INVESTIGATIONS INTO HYPERLIPIDEMIA AND ITS POSSIBLE ASSOCIATIONS WITH PANCREATITIS IN DOGS

A Dissertation

by

PANAGIOTIS XENOULIS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Biomedical Sciences

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ABSTRACT

Investigations into Hyperlipidemia and its Possible Associations with Pancreatitis in Dogs. (May 2011)

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Chair of Advisory Committee: Dr. Jörg M. Steiner

The relationship between hyperlipidemia and pancreatitis remains obscure in dogs. The aim of the present study was to investigate any possible association between hyperlipidemia and pancreatitis in dogs.

In the first part of the study, Miniature Schnauzers with hypertriglyceridemia were found to have significantly higher serum cPLI concentrations than Miniature Schnauzers with normal serum triglyceride concentrations (P=0.0001). Also, Miniature Schnauzers with severe hypertriglyceridemia (>862 mg/dL) had 4.5 times higher odds (P=0.0343) for having a serum cPLI concentration consistent with pancreatitis.

In the second part of the study, 17 Miniature Schnauzers prospectively enrolled with a history of pancreatitis were significantly more likely to have hypertriglyceridemia (71%) after resolution of pancreatitis than 34 age-matched Miniature Schnauzers without a history of pancreatitis (33%; odds ratio=5.02; P=0.0163).

For the third part of the study, assessment of the feasibility and usefulness of a novel density gradient ultracentrifugation method using NaBiEDTA for lipoprotein profiling in dogs was attempted. Density gradient ultracentrifugation using NaBiEDTA

was found to be useful for the study of lipoprotein profiles in dogs. Significant differences were detected in the lipoprotein profiles (mainly involving TRL and specific LDL fractions) among healthy Miniature Schnauzers, dogs of various other breeds, and hypertriglyceridemic Miniature Schnauzers.

In the fourth part of the study, the effect of a commercially available low-fat diet on serum lipid and pancreas-specific lipase (Spec cPL®) concentrations and lipoprotein profiles in Miniature Schnauzers with primary hypertriglyceridemia was evaluated. The study diet was found to be effective in significantly reducing serum triglyceride and cholesterol concentrations and changing the lipoprotein profiles of the dogs studied within 2 months. However, there was no significant effect of the study diet on serum Spec cPL concentrations.

In the last part of the study, serum triglyceride and cholesterol concentrations and lipoprotein profiles were compared between dogs with naturally occurring pancreatitis and healthy dogs. The majority of dogs with naturally occurring pancreatitis had normal serum triglyceride and cholesterol concentrations. Important differences were identified in lipoprotein profiles between dogs with pancreatitis (higher LDL_2, LDL_3, and LDL_4 fractions and lower TRL, HDL_{2a}, and HDL_{3c} fractions) and healthy control dogs.

DEDICATION

To Patricia

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Jörg Steiner, for his help and support throughout the course of this research, and also for the opportunities he has given me over the years that we have worked together. I would also like to thank my committee members, Dr. Rosemary Walzem, Dr. Craig Ruaux, Dr. Noah Cohen, and Dr. Jan Suchodolski, for their guidance and support. They have helped me a lot with their ideas and suggestions, and I have learned a lot from all of them.

I am especially grateful to Dr. Ronald Macfarlane for allowing me to do part of this research at the Laboratory for Cardiovascular Research, a component of the Department of Chemistry at Texas A&M University, and Paul Cammarata for his invaluable help with lipopoprotein profile analysis.

The people I worked with at the Gastrointestinal Laboratory at Texas A&M University deserve a special mention. Fellow Ph.D. students, technicians, and student workers, have all helped directly or indirectly with this project and they have made the time I spent with them fun and enjoyable. I thank all of them and wish them good luck with their lives.

I would also like to thank Royal Canin and especially Dr. Genie Thoni for providing funding for the dietary study of this project as well as for several other ongoing projects.

Several faculty members of the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University have also indirectly contributed positively to the

outcome of this research. They have been a source of inspiration and encouragement for me.

Many thanks to my family and friends, both in College Station and in Greece, for everything they have done and keep doing for me. Finally, I must thank my wife Patricia for her encouragement and support. Without her, none of this would be possible.

Panos Xenoulis

January 2011, College Station, Texas

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CHAPTER I

INTRODUCTION AND

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Lipoprotein metabolism and hyperlipidemia in dogs

History of the investigation of canine lipoproteins and lipoprotein metabolism

The first studies investigating canine lipoproteins were published in the 1940s and 1950s.[1-5] Without exception, these studies used dogs as an experimental animal model to study human disease. Most of these studies used a combination of methods to separate and classify the lipoprotein profiles of the dogs, including different forms of ultracentrifugation (e.g., sequential ultracentrifugation, analytical ultracentrifugation), flotation rates, precipitation with sulfated polysaccharides (e.g., dextran sulfate), and electrophoresis (e.g., paper electrophoresis). These methods are rarely used today, at least in the fashion that they were used in those initial studies, and their accuracy is now considered to be rather limited. Also, the majority of methods used in these initial studies were optimized to analyze lipoproteins in human serum, which, in contrast to dogs, predominantly contains low-density lipoproteins (LDL). Thus, complete separation of canine LDL and high-density lipoproteins (HDL) was not always achieved.[1-5]

Furthermore, information about food withholding was not always available, and

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This dissertation follows the style of *Journal of Veterinary Internal Medicine*.

the results of these initial studies are hard to interpret today mainly because of the different terminology and classification schemes used. Nevertheless, some basic information can be derived from those initial studies. For example, these studies showed that the majority of lipoproteins found in dogs (~75%) have a very low flotation rate (-3S) and thus would be classified as HDL molecules today (most likely HDL_2 and HDL_3).[1-5] Lipoproteins with higher flotation rates (-6S and -27S) were found to represent between 1% and 10% of the total lipoproteins of canine serum, and likely included LDL, VLDL, and chylomicron molecules.[1-5]

Subsequent studies in the 1970s by Solyom and colleagues[6,7] and Mahley and colleagues[8-10] provided additional information on lipoproteins in fasted healthy dogs, and showed that canine plasma lipoproteins consisted primarily of HDL_2, with much lower concentrations of HDL_1 and LDL. Chylomicrons and VLDLs were found only in dogs that were fasted for less than 12 hours. These researchers also determined the protein and lipid distribution of canine lipoproteins. They reported that 85% of total plasma cholesterol was carried by HDL_2, and 50% of plasma triglyceride was carried by VLDL. The above mentioned studies were also the first ones to describe in more detail some of the apoproteins found in each lipoprotein fraction. In their studies, both Solyom and colleagues and Mahley and colleagues used ultracentrifugation combined with electrophoresis for lipoprotein fractionation and characterization. In addition, immunohistochemical studies were performed to describe the apoproteins found in each lipoprotein fraction.

A series of subsequent publications from the late 1970s until recently, have investigated canine lipoprotein profiles in association with or in response to obesity,[11,12] diet change,[13,14] primary disorders of lipid metabolism,[15-17] experimentally induced pancreatitis,[18-20] and other (mainly endocrine) diseases.[21] These studies have used similar methodologies to previous studies (i.e., ultracentrifugation combined with electrophoresis), and provide information for both healthy dogs (which were used as controls) and dogs with several spontaneous or experimentally induced diseases. The information on lipoproteins in healthy dogs is similar to that reported by Solyom and colleagues and Mahley and colleagues in the 1970s. However, information on specific lipoprotein profile patterns associated with certain diseases or conditions has been new for these studies. For example, Whitney and colleagues[17] reported that idiopathic hypertriglyceridemia in Miniature Schnauzers is mainly characterized by increases in serum VLDL concentrations, with or without an increase in chylomicrons. Jeusette and colleagues[12] reported that obesity results in increased serum cholesterol and triglyceride concentrations in all lipoprotein fractions, and that appropriate dietary modification resulted in improvement of these concentrations. The studies investigating the effect of experimental pancreatitis on plasma lipids and lipoprotein concentrations,[18-20] are hard to compare because different methods of induction of pancreatitis were used, and the resulting pancreatic inflammation differed in severity between studies. However, some common trends can be identified. Experimental induction of pancreatitis did not generally lead to clinically significant changes in serum triglyceride or cholesterol concentrations. With regard to lipoproteins, a trend towards an increase in serum LDL

and HDL_1 concentrations and a decrease in serum HDL_2 concentration was evident in most studies.[18-20]

Finally, several relatively recent studies have been published that report the qualitative and quantitative apoprotein, enzyme, and/or fatty acid composition of lipoproteins in healthy and diseased dogs.[22-27] In addition to the standard ultracentrifugation and electrophoresis methods for lipoprotein fractionation, these studies used other methods, including gas chromatography, size-exclusion chromatography-mass spectrometry, and enzyme activity assays, depending on the goal of each study. Important information regarding the composition of canine lipoproteins was reported in these studies. For example, one study reported the identification of apoprotein A-II in canine HDL molecules, which until then was believed to be absent in this species.[26] In another study, it was confirmed that dogs have undetectable activities of cholesteryl-ester transfer protein (CETP), an enzyme responsible for transferring cholesterol among different types of lipoproteins that plays a key role in the so called reverse cholesterol transport.[24] The results of this study explain at least in part the uniqueness of dogs with regard to cholesterol metabolism.

The standard methodological approach for lipoprotein fractionation and characterization in the vast majority of the reported studies has been ultracentrifugation and electrophoresis. Several variations of these techniques have been reported; however, the general principles remain the same. Ultracentrifugation has proven to be extremely useful in the evaluation of lipoproteins. The underlying principle is that lipoproteins are naturally arrayed in a variety of classes and subclasses that differ in their density. One of

the most commonly used ultracentrifugation techniques, especially in older studies, is sequential ultracentrifugation. The principle of this technique is the stepwise adjustment of non-protein solvent density by the addition of a dense solute (e.g., KBr). The sequential addition of dense solutes allows for the sequential (from the less dense to the most dense) separation of chylomicrons, VLDL, LDL, and HDL as separate classes. Clear separation of triglyceride-rich lipoproteins (i.e., chylomicrons, VLDLs) and lipoprotein subclasses is not always possible to achieve with this method. Density-gradient ultracentrifugation is another method commonly used for lipoprotein fractionation studies. For this approach, solutions providing a stepwise decrement of density are layered in the tube, thus providing vertical stability. During ultracentrifugation, lipoproteins are distributed in the solution based on their densities. This technique is less time consuming than sequential ultracentrifugation, and with appropriate modifications allows for separation of subclasses of certain lipoproteins (LDL and HDL). The other standard methodology used for lipoprotein characterization (typically in combination with ultracentrifugation) is electrophoresis. Electrophoresis can be applied to whole serum or specific ultracentrifugation fractions. A variety of bands can be seen on the gel after electrophoresis (e.g., *β*, *pre-β*, *α*-lipoproteins). A complete lipoprotein analysis should include both ultracentrifugation and electrophoresis, and this combination has been used for the majority of studies on canine lipoproteins.

The above mentioned studies provide sufficient information on the major lipoprotein classes found in dogs. Healthy fasted dogs primarily have HDL molecules

and, in smaller concentrations, LDL and VLDL molecules. However, the existence and distribution of subclasses of these lipoproteins is less certain. Studies in humans using ultracentrifugation with novel density gradient solutions with or without affinity separation methods have revealed the existence of several subfractions of each of the major lipoproteins, with important health implications.[28-30] The existence and significance of such lipoprotein subfractions has yet to be determined in dogs.

Definitions

The term hyperlipidemia refers to an increased concentration of lipids (triglycerides, cholesterol, or both) in the blood.[31-33] More specifically, an increased blood concentration of triglycerides is referred to as hypertriglyceridemia, while an increased blood concentration of cholesterol is referred to as hypercholesterolemia. The term hyperlipoproteinemia refers to increased blood concentrations of lipoproteins, but it is often used interchangeably with the term hyperlipidemia.[34] However, the term hyperlipoproteinemia should ideally be limited to cases where measurements of lipoprotein concentrations have been conducted.[31,34]

The term lipemia is used to describe a turbid or lactescent appearance of serum or plasma.[31-33] Lipemia is a result of hypertriglyceridemia, but not hypercholesterolemia.[31,33-35] Mild hypertriglyceridemia does not cause lipemia. Usually, lipemia is apparent when serum triglyceride concentrations exceed 200 to 300 mg/dL.[34] As serum triglyceride concentrations increase, serum becomes turbid (cloudy) and then lactescent (milky).[31,33-35]

Hypertriglyceridemia is a relatively common clinicopathologic finding in dogs. In a study of 1,022 blood samples from both healthy and diseased dogs of various breeds, 5.4% had an increased serum triglyceride concentration, but the study did not include grossly lipemic samples.[36] Postprandial hypertriglyceridemia is normal, and typically resolves within 7-12 hours after a meal, depending on the fat content of the meal.[14,37] Persistent fasting hypertriglyceridemia is abnormal and can be either primary (most commonly idiopathic) or secondary to other diseases or drug administration.[37]

Overview of canine lipoproteins and lipoprotein metabolism

Canine lipoproteins can be divided into 4 major classes based on their hydrated density after ultracentrifugation: chylomicrons, very low density lipoproteins (VLDL), LDL, and HDL.[23,32,38] In humans, a class of intermediate density lipoproteins (IDL) and lipoprotein (a) have also been identified. These 2 classes of lipoproteins have not been described in dogs.[39,40] In addition, the existence of several other lipoprotein subclasses has been recently proposed for humans (LDL-1 through LDL-5, HDL-2a, HDL-2b, and HDL-3a through HDL-3b), but these subclasses have not been described in dogs to date.

Canine chylomicrons consist of triglycerides, phospholipids, free and esterified cholesterol, and apo B_{48}.[34,37,39-41] Human chylomicrons also contain apo A-I and apo A-IV, but this has not been reported in dogs.[34,37,39-41] Human and canine VLDL molecules are formed by endogenously synthesized triglycerides, cholesterol, phospholipids, and apo B_{100}, while canine VLDL molecules also contain B_{48}.[39-41] LDL molecules in humans contain mainly cholesteryl esters and B_{100}, but in dogs they also contain B_{48}.[37]

HDL molecules can be further subdivided into HDL_1, HDL_2, and HDL_3.[8,31,32,37-40,42] HDLs play an important role as donors and acceptors of apolipoproteins C (I, II, III), apo E, and various lipids from other lipoproteins in the circulation.[32,37,39,40,43] In humans, they also contain apo A-I, apo A-II, and apo A-IV. In dogs, HDL molecules have been reported to contain apo A-I,[8] while other apo A proteins were reported to be absent.[44] However, recent evidence suggests that canine HDLs also contain apo A-II and apo A-IV.[26] HDLs play a fundamental role in the metabolic pathway known as the reverse cholesterol transport pathway (see below).[32,37,39,45] Dogs appear to have predominantly HDLs (mainly HDL_2, but also HDL_3 and HDL_1), and the majority of cholesterol (~85%) is found in HDL, while LDL is found in very low concentrations.[8,26,37]

Lipid metabolism can be divided into 2 basic pathways: the exogenous pathway, which is associated with the metabolism of exogenous (dietary) lipids, and the endogenous pathway, which is associated with the metabolism of endogenously produced lipids.[37,39,40]

Exogenous pathway

The first step of dietary lipid metabolism is digestion.[41,46] Dietary lipids that reach the duodenum undergo emulsification and are then hydrolyzed by gastric and pancreatic lipases.[41,46,47] Hydrolysis products are transferred to the microvilli of the intestinal epithelial cell brush border in the form of micelles, where they diffuse through the epithelial cell membranes into the enteric mucosal cells.[41,46] In the intestinal mucosal cell, free fatty acids and monoglycerides reassemble to form new triglycerides, which

then combine with phospholipids, free and esterified cholesterol, and the Apo B_{48} protein to form chylomicrons.[34,37,39-41]

Chylomicrons are the lipoprotein class responsible for transfer of dietary lipids. After formation in the enterocytes, chylomicrons, which mainly contain triglycerides, are secreted into the lacteals and enter first the lymphatic and later the blood circulation where they acquire apolipoproteins C and apo E from circulating HDL molecules.[34,37,39-41] Apolipoprotein C-II, which is exposed on the chylomicron surface, activates the lipoprotein lipase attached to the capillary beds in adipose and skeletal muscle tissues, which then hydrolyzes triglycerides into free fatty acids and glycerol.[34,37,39-41] Free fatty acids enter the muscle cells (where they are used for energy production) and/or adipocytes (where they are re-esterified into triglycerides for storage). The cholesteryl-rich remaining particles (chylomicron remnants), return their apo C-II molecule to HDL and are recognized by specific hepatic apo E receptors that rapidly remove them from the circulation by endocytosis.[34,37,39-41] The cholesterol found in chylomicron remnants can be used for lipoprotein (VLDL) and/or bile acid formation or can be stored as cholesteryl esters.[34,41]

Endogenous pathway

While chylomicrons are responsible for transport of dietary lipids, VLDL, LDL, and HDL are mainly involved in the metabolism of endogenously produced lipids.[41] Endogenously synthesized triglycerides and cholesterol (and cholesteryl esters) combine with phospholipids, apo B_{100}, and apo B_{48} to form VLDL.[37,39-41] After VLDL molecules

reach the vasculature, they acquire apolipoproteins C and apo E from HDL.[34,37,39,40] VLDL apo C-II activates lipoprotein lipase located in the capillary beds, which in turn leads to hydrolysis of triglycerides and the production of free fatty acids and glycerol. The VLDL molecules remaining after hydrolysis of VLDL triglycerides (VLDL remnants), are either removed from the circulation in the liver or undergo further transformation by lipoprotein lipase and/or hepatic lipase to form LDL.[31,34,37,39-41]

LDL, which contains mainly cholesteryl esters, circulates in the blood and binds to specific receptors that are widely distributed throughout tissues in order to deliver cholesterol, which can be used for the synthesis of steroid hormones and cell membranes and for hepatic metabolism.[39-41]

HDL molecules, which are synthesized primarily in the liver, play an important role as donors and acceptors of apolipoproteins C, apo E, and various lipids from other lipoproteins in the circulation.[32,37,39,40] They play a fundamental role in the reverse cholesterol transport pathway, through which cholesterol is transferred from peripheral tissues to the small circulating discoid HDL molecules, thus converting them to nascent HDL_3 molecules.[32,37,39,45] Cholesterol is then esterified by the action of lecithin-cholesterol acyltranferase (LCAT), and cholesteryl esters move to the core of the HDL_3 molecule, thus allowing more free cholesterol to get absorbed onto their surface. Continued absorption of free cholesterol and subsequent esterification by LCAT leads to the formation of the larger, cholesteryl ester-rich HDL_2.[37,39,40,45] In humans, an additional enzyme (cholesteryl ester transfer protein; CETP) is involved in this process but the presence of this enzyme has not been documented in dogs.[24,31,32,37,39,42,48] The role of this

enzyme is to transfer triglycerides from LDL, VLDL, and chylomicrons to HDL_2 in exchange for cholesteryl esters. This results in the production of cholesteryl ester-rich LDL and triglyceride-rich HDL_2 molecules.[31,32,37,39,40] Due to the absence of this enzyme in dogs, HDL_2 molecules continuously acquire cholesteryl esters, resulting in the formation of HDL_1 molecules, which are unique to dogs. Cholesteryl esters are transferred from various tissues to the liver for disposal or reuse through HDL_1, rather than through LDL or VLDL molecules, which transfer cholesterol to peripheral tissues.[31,32,37] Thus, it is this function of HDL_1 that accounts for the lower incidence of atherosclerotic disorders in dogs compared to humans.[31]

Hyperlipidemia in dogs

Postprandial hyperlipidemia is physiologic and typically resolves within 7-12 hours after a meal, depending on the fat content of the meal.[14,31,37,49] Therefore, any determination of serum lipid concentrations should always follow a fast of at least 12 hours. Persistent fasting hyperlipidemia is abnormal and can be either primary or secondary to other diseases or drug administration.

Secondary causes of hyperlipidemia

Secondary hyperlipidemia is the most common pathologic form of hyperlipidemia in dogs.[50] Several diseases have been reported to cause hyperlipidemia.

Endocrine disease

Most commonly, canine hyperlipidemia is the result of an endocrine disorder, such as hypothyroidism, diabetes mellitus, or hyperadrenocorticism.[21,31,37,49,51,52] Increases of both serum triglyceride and cholesterol concentrations have been reported in dogs with hypothyroidism.[21,53-56] In 1 study, hypertriglyceridemia and hypercholesterolemia were found in 88% and 78% of dogs with hypothyroidism, respectively.[53] Usually, lipid abnormalities resolve after treatment of hypothyroidism.[21] In dogs with diabetes mellitus, hyperlipidemia is most commonly associated with hypertriglyceridemia, but hypercholesterolemia can also occur.[21,31,49,51,54,57] Hypertriglyceridemia usually resolves after successful management of diabetes mellitus, but hypercholesterolemia might persist despite therapy.[49,58] Finally, both naturally occurring hyperadrenocorticism and iatrogenic Cushing's disease have been associated with hyperlipidemia (hypertriglyceridemia and/or hypercholesterolemia) in dogs.[31,37,49,51,54,59,60] Hypertriglyceridemia might be present more frequently than hypercholesterolemia and increases of both types of lipids are usually mild or moderate.[31,37,51]

Pancreatitis

The presence of hyperlipidemia (hypertriglyceridemia and/or hypercholesterolemia) has long been associated with naturally occurring pancreatitis in dogs.[21,31,37,49,52,61-66] However, it remains uncertain whether hyperlipidemia develops as a result of pancreatitis or can be the cause of pancreatitis in some cases.[49,66] In models of

experimentally induced pancreatitis in dogs, hyperlipidemia does not develop after induction of pancreatitis.[18-20] However, the role of naturally occurring pancreatitis in the development of secondary hyperlipidemia is unknown. Thus, based on the current literature, it is possible either that hyperlipidemia is a preexisting abnormality in some dogs with naturally occurring pancreatitis, which might or might not contribute to the development of the disease, or that naturally occurring pancreatitis differs in its ability to produce hyperlipidemia from the experimental models of pancreatitis applied in the above mentioned studies.

Obesity

Increased serum triglyceride and/or cholesterol concentrations have been observed in obese dogs.[11,12,67] The most profound changes were associated with severe chronic obesity.[12] Weight loss in obese dogs leads to significant decreases of both serum triglyceride and cholesterol concentrations.[12,68]

Protein losing nephropathy (PLN)

Proteinuria associated with PLN, regardless of the cause, is often associated with hyperlipidemia in dogs. The typical lipid abnormality in dogs with PLN is hypercholesterolemia, which is usually mild or moderate.[69-73]

Hypercholesterolemia is usually part of a more complex syndrome, the nephrotic syndrome, which in addition to hypercholesterolemia, is characterized by hypoalbuminemia, proteinuria, and ascites.[31,37] Hypercholesterolemia has been reported

with varying frequencies in dogs with acquired glomerular disease and proteinuria, as well as in several hereditary forms of PLN (e.g., in Chinese Shar-Peis and Golden Retrievers).[69-73]

Cholestasis

Cholestasis has been reported to lead to mild or moderate hypercholesterolemia and mild hypertriglyceridemia in dogs.[49,74,75] Changes in lipoproteins, most importantly excessive esterification of lipoprotein cholesterol, have also been reported in dogs with experimentally induced cholestasis.[76,77]

Other causes

Several other causes of hyperlipidemia have been reported or suspected in dogs. These include high fat diets, lymphoma, infection with *Leishmania infantum*, congestive heart failure due to dilated cardiomyopathy, and administration of certain drugs (e.g., glucocorticoids, estrogen, phenobarbital, and potassium bromide).[14,78-81] Finally, in a recent study, significantly increased serum triglyceride concentrations were reported in association with other lipid abnormalities in dogs with parvoviral enteritis.[82]

Primary causes of hyperlipidemia in dogs

Primary lipid abnormalities are usually, but not always, associated with specific breeds. Depending on the breed, the prevalence of primary lipid abnormalities can vary widely.

Primary hyperlipidemia in Miniature Schnauzers

Primary hyperlipidemia in Miniature Schnauzers was the first breed-related primary lipid disorder described in dogs.[15,17,34,37,49,83] This condition was first reported more than 30 years ago in Miniature Schnauzers in the United States.[15,17,49,83] It is characterized by an abnormal accumulation of VLDL or a combination of VLDL and chylomicrons. Although hypercholesterolemia may also be present, this finding is not consistent.[17,49,83] A recent study has shown that primary hypertriglyceridemia is common in healthy Miniature Schnauzers in the United States, being present in 32.8% of 192 healthy Miniature Schnauzers investigated.[84] However, hypertriglyceridemia was much more prevalent among older dogs; more than 75% of healthy Miniature Schnauzers ≥ 9 years of age had hypertriglyceridemia. In addition, the severity of hypertriglyceridemia increased with age and the vast majority (> 80%) of Miniature Schnauzers with moderate to severe hypertriglyceridemia were ≥6 years of age.[84] There was no difference between male and female Miniature Schnauzers with regard to the prevalence of hypertriglyceridemia. These findings would suggest that hypertriglyceridemia of Miniature Schnauzers is the most common primary lipid disorder in dogs. It has been recommended that all Miniature Schnauzers in the United States should be evaluated for hypertriglyceridemia while they are healthy, because this information may be useful for the avoidance of misinterpretation of increased serum triglyceride concentrations when the dogs are presented for a clinical illness.[84] In addition, knowing the serum triglyceride status of the dog, the veterinarian might consider offering to switch the affected dog to a low-fat diet to avoid possible complications of hypertriglyceridemia. Due to the fact that

hypercholesterolemia was found only in association with hypertriglyceridemia, the presence of hypercholesterolemia alone in Miniature Schnauzers might require additional diagnostic investigation.

The cause of primary hypertriglyceridemia in Miniature Schnauzers is unknown. The fact that hypertriglyceridemia is highly prevalent within a single breed suggests a genetic cause.[15,17,83] Because lipoprotein lipase is the major enzyme involved in triglyceride clearance, deficiency of this enzyme has been considered as a possible cause of hypertriglyceridemia in this breed.[15,17,83] The crucial role of lipoprotein lipase in lipoprotein metabolism has been demonstrated in experimental animals (knockout mice), cats, dogs, and humans with lipoprotein lipase deficiency, which leads to severe hypertriglyceridemia.[85,86] However, a pilot study in Miniature Schnauzers with hypertriglyceridemia and pancreatitis failed to identify any mutations of the lipoprotein lipase gene, suggesting that inherited lipoprotein lipase dysfunction might not be the cause of hypertriglyceridemia in this breed.[87] In that particular study, however, dogs had both hypertriglyceridemia and pancreatitis, and the hypertriglyceridemia might have been a result of pancreatitis rather than the underlying condition.

Another plausible explanation for hypertriglyceridemia in Miniature Schnauzers is apo-CII deficiency. Deficiency of this enzyme was first described in 1978 by Breckenridge et al. in a human patient with severe hypertriglyceridemia.[88] Since then several reports of apo C-II deficient patients have been published and the abnormal apo C-II protein has been sequenced.[89-96] To date, at least 16 mutations of the apo C-II gene have been reported in humans.[97-111] In a recent preliminary study, the gene encoding

apolipoprotein C-II, which is an activator of lipoprotein lipase, was evaluated for the presence of possible mutations in Miniature Schnauzers with primary hypertriglyceridemia. However, no variants of the apo-CII gene were found to be associated with hypertriglyceridemia.[112] Further studies are warranted to identify the genetic basis of primary hypertriglyceridemia in Miniature Schnauzers.

Until recently, primary hypertriglyceridemia was considered to be a relatively benign condition in Miniature Schnauzers. However, recent studies indicate that hypertriglyceridemia in Miniature Schnauzers might be associated with pancreatitis, hepatobiliary disease, ocular disease, seizures, or possibly other conditions (for further discussion on this topic please see "Clinical signs and complications of hyperlipidemia" on page 18).

Primary hyperlipidemia in other dog breeds

Primary hypercholesterolemia without hypertriglyceridemia has been documented in 15 Briards from the United Kingdom (UK).[35] A similar condition has also been described in a family of rough-coated Collies also from the UK.[16] A slightly different condition of primary hypercholesterolemia with or without concurrent hypertriglyceridemia has been reported in Shetland Sheepdogs.[113] The cause of these lipid abnormalities that mainly involve cholesterol metabolism has not yet been determined, but hereditary factors were suspected.[16,35,113] In addition, primary hypercholesterolemia has been reported anecdotally in Doberman Pinschers and Rottweilers.[114]

Primary hyperlipidemia with hypercholesterolemia and hypertriglyceridemia has been reported in 2 related Beagles.[115] Finally, primary hypertriglyceridemia has been reported in 2 related Brittany Spaniels and 1 mixed-breed 28-day old puppy.[116,117]

Clinical signs and complications of hyperlipidemia

In general, dogs with secondary hyperlipidemia display clinical signs associated with the primary disorder. In contrast, dogs with primary lipid disorders are often asymptomatic for long periods or throughout their lives, depending on many factors, including the type and severity of hyperlipidemia. However, some dogs with hyperlipidemia develop secondary diseases as a result of hyperlipidemia that may account for the development of specific clinical signs.

Pancreatitis

For a detail discussion on this topic please see "Relationship between hyperlipidemia and pancreatitis" on page 47.

Hepatobiliary disease

Clinical studies and anecdotal observations suggest that 2 hepatic disorders might be associated with hypertriglyceridemia in dogs: vacuolar hepatopathy and gallbladder mucocele.[118,119] Hyperlipidemia-associated vacuolar hepatopathy has been anecdotally associated with hyperlipidemia in Miniature Schnauzers. Also, a gallbladder mucocele has been commonly reported in dog breeds that are predisposed to idiopathic

hyperlipidemia (e.g., Miniature Schnauzers and Shetland Sheepdogs), and hyperlipidemia has been implicated as a cause of gallbladder disease in humans.[120-122] In a recent study, an association between gallbladder mucocele formation and dyslipidemias (hypertriglyceridemia and hypercholesterolemia) was described in Shetland Sheepdogs.[121] In this study, many of the dogs with a gallbladder mucocele had no clinical signs or biochemical abnormalities, except for an increased serum ALP activity in some cases.[121] Although asymptomatic in many dogs, both vacuolar hepatopathy and gallbladder mucocele can be associated with significant morbidity and even mortality.

In a recent study, idiopathic hypertriglyceridemia (especially ≥ 400 mg/dL) was found to be associated with increased serum hepatic enzyme activities in healthy Miniature Schnauzers.[123] In that study, 60% and 45% of the Miniature Schnauzers with serum triglyceride concentrations ≥ 400 mg/dL had increased serum ALP and ALT activities, respectively. In contrast, 0% and 9% of the Miniature Schnauzers with normal serum triglyceride concentrations had increased ALP and ALT activities, respectively. Whether or not such cases require additional diagnostic investigation of the cause of the liver disease remains to be determined. Given the fact that in this particular study most dogs had increases in more than 1 serum liver enzyme activity that were considered significant (i.e., > 2 times the upper limit of the reference range), additional diagnostic work-up and/or retesting would appear to be appropriate.

Atherosclerosis

Although dogs appear to be resistant to atherosclerosis due to their lipoprotein composition and metabolism, they have been reported to develop atherosclerosis in both experimental and clinical studies.[9,10,124-126] Spontaneous atherosclerosis has been reported in dogs mainly in association with secondary hypercholesterolemia due to endocrinopathies.[124,126] In 1 study, 60% of 30 dogs with atherosclerosis had hypothyroidism and 20% had diabetes mellitus.[126]

Ocular disease

Several ocular manifestations of hyperlipidemia, such as lipemia retinalis, lipemic aqueous, and lipid keratopathy have been reported in dogs.[127] Recently, solid intraocular xanthogranuloma formation was reported as a unique disorder of hyperlipidemic Miniature Schnauzers.[128]

Other possible complications of hyperlipidemia

Seizures and other neurologic signs have been reported to occur potentially as a result of severe hyperlipidemia in dogs.[15,34,129,130] However, the relationship between these disorders remains obscure in dogs. Also, some authors report that hyperlipidemia can cause clinical signs of abdominal pain, lethargy, vomiting, and/or diarrhea without evidence of pancreatitis or other diseases.[33,83] This is highly speculative, however, because published reports are lacking and, given the difficulty in diagnosing pancreatitis especially in past decades, pancreatitis could have easily been missed in these patients.

Treatment of hyperlipidemia

The initial step in the treatment of hyperlipidemia is the determination of whether the patient has a primary or a secondary lipid disorder.[31,33,52] Thus, specific diagnostic investigations should be performed in order to diagnose or rule-out diseases that can cause secondary hyperlipidemia. Treatment of secondary hyperlipidemia relies on the successful treatment of the underlying disorder after which hyperlipidemia usually resolves.[31,33,49,52] Resolution of secondary hyperlipidemia after treatment of the cause should always be confirmed by laboratory testing (usually 4-6 weeks after correction of the primary disease). If hyperlipidemia has not resolved, a wrong diagnosis, ineffective treatment, or concurrent primary or secondary hyperlipidemia due to other causes should be considered.

After secondary causes of hyperlipidemia have been ruled out, a presumptive diagnosis of a primary lipid disorder can be made.[49] It has been anecdotally recommended that hypertriglyceridemia that exceeds 500 mg/dL should be treated in order to avoid possible complications.[33,49] It also has been recommended that the treatment goal should be to keep fasting serum triglyceride concentrations below 500 mg/dL.[33] Primary hypercholesterolemia is usually associated with less severe complications compared to hypertriglyceridemia. Treatment of hypercholesterolemia should aim to lower serum cholesterol concentrations below 500 mg/dL.

Dietary management

Typically, the first step in the management of primary hyperlipidemia is dietary modification.[31,33,49,52] Dogs with primary hyperlipidemia should be offered a low-fat diet throughout their lives.[33] Diets that contain less than 25 g of fat per 1,000 Kcal are recommended.[31,33,131] Many commercially available diets are suitable for dogs with primary hyperlipidemia. Also, many home-made low-fat diets (e.g., boiled lean turkey breast and rice) are suitable for these dogs, but care should be taken to make sure that these diets are balanced. Treats and table scraps should be avoided unless they are low in fat.[131] Serum lipid concentrations should be re-evaluated after feeding a low-fat diet for about 3-4 weeks.[33] If the serum triglyceride concentration has decreased to < 500 mg/dL, dietary therapy should be continued and the new diet should be offered for the rest of the animal's life, and serum triglyceride concentrations should be re-evaluated intermittently every 6 months.[33] In dogs that do not sufficiently respond to low-fat diets, an ultra low-fat diet (< 20 g of fat per 1,000 Kcal) can be offered, or medical treatment can be initiated.[131]

Medical management

Some dogs with primary hyperlipidemia will not sufficiently respond to feeding a low or ultra low-fat diet alone, especially when hypertriglyceridemia is due to endogenously formed triglycerides.[32-34,83] In these cases, medical treatment is required in addition to the low-fat diet in an effort to effectively reduce serum lipid concentrations.[32,83] It needs to be pointed out that no studies have evaluated the efficacy

of lipid-lowering drugs in dogs, and therefore, evidence-based recommendations cannot be made.

Polyunsaturated fatty acids of the n-3 series (omega-3 fatty acids) are abundant in marine fish.[132] Omega-3 fatty acid supplementation, usually in the form of fish-oil, has been shown to lower serum lipoprotein concentrations in humans with primary hypertriglyceridemia, normal humans, and experimental animals.[133,134] In a recent study in healthy dogs, fish-oil supplementation led to a significant reduction of serum triglyceride concentrations, suggesting that this supplement may be helpful in the treatment of primary canine hypertriglyceridemia.[135] No major side effects were observed.[135] However, studies evaluating the efficacy of fish-oil supplementation in dogs with hyperlipidemia are lacking and clinical experience is limited. Because side effects are rarely reported and because omega-3 fatty acids maybe effective in lowering lipidemia, some authors recommend that fish-oil should be administered in dogs with primary hypertriglyceridemia that do not respond to a low-fat diet alone.[31,34,132,135] Menhaden fish-oil capsules have been successfully used at doses ranging from 220-330 mg/kg of body weight once a day.[34,135] Periodic retesting of serum triglyceride concentrations is recommended during treatment.

Gemfibrozil belongs to the group of fibric acid derivatives and has been reported to reduce serum triglyceride concentrations in both healthy humans and patients with hypertriglyceridemia.[136,137] In dogs, its use is anecdotal and it is usually administered at a fixed dose of 200 mg/day.[34] Because side effects are believed to be minimal and only occur rarely, gemfibrozil is commonly recommended in combination with dietary

therapy when the latter fails to lower serum triglyceride concentrations below 500 mg/dL.[34,49]

Niacin is a vitamin that has been used successfully for the treatment of hyperlipidemia in humans for many years.[138] In dogs, niacin treatment has been reported in very few patients with primary hypertriglyceridemia. Niacin reduced serum triglyceride concentrations for several months without causing any side effects.[31,34,49] However, large clinical trials regarding the efficacy and safety of niacin in dogs with primary hypertriglyceridemia are lacking. As is the case in humans, niacin administration in dogs is potentially associated with side effects such as erythema and pruritus.[34,138] Niacin is usually administered at a dose of 25-100 mg/day.[34]

Pancreatitis in dogs

History of the study of pancreatitis in dogs

The first known studies that involved the canine pancreas were reported several centuries ago. In 1682, J. C. Brunner published his book *Experimenta Nova Circa Pancrease*, in which he described his interesting experiments on dogs, in an effort to determine the role the pancreas in digestion.[139] Since then, several other studies, including the famous work published by J.P. Pawlow in 1897 entitled *The Work of the Digestive Glands*, have investigated mainly the function of the canine pancreas, but always as model for understanding human pancreatic physiology.[139] Although pathologic conditions of the human pancreas (most likely representing inflammatory diseases of the pancreas) had been noticed and described in antiquity, centuries before

the understanding of the function of the pancreas, pancreatitis in humans was described in more detail as a disease entity in the late 19th century.[139] Following that, several studies of experimentally induced pancreatitis in dogs were reported, which initially had the sole goal to study human disease in canine models.[140-143] However, later, experimental studies were also designed to study canine disease.[144-149] Although these experimental studies provided some initial information about pancreatitis in dogs, it was later recognized that experimentally-induced pancreatitis does not mirror the spontaneous disease. This lack of clinical applicability of findings from experimental studies as well as the invasiveness of experimentally-induced pancreatitis dramatically limited the number of such studies.

Before the 1930s, canine pancreatitis was either not mentioned as a disease entity in veterinary textbooks at all or it only was mentioned in veterinary pathology textbooks as an extremely rare condition that was only diagnosed during necropsy.[150] The first reports of spontaneous canine pancreatitis in English were published in the 1930s and 1940s, and up until the 1950s each described isolated or a very small number of cases.[151-153] The first published case series that investigated canine pancreatitis in more detail appeared in the 1960s.[61] These studies provided the first comprehensive information on clinical presentation and histopathology of canine pancreatitis. Due to the lack of sensitive and specific tests for the diagnosis of canine pancreatitis in a clinical setting at that time, the diagnosis was based almost solely on necropsy and histopathologic findings and therefore these studies included mainly patients with severe pancreatitis that either died or were euthanized as a result of pancreatitis. Several studies

followed from the 1970s until today that investigated the etiology, clinical presentation, diagnosis, prediction of severity, and histopathology of spontaneous canine pancreatitis. In contrast, there has almost been a complete lack of studies evaluating treatment modalities in canine pancreatitis.

Definitions

Strictly speaking, pancreatitis refers to an inflammation (i.e., infiltration with inflammatory cells) of the exocrine pancreas. However, the term pancreatitis is commonly expanded also to include diseases of the exocrine pancreas characterized mainly by necrosis that may have a minimal inflammatory component (often referred to as acute pancreatic necrosis or necrotizing pancreatitis).[154] It is widely believed that pancreatic necrosis is associated with a severe and often fatal course of disease, while pancreatitis without necrosis (e.g., edematous interstitial pancreatitis) is usually mild. However, no convincing evidence currently exists to support this assumption in clinical cases of canine pancreatitis.

Pancreatitis is generally divided into acute (which typically includes acute pancreatic necrosis) and chronic forms, based on the absence or presence of permanent histopathologic lesions, such as pancreatic fibrosis and/or atrophy. The term recurrent acute pancreatitis is sometimes used to describe recurrent episodes of pancreatitis that are not associated with permanent histopathologic changes. A plethora of other clinical (e.g., mild or severe, fatal or non-fatal) and histopathological (e.g., edematous, interstitial, necrotizing, neutrophilic, lymphocytic) terms have been used to further

classify pancreatitis in dogs. However, no universally standardized terminology for pancreatitis has been agreed upon for veterinary species, and different authors classify pancreatitis differently. It is not clear at this time whether the different forms of pancreatitis (e.g., acute edematous pancreatitis, acute pancreatic necrosis, chronic pancreatitis) represent different phenotypes of the same disease or distinct disease entities, whether they share the same etiologic and pathogenetic mechanisms, or which factors determine the development of each form.

Pathogenesis and pathophysiology

Most of our understanding regarding the pathogenesis of pancreatitis is based on animal models and some clinical studies in humans. There is mounting evidence that genetic and possibly environmental factors may sensitize the pancreas to injury induced by one or more etiologic factors.[155,156] Regardless of the actual etiology, there appears to be a common pathogenetic mechanism in most cases of acute pancreatitis. Evidence supports that the initiating events that lead to pancreatitis take place in the acinar cell. Two well-documented early intracellular events that have been shown to precede the development of acute pancreatitis are retention and intracellular activation of zymogens.[155,156] Zymogens are inactive precursors of pancreatic enzymes that are stored in zymogen granules and are normally secreted into the pancreatic duct through the apical membrane of the acinar cell. The factors that lead to retention of zymogen granules and to premature intracellular activation of the zymogens are not fully elucidated. One of the most popular theories is the co-localization theory.[155,156] Based on

this theory, zymogen granules that are accumulating in acinar cells co-localize with lysosomes. Lysosomal enzymes, such as cathepsin B, are then thought to activate trypsinogen into trypsin, which subsequently activates other zymogens. Evidence from other studies indicate that the cytosolic concentration of free ionized calcium also plays an important role in the intracellular activation of zymogens.[157-159] In addition to the decreased secretion and intracellular activation of pancreatic enzymes, there is evidence of disruption of the paracellular barrier in the pancreatic duct that allows its contents to leak into the paracellular space, and also redirect the secretion of zymogen granules from the apical pole to the basolateral region of the acinar cell and into the interstitial space.[156] Once intracellular activation of pancreatic enzymes has taken place, autodigestion of the acinar cell follows and activated enzymes escape, initially into the pancreatic tissue (leading to local effects) and subsequently into the peritoneal cavity and the systemic circulation (potentially contributing to systemic effects). Local effects vary and can range from mild interstitial edema to severe acinar cell necrosis, hemorrhage, and peripancreatic fat necrosis. The extent and severity of local effects determine to a large degree the systemic response. Acinar cell injury leads to recruitment and activation of inflammatory cells (most importantly neutrophils and macrophages), which release proinflammatory cytokines and other inflammatory mediators (such as interleukin [IL]-1, IL-2, IL-6, IL-18, tumor necrosis factor-α, substance P, platelet-activating factor [PAF]) that play a crucial role in modulating systemic complications of the disease.[155,160] Such systemic complications can include cardiovascular shock, acute renal failure, neurologic abnormalities, disseminated intravascular coagulation (DIC), systemic

inflammatory response syndrome (SIRS), acute respiratory failure, and/or multiple organ failure, and can be seen in cases of severe acute pancreatitis.[155,160]

Etiologic and risk factors

In contrast to humans, in whom an etiology of pancreatitis can be identified in the vast majority of cases, the etiology of pancreatitis in dogs usually remains unknown (idiopathic pancreatitis).[161,162] It is expected, however, that recognition of new causes of canine pancreatitis will allow etiologic classification in a bigger proportion of cases in the future. Several factors have been reported as risk factors for pancreatitis in dogs, but the majority of these factors have been implicated by association, and therefore, very few definitive causes of pancreatitis have actually been reported.[161] The main causes of human pancreatitis (i.e., biliary obstruction and alcoholism) do not represent common problems in small animals.[155,162] Other well defined causes of human pancreatitis (e.g., autoimmune pancreatitis) have not been proven in dogs as of yet.

Breed

Several dog breeds have been reported to be at increased risk for pancreatitis, although findings among studies are not always in agreement with each other. A breed predisposition most likely reflects either a genetic cause of pancreatitis or a predisposition to other diseases or conditions that are risk factors for pancreatitis (e.g., hypertriglyceridemia in Miniature Schnauzers). Differences with regard to breed predisposition probably exist among different geographic regions, as blood-lines might

be quite different, especially in older breeds that were introduced to a geographic region several decades ago. Miniature Schnauzers, Yorkshire Terries, and Terriers in general have all been consistently shown to have a higher odds of pancreatitis.[63-65,163] Boxers, Cavalier King Charles Spaniels, Cocker Spaniels, and Collies have been shown to be overrepresented in a cohort of dogs with chronic pancreatitis in the United Kingdom.[164]

Hypertriglyceridemia

Hypertriglyceridemia has long been suspected to be a risk factor for pancreatitis in dogs, but convincing evidence is lacking.[64,65,165] A definitive etiological association between hypertriglyceridemia and pancreatitis has been difficult to prove, mainly due to the fact that hypertriglyceridemia may be the result of pancreatitis rather than the cause. (For the association between hypertriglyceridemia and pancreatitis please see corresponding section on page 47).

Hereditary pancreatitis

In a recent study, a combination of 3 variants of the serine protease inhibitor Kazal type 1 (SPINK1) gene was identified in Miniature Schnauzers, and an association of these variants with pancreatitis was demonstrated.[166] Mutations of the SPINK1 gene, although different from those described in Miniature Schnauzers, have also been described and associated with pancreatitis in humans.[167] The product of the SPINK1 gene, pancreatic secretory trypsin inhibitor (PSTI), is found in acinar cells and acts as one of the defensive mechanisms against prematurely activated trypsin. It is possible that

the mutant protein lacks this function, thereby leaving the acinar cell more susceptible to injury, although this has not been convincingly shown in either humans or dogs. Thus, the exact role of variants of the SPINK1 gene in the development of pancreatitis in Miniature Schnauzers remains to be determined. It has been hypothesized that mutations of this gene may not actually cause pancreatitis, but may sensitize the pancreas to injury through other factors. Based on the fact that some breeds have been found to be overrepresented in some reports, genetic causes of pancreatitis have also been suspected in other breeds (e.g., Yorkshire Terriers).[64]

Diet

The role of diet, and more specifically the fat content of the diet, in the development of canine pancreatitis remains largely unclear. Based on anecdotal clinical observations, foods high in fat increase the risk of pancreatitis. Older experimental studies have suggested that diets with a very high fat content may induce pancreatitis and may increase the severity of experimentally induced pancreatitis in dogs.[144,165] The mechanism by which high-fat diets increase the risk for pancreatitis is not known, but it is possible that high-fat diets may predispose dogs to pancreatitis through hypertriglyceridemia as a result of a fatty meal. In a recent retrospective case-control study of dogs, several factors such as getting into the trash, consuming table scraps, and ingestion of "unusual" food were found to be associated with increased odds of pancreatitis.[163] However, no specific foods were identified that could be associated with an increased risk of pancreatitis.

Drugs

As in humans, drug-induced pancreatitis has been reported mainly in the form of case reports or case series, but a cause-and-effect relationship has not been established for most cases.[168] Nevertheless, a history of drug administration in conjunction with compatible findings should raise a concern for pancreatitis due to a pharmacological substance. Based on the remarkably large number of drugs prescribed in both human and veterinary medicine, and the fact that drug-induced pancreatitis appears to be quite rare, most drugs implicated are thought to cause pancreatitis in an idiosyncratic fashion. Theoretically, any drug can potentially cause pancreatitis. However, pancreatitis seems to be more commonly associated with the use of certain drugs. Drugs believed to be most commonly associated with pancreatitis in dogs include potassium bromide, phenobarbital, L-asparaginase, azathioprine, and meglumine antimonate.[169-171]

Endocrine disease

In 1 study, hyperadrenocorticism, hypothyroidism, and diabetes mellitus were reported to be more commonly present in dogs with pancreatitis than in dogs with no evidence of pancreatitis.[65] In another study, 13% of 221 dogs with diabetes mellitus were reported to have pancreatitis.[172] However, evidence is currently far from convincing that these endocrine diseases represent risk factors for canine pancreatitis. It has been hypothesized that hypertriglyceridemia associated with these endocrine diseases might be a more significant risk factor for pancreatitis in dogs than the conditions themselves. Also, diabetes mellitus may be an effect of pancreatitis rather than a cause.

Obesity

A relationship between obesity and pancreatitis has been suggested for dogs. Studies have shown that dogs diagnosed with pancreatitis are more frequently obese than dogs that do not have pancreatitis.[64,65,163] However, a pathogenetic link between obesity and pancreatitis has not been convincingly shown to date.

Other factors

Age is often listed as a risk factor for pancreatitis because most dogs with pancreatitis are middle aged or older. No clear sex predisposition has been identified to date. Hypotension (e.g., during anesthesia or after severe blood loss), hypercalcemia (both iatrogenic and as a result of diseases such as neoplasia and hyperparathyroidism), abdominal trauma, extensive surgical manipulation of the pancreas, certain infections (e.g., an infection with certain *Babesia* spp. strains) and obstruction of the pancreatic duct (e.g., due neoplasia) are also suspected risk factors for pancreatitis in dogs, but scientific evidence is weak or lacking at this point.[161,173] Chronic gastrointestinal disease might also be a risk factor for pancreatitis in dogs.[174] Primary or metastatic neoplasia of the pancreatic parenchyma is often associated with secondary inflammation of the exocrine pancreas. Previous surgery and epilepsy have also been reported as potential risk factors for canine pancreatitis.[65,163]

Signalment

Dogs of any age, breed, or sex can develop pancreatitis. In several reports, most affected dogs are middle-aged to older.[63,65] Miniature Schnauzers and Yorkshire Terriers appear to be at increased risk for pancreatitis, while a predisposition for other breeds is less clear.[64,65,161,163] In 1 study some other breeds (e.g., Boxers, Cavalier King Charles Spaniels, Cocker Spaniels, and Collies) have been suggested to be predisposed to chronic pancreatitis, but this has not been confirmed by other studies.[164] No clear sex predisposition has been identified.

Clinical signs and physical examination findings

It has been recognized that dogs with pancreatitis can be subclinical or present with a wide variety of clinical signs, ranging from mild partial anorexia with no apparent gastrointestinal signs to severe systemic signs with cardiovascular shock and DIC. There is no single clinical sign or combination of clinical signs that is pathognomonic for pancreatitis in dogs. Clinical signs have been well described in dogs with severe acute pancreatitis and may include anorexia (91%), vomiting (with or without blood; 90%), weakness (79%), polyuria and polydipsia (50%), and diarrhea (with or without blood; 33%).[64] Many of the clinical signs reported in dogs with pancreatitis are likely to be the result of complicating or concurrent diseases rather than pancreatitis *per se* (e.g., polyuria and polydipsia are more likely to be the result of concurrent diabetes mellitus). The most common physical examination findings in dogs with severe acute pancreatitis include dehydration (97%), abdominal pain (58%), fever (32%), and icterus (26%).[64]

The combination of vomiting and abdominal pain, although suggestive of pancreatitis, is also seen with other diseases (e.g., gastrointestinal foreign bodies, peritonitis). Other possible findings include shock, hypothermia, a cardiac murmur, tachycardia, bleeding diathesis, ascites, a palpable abdominal mass, and/or harsh lung sounds.[64] Patients with less severe or chronic pancreatitis typically manifest less profound clinical signs, such as anorexia and depression, or might even be subclinical.

Clinical pathology[64,175]

Results of complete blood count (CBC), serum biochemistry profile, and urinalysis are nonspecific, and thus of limited usefulness for the diagnosis of pancreatitis in dogs. However, these tests should always be performed in dogs with suspected pancreatitis because they are useful for ruling out other differential diagnoses and also provide important information about the systemic condition of the animal.

Often, especially in mild cases, the CBC, serum biochemistry profile, and urinalysis are normal. Possible hematologic findings in dogs with pancreatitis include anemia or hemoconcentration, leukocytosis or leucopenia, and thrombocytopenia. Evidence of coagulopathy, such as prolonged activated clotting time (ACT), prothrombin time (PT), and/or partial thromboplastin (PTT) time, are seen in some cases, and may or may not be associated with spontaneous bleeding. In other cases, there might be clinical evidence suggestive of DIC, such as thrombocytopenia, prolongation of clotting times (i.e., ACT, PT, PTT), and a positive d-dimer test. Different combinations of increases in liver enzyme activities and hyperbilirubinemia are common and might

erroneously direct the clinician to suspect primary liver disease. Increases in serum creatinine and blood urea nitrogen (BUN) concentrations are variable and most often associated with dehydration due to vomiting, diarrhea, and/or decreased water intake. In severe cases, azotemia might be the result of secondary renal failure. Other possible findings include hypoalbuminemia, hypertriglyceridemia, hypercholesterolemia, and hyperglycemia or hypoglycemia. Electrolyte abnormalities are commonly present and variable, with hypokalemia, hypochloremia, and/or hyponatremia being most common.

Clinical enzymology

Serum pancreatic lipase immunoreactivity (PLI) concentration

The only cell type known to synthesize pancreatic lipase is the pancreatic acinar cell. An immunoassay for specific measurement of canine pancreatic lipase has been developed and analytically validated.[176] In contrast to the traditional activity assays for lipase, which indiscriminately measure the activity of lipases of a variety of different cellular origins, this immunoassay specifically quantifies pancreatic lipase based on its unique antigenic structure using specific antibodies. The original in-house immunoassay for canine pancreatic lipase (cPLI) has been replaced by a widely available commercial immunoassay (Spec cPL®), which performs similarly to the original cPLI ELISA.[177,178]

Clinical studies suggest that serum cPLI (or Spec cPL) has high specificity for canine pancreatitis. In one study of 31 dogs with a normal pancreas on histopathology the specificity of Spec cPL was very high (96.8%).[179] In a recent multicenter study in which dogs with clinical evidence of pancreatitis were studied, the specificity of this

assay was reported at 78%.[180] Experimentally induced chronic renal failure and prednisone administration were not found to have a clinically significant effect on serum cPLI concentration.[181,182] The specificity of cPLI needs further investigation in dogs with various gastrointestinal diseases but no pancreatitis. It also remains to be determined whether serum cPLI concentration can be increased in patients with histopathologically mild pancreatitis that might be of minor clinical importance and does not contribute to the development of clinical signs. Overall, compared to other serum tests currently available, serum cPLI is considered to have the highest specificity for pancreatitis.[179-185]

Studies also show that serum cPLI concentration is sensitive for the diagnosis of pancreatitis in dogs.[177,180,186,187] The reported sensitivity of cPLI for the diagnosis of canine pancreatitis ranges between 64% and 93%, possibly depending on the severity of the disease in the patients studied. Overall, the sensitivity of serum cPLI is higher than any other serum test currently available.[177,180,186,187] However, false negative results are likely to occur especially in mild cases.

Overall, serum cPLI concentration appears to be a sensitive and specific marker of canine pancreatitis, and is currently considered to be the serum test of choice for the diagnosis of pancreatitis in this species.

Based on clinical observations and the results of studies available to date,[177] serum cPLI concentration does not appear to correlate with the severity of pancreatitis. Therefore, an individual measurement of serum cPLI concentration cannot be used to predict the severity of pancreatitis. No controlled studies have looked at the significance

of changes over time in serum Spec cPL concentrations in individual patients with pancreatitis.

A point-of-care test for the estimation of pancreatic lipase in serum (SNAP cPL®) has become available. Published studies evaluating the performance of this test are currently lacking but it is claimed to show the same clinical performance as the serum Spec cPL assay. The recommended use of this test is for the rule-out of pancreatitis in dogs presenting for acute clinical signs that are consistent with pancreatitis. Due to the high sensitivity of Spec cPL, a negative result makes a diagnosis of pancreatitis unlikely. However, false negative results might occur in some cases. A positive test result should be followed by laboratory measurement of serum Spec cPL concentration.

Serum amylase and lipase activities

Serum amylase and lipase activities have been considered as markers for pancreatitis in dogs for several decades, but several studies have shown that they are not good markers for spontaneous canine pancreatitis due to their low sensitivity and specificity.[183,188] In one study, about 50% of dogs with increased activity of either serum amylase or lipase activity had no histopathologic evidence of pancreatitis.[183] This means that a large proportion of dogs that have diseases other than pancreatitis (e.g., certain renal, hepatic, intestinal, and neoplastic diseases) might have increased serum lipase and/or amylase activities.[183] This is due to the fact that there are many lipases and amylases of different cellular origins that cannot be specifically analyzed by use of

enzymatic assays. Even significant increases of amylase and lipase activities can result from non-pancreatic disorders, and should always be followed by the use of more specific and sensitive tests.[165,183,184,189] In addition, the sensitivity of serum amylase and lipase activities for spontaneous canine pancreatitis varies but is generally low (14%-73% for serum lipase activity and 18%-69% for serum amylase activity).[64,177,187] Therefore, pancreatitis cannot be definitively diagnosed or ruled out based on serum amylase and/or lipase activities.[64,183]

Serum trypsin-like immunoreactivity (TLI) concentration

The canine TLI assay is a species-specific immunoassay that measures trypsinogen, trypsin, and also potentially trypsin molecules that are bound to trypsin inhibitors in serum. Trypsinogen is the inactive precursor of trypsin and is synthesized exclusively in pancreatic acinar cells. The sensitivity of serum cTLI for the diagnosis of canine spontaneous pancreatitis is low (36%-47%), probably due to its short half-life.[177,184,187] In addition, although there is strong evidence that trypsinogen is exclusively of pancreatic origin,[190] it is believed that it is cleared by glomerular filtration in dogs, and serum cTLI concentration can be increased in dogs with renal failure.[184,185]

Other diagnostic tests

Several other tests have been developed and evaluated for the diagnosis of canine pancreatitis. However, none of these tests can currently be recommended for clinical use, either because their clinical usefulness has not been sufficiently determined or because

they have been shown to have a low specificity and/or sensitivity. In addition, most of these tests have limited availability or are only available for experimental use.

Trypsinogen activation peptide (TAP) is a small peptide that is released when trypsinogen is activated to trypsin.[184] Under physiologic conditions, trypsinogen is activated mainly in the intestinal lumen, and thus serum TAP concentrations are low or undetectable.[184] During pancreatitis, trypsinogen is prematurely activated in the pancreas and TAP is released into the circulation.[184,191] Plasma and urinary TAP concentrations have been evaluated in healthy dogs, dogs with spontaneous pancreatitis, and dogs with other systemic diseases in a single study.[184] In this study, plasma TAP concentrations showed a fairly good specificity (87.9%) but a low sensitivity (53.3%) for the detection of pancreatitis. Urine TAP concentrations did not show any advantage over serum TAP concentrations in diagnosing pancreatitis. Both tests showed increases in dogs with severe pancreatitis, but were normal or even decreased in cases of mild pancreatitis. However, this study suggested that, as in humans, serum and urine TAP concentrations might be more useful as a prognostic indicator in dogs with pancreatitis.[184]

Measurement of lipase activity in peritoneal fluid and comparison with serum lipase activity has been evaluated as a tool for the diagnosis of acute pancreatitis in dogs.[192] However, further well-designed studies are needed before such a tool can be recommended for clinical use in canine patients. Other tests that have been evaluated for the diagnosis of canine pancreatitis include serum concentrations of trypsin-α_1-proteinase inhibitor complexes or α_2-macroglobulin, but neither of these tests proved to be particularly sensitive or specific for the diagnosis of canine pancreatitis.[193-196]

Diagnostic imaging

Abdominal radiography

Conclusive diagnosis or exclusion of pancreatitis is not possible based on abdominal radiography alone.[64] In the majority of cases of canine pancreatitis, abdominal radiographs are normal or only show non-specific changes.[64] Despite that, abdominal radiography remains a logical initial approach for patients suspected of having pancreatitis, because it is useful for the rule-out of other differential diagnoses.

Non-specific radiographic findings that may be found in dogs with pancreatitis include an increased soft tissue opacity and decreased serosal detail in the cranial right abdomen, displacement of the stomach and/or duodenum from their normal positions, dilation of bowel loops adjacent to the pancreas, and the presence of a cranial abdominal mass.[64]

Abdominal ultrasound

Abdominal ultrasound is considered the imaging modality of choice for the diagnosis of pancreatitis in dogs. However, abdominal ultrasonography is also associated with disadvantages, and its clinical utility in the diagnosis of pancreatitis is highly dependent on the experience of the ultrasonographer and the quality of the equipment used. It has been reported to have a relatively high sensitivity of about 68% for severe acute pancreatitis in dogs.[64] In a recent study where ultrasonography was performed in 26 animals (both dogs and cats) with suspected gastrointestinal disease, 6 (23.1%) of the animals had ultrasonographic evidence consistent with pancreatitis, while histopathology

revealed either a normal pancreas or pancreatic hyperplasia.[197] In the same study, there was only 22% agreement between the ultrasonographic and the histopathologic diagnoses.[197] Although not free of limitations, this study highlights that ultrasonographic findings in animals with suspected pancreatitis should be interpreted with caution. It is also important to note that a normal ultrasonographic appearance of the pancreas does not rule-out pancreatitis.[64,198] If stringent criteria are applied, the specificity of abdominal ultrasonography for pancreatitis is considered to be relatively high, although other diseases of the pancreas (e.g., neoplasia, hyperplastic nodules, pancreatic edema due to portal hypertension or hypoalbuminemia) may display similar ultrasonographic findings and sometimes cannot be definitively differentiated from pancreatitis.[199,200]

The most important ultrasonographic findings suggestive of pancreatitis in dogs include hypoechoic areas within the pancreas, increased echogenicity of the surrounding mesentery (due to necrosis of the peripancreatic fat), fluid around the pancreas, and enlargement and/or irregularity of the pancreas.[64,198,201] Differentiation between necrotizing and edematous pancreatitis might be possible based on ultrasonographic examination, although this has not been confirmed in clinical studies. On occasion, hyperechoic areas of the pancreas possibly indicating the presence of pancreatic fibrosis may be present. Less specific findings may include a dilation of the pancreatic or biliary duct, and abdominal effusion. Abdominal ultrasonography is also very useful for the diagnosis of local complications of pancreatitis such as pancreatic abscesses, pancreatic pseudocysts, and biliary obstructions.[201] In addition, ultrasound-guided fine-needle aspiration is a useful tool for the management of non-infectious fluid accumulations of

the pancreas (e.g. pancreatic pseudocyst), and for obtaining pancreatic specimens for cytological evaluation.[202]

Other imaging modalities

Several other imaging modalities are routinely used to diagnose or evaluate pancreatitis in human patients. Contrast enhanced computed tomography (CECT) is an extremely valuable tool for the evaluation of human patients with suspected pancreatitis and might also prove to be useful in dogs, but it has not been evaluated in an adequate number of canine pancreatitis cases.[203] Other imaging modalities (e.g., endoscopic retrograde cholangiopancreatography (ERCP), endoscopic ultrasonography), have been studied in healthy dogs and dogs with gastrointestinal diseases with varying results.[204,205] However, due to the lack of standardized criteria for the diagnosis of pancreatitis, the complexity of these modalities, their limited availability, and the cost of the equipment they cannot be currently recommended for the diagnosis of canine pancreatitis.

Pathology

Certain macroscopic lesions identified during surgery, laparoscopy, or necropsy are highly suggestive of pancreatitis and are preferred sites for biopsy collection.[177] Macroscopic pancreatic lesions suggestive of pancreatitis may include peripancreatic fat necrosis, pancreatic hemorrhage and congestion, and a dull granular capsular surface.[177] However, gross pathologic lesions may not always be apparent in dogs with pancreatitis, and in some cases, they might be difficult to differentiate from nodular hyperplasia.[206]

At present, a definitive diagnosis of pancreatitis can only be made by histopathologic examination of the pancreas. Histopathology is also the only way to differentiate acute and chronic pancreatitis and, in some cases, pancreatitis from pancreatic neoplasia. Although not clearly defined in veterinary species, the presence of permanent histopathologic changes (such as fibrosis and acinar atrophy) is considered suggestive of chronic pancreatitis.[165,206] Acute pancreatitis is characterized by the absence of permanent histopathologic lesions. The predominant inflammatory cellular infiltrate (neutrophils or lymphocytes) is often used to describe pancreatitis as suppurative or lymphocytic, respectively, and a significant degree of necrosis is usually used to characterize the pancreatitis as necrotizing.

Several limitations are associated with pancreatic histopathology as a definitive diagnostic tool for pancreatitis. First, determining the clinical significance of histopathologic findings may be challenging. At the same time, exclusion of pancreatitis based on histopathology is difficult because inflammatory lesions of the pancreas are often highly localized and can easily be missed.[206] Therefore, multiple sections of the pancreas must be evaluated in order to increase the likelihood of finding microscopic lesions, although this is not always feasible in clinical cases.[206] Finally, although pancreatic biopsy *per se* is considered safe, it requires invasive procedures that are expensive and potentially detrimental in patients that are hemodynamically unstable.[197]

Cytology

Fine-needle aspiration (FNA) of the pancreas and cytological examination has been recently introduced as a diagnostic tool for pancreatitis in small animals. It should be performed either under ultrasonographic guidance or during laparotomy.[207] To date there are no studies that have evaluated the sensitivity and specificity of this diagnostic modality for the diagnosis of canine pancreatitis, but the finding of acinar cells and inflammatory cells in the aspirate is considered specific for pancreatitis. Pancreatic acinar cells constitute the majority of the cells found in FNA smears from a normal pancreas.[207] In patients with acute pancreatitis the cytological picture is mainly characterized by hypercellularity and the presence of intact and degenerated neutrophils and degenerated pancreatic acinar cells. It should be noted that, as for histopathology, localized lesions might be missed. Therefore, negative results are not sufficient to rule-out pancreatitis. Fine-needle aspiration cytology might also be useful in differentiating other conditions of the pancreas (e.g., neoplasia) from pancreatitis.

Treatment

The etiology of pancreatitis remains unknown in the majority of cases, and therefore, treatment of pancreatitis remains almost exclusively supportive. It is expected that future recognition of specific causes of canine pancreatitis will lead to the development of more specific treatments for different forms of pancreatitis that are now classified as idiopathic. Until then, the presence of possible risk factors or etiologic

factors should always be investigated in dogs with pancreatitis. If any of these factors are present, they should be managed as possible.

Other than that, the treatment of pancreatitis in dogs remains almost exclusively supportive and symptomatic. Fluid therapy, in some cases including plasma transfusion, is considered important in both humans and dogs with severe disease and hypovolemia and/or dehydration.[165,196,208-215] Pain management is also considered crucial in cases of both acute and chronic pancreatitis, when pain is present.[216-218] The use of antiemetic medications is recommended in cases of pancreatitis where vomiting is present.[219-221] Routine antibiotic therapy is highly controversial and it is usually not recommended in dogs unless an infection is present.[155,208,222-225] Finally, surgical intervention of pancreatitis is rarely recommended in dogs unless certain complications occur (such as pancreatic abscess, pancreatic pseudocyst, or extrahepatic biliary tract obstruction).[226-237] A plethora of other therapeutic agents have been recommended by some authors in both veterinary and human medicine.[238-240] but there is currently no convincing evidence that any of these agents is beneficial for the treatment of spontaneous pancreatitis in dogs.

The role of nutrition in the management of pancreatitis needs some attention. The nutritional approach of humans with pancreatitis (especially acute pancreatitis) has been the focus of extensive basic and clinical research for several decades. [241-243] Unfortunately, studies in dogs suffering specifically from either acute or pancreatitis are sparse and therefore the nutritional approach in these cases is based on clinical experience.[244-251] Questions regarding the nutritional approach of dogs with pancreatitis have to do with the timing of feeding during an episode of acute pancreatitis, the route of

nutrient delivery (which also mainly is related to dogs with acute pancreatitis), and the type of diet administered, which might be important in dogs with both acute and chronic pancreatitis. Although the diet of choice for the management of dogs with pancreatitis has not been systematically studied to date, based on anecdotal experience, a balanced ultra-low-fat diet is currently the preferred choice for dogs. This is mainly based on studies that suggest that high-fat diets (typically table scraps) might be related to the development of pancreatitis in some dogs.[252] This is further supported by studies that implicate hypertriglyceridemia in the development of pancreatitis in humans and experimental models.[252] However, no studies have evaluated whether the fat content of commercially available canine diets is important in treating or preventing acute and chronic pancreatitis. While several supportive measures are important in the management of acute pancreatitis, nutritional management might be the only measure for the long-term management of dogs with chronic pancreatitis.

The association between hyperlipidemia and pancreatitis

An association between hyperlipidemia and pancreatitis was first noted in humans by Speck in 1865.[253] Several clinical and experimental studies in humans and animals, respectively, have been conducted since then, and today, severe hypertriglyceridemia is a well recognized risk factor for pancreatitis in humans.[254-257] Hypertriglyceridemia is believed to be the 3rd most common cause of pancreatitis in humans, after alcohol- and gallstone-induced pancreatitis, accounting for up to 10% of cases.[258] However, within specific groups, the prevalence of hypertriglyceridemia-

associated pancreatitis is much higher; for example, hypertriglyceridemia has been listed as a cause in 56% of patients with gestational pancreatitis and up to 41% of patients with primary hypertriglyceridemia.[258] Hypertriglyceridemia-associated pancreatitis can occur as a result of primary hypertriglyceridemia in humans (mainly types I, IV, and V, based on the classification by Fredrickson), but also as a result of hypertriglyceridemia secondary to other diseases (most commonly poorly controlled diabetes mellitus, alcoholism, or obesity), special physiologic states (e.g., pregnancy), or drug administration (e.g., estrogens, tamoxifen).[258]

An increased risk for pancreatitis due to hypertriglyceridemia has been conventionally thought to exist when serum triglyceride concentrations exceed 1,000 mg/dL.[254] In contrast, hypercholesterolemia does not constitute a risk factor for pancreatitis in humans.[254] The mechanism by which hypertriglyceridemia induces pancreatitis is not clear, but it has been suggested that serum triglycerides are hydrolyzed by the action of pancreatic lipase, leading to excessive production of free fatty acids, which are toxic to the pancreatic acinar cell.[259,260] Another possible hypothesis is that hyperviscosity develops as a result of increased chylomicrons and/or VLDLs in the capillaries, which in turn leads to ischemia of pancreatic tissue.[258] Finally, genetic factors, such as mutations of the cystic fibrosis transmembrane conductance regulator gene or the SPINK1 gene may play a role by sensitizing the pancreas to the effects of hypertriglyceridemia.[258]

A similar relationship between hypertriglyceridemia and pancreatitis has been suggested for dogs, and an association between the 2 conditions has been shown in

several studies.[15,33,34,37,49,52,66,83,165] However, this association remains obscure in dogs as the etiology of hypertriglyceridemia cannot always be determined in these studies. The presence of hypertriglyceridemia in dogs with pancreatitis might be due to a pre-existing disorder of lipid metabolism, which may or may not be related to the etiology of pancreatitis, but might also be the result of pancreatitis, or it might just be an incidental finding in some cases.[252]

Although it is widely believed that hypertriglyceridemia can develop as a result of pancreatitis, this has not been convincingly shown in dogs with naturally occurring pancreatitis. Furthermore, with the exception of the results of 1 older study,[261] hypertriglyceridemia does not seem to be a consequence of experimental pancreatitis in dogs.[18-20] In order for hypertriglyceridemia to be considered as a possible etiologic factor for pancreatitis it should precede the development of pancreatitis.

Miniature Schnauzers have been reported to develop pancreatitis more commonly than dogs of other breeds, and this high prevalence of pancreatitis in Miniature Schnauzers has been attributed to the fact that dogs of this breed commonly develop hypertriglyceridemia.[33,49,66,165] Available clinical and experimental data to support the above hypotheses are limited, however. In experimental studies, pancreatitis has been shown to develop in dogs after feeding a high-fat, low-protein diet, and is more severe when induced in dogs being fed a high-fat diet.[144,146] Also, *in vitro* studies on an isolated canine pancreas showed that high triglyceride concentrations can induce pancreatitis possibly through the release of free fatty acids.[260] Clinical studies have shown an association between hyperlipidemia and pancreatitis in dogs, although it is not

clear whether hyperlipidemia was the cause or a result of pancreatitis, or just an incidental finding in some cases.[15,19,21,63-66] Secondary hyperlipidemia seen in dogs with some endocrinopathies (e.g., hyperadrenocorticism) or obesity may be responsible for the increased risk for pancreatitis associated with these diseases.[11,65,65,172] Based on these studies, there is strong evidence for an association between hypertriglyceridemia and pancreatitis in dogs, but the exact nature of this relationship has not been established.

CHAPTER II

ASSOCIATION BETWEEN SERUM TRIGLYCERIDE AND CANINE PANCREATIC LIPASE IMMUNOREACTIVITY CONCENTRATIONS IN MINIATURE SCHNAUZERS*

Introduction

Primary (idiopathic) hypertriglyceridemia is a common condition in Miniature Schnauzers in the United States.[84] In a recent study, hypertriglyceridemia was present in 32.8% of 192 Miniature Schnauzers investigated. Also, hypertriglyceridemia was shown to be age-related, with a much higher prevalence in older Miniature Schnauzers.[84] Hypertriglyceridemia in Miniature Schnauzers has previously been shown to be associated with an abnormal accumulation of VLDL (very low density lipoproteins) or a combination of VLDL and chylomicrons, with or without hypercholesterolemia.[17,83] The fact that hypertriglyceridemia is especially prevalent within a certain breed suggests a hereditary mechanism, but the metabolic and genetic bases of this disorder have not been identified.[49,83]

Investigations into the disease conditions potentially associated with hypertriglyceridemia have only recently been reported in dogs. For example, as is the case in humans, hypertriglyceridemia has been recently reported to be associated with

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*Xenoulis PG, et al. Association between serum triglyceride and canine pancreatic lipase immunoreactivity concentrations in miniature schnauzers. *J Am Anim Hosp Assoc* 2010;46(4):229-34.
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hepatobiliary disease in both Miniature Schnauzers and dogs of other breeds.[121,123] In humans, severe hypertriglyceridemia is also a known risk factor for pancreatitis.[254,255,262] Miniature Schnauzers are generally considered to have a high incidence of pancreatitis.[63,163,165,216] In a recent study, bivariable screening showed that Miniature Schnauzers were 4.1 times (95% CI, 1.9 to 9.2; $P < 0.001$) more likely to have pancreatitis than dogs of other breeds.[163] The basis for this predisposition is unknown, but hypertriglyceridemia has long been considered as a possible cause of pancreatitis in the Miniature Schnauzer.[17,83,165] However, studies investigating any association between these 2 disorders in dogs are lacking, and this consideration is mainly based on clinical impressions. *In vitro* studies support the concept that hypertriglyceridemia can initiate pancreatic inflammation, and there is evidence that high fat diets or even a single fatty meal can induce pancreatitis in dogs.[144,165,260] In contrast to hypertriglyceridemia, hypercholesterolemia does not appear to be a risk factor for pancreatitis in humans or dogs.[33,254,255] Recognition of an association between hypertriglyceridemia and pancreatitis in Miniature Schnauzers is clinically important because management of hypertriglyceridemia with low-fat diets and/or lipid-lowering drugs may prevent or resolve pancreatitis in these dogs.

Until recently, a clinical diagnosis of pancreatitis was problematic in dogs because no specific and sensitive tests were available. However, the recent development and analytical validation of an ELISA specific for canine pancreatic lipase, canine pancreatic lipase immunoreactivity (cPLI), has facilitated the diagnosis of pancreatitis in dogs as it has been reported to be sensitive (reported sensitivity ranging from 64% for

mild pancreatitis to 82% for severe pancreatitis) and specific for the diagnosis of canine pancreatitis.[a,b,c,d,176,177,182,263,264]

The aim of this study was to investigate possible associations between serum triglyceride and cPLI concentrations in a large group of Miniature Schnauzers.

Materials and methods

Serum samples from 195 Miniature Schnauzers were collected and triglyceride and cPLI concentrations measured. Serum samples were collected on a sequential basis; no bias was given to lipemic samples. Based on the submission form, food needed to be withheld from dogs for at least 12 hours before blood collection. Serum triglyceride concentrations were measured by an enzymatic *in vitro* assay (reference range 26 to 108 mg/dL).[e] Serum cPLI concentration was measured using an in-house ELISA as described elsewhere.[176] The reported reference range for cPLI is 2.2 to 102.1 μg/L, and serum cPLI concentrations ≥200 μg/L are considered consistent with canine pancreatitis.[176] Serum cPLI concentrations that fall between 102.1 μg/L and 200 μg/L are considered equivocal, and retesting is suggested. It should be noted that cPLI is currently measured as Spec cPL™, which shows the same clinical performance as the original cPLI assay, but has a different reference range (0 to 200 μg/L) from the assay used in the present study.

Dogs were divided into 2 groups according to their serum triglyceride concentration. Group 1 consisted of dogs with serum triglyceride concentrations within

the reference range, while Group 2 consisted of dogs with serum triglyceride concentrations above the upper limit of the reference range (>108 mg/dL).

Statistical analysis

The data were analyzed for normal distribution using the Kolmogorov-Smirnov test. All data failed normality testing and non-parametric methods were used for further analysis. The data were analyzed for correlation between serum cPLI and triglyceride concentrations using the Spearman rank correlation coefficient. The median serum cPLI concentration was compared between groups with the Mann-Whitney *U* test. Receiver operator characteristic (ROC) analysis was performed and the likelihood ratio, which indicates an estimate of how much a positive or negative result affects the likelihood that a patient would have the disease, was calculated.[265] Proportions of dogs with increased serum cPLI concentrations were compared between groups using Fisher's exact tests. Odds ratios and their 95% CI were also calculated. Dogs with serum cPLI concentrations in the equivocal range (e.g., neither within the reference range nor consistent with pancreatitis; n=33), were excluded from this analysis as this could potentially affect the validity of the results. All statistical analyses were performed using a statistical software package[f] and a p value of < 0.05 was considered significant.

Results

There was a significant but weak positive correlation between serum cPLI and triglyceride concentrations in all dogs (Spearman r=0.3215; P<0.0001; Figure 1).

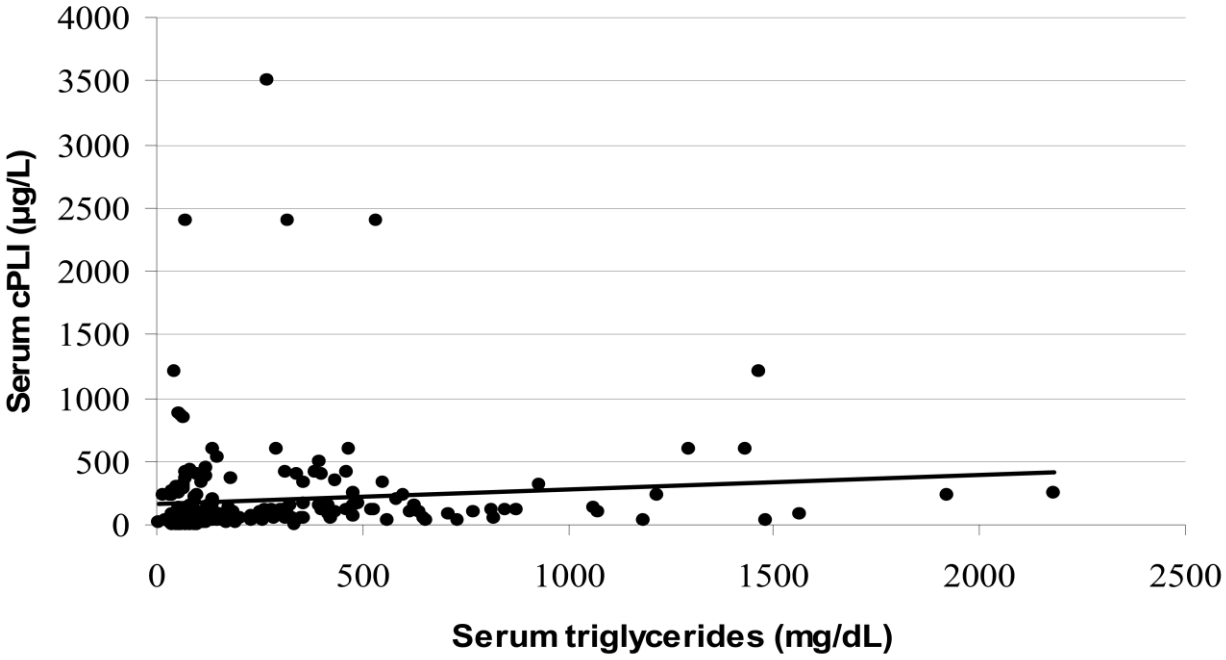

Figure 1. Correlation between serum triglyceride and cPLI concentrations in 195 Miniature Schnauzers. There was a significant positive correlation between the 2 parameters (Spearman r=0.3215; P<0.0001).

ROC analysis and calculation of likelihood ratios at different triglyceride concentrations for serum cPLI concentrations consistent with pancreatitis revealed that the likelihood ratio remained close to 1.0 for serum triglyceride concentrations below approximately 800 mg/dL and increased sharply at serum triglyceride concentrations of more than 800 mg/dL (Figure 2). The lowest serum triglyceride concentration that produced a statistically significant increased risk for a cPLI ≥200 μg/L (a serum cPLI concentration that is considered consistent with pancreatitis) was 862 mg/dL. This concentration was used for further statistical analysis. The likelihood ratio at this concentration for a cPLI ≥200 μg/L was 3.3.

Eighty of the 195 dogs (41%) had serum triglyceride concentrations below the upper limit of the reference range and these dogs were designated as Group 1. One hundred and fifteen of the 195 dogs (59%) had serum triglyceride concentrations above the upper limit of the reference range and were designated as Group 2.

Dogs in Group 2 had a significantly higher median serum cPLI concentration (99.5 μg/L) than dogs in Group 1 (39.3 μg/L; P=0.0001; Figure 3). In addition, dogs in Group 2 with serum triglyceride concentrations ≥862 mg/dL (as determined by ROC analysis) had significantly higher median serum cPLI concentrations (223.4 μg/L) than dogs in Group 1 (P=0.0077; Figure 4).

After excluding the dogs with serum cPLI concentrations in the equivocal range (n=33), there were 75 dogs with normal serum triglyceride concentrations and 87 dogs with hypertriglyceridemia.

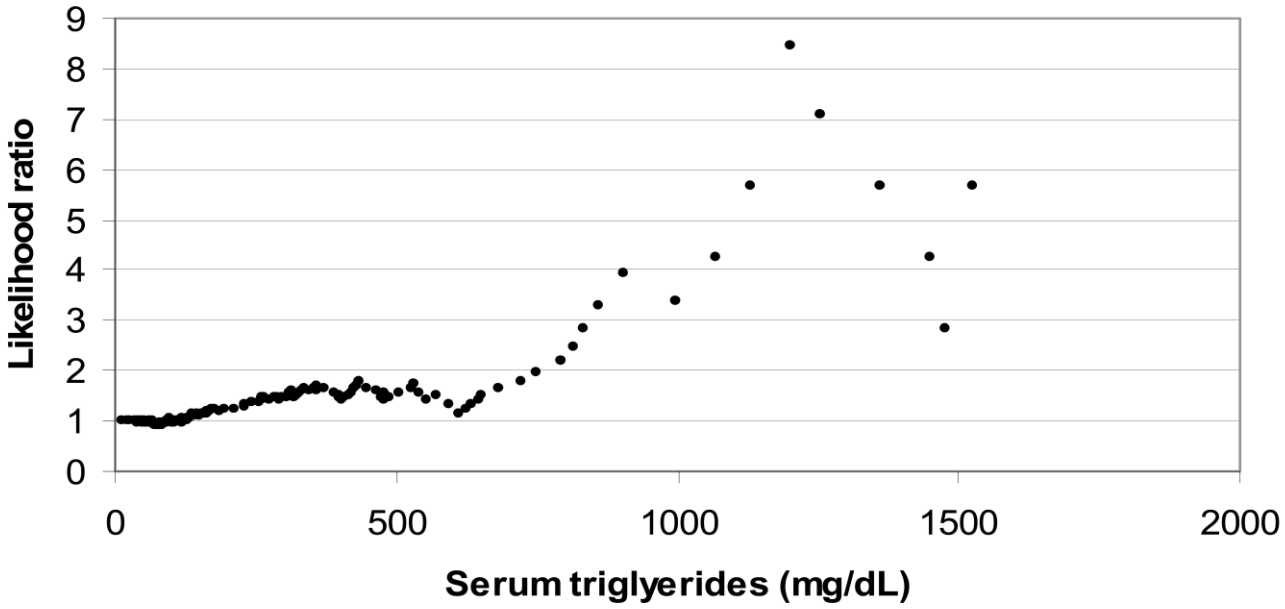

Figure 2. Likelihood ratios for different serum triglyceride concentrations for serum cPLI concentrations consistent with pancreatitis (≥200 µg/L). The likelihood ratio remained between 1.0 and 2.0 for serum triglyceride concentrations below approximately 800 mg/dL, and increased sharply for serum triglyceride concentrations of more than 800 mg/dL. The lowest serum triglyceride concentration that produced a statistically significant likelihood for a cPLI ≥ 200 µg/L was 862 mg/dL.

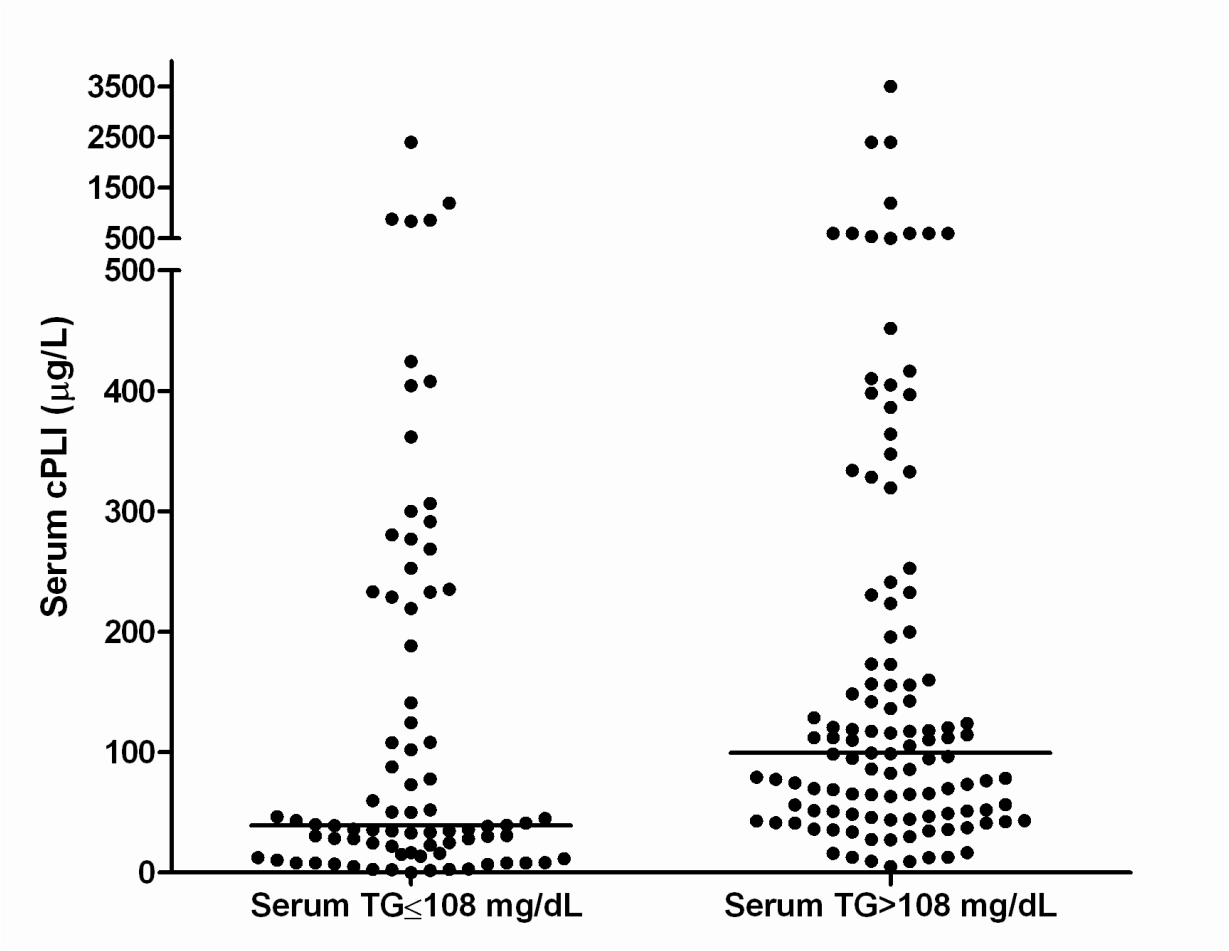

Figure 3. Serum cPLI concentrations in Miniature Schnauzers with normal serum triglyceride concentrations (TG≤108 mg/dL) and in Miniature Schnauzers with serum triglyceride concentrations above the upper limit of the reference range (TG>108 mg/dL). The lines represent the median of each group. There was a statistically significant difference in the median serum cPLI concentrations between the 2 groups (P=0.0001).

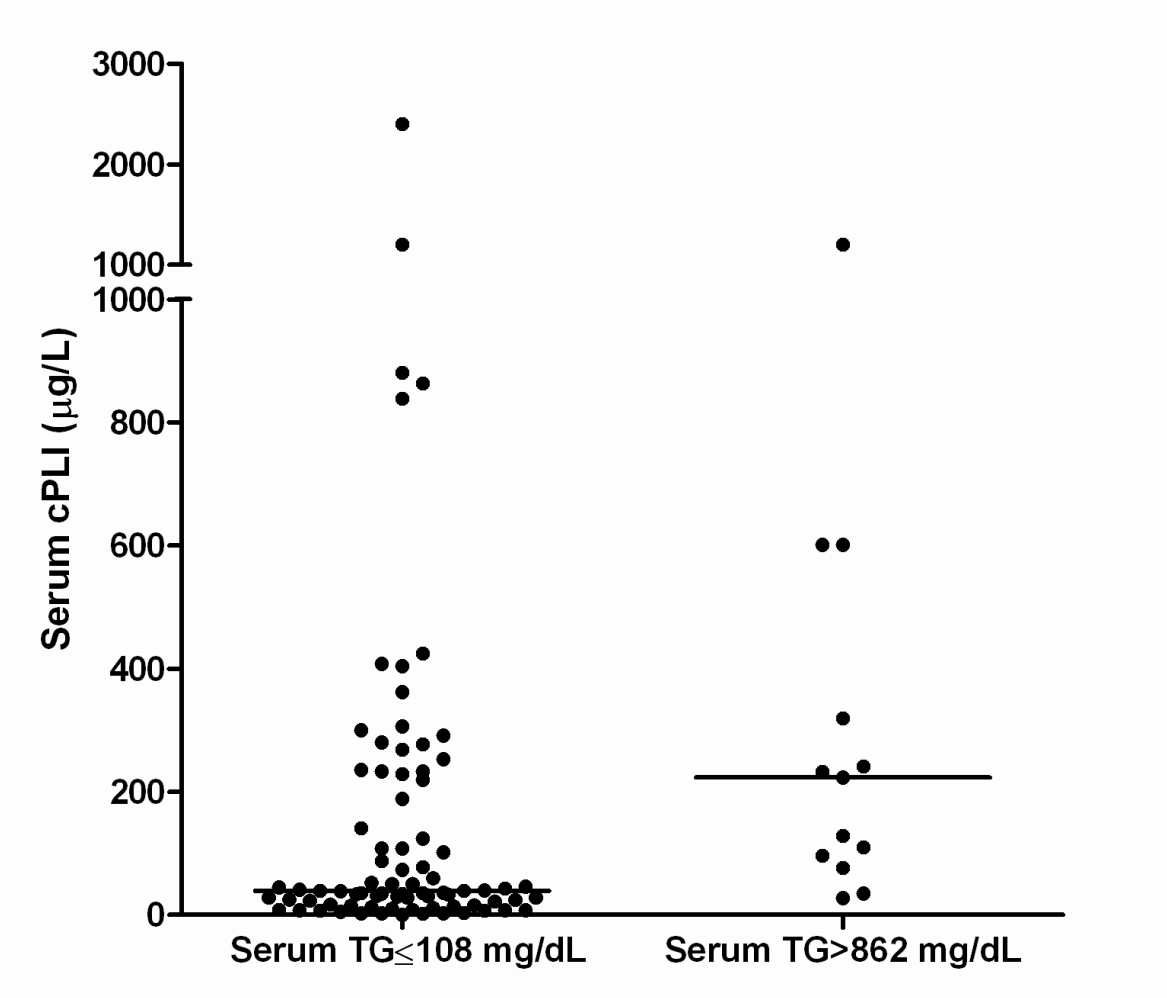

Figure 4. Serum cPLI concentrations in Miniature Schnauzers with normal serum triglyceride concentrations (TG≤108 mg/dL) and in Miniature Schnauzers with a severely increased serum triglyceride concentration (TG>862 mg/dL). The lines represent the median of each group. There was a statistically significant difference in the median cPLI concentration between the 2 groups (P=0.0077).

There was no significant difference in the proportion of dogs with serum cPLI concentrations consistent with pancreatitis between dogs with hypertriglyceridemia (29 of 87 dogs; 33.3%) and dogs with normal triglyceride concentrations (21 of 75 dogs; 28%; P=0.4986). However, of the 87 dogs with hypertriglyceridemia, 11 had serum triglyceride concentrations ≥862 mg/dL, and there was a statistically significant difference in the proportion of dogs with serum cPLI concentrations consistent with pancreatitis between the dogs with serum triglyceride concentrations ≥862 mg/dL (7 of 11 dogs; 63.6%) and dogs with serum triglyceride concentrations in the reference range (21 of 75 dogs; 28%; P=0.0343; odds ratio, 4.5; 95% CI, 1.2 to 17.0).

Discussion

Results of the present study suggest an association between serum triglyceride and cPLI concentrations in Miniature Schnauzers. Miniature Schnauzers with hypertriglyceridemia were found to have significantly higher serum cPLI concentrations than Miniature Schnauzers with normal serum triglyceride concentrations. In addition, Miniature Schnauzers with serum triglyceride concentrations ≥862 mg/dl were 4.5 times (95% CI, 1.2 to 17.0) more likely to have serum cPLI concentrations considered consistent with pancreatitis (>200 μg/L) than Miniature Schnauzers with normal serum triglyceride concentrations. Although previous studies have shown a frequent co-existence of hypertriglyceridemia and pancreatitis in dogs, the present study is the first to implicate the degree of hypertriglyceridemia as a cause of increased serum cPLI concentrations consistent with pancreatitis in Miniature Schnauzers.[19,63,65]

A serum triglyceride concentration of 862 mg/dL was selected based on ROC analysis, and this concentration was associated with a greater than 3-fold likelihood for having a serum cPLI concentration that is considered consistent with pancreatitis compared to the likelihood for dogs with triglyceride concentrations ≤ 862 mg/dL. The likelihood ratio for having a serum cPLI concentration that is consistent with pancreatitis was often higher (up to 8.5) when higher serum triglyceride concentrations were selected (Figure 2). Increases of serum triglyceride concentrations ≥ 862 mg/dL are generally considered to be rather severe. Results of the present study suggest that the severity of hypertriglyceridemia is an important factor affecting the risk for increased cPLI concentration consistent with pancreatitis in Miniature Schnauzers, and that only severely increased serum triglyceride concentrations are associated with an increased risk for an increased cPLI concentration consistent with pancreatitis in this breed. Mildly or moderately elevated serum triglyceride concentrations (e.g., < 800 mg/dL) did not appear to represent a risk factor for a serum cPLI concentration suggestive of pancreatitis in this study. These findings are in agreement with studies in humans, in which only severe hypertriglyceridemia is recognized as a cause of pancreatitis.[254,255] In humans, it has been reported that a serum triglyceride concentration above 1,000 mg/dL is an identifiable risk factor for pancreatitis.[255]

In human patients, it has been described that hypertriglyceridemia can either be the cause or the result of pancreatitis, and the same has also been suggested for dogs.[19,165,255] When hypertriglyceridemia is the result of pancreatitis in humans it is usually mild to moderate, while hypertriglyceridemia that causes pancreatitis is usually

severe (typically ≥1,000 mg/dl).[255] In the present study, many dogs had serum triglyceride concentrations above the upper limit of the reference range and it is possible that some of these dogs might have had hypertriglyceridemia secondary to pancreatitis rather than the opposite. However, this speculation is not supported by the current literature. In 1 study where acute pancreatitis was experimentally induced in dogs, hypertriglyceridemia did not develop up to 96 hours after induction of pancreatitis.[19] Similar results were reported in another study where pancreatitis was experimentally induced in dogs by ligation of the major and minor pancreatic ducts.[18] In this study, pancreatitis did not result in hypertriglyceridemia or hypercholesterolemia up to 14 days after induction of pancreatitis.[18] In a more recent study, mild increases in serum triglyceride concentrations were noted in dogs after induction of pancreatitis.[20] Although serum triglyceride concentrations significantly increased, they remained within the reference range even after induction of pancreatitis.[20] However, this was not clearly discussed in this report.[20] Based on these studies, it can be concluded that hypertriglyceridemia is not a feature of experimentally induced pancreatitis in dogs. However, it remains unknown whether this is also the case for spontaneous canine pancreatitis. It should be mentioned that in another study of 56 dogs of different breeds (not including Miniature Schnauzers) with serum cPLI concentrations diagnostic for pancreatitis, only 14 (25%) had hypertriglyceridemia and of these only 1 (1.8%) had serum triglyceride concentration ≥862 mg/dL.[g] Collectively, the above data indicate that severe hypertriglyceridemia with triglyceride concentrations above 862 mg/dL, which in this study showed a significant association with serum cPLI concentrations consistent

with pancreatitis, appears to be unlikely to be the result of pancreatitis. Furthermore, in our clinical experience with Miniature Schnauzers, the vast majority of Miniature Schnauzers with pancreatitis and severe hypertriglyceridemia have preexisting hypertriglyceridemia, and most of these patients also have persistent hypertriglyceridemia even after recovery from pancreatitis, unless a low-fat diet is being offered.

Because of the high incidence of idiopathic hypertriglyceridemia in Miniature Schnauzers,[84] many of the dogs with elevated serum triglyceride concentrations enrolled in this study are likely to have this condition. However, secondary causes of hypertriglyceridemia, mostly endocrinopathies such as diabetes mellitus, hyperadrenocorticism, and hypothyroidism cannot be excluded as potential causes of the hypertriglyceridemia in these dogs.[49] There is no evidence to support that the source (rather than the severity) of hypertriglyceridemia can affect the risk for pancreatitis, and severe hypertriglyceridemia of any cause (primary or secondary) should be considered a risk factor for high cPLI concentrations consistent with pancreatitis in Miniature Schnauzers.[65] This is also the case in human patients, where severe hypertriglyceridemia secondary to other diseases (e.g., uncontrolled or untreated diabetes mellitus, diabetic ketoacidosis) has also been associated with pancreatitis.[257,266]

One limitation of the present study is the fact that the medical histories of the dogs enrolled were not available. Because of this, the true disease status of these patients (e.g., whether they had clinical signs of pancreatitis) was unknown. However, the majority of samples submitted the authors' laboratory come from animals with

gastrointestinal signs or suspected gastrointestinal disease. In addition, any concurrent conditions or medications administered cannot be excluded as factors contributing to the serum cPLI concentrations observed. It is important to note, however, that there are currently no known factors other than pancreatitis that have been shown to increase serum cPLI concentrations above 200 µg/L (the current cut-off value for pancreatitis).[c,d,182] Therefore, the finding of this study that there is an association between serum triglyceride and cPLI concentrations cannot be arbitrarily translated into an association between serum triglycerides and clinical pancreatitis, though such an association remains possible. To definitely make such a conclusion further studies are needed to prospectively evaluate the risk for increased cPLI concentrations and pancreatitis in Miniature Schnauzers with hypertriglyceridemia. In addition, further studies are warranted to investigate the association between hypertriglyceridemia and pancreatitis in dogs of other breeds.

In conclusion, this is the first clinical study supporting an association between hypertriglyceridemia, especially when severe (≥862 mg/dL), and increased serum cPLI concentrations consistent with pancreatitis in Miniature Schnauzers. Miniature Schnauzers with severe hypertriglyceridemia (≥862 mg/dl) were 4.5 times more likely to have a serum cPLI concentration consistent with pancreatitis than Miniature Schnauzers with a normal serum triglyceride concentration.

Footnotes

[a]Steiner JM, Broussard J, Mansfield CS, Gumminger SR, Williams DA: Serum canine pancreatic lipase immunoreactivity (cPLI) concentrations in dogs with spontaneous pancreatitis. J Vet Int Med, 15: 274, 2001 (abstract)

[b]Sinclair HM, Fleeman LM, Rand JS, Williams DA, Steiner JM. Continuing pancreatic inflammation or reduced exocrine function are common in dogs after acute pancreatitis. J Vet Intern Med, 20:750, 2006 (abstract)

[c]Steiner JM, Finco DR, Gumminger SR, Williams DA. Serum canine pancreatic lipase immunoreactivity (cPLI) in dogs with experimentally induced chronic renal failure. J Vet Int Med, 15: 311, 2001 (abstract)

[d]Steiner JM, Gumminger SR, Ruaux CG, Williams DA. The influence of feeding on serum canine pancreatic lipase immunoreactivity (cPLI) concentrations. J Vet Intern Med, 16: 382, 2002 (abstract)

[e]Hitachi 911 Automatic Analyzer, Boehringer Mannheim, Indianapolis, IN

[f]Prism 5, GraphPad Software Inc., San Diego, CA

[g]Xenoulis et al., unpublished data

CHAPTER III

SERUM TRIGLYCERIDE CONCENTRATIONS IN MINIATURE SCHNAUZERS WITH AND WITHOUT A HISTORY OF PROBABLE PANCREATITIS*

Introduction

The cause of pancreatitis usually remains unknown in dogs.[65,162] Several risk factors for pancreatitis have been indentified or suspected in dogs including trauma, certain drugs, endocrine diseases, obesity, and dietary indiscretion, but a definitive cause-and-effect relationship has not been established for most of them.[63,65,163] Hypertriglyceridemia has long been considered a possible cause of or risk factor for pancreatitis in dogs.[19,63,65,165,252] Furthermore, it is widely believed that a suspected high prevalence of pancreatitis in Miniature Schnauzers[63,163] might be associated with the documented high prevalence of hypertriglyceridemia in this breed.[15,17,19,84,165,252]

The association between hypertriglyceridemia and pancreatitis remains obscure in dogs. Hypertriglyceridemia is commonly reported in dogs with pancreatitis, but the etiology of hypertriglyceridemia is not always possible to determine in these cases.[19,21,63-65]

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*Xenoulis PG, et al. Serum triglyceride concentrations in Miniature Schnauzers with and without a history of probable pancreatitis. *J Vet Intern Med* 2011;25(1):20-25.
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The presence of hypertriglyceridemia in dogs with pancreatitis might be due to a pre-existing disorder in lipid metabolism, which may or may not be related to the etiology of pancreatitis, or it might be the result of pancreatitis.[252] Although it is widely believed that hypertriglyceridemia can develop as a result of pancreatitis, this has not been convincingly shown in dogs with naturally occurring pancreatitis. Furthermore, with the exception of the results of an older study,[261] hypertriglyceridemia does not seem to be a consequence of experimental pancreatitis in dogs.[18-20] In order for hypertriglyceridemia to be considered as a possible etiologic factor for pancreatitis it should precede the development of pancreatitis. Hypertriglyceridemia that precedes the development of pancreatitis might be primary such as hypertriglyceridemia of certain breeds such as Miniature Schnauzers or secondary to other diseases including diabetes mellitus, hyperadrenocorticism, hypothyroidism or drug administration (such as glucocorticoids).[252] A possible role of hypertriglyceridemia as a cause of pancreatitis in Miniature Schnauzers or other dog breeds has been suspected based on knowledge extrapolated from human medicine, where severe hypertriglyceridemia is a well recognized cause of pancreatitis,[255,257,258,267] *in vitro* or in experimental studies,[144,260] and anecdotal clinical impressions.[165]

The possibility of hypertriglyceridemia potentially developing as a result of pancreatitis complicates the determination of the role of hypertriglyceridemia as an etiologic factor of pancreatitis in both humans and dogs.[252,255] One approach that is often used in human studies in order to overcome this obstacle is the measurement of serum triglyceride concentrations after pancreatitis has resolved.[256,267-270] This is based on the

logical assumption that a defect in lipid metabolism causing hypertriglyceridemia must be a pre-existing condition in order for a patient to develop pancreatitis as a result of hypertriglyceridemia. If this is the case, hypertriglyceridemia should also persist after resolution of pancreatitis.[256,267-270] In contrast, hypertriglyceridemia secondary to pancreatitis should resolve when pancreatitis is no longer present. Therefore, the presence of hypertriglyceridemia soon after resolution of pancreatitis provides retrospective evidence of a pre-existing condition, which if severe enough, could be considered as an etiologic factor for pancreatitis.[256,267-270]

Studies investigating disorders of lipid metabolism in patients with a history of naturally occurring pancreatitis have been reported in humans but not in veterinary species. The aim of this study was to compare serum triglyceride concentrations between Miniature Schnauzers with a recent history of pancreatitis and those without such a recent history of pancreatitis.

Materials and methods

Study design

This study is a prospective case-control study.

Dogs with pancreatitis (Group 1)

Over a 2-year period (November 2006 – November 2008), a total of 35 Miniature Schnauzers with a diagnosis of pancreatitis were initially considered for prospective enrollment into this study. The diagnosis of pancreatitis was based on the presence of at

least 2 clinical signs compatible with pancreatitis (vomiting, anorexia, depression, abdominal pain, diarrhea) and a serum Spec cPL® concentration ≥400 μg/L (the currently recommended cut-off value for pancreatitis).[a,b,178] The dogs were enrolled on a sequential basis based solely on whether the owner had agreed to submit a follow-up sample after clinical resolution of pancreatitis. The 35 dogs considered for enrollment into the study were from various regions of the United States of America.

After clinical recovery from pancreatitis, the owners of these dogs were asked to have a follow-up blood sample collected. The person communicating with the owners concerning the request for a follow-up sample was blinded to the serum triglyceride concentration of the dog during the initial evaluation. Thus, no preference was given to dogs with hypertriglyceridemia. The dogs were eventually included in the study if at the time the follow-up sample was collected they had: a) complete absence of any clinical signs, b) a serum Spec cPL concentration within the reference interval (<200 μg/L),[178] c) a serum free T4 or total T4 concentration within the reference interval, and d) no history of diseases or use of drugs known to affect lipid metabolism (other than pancreatitis). Dogs fulfilling these enrollment criteria were designated as Group 1 (dogs with clinically resolved pancreatitis and a serum Spec cPL concentration within the reference interval). Only the follow-up blood sample, which was obtained after clinical and biochemical resolution of pancreatitis, was used for statistical comparison with the control group. The initial sample (during pancreatitis) was excluded from the analysis because it would not have been possible to determine whether possible hypertriglyceridemia in this initial sample was primary or had developed secondary to

pancreatitis. For the same reason, serum Spec cPL concentration was measured in the follow-up sample of each dog, and dogs with serum Spec cPL concentrations outside the reference interval (i.e., >200 μg/L) were excluded from further analysis. Serum triglyceride and cholesterol concentrations were evaluated in both the initial (during pancreatitis) and the follow-up sample for all dogs in Group 1.

Control dogs (Group 2)

Each dog enrolled into Group 1 was age-matched with 2 clinically healthy Miniature Schnauzers with no history of pancreatitis (Group 2). The selection of dogs into this group was based solely on their age (which had to match the age of each one of the dogs in Group 1). The person selecting the dogs was blinded to the serum triglyceride concentration of the dog. Inclusion criteria for dogs in Group 2 were identical to those for dogs of Group 1, with the exception of the history of pancreatitis. Therefore, dogs in Group 2 were included in the study if they had: a) absence of any clinical signs at the time of blood collection, b) a serum Spec cPL concentration within the reference interval, c) a serum free T4 or total T4 concentration within then reference interval, and d) no history of diseases or current drug use known to affect lipid metabolism. Serum triglyceride and cholesterol concentrations were measured in all dogs of Group 2.

Blood collection and handling

Owners that decided to participate in the study were sent styrofoam boxes containing ice packs and the material necessary for blood collection, and were asked to schedule an appointment with their veterinarian. Veterinarians were instructed to collect 10 ml of blood into a red-top tube (with no additive), wait for clot formation, centrifuge the sample, separate the serum from the clot, transfer the serum to another red-top tube, and send the samples to the Gastrointestinal Laboratory packed on ice by overnight courier. Owners had been instructed not to feed their dogs for at least 12 hours before the scheduled blood collection. Upon receipt, serum samples were immediately aliquoted and stored at -80^{o}C until further use.

Questionnaires and consent forms

Owners were asked to complete a questionnaire for each dog. Questions covered date of birth, sex, body weight, current diet(s), current medications, and current and past health history of the dogs. Questionnaires from all dogs were reviewed to determine whether the dogs fit the inclusion criteria for each group. All owners had to sign an informed owner consent form. The study protocol was reviewed and approved by the Clinical Research Review Committee at Texas A&M University.

Assays

Serum triglyceride (reference range: 26 to 108 mg/dL) and cholesterol (reference range: 124 to 335 mg/dL) concentrations were measured by enzymatic assays.[c] Serum

Spec cPL concentrations were measured using an analytically validated immunoassay as described elsewhere.[178,271] Serum free T4 concentration was measured using a commercial equilibrium dialysis radioimmunoassay.[d] Serum total T4 concentrations were measured using a solid-phase chemiluminescent competitive assay.[e]

Statistical analyses

A commercial statistical software package was used for all statistical analyses.[d] Data were analyzed for normal distribution using the Shapiro-Wilk test. Normally distributed data were analyzed using t-tests or paired t-tests where appropriate. Non-normally distributed data were analyzed using Wilcoxon tests or Wilcoxon rank sum tests. Proportions were compared between groups using the Fisher's exact test. Significance was set at $P<0.05$ for all analyses.

Results

Dogs in Groups 1 and 2

All dogs were prospectively enrolled into this study. Of the 35 dogs initially considered for enrollment in Group 1, 17 met the inclusion criteria for this group. Five dogs were excluded because they had clinical sings of anorexia, diarrhea, and/or abdominal pain at the time of the second blood collection, 4 dogs were excluded because they had been diagnosed with diabetes mellitus (2) or hypothyroidism (2), and 7 dogs were excluded because of a Spec cPL concentration in the follow-up sample above 200 μg/L (3 of these dogs also had clinical signs associated with pancreatitis). Follow-up

samples from the 17 Miniature Schnauzers that were included in Group 1 were collected within a median interval of 12 weeks (range: 2 to 41 weeks) after the first sample collected at the time of diagnosis of pancreatitis. Group 1 consisted of 9 female (8 spayed) and 8 male (6 castrated) dogs.

Group 2 consisted of 34 Miniature Schnauzers that had fulfilled the inclusion criteria outlined above and had been age-matched with the dogs in Group 1. Group 2 consisted of 22 female (8 spayed) and 12 male (3 castrated) dogs.

Comparisons between Groups 1 and 2

All comparisons between Groups 1 and 2 were made using the information and test results that corresponded to the follow-up sample (which was collected after clinical and biochemical resolution of pancreatitis) for Group 1. The weight was known for 16/17 dogs in Group 1 and 30/34 dogs in Group 2; there was no significant difference of the mean body weight between Group 1 (7.8 kg) and Group 2 (7.6; P=0.6702). As outlined in the study's inclusion criteria, all dogs had a serum free T4 (15 dogs in Group 1 and 32 dogs in Group 2) or total T4 (2 dogs in each Group) within the respective reference interval. Of the dogs in Group 1, 8/16 (50.0%) were mainly on a home-made (n=1) or commercial (n=7) diets labeled as low-fat, including prescription (n=5) and non-prescription (n=2) diets. Of the 34 dogs in Group 2, 1 was mainly on a home-made low-fat diet, none were on prescription low-fat diets at the time of blood collection, while 4 dogs (11.8%) were on commercial diets labeled as low-fat. Dogs in Group 1

were significantly more likely to be on a low-fat diet than dogs in Group 2 (odds ratio=5.8, 95% CI, 1.5 to 22.7, P=0.0143).

Of the 17 Miniature Schnauzers in Group 1, 12 (71%) had serum triglyceride concentrations above the reference interval in the follow-up sample, while only 11 (33%) of the 34 Miniature Schnauzers in Group 2 had serum triglyceride concentrations above the upper limit of the reference interval. Miniature Schnauzers in Group 1 were significantly more likely to have hypertriglyceridemia than Miniature Schnauzers in Group 2 (odds ratio=5.02; 95% CI, 1.4 to 17.8; P=0.0163). To take into account the severity of hypertriglyceridemia, the proportion of dogs with moderate to severe hypertriglyceridemia (>500 mg/dL)[252,257] was compared between groups. Ten out of 17 (59%) dogs in Group 1 and 3/34 (9%) in Group 2 had serum triglyceride concentrations >500 mg/dL (odds ratio=14.8; 95% CI, 3.2 to 68.1; P=0.0003). Serum triglyceride concentrations were significantly higher in dogs of Group 1 (median: 605.0 mg/dL; range: 41.0 to 2,134.0 mg/dL) than in dogs of Group 2 (median: 73.0 mg/dL; range: 23.0 to 3,975.0; P=0.002; Figure 5).

Serum cholesterol concentrations were significantly higher in dogs of Group 1 (median: 287.6 mg/dL) than in those of Group 2 (median: 204.0 mg/dL; P=0.0001; Figure 6). Five dogs in Group 1 (29.4%) and 1 dog in Group 2 (2.9%; P=0.0136) had serum cholesterol concentrations above the upper limit of the reference interval, and hypercholesterolemia was associated with hypertriglyceridemia in all cases.

Comparisons within Group 1

Within Group 1, serum triglyceride concentrations were not significantly different during (median: 215.5 mg/dL) and after resolution of pancreatitis (median: 580.0 mg/dL; P=0.552). However, great variation of values within the same dog existed between the two time-points for many of the dogs (Figure 7). Interestingly, serum triglyceride concentrations were normal or below the lower limit of the reference interval in 3 of the dogs during pancreatitis (20, 51, and 85 md/dL) and increased (429, 2,134, and 1,851 mg/dL, respectively) after resolution of pancreatitis (Figure 7). These dogs were each on the same diet (n=2) or on diets with a similar fat content (n=1) both time-points.

In the same group, mean serum cholesterol concentrations were not significantly different during (251.7 mg/dL) and after resolution of pancreatitis (272.7 mg/dL; P=0.3523).

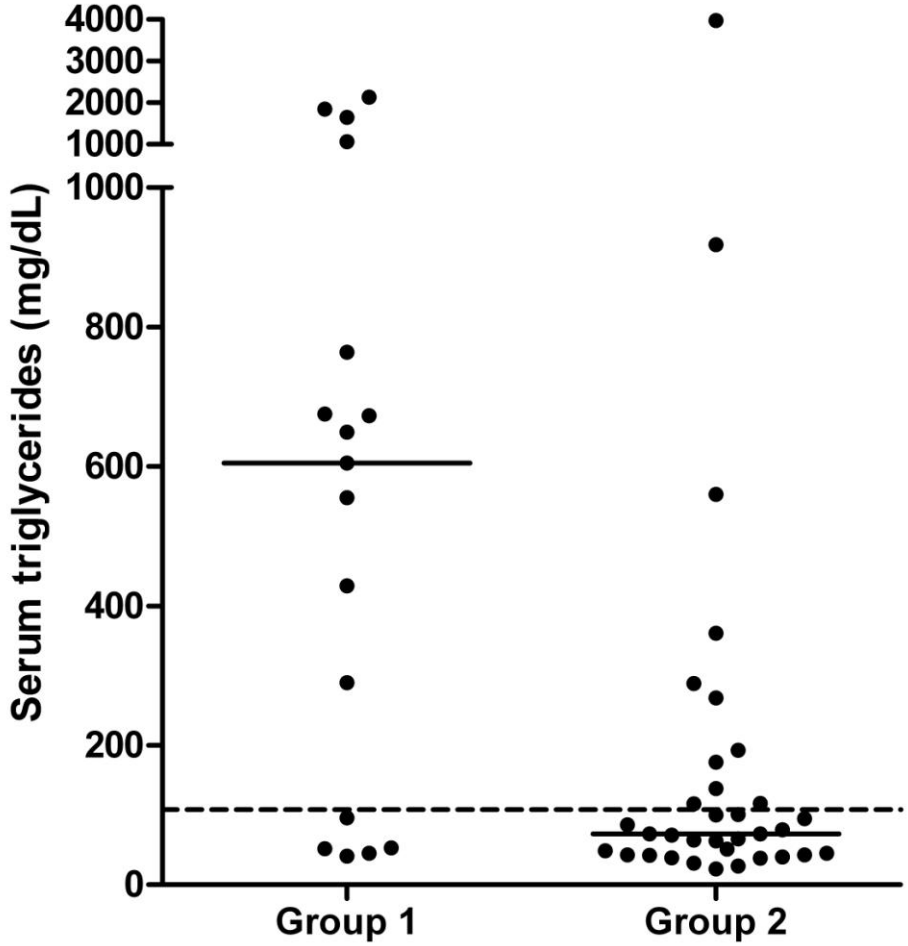


Figure 5. Comparison of serum triglyceride concentrations between Miniature Schnauzers with a history of pancreatitis (Group 1) and Miniature Schnauzers with no history of pancreatitis (Groups 2). Serum triglyceride concentrations were significantly higher in dogs of Group 1 (median: 605.0 mg/dL) than those in dogs of Group 2 (median: 73.5.0 mg/dL; P=0.0108). The dashed line represents the upper limit of the reference interval. Note: the Y-axis in split.

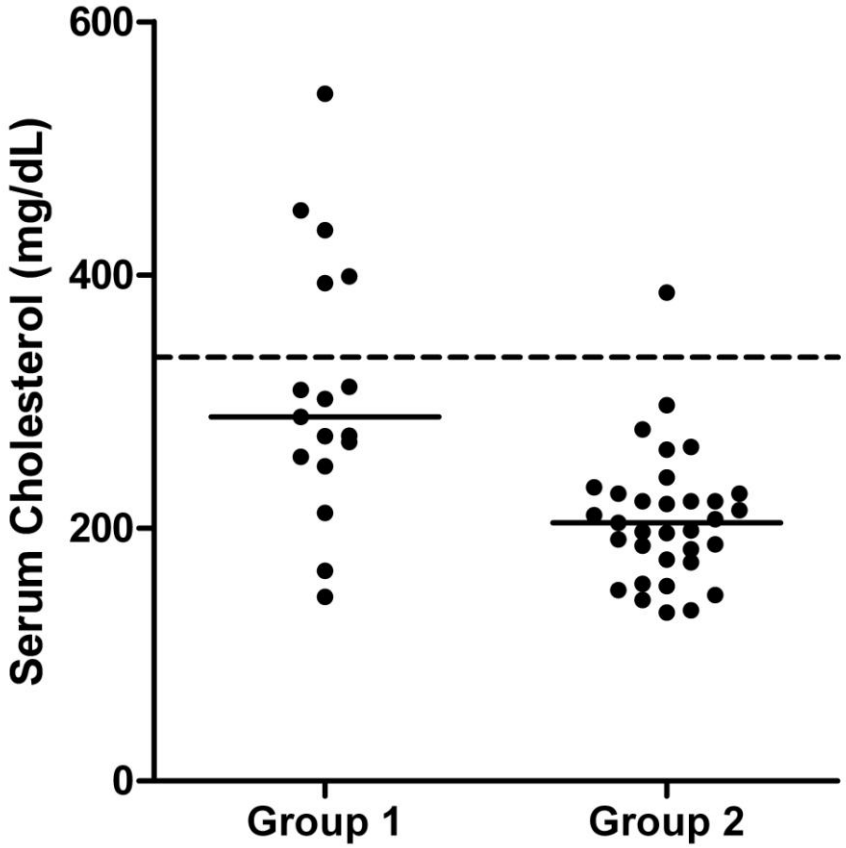


Figure 6. Comparison of serum cholesterol concentrations between Miniature Schnauzers with a history of pancreatitis (Group 1) and Miniature Schnauzers with no history of pancreatitis (Groups 2). Serum cholesterol concentrations were significantly higher in dogs of Group 1 (median: 287.6 mg/dL) than of those in Group 2 (median: 214.0 mg/dL; P=0.002). Only 5 dogs in Group 1 and 2 dogs in Group 2 had serum cholesterol concentrations above the upper limit of the reference interval. The dashed line represents the upper limit of the reference interval.

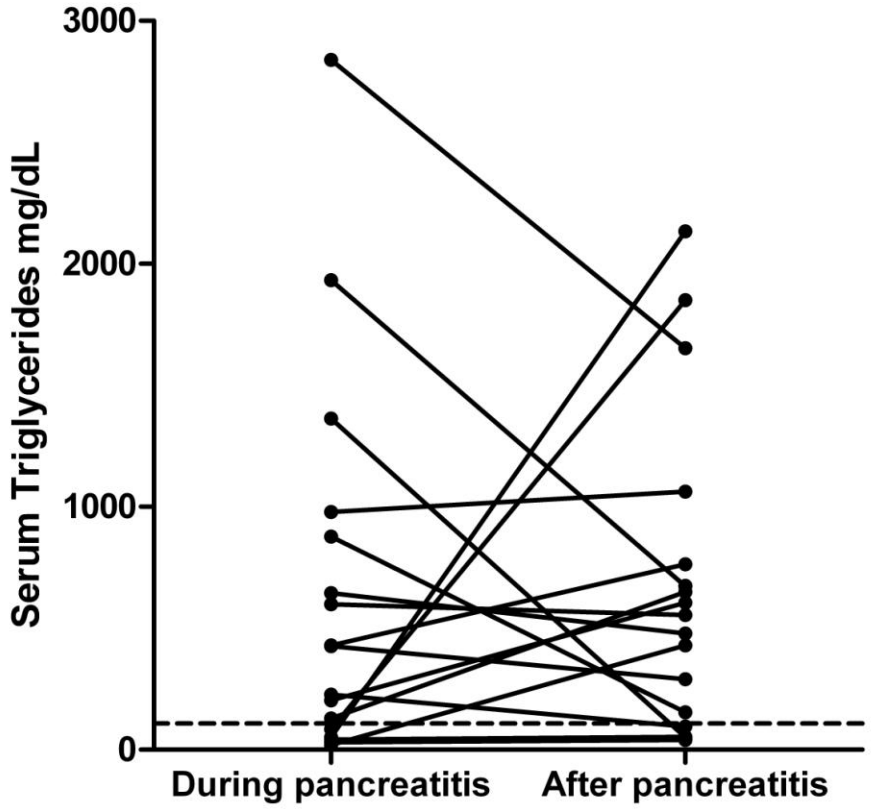


Figure 7. Comparison of serum triglyceride concentrations during pancreatitis and after resolution of pancreatitis. This graph shows the distribution and comparison of serum triglyceride concentrations in dogs of Group 1 at the time of diagnosis of pancreatitis and also after clinical and biochemical resolution of pancreatitis. Serum triglyceride concentrations were not significantly different at the time of diagnosis of pancreatitis (median: 215.5 mg/dL) than after resolution of pancreatitis (median: 580 mg/dL; P=0.552). For many of the dogs great variation of serum triglyceride concentrations existed between the 2 time-points. The dashed line represents the upper limit of the reference interval.

Discussion

In the present study, Miniature Schnauzers with a history of pancreatitis were 5 times more likely to have hypertriglyceridemia and almost 15 times more likely to have moderate to severe hypertriglyceridemia (defined as >500 mg/dL) than age-matched Miniature Schnauzers with no history of pancreatitis. The majority of Miniature Schnauzers with a history of pancreatitis (58.8%) had moderate to severe hypertriglyceridemia as compared to only 8.8% in the control group. The presence of hypertriglyceridemia after resolution of pancreatitis is considered to provide retrospective evidence of a pre-existing disorder in lipid metabolism.[256,267,268,270] Knowing whether severe hypertriglyceridemia is a risk factor or even a cause of pancreatitis is of major importance in dogs, because control of hypertriglyceridemia might prevent the development or recurrence of pancreatitis. This has been confirmed in human patients with severe hypertriglyceridemia and pancreatitis, in whom control of hypertriglyceridemia limits the risk of recurrence of pancreatitis.[267,272] People with uncontrolled severe hypertriglyceridemia commonly have recurrent pancreatitis.[257,272]

It remains to be determined whether pre-existing hypertriglyceridemia in this group of Miniature Schnauzers predisposed them to develop pancreatitis. A clear causal association between hypertriglyceridemia and pancreatitis in Miniature Schnauzers is very challenging to illustrate in clinical studies. Ideally, samples before, during, and after resolution of pancreatitis should be collected in order to clearly determine the association between hypertriglyceridemia and pancreatitis. Although this is possible to do in experimental models of pancreatitis, it is impossible in cases of naturally occurring

pancreatitis as it cannot be predicted which dogs will develop pancreatitis. Therefore, evidence of pre-existing hypertriglyceridemia in this study was based on the presence of hypertriglyceridemia after recovery from pancreatitis. Most follow-up samples were collected soon after pancreatitis was diagnosed (within 1-4 months) and thus it is very likely that hypertriglyceridemia was present before the development of pancreatitis. This has been shown in a large number of human studies using different methods, in which disorders of lipid metabolism that persist after recovery from pancreatitis were considered to be pre-existing.[256,267,268,270] Due to the fact that follow-up samples in Group 1 were collected after resolution of pancreatitis (based on both clinical and biochemical evidence), it is unlikely that hypertriglyceridemia in these samples was the result of pancreatitis. There is a theoretical possibility that pancreatitis could have permanently changed lipid metabolism in those dogs but, to the authors' knowledge, this has not been reported or even hypothesized in dogs or any other species.

It needs to be pointed out that, in this study, Miniature Schnauzers with a history of pancreatitis were significantly more likely to be on diets labeled as low-fat than dogs of the same breed without such a history. This is very important because the reported serum triglyceride concentrations after recovery from pancreatitis likely underestimate the true prevalence and severity of serum triglyceride concentrations before the development of pancreatitis. Despite that, Miniature Schnauzers with a history of pancreatitis were significantly more likely to have hypertriglyceridemia than age-matched Miniature Schnauzers with no history of pancreatitis. It is very important to note, however, that the diets were considered to be low-fat only based on their label and

not their actual fat content. Although this is a rather inaccurate way to determine the fat content of a diet, such an approach was chosen because several dogs were on home-made diets, while other dogs were on more than one diets at the same time, and it would be impossible to determine that fat content of the diet for each dog.

Many dogs in the control group had hypertriglyceridemia and yet no known history of pancreatitis. This is also commonly reported in humans, and the reason for this is unknown.[255,258] There are several possible explanations for this observation. First, as mentioned above, a causal association between hypertriglyceridemia and pancreatitis in dogs remains to be determined. If a causal association exists, it is possible that there are differences in lipoprotein composition or metabolism between individuals, which may or may not predispose to pancreatitis. In addition, individual cases of mild pancreatitis might have escaped diagnosis, or some of these patients will develop pancreatitis in the future. It should also be pointed out that in the case of the present study, most hypertriglyceridemic dogs (91%) in the control group had mild (<500 mg/dL) hypertriglyceridemia, which might be less likely to be associated with pancreatitis. Several studies in humans have evaluated various methods to predict which hypertriglyceridemic individuals will develop pancreatitis but results of these studies are inconclusive.[256,268,270] Such studies have not been reported, but based on the results reported here, are clearly warranted in dogs, in order to provide a better understanding of the possible lipid disorders associated with pancreatitis.

Great variation in serum triglyceride concentrations existed within some individual dogs in Group 1 between the 2 time-points (during and after resolution of

pancreatitis). Although these results must be interpreted with caution because dietary information was not available for all dogs during pancreatitis and because the diet was different between the two time-points for some of the dogs, an interesting observation can be made. Serum triglyceride concentrations were normal or below the lower limit of the reference interval in 3 of the dogs during pancreatitis (20, 51, and 85 md/dL) and increased or even dramatically increased (429, 2,134, and 1,851 mg/dL, respectively) after resolution of pancreatitis (Figure 7). These dogs were each on the same diet (n=2) or on diets with a similar fat content (n=1) during both time-points. Although the reason for this finding is unknown, it might be that extensive anorexia (all 3 dogs were reported to be anorexic) and/or fasting during pancreatitis led to normalization of serum triglyceride concentrations in these dogs during this time-period. Similar findings have been reported in humans with pancreatitis, where even profound increases in serum triglyceride concentrations (>1,750 mg/dL) returned to normal within 72 hours of initial presentation.[267,269,270] On the other hand, one dog that was on the same diet at both time-points had severely increased serum triglyceride concentrations during pancreatitis (1,363 mg/dL) and normal serum triglyceride concentrations after resolution of pancreatitis (52 mg/dL). Regardless of the possible explanation, these findings suggest that serum triglyceride concentrations during the course of pancreatitis may not accurately reflect the true triglyceride concentrations.[255,258,267] Therefore, it might be recommended that serum triglyceride concentrations be measured after resolution of pancreatitis to make sure that the measurements accurately reflect the condition of each dog.

Although serum cholesterol concentrations were significantly higher in Miniature Schnauzers with a history of pancreatitis compared to controls, the medians of both groups were within the reference interval. In addition, only 5 dogs in Group 1 had hypercholesterolemia, which was always mild and seen only in association with hypertriglyceridemia. This is in agreement with the results of a previous study in healthy Miniature Schnauzers.[84] To the authors' knowledge, there is no evidence to suggest that hypercholesterolemia might play a role in the development of pancreatitis in dogs or any other species, and this is supported by the findings of the present study.

Due to the fact that the dogs in this study had normal serum free or total T4 concentrations, no clinical signs, and no history of a disease or drug administration known to affect lipid metabolism, it is logical to assume that hypertriglyceridemia in these dogs was likely idiopathic.[84,252] Although an association between endocrinopathies commonly accompanied by secondary hypertriglyceridemia and pancreatitis has been previously reported, the retrospective design of these studies does not allow determination of whether this was due to hypertriglyceridemia or not.[64,65] Further studies are needed to determine what proportion of dogs with pancreatitis has persisting hypertriglyceridemia secondary to other diseases.

One limitation of the present study is that even with a normal Spec cPL concentration and the absence of clinical signs of pancreatitis, mild residual inflammation of the pancreas cannot be definitively excluded. The only way to exclude this possibility would be through histopathologic examination of multiple pancreatic biopsies, a very invasive procedure that cannot be ethically justified in patients that have

recovered from pancreatitis. In addition, even if some mild residual inflammation of the pancreas was present in some dogs, it is not known whether this can affect lipid metabolism. Theoretically, longer intervals between pancreatitis and the follow-up sample could have been used, but then it would have been be more likely that, with increasing age, hypertriglyceridemia would develop, because previously reported data suggest that hypertriglyceridemia in the Miniature Schnauzer should be regarded as an age-related condition.[84]

In conclusion, Miniature Schnauzers with a history of pancreatitis were 5 times more likely to have hypertriglyceridemia than age-matched control dogs of the same breed. In addition, Miniature Schnauzers with a history of pancreatitis were almost 15 times more likely to have moderate to severe hypertriglyceridemia (>500 mg/dL) than controls. It is logical to assume that hypertriglyceridemia was a pre-existing condition in most Miniature Schnauzers with a history of pancreatitis in this study. This study shows that, in contrast to control dogs, the majority of Miniature Schnauzers with a history of pancreatitis have hypertriglyceridemia. Additional studies are needed to further clarify the role of hypertriglyceridemia in the development of pancreatitis in Miniature Schnauzers as well as other dog breeds.

Footnotes

[a]Spec cPL®, IDEXX Laboratories, Inc., Westbrook, ME

[b]McCord, K., Davis, J, Leyva, F.et al. A multi-institutional study evaluating diagnostic utility of Spec cPL™ in the diagnosis of acute pancreatitis in dogs. J Vet Int Med 23(3), 734. 2009.

[c]Roche/Hitachi MODULAR *ANALYTICS* D 2400 module, Roche Diagnostics, Indianapolis, IN

[d]Free T4 (by ED), Antech Diagnostics, Irvine, CA

[e]Immulite 2000 Canine Total T4, Siemens Healthcare Diagnostics, Deerfield, IL

[f]Prism5, GraphPad, San Diego, CA

CHAPTER IV

LIPOPROTEIN PROFILING USING DENSITY GRADIENT ULTRACENTRIFUGATION IN HEALTHY DOGS OF VARIOUS BREEDS, HEALTHY MINIATURE SCHNAUZERS, AND MINIATURE SCHNAUZERS WITH HYPERLIPIDEMIA

Introduction

The investigation of lipoprotein profiles in serum or plasma from healthy dogs has been the subject of research since the 1940s.[1,2,6,8] Much of our current knowledge on canine lipoproteins in dogs originates from studies reported in the 1970s, which investigated dogs as possible models for human cardiovascular disease.[6,8,21] More recent studies have investigated canine lipoproteins in association with several disease conditions or physiologic stages.[11,12,17,19-21,273] These studies have provided important information on the major serum lipoprotein fractions found in dogs, namely chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). With the development and increased availability of advanced methodologies (e.g., nuclear magnetic resonance spectroscopy,[274] newer density gradient ultracentrifugation techniques,[28,275] surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS)[276]), more detailed, accurate, and effortless characterization of lipoprotein profiles in dogs should be possible. Some studies in humans have suggested that the major serum lipoprotein classes are each a very heterogeneous group of molecules that can be subdivided into

several subfractions, with different disease associations for each subfraction.[277,278] A significant body of research has been devoted to defining the role of these lipoprotein subfractions in a number of human diseases.[277,278] Novel methodologies developed for the effective separation and study of lipoprotein subfractions have not previously been applied to dogs, and therefore the feasibility of application and usefulness of these techniques is not known in this species.

Diseases that affect lipoprotein metabolism are common in dogs.[252] By far, the most common disorders of lipoprotein metabolism in dogs are secondary to other diseases, such as diabetes mellitus, hypothyroidism, and hyperadrenocorticism.[252] Miniature Schnauzers are particularly interesting with regard to their serum lipids and lipoprotein profiles. Primary hypertriglyceridemia is a common condition in Miniature Schnauzers in the United States. In 1 study, hypertriglyceridemia was present in 32.8% of 192 Miniature Schnauzers investigated.[84] In this breed, hyperlipidemia, and more specifically hypertriglyceridemia, might be associated with diseases such as hepatobiliary disease, pancreatitis, insulin resistance, and ocular disease.[123,252,279,280] The biochemical, metabolic, and genetic bases of hypertriglyceridemia in Miniature Schnauzers have not been identified yet. Previous studies have shown that hypertriglyceridemia in Miniature Schnauzers is mainly characterized by an abnormal accumulation of VLDL with or without hyperchylomicronemia.[17]

Detailed characterization of serum lipids and lipoproteins in Miniature Schnauzers can provide a greater insight into the biochemical and metabolic bases of primary hypertriglyceridemia in this breed. In addition, characterization of serum lipids

and lipoproteins in healthy dogs of various breeds is important as a basis for further research in dogs with hyperlipidemia of different causes and the development of disorders as a result of hyperlipidemia. Despite the importance of lipoprotein research in dogs, the fact that most previously used methods for lipoprotein profiling are rather laborious and time-consuming has been a major obstacle to the wide application and use of lipoprotein profiling in this species. To this end, a convenient, economical, and robust method of lipoprotein profiling would be highly desirable. The aim of the present study was to assess the feasibility of a novel density gradient ultracentrifugation method in dogs, and to characterize and compare the lipoprotein profiles of healthy dogs of various breeds, healthy Miniature Schnauzers, and Miniature Schnauzers with primary hypertriglyceridemia using the same method.

Materials and methods

Dogs of various breeds (Group 1)

A total of 35 healthy dogs of various breeds with serum triglyceride and cholesterol concentrations within their respective reference intervals were selected for enrollment into the study. Inclusion criteria included absence of any clinical signs at the time of blood collection and no history of diseases or current use of drugs known to affect lipid metabolism. Owners had been instructed not to feed their dogs for at least 12 hours before the scheduled blood collection. Serum triglyceride and cholesterol concentrations were available for all dogs.

Miniature Schnauzers (Group 2)

Thirty-one Miniature Schnauzers with serum triglyceride and cholesterol concentrations within their respective reference intervals (Group 2A) and 31 Miniature Schnauzers with hypertriglyceridemia (Group 2B) were also included in the study. Samples from these dogs were selected from a pool of >300 samples from Miniature Schnauzers that were collected as part of several ongoing projects related to hypertriglyceridemia in this breed. Dogs that were included in Group 2 had to have absence of any clinical signs at the time of blood collection and no history of diseases or current use of drugs known to affect lipid metabolism. Miniature Schnauzers in Group 2A had to be 7 years of age or older in order to minimize the possibility of them developing hyperlipidemia in the near future. In addition, for the Miniature Schnauzers with hypertriglyceridemia (Group 2B), serum Spec cPL, glucose, total T4, and free T4 (in cases in which serum total T4 was below the lower limit of the reference interval) concentrations were measured. Based on the historical information for each dog and the results of the tests performed, all hypertriglyceridemic dogs enrolled in this study were diagnosed as having primary idiopathic hypertriglyceridemia of Miniature Schnauzers. Serum cholesterol concentrations were measured in all dogs of Group 2. Owners of dogs in Group 2 had been instructed not to feed their dogs for at least 12 hours before the scheduled blood collection.

Blood collection and handling

Owners living in relative proximity to the Gastrointestinal Laboratory at Texas A&M University were instructed to schedule an appointment for blood collection at that location. Owners that could not come to Texas A&M for blood collection were each sent a styrofoam box containing ice packs and the material necessary for blood collection, and were asked to schedule an appointment with their veterinarian for blood collection. Ten milliliters of blood were collected from each dog into a red-top tube (with no additive). Immediately after clot formation, the samples were centrifuged and the serum was separated from the clot. Samples not collected at Texas A&M University were sent to the Gastrointestinal Laboratory packed on ice by overnight courier. Serum samples were stored at -80^{o}C until further use.

Questionnaires and consent forms

Owners of all dogs were asked to complete a questionnaire for each dog. Questions covered date of birth, sex, body weight, current diet(s), current medications, and current and past health history of the dogs. Questionnaires from all dogs were reviewed to determine whether the dogs fit the inclusion criteria for each group. All owners signed an informed owner consent form. The study protocol was reviewed and approved by the Clinical Research Review Committee at Texas A&M University.

Assays

Serum triglyceride (reference interval: 26 to 108 mg/dL), cholesterol (reference interval: 124 to 335 mg/dL), and glucose (reference interval: 60 to 120 mg/dL) concentrations were measured by automated enzymatic assays.[a] Serum Spec cPL concentrations (reference interval: ≤200 μg/L) were measured using an analytically validated immunoassay as described elsewhere.[178] Serum total T4 concentrations were measured by a solid-phase chemiluminescent competitive assay.[b] Serum free T4 concentration was measured using a commercial equilibrium dialysis radioimmunoassay.[c]

Lipoprotein profile analysis

Lipoprotein profiling was carried out using a bismuth sodium ethylenediaminetetraacetic acid (NaBiEDTA or NaBiY) density gradient ultracentrifugation method as previously described with some modifications.[281] The sodium salt of BiEDTA has been described as a novel solute forming self-generating density gradient during ultracentrifugation of serum samples for the separation of lipoproteins.[281] Briefly, for each sample, 1,284 μL of a 0.18M NaBiEDTA[d] gradient solution was added into a 1.5 mL tube. The fluorescent probe 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine (NBD C_6-ceramide)[e] was reconstituted with 1 g/mL DMSO, and 10 μL of the 1 mg/mL solution were added to each tube to label the lipoproteins.

Finally, 6 µL of serum were added to each tube. The mixture was vortexed at 1,400 rpm for 10 seconds and 1,150 µL of it were transferred into an ultracentrifuge tube.[f] The mixture was allowed to incubate for 30 minutes at 5°C to allow for the fluorescent probe to saturate the lipoproteins. The solution was then centrifuged at 120,000 rpm, at 5°C, for 6 hours in a Beckman Optima ultracentrifuge (TLX-110)[g] with a 30° fixed angle TLA 120.2 rotor[h]. A quality-control sample was included in each run to verify proper operating conditions were achieved. Immediately after ultracentrifugation, the top of each sample was carefully layered with 250 mL of hexane and imaged without delay.

For imaging, each tube was placed in a custom, in-house imaging instrument as previously described.[281] The samples were imaged using a custom-built fluorescence imaging system consisting of a digital camera[i] with a MH-100 metal halide continuous light source[j], located in a dark room (Figure 8).

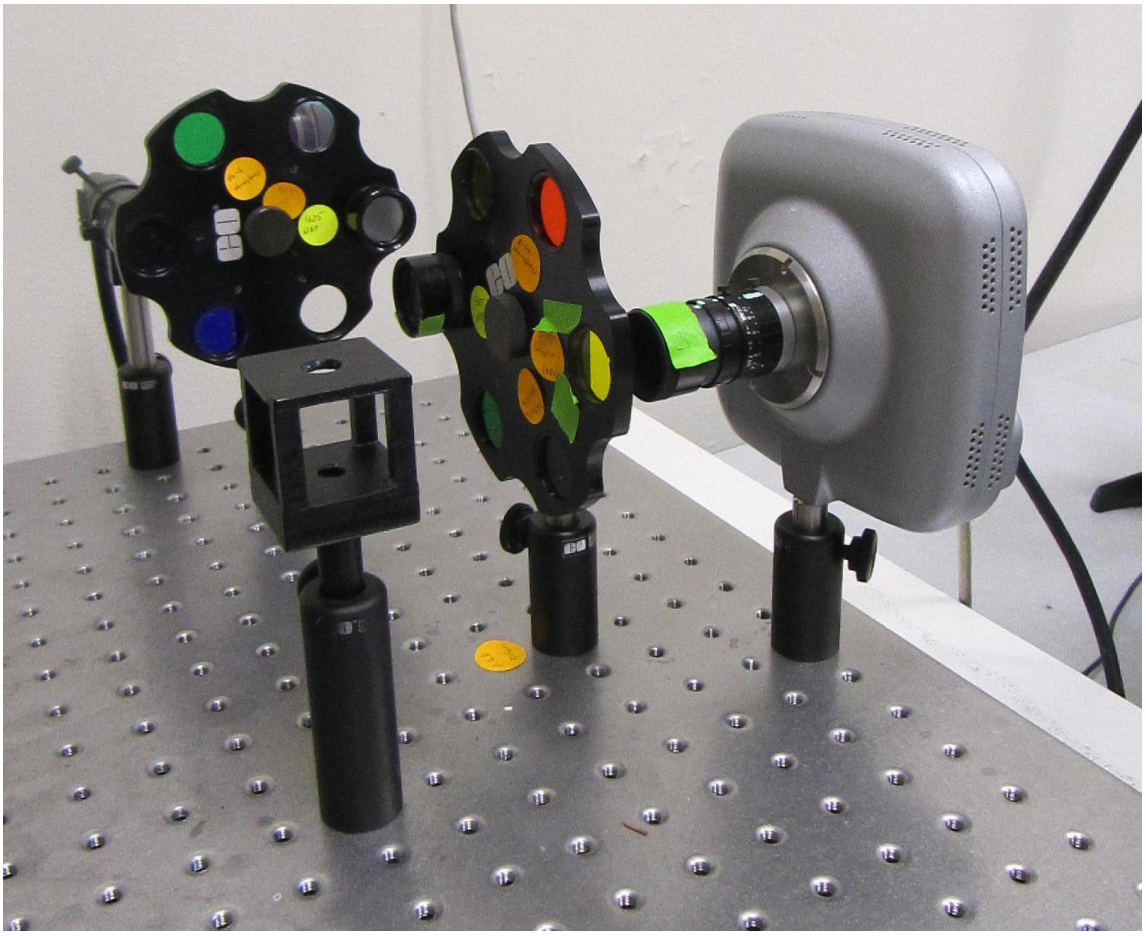


Figure 8. Custom-built fluorescence imaging system consisting of a digital camera with a MH-100 metal halide continuous light source used for imaging of the tubes after ultracentrifugation.

Two filters[k] matching the excitation (blue-violet filter centered at 407 nm) and emission (a yellow emission long pass filter with a cut-on wavelength of 515 nm) characteristics of NBD C_6-cermide were used. A gain of 1.0000, a target intensity of 30%, and an exposure time of 53.3 ms were selected. In order to be analyzed, the image of the each tube following ultracentrifugation was converted to a density profile using a commercially available software program.[l] A tube coordinate scale was established where 0.0 mm was the top of the tube and 34.0 mm was the bottom of the tube.[281] The average intensity was then plotted as a function of tube coordinate.

The ultracentrifugation method described above is able to identify 11 separate density lipoprotein fractions (TRL, 5 LDL fractions, and 5 HDL fractions). This separation is based solely on the density characteristics of the lipoprotein fractions and not on their functional properties. Therefore, this method was used to develop lipoprotein fingerprinting in canine serum based on lipoprotein densities alone. Density ranges were based on those previously published by several authors.[282] Specifically, the 11 fractions and their corresponding densities (*d*) were as follows: chylomicrons and VLDL (*d*<1.017 g/mL), LDL_1 (*d*=1.019 to 1.023 g/mL), LDL_2 (*d*=1.023 to 1.029 g/mL), LDL_3 (*d*=1.029 to 1.039 g/mL), LDL_4 (*d*=1.039 to 1.050 g/mL), LDL_5 (*d*=1.050 to 1.063 g/mL), HDL_{2b} (*d*=1.063 to 1.091 g/mL), HDL_{2a} (*d*=1.091 to 1.110 g/mL), HDL_{3a} (*d*=1.110 to 1.133 g/mL), HDL_{3b} (*d*=1.133 to 1.156 g/mL), and HDL_{3c} (*d*=1.156 to 1.179 g/mL).[282] HDL_1 is typically not found in healthy humans but it has been thought to occur in healthy dogs.[8,282] However, it has not been convincingly shown that the previously described canine HDL_1 molecule has the same function as human HDL_1, and its density

range HDL_1 has not been accurately determined (previously published densities vary between 1.025 and 1.1).[8] Studies in humans that have determined the density of HDL_1 suggest that the density of this molecule is around 1.08 g/mL, which would suggest that it corresponds with HDL_{2b} and possibly HDL_{2a}.[283]

Statistical analysis

Commercial statistical software packages were used for all statistical analyses.[m,n,o] Data were analyzed for normal distribution using the Shapiro-Wilk test. Normally distributed data were analyzed using t-tests, while not normally distributed data were analyzed using Mann-Whitney tests. Sliced-inverse regression was used to test the hypothesis that there is a relationship between the group and the lipoprotein profiles. Non-parametric correlations were used to test a linear relationship between parameters. Significance was set at $P<0.05$ for all analyses.

Results

Dogs in Group 1

The 35 dogs with serum triglyceride and cholesterol concentrations within the reference interval belonged to 19 different breeds (23 dogs), while 12 dogs were mixed-breed. Sixteen dogs were female (15 spayed) and 19 dogs were male (16 castrated). The body condition score (BCS) of the dogs in this group ranged from 3.5/9 to 6/9 (median: 5). The median age of the dogs was 4.4 years (range: 1.3 to 11.9 years).

Dogs in Group 2

Group 2A consisted of 31 Miniature Schnauzers with serum triglyceride and cholesterol concentrations within the reference interval. Fifteen dogs were female (9 spayed) and 16 dogs were male (5 castrated). The BCS of the dogs in this group ranged from 3/9 to 7/9 (median: 5). The average age of the dogs in this group was 9.3 years (range: 7 to 12 years).

Group 2B consisted of 28 Miniature Schnauzers with hypertriglyceridemia. Ten of the dogs also had hypercholesterolemia. Nineteen dogs were female (16 spayed) and 9 dogs were male (all castrated). The BCS of the dogs in this group ranged from 4/9 to 7/9 (median: 5). The average age of the dogs in this group was 9.5 years (range: 4.5 to 14 years).

There were no statistically significant differences in BCS among the 3 groups ($P=0.478$). Dogs in Group 1 were significantly younger than dogs of Group 2A ($P<0.0001$) and Group 2B ($P<0.0001$). There was no statistically significant difference in age between Groups 2A and 2B ($P=1.0$).

Serum triglyceride and cholesterol concentrations of dogs in Groups 1 and 2A (dogs with serum triglyceride and cholesterol concentrations within the reference interval)

Serum triglyceride concentrations of dogs in Group 1 (median: 52 mg/dL; range: 27 to 105 mg/dL), were not significantly different from those of dogs in Group 2A (median: 54 mg/dL; range: 19 to 108 mg/dL; p value > 0.05; Figure 9). Serum cholesterol concentrations of dogs in Group 1 (median: 221 mg/dL; range: 97 to 308

mg/dL) were significantly higher than those of dogs in Group 2A (median: 168 mg/dL; range: 74 to 316 mg/dL; p value= 0.003; Figure 10), although all values for both groups were within the reference interval.

Serum triglyceride and cholesterol concentrations of dogs in Group 2B (Miniature Schnauzers with hypertriglyceridemia)

Group 2B consisted of 31 Miniature Schnauzers with hypertriglyceridemia. Serum triglyceride concentrations ranged from 218 mg/dL to 3,975 mg/dL (median: 750 mg/dL). Eleven of the 31 dogs (35%) also had hypercholesterolemia. Serum cholesterol concentrations ranged 158 mg/dL to 575 mg/dL (median: 296 mg/dL). As expected, both serum triglyceride ($P<0.05$) and cholesterol ($P<0.05$) concentrations were significantly higher in dogs of Group 2B than dogs of Group 2A (Figures 9 and 10). Similarly, both serum triglyceride ($P<0.05$) and cholesterol ($P<0.05$) concentrations were significantly higher in dogs of Group 2B than dogs of Group 1 (Figures 9 and 10).

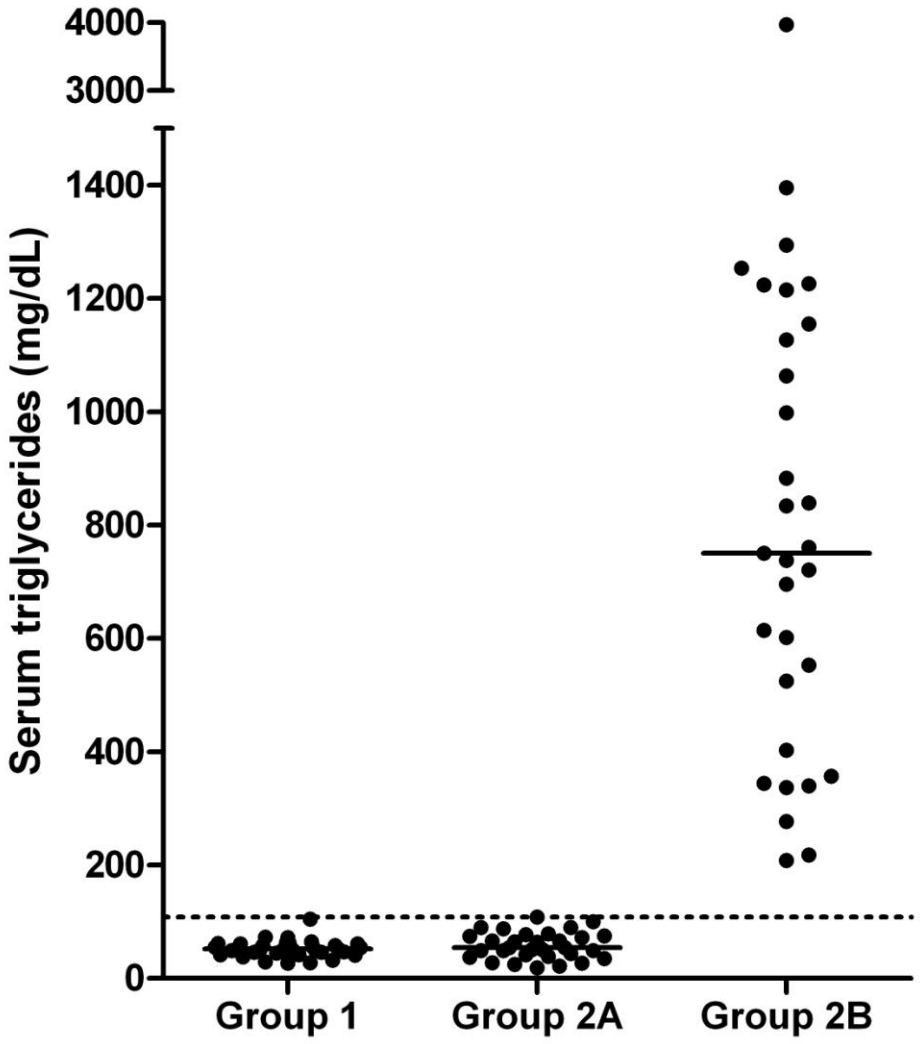


Figure 9: Serum triglyceride concentrations in dogs of other breeds (Group 1), Miniature Schnauzers with normal serum triglyceride concentrations (Group 2A), and Miniature Schnauzers with hypertriglyceridemia (Group 2B). Triglyceride concentrations of dogs in Groups 1 and 2A were all within the reference interval. Triglyceride concentrations of dogs in Group 2B were all above the reference interval. The dashed line represents the median for each group. Note: the Y-axis is split.

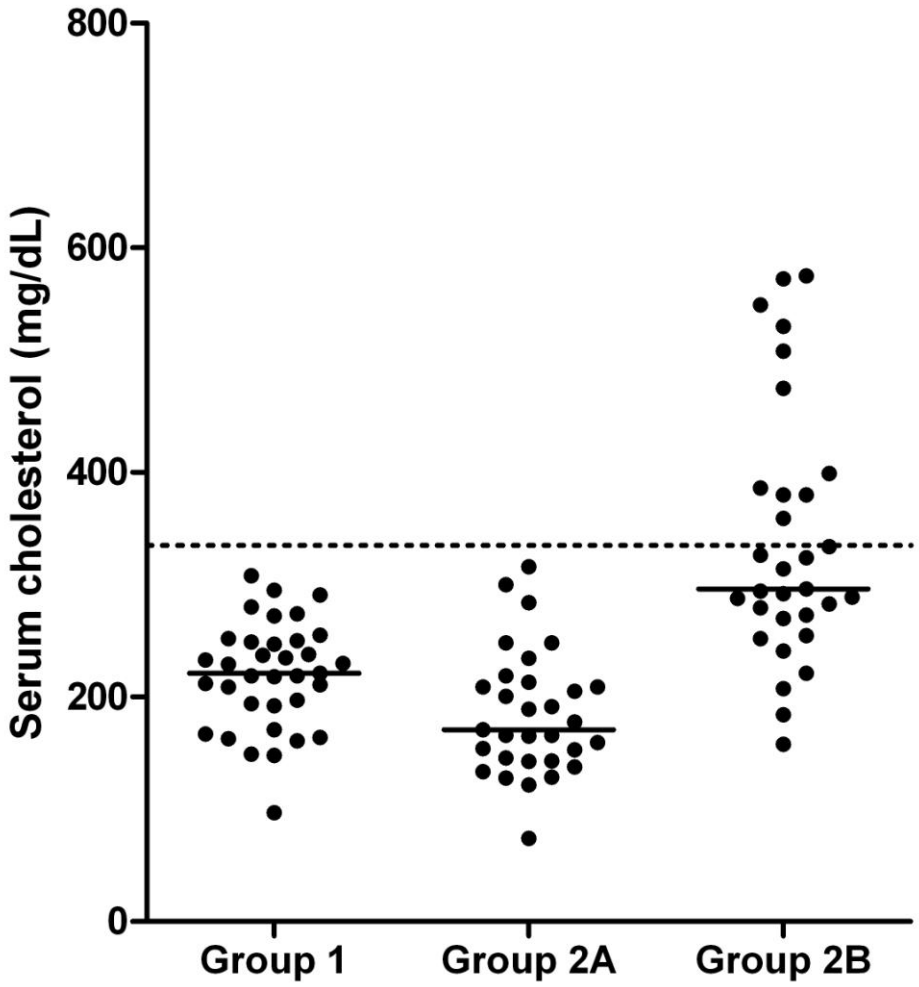


Figure 10: Serum cholesterol concentrations in dogs of other breeds (Group 1), Miniature Schnauzers with normal serum triglyceride concentrations (Group 2A), and Miniature Schnauzers with hypertriglyceridemia (Group 2B). Serum cholesterol concentrations of dogs in Groups 1 and 2A were all within the reference interval. Serum cholesterol concentrations were above the upper limit of the reference interval for 11 dogs in Group 2B. The dashed line represents the median for each group.

Lipoprotein profiles of dogs in Groups 1 and 2A (dogs with serum triglyceride and cholesterol concentrations within the reference interval)

Figure 11 on page 102 shows a representative lipoprotein profile from a dog in Group 1. It is clearly evident that the most abundant lipoprotein fraction is HDL, mainly HDL2 and HDL3, seen at a tube coordinate between 23 mm and 31 mm (Figure 11). LDL fractions are seen at a tube coordinate between 9 mm and 23 mm, but they were present in very small amounts, with the exception of LDL4 and LDL5. The fraction of triglyceride-rich lipoproteins (TRL) seen at a tube coordinate of 6 mm to 9 mm contains mainly chylomicrons, VLDL, and chylomicron and VLDL remnants. Triglyceride-rich lipoproteins were also found in very small amounts.

Figure 12 on page 103 shows a representative lipoprotein profile from a dog in Group 2A. The lipoprotein profiles of Miniature Schnauzers with serum triglyceride and cholesterol concentrations within the reference interval (Group 2A) were generally similar to the ones seen in dogs of Group 1 with regard to the abundance of major lipoprotein classes. However, most dogs in Group 2A showed some distinct differences in some lipoprotein fractions and subfractions.

Sliced inverse regression analysis was used to predict if differences in lipoprotein profiles were present between Groups 1 and 2A, and also to test whether lipoprotein profiles were effective in predicting to which group each dog belonged. Based on the classification table that documents the validity of predicted probabilities, the group to which each dog belonged (i.e., Miniature Schnauzer versus other breed) could be accurately predicted based on their lipoprotein profiles in 85% of the cases

(Eigenvalues=0.5455; P=0.00017; Figure 13). Specifically, 90% of Miniature Schnauzers could be classified as Miniature Schnauzers, and 80% of dogs of other breeds could be classified as other breeds based on their lipoprotein profiles alone. The most important lipoprotein fractions that served as predictors were the TRLs and the LDL fraction corresponding to LDL_4 and LDL_5 (Figures 11 and 12). Miniature Schnauzers had more prominent TRL peaks than dogs of other breeds, while dogs of other breeds had more prominent LDL_4 and LDL_5 peaks.

Lipoprotein profiles of dogs in Group 2B (Miniature Schnauzers with hypertriglyceridemia)

Figures 14A and 14B show representative lipoprotein profiles from 2 dogs in Group 2B. Similarly to dogs in Groups 1 and 2A, HDLs were abundant and LDLs were low in this group. However, dogs of this group had prominent peaks corresponding to the TRL area.

Sliced inverse regression analysis was used to predict if differences in lipoprotein profiles were present between Groups 2A and 2B, and also to test whether and which lipoprotein profiles were effective in predicting which group each dog belonged to. The SIR model showed that the group to which each dog belonged (i.e., Miniature Schnauzers with serum triglyceride concentrations versus hypertriglyceridemic Miniature Schnauzers) could be accurately predicted based on their lipoprotein profiles in 95% of cases (Eigenvalues=0.7638; P=0.000002; Figure 15).

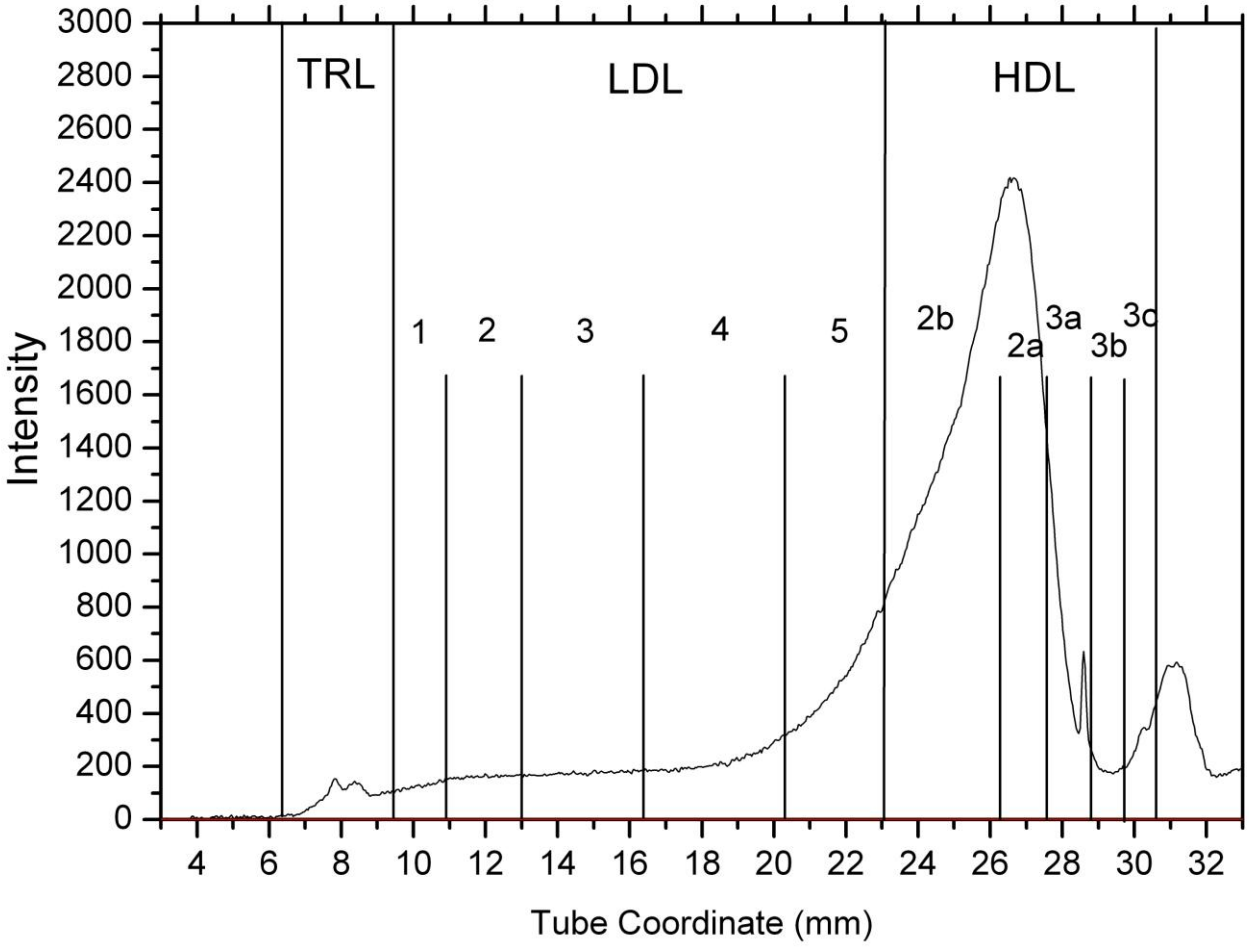


Figure 11: Representative lipoprotein density profile from a healthy dog of non-Miniature Schnauzer breed (Group 1). This dog had serum triglyceride and cholesterol concentrations within the respective reference intervals. The most abundant lipoprotein fraction is HDL, mainly HDL2 and HDL3, seen at a tube coordinate between 23 mm and 31 mm. LDL fractions are present in very small amounts and are seen at a tube coordinate between 9 mm and 23 mm. The fraction of TRLs seen at a tube coordinate of 6 mm to 9 mm contains mainly chylomicrons, VLDL, and chylomicron and VLDL remnants. Triglyceride-rich lipoproteins were also found in very small amounts.

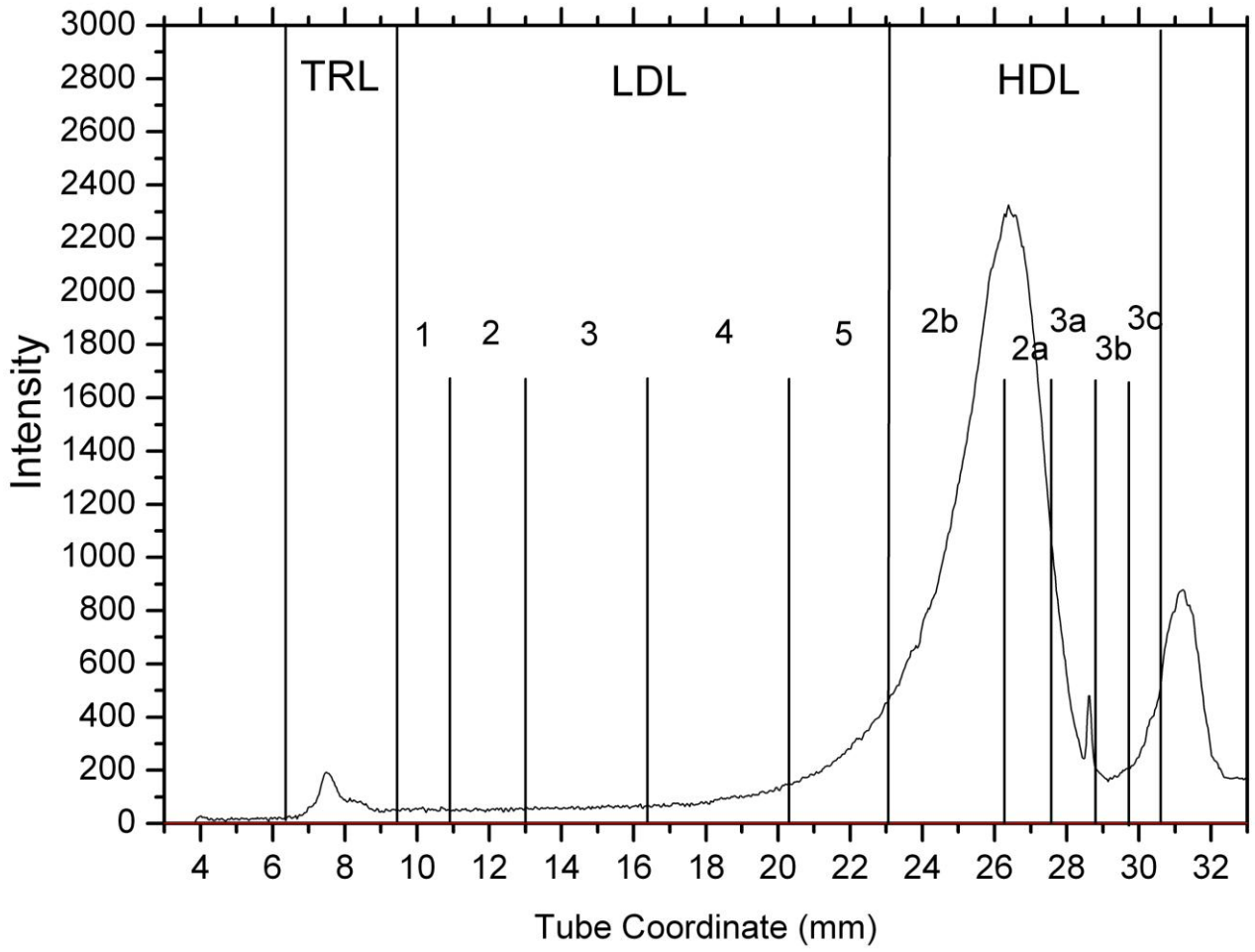


Figure 12: Representative lipoprotein density profile of a Miniature Schnauzer with a serum triglyceride concentration within the reference interval (Group 2A). This Miniature Schnauzer also had a serum cholesterol concentration within the reference intervals. The lipoprotein profiles of dogs in Group 2A were generally similar to the ones seen in dogs of Group 1 (Figure 11) with regard to the abundance of major lipoprotein classes. However, most dogs in Group 2A showed some distinct differences in some lipoprotein fractions and subfractions (e.g., more abundant TRLs and less abundant LDLs).

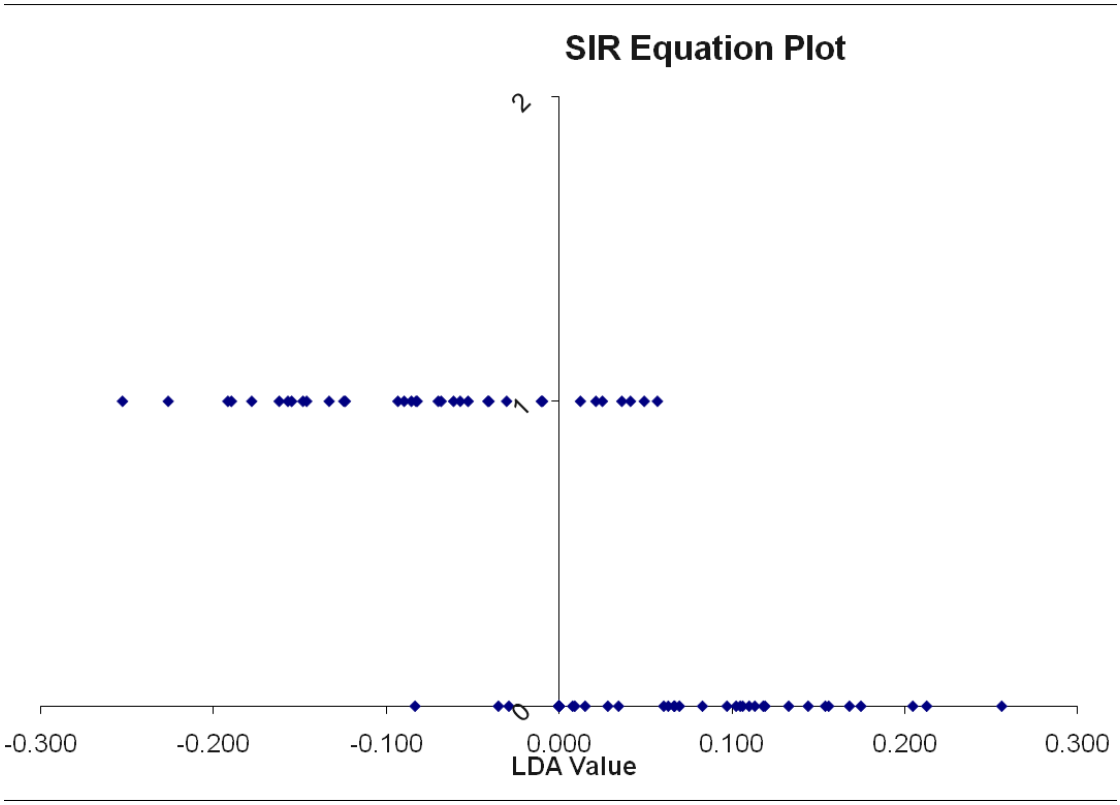
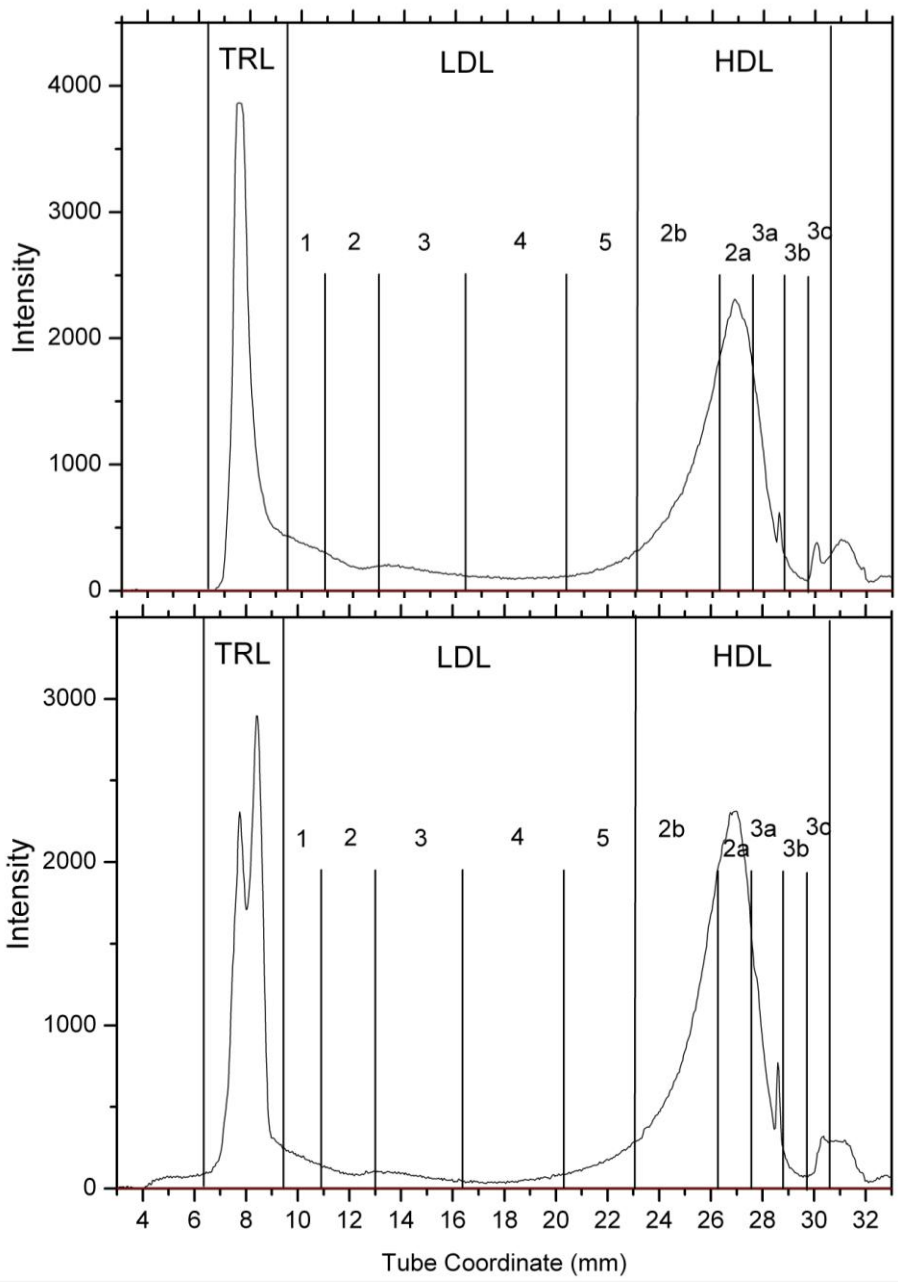


Figure 13. 1-dimensional **s**liced inverse regression plot showing classification of dogs into groups based on lipoprotein profile analysis (Group 1 vs Group 2A). The vertical line represents the line that separates the 2 groups based on their lipoprotein profile analysis. The LDA value provides a ranking value for each dog. The dogs represented by the dots that are at the bottom of the graph are the Miniature Schnauzers of Group 2A. Their lipoprotein profiles plot them all to the right of the vertical line, with the exception of 3 dogs (90% of dogs classified correctly). The dogs represented by the dots at the top of the graph are the dogs of other breeds (Group 1). Twenty-eight of the 35 dogs (80%) are classified as a separate group to the left of the vertical line. Seven of the 35 dogs are classified to the right of the vertical line together with the Miniature Schnauzers.



Figures 14A and 14B: Representative lipoprotein density profiles of Miniature Schnauzers with hypertriglyceridemia (Group 2B). Similarly to dogs in Groups 1 and 2A, HDLs were abundant and LDLs were low in this group. However, dogs of this group had prominent peaks corresponding to the TRL area. Note the difference in the TRL fractions between the 2 dogs.

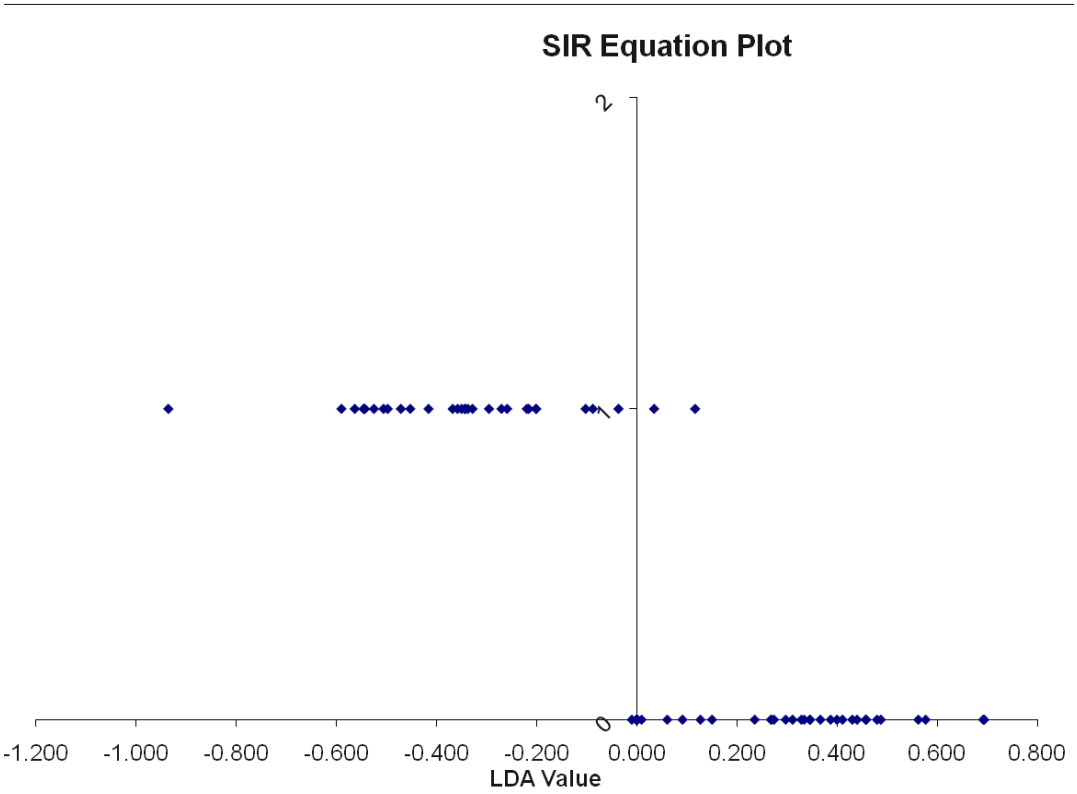


Figure 15. 1-dimensional **s**liced inverse regression plot showing classification of dogs into groups based on lipoprotein profile analysis (Group 2A vs Group 2B). The vertical line represents the line that separates the two groups based on their lipoprotein profile analysis. The LDA value provides a ranking value for each dog. The dogs represented by the dots that are at the bottom of the graph are the Miniature Schnauzers with hypertriglyceridemia. Their lipoprotein profiles plot them all to the right of the vertical line, with the exception of one dog with mildly increased serum triglyceride concentration (97% of dogs classified correctly). The dogs represented by the dots at the top of the graph are the Miniature Schnauzers with serum triglyceride concentrations within the reference interval. All but 2 dogs are classified as a separate group to the left of the vertical line (94% of dogs classified correctly).

Specifically, 97% of non-hypertriglyceridemic Miniature Schnauzers could be classified as correctly, and 94% of hypertriglyceridemic Miniature Schnauzers could be classified correctly based on their lipoprotein profiles alone. By far, the most important lipoprotein fraction that served as a predictor was the TRL fraction, which was more prominent in the dogs with hypertriglyceridemia. Fractions corresponding to LDL_2, LDL_3, LDL_4, were more prominent in Miniature Schnauzers with serum triglyceride concentrations within the reference interval (Figures 14A and 14B).

Correlations

Non-parametric correlation tests were used to test whether there was a linear relationship between lipoprotein fractions that showed significance in the SIR models and serum triglyceride and/or cholesterol concentrations. There was a significant positive correlation between TRL intensity and serum triglyceride concentration (Spearman r=0.81; 95% CI, 0.73 to 0.87; P<0.0001) and TRL intensity and serum cholesterol concentration (Spearman r=0.61; 95% CI, 0.46 to 0.72; P<0.0001). There were also significant, but weak, positive correlations between serum cholesterol concentration and LDL_2 (Spearman r=0.42; 95% CI, 0.24 to 0.58; P<0.0001) and LDL_4 (Spearman r=0.31; 95% CI, 0.11 to 0.48; P=0.0023).

Discussion

The results of the present study suggest that density gradient ultracentrifugation using NaBiY is a useful method for lipoprotein fingerprinting in dogs. This method was found to be easy to perform and proved to be a quick and accurate screening method for lipoprotein analysis in dogs. Important differences in lipoprotein profiles between different groups of dogs were detected with this method. An important and novel finding of the present study is that Miniature Schnauzers with serum triglyceride and cholesterol concentrations within the reference interval have significantly different lipoprotein profiles than those of dogs of various other breeds. In addition, the present study confirmed and expanded the findings of previous studies reporting that specific lipoprotein classes are associated with hypertriglyceridemia in Miniature Schnauzers.

To our knowledge, this is the first study showing that Miniature Schnauzers with normal serum triglyceride and cholesterol concentrations differ significantly in certain lipoprotein fractions (TRL and LDL4) from dogs of various other breeds. A previous study[17] identified a small number of healthy Miniature Schnauzers (4 out of 11 studied) that differed in some lipoprotein fractions (LDL and VLDL) compared with dogs of other breeds. However, in that particular study, Miniature Schnauzers were classified as non-lipemic based on the gross appearance of the plasma and not on a normal plasma triglyceride concentration. Indeed, all 4 of the non-lipemic Miniature Schnauzers classified as “different” had mild increases in plasma triglyceride concentrations, and therefore, mild lipid metabolism alterations were present.

The fraction of TRLs was a significantly higher in Miniature Schnauzers of Group 2A than in dogs of other breeds, despite the fact that there was no significant difference in serum triglyceride concentrations between the 2 groups and serum triglyceride and cholesterol concentrations were all within the reference interval. Interestingly, serum cholesterol concentrations were found to be significantly higher in dogs of other breeds compared to Miniature Schnauzers. This difference might be related to the fact that LDL4 was significantly higher in dogs of other breeds compared to Miniature Schnauzers. However, similarly to serum triglyceride concentrations, serum cholesterol concentrations were all within the reference interval. The marked differences in lipoprotein profiles between Miniature Schnauzers and dogs of other breeds despite the normal serum triglyceride and cholesterol concentrations clearly suggest that serum triglyceride and cholesterol concentrations are insensitive markers for detecting differences in lipoprotein metabolism in dogs. These differences in lipoprotein profiles could be identified in the vast majority of dogs, as about 90% of them could be classified to the correct group based on their lipoprotein profile alone.

The reason for the differences in lipoprotein profiles between Miniature Schnauzers and dogs of other breeds with normal serum triglyceride and cholesterol concentrations is unknown. The clinical importance of such finding is also unknown. One plausible scenario for such difference is that some Miniature Schnauzers might have an early disorder in lipoprotein metabolism that has not yet affected the overall serum triglyceride and cholesterol concentrations, but these concentrations might be affected in the future. This hypothesis is supported by the findings of a previous study that

suggested that hyperlipidemia is an age-related condition in Miniature Schnauzers.[84] However, this explanation does not seem very likely because all Miniature Schnauzers with serum triglyceride concentrations within the reference interval enrolled into this study were >7 years of age (median age: 9.3 years). Therefore, these dogs were already of a rather advanced age and it would seem unlikely that these dogs would develop hypertriglyceridemia later in life.[84] Another possibility is that the majority of Miniature Schnauzers differ in their basic lipoprotein metabolism from dogs of other breeds but only a portion of these dogs have severe enough lipid metabolism disorders leading to hyperlipidemia. Clearly, further studies are needed to determine the reason for this finding and whether it has any clinical significance.

Lipoprotein profiles of Miniature Schnauzers with hypertriglyceridemia were in agreement with the findings of previous studies.[17] It is interesting to note that the main difference between hyperlipidemic and normolipidemic Miniature Schnauzers involved significant increases of the TRL fraction in hyperlipidemic Miniature Schnauzers, and there was a strong correlation between TRLs and serum triglyceride concentrations. Similarly to previous studies, there was no difference in the HDL fractions.[17] Interestingly, the present study showed that a specific LDL fraction (LDL2) was significantly decreased in Miniature Schnauzers with hyperlipidemia. These findings support the findings of previous studies regarding increases in TRLs and decreases in LDLs, but also provide additional information about the specific LDL fraction affected.

Another interesting observation is that the group of Miniature Schnauzers with hyperlipidemia, although different in their lipoprotein profiles from Miniature

Schnauzers without hyperlipidemia, was rather diverse and there were some distinct differences among dogs in the same group. These differences were not always related to the different degrees of hyperlipidemia in these dogs. For example, many hyperlipidemic Miniature Schnauzers had 2 distinct peaks in their TRL fractions (Figure 14B), while others only had 1 (Figure 14A). These 2 peaks likely represent chylomicrons and VLDLs, which have slightly different densities. This is in agreement with findings of an older study,[17] in which it was shown that some hyperlipidemic Miniature Schnauzers had increases in VLDLs only, while others had increases in both VLDLs and chylomicrons. It is not known why some of the hyperlipidemic Miniature Schnauzers have only 1 TRL fraction affected while others have 2. In addition, as shown in previous studies,[17,84] a fraction if these dogs had increases in serum cholesterol concentrations. Thus, it is obvious that hyperlipidemia in Miniature Schnauzers is not a phenotypically uniform disease. This might be the result of the effect of environmental factors or maybe due to genetic heterogeneity.

Miniature Schnauzers of either group were significantly older that dogs of other breeds. This was the result of specific selection criteria. Hyperlipidemia in Miniature Schnauzers is known to be an age-related condition and, therefore, dogs in Group 2 had to be of older age in order to either have developed hyperlipidemia or to minimize the possibility that they would develop hyperlipidemia in the near future. For example, in one study, only 16% of Miniature Schnauzers between 1 and 3 years of age were hypertriglyceridemic, while 78% of Miniature Schnauzers >9 years of age were hypertriglyceridemic. No association between age and serum triglyceride or cholesterol

concentrations have been reported or suspected in dogs of breeds other than Miniature Schnauzers.

In conclusion, the results of the present study suggest that density gradient ultracentrifugation using NaBiY is a useful screening method for the study of lipoprotein profiles in dogs. Important differences in lipoprotein profiles can be detected with this method even among dogs that have serum triglyceride and cholesterol concentrations within the reference interval. Miniature Schnauzers with serum triglyceride and cholesterol concentrations within the reference interval had significantly different lipoprotein profiles (mainly with regard to TRL and LDL4) than dogs of various other breeds. In addition, it was further established that specific lipoprotein classes (TRL and specific LDL fractions, mainly LDL2) are associated with hypertriglyceridemia in Miniature Schnauzers. Changes in these lipoprotein classes are not always uniform among Miniature Schnauzers with hyperlipidemia. Further studies are needed to evaluate the usefulness of density gradient ultracentrifugation using NaBiY in evaluating hyperlipidemia of other causes in dogs, and to establish the clinical significance of differences in lipoprotein profiles in Miniature Schnauzers.

Footnotes

[a] Roche/Hitachi MODULAR ANALYTICS D 2400 module, Roche Diagnostics, Indianapolis, IN

[b] Immulite 2000 Canine Total T4, Siemens Healthcare Diagnostics, Deerfield, IL

[c] Free T4 (by ED), Antech Diagnostics, Irvine, CA Lipoprotein profiling was carried out using a [d] Bismuth Sodium Ethylenediaminetetraacetate, TCI AMERICA, Portland, OR

[e]NBD C_6-ceramide, Molecular Probes, Inc. Eugene, OR

[f] Tube, Thickwall, Polycarbonate (1 mL, 11 x 34 mm), Beckman Coulter Inc., Brea, CA

[g]Beckman Coulter Optima TLX-120 Ultracentrifuge, Beckman Coulter Inc., Brea, CA

[h]TLA-120.2 rotor**,** Beckman Coulter Inc, Brea, CA

[i]Digital Microfire Camera, Optronics, Coleta, CA

[j]MH-100, Dolan-Jenner Industries, Boxborough, MA

[k]SCHOTT North America, Inc., Elmsford, NY

[l]Origin 7.0, Microcal Software Inc., Northampton, MA

[m]SPSS 16.0, SPSS Inc., Chicago, IL

[n] Prism5, GraphPad, San Diego, CA

[o]R, http://www.r-project.org/

CHAPTER V

EFFECT OF A LOW-FAT DIET ON SERUM LIPID AND PANCREATIC LIPASE CONCENTRATIONS AND LIPOPROTEIN PROFILES IN MINIATURE SCHNAUZERS WITH HYPERLIPIDEMIA

Introduction

Canine primary hyperlipidemia is a metabolic disorder of likely diverse etiology that is more commonly seen in certain breeds.[252] Primary hyperlipidemia of Miniature Schnauzers was the first primary hyperlipidemia disorder described in dogs, and to date, it has been reported in dogs of this breed in the United States and Japan.[15,84,284] In 1 study from the United States, hypertriglyceridemia was present in 32.8% of 192 Miniature Schnauzers investigated.[84] Hyperlipidemia of Miniature Schnauzers is typically characterized by hypertriglyceridemia of various degrees due to increases in serum very low density lipoproteins (VLDL) and chylomicrons, with or without the presence of hypercholesterolemia.[17,84] In this breed, hypetriglyceridemia might be etiologically linked to disorders such as hepatobiliary disease, pancreatitis, insulin resistance, and ocular disease.[123,252,285] The association between hypertriglyceridemia and pancreatitis is of particular interest in Miniature Schnauzers as studies have suggested that Miniature Schnauzers are also predisposed to pancreatitis.[63,163] In fact, it is widely believed that hypertriglyceridemia predisposes Miniature Schnauzers to develop chronic pancreatitis based in part on the observation that dogs of this breed often appear to have multiple episodes of pancreatitis throughout their lives.[165,216]

The biochemical, metabolic, and genetic bases of hypertriglyceridemia in Miniature Schnauzers have not been elucidated. Therefore, definitive recommendations for effective prevention and specific management of this condition are currently unavailable. The most commonly recommended initial approach in the management of primary hypertriglyceridemia in Miniature Schnauzers is the use of a low-fat diet.[131,252] Feeding a low-fat diet is also commonly the recommended approach in the management of dogs suspected of having chronic pancreatitis.[286] There are several commercially available diets marketed as "low-fat", although their actual lipid content might vary substantially between one another. To our knowledge, none of these diets have been evaluated with regard to their effectiveness in the management of primary hypertriglyceridemia in Miniature Schnauzers. Also, the effect of low-fat diets on serum concentrations of markers for pancreatitis has not been evaluated. Therefore, the aim of the present study was to evaluate the effect of a commercially available low-fat diet on serum lipid and pancreas-specific lipase (Spec cPL®) concentrations and lipoprotein profiles in Miniature Schnauzers with primary hypertriglyceridemia.

Materials and methods

Dogs

Miniature Schnauzers with hypertriglyceridemia of various degrees were included in the study (Group 1). These dogs were selected from a pool of >300 Miniature Schnauzers that were enrolled as part of several ongoing projects related to hypertriglyceridemia in this breed. The requirements for inclusion in the present study

were a diagnosis of hypertriglyceridemia and willingness of each dog's owners to enroll their dog in the study. All dogs included in the study had to have absence of any clinical signs at the time of blood collection and no history of diseases or current use of drugs known to affect lipid metabolism. They also had to have a normal body condition score (BCS; between 4 and 6 on a scale of 1 to 9). In addition, for each dog, serum Spec cPL, glucose, total T4, and free T4 (by equilibrium dialysis) concentrations were measured. Based on the historical information for each dog and the results of the tests performed, all hypertriglyceridemic dogs enrolled in this study were diagnosed as having primary idiopathic hypertriglyceridemia.

A group of healthy Miniature Schnauzers with serum triglyceride and cholesterol concentrations within the respective reference intervals (Group 2) was used as a control group for the lipoprotein profile portion of the study. Dogs in Group 2 were used in order to obtain lipoprotein profiles from a group of healthy Miniature Schnauzers that could be used for illustration of whether lipoprotein profiles of hypertriglyceridemic Miniature Schnauzers tended to approach those of the healthy ones after the diet change. Dogs of Group 2 had to have absence of any clinical signs at the time of blood collection and no history of diseases or current use of drugs known to affect lipid metabolism. They also had to have a normal BCS (between 4 and 6 on a scale of 1 to 9) and be on diets that were not labeled as low-fat diets at the time of blood collection.

Study design

Each one of the dogs in Group 1 had a total of 4 blood samples collected. The first sample (sample 1) was used to diagnose primary hypertriglyceridemia. Then, in order to confirm the results of the initial sample and to investigate the variability of the findings, the owners were instructed to have a second sample (sample 2) collected 1 to 2 months after the collection of the initial sample, and without making any changes to the diet of their dogs. If hypertriglyceridemia was confirmed in the second sample, the dogs were put on the study diet (see below). Owners were instructed to gradually switch their dogs from the original to the study diet over 7 days. Since all dogs had a normal BCS, the amount of diet was determined based on the normal weight guide on the package. In addition, the owners were instructed to weigh their dogs at weekly intervals and adjust the amount of food accordingly in order to maintain a relatively stable body-weight for the duration of the study. Additional instructions given to the owners involved ensuring that the study diet was fed exclusively to the dogs enrolled in the study, with the exception of small quantities of steamed vegetables (carrots and broccoli) that could be used as treats. Approximately 7 to 9 weeks after the dogs had been exclusively on the study diet, a third blood sample (sample 3) was collected. Finally, a fourth sample (sample 4) was collected approximately 2 to 4 weeks after the third sample.

Serum triglyceride, cholesterol, and Spec cPL concentrations were measured in all samples and compared between time-points. Lipoprotein profiles were also determined and compared between time-points and between groups.

Dogs of Group 2 were enrolled on a one-time basis and no follow-up samples were collected from these dogs. As mentioned above, these dogs were only used for the lipoprotein profile portion of the study. Serum triglyceride, cholesterol, and Spec cPL concentrations were also measured in all samples of dogs in this group but were not used for any comparison with Group 1 (see above).

Study diet

The diet selected for the present study was a commercially available therapeutic diet labeled as "low-fat" in dry form.[a] The fat content of the study diet was 18.6 grams of fat per 1,000 Kcal.

Blood collection and handling

Owners that agreed to participate in the study were each sent a styrofoam box containing ice-packs and the material necessary for blood collection, and were asked to schedule an appointment with their veterinarian for the blood collection. Ten milliliters of blood were collected from each dog into a red-top tube (with no additive). Immediately after clot formation, the samples were centrifuged and the serum was separated from the clot. The samples were sent to the Gastrointestinal Laboratory packed on ice by overnight courier. Serum samples were stored at -80^{o}C until use.

Questionnaires and consent forms

Owners of all dogs were asked to complete a questionnaire for each dog. Questions covered date of birth, sex, body-weight, BCS, current diet(s), current medications, and current and past health history of the dogs. Questionnaires from all dogs were reviewed to determine whether the dogs fit the inclusion criteria for each group. All owners signed an informed owner consent form. The study protocol was reviewed and approved by the Clinical Research Review Committee at Texas A&M University.

Assays

Serum triglyceride (reference interval: 26 to 108 mg/dL), cholesterol (reference interval: 124 to 335 mg/dL), and glucose (reference interval: 60 to 120 mg/dL) concentrations were measured by automated enzymatic assays.[b] Serum Spec cPL concentration (reference interval: ≤200 μg/L) was measured using an analytically validated immunoassay as described elsewhere.[178] Serum total T4 concentration was measured by a solid-phase chemiluminescent competitive assay.[c] Serum free T4 concentration was measured using a commercial equilibrium dialysis radioimmunoassay.[d]

Lipoprotein profile analysis

Lipoprotein profiling was carried out using a bismuth sodium ethylenediaminetetraacetic acid (NaBiEDTA or NaBiY) density gradient

ultracentrifugation method as previously described, with some modifications.[281] The sodium salt of BiEDTA has been described as a novel solute forming a self-generating density gradient during ultracentrifugation of serum samples for the separation of lipoproteins.[281] Briefly, for each sample, 1,284 µL of a 0.18M NaBiEDTA[e] gradient solution was added into a 1.5 mL tube. The fluorescent probe 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine (NBD C_6-ceramide)[f] was reconstituted with 1 g/mL DMSO and 10 µL of the 1 mg/mL solution were added to each tube to label the lipoproteins. Finally, 6 µL of serum were added to each tube. The mixture was vortexed at 1,400 rpm for 10 seconds and 1,150 µL of the mixture were transferred into an ultracentrifuge tube.[g] The mixture was allowed to incubate for 30 minutes at 5^{o}C to allow for the fluorescent probe to saturate the lipoproteins. The solution was then centrifuged at 120,000 rpm, at 5^{o}C, for 6 hours in a Beckman Optima ultracentrifuge (TLX-110)[h] with a 30^{o} fixed angle TLA 120.2 rotor[i]. A quality control sample was included in each run to verify that proper operating conditions were achieved. Immediately after ultracentrifugation, the top of each sample was carefully layered with 250 µL of hexane and imaged without delay.

For imaging, each tube was placed in a custom, in-house imaging instrument as previously described.[281] The samples were imaged using a custom-built fluorescence imaging system consisting of a digital camera[j] with a MH-100 metal halide continuous light source[k], located in a dark room. Two filters[l] matching the excitation (blue-violet filter centered at 407 nm) and emission (a yellow emission long pass filter with a cut-on wavelength of 515 nm) characteristics of NBD C_6-cermide were used. A gain of 1.0000,

a target intensity of 30%, and an exposure time of 53.3 ms were selected. In order to be analyzed, the image of the each tube following ultracentrifugation was converted to a density profile using a commercially available software program.[m] A tube coordinate scale was established where 0.0 mm was the top of the tube and 34.0 mm was the bottom of the tube.[281] The average intensity was then plotted as a function of the tube coordinate.

The ultracentrifugation method described above has been described as being able to identify 11 distinct density lipoprotein fractions in humans (TRL, 5 LDL fractions, and 5 HDL fractions). This separation is based solely on the density characteristics of the lipoprotein fractions and not on their functional properties. Therefore, this method was used to develop lipoprotein fingerprinting in canine serum based on lipoprotein densities alone. Density ranges were based on those previously published for humans by several authors.[282] Specifically, the 11 fractions and their corresponding densities (*d*) were as follows: chylomicrons and VLDL (*d*<1.017 g/mL), LDL_1 (*d*=1.019 to 1.023 g/mL), LDL_2 (*d*=1.023 to 1.029 g/mL), LDL_3 (*d*=1.029 to 1.039 g/mL), LDL_4 (*d*=1.039 to 1.050 g/mL), LDL_5 (*d*=1.050 to 1.063 g/mL), HDL_{2b} (*d*=1.063 to 1.091 g/mL), HDL_{2a} (*d*=1.091 to 1.110 g/mL), HDL_{3a} (*d*=1.110 to 1.133 g/mL), HDL_{3b} (*d*=1.133 to 1.156 g/mL), and HDL_{3c} (*d*=1.156 to 1.179 g/mL).[282] HDL_1 is typically not found in healthy humans but it has been thought to occur in healthy dogs.[8,282] However, it has not been convincingly shown that the previously described canine HDL_1 molecule has the same function as human HDL_1, and its density range has not been accurately determined (previously published densities vary between 1.025 and 1.1).[8] Studies in humans that

have determined the density of HDL_1 suggest that the density of this molecule is around 1.08 g/mL, which would suggest that it corresponds to HDL_{2b} or possibly HDL_{2a}.[283]

Statistical analysis

Commercial statistical software packages were used for all statistical analyses.[n,o,p] Data were analyzed for normal distribution using the Shapiro-Wilk test. Normally distributed data were analyzed using t-tests, while not normally distributed data were analyzed using Mann-Whitney tests. For multiple comparisons, repeated measures one-way ANOVA was used for normally distributed data followed by Bonferroni analysis, while the Friedman's test was used for not normally distributed data followed by Dunn's multiple comparison tests. Proportions were compared between groups using Fisher's exact tests. Sliced inverse regression or logistic regression was used to test whether there was a relationship between group and lipoprotein profiles. Significance was set at $P<0.05$ for all analyses.

Results

Dogs

A total of 39 dogs were initially considered for enrollment in Group 1. The owners of 11 of these 39 dogs submitted the initial 2 samples needed for the study (before the diet change), but decided to not proceed with the study at that time. Another 12 dogs were excluded from the study because they were diagnosed with conditions that

might affect lipid metabolism (hypothyroidism, pancreatitis, pregnancy). Thus, 16 dogs completed the study.

The 16 dogs that completed the study had a median BCS of 5 (range: 4 to 6) and a median age of 8.5 years (range: 6.7 to 11.9 years). Eleven dogs were female (all spayed) and 5 dogs were male (all castrated).

Group 2 consisted of 28 Miniature Schnauzers that had a median BCS of 5 (range: 4 to 6) and a median age of 9.1 years (range: 7.1 to 12.2 years). Fourteen dogs were female (8 spayed) and 14 dogs were male (5 castrated).

Serum triglyceride, cholesterol, and Spec cPL concentrations in Group 1

Analysis using the Friedman's test (repeated measures non-parametric 1-way ANOVA) showed that serum triglyceride concentrations before the diet change (median of sample 1: 480 mg/dL; median for sample 2: 493 mg/dL) were significantly higher than after the diet change (median of sample 3: 177 mg/dL; median for sample 4: 168 mg/dL; P=0.0001; Figure 16). Dunn's post-test indicated that there were no significant differences between samples 1 and 2 (i.e., while dogs were on their original diets) or between sample 3 and 4 (i.e., while dogs were on the study diet). There were significant differences between samples before and after the diet change ($P<0.05$ for all). Significantly more dogs had hypertriglyceridemia in Group 1 before (16/16) than after the diet change (11/16; odds ratio: 15.8; 95% CI, 0.8 to 314.8; P=0.04). Also, significantly more dogs had serum triglyceride concentrations >500 mg/dL in Group 1

before (8/16) than after the diet change (0/16; odds ratio: 33.0; 95% CI, 1.7 to 643.6; P=0.002).

Serum cholesterol concentrations were available for all 4 time-points for 14 of the 16 dogs in Group 1. Repeated measures 1-way ANOVA analysis showed that serum cholesterol concentrations before the diet change (mean of sample 1: 381 mg/dL; mean for sample 2: 380 mg/dL) were significantly higher than after the diet change (mean of sample 3: 257 mg/dL; mean for sample 4: 178 mg/dL; P<0.0001; Figure 17). Post-hoc ANOVA analysis using Bonferroni's correction for multiple comparisons indicated that there were significant differences between all individual comparisons before and after the diet change (Figure 17). However, there was no difference in the proportion of dogs that had hypercholesterolemia before (8/16) and after the diet change (3/16; P=0.14).

Serum Spec cPL concentrations were available for all 4 time-points for 14 of the 16 dogs in Group 1. Analysis using the Friedman's test showed that serum Spec cPL concentrations before the diet change (mean of sample 1: 173 µg/L; mean for sample 2: 109 µg/L) were not significantly different than after the diet change (mean of sample 3: 144 µg/L; mean for sample 4: 137 µg/L; P=0.12; Figure 18). There was no statistically significant difference in the proportion of dogs that had Spec cPL concentration above the cut-off for a diagnosis of pancreatitis (>400 µg/L) before (2/16) and after the diet change (1/16; P=1.0).

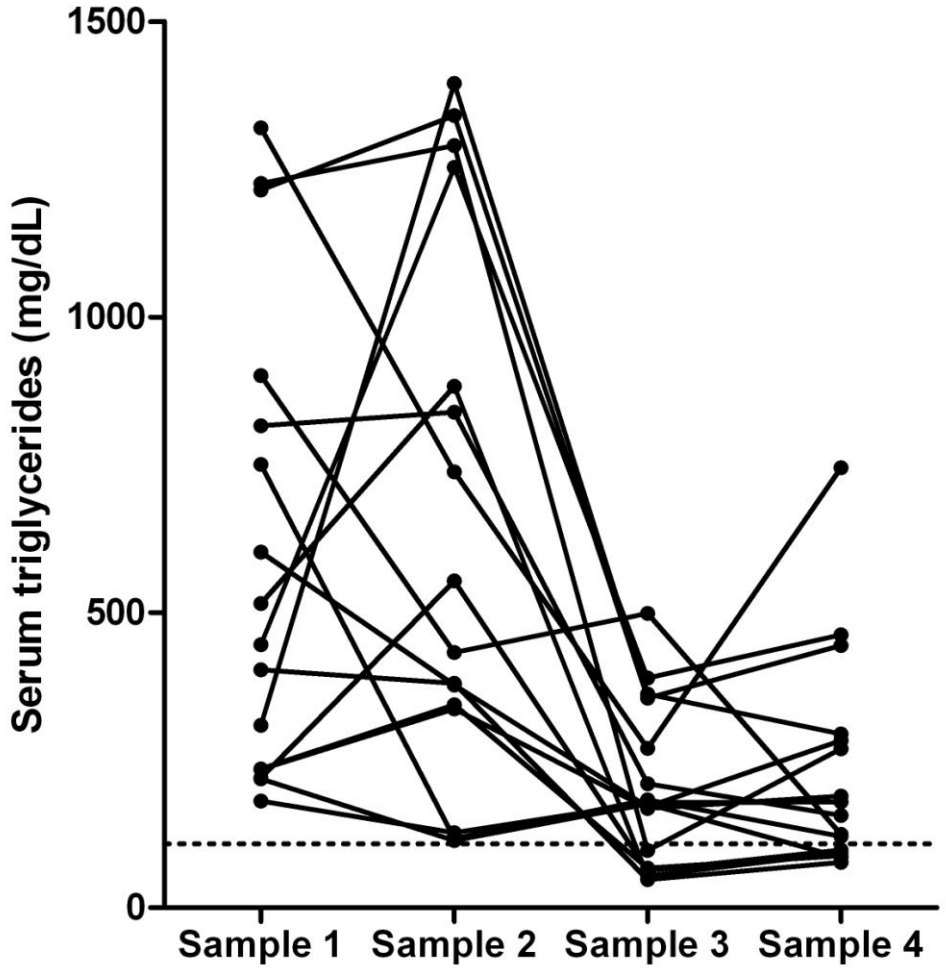


Figure 16. Serum triglyceride concentrations in dogs with hypertriglyceridemia before and after diet change. Samples 1 and 2 were collected while dogs were on their original diets. Samples 3 and 4 were collected approximately 8 and 12 weeks after the dogs were placed on the study diet, respectively. There was a significant reduction in serum triglyceride concentration after the diet change ($P<0.0001$). The dashed line represents the upper limit of the reference interval.

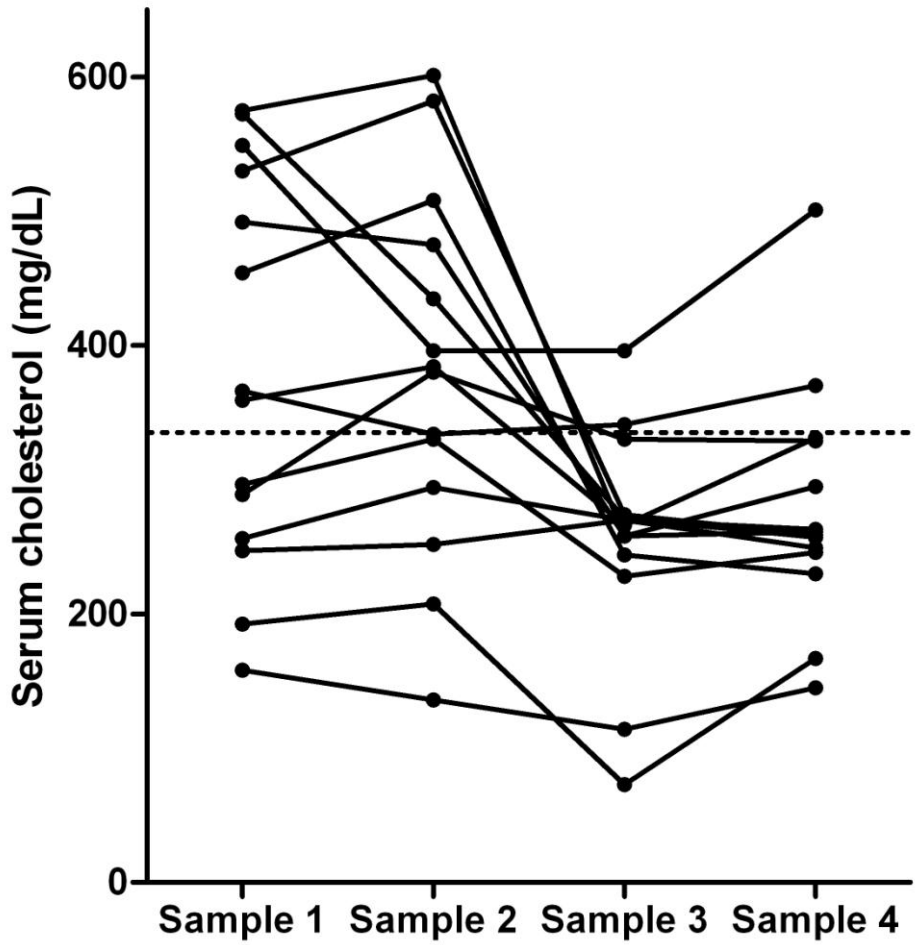


Figure 17. Serum cholesterol concentrations in dogs with hypertriglyceridemia before and after diet change. Samples 1 and 2 were collected while dogs were on their original diets. Samples 3 and 4 were collected approximately 8 and 12 weeks after the dogs were placed on the study diet, respectively. There was a significant reduction in serum cholesterol concentration after the diet change ($P<0.0001$). The dashed line represents the upper limit of the reference interval.

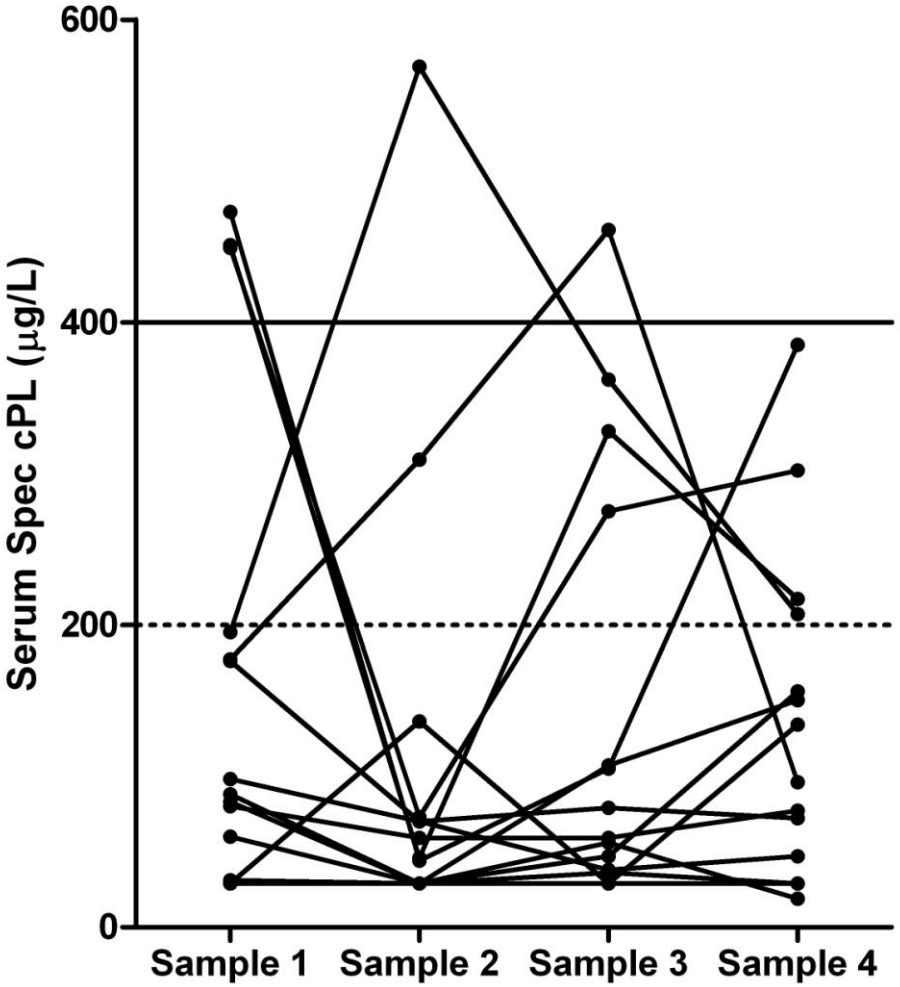


Figure 18. Serum Spec cPL concentrations in dogs with hypertriglyceridemia before and after diet change. Samples 1 and 2 were collected while dogs were on their original diets. Samples 3 and 4 were collected approximately 8 and 12 weeks after the dogs were placed on the study diet, respectively. There was no significant difference in serum Spec cPL concentrations after the diet change (P=0.14). The dashed line represents the upper limit of the reference interval. The solid line represents the cut-off for pancreatitis.

Lipoprotein profile analysis

For analysis of the lipoprotein profiles in response to diet change, the lipoprotein profiles after the diet change were compared with those of the same dogs before the diet change as well as those of the group of healthy dogs. Before the diet change, there was a 98% separation between Group 1 and Group 2 using SIR analysis (Eigenvalues=0.7748; P=0.0003; Figure 19). One Miniature Schnauzer from Group 1 had only a very mildly increased serum triglyceride concentration, and this dog was misclassified as a control dog). Therefore, 15/16 (94%) of hyperlipidemic Miniature Schnauzers were classified as hyperlipidemic based on their lipoprotein profiles alone. After the diet change, significantly fewer Miniature Schnauzers (7/16; 44%; odds ratio: 19.3; 95% CI, 2.0 to 184.0; P=0.006) were still classified as hyperlipidemic by lipoprotein profile analysis, while the majority of the dogs of this group (56%) were classified as normal (Figure 20). This means that with the diet change >50% of hyperlipidemic dogs no longer had evidence of lipoprotein abnormalities based on their lipoprotein profiles. Logistic regression analysis of the baseline lipoprotein profiles (before the diet change) of dogs that eventually responded and dogs that did not respond to the diet change showed that dogs that responded to the diet change could be separated with 88% accuracy from the ones that did not respond based on lipoprotein profile analysis even before the diet change. The logistic regression model fit the data well (chi-square=8.99; P=0.003; -2 log likelihood=4.09; Nagelkerke R^2=0.9; Hosmer-Lemeshow test p =1.0).

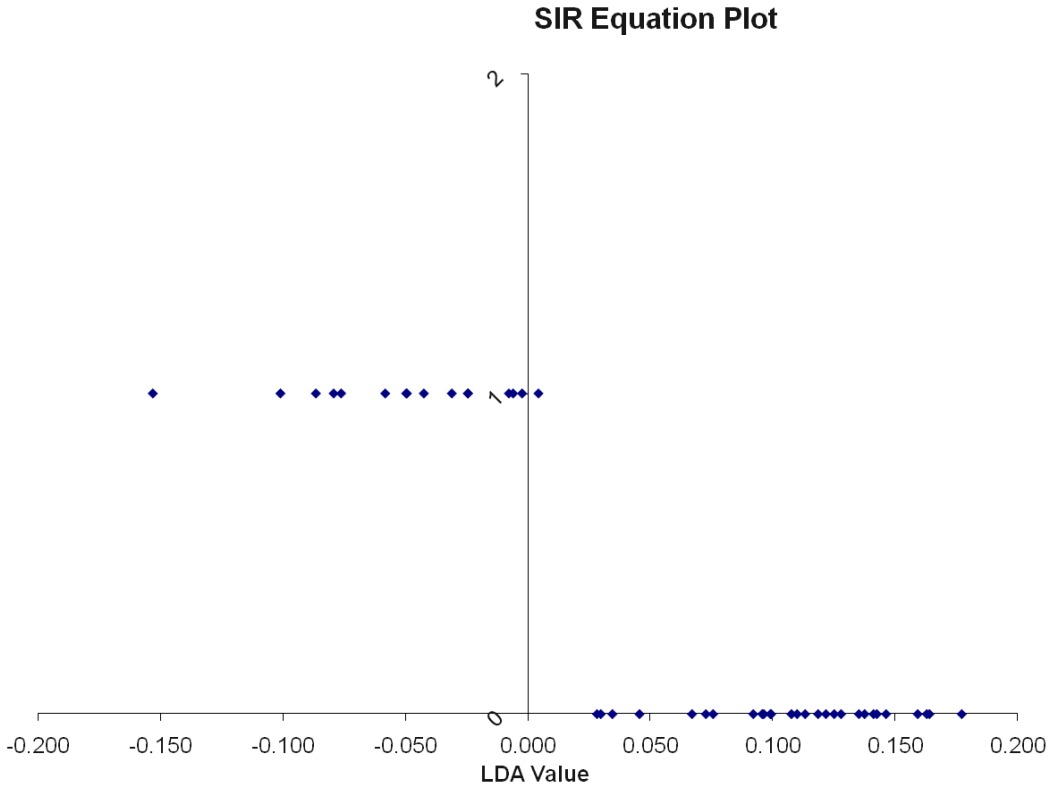


Figure 19. 1-dimensional **s**liced inverse regression plot showing classification of dogs into groups based on lipoprotein profile analysis before the diet change. The vertical line represents the line that separates the 2 Groups based on lipoprotein profile analysis. The LDA value provides a ranking value for each dog. The dogs represented by the dots that are at the bottom of the graph are the dogs of the control group. Their lipoprotein profiles plot them all to the right of the vertical line. The dogs represented by the dots at the top of the graph are the dogs of Group 1 (with hypertriglyceridemia) before the diet change. They are all classified as a separate group from the control dogs with the exception of 1 dog that is classified as borderline normal.

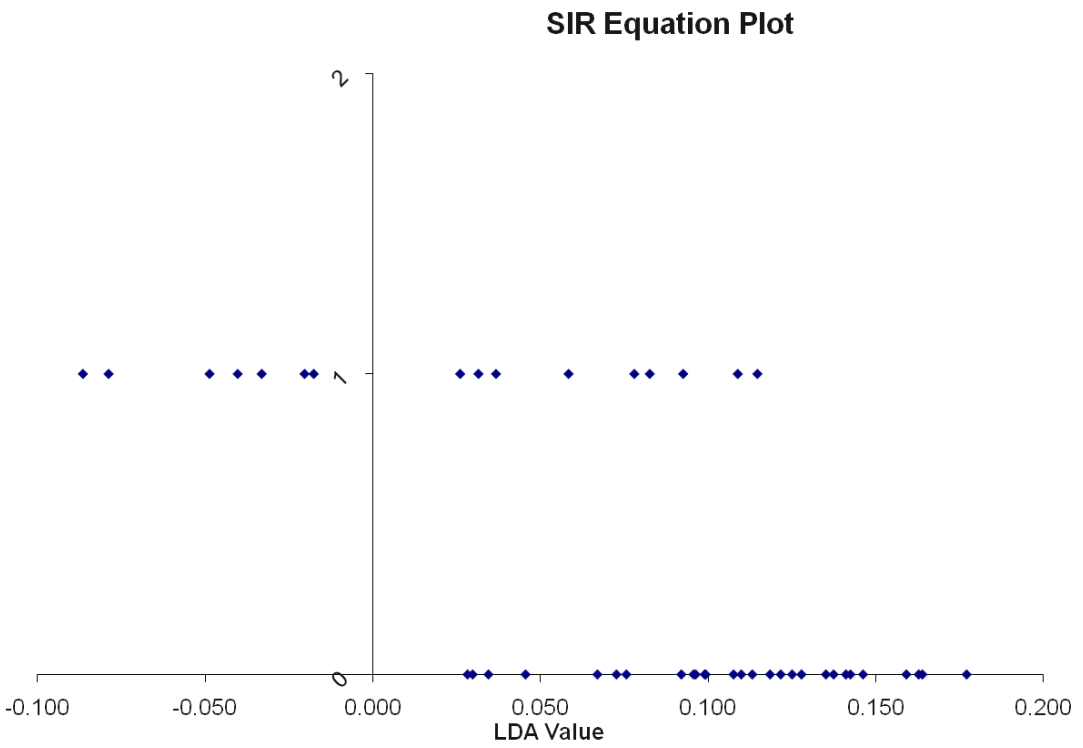


Figure 20. 1-dimensional **s**liced inverse regression plot showing classification of dogs into groups based on lipoprotein profile analysis after the diet change. The vertical line represents the line that separates the 2 groups based on their lipoprotein profile analysis. The LDA value provides a ranking value for each dog. The dogs represented by the dots that are at the bottom of the graph are the dogs of the control group. Their lipoprotein profiles plot them all to the right of the vertical line. The dogs represented by the dots at the top of the graph are the dogs of Group 1 (with hypertriglyceridemia) after the diet change. Nine of the 16 dogs are now classified to the right of the vertical line together with the control dogs, suggesting that they are considered normal based on their lipoprotein profile analysis. The remaining 7 dogs are classified separately from the control dogs even after the diet change. Note that there is a clear separation between dogs of Group 1 that responded to the diet change and those that did not.

Dogs that did not respond to the diet change tended to have lower LDL fractions (mainly LDL_1 and LDL_2) and higher HDL fractions (mainly HDL_{2a}, HDL_{3b}, and HDL_{3c}) than the ones that responded.

The most important changes in lipoprotein fractions in response to diet change as determined by SIR were decreases in TRL and LDL_1 and increases in LDL_4 and HDL_{3c}. Figures 21A and 21B show the lipoprotein density profiles from a representative dog in Group 1 before and after the diet change.

Discussion

To our knowledge, this is the first study evaluating the effect of a commercially available low-fat diet on serum lipid concentrations and lipoprotein profiles in Miniature Schnauzers with hyperlipidemia. The results of this study suggest that the study diet significantly reduced serum triglyceride and cholesterol concentrations in hyperlipidemic Miniature Schnauzers within 2 months. In addition, the lipoprotein profiles of the hyperlipidemic Miniature Schnauzers changed significantly 2 months after the initiation of the study diet, and the majority of dogs could be classified as normal based on their lipoprotein profile analysis at that point. Lipoprotein fractions mainly affected by the diet change were the TRLs and LDL_1 (which both decreased in response to feeding the low-fat diet) and LDL_4 and HDL_{3c} (which both increased in response to feeding the low-fat diet).

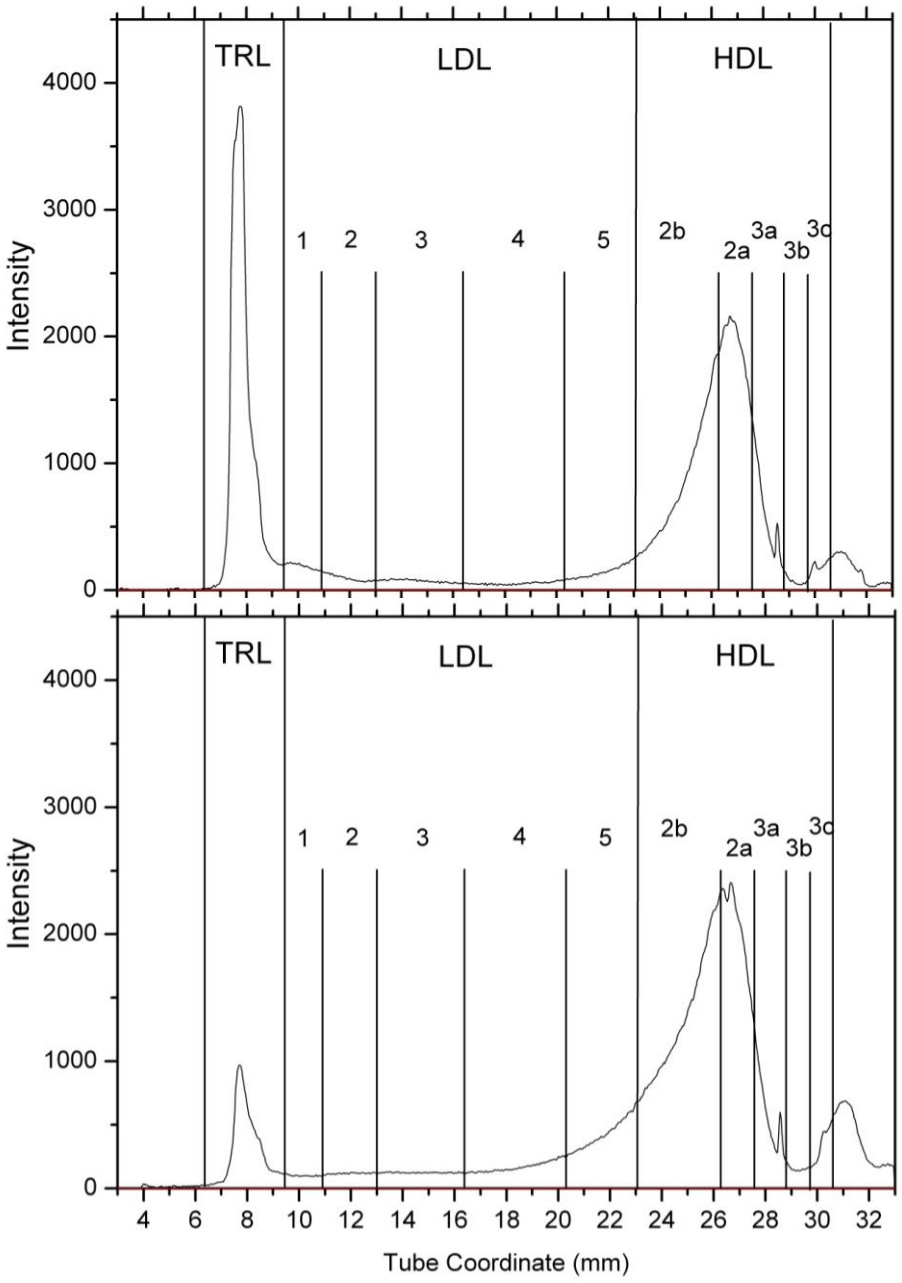


Figure 21. Lipoprotein density profiles from a representative dog with hypertriglyceridemia (Group 1) before and after the diet change. A. Lipoprotein profile of a dog with hypertriglyceridemia before the diet change. B. Lipoprotein profile of the same dog 8 weeks after the diet change. This dog had normal serum triglyceride and cholesterol concentrations at that point. Note the dramatic decrease in TRL. A decrease in LDL_1 and increases in HDL_{2b} and HDL_{2a} are also evident.

Hypertriglyceridemia has been linked to several conditions in dogs (especially Miniature Schnauzers), including pancreatitis, insulin resistance, hepatobiliary disease, and ocular disease.[252] The degree of hypertriglyceridemia seems to play an important role in most conditions.[252,279] Therefore, management of hypertriglyceridemia with a goal to reduce serum triglyceride concentrations below 500 mg/dL is usually recommended even when clinical sings are not present.[252] The results of the preset study suggest that the study diet is effective in lowering serum triglyceride concentrations to values below the ones considered to be associated with risk for disease (a recommended cut-off of 500 mg/dL was used in the present study), and in some cases it even led to normalization of serum triglyceride concentrations.

From each dog in Group 1, 2 samples were collected before the diet change and 2 after the diet change. This was done to ensure that dogs had persistent hyperlipidemia before the diet change and that some dogs would not spontaneously have serum and triglyceride concentrations within the reference range. For samples collected after the diet change, this was done to ensure that any reduction in serum and cholesterol concentration was not temporary or random. Therefore, each dog served as its own control. There was no significant difference in serum triglyceride or cholesterol concentrations between samples 1 and 2 (i.e., before the diet change) or between samples 3 and 4 (i.e., after the diet change). The fact that significant differences were detected only when serum triglyceride and cholesterol concentrations were compared between time-points before and after the diet change strongly suggests that this was due to an effect of the diet.

It is interesting to note that there was great variation in serum triglyceride concentrations between samples 1 and 2 (i.e., sample before the diet change). There also was variation between samples 3 and 4 (i.e., after the diet change) but it was not as prominent as compared to before the diet change. As mentioned above, there was no statistically significant difference between these time-points overall, but there was considerable variation within individual dogs. This was despite the fact that the dogs had been fasted for >15 hours. A plausible explanation is that, because the diet was not controlled during the first 2 sample collections, food of a different fat content might have been consumed before each of the blood collections. Alternatively, this variation might be normal for this condition as no long-term studies have evaluated the fluctuation of serum triglyceride concentration over time in Miniature Schnauzers with hyperlipidemia. A similar observation of such variation has also been described in another study with Miniature Schnauzers with pancreatitis.[280] This might suggest that at least 2 samples should be collected and evaluated during the diagnostic evaluation Miniature Schnauzers for hyperlipidemia to ensure that an accurate estimate of the severity of hyperlipidemia is obtained.

Another observation was that, although >50% of the dogs were classified as normal after the diet change based on their lipoprotein profiles, a considerable percentage (44%) of dogs was still classified as hyperlipidemic based on their lipoprotein profiles. This is in agreement with the observation that serum triglyceride concentrations were significantly reduced but not normalized in some of the dogs (Figure 22). It is not known why the dogs responded to the diet change to different

degrees. One possible explanation is that some dogs might need more time than 2 to 3 months to fully respond to the diet. Although there are no studies convincingly showing how many weeks are required for normalization of serum triglyceride concentrations after feeding a low-fat diet, clinical experience suggests that 2 to 3 months is usually sufficient. It is noteworthy that the baseline lipoprotein profiles (i.e., before the diet change) of the dogs that eventually responded to the diet were distinctly different from the ones of dogs that did not fully respond to the diet. This might suggest that hyperlipidemia in Miniature Schnauzers is a clinically diverse condition, which might have different underlying biochemical bases, and that certain phenotypes of the disease might not be as responsive to dietary management with low-fat diets. Data from the present study but also from older studies suggest that Miniature Schnauzers often have different biochemical abnormalities associated with hyperlipidemia. For example, in 1 study it was shown that some hyperlipidemic Miniature Schnauzers have hyperchylomicronemia while others do not.[17] Similarly, some Miniature Schnauzers with hypertriglyceridemia also have hypercholesterolemia while others do not.[17,84] Chylomicrons are dietary in origin and hyperchylomicroniemia is usually expected to respond to low-fat diets.[252] Increases in VLDL are not necessarily responsive to low-fat diets because VLDLs are produced through the endogenous pathway.[252] This finding further suggests that lipoprotein profile analysis might be useful in clinical practice as it provides important additional information to serum triglyceride and cholesterol concentration, and it might affect clinical decision making with regard to the management of dogs with hyperlipidemia.

There was no significant effect of diet on serum Spec cPL concentration. This finding was not surprising, however, because the enrolled dogs did not have any clinical signs of pancreatitis and the majority of them had a normal serum Spec cPL concentration at time of enrollment. Thus, the effect of low-fat diets on the clinical course of pancreatitis needs to be evaluated in additional studies.

In conclusion, this study evaluated the effect of a commercially available low-fat diet on serum lipid concentrations and lipoprotein profiles in Miniature Schnauzers with hyperlipidemia. The study diet was found to be effective in significantly reducing serum triglyceride and cholesterol concentrations within 2 months. The lipoprotein profiles of most hyperlipidemic dogs were shifted towards those of non-hyperlipidemic dogs within 2 months. A subgroup of dogs did not fully respond to the diet change as indicated mainly by their serum triglyceride concentrations and lipoprotein profiles. The reason for this finding is not known at this point, but differences in the pathogenetic basis of hyperlipidemia among dogs might have played a role. Given the fact that the dogs enrolled in the present study had naturally occurring hyperlipidemia, the study diet should be expected to be beneficial in clinical practice. However, it is not clear whether other low-fat diets would have the same effect on the management of hyperlipidemia in Miniature Schnauzers.

Footnotes

[a]Royal Canin Gastrointestinal™ Low-fat™, Royal Canin USA, Inc., St. Charles, MO

[b]Roche/Hitachi MODULAR ANALYTICS D 2400 module, Roche Diagnostics, Indianapolis, IN

[c]Immulite 2000 Canine Total T4, Siemens Healthcare Diagnostics, Deerfield, IL

[d]Free T4 (by ED), Antech Diagnostics, Irvine, CA

[e]Bismuth Sodium Ethylenediaminetetraacetate, TCI AMERICA, Portland, OR

[f]NBD C_6-ceramide, Molecular Probes, Inc. Eugene, OR

[g]Tube, Thickwall, Polycarbonate (1 mL, 11 x 34 mm), Beckman Coulter Inc., Brea, CA

[h]Beckman Coulter Optima TLX-120 Ultracentrifuge, Beckman Coulter Inc., Brea, CA

[i]TLA-120.2 rotor, Beckman Coulter Inc, Brea, CA

[j]Digital Microfire Camera, Optronics, Coleta, CA

[k]MH-100, Dolan-Jenner Industries, Boxborough, MA

[l]SCHOTT North America, Inc., Elmsford, NY

[m]Origin 7.0, Microcal Software Inc., Northampton, MA

[n]SPSS 16.0, SPSS Inc., Chicago, IL

[o]Prism5, GraphPad, San Diego, CA

[p]R, http://www.r-project.org/

CHAPTER VI

SERUM TRIGLYCERIDE AND CHOLESTEROL CONCENTRATIONS AND LIPOPROTEIN PROFILES IN DOGS WITH NATURALLY OCCURRING PANCREATITIS AND HEALTHY CONTROL DOGS

Introduction

The association between hyperlipidemia and pancreatitis remains obscure in dogs, but has been speculated to be bidirectional.[252] Hyperlipidemia, and more specifically hypertriglyceridemia, has been hypothesized to be able to cause pancreatitis in some circumstances.[252] This hypothesis is supported by the results of 2 recent clinical studies in dogs,[279,280] and is further supported by *in vitro* studies[260] and clinical studies in humans.[254,267] On the other hand, hyperlipidemia has also been hypothesized to be the result of pancreatitis.[165,254] Although this hypothesis is widely believed to be true, evidence supporting this hypothesis has not been documented in dogs with naturally occurring pancreatitis. Hypertriglyceridemia has been commonly reported in dogs with pancreatitis, but the etiology of hypertriglyceridemia often remains unknown in these cases.[19,21,63-65] Furthermore, with the exception of the results of 1 older study,[261] hypertriglyceridemia has not been reported to be a consequence of experimental pancreatitis in dogs.[18-20] However, experimental models of pancreatitis do not always mirror the pathophysiologic mechanisms that apply to spontaneous disease.

Studies specifically investigating the lipid status and lipoprotein profiles of dogs with spontaneous pancreatitis have not been reported. Characterization of the lipid

concentrations and lipoprotein profiles of dogs with pancreatitis might help to clarify the association between hyperlipidemia and pancreatitis. Therefore, the aims of the present study were: a) to measure serum triglyceride and cholesterol concentrations in dogs with naturally occurring pancreatitis and compare them with those of healthy dogs, and b) to determine the lipoprotein profiles of dogs with naturally occurring pancreatitis and compare them with those of healthy dogs.

Materials and methods

Dogs with pancreatitis (Group 1)

Dogs with a clinical diagnosis of pancreatitis were enrolled into this study. Serum samples from these dogs had been submitted to the Gastrointestinal Laboratory at Texas A&M University by veterinarians located throughout the United States as part of their diagnostic evaluation, and were stored at -80^{o}C until use. The submitting veterinarians were contacted and asked to complete a questionnaire for each dog. Questions covered the date of birth, sex and sexual status, body-weight and body condition score (BCS), current diet(s), current medications, and the current and past health history of the dogs. Questionnaires from all dogs were reviewed to determine whether the dogs fit the inclusion criteria for the study. Food had to be withheld for ≥12 hours prior to blood collection for all dogs enrolled into the study. The dogs were enrolled on a sequential basis based on whether the submitting veterinarian had agreed to complete the questionnaire and whether the dogs fit the inclusion criteria. No preference was given to lipemic samples.

The diagnosis of pancreatitis was based on: a) the presence of compatible historical findings of at least 2 of the following clinical signs (vomiting, anorexia, depression, abdominal pain, or diarrhea) and b) a serum Spec cPL® concentration ≥400 μg/L (the currently recommended cut-off value for pancreatitis).[178,180] Although dogs with concurrent diseases or those receiving medications that might affect lipid metabolism were initially included in this group in order to determine the overall prevalence of hyperlipidemia in this group, they were subsequently excluded from the analysis and only dogs that had pancreatitis without any recognized risk factors for hyperlipidemia were eventually included in this group. These dogs were designated as Group 1. Dogs with hyperlipidemia secondary to diseases other than pancreatitis or medications[252] were identified based on the clinical history, physical examination, and results from the serum chemistry profile (without the results for serum cholesterol and triglyceride concentrations) and specific tests (such as serum total and free T4 concentrations).

Healthy control dogs (Group 2)

Healthy dogs were enrolled into the study and served as controls. The dogs belonged to students and staff of the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University. The owners were asked to complete a questionnaire for each dog. Questions covered date of birth, sex and sexual status, body-weight and BCS, current diet(s), current medications, and the current and past health history of the dogs. Questionnaires from all dogs were reviewed to determine whether the dogs fit the

inclusion criteria for the study. All owners signed an informed owner consent form. The study protocol was reviewed and approved by the Clinical Research Review Committee at Texas A&M University.

Inclusion criteria for the control group were: a) absence of any clinical signs at the time of blood collection, b) no major abnormalities on the serum chemistry profile, c) a serum Spec cPL concentration within the reference interval, and d) no history of diseases or current drug use known to affect lipid metabolism.[252] Blood samples were collected from these dogs after food had been withheld for at least 12 hours. The blood samples were collected into red-top tubes (with no additive), allowed to clot for 20 minutes, centrifuged, and the serum was aliquotted out and stored at -80^{o}C until analysis.

Assays

Serum triglyceride (reference interval: 26 to 108 mg/dL), cholesterol (reference interval: 124 to 335 mg/dL), and glucose (reference interval: 60 to 120 mg/dL) concentrations were measured by automated enzymatic assays.[a] Serum Spec cPL concentrations (reference interval: ≤200 µg/L) were measured using a commercially available immunoassay that has been described elsewhere.[178] Serum total T4 concentrations were measured by a solid-phase chemiluminescent competitive assay.[b] Serum free T4 concentration was measured using a commercially available equilibrium dialysis radioimmunoassay.[c]

Lipoprotein profile analysis

Lipoprotein profiling was carried out using a bismuth sodium ethylenediaminetetraacetic acid (NaBiEDTA or NaBiY) density gradient ultracentrifugation method as previously described with some modifications.[281] The sodium salt of BiEDTA has been described as a novel solute forming a self-generating density gradient during ultracentrifugation of serum samples for the separation of lipoproteins.[281] Briefly, for each sample, 1,284 µL of a 0.18M NaBiEDTA[d] gradient solution was added into a 1.5 mL tube. The fluorescent probe 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine (NBD C_6-ceramide)[e] was reconstituted with 1 g/mL DMSO and 10 µL of the 1 mg/mL solution were added to each tube to label the lipoproteins. Finally, 6 µL of serum was added to each tube. The mixture was vortexed at 1,400 rpm for 10 seconds and 1,150 µL of it were transferred into an ultracentrifuge tube.[f] The mixture was allowed to incubate for 30 minutes at 5^{o}C to allow for the fluorescent probe to saturate the lipoproteins. The solution was then centrifuged at 120,000 rpm and 5^{o}C for 6 hours in a Beckman Optima ultracentrifuge (TLX-110)[g] with a 30^{o} fixed angle TLA 120.2 rotor.[h] A quality control sample was included in each run to verify that proper operating conditions had been achieved. Immediately after ultracentrifugation, the top of each sample was carefully layered with 250 µL of hexane and imaged without delay.

For imaging, each tube was placed in a custom, in-house imaging instrument as previously described.[281] The samples were imaged using a custom-built fluorescence imaging system consisting of a digital camera[i] with a MH-100 metal halide continuous

light source[j], located in a dark room. Two filters[k] matching the excitation (blue-violet filter centered at 407 nm) and emission (a yellow emission long pass filter with a cut-on wavelength of 515 nm) characteristics of NBD C_6-cermide were used. A gain of 1.0000, a target intensity of 30%, and an exposure time of 53.3 ms were selected. In order to be analyzed, the image of the each tube following ultracentrifugation was converted to a density profile using a commercially available software program.[l] A tube coordinate scale was established where 0.0 mm is the top of the tube and 34.0 mm is the bottom of the tube.[281] The average intensity was then plotted as a function of the tube coordinate.

The ultracentrifugation method described above has been shown to identify 11 distinct density lipoprotein fractions in human serum (TRL, 5 LDL fractions, and 5 HDL fractions). This separation is based solely on the density characteristics of the lipoprotein fractions and not on their functional properties. Therefore, this method was used to develop lipoprotein fingerprinting in canine serum based on lipoprotein densities alone. Density ranges were based on those previously published for humans by several authors.[282] Specifically, the 11 fractions and their corresponding densities (*d*) were as follows: chylomicrons and VLDL (d<1.017 g/mL), LDL_1 (d=1.019 to 1.023 g/mL), LDL_2 (d=1.023 to 1.029 g/mL), LDL_3 (d=1.029 to 1.039 g/mL), LDL_4 (d=1.039 to 1.050 g/mL), LDL_5 (d=1.050 to 1.063 g/mL), HDL_{2b} (d=1.063 to 1.091 g/mL), HDL_{2a} (d=1.091 to 1.110 g/mL), HDL_{3a} (d=1.110 to 1.133 g/mL), HDL_{3b} (d=1.133 to 1.156 g/mL), and HDL_{3c} (d=1.156 to 1.179 g/mL).[282] HDL_1 is typically not found in healthy humans, but has been speculated to be present in healthy dogs.[8,282] However, it has not been convincingly shown that the previously described canine HDL_1 molecule has the

same function as human HDL_1, and its density range has not been accurately determined (previously published densities vary between 1.025 and 1.1).[8] Previous studies in humans that have reported the density of HDL_1 suggest that the density of this molecule is around 1.08 g/mL, which would suggest that it corresponds to HDL_{2b}, or possibly HDL_{2a}.[283]

Statistical analyses

Commercially available statistical software packages were used for all statistical analyses.[m,n,o] Data were analyzed for normal distribution using the Shapiro-Wilk test. Normally distributed data were analyzed using t-tests. Non-normally distributed data were analyzed using Mann-Whitney tests. Proportions were compared between groups using the Fisher's exact test. Sliced inverse regression (SIR) was used to test whether there was a relationship between group and lipoprotein profiles. Significance was set at $P<0.05$ for all analyses.

Results

Dogs with pancreatitis (Group 1)

Twenty-eight dogs with a clinical diagnosis of pancreatitis were initially evaluated for inclusion into the study. Of these 28 dogs with pancreatitis, 11 had concurrent diseases that might have affected lipid metabolism and thus were excluded from the study. Specifically, 5 dogs had been diagnosed and/or were treated for diabetes mellitus, 3 dogs had been diagnosed and treated for hypothyroidism, 1 dog had been

diagnosed with hyperadrenocorticism and protein losing nephropathy, 1 dog had severe protein losing enteropathy, and 1 dog was being treated with prednisone.

Thus, Group 1 consisted of 17 dogs that had pancreatitis, but did not have any recognized risk factors for hyperlipidemia. These dogs belonged to 10 breeds while 2 dogs were mixed-breed. Nine dogs were female (all spayed) and 8 dogs were male (7 castrated). The body condition score (BCS) of the dogs in this group ranged from 3/9 to 7/9 (median: 4.5). The mean age of the dogs was 7.4 years (range: 1 to 14 years).

Healthy dogs

A total of 53 healthy dogs were enrolled in Group 2 as controls. Dogs of Group 2 belonged to 20 different breeds (34 dogs), while 19 dogs were mixed-breed. Twenty-six dogs were female (23 spayed) and 27 dogs were male (23 castrated). The body condition score (BCS) of the dogs in this group ranged from 4/9 to 7/9 (median: 5). The mean age of the dogs was 5.2 years (range: 1 to 13.5 years).

Serum triglyceride and cholesterol concentrations of all 53 dogs were compared to those of dogs in Group 1. However, lipoprotein profile analysis was only performed on 29 of the 53 healthy dogs of Group 2, that were selected to match the age of the dogs in Group 1.

Serum triglyceride and cholesterol concentrations

Twelve of the 28 dogs (43%) with pancreatitis initially considered for enrollment into Group 1 of the study had an increased concentration of serum triglycerides, serum cholesterol, or both.

Five of 17 dogs (29%) of Group 1 had an increased concentration of serum triglycerides, serum cholesterol, or both. In contrast, only a total of 5 of the 53 healthy dogs (9%) had an increased concentration of serum triglycerides, serum cholesterol, or both. This difference approached but did not reach statistical significance (P=0.055).

In Group 1 (dogs with pancreatitis, but no risk factors of hyperlipidemia), 3 of the 17 dogs (18%) had hypertriglyceridemia (Figure 22 on page 148). Hypertriglyceridemia was mild (<350 mg/dL) in all 3 cases. The median serum triglyceride concentration was 67 mg/dL (range: 48 – 324 mg/dL). Of the 53 control dogs in Group 2, 4 (7.5%) had hypertriglyceridemia. Hypertriglyceridemia was mild in all 4 cases (<300 mg/dL). There was no statistically significant difference in the proportion of dogs that had hypertriglyceridemia between dogs with pancreatitis (18%) and healthy controls (7.5%; P=0.35). However, there was a statistically significant difference in serum triglyceride concentrations between Group 1 (median: 67 mg/dL; range: 48 – 324 mg/dL) and Group 2 (median: 54 mg/dL; range: 26 – 257 mg/dL; P=0.0026; Figure 22). As mentioned above, serum triglyceride concentrations were within the reference interval for the majority of dogs in both groups.

Of the 17 dogs in Group 1, 4 (24%) had hypercholesterolemia. The median serum cholesterol concentration was 209 mg/dL (range: 102 – 849 mg/dL; Figure 23).

Of the 53 control dogs in Group 2, 1 (1.9%) had hypercholesterolemia. There was a statistically significant difference in the proportion of dogs that had hypercholesterolemia between dogs with pancreatitis (Group 1) and healthy control dogs (P=0.011). However, there was no statistically significant difference in serum cholesterol concentrations between Group 1 (median: 209 mg/dL; range: 142 – 849 mg/dL) and Group 2 (median: 227 mg/dL; range: 97 – 338 mg/dL; P=0.565; Figure 23).

Lipoprotein profile analysis

All 17 dogs of Group 1 were used for lipoprotein profile analysis. In contrast, only a fraction of dogs in Group 2 (29 dogs) with ages similar to the dogs of Group 1 were selected for lipoprotein profile analysis.

Sliced inverse regression analysis showed that lipoprotein profiles were distinctly different between dogs with pancreatitis and healthy control dogs (Eigenvalues = 0.6719; P=0.0012). Dogs could be classified in the correct group (healthy versus pancreatitis) with 89% accuracy based on their lipoprotein profiles alone (Figure 24). The most important differences in the lipoprotein profiles between dogs with pancreatitis and healthy dogs involved increases in LDL_2, LDL_3, and LDL_4, and less prominent decreases in TRL, HDL_{2a}, and HDL_{3c}. Figure 25A shows the lipoprotein density profile of a representative dog with pancreatitis. Figure 25B shows the lipoprotein density profile of a representative healthy dog. It is clear that the main differences between these two exemplary lipoprotein profiles involved the LDL fractions, while the differences in TRLs and HDL fractions were less prominent.

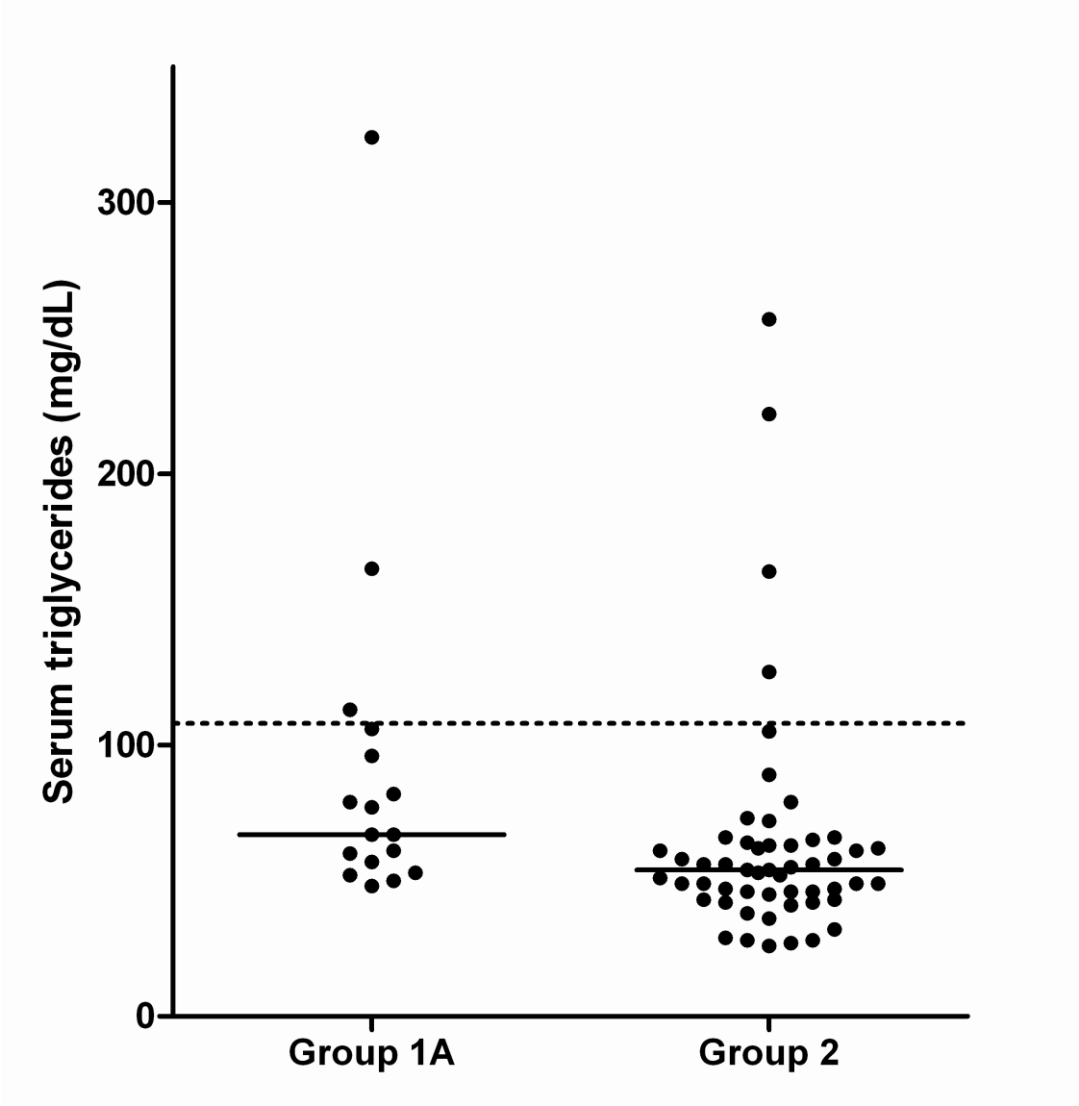


Figure 22. Serum triglyceride concentrations in dogs with pancreatitis (Group 1) and healthy control dogs (Group 2). Dogs in Group 1 had significantly higher (P=0.0026) serum triglyceride concentrations than dogs in Group 2. However, the majority of dogs in both groups had serum triglyceride concentrations within the reference interval. Hypertriglyceridemia, when present, was always mild. The dashed line represents the upper limit of the reference interval (108 mg/dL).

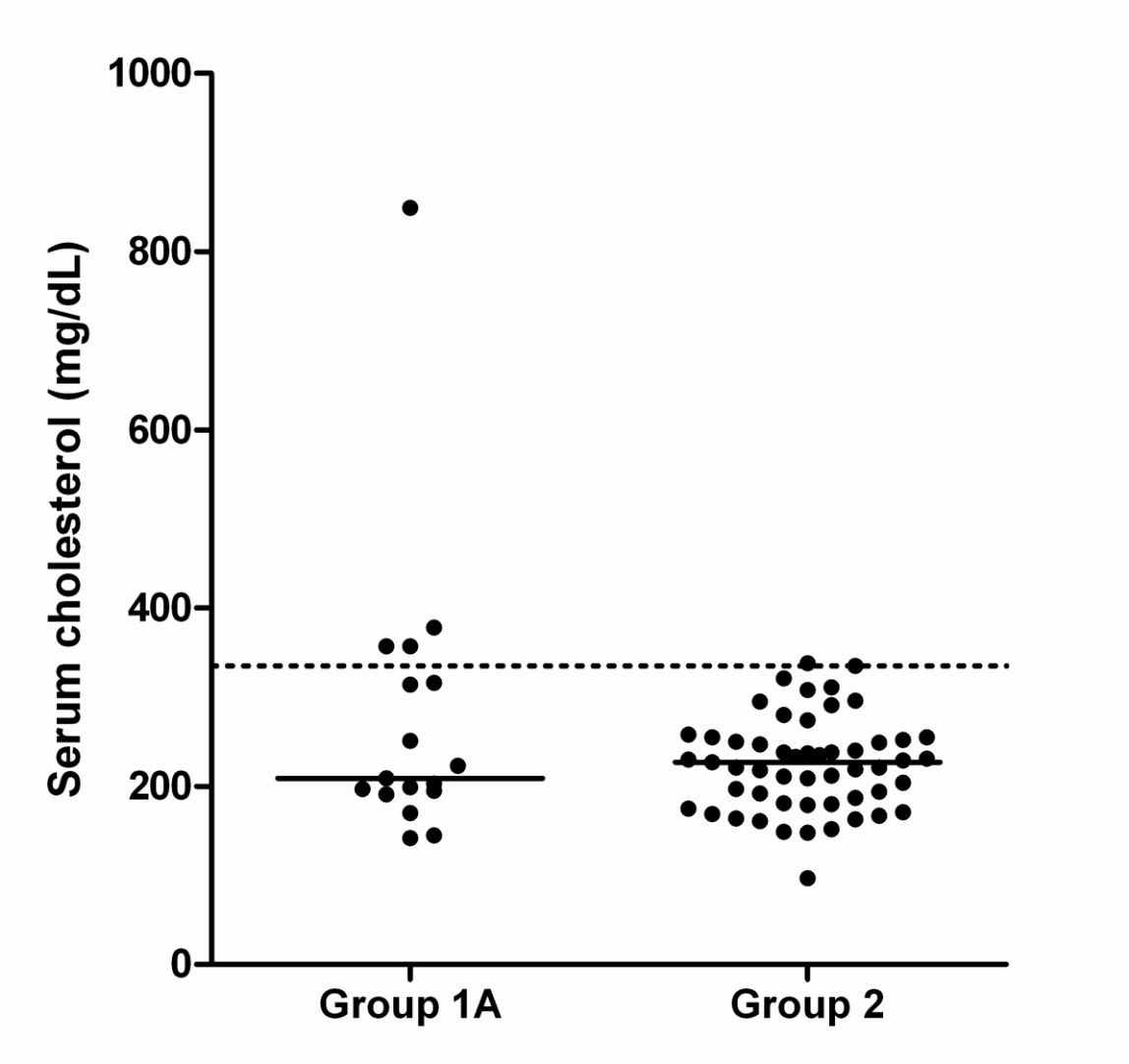


Figure 23. Serum cholesterol concentrations in dogs with pancreatitis (Group 1) and healthy control dogs (Group 2). There was no statistically significant difference in serum cholesterol concentration between the 2 groups (P=0.565). The majority of dogs in both groups had serum cholesterol concentrations within the reference interval, while a small number of dogs had a mildly increased serum cholesterol concentration. The dashed line represents the upper limit of the reference interval (335 mg/dL).

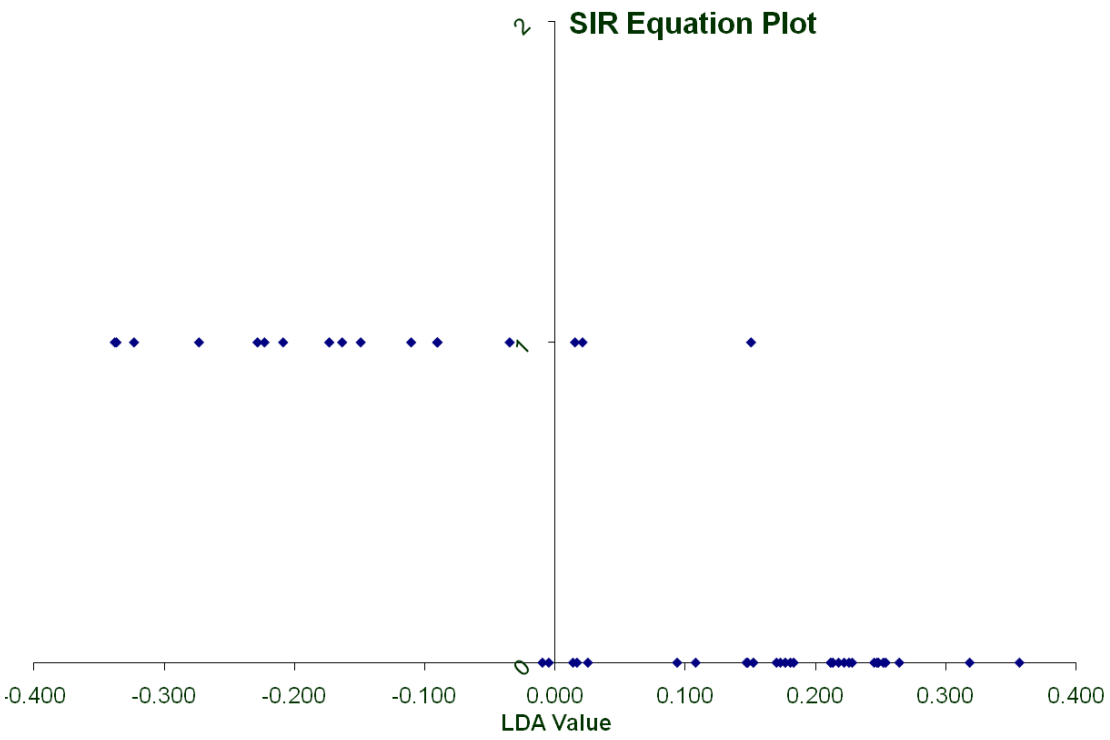


Figure 24. 1-dimensional **s**liced inverse regression plot showing classification of dogs into groups based on lipoprotein profile analysis (Group 1 vs Group 2). The vertical line represents the line that separates the two groups based on lipoprotein profile analysis. The LDA value provides a ranking value for each dog. The dogs represented by the dots that are at the bottom of the graph are healthy (Group 2). The majority of dogs (except for 2 dogs) have a lipoprotein profile that plots to the right of the vertical line. The dogs represented by the dots at the top of the graph are dogs with pancreatitis (Group 1). The majority of these dogs (with the exception of 3) have a lipoprotein profile that is different from that of the healthy dogs. Sliced inverse regression analysis correctly classified approximately 90% of the dogs based on their lipoprotein profiles.

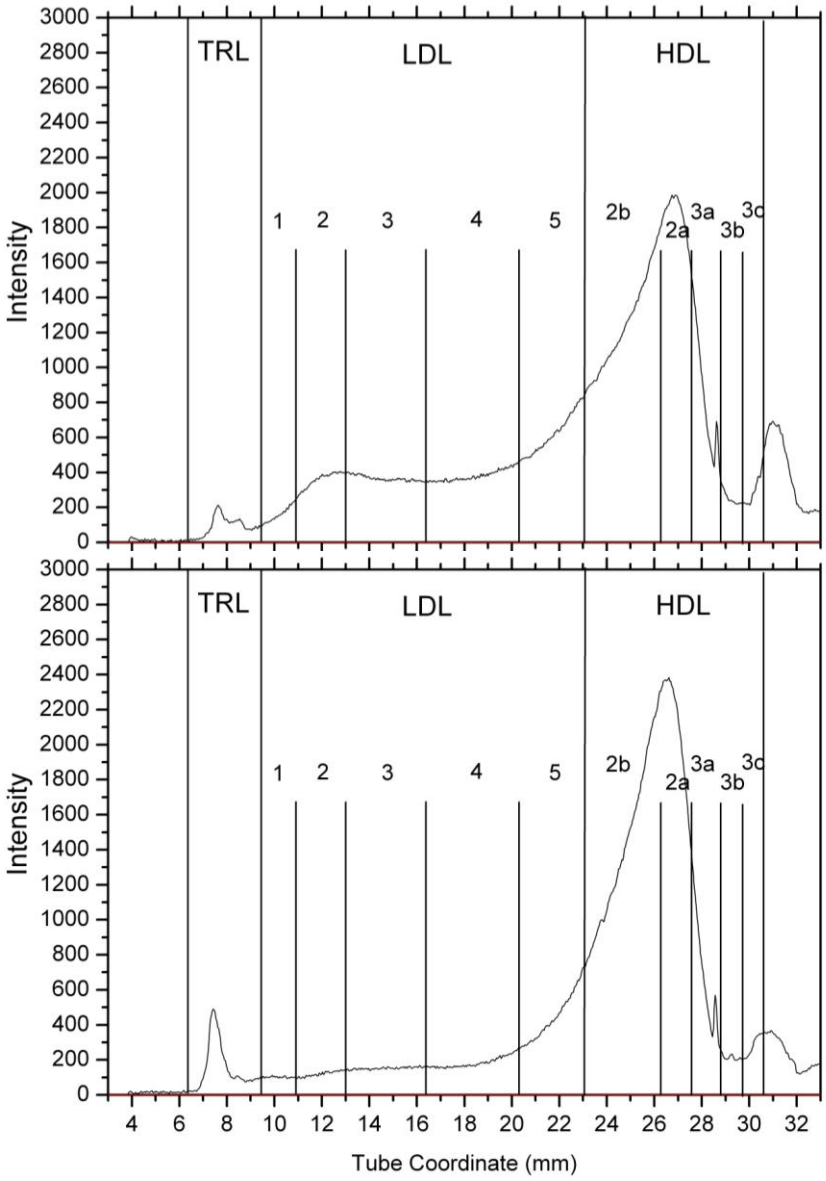


Figure 25. Lipoprotein density profiles from a representative dog with pancreatitis (A) and a representative healthy control dog (B). The dog in panel A shows profound increases in the LDL fractions (mainly LDL_2 through LDL_5) compared to the healthy control dog shown in panel B. Decreases in the TRL, HDL_{2b}, and HDL_{2a} fractions might also be present in the dog in panel A compared to the dog in panel B. Both dogs had serum triglyceride and cholesterol concentrations within their respective reference intervals.

Discussion

The purpose of the present study was to investigate the serum lipid and lipoprotein abnormalities associated with naturally occurring pancreatitis in dogs. Mild increases in serum triglyceride and/or cholesterol concentrations were seen in a relatively small proportion of dogs with pancreatitis. The majority of dogs (>70%) with pancreatitis had serum triglyceride and cholesterol concentrations within the respective reference intervals. In contrast, important differences were identified in lipoprotein profiles between dogs with pancreatitis and healthy dogs. Specifically, dogs with pancreatitis had higher LDL fractions (mainly LDL_2, LDL_3, and LDL_4) and lower TRL and HDL fractions (mainly HDL_{2a} and HDL_{3c}) than healthy dogs. These changes in lipoprotein profiles were evident in the majority of dogs with pancreatitis, even in cases in which serum concentrations of triglyceride and cholesterol were normal.

In the group of dogs investigated here, those with pancreatitis tended to more commonly have an increased concentration of serum triglycerides, serum cholesterol, or both (about 30%) than healthy dogs (about 10%), although this difference did not reach statistical significance. Serum triglyceride concentrations overall were higher among dogs with pancreatitis than among healthy dogs. However, this difference was not clinically important because the majority of dogs (>80%) had serum triglyceride concentrations within the reference interval, and there was no statistically significant difference in the proportion of dogs with hypertriglyceridemia between the 2 groups. On the other hand, a higher proportion of dogs with pancreatitis had hypercholesterolemia (24%) compared to healthy dogs (1.9%). It is of note, however, that > 75% of dogs with

pancreatitis had normal serum cholesterol concentrations. Also, overall, serum cholesterol concentration was not significantly higher in dogs with pancreatitis, suggesting that hypercholesterolemia was generally mild in those cases. In fact, only 1 dog had a serum cholesterol concentration >800 mg/dL, while the majority of dogs had normal serum cholesterol concentrations.

The above findings contradict a commonly held belief that pancreatitis is commonly associated with increases in serum triglyceride and/or cholesterol concentrations in dogs. This belief is most likely based on clinical studies in dogs that show a relatively high prevalence of hyperlipidemia in dogs with pancreatitis.[63,64] For example, in 1 study[64], 48% and 26% of dogs with pancreatitis were reported to have hypercholesterolemia and grossly lipemic serum, respectively. However, in those studies, dogs with secondary hyperlipidemia (e.g., due to diabetes mellitus or hypothyroidism) were not excluded from the calculation of the prevalence of hyperlipidemia, and it is very likely that most dogs had hyperlipidemia as a result of diseases other than pancreatitis. This is supported by the findings of the present study; before the exclusion of dogs that had secondary causes of hyperlipidemia (mainly diabetes mellitus and hypothyroidism), the overall prevalence of hyperlipidemia was 43%. However, when only dogs with pancreatitis and no other diseases were included in the present study, the prevalence of hyperlipidemia was much lower (29%) and it was mild in almost all cases.

The present study suggests that the majority of dogs (>70%) with pancreatitis have normal serum triglyceride and cholesterol concentrations, while only a small

fraction of these dogs (about 30% overall) have increases in serum triglyceride and/or cholesterol concentrations. About 20% of dogs had hypertriglyceridemia, and another 20% of dogs had hypercholesterolemia. It is equally important to note that both hypertriglyceridemia and hypercholesterolemia were mild when present in dogs with pancreatitis (typically <200 mg/dL and <400 mg/dL, respectively; Figures 22 and 23). Therefore, it might be recommended that when dramatic increases in serum triglyceride and/or cholesterol concentrations are present in dogs with pancreatitis, other causes of secondary hyperlipidemia or primary hyperlipidemia should be investigated.

The findings of the present study are in accordance with findings of studies on experimentally induced pancreatitis in dogs.[18-20] In general, most of those studies have come to the conclusion that hypertriglyceridemia is not a consequence of experimentally induced pancreatitis in dogs.[18-20] In 1 study, statistically significant increases in serum triglyceride concentrations were noted, but serum triglyceride concentrations remained within the reference interval throughout the study period.[20] Similar findings were reported regarding hypercholesterolemia in those studies.[18-20]

Although major changes in serum triglyceride and cholesterol concentrations were not found in dogs with pancreatitis in the present study, there were profound differences in lipoprotein profiles between dogs with pancreatitis and healthy controls. The dogs could be correctly classified in almost 90% of the cases as having or not having pancreatitis based on their lipoprotein profile alone. The main changes in the lipoprotein profiles involved increases in the LDL fractions (mainly LDL_2, LDL_3, and LDL_4). Decreases in HDL fractions such as HDL_{2a}, HDL_{3c}, and TRLs were also present.

Both LDL and HDL fractions contain mainly cholesterol.[252] The fact that the overall serum cholesterol concentration was normal in the majority of dogs with pancreatitis might be explained by the fact that increases in LDL fractions were balanced by a concurrent decrease in the HDL fractions. It is possible that during pancreatitis lipoprotein metabolism is altered in a way that facilitates redistribution of lipid components (mainly cholesterol) between LDL and HDL fractions. Further studies are needed to elucidate the mechanism of lipoprotein metabolism alterations in dogs with pancreatitis.

The changes in lipoprotein profiles in dogs with pancreatitis described in the present study are in agreement with the findings of studies on experimentally induced pancreatitis in dogs.[18-20] Those studies have all reported similar findings, including increases in LDL and decreases in HDL fractions. Based on these findings, serum alterations in the major lipoprotein fractions are similar between naturally occurring and experimentally induced pancreatitis. However, changes in lipoprotein subfractions as a result of experimental pancreatitis have not been reported in other studies and differences might be present.

TRLs were overall decreased in dogs with pancreatitis in the present study. To the authors' knowledge, this has not been described before in dogs with pancreatitis without concurrent diseases affecting the lipoprotein metabolism. Since TRL are the main lipoprotein fractions containing triglycerides, this finding contradicts another finding of the present study, which showed that serum triglyceride concentrations were significantly higher in dogs with pancreatitis. This finding might suggest that the content

of lipoproteins might also have changed in dogs with pancreatitis, and that TRL, LDL, and/or HDL contained higher concentrations of triglycerides than those in healthy dogs. Further studies are needed to describe the lipid content of the lipoprotein fractions of dogs with pancreatitis.

Overall, some of the changes observed in the present study with regard to serum lipid concentrations and lipoprotein profiles are similar to the changes reported in humans or animal models with various types of inflammation or infection.[287-289] Similar to the changes in acute phase proteins that occur during inflammation, major changes take place in lipoprotein metabolism in response to inflammation. These changes affect both the concentration and composition of serum lipoproteins and are mainly mediated by hormones and cytokines.[287-289] Typical lipoprotein changes in response to inflammation in humans include an increase in serum triglyceride concentration (due to an increased production of triglycerides and VLDL by the liver), and a decrease in HDL cholesterol.[287-289] Similar changes were observed in the present study. Changes in LDL molecules are more difficult to compare between humans and dogs because these 2 species differ substantially in their LDL metabolism. Nevertheless, inflammation in humans appears to induce a shift in LDL profiles leading to the appearance of small dense LDL molecules that correspond to fractions LDL_4 and LDL_5, a finding that was also observed in the dogs of Group 1 in the present study. It seems likely that, as in humans, changes in serum lipid concentrations and lipoprotein profiles in dogs with pancreatitis might be the result of a general inflammatory response rather than a

pancreatitis-specific change. Additional research is required to compare the lipoprotein profiles between dogs with different inflammatory conditions.

In conclusion, the majority of dogs with naturally occurring pancreatitis in the present study (>70%) had serum triglyceride and cholesterol concentrations within their respective reference intervals. In the relatively small percentage of dogs that showed increases in serum triglyceride and/or cholesterol concentrations those increases were generally mild. Therefore, profound increases in serum triglyceride and/or cholesterol concentrations in dogs with pancreatitis are unlikely to be the result of pancreatitis, and warrant further diagnostic investigation. In sharp contrast, important differences were identified in lipoprotein profiles between dogs with pancreatitis and healthy control dogs. Dogs with pancreatitis had higher LDL fractions (mainly LDL_2, LDL_3, and LDL_4) and lower TRL and HDL fractions (mainly HDL_{2a} and HDL_{3c}) than healthy control dogs. These changes in lipoprotein profiles were evident in the majority of dogs with pancreatitis, even in cases in which serum concentrations of triglyceride and cholesterol were normal. Further studies are needed to elucidate the mechanisms responsible for the changes in lipoprotein metabolism in dogs with pancreatitis. Also, additional research is required to determine whether these changes are pancreatitis-specific or a result of an inflammatory state in dogs.

Footnotes

[a]Roche/Hitachi MODULAR ANALYTICS D 2400 module, Roche Diagnostics, Indianapolis, IN

[b]Immulite 2000 Canine Total T4, Siemens Healthcare Diagnostics, Deerfield, IL

[c]Free T4 (by equilibrium dialysis), Antech Diagnostics, Irvine, CA

[d]Bismuth Sodium Ethylenediaminetetraacetate, TCI AMERICA, Portland, OR

[e]NBD C_6-ceramide, Molecular Probes, Inc. Eugene, OR

[f]Tube, thickwall, Polycarbonate (1 mL, 11 x 34 mm), Beckman Coulter Inc., Brea, CA

[g]Beckman Coulter Optima TLX-120 Ultracentrifuge, Beckman Coulter Inc., Brea, CA

[h]TLA-120.2 rotor, Beckman Coulter Inc, Brea, CA

[i]Digital Microfire Camera, Optronics, Coleta, CA

[j]MH-100, Dolan-Jenner Industries, Boxborough, MA

[k]SCHOTT North America, Inc., Elmsford, NY

[l]Origin 7.0, Microcal Software Inc., Northampton, MA

[m]SPSS 16.0, SPSS Inc., Chicago, IL

[n]Prism5, GraphPad, San Diego, CA

[o]R, http://www.r-project.org/

CHAPTER VII

SUMMARY AND CONCLUSIONS

An association between hyperlipidemia and pancreatitis was first noted in humans by Speck in 1865.[253] Several clinical and experimental studies in humans and animals, respectively, have been conducted since then, and today, severe hypertriglyceridemia is a well recognized risk factor for pancreatitis in humans.[254-257] The mechanism by which hypertriglyceridemia induces pancreatitis is not clear, but it has been suggested that serum triglycerides are hydrolyzed by the action of pancreatic lipase, leading to excessive production of free fatty acids, which are toxic to the pancreatic acinar cell.[259,260] Another possible hypothesis is that hyperviscosity develops as a result of increased chylomicrons and/or VLDLs within the capillaries, which in turn leads to ischemia of pancreatic tissue.[258] Finally, genetic factors, such as mutations of the cystic fibrosis transmembrane conductance regulator gene or the SPINK1 gene may play a role by sensitizing the pancreas to the effects of hypertriglyceridemia.[258]

A similar relationship between hypertriglyceridemia and pancreatitis has been suggested for dogs, and co-existence of the 2 conditions has been frequently described.[15,33,34,37,49,52,66,83,165] However, this association remains obscure in dogs because the etiology of hypertriglyceridemia cannot always be determined in these studies. It has been speculated that the presence of hypertriglyceridemia in dogs with pancreatitis might be due to a pre-existing disorder of lipid metabolism, which may or may not be related to

the etiology of pancreatitis, but might also be the result of pancreatitis, or it might just be an incidental finding in some cases.[252]

Miniature Schnauzers are a unique breed in regards to both hyperlipidemia and pancreatitis. Miniature Schnauzers have been reported to develop pancreatitis more commonly than dogs of other breeds, and this high prevalence of pancreatitis in Miniature Schnauzers has been attributed to the fact that dogs of this breed commonly develop hypertriglyceridemia.[33,49,66,165] Available clinical and experimental data to support these hypotheses are limited, however. Clinical studies have shown an association between hyperlipidemia and pancreatitis in dogs, although it is not clear whether hyperlipidemia was the cause or the result of pancreatitis, or even just an incidental finding in some cases.[15,19,21,63-66] Secondary hyperlipidemia seen in dogs with some endocrinopathies (e.g., hyperadrenocorticism) or obesity may be responsible for the increased risk for pancreatitis associated with these diseases.[11,65,65,172]

The hypotheses of the present study were that: 1) hypertriglyceridemia, especially when severe, is a risk factor for pancreatitis in Miniature Schnauzers, 2) primary hypertriglyceridemia and possibly subclinical pancreatic inflammation in Miniature Schnauzers will respond, at least partially, to feeding an ultra low-fat diet, and 3) dogs with pancreatitis will exhibit changes in their serum triglyceride and cholesterol concentrations, as well as in their lipoprotein profiles when compared to healthy dogs.

The objectives of the present study were: 1) to investigate a possible associations between serum triglyceride and canine pancreatic lipase immunoreactivity (cPLI) concentrations in Miniature Schnauzers, 2) to compare serum triglyceride concentrations

in Miniature Schnauzers with a recent history of pancreatitis to those without a history of pancreatitis, 3) to evaluate the feasibility and assess the usefulness of a novel method for ultracentrifugal separation of lipoproteins as a means for lipoprotein fingerprinting in dogs, 4) to compare the lipoprotein profiles among dogs of various breeds, healthy Miniature Schnauzers, and Miniature Schnauzers with hyperlipidemia, 5) to evaluate the effect of a commercially available ultra-low-fat diet on serum triglyceride, cholesterol, and Spec cPL concentrations, as well as the lipoprotein profiles, in Miniature Schnauzers with suspected primary hypertriglyceridemia, and 6) to evaluate serum triglyceride and cholesterol concentrations and describe the lipoprotein profiles in dogs with and without pancreatitis.

In the first part of the study, the objective was to investigate a possible association between serum triglyceride and canine pancreatic lipase immunoreactivity (cPLI) concentrations in Miniature Schnauzers. One hundred and ninety-five Miniature Schnauzers were enrolled and divided into 2 groups based on whether they had normal (Group 1) or increased (Group 2) serum triglyceride concentrations. Serum triglyceride and cPLI concentrations were measured and compared between groups. There was a significant but weak positive correlation between serum triglyceride and cPLI concentrations ($r=0.321$; $P<0.0001$). Miniature Schnauzers with hypertriglyceridemia had significantly higher serum cPLI concentrations (median: 99.5 μg/L) than Miniature Schnauzers with normal serum triglyceride concentrations (median: 39.3 μg/L; $P=0.0001$). A cut-off value of 862 mg/dL was selected for serum triglyceride concentration based on ROC analysis, and this concentration had an increased risk of 4.5

times (P=0.0343) for a serum cPLI concentration that is considered to be consistent with a diagnosis of pancreatitis with the assay used (>200 μg/L). Thus, this study supports an association between hypertriglyceridemia, especially when severe, and high cPLI concentrations in Miniature Schnauzers.

The objective of the second part of the study was to compare serum triglyceride concentrations between Miniature Schnauzers with and without a recent history of pancreatitis. To that end, 17 Miniature Schnauzers with a history of pancreatitis (Group 1) and 34 age-matched Miniature Schnauzers without a history of pancreatitis (Group 2) were prospectively enrolled. Two samples were collected from each of the 17 Miniature Schnauzers with pancreatitis: 1 during pancreatitis and 1 after clinical and biochemical resolution of pancreatitis. Serum triglyceride and cholesterol concentrations were compared between Group 1 (after resolution of pancreatitis) and Group 2. Miniature Schnauzers in Group 1 were significantly more likely to have hypertriglyceridemia (71%) after resolution of pancreatitis than Miniature Schnauzers in Group 2 (33%; odds ratio=5.02; 95% CI, 1.4 to 17.8; P=0.0163). Serum triglyceride concentrations were significantly higher in dogs of Group 1 (median: 605.0 mg/dL) after resolution of pancreatitis than in dogs of Group 2 (median: 73.5 mg/dL; P=0.002). Miniature Schnauzers with a history of pancreatitis were 5 times more likely to have hypertriglyceridemia than controls. Based on the results of this part of the study, hypertriglyceridemia appears to be associated with the development of pancreatitis in some dogs of this breed.

In the third part of the study, the goal was to assess the feasibility and usefulness of a novel, convenient, and economical density gradient ultracentrifugation method as a means for lipoprotein fingerprinting in dogs, and to characterize and compare the lipoprotein profiles of healthy dogs of various breeds, healthy Miniature Schnauzers, and Miniature Schnauzers with primary hypertriglyceridemia using the same method. Thirty-five healthy dogs of various breeds with serum triglyceride and cholesterol concentrations within their respective reference intervals (Group 1), 31 Miniature Schnauzers with serum triglyceride and cholesterol concentrations within their respective reference intervals (Group 2A), and 31 Miniature Schnauzers with hypertriglyceridemia (Group 2B) were included in the study. The most abundant lipoprotein fraction in dogs of Group 1 was HDL, mainly HDL_2 and HDL_3. LDL fractions were present in very small amounts, with the exception of LDL_4 and LDL_5. Triglyceride-rich lipoproteins were also found in very small amounts. The lipoprotein profiles of Miniature Schnauzers with serum triglyceride and cholesterol concentrations within the reference interval (Group 2A) were generally similar to the ones seen in dogs of Group 1 with regard to the abundance of major lipoprotein classes. However, most dogs in Group 2A showed some distinct differences in some lipoprotein fractions and subfractions. Using sliced inverse regression analysis, the group to which each dog belonged (i.e., Miniature Schnauzer versus other breeds) could be accurately predicted based on their lipoprotein profiles in 85% of the cases (P=0.00017). The most important lipoprotein fractions that served as predictors were the TRLs and the LDL fraction corresponding to LDL_4 and LDL_5. Miniature Schnauzers had more prominent TRL

peaks than dogs of other breeds, while dogs of other breeds had more prominent LDL_4 and LDL_5 peaks. Sliced inverse regression analysis in Groups 2A and 2B showed that the group to which each dog belonged (i.e., Miniature Schnauzers with normal serum triglyceride concentration versus Miniature Schnauzers with hypertriglyceridemia) could be accurately predicted based on their lipoprotein profiles in 95% of cases (P=0.000002). By far, the most important lipoprotein fraction that served as a predictor was the TRL fraction, which was more prominent in the dogs with hypertriglyceridemia. Fractions corresponding to LDL_2, LDL_3, LDL_4, were more prominent in Miniature Schnauzers with serum triglyceride concentrations within the reference interval.

The aim of the fourth part of the study was to evaluate the effect of a commercially available low-fat diet on serum lipid and pancreas-specific lipase (Spec cPL®) concentrations and lipoprotein profiles in Miniature Schnauzers with primary hypertriglyceridemia. Sixteen Miniature Schnauzers with hypertriglyceridemia of various degrees were included in the study (Group 1). A group of 28 healthy Miniature Schnauzers with serum triglyceride and cholesterol concentrations within the respective reference intervals (Group 2) was used as a control group for the lipoprotein profile analysis portion of the study. Dogs in Group 2 were used in order to obtain lipoprotein profiles from a group of healthy Miniature Schnauzers that could be used for illustration of whether lipoprotein profiles of hypertriglyceridemic Miniature Schnauzers tended to approach those of the healthy ones after the diet change. Each one of the dogs in Group 1 had a total of 4 blood samples collected. The first sample (sample 1) was used to diagnose primary hypertriglyceridemia. Then, in order to confirm the results of the

initial sample and to investigate the variability of the findings, the owners were instructed to have a second sample (sample 2) collected 1 to 2 months after the collection of the initial sample, and without making any changes to the diet of their dogs. If hypertriglyceridemia was confirmed in the second sample, the dogs were put on the study diet. Approximately 7 to 9 weeks after the dogs had been exclusively on the study diet a third blood sample (sample 3) was collected. Finally, a fourth sample (sample 4) was collected approximately 2 to 4 weeks after the third sample. Serum triglyceride concentrations before the diet change (median of sample 1: 480 mg/dL; median of sample 2: 493 mg/dL) were significantly higher than after the diet change (median of sample 3: 177 mg/dL; median of sample 4: 168 mg/dL; P=0.0001). Serum cholesterol concentrations before the diet change (mean of sample 1: 381 mg/dL; mean of sample 2: 380 mg/dL) were significantly higher than after the diet change (mean of sample 3: 257 mg/dL; mean of sample 4: 178 mg/dL; P<0.0001). Serum Spec cPL concentrations before the diet change (mean of sample 1: 173 µg/L; mean of sample 2: 109 µg/L) were not significantly different than after the diet change (mean of sample 3: 144 µg/L; mean of sample 4: 137 µg/L; P=0.12). For analysis of the lipoprotein profiles in response to the diet change, the lipoprotein profiles after the diet change were compared with those of the same dogs before the diet change as well as those of the group of healthy dogs. Before the diet change, there was a 98% separation between Group 1 and Group 2 using SIR analysis (P=0.0003). Therefore, 15/16 (94%) of hyperlipidemic Miniature Schnauzers were classified as hyperlipidemic based on their lipoprotein profiles alone. After the diet change, significantly fewer Miniature Schnauzers (7/16; 44%; odds ratio:

19.3; 95% CI, 2.0-184.0; P=0.006) were still classified as hyperlipidemic by lipoprotein profile analysis, while the majority of the dogs of this group (56%) were classified as normal. Logistic regression analysis of the baseline lipoprotein profiles (before the diet change) of dogs that eventually responded and dogs that did not respond to the diet change showed that dogs that responded to the diet change could be separated with 88% accuracy from the ones that did not respond, based on lipoprotein profile analysis even before the diet change. Dogs that did not respond to the diet change tended to have lower LDL fractions (mainly LDL_1 and LDL_2) and higher HDL fractions (mainly HDL_{2a}, HDL_{3b}, and HDL_{3c}) than the ones that responded.

The aims of the last part of the study were: a) to measure serum triglyceride and cholesterol concentrations in dogs with naturally occurring pancreatitis and compare them with those of healthy dogs, and b) to determine the lipoprotein profiles of dogs with naturally occurring pancreatitis and compare them with those of healthy dogs. Seventeen dogs with pancreatitis and 53 healthy control dogs were enrolled. There was no statistically significant difference in the proportion of dogs that had hypertriglyceridemia between dogs with pancreatitis (18%) and healthy controls (7.5%; P=0.35). However, there was a statistically significant difference in serum triglyceride concentrations between dogs with pancreatitis (median: 67 mg/dL; range: 48 – 324 mg/dL) and dogs healthy control dogs (median: 54 mg/dL; range: 26 – 257 mg/dL; P=0.0026). There was a statistically significant difference in the proportion of dogs that had hypercholesterolemia between dogs with pancreatitis (Group 1) and healthy control dogs (P=0.011). However, there was no statistically significant difference in serum

cholesterol concentrations between dogs with pancreatitis (median: 209 mg/dL; range: 142 – 849 mg/dL) and healthy control dogs (median: 227 mg/dL; range: 97 – 338 mg/dL; P=0.565). Sliced inverse regression analysis showed that lipoprotein profiles were distinctly different between dogs with pancreatitis and healthy control dogs (P=0.0012). Dogs could be classified in the correct group (healthy versus pancreatitis) with 89% accuracy based on their lipoprotein profiles alone. The most important differences in the lipoprotein profiles between dogs with pancreatitis and healthy dogs involved increases in LDL_2, LDL_3, and LDL_4, and less prominent decreases in TRL, HDL_{2a}, and HDL_{3c}.

In summary, the main conclusions of the present study were:

1) Hypertriglyceridemia, especially when severe, is a risk factor for pancreatitis in Miniature Schnauzers. Therefore, it might be recommended that when hypertriglyceridemia is detected in dogs of this breed it should be treated and monitored.

2) Density gradient ultracentrifugation using NaBiEDTA as described in the present study is a useful screening method for the study of lipoprotein profiles in dogs. Important differences in lipoprotein profiles can be detected with this method even among dogs that have serum triglyceride and cholesterol concentrations within the reference interval.

3) Miniature Schnauzers with serum triglyceride and cholesterol concentrations within the reference interval appear to have significantly different lipoprotein profiles (mainly with regard to TRL and LDL_4) than dogs of various other breeds.

4) Specific lipoprotein classes (TRL and specific LDL fractions, mainly LDL_2) are associated with hypertriglyceridemia in Miniature Schnauzers. Changes in these lipoprotein classes are not always uniform among Miniature Schnauzers with hyperlipidemia.

5) The commercially available ultra-low-fat diet used in the present study was found to be effective in significantly reducing serum triglyceride and cholesterol concentrations within 2 months.

6) The commercially available ultra-low-fat diet used in the present study was found to be effective in shifting the lipoprotein profiles of most hyperlipidemic dogs towards those of non-hyperlipidemic dogs within 2 months.

7) A subgroup of Miniature Schnauzers appeared to not fully respond to the diet used in the present study as indicated mainly by their serum triglyceride concentrations and lipoprotein profiles. The reason for this finding is not known at this point, but differences in the pathogenetic basis of hyperlipidemia among dogs might have played a role.

8) The majority of dogs with naturally occurring pancreatitis appear to have serum triglyceride and cholesterol concentrations within their respective reference intervals. In the relatively small percentage of dogs that showed increases in serum triglyceride and/or cholesterol concentrations, those increases were generally mild. It might be recommended that profound increases in serum triglyceride and/or cholesterol concentrations in dogs with pancreatitis are unlikely to be the result of pancreatitis, and warrant further diagnostic investigation.

9) Important differences exist in lipoprotein profiles between dogs with pancreatitis and healthy control dogs. Dogs with pancreatitis have higher LDL fractions (mainly LDL_2, LDL_3, and LDL_4) and lower TRL and HDL fractions (mainly HDL_{2a} and HDL_{3c}) than healthy control dogs. These changes in lipoprotein profiles are evident in the majority of dogs with pancreatitis, even in those dogs with serum concentrations of triglyceride and cholesterol within the reference interval.

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