1991). There has also been a case report of anemia due to cobalamin deficiency in a Border collie (Battersby et al. 2005; Morgan et al. 1999). The dog was found to have erythroblastic anemia and methylmalonic aciduria. Additionally, juvenile selective cobalamin malabsorption in Australian Shepherds and Beagles has been reported (Battersby et. al 2005; Fordyce et. al.2000). These dogs presented with clinical signs of hyperammonemia in one report and hypoglycemia associated with vomiting, diarrhea, and seizure activity in another (Fordyce et al. 2000; Battersby et. al 2005).

Shar Peis with cobalamin deficiency often present with clinical signs of chronic gastrointestinal disease (usually small bowel diarrhea and weight loss), frequently with gastrointestinal protein loss. However, this syndrome has not yet been extensively characterized in this breed. Unfortunately, all evidence is purely anecdotal (Williams 1991; Peterson and Willard 2003; Bishop 2007). Cobalamin deficiency in

Shar Peis appears to differ from other cobalamin deficiency syndromes reported in dogs (Fyfe et al. 1991; Fordyce et al. 2000) and humans (Carmel et al. 2000) where gastrointestinal symptoms have not been described. The lack of the classical signs of cobalamin deficiency (Fyfe et al. 1991; Fordyce et al. 2000; Carmel et al. 2000) suggests that other mechanisms may be responsible for this condition in Shar Peis, such as malabsorption interference or defective transport of cobalamin, and a slow-onset of cobalamin deficiency may lead to a clinical syndrome causing gastrointestinal disease.

The success of linkage studies is crucially dependent on correct assignment of phenotypes. In order to prevent false classification of affected dogs, dogs with serum cobalamin concentrations in the lower range of the reference range (≤ 400 ng/L) and dogs younger than 2 years of age were excluded from the control group. This was done because cobalamin deficiency has been mostly characterized in juvenile dogs (Fyfe et al. 1991; Fordyce et al. 2000; Battersby et al. 2005) and clinical experience (Williams 1991; Peterson and Willard 2003; Bishop 2007) suggests that Shar Peis usually do not develop cobalamin deficiency until later on in life. Thus, our goal was to exclude any dogs with potentially subclinical or preclinical cobalamin deficiency from the control group. This exclusion of young dogs may also explain the discrepancy in ages between the two groups. Furthermore, data collected from the questionnaires regarding the current health status for each dog also suggested that control dogs (> 400 ng/L) enrolled into this study were clinically healthy, which provides evidence that cobalamin deficiency in this breed is mainly manifested in association with gastrointestinal disease.

The linkage analysis approach uses a nonrandom association of alleles, which allows for the use of smaller numbers of affected and unaffected individuals (Nolte and Te Meerman 2002; Nordborg and Tavare, 2002). Several veterinary studies have shown that even small sample sizes are useful for studying genetic diseases in pure bred dog populations (Hyun et al. 2003; Clark et al. 2006; Lippmann et al. 2007; Awano et al. 2008). As a first step, a genome wide scan can be performed using a relatively small number of markers in order to identify regions of association (Sutter et al. 2004) and such an approach has been conducted in this study. However, an association is

dependent upon the respective population, and linkage disequilibrium can extend from 400 to 700 kb in popular breeds (e.g., GSD, Labrador Retriever), whereas it can range from 3 to 3.2 Mb in breeds with a smaller population size such as the Chinese Shar Pei (Sutter et al. 2004; Guyon 2003). In the United States for instance, Shar Peis appear to be a rare breed, ranked 45th by the American Kennel Club in 2008 (compared to position 3 for the GSD). Therefore, in Shar Peis a potentially affected genomic locus, such as a mutant gene, could potentially be identified using the MSS-2 set and may be located up to 3 Mb up- or down-stream of the respective microsatellite marker.

However, this study may not conclusively narrow down the region on chromosome 13 as the major locus or primary gene responsible for cobalamin deficiency in the Shar Pei, because the MSS-2 set contains only 327 microsatellite markers with an average marker spacing of 9 Mb, and thus large gaps may be missed. However, 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 (Qureshi et al. 1994; Tanner et al. 2005). Therefore, further studies to fine map this region as well as the whole genome using a SNP array are warranted.

Allele 356 (bp) of microsatellite marker DTR13.6, which is part of the canine MSS-2, was found significantly more frequently in cobalamin-deficient Shar Peis than in Shar Peis of the control group. This marker is well characterized in other dog breeds, such as the Boykin Spaniel, German Shepherd dog, Shetland Sheepdog, Great Dane, Collie, and Havanese. The published PIC for the microsatellite marker DTR13.6 is 0.65 (Clark et al. 2004b). The PCR product size for this marker ranged from 316 to 356 bp (316, 336, 340, 344, 348, 352, and 356, respectively) (Clark et al. 2004b). We also found an additional canine microsatellite marker (REN13N11) that is not included in the canine MSS-2, whose allele 315 (bp) was also significantly associated with cobalamin deficiency in Shar Peis. Microsatellite marker REN13N11 was evaluated for PCR product size and PIC value. The PIC describes the usefulness of genetic markers for linkage studies when attempting to localize the gene locus on the chromosome, which may be involved in a rare disease (Guo and Elston 1999). Because the PIC values for both canine microsatellite markers that appeared to be

associated with cobalamin deficiency in Shar Peis, DTR13.6 and REN13N11, were similar, both markers are equally informative.

In this study, we present the first evidence of an association in 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. According to the data that is publicly available on the Ensemble Genome Browser, only one gene was identified near the region of the two microsatellite markers on canine chromosome 13. This gene, MYC_CANFA, is located between the two microsatellite markers that showed linkage disequilibrium, which is located approximately 0.06 Mb from microsatellite marker DTR13.6 and 1.01 Mb from REN12N11. Regec et al. (1995) have shown that the human transcobalamin II gene contains at least one binding motif for transacting oncogene products such as c-myc protein, the product of the MYC_CANFA gene. Hence, further investigation is warranted to determine whether the MYC_CANFA gene represents a potential candidate gene for cobalamin deficiency in Shar Peis.

In summary, we present evidence for an association of canine microsatellite markers DTR13.6 and REN13N11, located on chromosome 13, with cobalamin deficiency in Chinese Shar Peis. The future aim is to utilize a finer mapping tool, such as single nucleotide polymorphisms (SNP), to both verify and to fine map this region that is associated with this syndrome in the Shar Pei to locate candidate genes for further investigation.

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1.9 Reference

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IV Discussion

In this association study, serum cobalamin, folate, and cTLI concentrations were evaluated in Shar Peis. Fourteen of the enrolled Shar Peis had an undetectable serum cobalamin concentration. The median serum cobalamin concentration of the 28 Shar Peis with a normal serum cobalamin concentration that were enrolled was 614.5 ng/L and was similar to the median previously reported for 20 healthy Shar Peis (529.6 ng/L) and 61 healthy dogs of other breeds (500.7 ng/L) (BISHOP et al., 2007). Similar to a previous study, serum folate concentrations in cobalamin-deficient Shar Peis (median: 11.2 µg/L) were not significantly different from those in Shar Peis with normal serum cobalamin concentrations (median: 11.9 µg/L; *p* - value: 0.650) (unpublished data and BISHOP et al., 2007). Serum cTLI concentrations in all dogs of both groups (cobalamin-deficient Shar Peis and Shar Peis with normal serum cobalamin concentrations) were within the reference interval for the assay. However, compared to Shar Peis with normal serum cobalamin concentrations, cobalamin-deficient Shar Peis showed significantly higher serum cTLI concentrations (medians: 12.3 µg/L and 19.1 µg/L, respectively; *p* - value: < 0.010; unpublished data). The reason for this finding is not clear and would require further studies of exocrine pancreatic function and pathology in Shar Peis, including pancreatic histopathology in dogs from both groups. However, both medians were within the limits of the reference interval for serum cTLI concentrations and thus this finding does not appear to be of any clinical relevance.

The genetic portion of this study was to identify a genomic region or locus that cosegregates with the phenotype of cobalamin deficiency in Shar Peis. It is generally accepted that the success of genetic linkage studies is dependent on the correct assignment of phenotypes. In order to prevent an erroneous classification of affected dogs, dogs with serum cobalamin concentrations in the lower range of the reference interval (i.e., < 400 ng/L) and dogs less than 2 years of age were excluded as controls. Also, the data collected from questionnaires regarding the current health status of each dog suggested that all dogs enrolled into this association study were clinically and thus phenotypically stable.

In this association study, allele 356 (bp) of the canine MSS-2 microsatellite marker DTR13.6 was found significantly more frequently in cobalamin-deficient Shar Peis than in Shar Peis with a normal serum cobalamin concentration. This marker is well characterized in other dog breeds, such as the Boykin Spaniel, German Shepherd dog, Shetland Sheepdog, Great Dane, Collie, and Havanese. The published PIC of the microsatellite marker DTR13.6 is 0.65 (CLARK et al., 2004). The PCR product sizes for this marker range from 316 to 356 bp (316, 336, 340, 344, 348, 352, and 356 bp, respectively) in different dog breeds (CLARK et al., 2004). We found an additional canine microsatellite marker (REN13N11, not included in the canine MSS-2), for which allele 315 (bp) was also significantly associated with cobalamin deficiency in Shar Peis. Microsatellite marker REN13N11 was evaluated for PCR product size and revealed a PIC value of 0.79. Because the PIC values of both canine microsatellite markers co-segregating with cobalamin deficiency in Shar Peis, DTR13.6 and REN13N11, are comparable, both markers are considered equally genetically informative. This study in Shar Peis identified a critical region on canine chromosome 13 that was associated with cobalamin deficiency, but does not contain any of the genes previously reported to be associated with cobalamin deficiency in dogs or any other species.

The genomic map of a comprehensive database was used to identify genes that are located in close proximity to these two microsatellite markers located on canine chromosome 13, and revealed only one gene, the MYC_CANFA gene at location 28,240,103 - 28,242,545 with a distance of approximately 0.06 Mb to the microsatellite marker DTR13.6. In humans, mutations of this gene have been proposed as a cause of abnormal plasma transcobalamin II concentrations in some patients with malignant disorders and lymphoproliferative diseases, which are believed to be a product of the over expressed myc gene (REGEC et al., 1995). It is interesting to note that there appears to be a homology of portions of this gene and the human transcobalamin II gene (REGEC et al., 1995). In general, past studies have reported consistently normal serum cobalamin concentrations in human patients with transcobalamin II deficiency (FOWLER, 1998; QURESHI et al., 1994). However, a single case report of congenital transcobalamin II deficiency in a human patient was associated with a low serum cobalamin concentration (CARMEL and

RAVINDRANATH, 1984). A possible explanation for this finding could be that the subnormal serum cobalamin concentration reflects abnormalities of other serum cobalamin-binding proteins such as the R-binder. Because it is well known that endogenous cobalamin is usually carried by R-binders and not by transcobalamin II (HALL, 1977), normal serum cobalamin concentrations are to be expected in this condition. Also, this patient had no methylmalonic aciduria, which has been shown a typical consequence of congenital transcobalamin II deficiency (HALL, 1981). The finding of this case report illustrates that transcobalamin II deficiency in humans is more complex than merely a lack of transcobalamin II. The MYC_CANFA gene was sequenced in three Shar Peis with cobalamin deficiency and three Shar Peis with a normal serum cobalamin concentration (GRÜTZNER et al., 2008). No differences in the DNA sequences of the MYC_CANFA gene were identified when compared between the published canine sequence, the cDNA sequence, and between both groups of Shar Peis (GRÜTZNER et al., 2008). Thus, this study suggests that cobalamin deficiency in Shar Peis is not associated with a mutation of the MYC_CANFA gene (GRÜTZNER et al., 2008). However, there might be an additional exon of the MYC_CANFA gene in dogs, which has also been described in humans and cats, which might carry a mutation (MIYAMOTO et al., 2000).

Despite there being 327 microsatellite markers in the canine MSS-2, the average microsatellite marker spacing is 9 Mb (with no gaps larger than 17.1 Mb) (CLARK et al., 2004). Due to those gaps between the microsatellite markers it is possible that an entirely different area or single genes on the canine genome are associated with cobalamin deficiency in Shar Peis. Also, genes encoding for cobalamin-binding proteins and the regions around published genes have not been definitively elucidates in dogs as of yet. Thus it remains unknown whether the locus on canine chromosome 13 represents a major locus for cobalamin deficiency in this breed. A future aim could be to utilize a more detailed mapping tool, such as screening for single nucleotide polymorphisms (SNP), to both verify and to fine map this region that is associated with this syndrome in the Shar Pei as well as locate candidate genes for further investigation.

Cobalamin has been shown to be involved in many enzymatic reactions by mammalian cells (CARMEL et al., 2003). Both human and veterinary studies have suggested that an increased serum methylmalonic acid concentration reflects cobalamin deficiency at the cellular level (RUAUX et al., 2009; BERGHOFF et al., 2009). A combination of the measurement of serum cobalamin and serum methylmalonic acid concentration might therefore be considered to be stronger evidence of cobalamin deficiency at the cellular level than a decreased serum cobalamin concentration alone. This, 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.

Two other conditions have been reported to occur frequently in this breed, Shar Pei fever and cutaneous mucinosis. Shar Pei fever is an autoimmune disorder causing periodic flare-ups of fever. Cutaneous mucinosis is characterized by the deposition of excessive amounts of mucin in the dermis. Hyaluronic acid is the main component of mucin and its concentration was shown to be significantly higher in serum samples from Shar Peis with cutaneous mucinosis than in healthy controls. However, to date, a possible association between Shar Pei fever and/or cutaneous mucinosis and cobalamin deficiency has not been investigated in Shar Peis. Thus, further studies to evaluate a possible connection between these conditions are needed. A logical first step would be to investigate serum cobalamin and methylmalonic acid concentrations in patients with Shar Pei fever and cutaneous mucinosis.

In summary, the association of cobalamin deficiency in Shar Peis with alleles 356 (bp) and 315 (bp) of the canine microsatellite markers DTR13.6 and REN13N11, respectively, will facilitate further evaluation of the genomic loci in close proximity to both of these microsatellite markers on canine chromosome 13. Also, the data suggest that cobalamin deficiency in Shar Peis is not associated with a mutation of the MYC_CANFA gene. However, a phenotypic re-classification based on serum methylmalonic acid concentrations may lead to the identification of a different region of canine chromosome 13 or even on a different chromosome.

V Summary

Cobalamin (vitamin B_{12}) deficiency is a common disorder in the Chinese Shar Pei (Shar Pei), although the severity of clinical sings and the age of onset vary widely. Cobalamin deficiency in the Shar Pei is suspected to be hereditary. Therefore, the objective of this study was to investigate a potential genetic linkage of microsatellite markers to cobalamin deficiency in Shar Peis. Whole blood and serum samples were collected from a total of 42 unrelated Shar Peis. Serum cobalamin concentration was measured for each dog and genomic DNA was extracted from whole blood. The 327 microsatellite markers from the canine minimal screening set-2 (MSS-2) and four additional canine microsatellite markers not contained in the MSS-2, were amplified by polymerase chain reaction (PCR). The PCR products were resolved by automated capillary electrophoresis, and sized relative to an internal standard followed by analysis of genotype data using a commercially available software package. Linkage analysis was conducted by use of a Fisher's exact test, and statistical significance was set at $p < 1\times10^{-4}$.

Undetectable serum cobalamin concentrations (< 150 ng/L) were observed in 14 of 42 dogs (33.3 %) and these dogs were considered to be severely cobalamin deficient. Serum cobalamin concentrations were within the reference range in the remaining 28 dogs. Allele 356 of the microsatellite marker DTR13.6 on chromosome 13 was found significantly more frequently in cobalamin-deficient Shar Peis (19 of 28 alleles, or 67.9 %) than in Shar Peis with a serum cobalamin concentration in the reference interval (10 of 56 alleles, or 17.9 %; $p = 8\times10^{-6}$). Allele 315 of microsatellite REN13N11 on chromosome 13, which is not part of the MSS-2, was also found significantly more frequently in cobalamin-deficient Shar Peis (18 of 28 alleles, or 64.3 %) than in Shar Peis with a normal serum cobalamin concentration (10 of 56 alleles, or 17.9 %; $p = 3\times10^{-5}$). The genomic map of a comprehensive database was used to identify genes that are located in close proximity to these two microsatellite markers on canine chromosome 13, and revealed only one gene, the MYC_CANFA gene, at location 28,240,103 - 28,242,545 with a distance of approximately 0.06 Mb to the microsatellite marker DTR13.6. Primers for the MYC_CANFA gene were chosen to amplify exons I and II, respectively. The DNA sequencing results were

compared between three cobalamin-deficient Shar Peis, three Shar Peis with a normal serum cobalamin concentration, and also with the published cDNA sequences as part of the Ensemble Genomic map. No difference in the entire DNA sequence of the MYC_CANFA gene was found between any of the dogs belonging to either of the two groups, as well as the published canine sequence or the cDNA sequence.

Two microsatellite markers on canine chromosome 13, DTR13.6 and REN13N11, showed significant evidence of linkage to serum cobalamin deficiency in Shar Peis. Also, cobalamin deficiency in Shar Peis does not appear to be associated with a mutation of the MYC_CANFA gene. Further studies are warranted to investigate other genomic loci in proximity to these microsatellite markers on canine chromosome 13 for possible mutations in Shar Peis with cobalamin deficiency.

VI Zusammenfassung

Cobalaminmangel (vitamin B_{12}-Mangel) stellt eine beim Chinesischen Shar-Pei (Shar-Pei) häufig vorkommende Erkrankung dar, wobei das Ausmaß der klinischen Symptomatik sowie das erstmalige Auftreten des Cobalaminmangels beträchtlich variieren. Dem Cobalaminmangel beim Shar-Pei wird eine hereditäre Komponente zugeschrieben. Daher bestand das Ziel dieser Studie in der Identifikation der genetischen Verknüpfung zwischen Allelen von Microsatellitenmarkern und einem Cobalaminmangel beim Shar-Pei. Vollblut- und Serumproben wurden von insgesamt 42 nicht miteinander verwandten Shar-Peis gesammelt. Für jeden in die Studie einbezogenen Hund wurde die Serumkonzentration von Cobalamin bestimmt und die Vollblutprobe zur Extraktion von DNA verwendet. Die 327 Microsatellitenmarker des caninen Minimal Screening Set-2 (MSS-2), sowie vier der nicht im MSS-2 enthaltenen Microsatellitenmarker, wurden mit Hilfe der Polymerasekettenreaktion (PCR) amplifiziert. Die PCR-Produkte wurden anschließend anhand automatischer Kapillarelektrophorese sowie eines internen Standards ihrer Größe nach aufgetrennt, und mit Hilfe einer kommerziell verfügbaren Software analysiert. Die statistische Auswertung erfolgte anhand des Vierfeldertests nach Fisher, wobei die statistische Signifikanzgrenze mit $p < 1\times10^{-4}$ festgelegt wurde.

Vierzehn der 42 Shar-Peis (33,3 %) wiesen keine messbare Konzentration von Cobalamin im Serum (< 150 ng/L) auf und wurden daher der Gruppe von Hunden mit schwerem Cobalaminmangel zugeordnet. Bei den verbleibenden 28 Hunden lag die Serumkonzentration von Cobalamin innerhalb des Referenzintervals. Allel 356 (bp) des auf Chromosom 13 befindlichen Microsatellitenmarkers DTR13.6 wies einen signifikanten Unterschied in der Verteilung zwischen Shar-Peis mit Cobalaminmangel (19 von 28 Allelen oder 67,9 %) und Shar-Peis mit einer physiologischen Serumkonzentration von Cobalamin auf (10 von 56 Allelen oder 17,9 %; $p = 8\times10^{-6}$). Für Allel 315 des ebenfalls auf Chromosom 13 befindlichen Microsatellitenmarkers REN13N11 ergab sich ebenso ein signifikanter Unterschied in der Verteilung zwischen Shar-Peis mit Cobalaminmangel (18 von 28 Allelen, oder 64,3 %) und Shar-Peis mit physiologischer Cobalaminkonzentration im Serum (10 von 56 Allelen, oder 17,9 %; $p = 3\times10^{-5}$). Anhand einer genetischen Datenbank ließ sich nur ein einziges

Gen in der Nähe dieser beiden Microsatellitenmarker auf Chromosom 13 ausmachen. Dieses Gen, MYC_CANFA, befindet sich etwa 0.06 Mb vom Microsatellitenmarker DTR13.6 entfernt in Position 28,240,103 - 28,242,545. Im Anschluss an die Auswahl von Primern für die zwei bekannten Exons des MYC_CANFA Gens wurden diese beiden Exons sequenziert. Die Resultate der DNA-Sequenzierung wurden anschließend zwischen Shar-Peis mit Cobalaminmangel und solchen mit normaler Serumcobalaminkonzentration sowie mit in Datenbanken publizierten DNA-Sequenzen verglichen. Hinsichtlich der DNA-Sequenzen des MYC_CANFA-Gens beider Shar-Pei Gruppen, sowie der für den Hund publizierten DNA-Sequenz, als auch der cDNA-Sequenz waren jedoch keine signifikanten Unterschiede festzustellen.

Für die auf Chromosom 13 befindlichen Microsatellitenmarker DTR13.6 und REN13N11 bestand ein signifikanter Zusammenhang mit dem Cobalaminmangel beim Shar-Pei. Zudem scheint der Cobalaminmangel beim Shar-Pei nicht mit Veränderungen auf dem MYC_CANFA-Gen verbunden zu sein. Weitere Studien zum Cobalaminmangel beim Shar-Pei sind nötig, um genetische Loci in unmittelbarer Nähe dieser beiden Microsatellitenmarker auf Chromosom 13 näher zu untersuchen.

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VIII Table and figure legends

Table and figure legends for the publication

Association Study of cobalamin deficiency in the Chinese Shar Pei

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