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Identifizierung der Basensequenz des Lipoprotein-Lipase-Gens sowie dessen Bedeutung bei der Entwicklung von Hyperlipidämie und Pancreatitis beim Zwergschnauzer

Identification of the nucleotide sequence of the lipoprotein lipase gene as well as its role in the development of hyperlipidemia and pancreatitis in the Miniature Schnauzer

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1. Introduction

Canine gastrointestinal diseases are among the most common disorders encountered in veterinary practice, and pancreatitis in particular is found in dogs at a high incidence (Hänichen and Minkus, 1990). Factors incriminated in the pathogenesis of pancreatitis are numerous, including nutritional imbalances, drugs, pancreatic ischaemia, infectious diseases and hyperlipidemia. Based on the high prevalence of pancreatitis in the Miniature Schnauzer, several authors have also postulated a genetic predisposition for pancreatitis in this breed (Zawie, 1996). Hereditary pancreatitis is a well-known disease in human beings. A variety of different genes can be involved in the development of pancreatitis in humans. The present study evaluated the role of the lipoprotein lipase gene in the development of pancreatitis and hyperlipidemia in the Miniature Schnauzer.

Lipoprotein Lipase (LPL) is one of the key enzymes within the lipid turnover in the blood. In humans, malfunction of the LPL results in decreased clearance of lipoproteins from the blood leading to hyperlipidemia and pancreatitis. According to the high incidence of idiopathic hyperlipidemia in Miniature Schnauzers in the United States a disruption within the lipid metabolism was proposed to be the inciting event of the disease process in Miniature Schnauzers rather than a pancreatic disease itself (Williams, 1996).

This study used the lipoprotein lipase gene as a candidate gene to identify mutations causing hyperlipidemia and pancreatitis in Miniature Schnauzers

2. Literature Review

2.1. Lipoprotein Lipase

2.1.1. Introduction

In 1943 Paul Hahn first postulated the existence of a heparin-releasable clearing factor. He noticed that injection of heparin abolished lipemia after a fatty meal (Hahn, 1943). In 1955 this clearing factor could then be further characterized as a heparin-responsive lipase, termed clearing factor lipase (Afinsen and Boyle, 1952; Korn, 1955). Apolipoprotein C2 (apo C2) was identified as part of very low density lipoproteins in 1966 (Scanu, 1966) and was shown to be an important co-factor for clearing factor lipase. Subsequently, clearing factor lipase was renamed as lipoprotein lipase (EC 3.1.1.34; LPL). The enzyme consists of 3 major domains that are responsible for the interaction with different molecules. The catalytic function of the enzyme is dependant on interactions with proteoglycans of the capillary endothelium as well as lipoproteins and apolipoprotein C2 present in blood (Cryer, 1981; Wang and Hartsuck, 1992).
This chapter describes the structure and function of lipoprotein lipase, which is a key enzyme of lipid turnover in the body.

2.1.2. Function of the LPL

Triacylglycerols (TAGs) are transported in the blood as a component of chylomicrons and very low density lipoproteins (VLDL). Dietary TAGs are absorbed by the small intestine and packed into chylomicrons in the intestinal epithelial cells, whereas TAG that is synthesized in the liver is released into the blood stream as a part of VLDL. TAGs are transported to peripheral tissues. Lipoproteins are too large to cross the capillary endothelium and LPL hydrolyzes TAG thus making non-esterified fatty acids and monoacylglycerol available for tissue uptake.
Further functions of the LPL are facilitation of monocyte adhesion (Mamputu and Desfraits, 1997),

promotion of the proliferation of vascular smooth muscle cells (Mamputu and Desfraits, 2000), and induction of the expression of the tumor necrosis factor-α gene (Renier and Skamene, 1994).

2.1.3. Molecular Interaction and Hydrolysis

The physiological site of action of LPL is the luminal surface of capillary endothelial cells. The adhesion of the enzyme is facilitated by highly charged, membrane bound heparan sulfated proteoglycans (HSPG) (Cryer, 1981; Wang and Hartsuck, 1992). This interaction can be competed out by heparin. Apolipoprotein C2 is part of VLDL and chylomicrons and facilitates the interaction with LPL and also serves as an important co-factor for the hydrolytic action of LPL (Cryer, 1981; Wang and Hartsuck, 1992). After lipoproteins attach to LPL it undergoes a conformational change thereby exposing it's hydrolytic site. Through this action of LPL TAG is separated from the lipoprotein particle and hydrolyzed into non-esterified fatty acids and 2-monoacyl glycerides (Mahmood Hussain and Kancha, 1996), which are now available for tissue utilization. In adipose tissue fatty acids are re-esterified for energy storage. In the heart muscle fatty acids are oxidized to serve as an energy source (Cryer, 1981). Remnants of chylomicrons are transported to the liver where one of their components, apolipoprotein E (apo E), regulates their uptake into hepatocytes. Twelve to fourteen hours after a meal all chylomicrons are cleared from the blood stream (Mahmood Hussain and Kancha, 1996). Remnants of VLDL are called low density lipoproteins (LDL). They contain cholesterol, play an important role for cell membrane stability and are an important source of cholesterol for the synthesis of steroids and bile acids (Fielding and Fielding, 1991). Hydrolysis of lipoproteins is the result of a series of attachments and detachments of LPL to the lipoprotein particle (Eisenberg and Rachmilewitz, 1975). At each locus of attachment up to 40 LPL molecules may act simultaneously on a lipoprotein substrate, maximizing the rate of hydrolysis (Scow and Olivecrona, 1977). In addition, it has been shown, that LPL itself can dissociate from the endothelium or may remain attached to the remnant particle (Saxena and Witte, 1989; Vilella and Joven, 1993). This displacement of functional LPL prevents an oversupply of fatty acids to peripheral tissues under conditions of excessive biolysis (Braun and Severson, 1992).

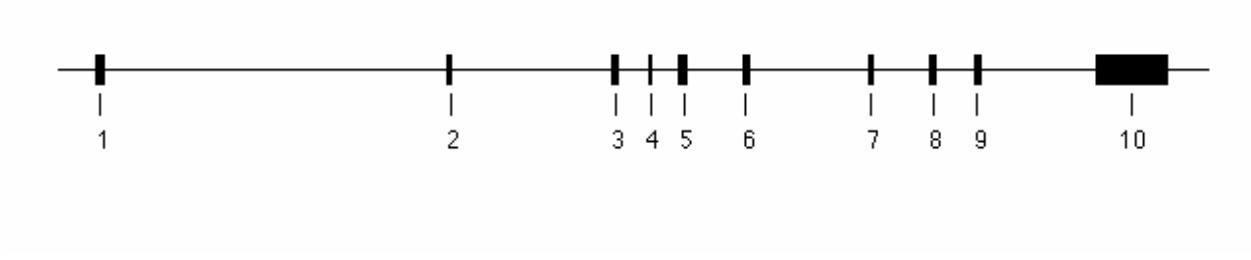
2.1.4. LPL Gene

Lipoprotein lipase is an extra hepatic enzyme synthesized in adipose tissue, heart muscle, skeletal muscle, and the lactating mammary gland (Braun and Severson, 1992; Camps and Reina, 1990; Camps and Reina, 1991). The genetic sequence of the LPL gene shares many similarities with genes encoding for classical pancreatic and hepatic lipase, and it is hypothesized that all 3 derive from a common ancestral gene (Hide et al., 1992).

The LPL gene has been sequenced in a number of species including human, guinea pig, mouse, rat, chicken, baboon, ox, sheep, pig, and fish (Enerback and Gimble, 1993; Oku and Ogata, 2002; Raisonnier and Etienne, 1995). Homology of the primary protein sequence of LPL between different mammalian species is in excess of 90%, except in the case of the guinea pig, where the homology with LPL in other mammalian species is 80%. Comparison of the avian and mammalian sequence of LPL shows a slightly lower degree of homology of 70%. On the basis of nucleotide sequence, the homology of the LPL gene among different species of mammals is 77–82% and that between birds and mammals 61% (Enerback and Gimble, 1993; Raisonnier and Etienne, 1995).

The LPL gene consists of ten exons and nine introns. The size of the individual exons is highly conserved across species (Enerback and Gimble, 1993; Raisonnier and Etienne, 1995). The human LPL gene is more than 30kb long and is situated on chromosome 8p22 (www.ncbi.nlm.nih.gov/entrez). The major part of the genetic sequence is formed by the nine introns with a 9kb intron being one of the longest introns (fig. 2.1). Exon 1 – 9 encode for a protein with 475 amino acids, whereas exon 10 forms the untranslated 3' end.

Figure 2.1: Structure of the human LPL gene: Exons 1 - 10 are interrupted by introns of various sizes



2.1.5. Synthesis and Structure of Lipoprotein Lipase

Although LPL-mediated hydrolysis occurs at the capillary endothelium no mRNA could be identified within endothelial cells. However, mRNA was present in many other cell types, such as adipose tissue, skeletal muscle, heart muscle, and the lactating mammary gland (Braun and Severson, 1992; Camps and Reina, 1990; Camps and Reina, 1991). LPL is also synthesized by hepatocytes during the fetal stage, but its production is suppressed shortly after birth (Staels and Auwerx, 1992). Therefore, LPL is referred to as an extra hepatic enzyme.

The initial step in LPL synthesis is the transcription of the LPL gene in the nucleus of parenchymal cells. Translation of the LPL mRNA into a nascent polypeptide occurs in the rough endoplasmatic reticulum (ER), followed by post-translational processing. During translation of the polypeptide chain, glycosylation is initiated. During transport of the protein to the Golgi apparatus this oligosaccharide is further modified. N-linked glycosylation is crucial for the catalytic activity of the mature enzyme. After sorting of the enzyme in the Golgi apparatus the peptide is directed to secretory vesicles, from where it either gets passed on to lysosomes for degradation or to the parenchymal cell surface where it binds to HSPG (Ben-Zeev and Mao, 2002; Braun and Severson, 1992). LPL is ultimately translocated to HSPG binding sites on the luminal surface of the capillary endothelium. Two complex oligosaccharide chains form the majority of the carbohydrates of the mature enzyme, which has an overall carbohydrate content of 12 % (Masuno and Schultz, 1991; Vannier and Ailhaud, 1989).

The three dimensional structure of LPL has been modeled based on the crystal structure of classical pancreatic lipase (Van Tilbeurgh et al., 1994; Winkler and D'Arcy, 1990). In its active form, human lipoprotein lipase is a homo dimer and consists of 448 amino acids. The enzyme contains a large N-terminal domain (312 amino acids) and a small C-terminal domain (135 amino acids) that are connected by a flexible region. Binding to the lipoprotein substrate is mediated by the C-terminus, whereas catalysis is a function of the N–terminal portion of the enzyme. Functionally important are the highly conserved active site triad (Ser 132, Asp 156, His 241), the oxyanion hole (Trp 55, Leu 133), a polypeptide lid (residues 216-239) and a β5 loop (residues 54-64) (Dugi and Dichek, 1992; Faustinella and Smith, 1992). Based on these findings Mead et. Al. derived a model, in which the

access of the substrate to the catalytic site is blocked by the polypeptide lid. When the lipoprotein substrate binds to LPL a conformational change is induced that leads to an opening of the lid. Additionally, the β5 loop folds back, making the active site even more accessible and also bringing the oxyanion hole into position. These changes increase the hydrophobicity of the catalytic triad attracting fatty acid side chains of potential substrate molecules. The conformation of LPL forces TAG into a position that makes the glycerol backbone accessible to the oxyanion hole for hydrolysis (Mead et al., 2002). Similar models have been described for classical pancreatic lipase as well as other lipases (Derewenda and Brzozowski, 1992; Grochulski and Li, 1993).

Several heparin binding sites have been identified in both subunits of the enzyme. Most important sites in the N-terminus are in the regions 279–282 and 292–304. About 50 different binding sites have been identified in the C-terminal region with Lys 319, Lys 403, Arg 405, Lys 407 and Lys 413 being most important (Berryman and Bensadoun, 1993; Hata and Ridinger, 1993; Lookene and Nielsen, 2000).

For maximal activity LPL requires apolipoprotein C2 (apoC2) as a co-factor. A charge/charge interaction has been proposed between the two proteins (Fielding and Fielding, 1976). The apoC2 binding site is located at Lys 147/Lys 148 in the N-terminal subunit (Murthy et al., 1996; Yang and Gu, 1989).

Five disulfide bridges are formed by ten cysteine residues (Cys 27 - Cys 40, Cys 216 - Cys 239, Cys 264 - Cys 283, Cys 275 - Cys 278, Cys 418 - Cys 438) (Raisonnier and Etienne, 1995). Other amino acids important for dimerisation of LPL are Ala 176, Gly 188, and Gly 195 (Hata and Ridinger, 1992; Keiper and Schneider, 2001).

Mutations of the LPL gene in regions encoding for most of these functional domains can lead to LPL malfunction. The following section explores the effect of LPL gene mutations on the lipid turnover.

2.1.6. Regulation of LPL Gene Expression

LPL tissue expression is regulated based on specific metabolic demands. During lactation LPL expression is shifted from the adipose tissue to the lactating mammary gland, whereas feeding results in an increase in enzyme synthesis in adipose tissue and a decreased synthesis in muscular tissue

(Hamosh and Clary, 1970; Lithell and Boberg, 1978). A variety of hormones are responsible for changes in LPL expression and activity, including insulin, catecholamines, growth hormone, and estrogen.

Altered LPL expression is also found in patients with certain disease states, such as atherosclerosis, diabetes, cachexia, or infection (Beauchamp and Renier, 2002; Michaud and Renier, 2001; Sartippour and Lambert, 1998).

2.1.7. Pathophysiological Importance of LPL

Due to its central role in the lipid turnover LPL is involved in the pathogenesis of several diseases. Hyperlipidemia is characterized by insufficient clearance of lipoproteins from the blood. Increased serum concentrations of TAGs result in clinical abnormalities, such as abdominal pain or hepatomegaly, or clinical syndromes such as pancreatitis, xanthoma, and/or lipemia retinalis. Major causes include familial LPL deficiency, untreated diabetes mellitus, certain pharmaceutical agents, and alcohol abuse (Santamarina-Fojo, 1998).

Obesity is a complex disorder that involves multiple factors including genetic, metabolic, and behavioral factors. The central role of LPL in lipid metabolism and it's effect on energy storage and utilization have been described by various authors (Eckel, 1989; Greenwood, 1985; Kern, 1997), indicating that LPL is one of several factors in this multifactorial disorder of obesity.

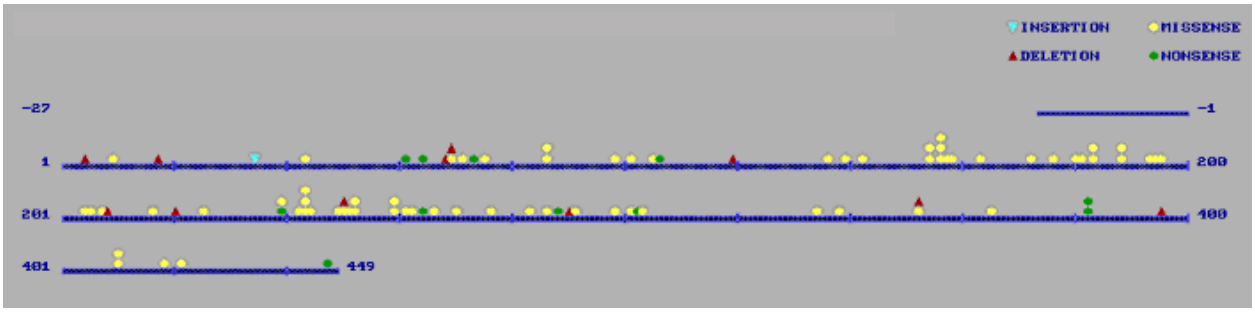
Alzheimer's disease, atherosclerosis, as well as dyslipidemia associated with diabetes mellitus, insulin resistance, infection, or cancer are other disease conditions where LPL is thought to play an important role (Baum and Chen, 1999; Hardardottir and Grunfeld, 1994; O'Brien and Gordon, 1992; Renier and Skamene, 1993; Tisdale, 1999).

2.1.8. Mutation of the LPL Gene and Familial LPL Deficiency

A total of 107 different mutations of the LPL gene have been described in humans, with 71 of these mutations resulting in complete absence of LPL activity and the other 36 leading to partial LPL deficiency with less severe hyperlipidemia (Human Gene Mutation Database (HGMD),

http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). Nonetheless, complete LPL deficiency is a rare condition (1/1 Mio worldwide) in humans. Partial LPL deficiency on the other hand is common (3 – 5%) in populations of European descent and due to alterations in the lipid profile may, as was mentioned earlier, lead to conditions such as atherosclerosis (Bijvoet and Gagne, 1996; Jukema, 1996; Reymer and Gagne, 1995). The majority (79) of these 107 mutations are missense or nonsense substitutions, 7 substitutions affect splicing, and while others are characterized as deletions or insertions. Mutations have been identified all across the LPL gene (fig. 2.2) with a tendency to cluster within regions encoding for the major protein domains.

Figure 2.2: Mutations of the human LPL gene superimposed on the LPL amino acid sequence (HGMD 2005)



Familial LPL deficiency refers to a condition, where the genetic cause of LPL deficiency can be traced back within a certain pedigree. Several of the above mentioned mutations can represent an inherited defect. Familial LPL deficiency has been shown to follow an autosomal recessive trait and is characterized by hyperlipoproteinemia, recurrent acute pancreatitis, exocrine pancreatic insufficiency, diabetes mellitus, and xanthomas (Murthy et al., 1996).

Animal models for LPL deficiency have been developed in the mouse and the cat. Transgenic and gene-targeted mice have been engineered to study the role of LPL in lipid metabolism, and have been proven to be a useful model. But mice homozygous for an LPL defect do not survive beyond the first day of life (Coleman and Seip, 1995; Weinstock, 1995). However, a population of cats with heterozygous as well as homozygous carriers of LPL deficiency has been successfully established. The LPL deficiency in these cats results in a lipid and lipoprotein phenotype that predominantly parallels human LPL deficiency (Ginzinger and Clee, 1999).

2.2. Canine Genetics

2.2.1. Genetic Defects in the Dog

Molecular genetics are now widely used to identify the genotypic background for certain phenotypic expressions in dogs. This includes traits such as coat color, but also a variety of diseases. Naturally occurring genetic disorders are believed to be more common in dogs than in any other non-human species (Dukes-McEwan, 2002; Patterson, 2000). There are documented breed predispositions for various diseases, which are believed to occur due to unique breed population structures (Ostrander and Galibert, 2000). The separation of dogs into different breeds with closed studbooks has created a barrier against the mixing of genes, resulting in isolated breeding populations, in which certain genetic defects can accumulate(Dukes-McEwan, 2002). Another reason for this accumulation is the so called “popular sire effect”, where dogs carrying a genetic defect may be bred repeatedly because of their outstanding conformity to breed standards (Ostrander, 2000). However, the documented multi-generational pedigrees available for the dog provide a statistical advantage for genetic studies, and since many genetic diseases in the dog resemble those in humans, the dog has become an attractive model for research on hereditary diseases in humans (Dukes-McEwan, 2002; Galibert, 1998). Veterinary clinicians commonly encounter diseases that are associated predominantly with a specific breed. More than 450 hereditary diseases have been described in the dog, and approximately half of them model a human disease (Online Mendelian Inheritance in Animals: www.angis.org.au/omia). Most of these diseases follow a recessive trait, are a consequence of genetic homogeneity in purebred dog populations, and constitute a major health problem in purebred dogs (Dukes-McEwan, 2002; Patterson, 2000). Unlike in humans, most autosomal dominant diseases in the dog are not propagated because breeders choose not to breed affected dogs. In contrast, recessive diseases are much more difficult to control because breeders are generally unable to distinguish between normal dogs and asymptomatic carriers or breeders underestimate the significance of breeding an asymptomatic carrier.

Hereditary diseases are a major concern for breeders of purebred dogs (Mellersh and Langston, 1997). Thus veterinarians are frequently consulted for advice. The veterinary practitioner should

therefore be able to discuss available screening methods and how they can best be used to benefit the individual dog as well as the breeding population (Metallinos, 2001).

2.2.2. Genetic Analysis in the Dog

2.2.2.1. Genetic Markers

A genetic marker is like a fixed landmark that helps a geneticist to determine certain positions on the genome and is not necessarily linked to a disease. The genetic markers most commonly used are microsatellites. Microsatellite markers are widely distributed throughout the entire genome and consist of a repeating DNA sequence (e.g. CAACAACAA). While the pattern of each microsatellite stays the same, each marker can show significant variation in size due to a variable number of repeats between individuals (e.g. CAACAACAA vs. CAACAACAACAACAACAACAA). The different sizes in which a specific marker appears are the so called alleles of that marker(Lewin, 1997). When performing a whole genome screen, known markers are evaluated and the allele of each marker is determined for each dog. Since evaluating all of the 3,270 markers of the canine genome that have been described to date is impractical, the canine genetics community has developed two Minimal Screening Sets (MSS-1 and MSS-2) (Guyon, 2003). The MSS-1 consists of 172 microsatellite markers, while the MSS-2 is composed of 327 microsatellite markers (Richman et al., 2001). By performing polymerase chain reactions (PCRs) using genetic material (i.e. DNA) extracted from dogs with a known phenotype (e.g. pancreatic acinar atrophy) each marker-allele is determined. Evaluating several microsatellites within the same PCR reaction can accelerate data analysis and is called “multiplexing”. Reports concerning the use of multiplexing are available for both the MSS-1 and the MSS-2 (Clark L et al., 2004, in press; (Cargill et al., 2002)). Both the multiplexed MSS-1 and MSS-2 are now widely used to perform whole genome screens in dogs.

Sequencing of the canine genome, termed canine genome project, is currently under way by Ostrander et. al. This project will offer new possibilities, for example the identification of new genetic markers such as SNPs (single nucleotide polymorphisms). This work will also allow high resolution mapping of the canine genome for certain diseases (Kirkness and Bafna, 2003).

2.2.2.2. Linkage Analysis

Linkage analysis is one form of genetic analysis. The first successful linkage analysis for a canine genetic disease was carried out by Yuzbasiyan-Gurkan et al., who identified a marker for copper toxicosis in the Bedlington Terrier (Yuzbasiyan-Gurkan, 1997). The basic concept of linkage analysis is that a genetic marker close to or within a disease gene is inherited along with that gene. Whenever a mutation is present in the gene of interest the marker also shows a different allele. The geneticist then tries to identify a correlation between the appearance of a certain marker allele and the disease without looking at the gene itself.

A whole genome screen is one approach to carry out linkage analysis. For example, the MSS-1 and MSS-2 are used to analyze known genetic markers in the genome of a given pedigree(Dukes-McEwan, 2002; Greer et al., 2003; Richman et al., 2001). Statistical methods are then used in order to identify one or more markers with an allele that is significantly correlated with a specific disease phenotype. The position of this linked marker on a physical map of the canine genome can then be determined and the gene segregating with the marker can be identified in some cases.

A slightly different approach is used if a multigenerational pedigree cannot be established. By using linkage disequilibrium analysis the individuals are divided into two groups: affected and unaffected. Once again, a whole genome scan is performed to identify a marker allele that segregates with the affected group of dogs. However, this method is statistically not as informative as linkage analysis(Greer et al., 2003).

2.2.2.3. Candidate Gene Approach

The most direct method for genetic analysis is the candidate gene approach, where a selected number of genes are evaluated. A candidate gene is chosen either based on its role for an analogous disease in another species, based on pedigree-analysis, and also based on the phenotype of affected and non-affected individuals. (Greer et al., 2003; Kijas and Miller, 2003). The gene is then evaluated either by direct sequencing and identification of specific mutations or by linkage analysis with an associated marker.

An example for a successful candidate gene approach is the evaluation of the canine rhodopsin gene

to determine the genetic cause of progressive retinal atrophy in the English Mastiff (Kijas and Miller, 2003).

2.2.2.4. The LPL Gene as a Candidate Gene for Pancreatitis in the Miniature Schnauzer

This work focuses on the evaluation of the LPL gene and its role in the development of hyperlipidemia and pancreatitis in the Miniature Schnauzer. The LPL gene has been chosen as a candidate gene for the following reasons.

First, based on the high prevalence of hyperlipidemia and chronic pancreatitis in the Miniature Schnauzer, several authors have postulated a genetic predisposition for pancreatitis in this breed (Hänichen and Minkus, 1990; Williams, 1996). One genetic study has been carried out already to identify the genetic basis for chronic pancreatitis in this breed, excluding the cationic trypsinogen gene as a possible cause for this condition (Bishop and Steiner, 2002). Thus, additional genetic studies are warranted.

Second, and as mentioned earlier, mutations of the LPL gene have been shown to result in hyperlipidemia and pancreatitis in humans, cats, and mice. Phenotypes resulting from LPL mutations are very similar among these species (Ginzinger and Clee, 1999), and parallel the condition found in Miniature Schnauzers. Due to the similarities in the phenotype we propose that mutations of the LPL gene are the underlying cause for hyperlipidemia and pancreatitis in the Miniature Schnauzer.

Third, the LPL gene has been shown to be well conserved between mammalian species. Therefore, mutations of the LPL gene in Miniature Schnauzers are expected to result in similar phenotypes as found in familial LPL deficiency in humans, cats, and mice.

2.3. The Exocrine Pancreas of the Dog

2.3.1. Anatomy

The pancreas of dogs consists of two lobes that diverge from the vicinity of the pylorus. Both lobes are connected by a small central body. The left lobe is directed caudo-medially and follows the pyloric part of the stomach. It is situated in the deep leaf of the greater omentum with direct contact to the liver and the transverse colon (Dyce, 1996; Evans and Christensen, 1979; Schummer et al., 1979). The longer right lobe is directed caudo-dorsally and accompanies the dorsal surface of the descending duodenum. It is situated in the mesoduodenum and makes contact to the liver. The pancreas develops from the ventral and dorsal primordia that arise from the embryonic small intestine and can be viewed as an extension of the duodenal glandular mucosa (Schummer et al., 1979). Two secretory ducts form the remnant connection between the pancreas and the duodenum. The duct of the ventral primordium develops into the pancreatic duct and joins the bile duct on the major duodenal papilla, 3 to 6 cm distal to the pylorus. The accessory pancreatic duct, which serves as the main secretory channel in the dog, emerges from the dorsal primordium. It opens on the minor duodenal papilla 3 to 5 cm further distal into the duodenum (Dyce, 1996). The color of the pancreas depends on the amount of blood it contains and ranges from pale pink during the fasting state to dark red following a meal (Bernard, 1985). The exocrine tissue accounts for more than 98 % of the pancreas and is mainly composed of acinar cells that are responsible for the synthesis and storage of the digestive enzymes. Additional components of the exocrine pancreas are the branching duct system, blood vessels arising from the celiac and cranial mesenteric arteries, veins draining into the portal vein, and nerve fibers derived from the vagus and splanchnic nerves (Holst, 1993; Williams and Goldfine, 1993).

2.3.2. Physiology

The pancreas synthesizes and secretes a fluid that is rich in digestive enzymes into the duodenum to facilitate the digestion of proteins, lipids, and polysaccharides. There is a wide variety of pancreatic

enzymes secreted by pancreatic acinar cells, including trypsin, chymotrypsin, elastase, carboxypeptidase, phospholipase A2, pancreatic lipase, and colipase (Rinderknecht, 1993). To prevent autodigestion some of these enzymes are synthesized and secreted as catalytically inactive zymogens, and stored in zymogene granules within the acinar cells. Activation of trypsinogen, the zymogen of trypsin, occurs after secretion into the duodenum through cleavage of trypsinogen activation peptide by another enzyme, enteropeptidase. Once trypsin is activated an activation cascade is initiated, resulting in sequential activation of other digestive zymogens through trypsin. Another defense mechanism against autodigestion is the cosynthesis, costorage, and cosecretion of pancreatic secretory trypsin inhibitor (PSTI), which is synthesized, stored, and secreted along with pancreatic enzymes. PSTI inhibits active trypsin to prevent initiation of the activation cascade within the pancreas, and therefore prevents self digestion of the organ (Eddeland and Ohlsson, 1976; Laskowski and Kato, 1980; Rinderknecht, 1998). Pancreatic juice is also a major source of bicarbonate necessary for adjustment of the pH in the duodenal lumen. It is secreted by the centroacinar cells of the pancreatic duct system. A variety of nervous and hormonal mechanisms regulate pancreatic secretion in response to cephalic stimulation in anticipation of food, as well as gastric and intestinal stimulation in the presence of food (Chey, 1993; Singer, 1993). Pancreatic secretion is biphasic with a first peak after one to two hours after feeding and a second peak after eight to eleven hours after feeding. Pancreatic juice is rich in enzymes during the first peak, and rich in bicarbonate during the second peak (Singer, 1993).

2.3.3. Diseases of the Exocrine Pancreas

2.3.3.1. Pancreatitis

Pancreatic inflammation in the dog can be acute or chronic. Acute pancreatitis is characterized as a sudden onset of pancreatic inflammation that may continue to occur in repeated bouts. If inflammation persists leading to permanent morphological changes and impairment of organ function, the condition is referred to as chronic pancreatitis (Banks, 1994; Bradley, 1993; Sarner, 1993). Depending on the severity of the disease the affected pancreas appears edematous and swollen. Also, accumulation of fluid may be found within the peritoneal cavity. Areas of hemorrhage

and chalky fat necrosis also can be found in the pancreas as well as the surrounding area. Fibrous adhesions to adjacent organs may be present (Jubb et al., 1985). Inappropriate proenzyme activation and auto digestion are believed to be the underlying mechanisms for pancreatitis (Reber et al., 1993; Steer and Saluja, 1993). Pancreatic elastase and phospholipase A promote coagulation necrosis and vascular injury, while trypsin and chymotrypsin promote pancreatic edema and proteolysis and further aggravate the activation cascade. Proteolytic enzymes also spill into the vascular space, where plasma protease inhibitors act as scavengers of these proteases. However, an oversupply of pancreatic enzymes may lead to depletion of these protease inhibitors, leading to unbound proteases in the vascular space that can activate the kinin, coagulation, fibrinolytic, and complement cascade systems resulting in disseminated intravascular coagulation and shock (Lasson, 1984; Lasson and Ohlsson, 1984; Ohlsson et al., 1971).

The inciting events resulting in acute and chronic pancreatitis usually remain unknown. However several potential factors have been identified. Ingestion of a high fat meal and concurrent hyperlipidemia can trigger the disease. It has been suggested, that abnormally high TAG concentrations may lead to the release of toxic fatty acids, resulting in damage of pancreatic capillaries (Guzman et al., 1985; Pitchumoni and Scheele, 1993; Saharia et al., 1977). Hypercalcemia due to hyperparathyroidism has been associated with the development of pancreatitis in some dogs (Simpson, 1993). Certain drugs such as azathioprine, thiazide diuretics, sulfonamides, tetracyclines, L-asparaginase, and potassium bromide are also suspected to cause pancreatitis in dogs (Gaskell et al., 1975; Mallory and Kern, 1980). Pancreatitis can be induced experimentally by pancreatic duct obstruction, pancreatic trauma, duodenal reflux, and pancreatic ischemia. However, their importance in the development of spontaneous disease remains unknown (Jacobs et al., 1985; Lerch and Adler, 1994; Westermarck and Saario, 1989). Furthermore, bacterial, viral, mycoplasmal, and parasitic infections may be associated with pancreatitis (Steer, 1986).

The clinical picture of acute pancreatitis is characterized by a sudden onset of vomiting, anorexia, depression, dehydration, abdominal pain or discomfort, and in some cases diarrhea. Abdominal discomfort may only be evident upon abdominal palpation. In dogs with systemic involvement, signs of shock, respiratory distress, bleeding disorders, and cardiac arrhythmias may also be present. Signs of chronic pancreatitis are more variable and non-specific (Pidgeon, 1987b; Rutgers et al., 1985).

Diagnostic imaging studies can include radiography, ultrasonography, or computed tomography of the abdomen. Radiographic signs of pancreatitis include dilated intestinal loops, an increased density, diminished contrast, and granularity in the right cranial abdomen, and transposition of abdominal organs. However, these findings are not very sensitive or specific. In contrast, abdominal ultrasonography is highly specific for pancreatitis if stringent criteria are applied (Steiner, 2003). Pancreatic enlargement alone is not sufficient to make a diagnosis of pancreatitis, because pancreatic edema can also be observed in other conditions. The pancreas may appear hypoechoic when pancreatic necrosis is present and may appear hyperechoic in cases where pancreatic fibrosis has developed (Hess et al., 1998). Also, pancreatitis is often associated with various degrees of peripancreatic fluid accumulation. A loss of echodensity is indicative of pancreatic necrosis (Nyland et al., 1983). Serum chemistry findings may vary and usually include leucocytosis, an increased packed cell volume, azotemia, hypercholesterolemia, and fasting hypertriglyceridemia (Hill and Van Winkle, 1993; Schaer, 1979). Additionally, the release of high concentrations of toxic substances from the pancreas into the portal blood may result in increased hepatic enzyme activities (Jacobs et al., 1985). Necrotizing pancreatitis in dogs is often accompanied by hyperglycemia, potentially in response to stress-related increases in catecholamines and cortisol (Hill and Van Winkle, 1993).

Serum lipase activity has been used for the diagnosis of pancreatitis for several decades but is neither very sensitive nor very specific for the diagnosis of pancreatitis in the dog (Strombeck et al., 1981). Measurement of serum pancreatic lipase immunoreactivity is the most sensitive and specific diagnostic test for canine pancreatitis currently available. This assay is now widely used for the diagnosis of pancreatitis in the dog (Steiner et al., 2003).

Withholding oral intake of food and water for 3 to 5 days has been recommended as standard therapy of pancreatitis in the dog. However, recent studies in human patients with pancreatitis would suggest that this practice may not only be unnecessary but detrimental to the patient (Kahl et al., 2003). If the patient is vomiting such practice may be justified and slow introduction of a low-fat maintenance diet should be attempted after the animal stops to vomit. If drugs or other agents are suspected to be the cause of the condition, these substances should be withdrawn and replaced by alternative agents. Fluid therapy over several days is required to counterbalance dehydration as well as fluid and electrolyte losses (Drazner, 1986; Pidgeon, 1987a).

2.3.3.2. Chronic Pancreatitis in the Miniature Schnauzer

The clinical picture in this breed is characterized by recurrent signs of pancreatitis (Lasson and Ohlsson, 1984; Rogers et al., 1975), and based on the high prevalence of pancreatitis in the Miniature Schnauzer (Williams, 1996), several authors have also postulated a genetic predisposition for pancreatitis in this breed (Zawie, 1996). Hereditary pancreatitis is a well-known disease in humans. Genes involved in the development of chronic pancreatitis are the cationic trypsinogen gene (Whitcomb, 2000), lipoprotein lipase gene (Peterson and Amir, 2002), cystic fibrosis gene (Sharer and Schwarz, 1998) and pancreatic secretory trypsin inhibitor gene (Hirota and Kuwata, 2003). The role of the cationic trypsinogen gene in Miniature Schnauzers with chronic pancreatitis has been evaluated (Bishop et al., 2004), however, no evidence was found that mutations within that gene were responsible for the disease in Miniature Schnauzers. As mentioned earlier mutations within the human LPL gene can cause changes at the major binding sites of the enzyme, thereby leading to a lack in lipase activity of LPL. Humans having these mutations showed severe hyperlipoproteinemia and pancreatitis (Peterson and Amir, 2002). According to the high incidence of idiopathic hyperlipidemia in Miniature Schnauzers in the United States a disruption within the lipid metabolism was proposed to be the inciting event of the disease process in Miniature Schnauzers rather than a pancreatic disease itself (Williams, 1996; Zawie, 1996).

2.3.3.3. Exocrine Pancreatic Insufficiency (EPI)

The pancreas is known to have a large functional reserve, and clinical signs due to exocrine pancreatic insufficiency do not occur until a significant portion of pancreatic function, approximately 90%, has been lost (DiMagno et al., 1973). The most common cause of EPI in the dog is pancreatic acinar atrophy, whereas EPI due to chronic pancreatitis, which is the most common cause of EPI in people, occurs less commonly in the dog (Holroyd, 1968; Rimaila-Pärnänen and Westermarck, 1982). Pancreatic acinar atrophy (PAA) is most commonly recognized in German Shepherd dogs (GSDs) and rough-coated Collies, but other breeds may also be affected. The prevalence of PAA in the German Shepherd dog is higher than in any other breed (Westermarck et al., 1989; Westermarck et al., 1993). Initial studies evaluating several pedigrees suggested an autosomal recessive mode of

inheritance (Westermarck, 1980). Recently, statistical analysis has been carried out in 2 unrelated multigenerational pedigrees of 135 German Shepherd dogs and the results strongly suggest an autosomal recessive mode of inheritance in these pedigrees (Moeller et al., 2000). However, after 10 years of follow up only 2 of 6 dogs from a litter bred from 2 affected parents developed PAA (Elias Westermarck , personal communication, 2004), observations that are not consistent with such a mode of inheritance. Thus the trait of PAA, at least in some lines, may well be polygenic. Linkage studies using the MSS-1 and the MSS-2 are under way in order to identify a genetic marker for PAA in the German Shepherd dog (Clark et.al., personal communication, 2004).

PAA is characterized by progressive atrophy of pancreatic acinar tissue along with scattering and disorganization of pancreatic acinar cells (Westermarck et al., 1993). Islets of Langerhans are disorganized, but remain otherwise unaffected. Concurrent diabetes mellitus is not a feature of dogs with PAA.

There is considerable evidence that immune-mediated mechanisms play a major role in the pathogenesis of PAA (Wiberg et al., 1999). Once acinar cell atrophy is almost complete, the lack of digestive enzymes results in malabsorption and eventually the classical clinical signs of EPI. Not only are pancreatic enzymes crucial for nutrient digestion, but they also affect small intestinal mucosal function, brush border enzyme activity, and the small intestinal microflora. Concurrent small intestinal bacterial overgrowth (SIBO) is observed in more than 70% of the German Shepherd dogs diagnosed with PAA (Sorensen et al., 1988; Williams et al., 1985).

At the time of diagnosis dogs are usually between 1 and 5 years of age. Feces are soft and voluminous with a grey-yellowish color, and borborygmus and flatulence may be marked. Severe weight loss and polyphagia are common findings in cases that are not diagnosed early in the disease process. Additionally, vomiting and bouts of anorexia have been reported in some GSDs with PAA (Raiha and Westermarck, 1989; Rogers et al., 1983; Westermarck et al., 1989).

Canine trypsin like immunoreactivity (cTLI) is the diagnostic test of choice for diagnosing EPI (Williams and Batt, 1988). Recently, an assay for the measurement of elastase in feces has been developed, but this assay has a poor positive predictive value for canine EPI (Spillmann et al., 1998). Affected dogs generally respond well to supplementation of the diet with powdered pancreatic extract (2 tsp/20 kg/meal) (Pidgeon and Strombeck, 1982). Additionally, supplementation with

vitamin E (400-500 IU/20 kg once daily with food for 1 month initially) and cobalamin (250-1000 µg SC once a week for 4-6 weeks initially) should be considered, since deficiencies of these vitamins are common, may not resolve with enzyme supplementation alone, and yet are safely and inexpensively supplemented. Additional therapeutic measures are required in a minority of patients (Sarner, 2003).

2.3.3.4. Pancreatic Neoplasia

Pancreatic neoplasia is uncommonly diagnosed in dogs and is mainly found in older animals. Adenocarcinomas originating from acinar or duct cells have both been described, and the Airedale terrier appears to have an increased incidence for pancreatic adenocarcinomas (Withrow, 1996). At the time of diagnosis metastases are usually present in the duodenum, the liver, and/or the local lymph nodes. Clinical signs are mainly nonspecific and may include weight loss, anorexia, depression, and vomiting. Additionally, metastases obstructing the bile ducts or pancreatic ducts might result in icterus and/or EPI (Bright, 1985). Abdominal radiographs and ultrasonic imaging are helpful to identify pancreatic masses. However, pancreatic biopsy is required for a definitive diagnosis. The prognosis for animals with carcinomas of the exocrine pancreas is extremely poor (Banks, 1993), and supportive therapy should be targeted at associated conditions, such as diabetes mellitus and EPI (Bright, 1985).

3. Materials and Methods

3.1. Study Subjects

The database of the Gastrointestinal Laboratory at Texas A&M University was screened for Miniature Schnauzer dogs for which a serum sample was available. Serum sample were available from 170 Miniature Schnauzers and serum cPLI concentrations were measured in each sample using an in-house ELISA (Steiner et al., 2003). Serum cPLI exclusively measures lipase that originates from the exocrine pancreas. Serum cPLI has been shown to be specific for exocrine pancreatic function in the dog and is also highly sensitive for canine pancreatitis (82%). Serum cPLI is the most sensitive and specific diagnostic test currently available for the diagnosis of canine pancreatitis. A serum cPLI concentration above 200.0 µg/L was considered diagnostic for a diagnosis of pancreatitis. A value of 102.1 µg/L and below was considered normal and dogs with serum cPLI concentrations below this value served as control dogs.

Two study groups were established. The first group consisted of 12 affected dogs based on a serum cPLI concentration above 200.0 µg/L, a clinical history compatible with pancreatitis and a serum triglyceride concentration above the upper limit of the reference range. Also, 9 healthy control dogs were chosen based on a serum cPLI concentration of 102.1 µg/L or less, a lack of clinical signs compatible with pancreatitis, and a serum triglyceride concentration within the reference range.

DNA was then collected from these 21 dogs using DNA extraction techniques as described below.

3.2. Isolation of DNA

3.2.1. DNA Extraction from White Blood Cells

DNA extraction from white blood cells using whole blood samples was performed using Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Briefly, a 300 µl peripheral blood sample was taken from the jugular vein and

anticoagulated in EDTA. Red blood cell lysis solution (Puregene, Gentra Systems), 900 µl, was added and the sample was incubated at room temperature for 10 min, then centrifuged for 30 seconds at 15,000 x g (Centrifuge 5417C, Eppendorf). The supernatant was discarded and the pellet was vigorously vortexed to resuspend the white blood cells. The cells were lysed by addition of 300 µl cell lysis Solution (Puregene, Gentra Systems). The protein was then precipitated by addition of 100 µl protein precipitation solution (Puregene, Gentra Systems) followed by vigorous vortexing for 20 sec and centrifugation for 3 min at 15,000 x g. The resulting supernatant, containing the DNA, was retained and transferred into a new tube. DNA was precipitated by addition of 300 µl isopropanol (100%), followed by centrifugation for 4 min at 15, 000 x g. The resulting DNA pellet was dried by addition of 300 µl ethanol (70 %) followed by centrifugation for 2 min at 15,000 x g. Samples were air dried for 15 min. The DNA was rehydrated by incubation of the sample with 100 µl of DNA Hydration Solution (Puregene, Gentra Systems) overnight at room temperature. The DNA samples were stored at -20ºC until use.

3.2.2. DNA Extraction from Mucosal Cells

DNA extraction from buccal mucosal cells was performed using Puregene DNA Purification Kit (Gentra Systems) according to the manufacturer's instructions. Briefly, samples were collected by rolling cytology brushes (CytoSoft, Medical Packing Corporation) over the buccal mucosa of the dogs. Four samples were collected from each dog. The brush was placed into a tube and 300 µl of Cell Lysis Solution (Puregene, Gentra Systems) and 1.5 µl Proteinase K (20 mg/ml) (Puregene, Gentra Systems) were added. The tube was inverted several times to ensure distribution of the solution over the entire brush, and the sample was incubated over night at 55˚C (Branson 2210, Branson Ultrasonic Corp., CT, USA). The brush was removed and 100µl protein precipitation solution (Puregene, Gentra Systems) were added and votexed for 20 sec prior to incubation for 15 min at -20˚C. The sample was centrifugation for 5 min at 15, 000 x g. The resulting supernatant, containing the DNA, was retained and transferred into a new tube. The DNA was precipitated by addition of 500 µl isopropanol (100%) and 4 µl glycogen (12 mg/ml; Purescript, Gentra Systems), followed by 30 min incubation at -80˚C and centrifugation for 5 min at 15, 000 x g. The resulting

DNA pellet was dried by addition of 300 µl ethanol (70 %), followed by centrifugation for 2 min at 15,000 x g. Samples were air dried for 15 min. The DNA was rehydrated by incubation of the sample with 40 µl of DNA Hydration Solution (Puregene, Gentra Systems) overnight at room temperature. The DNA samples were stored at -20ºC until use.

3.2.3. Quality Control and DNA Concentration

Gel electrophoresis

Gel electrophoresis was used to estimate the DNA yield and quality. One µl of each sample was run on a 2.5 % agarose gel (Agarose Low EEO, Fisher Scientific, Hampton, NH, USA) prepared in TAE – buffer (Tris – Acetate – EDTA Buffer, Sigma-Aldrich, St.Louis, MO, USA) in an electrophoresis chamber (BioMax MP1015, Kodak, Rochester, NY, USA) for 90 min at 90 volt (Model 300 Power Supply, VWR Scientific, West Chester, PA, USA). The gel was stained in an ethidium bromide (0.5 µg / ml) bath for 10 min, followed by a de-staining for 25 min in water. The DNA was cross-linked by exposure to UV light for 1 sec and its banding pattern was documented with a digital camera. Samples that exhibited degradation were not included in the analysis. An estimate of the sample concentration was made based on the comparison with a reference sample (2 – log Ladder, BioLabs Inc., Beverly, MA, USA).

Spectrophotometry

To standardize amplification the DNA was quantified by spectrophotometry. The extinction for each probe was measured at a wavelength of 260 nm (E_{260}) for DNA and 280 nm (E_{280}) for protein (Ultrospec 2000, Pharmacia/Pfizer, New York, NY, USA). Calculation of the DNA concentration:

$$C_{DNA} = E_{260} \times 50\ ng/\mu l \times 35$$

Samples were diluted or reconcentrated to 100 ng/µl. Protein contamination has the potential to interfere with amplification. Therefore, samples showing a high protein content (E_{260} / E_{280} ratio ≤

1.5) underwent on additional step of protein precipitation (see 3.1.1.). If protein content remained high after the second precipitation the samples were not included in the analysis.

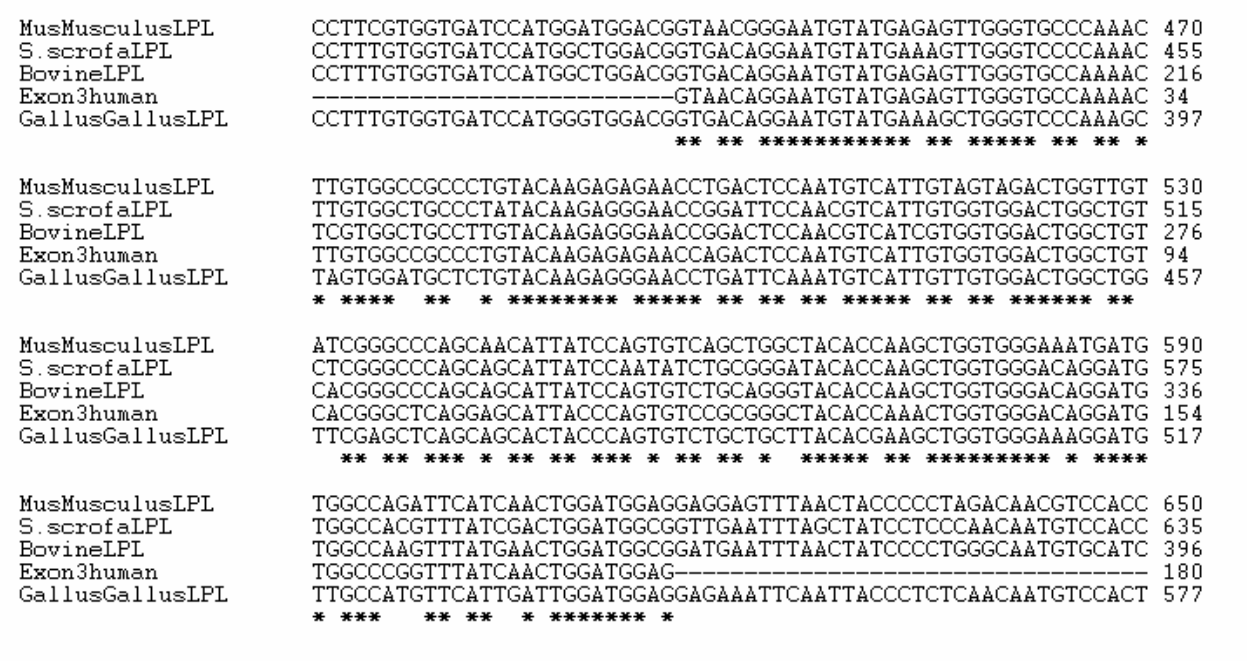
The collected DNA samples were then used to determine the nucleotide sequence of the LPL gene in the 21 canine subjects. Sequences were analyzed and compared between the affected dogs and the healthy control dogs to determine if sequence differences correlate with the affected phenotype. The methodology used is described in detail in the following section.

3.3. Primer Design

3.3.1. Nucleotide Sequence of the Canine LPL Gene

The nucleotide sequence of the lipoprotein lipase gene (LPL) has been published for a variety of species, but not for the dog. Therefore, coding DNA sequences of the human (www.ncbi.nlm.nih.gov/entrez, NM_000237), bovine (www.ncbi.nlm.nih.gov/entrez, AY216661, M16966), porcine (www.ncbi.nlm.nih.gov/entrez, NM_214286), murine (www.ncbi.nlm.nih.gov/entrez, M63335) and avian (www.ncbi.nlm.nih.gov/entrez, NM_205282) LPL gene were used for reference in this study. The LPL gene is very well conserved among species (see 2.1.4.). By comparing (ClustalW, www.ebi.ac.uk/clustalw/) the cDNA of the non-human species against the human exons, it was possible to clearly identify the respective exonic regions within the other species (fig. 3.1). The same approach was used to identify the unknown sequence of exon 1 – 9 in the canine genome. Human LPL cDNA was used as a reference. The complete sequence of the canine genome has recently been identified and is available in an on-line database (Trace Archive, www.ncbi.nlm.nih.gov/Traces/trace.cgi). However, the majority of canine genes, including the LPL gene, had not been characterized at the beginning of this study. Each exon of the human LPL gene was compared with the canine genome to identify clones of high homology with the respective exon. Specifically, the on-line service Megablast (www.ncbi.nlm.nih.gov/BLAST/mmtrace.shtml) was used to screen the Trace Archive database and retrieve the sequence for exon 1 through 9 of the canine LPL gene.

Figure 3.1: Example of a multiple sequence alignment among the different species



The results were then compared to the entire NCBI database (www.ncbi.nlm.nih.gov) using Megablast to exclude the possibility that the identified nucleotide sequences encode for similar genes (e.g. pancreatic lipase, hepatic lipase) instead of the LPL gene.

3.3.2. Identification of Primer Sites

The Trace Archive blast provided a variety of canine clones for each exon as well as the neighboring intronic regions of the canine LPL gene. The respective clones of each exon were compared in a multiple sequence alignment (ClustalW, www.ebi.ac.uk/clustalw/) to verify the given sequence and to exclude amplification and sequencing errors. The intronic regions surrounding each exon were then searched for suitable sites for primer design. Regions with repetitive sequence motives were avoided. Goal of the primer design was to obtain primers that could be used to amplify the complete exon as well as the exon/intron boundaries, which give additional information about splicing sites.

3.3.3. Primer Design

The on-line software Netprimer (www.premierbiosoft.com/netprimer/index.html) was used for primer design for identified sites. Netprimer combines primer design algorithms with a web-based interface allowing the user to analyze primers over the Internet. All primers are analyzed for melting temperature (T_m) using the nearest neighbor thermodynamic theory to ensure accurate T_m prediction. Primers are analyzed for all secondary structures including hairpins, self-dimers, and cross-dimers in primer pairs. This ensures the availability of the primer for the reaction, as well as, minimizing the formation of primer dimers. Table 3.3 shows the designed primer pairs including their respective comprehensive analysis report. The following variables were used as the main decision making criteria.

Rating

The rating of a primer allows for simplified identification of the predicted efficiency of a primer as well as choosing between closely matched primers; the higher the rating of a primer, the higher is it's amplification efficiency. The rating of individual primers is based on the stability of its secondary structures and was calculated as:

Rating = 100 + (ΔG (Dimer) * 1.8 + ΔG (Hairpin) * 1.4)

Melting Temperature (Tm)

The melting temperature was calculated using the formula based on the nearest neighbor thermodynamic theory and is defined as the temperature at which half of the oligonucleotides are bonded (Freier et al., 1986). Tm was calculated as:

$$Tm = \Delta H / (\Delta S + R * \ln(C/4)) + 16.6 \log ([K^+] / (1 + 0.7\ [K^+])) - 273.15$$

ΔH = enthalpy for helix formation

ΔS = entropy for helix formation

R = molar gas constant (1.987 cal/°C * mol)

C = the nucleic acid concentration

[K+] is the potassium salt concentration

GC%

GC% is the percentage of G and C of the primer. It is calculated by dividing the sum of G and C with the total number of bases present in the primer. An optimal value of 50 – 60 % has been suggested.

3' end stability

The stability of the primer determines its false priming efficiency. An ideal primer has a stable 5' end and an unstable 3' end. If the primer has a stable 3' end, it may anneal to a site, which is complementary to, but different from the target with its 5' end unattached. This may lead to amplification of unwanted sequences.

Primers with low stability at the 3' ends function well because the 3' end annealing to false priming sites are too unstable to extend. The 3' end stability is expressed by the ΔG value of the 5 bases of the primer taken from its 3' end. The lower this value, numerically, the more liable the primer is to show secondary bands.

Free Energy (ΔG)

ΔG is the free energy of the primer calculated using the nearest neighbor method (Breslauer et al., 1986). ΔG is calculated by the formula $\Delta G = \Delta H - T\Delta S$. Here ΔH is the enthalpy of primer, T is the temperature, ΔS is the entropy of primer. T is set to 25ºC.

ΔH

ΔH is the enthalpy of the primer as calculated by the nearest neighbor method (Table 3.1) (Breslauer et al., 1986). ΔH is calculated from the nucleotide sequence. For example ΔH for a pentamer ATGCA is calculated as follows:

$\Delta H (ATGCA) = \Delta H (AT) + \Delta H (TG) + \Delta H (GC) + \Delta H (CA)$

Table 3.1: Enthalpy values ΔH of a nearest neighbor nucleotide (in -cal/°K/mol)

Second Nucleotide → First Nucleotide ↓	dA	dC	dG	dT
dA	9100	6500	7800	8600
dC	5800	11000	11900	7800
dG	5600	11100	11000	6500
dT	6000	5600	5800	9100

ΔS

ΔS is the entropy of the primer as calculated by the nearest neighbor method (Table 3.2) (Breslauer et al., 1986).

ΔS is calculated based on the nucleotide sequence of the primer. For example, ΔS for a pentamer ATGCA is calculated as follows:

ΔS (ATGCA) = ΔS (AT) + ΔS (TG) + ΔS (GC) + ΔS (CA)

An initiation value of 15.1 is added to the ΔS calculation.

Table 3.2: Entropy values ΔS of a nearest neighbor nucleotide (in -cal/°K/mol)

Second Nucleotide → First Nucleotide ↓	dA	dC	dG	dT
dA	24.0	17.3	20.8	23.9
dC	12.9	26.6	27.8	20.8
dG	13.5	26.7	26.6	17.3
dT	16.9	13.5	12.9	24.0

5' end stability

Stability of the 5' terminus allows for efficient annealing of the primer to the target site. This stable 5' region is called the GC clamp. It ensures adequate binding of the primer to the template. Use of primers with optimal stability allows for the use of lower annealing temperatures without the

production of secondary bands. Notice that the 3' end should not be very stable and the 5' end should have a strong GC clamp. The GC Clamp is the ΔG value of the 5 bases of the primer taken from its 5' end. The lower this value, numerically, the more efficient is the primer.

Repeats and Runs

Repeats and runs increase the likelihood of false priming. Primers having 3 or more dinucleotide repeats or 3 or more base runs were reported.

Secondary Structures of Primers

Hairpins

A hairpin loop is formed when primer folds back on itself and is held in place by intramolecular bonds. Because hairpin loop formation is an intramolecular reaction, it can occur with as few as 3 consecutive homologous bases. The free energy represents the stability of the hairpin loop. The free energy of the loop is based upon the energy of the intramolecular bond and the energy needed to twist the DNA to form the loop. If this free energy is greater than 0, the loop is too unstable to interfere with the reaction. However, if the free energy is less than 0, the loop could reduce the efficiency of the amplification rection.

Dimers and Cross Dimers

Dimers occur when a region of homology is present within a primer (self-dimer) or between the sense and anti-sense primer (cross-dimer). This results in annealing of the two primers, increasing production of the primer dimer artifact and reducing product yields.

This is particularly problematic when the homology occurs at the 3' end of either primer. The 3' end will extend readily leading to primer-dimer artifact.

Table 3.3: Forward and reverse primer for amplification of exon 1 of the canine LPL gene

Oligo Name	**Ex1-U(2)-F**	**Ex1-U(2)-R**
Sequence	**GAGTGGGAACAGTGTCAGACTCG**	**CCAACGCCTGAGGTTCTCC**
Bases	23	19
Rating	90	92
Tm (℃)	61.04	60.09
GC%	56.52	63.16
ΔG (kcal/mol)	-35.99	-34.53
3' end stability (kcal/mol)	-8.13	-7.82
ΔH (kcal/mol)	-157.9	-150
ΔS (kcal/mol)	-0.41	-0.39
5' end stability (kcal/mol)	-6.47	-8.31
Hairpins (kcal/mol)		
Dimers (kcal/mol)	-5.52	-4.17
Palindromes (kcal/mol)		
Repeats		
Runs	GGG	
Cross Dimers (kcal/mol)		-5.86

Table 3.4: Forward and reverse primer for amplification of exon 2 of the canine LPL gene

Oligo Name	**WGS-Ex2(2)-F**	**WGS-Ex2(2)-R**
Sequence	**TAGCATCGGTGGTAGTTGC**	**TTAGATTCCACAGTCCTCACC**
Bases	19	21
Rating	86	100
Tm (℃)	54.17	53.97
GC%	52.63	47.62
ΔG (kcal/mol)	-31.53	-32.01
3' end stability (kcal/mol)	-8.38	-7.94
ΔH (kcal/mol)	-143.9	-148.5
ΔS (kcal/mol)	-0.38	-0.39
5' end stability (kcal/mol)	-7.65	-6.08
Hairpins (kcal/mol)	-1.59	
Dimers (kcal/mol)	-6.09	
Palindromes (kcal/mol)		
Repeats		
Runs		
Cross Dimers (kcal/mol)		-7.37

Table 3.5: Forward and reverse primer for amplification of exon 3 of the canine LPL gene

Oligo Name	**Ex-3-U(1)-F**	**Ex-3-U(1)-R**
Sequence	**CAAGTTGTAAGTGGTTATTTTAGG**	**TTATCATAATGCTGCTTTCTGG**
Bases	24	22
Rating	92	92
Tm (℃)	53.73	55.58
GC%	33.33	36.36
ΔG (kcal/mol)	-35.63	-34.73
3' end stability (kcal/mol)	-7.57	-8.2
ΔH (kcal/mol)	-179.1	-165.2
ΔS (kcal/mol)	-0.48	-0.44
5' end stability (kcal/mol)	-6.84	-5.95
Hairpins (kcal/mol)		
Dimers (kcal/mol)	-3.91	-4.38
Palindromes (kcal/mol)		
Repeats		
Runs	TTTT	TTT
Cross Dimers (kcal/mol)		-4.38

Table 3.6: Forward and reverse primer for amplification of exon 4 of the canine LPL gene

Oligo Name	**WGS-Ex4(4)-F**	**WGS-Ex4(4)-R**
Sequence	**GAGTTAATTTTCAGCATTGCC**	**TCGCTTCTGACAGTAGGTGG**
Bases	21	20
Rating	88	100
Tm (℃)	54.82	55.88
GC%	38.1	55
ΔG (kcal/mol)	-33.97	-32.3
3' end stability (kcal/mol)	-10.11	-9.43
ΔH (kcal/mol)	-161.7	-145
ΔS (kcal/mol)	-0.043	-0.38
5' end stability (kcal/mol)	-6.46	-9.92
Hairpins (kcal/mol)	-0.32	
Dimers (kcal/mol)	-6.09	
Palindromes (kcal/mol)		
Repeats		
Runs	TTTT	
Cross Dimers (kcal/mol)		-5.13

Table 3.7: Forward and reverse primer for amplification of exon 5 of the canine LPL gene

Oligo Name	**Ex-5-U(1)-F**	**Ex-5-U(1)-R**
Sequence	**GATCCAATCACTACAGAATAAGG**	**CAAGTGCTATACATGTGACCAG**
Bases	23	22
Rating	91	85
Tm (℃)	53.12	53.37
GC%	39.13	45.45
ΔG (kcal/mol)	-33.46	-31.98
3' end stability (kcal/mol)	-7.57	-7.96
ΔH (kcal/mol)	-163.3	-150.2
ΔS (kcal/mol)	-0.44	-0.4
5' end stability (kcal/mol)	-7.69	-6.84
Hairpins (kcal/mol)		
Dimers (kcal/mol)	-4.62	-8.07
Palindromes (kcal/mol)		ACATGT
Repeats		
Runs		
Cross Dimers (kcal/mol)		-4.89

Table 3.8: Forward and reverse primer for amplification of exon 6 of the canine LPL gene

Oligo Name	**WGS-Ex6-F**	**WGS-Ex6-F**
Sequence	**TCGAGCTGTTAACTGCCACC**	**AGGCTGCTGCATAGAGTAGTGC**
Bases	20	22
Rating	86	84
Tm (℃)	58.61	59.25
GC%	55	54.55
ΔG (kcal/mol)	-34.21	-36.19
3' end stability (kcal/mol)	-9.43	-8.03
ΔH (kcal/mol)	-151.8	-164.6
ΔS (kcal/mol)	-0.39	-0.43
5' end stability (kcal/mol)	-8.36	-9.41
Hairpins (kcal/mol)		-1.99
Dimers (kcal/mol)	-7.53	-7.05
Palindromes (kcal/mol)	GTTAAC	
Repeats		
Runs		
Cross Dimers (kcal/mol)		-4.3

Table 3.9: Forward and reverse primer for amplification of exon 7 of the canine LPL gene

Oligo Name	**WGS-Ex7-F**	**WGS-Ex7-R**
Sequence	**CTGAATTGCCTGCTTATCTGG**	**CCTACTCTTCTTCTGTTCTAAAGACC**
Bases	21	26
Rating	90	86
Tm (℃)	57.79	57.25
GC%	47.62	42.31
ΔG (kcal/mol)	-35.02	-38.32
3' end stability (kcal/mol)	-8.2	-7.58
ΔH (kcal/mol)	-160.3	-187.4
ΔS (kcal/mol)	-0.42	-0.5
5' end stability (kcal/mol)	-7.07	-6.97
Hairpins (kcal/mol)		-2.02
Dimers (kcal/mol)	-5.36	-6.12
Palindromes (kcal/mol)		
Repeats		
Runs		AAA
Cross Dimers (kcal/mol)		-4.54

Table 3.10: Forward and reverse primer for amplification of exon 8 of the canine LPL gene

Oligo Name	**WGS-Ex8-F**	**WGS-Ex8-R**
Sequence	**CCTACATGCCATTGATCC**	**GGGAGGCTCCATTATCC**
Bases	18	17
Rating	89	86
Tm (℃)	50.74	51.58
GC%	50	58.82
ΔG (kcal/mol)	-29.08	-29.71
3' end stability (kcal/mol)	-7.69	-7.08
ΔH (kcal/mol)	-133.7	-136.6
ΔS (kcal/mol)	-0.35	-0.36
5' end stability (kcal/mol)	-6.97	-9.31
Hairpins (kcal/mol)		-1.34
Dimers (kcal/mol)	-5.62	-6.24
Palindromes (kcal/mol)		
Repeats		
Runs		GGG
Cross Dimers (kcal/mol)		-5.64

Table 3.11: Forward and reverse primer for amplification of exon 9 of the canine LPL gene

Oligo Name	**WGS-Ex9-F**	**WGS-Ex9-R**
Sequence	**GTCGCTGACCAGAATGG**	**AGCCACAAGAATCAGTGC**
Bases	17	18
Rating	88	91
Tm (°C)	51.11	50.46
GC%	58.82	50
ΔG (kcal/mol)	-28.27	-28.65
3' end stability (kcal/mol)	-8.44	-8.03
ΔH (kcal/mol)	-125.5	-130.8
ΔS (kcal/mol)	-0.33	-0.34
5' end stability (kcal/mol)	-9.67	-9.76
Hairpins (kcal/mol)	-0.82	-0.2
Dimers (kcal/mol)	-6.02	-4.3
Palindromes (kcal/mol)		
Repeats		
Runs		
Cross Dimers (kcal/mol)		-6.13

The designed primer pairs were then used to amplify exon 1 through 9 of the LPL gene in the 21 Miniature Schnauzers.

3.4. Polymerase Chain Reaction (PCR)

3.4.1. Primer Concentration

The commercially produced primer (Sigma–Genosys, St.Louis, MO, USA) were reconstituted in TAE – buffer and used at a final primer concentration of 10 µmol/L.

3.4.2. Optimization of Amplification Conditions

To determine the optimal amplification conditions for each primer pair 9 different premixes (#1-9), containing different concentrations of buffered salt solution with nucleotides, Mg^{2+} and MasterAmp PCR enhancer with betaine, were tested at different annealing temperatures. Reactions with a total

volume of 25 µl were prepared on ice. A mastermix [95 µl sterile water, 10 µl forward primer solution, 10 µl reverse primer solution, 5 µl DNA (100 ng/µl) and 5 µl DNA polymerase mix (MasterAmp Extra – Long DNA Polymerase Mix; 2.5 U / µl, Epicentre, WI, USA)] was used. The mastermix (12.5 µl) was combined with the respective premix (12,5 µl; MasterAmp Extra-Long PCR 2X Premixes 1 – 9). A negative control was prepared using 10 µl sterile water, 1 µl forward primer solution, 1 µl reverse primer solution, 5 µl DNA polymerase mix (MasterAmp Extra – Long DNA Polymerase Mix; 2.5 U / µl) and 12.5 µl premix. All samples were centrifuged at low speed for 5 sec. A Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) was programmed using the following variables:

- Initial denaturation at 94 ºC for 45 seconds.
- Denaturation at 94 ºC for 45 seconds for 30 cycles.
- A touchdown approach was used for annealing. The first cycle used an annealing temperature 3ºC above the calculated primer melting temperature. During each of the following 6 cycles the annealing temperature was decreased by 1 ºC, and then held constant at 3 ºC below the calculated melting temperature for the following 23 cycles. During each cycle the annealing temperature was held constant for 1 min.
- Extension at 72ºC for 2.5 min for 30 cycles.
- Final extension at 72ºC for 18 min.

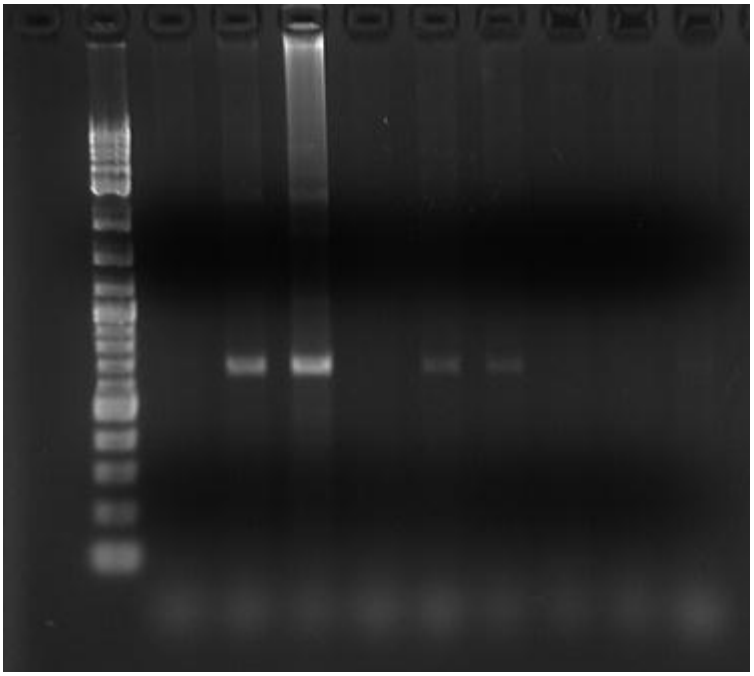
Additionally, a positive contol (21 µl sterile water, 3 µl Contol Lambda DNA Template/Primer, 1 µl DNA polymerase mix (MasterAmp Extra – Long DNA Polymerase Mix 2.5 U / µl) for each premix was prepared whenever a new batch of MasterAmp Extra-Long PCR Kit was purchased.
Amplification conditions were as follows:

- Initial denaturation at 98ºC for 1 min
- Denaturation at 98ºC for 20 seconds for 20 cycles
- Annealing at 56ºC for 1 min for 20 cycles
- Extension at 68ºC for 20 min for 20 cycles

An agarose gel was prepared as described in section 3.2.3. PCR tubes were put on ice, 5 µl running buffer (6X Type I Loading Solution, Sigma) were added and mixed with the sample. The gel was loaded with 17 µl of the mixture per well. A reference sample comprised of a log ladder (2 – log Ladder, BioLabs Inc.) was loaded into a separate well. Staining, de-staing and evaluation of the gel were performed as described in 3.2.3.

A premix was chosen that resulted in one band of the desired template size (fig. 3.2). Using this premix, PCR reactions were performed at different temperatures to determine the optimal annealing temperature. These conditions were then used for large scale amplification of the samples from the Miniature Schnauzer.

Figure 3.2: Example for PCR optimization: amplification of exon 3 using different premixes. The second column shows the reference sample.



If no specific amplification could be achieved the primer pair for the corresponding exon was discarded and a new primer pair was designed.

3.4.3. Sequencing

3.4.3.1. Amplification Conditions for Exon 1 - 9

Each exon was amplified in two 50 µl reactions containing:

- 2 µl forward primer
- 2 µl reverse primer
- 1.5 µl DNA
- 19 µl sterile water
- 1 µl Taq
- 25 µl Premix

Different conditions were used to amplify the 9 exons of the canine LPL gene (Table 3.4).

Table 3.12: Amplification conditions exon 1 - 9

	T_m (°C)	Premix
Exon 1	65	2
Exon 2	61	4
Exon 3	58	1
Exon 4	60	3
Exon 5	58	4
Exon 6	63	2
Exon 7	61	1
Exon 8	58	6
Exon 9	58	9

3.4.3.2. Direct Sequencing of Exon 1 - 9

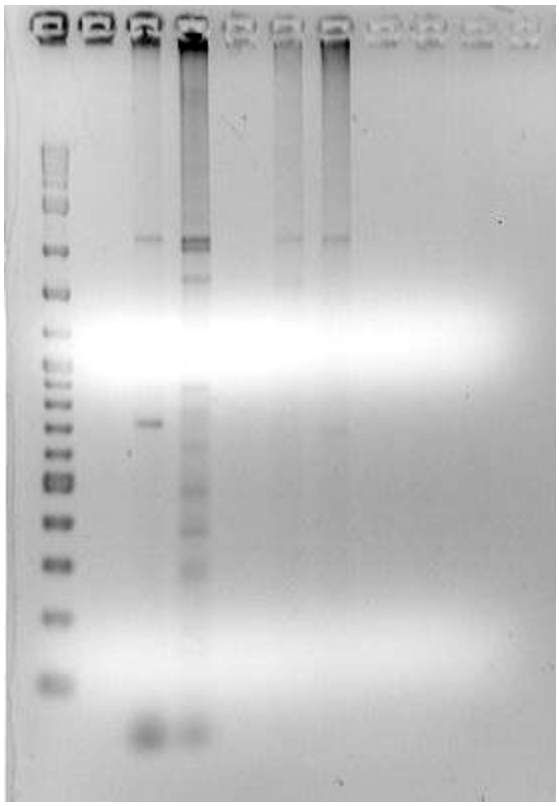
DNA fragments from PCR reactions were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) as specified by the manufacturer. The two identical PCR products from each exon were combined in this step to increase the final DNA concentration. Spectrophotometry was used to determine the concentration of the PCR product as specified in section 3.2.3.

Sequencing reactions were carried out in 6 µl volumes containing 100 – 200 ng PCR product, 2 µl Big Dye® Terminator v 1.1 Cycle Sequencing Mix (Applied Biosystems, Foster City, CA, USA), 10

pmol primer, and sterile water. Thermal Cycling was performed on a Mastercycler Gradient Thermal Cycler (Eppendorf) using 30 cycles of 96ºC for 30 seconds, 55ºC for 15 seconds and 60ºC for 4 min. Primer, shown in Tables 3.4 through 3.11, were used for sequencing reactions of exon 2 through 9. No specific amplification could be achieved for exon 1 (fig 3.3). Therefore, nested primers were used to sequence exon 1:

Ex1-S(1)-F: $^{5'}$TCTCGGAGGGAACCAGC
Ex1-S(2)-F: $^{5'}$CGAGATGGAGAGCAGAGC
Ex1-S(1)-R: $^{5'}$CCAACGCCTGAGGTTCTCC

Figure 3.3: Amplification of exon 1



Reactions were purified by diluting the samples with 25 µl sterile water, adding the solution onto Spin-50 Mini-Columns (USA Scientific, Ocala, FL, USA) and centrifuging it for 4 min at 1000 x g.

Samples were then dried completely using a vacuum centrifuge (SC 210A modular multicomponent system, Savant Instruments, Holbrook, NY, USA). Automated sequencing was performed on an ABI 3100 Genetic Analyzer (Hitachi, Tokyo, Japan).

4. Results

4.1. LPL Gene Sequence in the Miniature Schnauzer

The LPL gene was amplified and sequenced in 21 Miniature Schnauzers. 3 sequences were obtained from each dog. A triplicate that showed identical nucleotide sequences was compared to the other dogs in the study. If a triplicate was not homogeneous further sequencing was performed in order to verify those variations. Figures 4.1 through 4.9 show the complete cDNA sequence, the amino acid sequence (one letter code) and the sequence for the exon/intron boundaries (3' and 5' end of the respective intron) for the LPL gene that was common in all Miniature Schnauzers evaluated.

Figure 4.1: This figure shows the nucleotide and protein sequence of exon 1

Exon 1

```
atggagagcagagcctactcctggtggccctgggcatgtggctgcagagtctggccgcc
 M   E   S   R   A   L   L   L   V   A   L   G   M   W   L   Q   S   L   A   A
gccgcccgaattccag
 A   A   R   I   P
```

The 14 underlined nucleotides represent the area of the sequence for primer placement.

Figure 4.2: This figure shows the nucleotide and protein sequence of exon 2

Exon 2

Intron 1 (3'end) :

```
                                        CAACTTTTCCTTTTTTAGGAATTCCAG
```

Exon 2:

```
gaggaaatgattttgtagatatcgaaagtaaatttgctctaaggacccctgaagacacag
   G   N   D   F   V   D   I   E   S   K   F   A   L   R   T   P   E   D   T
ctgaggatacctgccacctcattcccggagtgatagaatctgtggctaactgccacttca
A   E   D   T   C   H   L   I   P   G   V   I   E   S   V   A   N   C   H   F
atcacaccagcaagacctttgtggtgatccatggctggacg
N   H   T   S   K   T   F   V   V   I   H   G   W   T
```

Intron 2 (5'end):

```
GTAAGACAGTTTCTTAGGGAAGGAGCAGATTGGGGTAGACCAGGCAT
```

Figure 4.3: This figure shows the nucleotide and protein sequence of exon 3

Exon 3

Intron 2 (3'end):

CAAGTTGTAAGTGGTTATTTTAGGAAAGCTTGTATCATCCTTTCCAG

Exon 3:

gtgacaggaatgtatgagagttgggtgccaaaacttgtggctgccctgtacaagagggaa
V T G M Y E S W V P K L V A A L Y K R E
ccggactccaatgtcattgtggtggactggctgtcacgagcccagcagcattatccagtg
P D S N V I V V D W L S R A Q Q H Y P V
tctgcagggtacaccaagctggtgggaaaagatgtggccaagttcatcaactggttggcg
S A G Y T K L V G K D V A K F I N W L A

Intron 3 (5'end): GTAAGGACTGGGGGAAGAAGACATGTGTCCAAAACATATCTCTTCAC
TAGTACTAAACAAAAAACTGGTTTTTATTACT

Figure 4.4: This figure shows the nucleotide and protein sequence of exon 4

Exon 4

Intron 3 (3'end):

AACCCCTTTTTCTTTTTCTCTTCCAAAG

Exon 4:

gaggaatttcagtatcctctggacaatgtccatcttttgggatacagccttggagcgcat
E E F Q Y P L D N V H L L G Y S L G A H
gctgctggcattgcaggaagtctgaccaataagaaggtcaatagaattactg
A A G I A G S L T N K K V N R I T

Intron 4 (5'end):

GTAAGAAGGCAATGCCAGTAGATTTATCATAGAAAAGTTGAGATGCCTGTCATTCTGAAA
GAGAATAGGATGCTTGTCAAATTCCCATATGTATGTGATGTTCC

Figure 4.5: This figure shows the nucleotide and protein sequence of exon 5

Exon 5

Intron 4 (3' end):

GGACAAAATGAATGGGAATTTAAATATCTGTGAGGACTTTTTTTTCCCCCCATTAAG

Exon 5:

gtctagatccagctggacctaactttgagtatgcagaagctccaagtcgtctttctcctg
L D P A G P N F E Y A E A P S R L S P
atgatgcagattttgtagatgtcttacacacattcacaagagggtcacctggccgaagta
D D A D F V D V L H T F T R G S P G R S
ttggaatccagaaaccagtaggacatgttgatatttatcctaatggaggcacttttcaac
I G I Q K P V G H V D I Y P N G G T F Q
caggatgtaacattggggaagccatccgtgtgattgcagagagaggccttggag
P G C N I G E A I R V I A E R G L G

Intron 5 (5' end):
GTAACTATGATTTAGAAGTTAATTAAACTGTTTCGTTCTTAATTCTTACTGATCTAGTCT
CCTACCCATTCCCCACAAGTAAGTAGTTTTAATATATACATTTAGCCAAGAAATG

Figure 4.6: This figure shows the nucleotide and protein sequence of exon 6

Exon 6

Intron 5 (3' end):
TGAAACACTTATTATGGACTTTCATGGAATTGAATTTTCTTTGTGCTTCTTTCTTCCCAG

Exon 6:
atgtggaccagctagtgaaatgctcccatgagcggtccattcacctctttattgactctc
V D Q L V K C S H E R S I H L F I D S
tgttgaatgaagaaaatccaagtaaggcctaccggtgcaactcaaaggaagcctttgaga
L L N E E N P S K A Y R C N S K E A F E
aagggctttgcctgagttgcagaaagaaccgttgcaacaacatgggctatgagatcaata
K G L C L S C R K N R C N N M G Y E I N
aggtcagagccaaaagaggcagcaaaatgtacctgaagactcgctctcagatgccttaca
K V R A K R G S K M Y L K T R S Q M P Y
aag
K

Intron 6 (5' end):
GTAGGCTGGAGAATGTTGTGAGTAGGGAAGATCAATTTGATCCTATTTTTTTGTCATGCT
CATTGCCTCCATGTACTGAGT

Figure 4.7: This figure shows the nucleotide and protein sequence of exon 7

Exon 7

Intron 6 (3' end):
TTGCATAAAAACTGATTAGCACTTGTTCCCTACATTTTCTCCCTACA

Exon 7 :
gtcttccattaccaagtaaagatacatttttctgggactgagagtgatgcacagaccaac
V F H Y Q V K I H F S G T E S D A Q T N
caggccttcgagatctctctgtatggcactgtggctgagagtgagaacatccctttacc
Q A F E I S L Y G T V A E S E N I P F T
ct

Intron 7 (5' end):
GTGAGTAGCCACATGGTTTAACC

Figure 4.8: This figure shows the nucleotide and protein sequence of exon 8

Exon 8

Intron 7 (3' end):

CCCAAAACAAAAAAACTTGTTTCTAAACTAACCAAATATGCTGATTTTTTTCTTCAG

Exon 8:

```
gcctgaagtttctgctaataagacatactcttttctaatttacacggaggtggatattgg
  P   E   V   S   A   N   K   T   Y   S   F   L   I   Y   T   E   V   D   I   G
agaactgctaatgttgaaactcaaatggaagagtgattcatacttcagctggtcagactg
  E   L   L   M   L   K   L   K   W   K   S   D   S   Y   F   S   W   S   D   W
gtggagcagccctggctttgctattgagaagatcagagtaaaagctggagagactcagaa
  W   S   S   P   G   F   A   I   E   K   I   R   V   K   A   G   E   T   Q   K
aaa
```

Intron 8 (5' end):

GTAATTAAATTTATTTTTT

Figure 4.9: This figure shows the nucleotide and protein sequence of exon 9

Exon 9

Intron 8 (3' end):

TAACCAAATCATATATTTTTTGAACAACTGTTTCTCTTTTCCCATATGACATGTTCACAT
TCATTTTCTTCTACAG

Exon 9:

```
ggtaatcttctgttccagggagaaagtgtctcatctgcagaaaggaaagtcgtctgtggt
  V   I   F   C   S   R   E   K   V   S   H   L   Q   K   G   K   S   S   V   V
atttgtgaaatgccatgacaagtctctgaataagaagtctggctg
  F   V   K   C   H   D   K   S   L   N   K   K   S   G
```

Intron 9 (5' end):

GTGAGCATCATGGGCTAAAGTTCCTTGGGTATCCTGAGCTTGCAGTTAGGGGACACGGCT
TTATACATTGCTCTTCATCCCATAACTTAAAGA

A multiple sequence alignment was performed to determine the similarity of the identified sequence in the Miniature Schnauzer with the sequence published on the Trace Archive database obtained from a boxer (fig 4.10). Two variations were identified in the Miniature Schnauzer. A nucleotide substitution 826 C>T within exon 6 as well as an insertion of a G at the beginning of exon 7.

Figure 4.10: Multiple sequence alignment Trace Archive vs. Miniature Schnauzer. Please note that the numbers for the position of the Trace Archive nucleotides are generic and therefore do not correlate with those of the Miniature Schnauzer.

```
gi|57105133-        TCTCTGCTGAATGAAGAAAATCCAAGTAAGGCCTACCGGTGCAACTCAAAGGAAGCCTTT 1140
Schnauzer-LPL       TCTCTGTTGAATGAAGAAAATCCAAGTAAGGCCTACCGGTGCAACTCAAAGGAAGCCTTT 803
                    ****** ************************************************************

gi|57105133-        GAGAAAGGGCTTTGCCTGAGTTGCAGAAAGAACCGTTGCAACAACATGGGCTATGAGATC 1200
Schnauzer-LPL       GAGAAAGGGCTTTGCCTGAGTTGCAGAAAGAACCGTTGCAACAACATGGGCTATGAGATC 863
                    ************************************************************

gi|57105133-        AATAAGGTCAGAGCCAAAAGAGGCAGCAAAATGTACCTGAAGACTCGCTCTCAGATGCCT 1260
Schnauzer-LPL       AATAAGGTCAGAGCCAAAAGAGGCAGCAAAATGTACCTGAAGACTCGCTCTCAGATGCCT 923
                    ************************************************************

gi|57105133-        TACAAAG-TCTTCCATTACCAAGTAAAGATACATTTTTCTGGGACTGAGAGTGATGCACA 1319
Schnauzer-LPL       TACAAAGGTCTTCCATTACCAAGTAAAGATACATTTTTCTGGGACTGAGAGTGATGCACA 983
                    ******* ***********************************************************
```

However, a comparison of the protein sequences (Protein Id: XP_534584.1) showed 100% sequence homology between the two.

4.2 Single Nucleotide Polymorphisms (SNPs) identified in the Miniature Schnauzers studied

All dogs in this study showed the nucleotide sequence shown in Figures 4.1 through 4.9. Additionally, 10 SNPs could be identified in exons of single dogs (Table 4.1 and Figures 4.11 through 4.15). SNPs are DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered. Each individual has many single nucleotide polymorphisms that together create a unique DNA pattern for that individual.

Table 4.1: Overview of SNPs found in exon 2, 3, 5, and 7 in 21 Miniature Schnauzers

		SNPs				
		Exon 2	**Exon 3**	**Exon 5**	**Exon 6**	**Exon 7**
Affected Dogs	1					
	2					
	4	84 T>C				
	5				826 T>C	
	6				826 T>C	
	8					
	15					
	16			546 A>C		
	17		269 A>C, Lys(90)Thr			
	18			690 T>C		
	20			699 C>T		
	21					
Healthy Control Dogs	3					
	7		302 A>G, Asp(101)Gly			
	9					
	10				967 A>G, Lys(323)Glu	
	11					
	12					
	13				957 A>G	
	14					1057 A>G
	19					

Exon 2

A 84 T>C substitution (fig. 4.11) was found in one of the affected dogs. This allele does not lead to a change in the protein sequence.

Figure 4.11: SNP in exon 2

```
SNP 84 (T>C):
gaggaaacgattttgtagatatcgaaagtaaatttgctctaaggacccctgaagacacagc
   G   N   D   F   V   D   I   E   S   K   F   A   L   R   T   P   E   D   T   A
tgaggatacctgccacctcattcccggagtgatagaatctgtggctaactgccacttcaat
  E   D   T   C   H   L   I   P   G   V   I   E   S   V   A   N   C   H   F   N
cacaccagcaagacctttgtggtgatccatggctggacg
 H   T   S   K   T   F   V   V   I   H   G   W   T
```

Exon 3

One nucleotide polymorphism 269 A>C (fig. 4.12) was identified in one affected dog, leading to a single amino acid substitution; Lys(90)Thr.

Figure 4.12: SNPs in exon 3

```
SNP 269 (A>C):
gtgacaggaatgtatgagagttgggtgccaacacttgtggctgccctgtacaagagggaa
 V   T   G   M   Y   E   S   W   V   P   T   L   V   A   A   L   Y   K   R   E
ccggactccaatgtcattgtggtggactggctgtcacgagcccagcagcattatccagtg
 P   D   S   N   V   I   V   V   D   W   L   S   R   A   Q   Q   H   Y   P   V
tctgcagggtacaccaagctggtgggaaaagatgtggccaagttcatcaactggttggcg
 S   A   G   Y   T   K   L   V   G   K   D   V   A   K   F   I   N   W   L   A
```

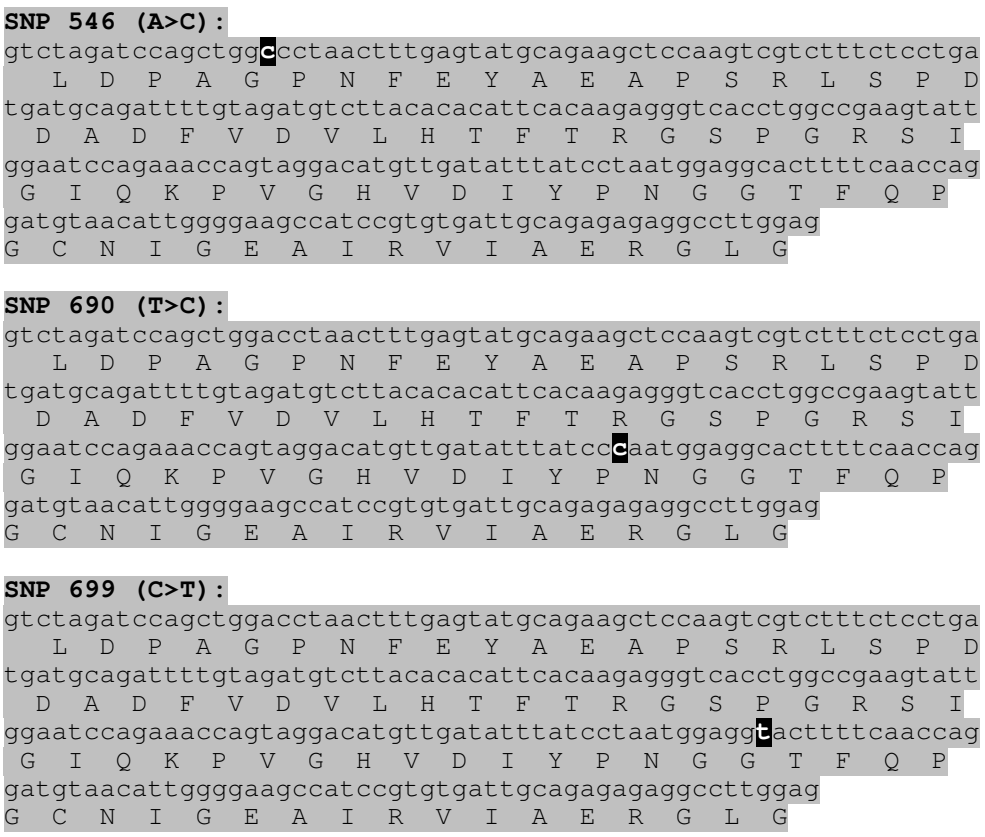
Another single nucleotide polymorphism 302 A>G was identified in one healthy control Miniature Schnauzer, causing a single amino acid substitution; Asp(101)Gly.

```
SNP 302 (A>G):
gtgacaggaatgtatgagagttgggtgccaaaacttgtggctgccctgtacaagagggaa
 V   T   G   M   Y   E   S   W   V   P   K   L   V   A   A   L   Y   K   R   E
ccggGctccaatgtcattgtggtggactggctgtcacgagcccagcagcattatccagtg
 P   G   S   N   V   I   V   V   D   W   L   S   R   A   Q   Q   H   Y   P   V
tctgcagggtacaccaagctggtgggaaaagatgtggccaagttcatcaactggttggcg
 S   A   G   Y   T   K   L   V   G   K   D   V   A   K   F   I   N   W   L   A
```

Exon 5

SNPs were found in three of the affected dogs. Each dog showed a different SNP. 546 A>C, 690 T>C, and 699 C>T (fig. 4.13) are all nucleotide substitutions that do not result in amino acid substitutions.

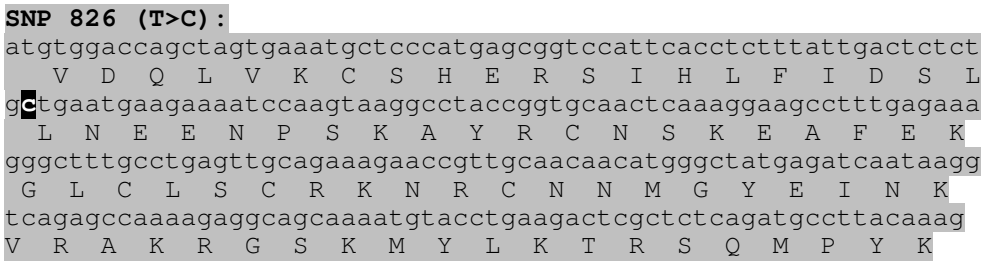
Figure 4.13: SNPs in exon 5



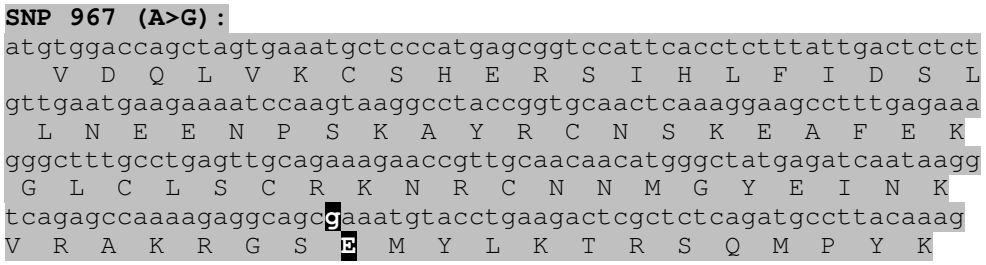
Exon 6

Two affected dogs showed the same nucleotide substitution 826 T>C. In one healthy control dog another SNP, 957 A>G (fig. 4.14), was found. None of these SNPs are associated with a change in the amino acid sequence.

Figure 4.14: SNPs in exon 6



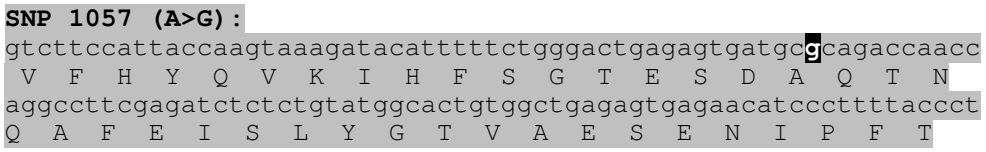
One healthy control dog showed a 967 A>G substitution, leading to a single amino acid substitution, Lys(323)Glu.



Exon 7

One normal control dog showed a 1057 A>G substitution (fig. 4.15). This allele does not lead to a change in the protein sequence.

Figure 4.15: SNP in exon 7



Also, variations within the adjacent intronic regions of exon 1 – 9 could be identified. Table 4.2 shows the nature and distribution of these findings.

Table 4.2: Overview of SNPs found in intron 1, 2, 3, 4, and 6 in 21 Miniature Schnauzers

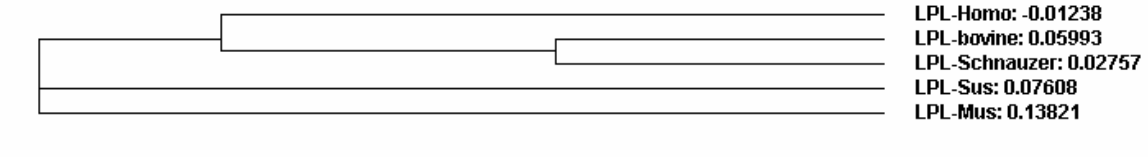
		Intronic Variations				
		Intron 1	**Intron 2**	**Intron 3**	**Intron 4**	**Intron 6**
Affected Dogs	1					
	2					
	4			G>A, 27 bp downstream Ex3		
	5					
	6					
	8					
	15					
	16	A>G, 8bp upstream Ex2				
	17					
	18					
	20	A>C, 8bp upstream Ex2			T insert 31 bp upstream Ex5	
	21					
Healthy Control Dogs	3					
	7					
	9					
	10				C>T, 12 bp upstream Ex5	CC>TT, 5,6 bp upstream Ex7
	11			A insert, 29 bp downstream Ex3		
	12	A>C, 8bp upstream Ex2	T>G, 36bp downstream Ex2			
	13					
	14					
	19					

5. Discussion

The lipoprotein lipase (LPL) gene was chosen as a possible candidate gene for the development of hyperlipidemia and pancreatitis in Miniature Schnauzers because of the central role of LPL in lipid metabolism. Also, LPL has been associated in humans affected with a similar syndrome that also leads to hypertriglyceridemia and chronic pancreatitis and is termed LPL deficiency. Finally, the LPL gene shows a highly conserved structure and function among mammals, which justified the evaluation of the LPL gene in the dog. Miniature Schnauzers with hypertriglyceridemia and concomitant pancreatitis were selected to make up the affected group. Goal was to sequence the LPL gene in the dog and to determine if a statistically significant difference exists in the LPL nucleotide sequence between affected Miniature Schnauzers and healthy control dogs. Additionally, intron/exon boundaries of the coding sequence were screened for nucleotide variations that could interfere with splicing.

We sequenced the complete LPL cDNA from 21 dogs. When compared to other species (fig. 5.1) closest similarity was found with the bovine and human sequence, as expected according to phylogenetic data.

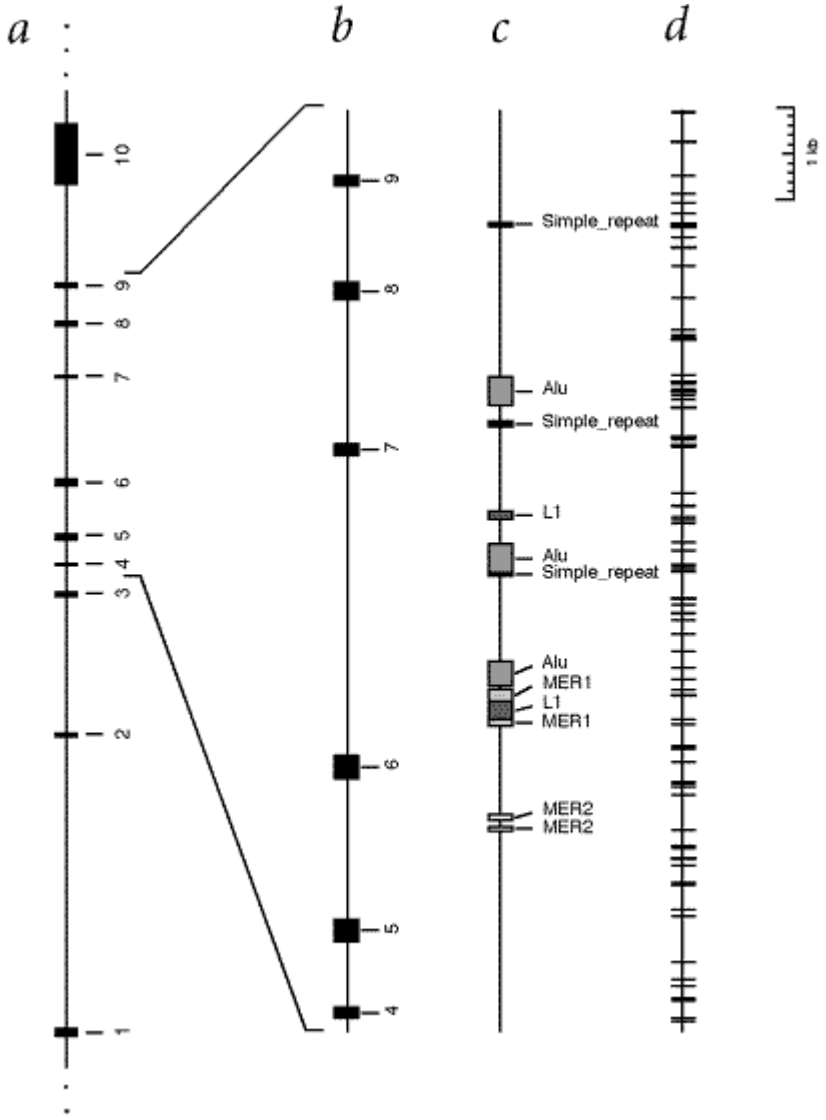
Figure 5.1: Phylogram tree of LPL genes in different species



Exons 2 through 9 showed a conserved length among all mammalian species recorded. Exon 1 in the dog showed four nucleotide deletions, resulting in a total length of 76 bp as compared to 88 bp in the human LPL gene. This lack of conservation can be attributed to less stringent requirements for this region, which is noncoding. Exon 1 of the LPL gene makes up the 5' untranslated region (5' UTR), while exon 2 starts with the initiator codon ATG (Murthy et al., 1996). Therefore, even though the lipoprotein lipase is very well conserved among species, sequence differences in exon 1 can occur.

107 reported mutations have been linked to LPL malfunction resulting in hyperlipidemia and pancreatitis in humans (2.1.8). These mutations mainly cluster around regions that encode for the major protein domains. Exon 4, 5, and 6 of the human LPL gene show so called “hot spots” (fig. 2.2). Mutations in these regions affect the enzyme function by interfering with the 3 major binding sites for HSPG, apo C2, and the triglyceride substrate as well as dimerization (Murthy et al., 1996).

Figure 5.2: DNA sequence diversity in a 9.7-kb region of the human lipoprotein lipase gene (Murthy et al., 1996)



a) The entire gene spans approximately 30 kb of genomic DNA. b) The region sequenced in 71 individuals spans 9.7 kb of the LPL gene from the 3´ end of intron 3 to 5´ end of intron 9. d), The types and distribution of repeat sequences within the target region identified by RepeatMasker. d) The location of the 88 DNA variants identified by sequencing the 71 individuals across the 9.7-kb region.

In a study of 71 humans unselected for health status 88 sequence variations had been found in the LPL gene (fig. 5.2), including those that would lead to amino-acid substitutions. The amount of DNA variation found in LPL highlights the potential problems associated with interpreting genetic studies (Nickerson et al., 1998). The variation found in this gene is considerably greater than generally appreciated in biomedical genetics. These data suggest that the levels and patterns of sequence variation found in the human LPL gene could pose a challenge in identifying sites, or a combination of sites, that influence variation in risk of disease within and among populations.
10 exonic SNPs and 9 intronic SNPs were identified in 21 Miniature Schnauzers. Upon analysis, none of the alleles identified in this study showed alterations that were associated with the disease status.

Twenty-one study dogs were chosen from all across the United States, and therefore are likely to be from different pedigree lines, although they are derived from a common ancestral stock. The appearance of SNPs among dogs from different ancestral lines is a common phenomenon in purebred dog populations. Our findings suggest that the nucleotide substitutions we identified in the canine LPL gene are due to pedigree line differences rather than disease status. Similar to microsatellites (2.2.2.), SNPs are commonly used markers for certain lines of origin as well as disease traits.

We also identified two differences in the LPL nucleotide sequence between the Trace Archive sequence derived from the Boxer and the Miniature Schnauzer. The sequence on Trace Archive has not yet been curated, and it is likely that these findings are due to sequences trace problems. Also, breed differences between the Boxer and the Miniature Schnauzer are a possible explanation. More importantly, upon further analysis, both nucleotide sequences resulted in the same amino acid sequence.

In conclusion, based on the study population examined here, the syndrome of hypertriglyceridemia and pancreatitis in the Miniature Schnauzer is not linked to mutations in the mRNA or the splicing regions of the lipoprotein lipase gene.

6. Summary

Lipoprotein Lipase (LPL) is a key enzyme in lipid transport. It catalyses the hydrolysis of the triacylglycerol component of chylomicrons and very low-density lipoproteins (VLDL), providing non-esterified fatty acids for tissue utilization. The gene encoding for LPL has already been identified in several species except the dog. Mutations of the human LPL-gene have been shown to cause partial or complete malfunction of the enzyme, resulting in accumulation of lipoproteins in the blood. This condition is called familial LPL deficiency. LPL malfunction results in hyperlipoproteinemia, recurrent acute pancreatitis, and ultimately pancreatic insufficiency.

Several authors have postulated a genetic cause for pancreatitis in the Miniature Schnauzer. An idiopathic increase in serum triglyceride concentration can also be found in this breed.

Based on these findings we were evaluating a possible role of the lipoprotein lipase gene in the development of pancreatitis and hyperlipidemia in the Miniature Schnauzer. First, we identified the genetic sequence of the LPL gene in the dog. We determined clones on the Trace Archive database for the canine genome project that contain the genomic sequence of a particular exon as well as its adjacent intronic regions. Based on these findings we designed primers for each exon using the software Netprimer (www.premierbiosoft.com/netprimer/index.html). Canine subjects were chosen from a pool of 170 Miniature Schnauzers from the database at the Gastrointestinal Laboratory at Texas A&M University. Based on clinical history, serum cPLI concentrations, and serum triglyceride concentrations 21 Miniature Schnauzers were chosen and were selected into a clinically normal control group (9 dogs) and an affected group (12 dogs). DNA was then collected from either white blood cells or mucosal cells of these dogs. After PCR optimization, exon 1 through 9 including the adjacent intronic regions were amplified in all dogs using MasterAmp Extra – Long PCR Kit (Epicentre, WI, USA) and were sequenced in triplicates. Differences in the nucleotide sequences were then compared among the two groups. 10 exonic SNPs and 9 intronic SNPs were identified. Upon analysis, none of these variations could be associated with the disease status.

We conclude that pancreatitis associated with hyperlipidemia in the Miniature Schnauzer is not linked to mutations of the lipoprotein lipase gene or its splicing regions.

7. Zusammenfassung

Die Lipoprotein Lipase (LPL) ist ein Enzym mit zentraler Bedeutung im Fettstoffwechsel. Das Enzym katalysiert die Hydrolyse der triglyzerid Komponente von Chylomikronen und Very Low Density Lipoproteinen (VLDL). Dabei werden unveresterte Fettsäuren freigesetzt, welche dann zur Gewebeaufnahme zur Verfügung stehen. Das Gen, welches für die LPL kodiert, wurde bereits bei zahlreichen Spezies identifiziert, jedoch noch nicht beim Hund. Ferner wurde gezeigt, dass Mutationen im humanen LPL Gen die Enzymfunktion teilweis oder vollständig einschränken, wodurch es zu einer Ansammlung von Lipoproteinen im Blut kommt. Dieses Krankheitsbild wird als familiärer LPL Mangel bezeichnet. Hyperlipoproteinämie, rezidivierende akute Pankreatitis, sowie im weiteren Verlauf exokrine Pankreasinsuffizienz sind die vorherrschenden Symptome dieser Erkrankung. Mehrere Autoren vermuten eine genetische Ursache für Pankreatitis beim Zwergschnauzer. Ebenso ist eine idiopathische Erhöhung der Triglyzeride im Serum bei dieser Rasse zu finden. Basierend auf diesen Beobachtungen haben wir das Lipoprotein Lipase Gen auf dessen potentielle Rolle bei der Entstehung von Pankreatitis und Hyperlipämie im Zwergschnauzer untersucht.

Zuerst wurde die Basensequenz des LPL Gens beim Hund bestimmt. Hierzu wurden Klone der Trace Archive Datenbank des Canine Genome Projects identifiziert, welche die Basensequenz für ein bestimmtes Exon sowie dessen angrenzende Introns enthielten. Die so bestimmten Gensequenzen dienten unter Verwendung der Software Netprimer (www.premierbiosoft.com/netprimer/index.html) als Vorlage bei der Gestaltung von Primerpaaren für die jeweiligen Exons.

Aus 170 Zwergschnauzern von der Datenbank des Gastrointestinal Laboratory an der Texas A&M University wurden anhand von klinischen Symptomen, cPLI Werten und Serum-Triglyzerid Konzentrationen 21 Hunde für diese Studie ausgewählt. In 12 Hunden war das Krankheitsbild ausgeprägt, während 9 Hunde klinisch gesund waren. Anschliessend wurde von allen Hunden DNA aus weissen Blutzellen oder Schleimhautzellen gewonnen. Nach Optimierung der PCR wurden under Verwendung eines MasterAmp Extra-Long PCR Kits (Epicentre, WI, USA) Exon 1 bis 9 einschliesslich der jeweils angrenzenden Intron Regionen in allen Hunden amplifiziert, und

anschliessend dreifach sequenziert.

Insgesamt konnten 10 SNPs in Exons, sowie 9 SNPs in den angrenzenden Intron Regionen identifiziert werden. Weitere Untersuchungen ergaben, dass keine dieser Variationen mit dem Krankheitsbild in Verbindung steht.

Aus unseren Ergebnissen schliessen wir, dass Pankreatitis verbunden mit Hyperlipämie beim Zwergschnauzer nicht mit Mutationen des Lipoprotein Lipase Gens oder dessen Splicing Regionen in Verbindung steht.

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Appendix

A.1. List of Figures

A.2. List of Tables

A.3. Abbreviations

LPL:	lipoprotein lipase
apo C2:	apolipoprotein C2
TAG:	triacyglycerol
VLDL:	very low density lipoprotein
HSPG:	heparin sulfated proteo glycans
apo E:	apolipoprotein E
kb:	kilobase
bp:	basepair
ER:	endoplasmatic reticulum
Lys:	Lysine
Arg:	Arginine
Cys:	Cysteine
Gly:	Glycine
Ala:	Alanine
Thr:	Threonine
Asp:	Aspartate
Glu:	Glutamate
HGMD:	human gene mutation database
MSS-1/2:	minimal screening set 1/2
PCR:	polymerase chain reaction
SNP:	single nucleotide polymorphism
PSTI:	pancreatic secretory trypsin inhibitor
EPI:	exocrine pancreatic insufficiency
PAA:	pancreatic acinar atrophy
SIBO:	small intestinal bacterial overgrowth
GSD:	German Shepherd dog
cTLI:	canine trypsin like immunoreactivity
cPLI:	canine pancreatic lipase immunoreactivity
cDNA:	complementary DNA
ΔH:	enthalpy for helix formation
ΔS:	entropy for helix formation
R:	molar gas constant (1.987 cal/°C * mol)
C:	the nucleic acid concentration
[K+]:	potassium salt concentration
Taq:	polymerase isolated from the bacteria Thermophilus aquaticus
Tm:	melting temperature
A:	adenine
G:	guanine
T:	thymine
C:	cytosine
Ex:	exon

A.4. Curriculum Vitae

Name:	Ralph Schickel
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