INVESTIGATION INTO POSSIBLE MUTATIONS OF THE SPINK1 GENE AS A CAUSE OF HEREDITARY PANCREATITIS IN THE MINIATURE SCHNAUZER

A Dissertation

by

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The Miniature Schnauzer has been anecdotally reported to have a hereditary predisposition to the development of pancreatitis. The aims of this study were to establish a true breed predisposition for the disease and to investigate a potential genetic etiology.

The first part of this study investigated breed predisposition for the development of pancreatitis. Miniature Schnauzers were found to have an odds ratio of 1.23 (P = 0.0240) for having an increased cPLI (as measured by an in-house ELISA or by Spec cPL®) serum concentration compared to the population as a whole.

The second part of this study investigated the SPINK1 gene in Miniature Schnauzers with and without evidence of pancreatitis. Three variants were found in the gene and Miniature Schnauzers that were homozygous for the variants had an odds ratio of 25 (P = 0.0067) for having clinical and biochemical evidence of pancreatitis compared to healthy individuals.

The third part of the study examined the entire canine genome using SNP scanning to investigate other genes or regions that may be associated with pancreatitis in the Miniature Schnauzer. The analysis revealed only a small region on chromosome 2 that was associated with the phenotype of pancreatitis. The SPINK1 gene is also located in this region.

The final part of the study attempted to determine if one of the variants found in the second part of the study altered the final cDNA transcript. mRNA was extracted
from the pancreas of Miniature Schnauzers known to have the variants and their cDNA was transcribed. It was found that this variant did not lead to exon skipping or truncation of the final protein transcript.

In conclusion, this study demonstrated an increased prevalence of the phenotype of pancreatitis in the Miniature Schnauzer and was able to statistically associate this phenotype with variants in the *SPINK1* gene.

Further investigation into the mechanisms that may alter the structure, function, and expression of the *SPINK1* variants found are warranted.
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chapter i
introduction and literature review

physiology of the exocrine pancreas

pancreatic enzymes can be divided into three major groups based on their function: enzymes that digest proteins, enzymes that digest carbohydrates, and those that digest lipids or fats. the main enzymes responsible for protein digestion are trypsin, chymotrypsin, carboxypeptidase, and elastase, among others. trypsin and chymotrypsin break proteins into small peptides while, carboxypeptidase cleaves off individual amino acids. carbohydrate digestion is accomplished principally by pancreatic amylase. amylase hydrolyzes starches, glycogen, and other carbohydrates primarily into disaccharides. lipids are digested by pancreatic lipase, phospholipase, and cholesterol esterase.

pancreatic secretion is under the influence of three main stimuli. the first is via acetylcholine, which is a neurotransmitter released from both the parasympathetic vagus and also from cholinergic nerves. acetylcholine is responsible for the cephalic and gastric phases of pancreatic secretion. however, it only allows for moderate release of zymogen granules into the acinar ducts. the other two stimuli are secretin and cholecystokinin. the peptide hormone secretin is released in response to acidic chyme in the duodenum by duodenal enteroendocrine cells. secretin causes the pancreas to secrete large amounts of sodium bicarbonate that, in turn, buffer the intraluminal pH. this subsequent pH change helps to create an environment that is conducive to
pancreatic enzyme activation.\textsuperscript{1} It should be noted that the intestinal mucosa is also able to secrete bicarbonate.\textsuperscript{5} Cholecystokinin is activated by the presence of food in the duodenum and its release stimulates the pancreas to release large amounts of both zymogen granules and active enzymes.\textsuperscript{2,4,5} Simultaneously, the intestinal mucosa releases enterokinase in response to the detection of chyme.\textsuperscript{5,6} Enterokinase cleaves the activation peptide off trypsinogen, leading to active trypsin.\textsuperscript{5} Trypsin, in turn, can activate more trypsinogen and also the remainder of the other zymogens.\textsuperscript{1,2,4,5,7}

Since several active enzymes can damage pancreatic acinar cells, many of these enzymes are secreted from the pancreas in an inactive pro-form known as zymogens.\textsuperscript{5} Trypsinogen and chymotrypsinogen are examples of such zymogens. Pro-elastase and prophospholipase are other examples.\textsuperscript{1} However, other enzymes such as amylase and lipase are secreted in their active form.\textsuperscript{4} In order to protect the pancreas further, the pro-enzymes and zymogens, upon being synthesized in the rough endoplasmic reticulum, are transported to the Golgi apparatus to undergo selective glycosylation.\textsuperscript{2,4,5} Lysosomal hydrolases are intracellular enzymes that could potentially lead to premature activation of zymogens and are packed separately from zymogen. The separation is based on the 6-phosphoryl mannose receptor that zymogens lack.\textsuperscript{2} Since zymogens do not have a mannose tag, they are transported separately for their eventual release into the ductal lumen by exocytosis.\textsuperscript{8} As an additional safeguard from auto-activation, pancreatic secretory trypsin inhibitor (PSTI) is co-synthesized, co-transported, and co-stored with zymogens.\textsuperscript{2,4,5} This enzyme binds prematurely activated trypsin as it is being activated.\textsuperscript{2,5}
Pathophysiology of pancreatitis

Retention and premature activation of trypsinogen within acinar cells has been suggested to be the initiating event leading to pancreatitis using electron microscopy as. A secretory block occurs on the apical membrane of acinar cells, which does not allow the release of zymogen granules from the cell. This sets the stage for the inappropriate co-localization of zymogen granules and lysosomes. During this fusion of zymogen granules and lysosomes, trypsinogen is thought to be activated to trypsin primarily by cathepsin B, although other mechanisms or mediators may also be involved. Additionally, pH in the co-localized giant vacuoles is also an important factor leading to trypsinogen activation, as a low pH can lead to auto-activation of trypsinogen. Although premature activation of trypsinogen within the acinar cell is the earliest abnormality seen, the initial intracellular trigger is unknown. There is evidence that the concentration of free calcium within the acinar cell may play an important role as an initial event. Calcium plays a role in the activation of trypsinogen to trypsin as pure inhibition of calcium signaling stops the activation of trypsinogen. Indeed, sustained increased acinar calcium concentrations are also toxic to cells. Many risk factors for pancreatitis including ductal hypertension, hypoxia, hyperlipidemia, and others have been shown to increase acinar calcium concentrations, many through an impairment of the acinar Ca²⁺-ATPase mechanism. In some instances, PSTI may stop further premature activation of trypsinogen and pancreatitis does not ensue. However, if the system and other protective mechanisms are overwhelmed, pancreatitis may develop.
The pathophysiology of pancreatitis is complex and is due not only to activated digestive enzymes causing local damage but also to systemic spread of enzymes and activation of the inflammatory and coagulation cascades, which may cause systemic inflammatory response syndrome (SIRS), disseminated intravascular coagulation (DIC), acute respiratory distress syndrome (ARDS), and multiple organ dysfunction syndrome (MODS) in severe cases. Inflammation of the pancreas leads to vasodilation and ultimately granulocyte migration. Neutrophil migration is mediated by chemokines, primarily IL-8, but can also be driven by the presence of trypsin and elastase. Additionally, neutrophils may cause a shift from apoptotic pancreatic cells to necrosis. Apoptosis, unlike necrosis, does not elicit an inflammatory response. Increased vascular permeability due to inflammation may also cause hypercoagulability, which can be a major cause of morbidity and mortality in humans.

The pancreas is very sensitive to ischemia and poor perfusion. Vasoconstriction of the pancreatic capillaries has been shown to worsen pancreatitis experimentally and is also considered to be a risk factor for the disease. Other substances such as bradykinin, C5a, substance P, TNF-α, and the activation of NF-kβ are also significant contributors to the severity and pathophysiology of pancreatitis and are currently being investigated. The role of these substances has been reviewed extensively elsewhere. Genetic mutations and variants also play a role in the pathophysiology of pancreatitis (see the following section for a comprehensive review of these mechanisms).

The pathophysiology of pancreatitis is complex and multifactorial and current investigations are also looking into the role of pancreatic duct cells. There is increasing
evidence that alterations in calcium signaling in pancreatic ductal cells play a major role in cellular damage during pancreatitis. The acinar cells secrete a viscous, proteinaceous, acidic, and Cl−-rich fluid, while pancreatic ductal cells secrete a bicarbonate-rich and low viscosity fluid that acts to neutralize protons secreted by acinar cells and keeps zymogens in their inactive form until they reach the duodenal lumen. Ductal cell secretion, as mentioned previously, is mediated in response to a meal due to cell membrane surface recognition of the two main agonists, secretin and acetylcholine. These agonists bind to G proteins via membrane receptors, which results in activation of adenylyl cyclase and eventual release of Ca2+ from intracellular stores. The basolateral surface of pancreatic ductal cells contains Na+/HCO3− cotransporters that transport both molecules into the cell in response to increased intracellular Ca2+. Additionally, CO2 is probably passively diffused through the same membrane and is converted to HCO3− via carbonic anhydrase. The luminal surface of ductal cells contains Cl−/HCO3− exchangers and the cystic fibrosis transmembrane conductance regulator protein (CFTR) channels. These allow for the transport of the intracellular HCO3− into the ductal lumen. As mentioned previously, CFTR mutations may impair this mechanism. Such mutations, as well as the presence of bile acids, ethanol, or other substances will also inhibit ductal secretion. These substances will induce intracellular bicarbonate secretion and calcium release, which is sustained. This chronic calcium release will inhibit the efficacy of physiologic secretagogues, leading to cell necrosis. Cellular death will lead to an inflammatory reaction that may further lead to premature trypsin activation closing a vicious cycle. In addition, Pallagi et al. demonstrated that
prematurely activated trypsin reduces pancreatic ductal bicarbonate secretion via PAR-2 inhibition of the apical exchanger and CFTR channel, which also decreases luminal pH leading to further activation of zymogens.\textsuperscript{35} It is likely that further discoveries of defective calcium signaling and pancreatic ductal cell malfunction will contribute to the understanding of the pathogenesis of pancreatitis.

**Hereditary pancreatitis in humans**

Despite being recognized as a potentially genetic disease in 1952, prior to the 1990’s, gallstones and alcoholism were considered the primary cause of pancreatitis.\textsuperscript{36-38} Indeed the first report of an association between alcohol and pancreatic disease was described in a 1788 case report of a thirty-four year old gentleman "who was accustomed to free living and strong corporeal exertions in the pursuit of country amusements".\textsuperscript{37} At necropsy, his pancreas was found to have a number of pancreatic calculi present.\textsuperscript{37} However, as time progressed it was increasingly recognized that children without cystic fibrosis (another recognized cause of pancreatitis) were being diagnosed with pancreatitis at an early age without the aforementioned risk factors. Additionally, cases of familial pancreatitis were being reported. In 1996 during the birth of modern genetics, using the candidate gene approach, the first genetic mutations that were associated with pancreatitis were reported and not only helped elucidate mechanisms behind the development of acute and chronic pancreatitis but also established the possible progression from acute to chronic pancreatitis.\textsuperscript{37,39}
Whitcomb et al. discovered a gain-in-function mutation in the gene (PRSS1), encoding for cationic trypsinogen that was associated with pancreatitis in families with pancreatitis. The variant discovered was a substitution mutation that replaced an arginine with a histidine at residue 117. One of the mechanisms of protecting the pancreas from autodigestion is the ability of trypsin to auto-digest itself by breaking peptide chains at arginine or lysine residues. X-ray crystallography demonstrated that the R117 residue site was critical for self-autolysis and, as such, a substitution would not allow for trypsin-like hydrolysis of the chain connecting the two domains required for trypsin activity, thus creating a “super-trypsin” that is resistant to auto-degradation. Since this initial discovery, approximately 20 gain-of-function mutations have been discovered in the gene and have been identified in 80% of families with hereditary pancreatitis. These discoveries have been pivotal in the understanding of ductal and acinar cell pathophysiology during both acute and chronic pancreatitis. Studies of these mutations have demonstrated that acute pancreatitis can lead to chronic pancreatitis and that trypsin is the key molecule responsible for the initiation of pancreatitis. Additionally, many of these mutations are near calcium binding sites on the protein and, as a result, the critical role of calcium as the key regulator of trypsin has also been further explained.

The SPINK1 (serum protease inhibitor, Kazal type 1) gene encodes for pancreatic secretory trypsin inhibitor (PSTI), which is an acute phase protein that is present in acinar cells, especially during times of inflammation. PSTI represents another protective mechanism against premature trypsin activation, by inhibiting prematurely activated
trypsin molecules and thus preventing further autoactivation.\textsuperscript{36,40} Witt and later Pfutzer \textit{et al.} demonstrated an association with the N34S mutation in children and adults with idiopathic pancreatitis.\textsuperscript{43,44} These and other studies demonstrated that while the homozygous variant was closely associated with the phenotype, an increased prevalence of the N34S variant (and others) and a high percentage of heterozygotes within pancreatitis cohorts raised some speculation as to \textit{SPINK1’s} role as a causative mutation.\textsuperscript{36,40,43} However, a meta-analysis of 24 studies of the importance of the N34S variant in chronic pancreatitis revealed strong evidence that mutations of \textit{SPINK1} are more closely tied to idiopathic pancreatitis (OR: 14.97) and chronic pancreatitis (OR: 11.00) than even alcohol use (OR: 4.98).\textsuperscript{21,45} Other studies have documented other variants including the Ans34Ser and a splice variant IVS3+2T>C, which are common.\textsuperscript{36} The role of \textit{SPINK1} is causative in homozygous individuals. However, heterozygocity is only associated with pancreatitis when other variants in other susceptibility genes (\textit{PRSS1}, \textit{CFTR}, etc.) are present. This is extremely important in the increased recognition of families with a variable number of multiple germline pathogenic gene variants that affect trypsin regulation.\textsuperscript{21,36,40,46}

In humans, variants of the cystic fibrosis transmembrane conductance protein, which is coded by the gene \textit{CFTR}, have also been associated with recurrent acute or chronic pancreatitis.\textsuperscript{47-49} \textit{CFTR} is an anion channel that allows for the movement of chloride and bicarbonate across the apical membrane of the pancreatic duct cell (but they also occur in lungs, sweat glands, and other organs), which causes an increase of pH and flushing of pancreatic zymogens into the duodenal lumen.\textsuperscript{40} There are over 2,000 known
mutations of this gene and, in humans mutations are classically associated with cystic fibrosis.\textsuperscript{36,48} Cystic fibrosis is a syndrome that is characterized by the presence of thick and abnormal secretions in the lungs. However, patients may also present with advanced chronic pancreatitis and malabsorption, depending on the mutations present in the gene.\textsuperscript{36,47,48} The main defect in this gene alters the protein, which acts as a protein channel that regulates the movement of chloride ions in and out of cells.\textsuperscript{47,48} In patients with genotypes that cause pancreatitis, the mutations result in defective bicarbonate secretion from pancreatic ductal cells, which is also responsible for flushing trypsin out of the pancreatic duct. Variants that cause dysfunction of this protein allow for retention of zymogens within the duct in which they can become activated and lead to autodigestion of the pancreas.\textsuperscript{47} In humans, mutations of the \textit{CFTR} gene are classified based on the severity of protein modification and the presence of a severe pathogenic variant (\textit{CFTR\textsubscript{SEV}}) on each allele leads to the clinical condition of cystic fibrosis. However, the presence of only one allele (\textit{CFTR\textsubscript{SEV}}) and a bicarbonate-defective variant (\textit{CFTR\textsubscript{BD}}) on the other allele, or also the presence of two bicarbonate-defective variants (\textit{CFTR\textsubscript{BD}/CFTR\textsubscript{BD}}) leads to pancreatitis.\textsuperscript{36} Additionally, in humans, hereditary pancreatitis is strongly associated with the N34S mutation of the \textit{SPINK1} gene. However, few people with mutations actually develop the clinical syndrome.\textsuperscript{48} Studies have now shown that the presence of the N34S mutation and a co-mutation with the \textit{CFTR} variant p.R75Q is significantly (OR: 3.4) associated with chronic pancreatitis.\textsuperscript{48} Thus, \textit{CFTR} variants on their own may cause pancreatitis as well as potentially acting as a modifier gene for \textit{SPINK1} mutations or vice versa. Indeed, the combination of a
mutation that increases trypsinogen activation (CFTR/PRSS1) along with a mutation that alters the ability of the pancreas to protect itself (SPINK1) appears to the common pattern responsible for potentially 1/3 of recurrent acute and chronic pancreatitis cases in humans.21,36,46 This is currently being investigated in the NAPS2 (North American Pancreatitis Studies) clinical study, which aims to evaluate a large cohort of patients with pancreatitis using genome-wide association studies and next generation sequencing to help elucidate the complex relationship between the three main genes implicated in hereditary pancreatitis (i.e., PRSS1, CFTR, and SPINK1).46

Several other genes have been evaluated in human patients with pancreatitis and are now largely considered disease modifiers as they do not cause pancreatitis on their own but play a role in families with complex and multiple germline gene variants.21,36,40,46 For example, the gene CASR encodes for the calcium sensing receptor that is located on the cell membrane of numerous cells.50 Its job is to sense calcium concentrations outside of the cell and to initiate a variety of cell mechanisms to respond to the calcium level.21,40,50 Variants of the CASR gene are commonly associated with pathogenic variants of CFTR and suggest a misinterpretation of the calcium level and premature opening of CFTR channels during times of high ductal calcium concentrations, which can cause premature activation of trypsin.21,40

Chymotrypsin C, which is encoded by CTRC, is a minor pancreatic enzyme that is co-synthesized with trypsinogen.40,51 It has been speculated that besides it’s digestive properties, chymotrypsin B is also able to degrade trypsin when activated, thus belonging to the body's defense against pancreatic autodigestion.40 Variants found within
this gene are not thought to be causative of pancreatitis alone, but they are often associated other mutations in the CFTR or SPINK1 gene and are part of the pathogenetic process in families with multiple gene variants that cumulatively put them at risk for pancreatitis.\textsuperscript{40,51,52}

Claudin-2, which is a tight junction protein expressed in the proximal pancreatic duct, forms cation-selective ion and water channels between endothelial cells and is thought to be responsible for allowing transport of water and sodium into the pancreatic duct to match chloride and bicarbonate secretion through the CFTR protein.\textsuperscript{3,53,54} A risk allele T of the gene CLDN2 is associated with nearly half of all men with alcoholic pancreatitis.\textsuperscript{3,21,53,54} Furthermore, immunohistochemical studies of surgical specimens show increased levels of this protein within acinar cells and an altered localization, but the definitive pathophysiology leading to pancreatitis has yet to be determined.\textsuperscript{21}

In conclusion, multiple genes have been implicated in humans with pancreatitis. Some genes are believed to be causative, while many others may be silent unless combined with other risk variants from other genes or environmental factors such as alcohol abuse. Indeed, human pancreatitis is emerging to be an exciting disease to study both complex genetic traits and epistasis genetics.\textsuperscript{21}

\textbf{Pancreatitis in the dog}

Pancreatitis in the dog has been divided into two categories, acute and chronic, which can only be differentiated based on histopathology.\textsuperscript{4,5,7,55,56} In contrast to humans, there is no consensus for the definitions of acute versus chronic pancreatitis in the
To complicate matters further, temporal relationships appear to exist in the clinical presentation of patients with “acute or chronic pancreatitis” and various other terms are commonly assigned to these manifestations. Thus, risk factors and etiologies overlap in the literature and for the purpose of this study they are not differentiated unless noted.

Acute pancreatitis usually considered to be a temporal and reversible disease, which is often associated with edema and neutrophilic infiltration, and in severe cases can also be associated with pancreatic necrosis. In contrast, chronic pancreatitis is characterized by permanent changes, such as fibrosis and parenchymal atrophy and is mostly associated with lympho-plasmacytic inflammation, yet patients with chronic pancreatitis may show an “acute” clinical manifestation. Generally, acute and chronic pancreatitis cannot be differentiated clinically and many cases may indeed represent “acute-on-chronic” disease. This is supported by a recent report where 58/63 dogs had concurrent acute and chronic changes on histopathology, while only 5 dogs had purely acute disease.

The etiologies and risk factors of acute pancreatitis in the dog are not very well understood. In the majority of dogs diagnosed with acute pancreatitis, an underlying condition cannot be identified and the cause is considered idiopathic. Signalment has been implicated as a risk factor for pancreatitis. In two older studies, dogs that were 6 years or older were identified to be at increased risk. Older studies did not identify a sex predilection. Another study found that neutering increased the odds of pancreatitis in dogs, similarly to the findings of a previous study.
Breed predisposition is generally anecdotal but a few reports can be found in the literature.8,59,60 There are only a few papers that discuss breed in association with histologically confirmed pancreatitis.3,17,61 Hess et al. only identified the Yorkshire Terrier (OR: 41.8; 95%CI: 1.0 - 1,731) in her study. Notwithstanding the confidence intervals found, the control group was significantly younger, more likely intact and of a larger breed, and were more likely underweight.8 In 1993 Cook et al. reported a retrospective study of 101 dogs and made the observation that “Terrier” breeds were at an increased risk. However, it should be noted that in that study, only 37 of the dogs had histopathological evidence and the other cases were diagnosed with pancreatitis using less than ideal criteria. Additionally, the results of histopathology were not discussed. However, dogs were placed in groups based on the AKC groups and it should be mentioned that the AKC grouping does not necessarily place genetically related dogs together but primarily assigns dogs based on their intended use and not their genetic relatedness.59 A very old paper mentions 10 dogs with chronic pancreatitis and their respective breed. However, there were no controls mentioned in this paper, nor does the author try to associate any specific breed with this condition.62 A recent paper evaluated 200 dogs at post-mortem in order to assess the prevalence of chronic pancreatitis in the UK.55 This study identified Cavalier King Charles Spaniels, Collies, and Boxers as having an increased risk for chronic pancreatitis, however in 24.5% of the cases, the pancreas was too autolyzed to interpret, especially in large breed dogs, potentially biasing the results.55 A unique syndrome in Europe has been recognized in English Cocker Spaniels. These dogs often have keratoconjunctivitis sicca or protein-losing
nephropathy and concurrent chronic pancreatitis. The histopathology in these dogs is reported to be unique and is characterized by perilobular and periductal progressive fibrosis and is characterized on immunohistochemistry by T-cell dominated lymphocyte infiltration with plasma cells that stain positive for IgG4. A recent study of chronic pancreatitis found that pure bred dogs were 3.35 times more likely to have chronic pancreatitis and also were more likely to belong to an AKC toy and non-sporting breed (OR: 2.83). These dogs were also 10.2 times more likely to have clinical as opposed to subclinical chronic pancreatitis. Lem et al. published a report examining dietary factors and pancreatitis, and found that Miniature Schnauzers, Yorkshire Terriers, and “Terriers” in general had an increased risk for developing pancreatitis. In these cases, pancreatitis was diagnosed based on a variety of clinical criteria. Interestingly, despite a strong clinical suspicion that Miniature Schnauzers may be predisposed to pancreatitis, this has not yet been substantiated in the literature. Bostrom et al. reported on a group of dogs with chronic pancreatitis that when compared to the general necropsy population were more likely to be neutered (OR: 4.64) and female (OR: 2.17).

Dietary indiscretion has long been implicated as a risk factor for pancreatitis in dogs. In a recent retrospective study showed that the ingestion of unusual food items and a history of eating table scraps increased the odds for pancreatitis, although no specific food was implicated. This same study also demonstrated that overweight dogs were predisposed to pancreatitis although this may have been related to more frequent dietary indiscretion in this group of dogs. It has been shown that high fat diets given
experimentally can induce pancreatic enzyme secretion, but histopathology was not performed to determine whether the pancreatitis had ensued.63

Metabolic and endocrine diseases are also implicated as a cause of pancreatitis.5,7,17 Conditions such as hyperadrenocorticism and hypothyroidism have been associated with an increased risk, but a cause and effect relationship has not been definitively proven.8,17,59,64 Diabetes mellitus is also associated with pancreatitis but may be more of consequence of chronic pancreatitis as the current literature suggests that as many as 30% of dogs with diabetes mellitus may have chronic pancreatitis.56 Dogs with chronic pancreatitis in one study were 13.5 times more likely to have concurrent endocrine diseases than the general necropsy population.57 Of the 61 dogs with chronic pancreatitis, 12 had diabetes mellitus, 4 had hypothyroidism, and 1 each had hyper- and hypoadrenocorticism.57 Increased serum triglyceride concentrations >900 mg/dl, at least in the Miniature Schnauzer, has also been shown to be associated with increased Spec cPL concentrations and several of the endocrine diseases mentioned are associated with secondary hyperlipidemia, which may be responsible for the increased risk for pancreatitis.17,65

Drugs are thought to play a role in acute pancreatitis in the dog, but conclusive evidence is lacking for many implicated drugs. Drugs that have been implicated in the etiology of pancreatitis include calcium, L-asparaginase, potassium bromide, phenobarbital, and antimonial drugs, however evidence for each is mostly anecdotal.17,66,67 It has recently been reported that dogs being treated with potassium bromide, phenobarbital, or both drugs in combination, had an increased risk for having
an increased serum cPLI concentration.\textsuperscript{67} Also, a case report implicated N-methyl-

glutamine as a cause of acute pancreatitis, however a subsequent abstract found no association of the administration of this drug with increased Spec-cPL concentrations in a larger population of dogs.\textsuperscript{68} Experimentally, a number of procedures involving the administration of caerulein, carbamylcholine, and scorpion venom induced pancreatitis in dogs.\textsuperscript{7} A study looking into naturally infected dogs with Leishmania that were treated with meglumine antimonite did not, however, reveal an increased serum Spec cPL concentration or clinical signs compatible with pancreatitis.\textsuperscript{68}

Infections only rarely are considered the cause of pancreatitis in the dog. However, isolated reports of \textit{Babesia canis}, \textit{Klebsiella pneumonia}, and \textit{Pseudomonas aeruginosa} pancreatic infections can be found.\textsuperscript{5,69,70} In a recent retrospective study of dogs, primarily in Texas, infected with \textit{Heterobilharzia americana} that had undergone necropsy, 35\% had trematode eggs in the pancreas associated with inflammation.\textsuperscript{71} This finding may implicate this organism as a potentially under-reported cause of pancreatitis but further studies are warranted. Finally, a recent study from Greece demonstrated a serum Spec cPL concentration suggestive of pancreatitis in 20\% of dogs with naturally infected \textit{Ehrlichia canis}, however clinical signs were minimal in affected dogs.\textsuperscript{72}

Hypocalcemia has been implicated as a risk factor for pancreatitis, but occurs uncommonly in acute pancreatitis.\textsuperscript{73} Approximately 40\% of dogs with chronic pancreatitis (diagnosed by histopathology) have a low serum ionized calcium.\textsuperscript{57} However, increasing evidence indicates that hypocalcemia is common in critically ill dogs and may not be specific to pancreatitis. Rather, hypocalcemia in critically ill
patients is considered to have a multi-factorial pathophysiology that includes derangement of parathyroid hormone and magnesium, changes in chelation or protein bound calcium, acid-base imbalances, and third-space loss, among others.\textsuperscript{73}

Also, hypoperfusion, ischemia, trauma, and mechanical duct obstruction can all cause pancreatitis in the dog.\textsuperscript{5,17} There is debate regarding whether handling at surgery can cause pancreatitis or whether the isolated cases reported are caused by poor perfusion of the pancreas during anesthesia. However, it is of note that pancreatitis was not found to be a complication in a published report involving laparoscopic pancreatic biopsy.\textsuperscript{74}

Finally, genetics are a likely risk factor for canine pancreatitis and are the topic of this thesis. A study evaluating the cationic trypsinogen gene in Miniature Schnauzers with clinical findings consistent with pancreatitis did not reveal any mutations or variants.\textsuperscript{75} Since the Miniature Schnauzer breed has frequently been associated with pancreatitis, the objective of this study was to evaluate if the breed is at risk and also what gene(s) may be associated with the phenotype of pancreatitis seen in these dogs.

The clinical presentation of pancreatitis can vary considerably in dogs. Dogs may be essentially asymptomatic, have mild clinical signs, or may develop life-threatening DIC and shock.\textsuperscript{76} Others may also develop icterus from extra-hepatic bile duct obstruction secondary to pancreatitis. There are no clinical findings that are pathognomonic for pancreatitis in the dog.\textsuperscript{76} Clinical signs have been reported in dogs with acute pancreatitis, which may include anorexia (91%), vomiting (90%), weakness (79%), polydipsia and polyuria (50%), and diarrhea (33%).\textsuperscript{8,59,77} Dogs with chronic
pancreatitis had similar clinical signs, including vomiting (88%), hyporexia (98%), lethargy (93%), diarrhea (49%), fever (24%), and abdominal pain (35%).\textsuperscript{57} Physical examination findings in dogs with fatal severe acute pancreatitis included dehydration (97%), abdominal pain (58%), fever (32%), and icterus (26%).\textsuperscript{77}

Serum lipase and amylase activities were the original serum markers used for the diagnosis of pancreatitis and are considered useful for the diagnosis of pancreatitis in humans.\textsuperscript{78-80} However, in dogs the reported sensitivity for lipase and amylase activities are 41-69\% and 32-73\%, respectively, while the specificities are closer to 50\% for each.\textsuperscript{7,17,78-83} Lipase originates from many cell types and organs, including the pancreas, liver, stomach, and others, and is thus not specific for exocrine pancreatic disease.\textsuperscript{3,16,56,78-80,84} Indeed, serum lipase activity can be increased in dogs with gastroenteritis, liver disease, renal failure, and various other conditions.\textsuperscript{3,16,78,79,85,86} Furthermore, lipase activity can be increased after the administration of dexamethasone. Therefore, the use of lipase activity is no longer recommended for the diagnosis of pancreatitis in the dog.\textsuperscript{7,16,56,79}

The serum cTLI (canine trypsin-like immunoreactivity) assay was developed and validated for the diagnosis of exocrine pancreatic insufficiency and is considered the gold standard for the diagnosis of this disease.\textsuperscript{7,16,17,79,80,84,87,88} Serum cTLI concentration is pancreas-specific and during pancreatitis serum cTLI concentration increases markedly. However, it also falls quite rapidly, thus limiting the clinical usefulness of this marker for the diagnosis of pancreatitis. The reported sensitivity of serum cTLI concentration is 36-47\% and this parameter is also not considered to be
specific for pancreatitis, thus it is generally not recommended for the diagnosis of pancreatitis in dogs.\textsuperscript{5,16,17,79,80,84,85}

Canine pancreatic lipase immunoreactivity (cPLI) measures the concentration of pancreatic lipase, as opposed to total lipase activity.\textsuperscript{80,91-93} The original assay for the measurement of cPLI was an in-house ELISA, but has now been replaced with the commercially available assay, Spec cPL.\textsuperscript{94} Information on the original assay has been extensively reviewed elsewhere.\textsuperscript{80} The Spec cPL differs from the original assay for the measurement of cPLI as the Spec cPL assay uses a monoclonal antibody as opposed to a polyclonal antibody used in the original assay.\textsuperscript{80,84} The assays show good correlation, but the reference intervals are different.\textsuperscript{16} A serum Spec cPL concentration of $>400$ µg/L is considered diagnostic of pancreatic inflammation.\textsuperscript{17,79,80,84} The in-house SNAP cPL exhibits good correlation with the Spec cPL, however all positive results should be confirmed by the measurement of Spec cPL in serum as an “abnormal” result of the SNAP cPL may indicate a serum concentration in the diagnostic range or also the gray or inconclusive range.\textsuperscript{17,76,80,84} Multiple studies, including a large multi-institutional study, have evaluated the Spec cPL for sensitivity and specificity using blinded clinical diagnosis by a panel of internists.\textsuperscript{58,79,95-97} The sensitivity of Spec cPL in this study was reported to range between 63.6 and 71\% with a specificity ranging between 86 and 97.5\% using 400 µg/L as the diagnostic cut-off value.\textsuperscript{16,58,79,95,97} These studies have been reviewed extensively elsewhere.\textsuperscript{80} Serum Spec cPL concentrations have been reported to be quite stable for days at room temperature, weeks at 4°C, and years at -20°C.\textsuperscript{17,57,80,98} The half-life of the assay is approximately 2 hours and thus increased concentrations can
be cleared in <24 hours if no further leakage occurs.\textsuperscript{99} Despite higher numbers that are clinically correlated with severity in some cases, there is no current evidence to suggest that the higher the serum cPLI concentration, the more severe the disease.\textsuperscript{76,80} Indeed a recent study demonstrated that the Spec cPL concentration must change by \( \sim 452.6\% \) to be considered significantly different. Unlike other GI function tests fasting is not essential for the performance of this assay.\textsuperscript{100} Studies have demonstrated that the use of steroids or the presence of chronic kidney disease do not affect results.\textsuperscript{98,101} However, a recent study demonstrated an increased Spec cPL in dogs with hyperadrenocorticism (as diagnosed by clinical signs and ACTH stimulation test) compared to healthy dogs, 491.1 µg/L vs. 74.2 µg/L), which may indicate a high rate of subclinical pancreatitis in dogs with hyperadrenocorticism.\textsuperscript{102} Haworth et al. looked at the diagnostic accuracy of the SNAP cPL and Spec cPL in dogs presenting for acute abdomen.\textsuperscript{103} This study reported an agreement between a positive SNAP cPL and a Spec cPL \( \geq 400 \) µg/L and a “clinical diagnosis” to be associated with \( \kappa = 0.33 \) and \( \kappa = 0.43 \), respectively, concluding that these assays may be associated with a false positive diagnosis of pancreatitis in dogs with abdominal disease.\textsuperscript{103} However, no histopathology was obtained from dogs from either study to rule out any evidence of pancreatitis.\textsuperscript{102,103} In a study evaluating Spec cPL and serum total lipase activity measured with the 1,2-o-dilauryl-rac-glycerol-3-glutaric acid-(6'-methylresorufin) ester (DGGR) substrate and abdominal ultrasonography, there was poor agreement of either assay with abdominal ultrasound.\textsuperscript{104} Other diagnostic tests have been evaluated for the diagnosis of pancreatitis but are not currently recommend
due the decreased sensitivity and specificity and/or limited clinical availability.\textsuperscript{17,84,85,105,106}

Abdominal radiography is commonly used in clinical patients for evaluation of acute vomiting and abdominal pain, however it is associated with a poor sensitivity (24\%) and specificity for pancreatitis.\textsuperscript{77} Classically, decreased serosal detail in the cranial right abdomen and displacement of other organs in the region have been associated with pancreatitis.\textsuperscript{76} The true value of abdominal radiography is probably to rule out other causes of acute abdomen when evaluating a dog with possible pancreatitis.

Ultrasonography is currently considered the imaging modality of choice for the diagnosis of pancreatitis in the dog. There is however considerable variation in the diagnostic sensitivity and specificity based on the skill and experience of the operator and the quality of the equipment. The specificity is thought to be high, however the highest sensitivity that has been reported is 68\% for severe disease.\textsuperscript{77} In a recent study of dogs undergoing abdominal ultrasound for suspicion of pancreatitis, some dogs diagnosed with pancreatitis by ultrasonography actually had no evidence of pancreatitis on histopathology.\textsuperscript{74} Indeed, the overall agreement between histopathology and ultrasound diagnosis was only 23.1\% in this study.\textsuperscript{74} Generally, ultrasound is thought of as supportive evidence with other findings on physical examination, history, and the measurement of cPLI concentration for making the clinical diagnosis of pancreatitis.\textsuperscript{16,17,84}

Computed tomography (CT) is the imaging modality of choice for diagnosing pancreatitis in human patients but has been poorly evaluated in dogs at this point and
Further studies are indicated.\textsuperscript{107} Other studies looking at endoscopic ultrasound and retrograde cholangio-pancreatography have been evaluated but are not generally available.\textsuperscript{108-110} In an experimental model of pancreatitis, quantitative contrast-enhanced ultrasonography (CEUS) was successfully used to differentiate pancreatitis from normal pancreas.\textsuperscript{111}

Histopathology is considered the gold standard for the diagnosis of pancreatitis. However, it has recently been questioned whether it should be considered the standard for “clinically significant” pancreatitis.\textsuperscript{57,61,97,112} Additionally, exclusion of pancreatitis may be difficult as studies have shown that lesions may be highly localized and missed with routine biopsy.\textsuperscript{61} Thus, multiple sections must be taken if feasible. Finally, pancreatic biopsy is considered invasive and costly and is not routinely performed. Further studies are needed to determine whether there is correlation with specific histopathology and clinical signs or severity. A recent study did however associate higher histological scores for pancreatic and peripancreatic fat necrosis in cases of clinical chronic pancreatitis as opposed to asymptomatic cases.\textsuperscript{57} A histological grading scheme has been developed for assessing pancreatitis in dogs.\textsuperscript{16,112}

The therapy for canine pancreatitis varies according to clinical severity but generally centers around fluid therapy, pain control, anti-emetic and -nausea therapy, and nutritional support.\textsuperscript{3-5,7,16,17,56,76} Fluid therapy is essential in severe cases because of an increased vascular permeability and progressive reduction of capillaries as a result of acinar injury.\textsuperscript{16} There are no clinical studies evaluating different fluid types in dogs with pancreatitis though one clinical study demonstrated that crystalloids may be insufficient.
and experimental work suggests that dextrans or hypertonic saline may be of benefit.\textsuperscript{16,113-115} Analgesic support is an essential component of the management of dogs with pancreatitis.\textsuperscript{4,5,7,16,17,56,76} Frequently, opioids are considered first line therapy tailored to the severity of disease. Anti-nausea drugs are commonly employed for the treatment of canine pancreatitis and include maropitant, ondansetron, dolasetron, and metoclopramide. There are no clinical studies evaluating the efficacy of these drugs in pancreatitis.

Traditionally, dogs treated for pancreatitis have often been fasted based on the assumption that decreased food in the duodenum would “rest” the pancreas. The majority of human medical and experimental evidence and one small study in dog suggest that enteral nutrition is preferred over parenteral for acute pancreatitis.\textsuperscript{116} Dogs are often fed low fat diets during and post recovery. though studies have demonstrated that there is no measurable difference in pancreatic secretion regardless of the fat content of food.\textsuperscript{16} There is evidence that Miniature Schnauzers who are prone to hypertriglyceridemia many benefit from a long-term low fat diet and may decrease the risk of recurrent pancreatitis.\textsuperscript{117} The treatment for pancreatitis dogs has been reviewed elsewhere and further studies are indicated for evaluation of treatment modalities.\textsuperscript{16}
CHAPTER II
ASSOCIATION BETWEEN BREED AND INCREASED SERUM SPEC CPL CONCENTRATIONS IN DOGS

Introduction

Breed predilection for pancreatitis is largely anecdotal in the literature. Very few papers exist that associate breed with histopathologically confirmed pancreatitis due to the invasiveness of the procedure. In an early study, Hess et. al identified only the Yorkshire Terrier as having a breed predisposition however the control group was significantly different in age and sex. Cook et. al reported a retrospective study of 101 dogs and noted that “terrier” type breeds were at an increased risk, although many cases were diagnosed with less than ideal criteria. A paper from the UK evaluated chronic pancreatitis and identified Cavalier King Charles Spaniels, Collies, and Boxers as being at an increased risk. Finally a recent epidemiological study that evaluated risk factors for pancreatitis identified Miniature Schnauzers and Yorkshire Terriers as being predisposed. There has been no study to date that examines breed association for pancreatitis in a large population of dogs.

In humans, pancreatitis was originally thought to be caused by either alcoholism or gallstones, however children and families were diagnosed with pancreatitis that did not have these predisposing factors. Subsequent studies were able to identify genetic mutations in the PRSS1 (cationic trypsinogen), SPINK1 (pancreatic secretory trypsin inhibitor), CFTR (cystic fibrosis transmembrane conductance protein), and others
that have proven to be causative. In dogs, there appears to be a breed-related tendency and it is likely that a gene or genes are involved in the pathogenesis of pancreatitis in some breeds. Little work has been done to search for candidate genes in dogs. However, previous studies did not yield any abnormalities in the cationic trypsinogen, anionic trypsinogen, or lipoprotein lipase gene in Miniature Schnauzers with pancreatitis.

Due to the difficulties in diagnosing pancreatitis, sensitive and specific markers for the diagnosis of pancreatitis have been developed. The original in-house cPLI was replaced with the commercially available Spec cPL assay. This assay has a reported sensitivity of 63.6 – 71.0% and a specificity of 86.0 – 97.5% at a diagnostic cut-off of 400 µg/L. Thus, this diagnostic marker may serve as an alternative and non-invasive diagnostic test for the purpose of larger epidemiological studies.

The aim of this study was to investigate breed associations with an increased Spec cPL concentration within a population of dogs being tested for suspicion of pancreatitis. We hypothesized that breeds with a statistically increased odds ratio for an increased Spec cPL may be breeds that are genetically predisposed to the development of pancreatitis and further investigation into genetic etiologies in these breeds may be warranted.

**Materials and methods**

The database at the Gastrointestinal Laboratory at Texas A&M University was reviewed from October 30, 2006 to October 30, 2009 for submissions for the
measurement of pancreatic lipase immunoreactivity concentrations by Spec cPL. Data was collected from dogs that had a serum Spec cPL concentration $\geq 400 \mu g/L$ (considered diagnostic for pancreatitis) and those with a serum cPLI concentration within the reference interval of the assay ($\leq 200 \mu g/L$). Additional data collected included the signalment information of breed, age, and gender. Cases were excluded if the breed, age, and gender were not reported as well as the submission form being marked as “mixed breed”. Patients with a serum Spec cPL concentration of 201 – 399 $\mu g/L$ were also excluded as these represent results within the grey zone of the assay. Finally, breeds with less than 15 individuals represented were excluded. This data was collected and put into spreadsheet form for statistical analysis.

All data were analyzed for normality using a Kolmogorov-Smirnov, D’Agostino and Pearson, and a Shapiro-Wilk test. A Mann-Whitney test was used to compare ages between the dogs with serum Spec cPL concentrations $\geq 400 \mu g/L$ to those with a serum Spec cPL within the reference interval. A two-sample test of proportion was used to compare proportions based on their gender.

A $\chi^2$ or Fisher’s Exact test was used to compare the proportion of dogs (within each individual breed) with increased Spec cPL concentrations compared to the proportion of dogs with increased Spec cPL concentrations within the total study population (excluding the breed of interest). For each test, odds ratios and their respective 95% confidence interval were also calculated. Due to the large number of breeds being evaluated, P values were adjusted for multiple testings using the Benjamini and Hochberg’s False Discovery Rate test.
In breeds where the odds ratio was found to be greater than one, a logistic regression model was developed and fitted to adjust for differences found in age and sex that may influence the results. Individual models were tested for Goodness-of-fit with a Hosmer-Lemeshow $\chi^2$ test. Each model was also tested for multicolinearity, overdispersion ($\psi$), and interaction between variables. All data were analyzed with two commercially available software packages.$^{b,c}$

Additionally, breeds with statistically significant odds ratios for not having an increased Spec cPL concentration compared to the breed population as a whole were also recorded and their 95% confidence intervals were calculated.

**Results**

In total, 16,404 dogs were identified and fulfilled the study criteria. This population of dogs was represented by 96 individual breeds. Within this population, 3,482 (21.2%) had a serum Spec cPL concentration $\geq$ 400 $\mu$g/L. Dogs with a serum Spec cPL concentrations $\geq$ 400 $\mu$g/L were significantly older (median age: 9 years; interquartile range: 6-12 years) than dogs with Spec cPL concentrations within the reference interval (median: 6 years; interquartile range: 3-9 years; $p <0.0001$)(Figure 1).
Figure 1: Comparison between ages of dogs stratified by Spec cPL concentration. The median age of the dogs with a Spec cPL $\leq$ 200 µg/L was 6 years (interquartile range: 6-12 years) versus 9 years (IQR: 3-9 years) of age in dogs with a Spec cPL $\geq$ 400 µg/L.
There were no differences in the proportion of males (50.7%) vs. females (49.3%) in the dogs with a serum Spec cPL concentration within the reference interval (= 0.1216). However, there were significantly more females (53.4%) than males (46.6%) in the dogs with a Spec cPL concentration µg/L (< 0.0001).

Models were constructed to adjust for the effects of age and gender. After model adjustment using logistic regression, 13 breeds were identified as having an increased odds ratio for having an increased serum Spec cPL concentration. The top five breeds with an increased serum cPLI concentration ≥ 400 µg/L were Miniature Pinschers (101/208; 48.6%; OR: 3.64), Boxers (236/669; 35.3%; OR: 3.49), Keeshonds (29/52; 55.8%; OR: 3.24), Pomeranians (106/249; 42.6%; OR: 2.53), and Eskimos (28/61; 45.9%; OR: 2.02). Other dog breeds with increased odds ratios included Fox Terriers, Shetland Sheepdogs, Cocker Spaniels, Dachshunds, Yorkshire Terriers, Rottweilers, Standard Schnauzers and Miniature Schnauzers. All P values remained significant after model adjustments for multiple testing with the exception of the Standard Schnauzer. The complete data set is summarized in Table 1. Additionally, 15 breeds were identified as having significant odds ratios for not having an increased Spec cPL proportion compared with the rest of the population (Table 2).

Discussion

This is the first paper to evaluate the association between breed and an increased serum Spec cPL concentration in a large number of dogs for which a sample had been
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<th>Adjusted Odds Ratio</th>
<th>95% Confidence Interval</th>
<th>Adjusted P value</th>
<th>Breed</th>
<th>N</th>
<th>GOF P value</th>
<th>Ψ</th>
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**Table 1:** Breeds with a significant odds ratio for having an increased Spec cPL concentration ≥400 µg/L. Note that the OR for Standard Schnauzers did not reach statistical significance after the differences in age and sex were taken into account. GOF = goodness-of-fit, Ψ = psi coefficient for overdispersion.
<table>
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<th>P value</th>
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<td>0.0352</td>
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</table>

**Table 2:** Odds ratios and P values for dog breeds with decreased odds for an increased Spec cPL concentration. N = number of individuals within the breed.
submitted for testing by their veterinarian. As these are clinical patients it is likely that the majority of these samples were submitted for clinical suspicion of pancreatitis although this cannot be verified. It is interesting to note that the percentage of “positive” patients within this population (21.2%) being tested was high. This could be due to increased recognition and acumen of veterinarians of the disease process, high prevalence of the disease or may reflect the increased sensitivity of this assay. False positive test results are another potential reason for the high percentage of positive patients, however recent studies evaluating the Spec cPL found the specificity to be between 86 and 97.5%.\textsuperscript{58,79,80,95-97} While histopathology is considered the gold standard for pancreatitis diagnosis, this has recently been questioned as to whether this should be the diagnostic standard for clinically significant disease.\textsuperscript{57,61,97,112} A recent study demonstrated that focal areas of pancreatitis may be easily missed on biopsy and many dogs that had been euthanized for other reasons actually had histopathological evidence of pancreatitis.\textsuperscript{61} Indeed, anecdotally there are many patients with abnormal serum Spec cPL concentrations that do not have any clinical signs of pancreatitis.\textsuperscript{80} In this study, we chose to use the most sensitive and specific test that could be performed in a large population of dogs.

Further studies looking at increased serum Spec cPL in large populations of healthy dogs would be interesting to compare the prevalence of pancreatitis as well as the breed association found in this study.

The findings of this study support earlier studies that dogs with evidence of pancreatitis tend to be older in age.\textsuperscript{8} We have also identified a higher proportion of
female dogs having an increased prevalence compared to male dogs, which was similar to a recent study. This study did not evaluate the effect of neutering. Due to these signalment influences found in this population, the authors used this data to construct models that take these effects into consideration when evaluating whether a certain breed had an increased prevalence. This study identified an interesting group of dogs with a breed predilection for an increased Spec cPL concentration.

Three of the top five breeds were Keeshonds, Eskimos, and Pomeranians, which are closely related. They appear to have all descended from a historic group of dogs known as German Spitzs. This may indicate a genetic etiology in these breeds and further investigation of this group of breeds is warranted. Other identified breeds were breeds that have been reported previously or anecdotally to be at an increased risk. Surprisingly, the Miniature Schnauzer, typically considered the prototype breed for pancreatitis, did not have the highest prevalence. However, the data on the Miniature Schnauzer may be influenced by bias and a resultant increased rate of testing by veterinarians due to clinical experience and anectodal reports. Miniature Schnauzers with clinical signs of pancreatitis and increased Spec cPL have been found to often be homozygous for several variants of the SPINK1 gene in an earlier study. A later study disputed this finding but this study did not use consistent criteria for diagnosis and also did not test the control group for the disease and the intron exon boundary variant was not evaluated.

Several dog breeds were identified with a lower risk for having an increased Spec cPL concentration. Most of these breeds were larger breeds and none were breeds that
have been previously identified or suggested as being predisposed. Models were not constructed for these breeds as they were not the primary aim of this study but were included for clinical interest.

There are several important shortcomings of this study. These include not identifying the reason for clinical testing of the dogs. Additionally, although an excellent assay, the Spec cPL assay does not have a 100% sensitivity and specificity.80,96,97 This particular study is also unable to separate those dogs that may have acute and suppurative inflammation from those with chronic lymphocytic and fibrotic disease as these conditions can only be differentiated by histopathology. As there is no way to clinically differentiate the two conditions, the authors acknowledge that there may be different breed predispositions between the two conditions. Finally, many clinical conditions can mimic the clinical signs of pancreatitis and pancreatitis can be secondary to another primary disease process, thus imaging would have been ideal to rule out other disease processes.

Due to the large number of dogs evaluated in this study there are likely some errors in the data, such as mixed breed dogs being mislabeled as a particular breed. Within a database of this size there also likely exist multiple human transcription errors. Additionally, due to the inherent properties of the database and the size of the population, cases that were resubmitted for follow-up testing were unable to be completely removed from the population. However, care was taken to remove duplicates and minimize transcription errors whenever noted. By evaluating a very large number of cases, it was hoped that these errors are negligible in their overall effects.
In conclusion, this study evaluated a large population of dogs for a breed predisposition for an increased serum Spec cPL concentration. Being female and of older age was found to be associated with this laboratory finding. Additionally, this study supports earlier findings that certain breeds may be predisposed to an increased Spec cPL concentration and thus possibly pancreatitis. This study also identified a closely related group of dogs, Pomeranians, Eskimos, and Keeshonds with a predisposition for an increased serum Spec cPL concentration. Further studies looking at genetic mechanisms in these dogs are warranted. Furthermore, comparing the breed associations found in this study to those in a large general population of healthy dogs would be interesting to evaluate.
Footnotes

\textsuperscript{a} Spec cPL\textsuperscript{®}, IDEXX Laboratories, Inc., Westbrook, ME

\textsuperscript{b} Stata 12, StataCorp, College Station, TX

\textsuperscript{c} Prism 5, GraphPad Software Inc., San Diego, CA

\textsuperscript{d} Fédération Cynologique Internationale, http://www.fci.be/en
CHAPTER III
INVESTIGATION OF THE VARIANTS OF THE SPINK1 GENE AND THEIR ASSOCIATION WITH PANCREATITIS IN MINIATURE SCHNAUZERS

Introduction

The human serine protease inhibitor, Kazal type 1 gene (SPINK1) encodes for a protein with a molecular mass of 6.5 kD. This protein, pancreatic secretory trypsin inhibitor (PSTI), is produced, stored, and secreted by pancreatic acinar cells. The gene is approximately 7.5 kb long, has 4 exons, and is located on chromosome 5. In humans, the product of the SPINK1 gene has been hypothesized to act as an important safety mechanisms that protects the pancreas from degradation by premature intrapancreatic trypsinogen activation.

Hereditary pancreatitis in humans is a well-known condition that, in a subgroup of patients, has been associated with mutations of the SPINK1 gene. Witt et al. hypothesized that mutations of the SPINK1 gene may lead to an alteration of protein function, which may lead to pancreatic autodigestion and subsequent pancreatitis.

There is evidence to suggest that Miniature Schnauzers have a hereditary predisposition towards the development of pancreatitis. A recent retrospective study showed that the odds ratio for Miniature Schnauzers to develop pancreatitis was 4.1 (95%CI: 1.9 – 9.2, p <0.001) when compared to case based controls. Additionally, a query of the Veterinary Medical Database from 1995 – 2003 showed that the prevalence

1 Reprinted with permission from “Identification of variants of the SPINK1 gene and their association with pancreatitis in Miniature Schnauzers” by Bishop MA, Xenoulis MD, Suchodolski JS, Steiner JM. American Journal of Veterinary Research, 71, 527-533, Copyright 2010 by Diane A. Fagen, AJVR
of a diagnosis of pancreatitis in this breed was 4.4% compared to a prevalence of this
diagnosis in the general population of 0.7%. Previous studies have shown that the high
prevalence of pancreatitis observed in this breed is not associated with any mutations of
the cationic or anionic trypsinogen genes.

Therefore, it was hypothesized that mutations of the \textit{SPINK1} gene may be the
cause of pancreatitis in Miniature Schnauzers. The aim of this study was to sequence and
evaluate the \textit{SPINK1} gene for variants or risk alleles in Miniature Schnauzers with and
without pancreatitis, and if such variants were identified, to determine if they are
associated with pancreatitis.

\textbf{Materials and methods}

Whole blood and serum samples were collected from healthy Miniature
Schnauzers, Miniature Schnauzers with pancreatitis, and healthy dogs of other breeds to
serve as controls.

The following criteria were used to define the diagnosis of pancreatitis: clinical
signs compatible with a diagnosis of pancreatitis (such as vomiting, anorexia, and/or
abdominal pain), an increased serum canine pancreatic lipase immunoreactivity
concentration (cPLI \( \geq 200 \ \mu\text{g/L} \)) or an increased serum specific canine pancreatic lipase
(Spec cPL \( \geq 400 \ \mu\text{g/L} \)) concentration. Dogs with increased serum cPLI or Spec cPL
concentrations were recruited from a search of the Gastrointestinal Laboratory database
at Texas A&M University. Each dog’s veterinarian was contacted and asked to fill out a
questionnaire regarding the clinical signs and overall health of the affected dog. Also,
the veterinarian was asked about the date of birth, gender, sexual status, body weight, current diet, current medications, and medical history of the dog. If the dogs met the previously mentioned criteria, veterinarians were asked to contact the owner for permission to enroll the dog. If permission was granted by the owner, signed informed consent (protocol reviewed and approved by the Texas A&M University’s Animal Care and Use Committee) was obtained and the veterinarian collected whole blood samples into tubes containing EDTA and serum. Owners were instructed not to feed their dog for at least 12 hours prior to the scheduled blood collection.

Samples from healthy Miniature Schnauzers were obtained as part of a separate study. These dogs were voluntarily enrolled by breeders located throughout the United States and samples were collected using the same collection protocol described above. Additionally, the owners were questioned and the dogs’ pedigrees were examined to ensure they were not related for at least two generations. In order to be considered healthy, dogs had to have the absence of any clinical sign of any disease for 3 months prior to blood collection and a normal serum cPLI or Spec cPL concentration. Additional exclusion criteria included the absence of any prior history of pancreatitis.

Samples from healthy dogs of other breeds were obtained from dogs enrolled in the study by students and staff at Texas A&M University College of Veterinary Medicine and Biomedical Sciences using the same protocol as described above. These dogs included: 1 Yorkshire Terrier, 2 Border Collies, 2 Beagles, 3 German Shepherd Dogs, 1 Coonhound, 3 Lundehunds, 1 Boston Terrier, 1 Alaskan Malamute, 1 Bull Mastiff, 3 Chinese Shar Peis, and 5 mixed breed dogs.
All serum samples were analyzed for canine pancreatic lipase immunoreactivity concentration using either an in-house ELISA (cPLI) as previously described or a commercially available assay (Spec cPL). Previous studies have shown that pancreatic lipase is specific for the pancreatic acinar cells and the measurement in serum is sensitive for the diagnosis of pancreatitis.

DNA was extracted from whole blood samples using a commercially available kit according to the manufacturer’s instructions.

Primer design, PCR, and sequencing

The SPINK1 gene in dogs encodes for a protein that is 80 amino acids in length and is located on chromosome 2. The gene consists of 4 exons and 3 introns, and has a total exon length of 363 base pairs (bp). Exons 1, 2, 3, and 4 consist of 98 bp, 35 bp, 107 bp, and 123 bp, respectively. The nucleotide sequence of the SPINK1 gene in dogs is publicly available (GenBank: XM_845464). This sequence was used to design primers to amplify all 4 exons and their respective intron boundaries. Primers were designed using a commercially available software program (Table 3).

PCR was performed and optimized for each of the 4 exons. Briefly, for the first 3 exons, the 25 µl reaction mixture contained approximately 50 ng of genomic DNA, 10X Buffer (15 mM Tris HCl, 50 mM KCl (pH 8.0)), 2.5 mM MgCl2, 10 µM of each dNTP, 0.4 µM of each sense and antisense primer, and 2 units of polymerase. The conditions for the fourth exon were similar, except for the addition of a greater concentration of
<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>F</strong> 5' TTCCAGGCTGCACTGTCTTTCTTT 3'</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong> 5' CCTGAGTCAAAGGCAGATGCTCAA 3'</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><strong>F</strong> 5' ATGTCTCTGCTCTCTCTCTGTGTCT 3'</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong> 5' ACAGCTTCACTGTGTGGAGTTGG 3'</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><strong>F</strong> 5' TACCACCTCTTGTACAGCCTT 3'</td>
<td>343</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong> 5' TGGTTTATTGGAAACTTAGAGGGA 3'</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><strong>F</strong> 5' TTTCTCCTATGGTTCAATTT 3'</td>
<td>251</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong> 5' CCCTGATCCCAAATCTA 3'</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3:** Primers for the amplification of canine *SPINK1*. Key: **F** = forward primer, **R** = reverse primer, bp = base pairs.¹²⁴
MgCl₂ (3.5 mM). PCR cycling was performed using a commercially available thermocycler according to the cycling conditions shown in Table 4.

Aliquots of the PCR products were electrophoresed on 1% agarose gels at 100 V. Gels were stained and exposed to UV light for visualization of purity and to ensure the correct size of the amplicons. For the initial study, PCR amplicons were ligated into a vector and Escherichia coli organisms were transformed with the ligants. Plasmids were extracted per manufacturer’s instructions using a commercially available plasmid purification kit. The purified amplicon was then sequenced in both directions using the ABI BigDye Terminator Sequencing Mix. The products were analyzed and separated on an ABI PRISM 337 DNA Sequencer. For the association study, PCR products were sequenced directly using the same materials and protocols as above.

Initially, all four exons and their intron boundaries were amplified from genomic DNA in 22 dogs, which included 8 healthy Miniature Schnauzers, 6 Miniature Schnauzers with pancreatitis, and 8 healthy dogs of other breeds. Sequences were compared to each other and to the published sequences using a commercially available software package.

DNA from 65 additional dogs was sequenced in the two regions of the SPINKI gene found to carry the three variants identified in the initial study. These additional dogs included 17 healthy Miniature Schnauzers (for a total of 25 healthy Miniature Schnauzers), 33 Miniature Schnauzers with pancreatitis (for a total of 39 Miniature Schnauzers with pancreatitis), and 15 healthy dogs of other breeds (for a total of 23 healthy dogs of other breeds), for a grand total of 87 dogs analyzed for the variants of
### Table 4: PCR cycling conditions for amplification of each exon.\textsuperscript{124}

<table>
<thead>
<tr>
<th></th>
<th>Exons 1 &amp; 2</th>
<th>Exon 3</th>
<th>Exon 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>94°C for 4:00</td>
<td>94°C for 5:00</td>
<td>-</td>
</tr>
<tr>
<td>Step 2</td>
<td>94°C for 0:15</td>
<td>94°C for 0:30</td>
<td>94°C for 0:30</td>
</tr>
<tr>
<td>Step 3</td>
<td>67°C for 0:30</td>
<td>67°C for 0:30</td>
<td>56°C for 0:30</td>
</tr>
<tr>
<td>Step 4</td>
<td>72°C for 0:45</td>
<td>72°C for 1:00</td>
<td>72°C for 0:30</td>
</tr>
<tr>
<td>Step 5</td>
<td>repeat 34X from step 2</td>
<td>repeat 34X from step 2</td>
<td>repeat 34X from step 2</td>
</tr>
<tr>
<td>Step 6</td>
<td>72°C for 1:00</td>
<td>72°C for 2:00</td>
<td>-</td>
</tr>
</tbody>
</table>
interest in the *SPINK1* gene. The sequences were compared among dogs and with the published sequence.

The proportions of Miniature Schnauzers with pancreatitis and variants of the *SPINK1* gene were compared with the number of healthy Miniature Schnauzers with variants using a Fisher’s exact test and the odds ratios (OR) and their 95% confidence intervals (CI) were calculated. The ages of the two groups of Miniature Schnauzers were compared using a Mann Whitney test and an unpaired t test. All data were tested for normal distribution using the Kolmogorov-Smirnov test. Significance was set at P < 0.05.

**Results**

The sequencing results of the initial 22 dogs revealed 3 different variants. In exon 2, two missense mutations were identified. The first was a *C* to *A* substitution at nucleotide 5, which causes an amino acid substitution for residue 20 (Asn → Lys, N20K). The second missense mutation is caused by an *A* to *C* substitution at nucleotide 19, which causes an amino acid substitution at residue 25 (Asn → Thr, N25T). The third variant is located on intron 3. This variant is a poly-T insertion and duplication mutation, 26 nucleotides from the end of exon 3 (IVS3+26-27ins(T)30-39,15_61dup11). All three variants were always found together in both healthy Miniature Schnauzers and those with pancreatitis. No variants were found in the initial six healthy dogs of other breeds evaluated.
In the larger association screening study, the 2 exonic variants were found to always appear together in all three dog groups. The intronic variant was only found in the two Miniature Schnauzer groups, where the three variants were found in almost complete linkage disequilibrium with each other. Interestingly, one healthy Miniature Schnauzer was heterozygous for the two exonic variants and did not have the intronic variant.

Overall, the 3 variants were significantly associated with pancreatitis in Miniature Schnauzers. Miniature Schnauzers with pancreatitis were 9.5 times (95%CI: 1.0 – 87.0; P = 0.0300) as likely to have at least one copy of each of the three variant alleles as healthy Miniature Schnauzers. Additionally, pancreatitis in Miniature Schnauzers was significantly associated with being homozygous for all three variants compared to healthy Miniature Schnauzers. When comparing the proportion of dogs that were homozygous for these variants versus the proportion of dogs with wild type alleles, the odds ratio was 13.9 (95%CI: 1.4 – 135.6; P = 0.0143). When comparing the number of dogs that were homozygous for the 3 variants versus the proportion of dogs that were heterozygous or wild type, the odds ratio was 3.4 (95%CI: 1.1 – 9.0; P = 0.0403). However, heterozygosity for all 3 variants was not significantly associated with pancreatitis in Miniature Schnauzers (P = 0.6046) compared to healthy controls. Finally, healthy Miniature Schnauzers were significantly more likely to harbor the exonic variants compared to the healthy control group of other breeds (OR: 9.1, 95%CI: 2.4 – 34.3; P = 0.0011).
There was a statically significant difference in the median age of Miniature Schnauzers with pancreatitis versus the healthy Miniature Schnauzer group (P = 0.0009). For that reason, a second control group was generated which included only dogs > 5 years of age, which have been shown to be at increased risk for pancreatitis. After exclusion of the younger dogs, a total of 11 healthy Miniature Schnauzers remained in the control group. An unpaired t test found that there was no significant difference in the mean ages of the two groups after excluding the dogs younger than 5 years of age (P = 0.6606). Table 5 illustrates the differences in ages and sex among groups.

Upon re-analysis of the data with the control dogs of > 5 years of age, it was found that Miniature Schnauzers with pancreatitis were 21.7 times (95%CI: 2.1 – 224.4; P = 0.0063) as likely to have at least one copy of each of the three variant alleles as healthy Miniature Schnauzers. Additionally, pancreatitis in Miniature Schnauzers was significantly associated with being homozygous for all three variants compared to healthy Miniature Schnauzers. When comparing the proportions of dogs that were homozygous for these variants versus the proportion of dogs with wild type alleles, the odds ratio was 25 (95%CI: 2.2 – 284.8; P = 0.0067). When comparing the proportion of dogs that were homozygous for the 3 variants versus the proportion of dogs that were heterozygous or wild type, the results of the Fisher’s exact test was no longer significant (P = 0.1658). As before, heterozygosity for all 3 variants was not significantly associated with pancreatitis in Miniature Schnauzers (P = 0.1345) compared to the new healthy control group. Table 6 illustrates the combined frequencies of the variants found in both
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Sex (M:F:UNK)</th>
<th>Mean age (yrs) (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS w/pancreatitis</td>
<td>39</td>
<td>16:15:8</td>
<td>8.6 (3.4)</td>
</tr>
<tr>
<td>Healthy MS</td>
<td>25</td>
<td>9:15:1</td>
<td>5.3 (3.1)</td>
</tr>
<tr>
<td>Healthy MS (only older dogs)</td>
<td>11</td>
<td>5:5:1</td>
<td>8.1 (1.8)</td>
</tr>
</tbody>
</table>

**Table 5:** Age and sex distribution. Key: MS = Miniature Schnauzer, n = total number of dogs, SD = standard deviation.¹²⁴
Discussion

Pancreatitis has been found to be the most common disease affecting the exocrine pancreas in the dog.\textsuperscript{17} Although acute pancreatitis has been traditionally believed to be more common in dogs, recent studies would suggest that, in contrast to previous suspicion, chronic pancreatitis may be more common in dogs than acute pancreatitis.\textsuperscript{17} Little is known about whether chronic pancreatitis is caused by recurrent bouts of acute pancreatitis or if this condition represents a separate syndrome. Some factors that can serve as potential risk factors for the development of pancreatitis in dogs include obesity, trauma, high fat diets, pharmaceuticals, endocrinopathies, and hypertriglyceridemia.\textsuperscript{17} However, most cases of pancreatitis in the dog are considered idiopathic as an etiology is rarely definitively identified.\textsuperscript{61} Because of the breed prevalence of pancreatitis in dogs, genetics are proposed to play an important role in the development of pancreatitis in some breeds, such as the Miniature Schnauzer, the Yorkshire Terrier, and maybe other breeds.\textsuperscript{5,8,55}

As mentioned above, in humans \textit{SPINK1} is thought to be one of the pancreas’ defensive mechanisms against prematurely activated trypsinogen.\textsuperscript{129} Mutations of the \textit{SPINK1} gene have been reported in several studies and are thought to be the cause of hereditary pancreatitis in humans.\textsuperscript{44,119-121} Still some authors maintain that the mutations found in \textit{SPINK1} do not cause hereditary pancreatitis, but merely act as disease
Table 6: Findings and frequencies of the combined association study. The first column gives the possible combinations of alleles seen in this study for each of the three mutations. The order corresponds to the presence of N20K/N25T/IVS3+ variants. For example, hm/hm/hm would be a dog that was homozygous for both exon variants (N20K and N25T) and the intron variant. Key: hm = homozygous, ht = heterozygous, wild = wild type, MS = Miniature Schnauzer, OB = other breeds.¹²⁴

<table>
<thead>
<tr>
<th>Allele combinations</th>
<th>MS w/pancreatitis</th>
<th>Healthy MS</th>
<th>Healthy MS 2</th>
<th>Healthy OB</th>
</tr>
</thead>
<tbody>
<tr>
<td>hm/hm/hm</td>
<td>25 (64.1%)</td>
<td>9 (36.0%)</td>
<td>4 (36.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>ht/ht/ht</td>
<td>13 (33.3%)</td>
<td>10 (40.0%)</td>
<td>3 (27.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>hm/hm/wild</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0</td>
<td>1 (4.3%)</td>
</tr>
<tr>
<td>ht/ht/wild</td>
<td>0 (0%)</td>
<td>1 (4.0%)</td>
<td>0</td>
<td>6 (26.1%)</td>
</tr>
<tr>
<td>wild/wild/wild</td>
<td>1 (2.6%)</td>
<td>5 (20.0%)</td>
<td>4 (36.4%)</td>
<td>16 (69.6%)</td>
</tr>
<tr>
<td>total</td>
<td>39</td>
<td>25</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>variant allele frequency</td>
<td>0.81</td>
<td>0.57</td>
<td>0.50</td>
<td>0.12</td>
</tr>
</tbody>
</table>
modifiers by lowering the threshold for pancreatitis or increasing the severity of the disease caused by other genetic or environmental factors.\textsuperscript{43,118} One study that would support this notion reported that when the most common mutation, N34S, was recombinantly expressed, the subsequent protein did not show any alteration in its ability to inhibit trypsin.\textsuperscript{132} However, this mutation is also in linkage with four other intronic mutations, one of which may be significant.\textsuperscript{44} Other mutations of the \textit{SPINK1} gene have been identified and have been shown to be detrimental to protein function or expression. An extensive review of these findings can be found elsewhere.\textsuperscript{133} Finally, mutations in other genes, such as the gene for cationic trypsinogen have been associated with pancreatitis in humans and may be related to, or act in conjunction, with \textit{SPINK1} mutations.\textsuperscript{39,122,134}

To the authors’ knowledge, this is the first study documenting the presence of variants of the \textit{SPINK1} gene that are associated with pancreatitis in dogs. In this study, the exonic variants, N20K and N25T, were always found together in all dogs, regardless of the group. However, the intronic variant could only be identified in Miniature Schnauzers and also co-segregated with the exonic variants in this breed with the exception of one healthy Miniature Schnauzer. This exception could be due to a random crossover event or an accidental miss-mating in the past with a dog of another breed. The first exonic variant is present at a highly conserved asparagine residue reported in many species (Figure 2). The second asparagine at position 25 appears to be unique to the dog. The intronic variant is located on intron 3, 26 nucleotides from the end of exon 3 and the wild type intronic sequence in the region of the variant is identical to its human
Figure 2: Orthologue comparison of the \textit{SPINK1} gene between species and location of the two exon variants found. Alignment with ClustalW2 (http://http://www.ebi.ac.uk; EMBL-EBI, Hinxton, UK). Key: $\ast$ = all residues are identical, \% = conserved substitution, ; = semi-conserved substitution, N = asparagine, K = lysine, T = threonine$^{124}$
orthologue, suggesting conservation. In this study, Miniature Schnauzers homozygous for the 3 variants were significantly more likely to have pancreatitis. In addition, none of the healthy dogs of other breeds had the intronic variant.

The variants found in the present study are different than those described in humans with hereditary pancreatitis and \textit{SPINK} mutations.\textsuperscript{133} However; an intron-exon boundary mutation has been reported in people near the location of the intronic variant found in the dogs described here. cDNA analysis of this mutation revealed that exon 3 was skipped in the final transcript.\textsuperscript{135}

There could be several reasons for healthy Miniature Schnauzers to have a high prevalence of the 3 variants. First, even though the dogs were clinically healthy at the time of sample collection, the variant may predispose them to development of pancreatitis later in life. This speculation is supported by the fact that the healthy Miniature Schnauzers were significantly younger than Miniature Schnauzers with pancreatitis. In this study, age matched controls chosen from the beginning would have been ideal; however, it was exceedingly difficult to find healthy older Miniature Schnauzers that fulfilled our criteria for inclusion and we felt it was important to a large number of dogs. The difficulties were due to the fact that the majority of the older Miniature Schnauzers had clinical signs of chronic diseases such as diabetes mellitus and urolithiasis, for which Miniature Schnauzers are also predisposed.\textsuperscript{136,137} However, upon exclusion of dogs younger than 5 years of age, it was found, despite having a smaller
population of dogs to analyze, that being homozygous for the intron variant was still significantly associated with pancreatitis, in fact, even more so.

Secondly, mild or subclinical pancreatitis may have easily been missed by the owner as mild pancreatitis may be manifested as occasional vomiting or other nonspecific clinical signs. Also, a prior episode of pancreatitis may have been misdiagnosed as another condition. It is also possible that the gene is highly prevalent, but has a low penetrance and it may be necessary for other genes or other environmental factors to be present in order for pancreatitis to develop. Additionally, Miniature Schnauzers are known to have a highly prevalent syndrome of primary hypertriglyceridemia, which itself could be the cause of pancreatitis or a necessary co-factor. The presence of hypertriglyceridemia was not evaluated in this study as it would have been difficult to classify the dogs as having hypertriglyceridemia prior to the onset of pancreatitis or if the hyperlipidemia was secondary to pancreatic inflammation. Further studies are in progress to investigate the role of hyperlipidemia in the development of pancreatitis in the Miniature Schnauzer. Finally, although the cPLI has been shown to be the most sensitive (64–82%) serum marker for pancreatitis and is believed to be very specific, it is not possible to exclude mild subclinical pancreatitis in some of the healthy dogs evaluated. Histopathological evaluation of the pancreas is considered to be the gold standard for the diagnosis of pancreatitis in the dog, but it was not considered feasible. Biopsy of the pancreas is an invasive, expensive procedure that is not routinely performed in dogs suspected of pancreatitis. While some dogs may have been missed with the measurement of the cPLI/Spec cPL, we feel that
the presence of an elevated serum cPLI/Spec cPL concentration plus clinical signs compatible with pancreatitis is the most feasible and economical method for phenotype assignment in such a large group of dogs that live throughout the United States.

In this study, other dog breeds were found to only carry the exonic variants and at a lower frequency than Miniature Schnauzers, although many breeds were not studied. It could be that the exonic variants are SNPs that have been passed on through generations and perhaps the intronic variant represents the etiologic mutation in Miniature Schnauzers with chronic pancreatitis.

One problem we encountered in this study is the fact that Miniature Schnauzers with pancreatitis were significantly older than healthy Miniature Schnauzers. Pancreatitis has been shown to develop more commonly in older dogs, and this might have accounted for the discrepancy of the prevalence of pancreatitis in the two populations of Miniature Schnauzers (healthy vs. sick). However, to more accurately compare the true frequency of the variants, as previously mentioned, healthy Miniature Schnauzers under 5 years of age were excluded and data was re-evaluated, which confirmed the original findings.

In addition, population stratification could be another cause of the higher variant allele frequency seen in the affected group of Miniature Schnauzers, as a relationship among those Miniature Schnauzers could not be conclusively excluded. However, sick Miniature Schnauzers enrolled in this study came from many different locations within the United States, and we feel that this helped to minimize the possibility of selection of a population that were more inbred than the general Miniature
Schnauzer population. Finally, only few other dog breeds were studied, and thus the variants identified here might be present in breeds that were not included in the present study.

Further studies are needed to further evaluate the pathogenetic impact of the 3 variants identified here. Such studies would include the evaluation of the prevalence of the 3 variants in the general population of healthy dogs, dogs with pancreatitis, as well as in distinct groups such as other breeds with a suspected hereditary predisposition, hypertriglyceridemic dogs, and dogs being treated with drugs associated with pancreatitis. Additionally, studies focusing on possible effects of these mutations on the structure and function of SPINK1, mode of inheritance, and penetrance studies are warranted. Finally, a genome-wide search for other genes that may be contributing to pancreatitis in this breed, as well as a follow-up of the healthy Miniature Schnauzers with variants in order to see if they will develop pancreatitis in the future, are needed and underway.

In conclusion, we have reported 3 closely associated variants (2 exonic and 1 intronic) of the SPINK1 gene in both healthy Miniature Schnauzers and Miniature Schnauzers with pancreatitis. These variants were significantly associated with pancreatitis in this study. The exonic variants were also found in healthy dogs of other breeds, but at a lower frequency than in healthy Miniature Schnauzers. We conclude that defects of the SPINK1 gene likely play a role in pancreatitis in the Miniature Schnauzer. However, we also hypothesize that other environmental or genetic factors may also be contributing to this disease. Further studies of these genetic variants are warranted.
Footnotes

\(^a\) Veterinary Medical Database, http://www.vmdb.org, Urbana, IL


\(^d\) PUREGENE® DNA Purification Kit, Gentra Systems, Inc., Minneapolis, MN

\(^e\) PrimerQuest software, http://www.idtdna.com, Integrated DNA Technologies, Coralville, IA

\(^f\) AmpliTaq Gold DNA Polymerase, Applied Biosystems, Foster City, CA

\(^g\) MasterCycler Gradient Thermocycler, Eppendorf, Hamburg, Germany

\(^h\) Gel Red, Biotium, Hayward, CA

\(^i\) pCR®4-TOPO, Invitrogen Corporation, Carlsbad, CA

\(^j\) One Shot TOP10 Escherichia coli organisms, Invitrogen Corporation, Carlsbad, CA

\(^k\) Perfectprep® BAC 96 Plasmid Purification Kit, Eppendorf, Hamburg, Germany

\(^l\) ABI BigDye Terminator Sequencing Mix, Applied Biosystems, Foster City, CA

\(^m\) ABI PRISM 337 DNA Sequencer, Applied Biosystems, Foster City, CA

\(^n\) ChromasPro, Technelysium Pty Ltd, Eden Prairie, MN

CHAPTER IV

GENOME WIDE SNP SCAN IN MINIATURE SCHNAUZERS WITH EVIDENCE OF PANCREATITIS

Introduction

Miniature Schnauzers have long been suspected to be predisposed to the development of pancreatitis. Indeed a recent epidemiological study investigating risk factors for pancreatitis identified the Miniature Schnauzer as having an increased odds ratio.\textsuperscript{60} Additionally, a previous candidate gene study identified Miniature Schnauzers, with a phenotype consisting of clinical signs and an increased Spec cPL concentration, that were associated with being homozygous for two exon and one intron/exon boundary variants in the serum protease inhibitor, Kazal type 1 (\textit{SPINK1}) gene.\textsuperscript{124} However that study suggested that the \textit{SPINK1} gene may not be the only gene involved in the etiology of pancreatitis in this breed and that the condition may be polygenic or that it might be multifactorial and that some environmental factor may also be playing a role. Previous evaluation of the cationic trypsinogen, anionic trypsinogen, and lipoprotein lipase genes did not reveal any mutations in Miniature Schnauzers with pancreatitis.\textsuperscript{17,75} In order to further investigate other candidate genes, a comprehensive genome-wide scan would be warranted.

Genome wide mapping using single nucleotide polymorphisms (SNPs) is now a common tool for identifying chromosomal regions and candidate genes of interest.\textsuperscript{138-142} After the publication of the canine genome, a high density SNP map has been
constructed that identifies approximately 2.5 million SNPs with a density average of 1 SNP per kilobase throughout the entire canine genome. Subsequently a SNP array using a high through-put platform was developed and validated. The original SNP array utilized a set of 26,578 SNPs using 10 different dog breeds. The commercially available chip was expanded to include 49,663 SNPs and is known as the v2 platinum array. With this methodology, PCR primers that code for the different SNP variations are bound to a chip and tagged with a reporter molecule. DNA from the patient is washed over the chip and hybridizes with the primers. Hybridization is communicated by reporter molecules and subsequently each patient is genotyped for each individual SNP. DNA is collected from dogs with the disease of interest and normal healthy controls and their genotypes are compared. Statistical associations between disease and areas of the genome may point to areas that contain the gene(s) of interest. Areas of interest can be further investigated by gene sequencing or more refined SNP evaluation.

The aim of this study was to identify a region of interest or candidate genes that are associated with the phenotype of clinical signs of pancreatitis and increased Spec cPL concentrations in Miniature Schnauzers. A secondary aim of the study is to evaluate whether the previously identified variants in the \textit{SPINK1} gene are identified in a genome wide scan.

**Materials and methods**

Whole blood and serum samples were collected from Miniature Schnauzers with a history of clinical signs compatible with pancreatitis (i.e., vomiting, abdominal pain,
and diarrhea) and an increased serum Spec cPL concentration as well as from healthy control dogs with an absence of clinical signs of disease and a serum Spec concentration within the reference interval. The dogs were client-owned animals that were solicited by calling Miniature Schnauzer breeders and searching the database at the Gastrointestinal Laboratory at Texas A&M University for samples from dogs submitted for the measurement of serum cPLI or Spec cPL concentration. History and clinical signs were obtained via mailed questionnaires or phone calls to referring veterinarians or breeders.

Pancreatitis was diagnosed on the basis of the following criteria: clinical signs compatible with a diagnosis of pancreatitis (i.e., vomiting, anorexia, abdominal pain, diarrhea, or a combination of these) and an increase in serum cPLI concentration as measured by the original in-house ELISA $\geq 200 \mu g/L$ or as measured by Spec cPL $\geq 400 \mu g/L$. In order to be considered healthy, Miniature Schnauzers had to have no clinical signs of any disease for 3 months prior to blood collection, no prior history of pancreatitis, and have a serum cPLI concentration within the reference interval.

DNA was extracted from whole blood using a commercially available DNA purification kit and spectrophotometry was performed for both quantification and to ensure high quality samples utilizing the $A_{260}/A_{280}$ ratio.$^{a,b}$ Only DNA with a $A_{260}/A_{280}$ ratio of 1.6 or greater was used for further analysis. DNA was frozen at -20°C until enough samples were collected for analysis. DNA was then shipped frozen to the Vanderbilt Microarray Shared Resource Laboratory for genotyping using the commercially available v2 Platinum Array.$^c$ Results were analyzed using a commercially available software package.$^d$ Association testing was performed via $\chi^2$ tests for each
SNP and because of the large amount of multiple testing in this study, a Bonferroni correction for multiple statistical comparisons was used to determine significance. Alpha was set 0.5 for individual tests, but the overall significance was set to a conservative $P < 0.00001 (P < 1.0 \times 10^{-6})$. Significant SNP’s were located on the canine genome assembly CanFam 2.0.\textsuperscript{6,138} The ages of the dogs belonging to the two groups were compared with an unpaired $t$ test as data was found to be parametric.

**Results**

In total, 50 dogs were used for SNP analysis. There were 39 Miniature Schnauzers that fulfilled the inclusion criteria of having an increased serum cPLI concentration and clinical signs of pancreatitis. There were a total of 25 Miniature Schnauzers that were considered healthy, however this group as a whole was much younger (mean ± standard deviation) (5.3 ± 3.1 years) compared to those with clinical signs (8.6 ± 3.4 years) ($P < 0.001$). Due to this discrepancy and in order to age match these two groups for analysis, only dogs that were greater than 5 years of age were included in the population of healthy dogs. This was based on a previous report that dogs over the age of 5 were at increased risk for pancreatitis.\textsuperscript{8} Therefore, in total 11 healthy dogs were analyzed and they had a mean age of 8.1 ± 1.8 years ($P = 0.6610$).

SNP analysis only returned one SNP that fulfilled the very conservative $P$ value criteria. The location of this SNIP was identified on chromosome 2 at location 40,905,350 and the returned $P$ value was $2.9 \times 10^{-7}$. Additionally, although not statistically significant per our criteria, there were 13 other SNPs with $P$ values < 0.0001
that were also located on chromosome 2. These other SNPs are very close to the statistically significant SNP and they encompass the region from positions 40,339,715 to 43,363,462. These locations were identified on the canine genome assembly on chromosome 2. There are 40 known genes which occupy this location and the \textit{SPINK1} gene is located at position 42,112,016 to 42,120,711. The \textit{SPINK1} gene is centered in the region identified by the SNP array. (Figure 3). Several similar genes that encode for \textit{SPINK5} and \textit{SPINK6} are in the same region. The remaining genes encode for g protein receptors and other intracellular functions. There were no other pancreatic specific proteins or other obvious candidate genes identified that occupy this region.

**Discussion**

The results of this study show an association of the disease phenotype to a small region of chromosome 2. This is based on clustering of the SNP results occurring around this locus. These results support a previously reported association of the phenotype with variants in the \textit{SPINK1} gene of Miniature Schnauzers.\textsuperscript{126} The \textit{SPINK1} gene codes for the protein, pancreatic secretory trypsin inhibitor (PSTI).\textsuperscript{128} This is an acute-phase protein that is thought to act as one of the fail-safe mechanisms to prevent auto-digestion of the pancreas by binding to prematurely activated trypsin within the acinar cell.\textsuperscript{9,17} In humans, a N34S missense mutation of this gene has been associated with idiopathic pancreatitis.\textsuperscript{40,43,44,119,135} Further studies showed an increased prevalence of the variant and others as well as a large number of heterozygous individuals within pancreatitis cohorts.\textsuperscript{36,43} This raised questions as to the role of the N34S mutation as a causative
**Figure 3**: Animation of the SNP’s identified on the canine chromosome 2 that were associated with the phenotype of pancreatitis. The red bar indicates the *SPINK1* gene and its relative location to other SNPs. Numbers are the location on the genome where SNPs are located.
mutation. A recent meta-analysis study found that variants were more closely tied to the development of idiopathic and chronic pancreatitis than alcohol usage.\textsuperscript{21,45} Other variants including a splice variant has been identified and currently \textit{SPINK1} is considered to be the causative mutation in homozygous individuals.\textsuperscript{36,135} However, in heterozygous individuals, \textit{SPINK1} is only considered the cause when no other mutations can be identified (\textit{PRSS1}, \textit{CFTR}, etc.).\textsuperscript{21,45}

The data of this study suggest that the region identified on the canine chromosome 2 may likely contain the gene(s) of interest in Miniature Schnauzers with evidence of pancreatitis. While it is of great interest that the \textit{SPINK1} gene is located in the region with disease-associated SNIPs, other genes in this region could be causative despite not finding any other obvious targets in this location. Further refined SNP mapping using informative SNPs that are not on the genotyping chip may help to narrow the region or possibly locate other candidate genes that could be sequenced. Alternatively, the \textit{SPINK1} gene may be playing a role in a more complex phenotype that may require other environmental or metabolic factors to be present (i.e., hyperlipidemia or dietary factors).\textsuperscript{60,76,91,124} Indeed, being a Miniature Schnauzers with a serum triglyceride concentration over 900 mg/dl has previously been associated with a significantly increased odds ratio for an increased Spec cPL concentration.\textsuperscript{76} Further studies including the construction of a logistic regression model containing information on Miniature Schnauzers \textit{SPINK1} genotype as well as their fasting triglyceride concentrations may help to clarify if such a connection exists.
The shortcomings of this project include the smaller population of healthy individuals. However, it was surprisingly difficult to find Miniature Schnauzers that fulfilled our requirements to be considered healthy. Indeed, Miniature Schnauzers appear to have a predisposition for several chronic conditions as they age.\textsuperscript{64,137} However, recent bottlenecking of the canine population allows for long linkage disequilibrium within breeds and short linkage within the population.\textsuperscript{138,141} This allows for a small number of dogs to be used in SNP genotyping studies. In fact, recent studies used only 20 dogs to identify a discrete region for a simple recessive trait while a dominant trait such as hyperparathyroidism in Keeshonds was genotyped with 70 dogs using SNP analysis.\textsuperscript{141} Also, spotting on boxer dogs was identified using SNP analysis with only 36 dogs.\textsuperscript{142} Studies as these in dogs and studies in humans have found SNP association to be valuable for locating both simple and complex genetic diseases.\textsuperscript{138,143,144} At this point, it appears that pancreatitis in Miniature Schnauzers may be polygenic and as a result, a larger number of dogs would be needed. Indeed, had a larger population been available, the other SNPs at the chromosome 2 location may have become statistically significant.

Additionally, the establishment of an exact phenotype is difficult with this disease process. The gold standard for diagnosis of pancreatitis is currently considered to be pancreatic biopsy although many experts are calling this into question.\textsuperscript{57,61,97,112} With the advent of assays for the measurement of cPLI many clinicians consider the presence of an increased serum concentration and clinical signs to be consistent with the diagnosis after having ruled out other differential diagnoses.\textsuperscript{80} Recent studies have demonstrated small focal pancreatic lesions in dogs without overt signs of pancreatitis.\textsuperscript{112} Indeed in
this study, some of the “healthy” dogs may have had clinically insignificant pancreatitis on histopathology. Additionally, the dogs with the phenotype of pancreatitis in this study may have had the acute neutrophilic form of the disease versus the more chronic lymphocytic-plasmacytic form. In humans, SPINK1 mutations are associated with histopathologic findings of chronic disease.36,40 Ideally, dogs in the study would have had a pancreatic biopsy to further classify these two different disease entities. However, pancreatic biopsy is invasive, costly, and generally impractical.5,17,65 While the phenotype established in this study should identify individuals with pancreatitis, we were unable to definitively rule out other disease processes. Also, while pancreatitis can be a primary condition, it can also be secondary to many disease conditions such as peritonitis or neoplasia.17

In this study we did utilize both the original ELISA for the measurement of serum cPLI concentration as well as the Spec cPL. Ideally the same assay would have been used to establish the phenotype for the entire study. However, during a period of sample collection for this study, only the original ELISA was available for use, while later on the original ELISA was discontinued and replaced with the Spec cPL.80 Both assays have been fully evaluated and are considered equivalent.96,97

In conclusion, this study has identified a region of interest on canine chromosome 2 in Miniature Schnauzers with a phenotype of an increased serum cPLI concentration and clinical signs compatible with pancreatitis. In the region identified the SPINK1 gene is located suggesting this gene to likely play a role in the etiology of pancreatitis in the Miniature Schnauzer. Further refined mapping of the region with a
larger population of dogs may identify other targets that may play a role in the etiology of pancreatitis in this and potentially other breeds.
Footnotes

a PUREGENE® DNA Purification Kit, Gentra Systems, Inc., Minneapolis, MN

b NanoDrop 1000. NanoDrop Products, Wilmington, DE

c Canine v2 Platinum Array, Affymetrix, Santa Clara, CA

d Plink v.1.05 http://pngu.mgh.harvard.edu/purcell/plink/

e *Canis lupus familiaris (dog) genome*
CHAPTER V
EVALUATION OF THE CDNA IN MINIATURE SCHNAUZERS WITH VARIANTS IN THEIR \textit{SPINK1} GENE

Introduction

Previous studies have identified variants in the \textit{SPINK1} gene of Miniature Schnauzers with clinical signs of pancreatitis and an increased serum cPLI concentration.\textsuperscript{124} Two of these variants reflect single nucleotide substitutions that would change the cDNA of the second exon of the \textit{SPINK1} gene (Figure 4). The first missense mutation, N20K, changes the sequence from the uncharged polar amino acid asparagine to the charged polar amino acid lysine (Figure 5). The second variant, N25T, replaces asparagine with threonine (Figure 6). Besides these two variants, another abnormality was noted along the intron-exon boundary between intron 3 and exon 4 of the gene. This is an insertion and duplication mutation, known as IVS3+26-27ins(T)30-39,15\_16dup11. This variant inserts a variable poly-t sequence and then reduplicates 11 nucleotides upstream from the poly-t insertion and repeats them after the variable strand of thymine (Figure 7). It was also noted that these three missense mutations were in linkage in Miniature Schnauzers and while some dogs of other breeds did have the exon variants, the boundary mutation was unique to the Miniature Schnauzer.\textsuperscript{124} Additionally, Miniature Schnauzers were more likely to be homozygous for all three variants. It was hypothesized that this intron exon boundary mutation could be causative for pancreatitis by potentially interfering with possible splice sites and may yield a truncated mRNA
Figure 4: Sequencing results of the second exon of the SPINK1 gene. The wild type sequence is shown in the bottom graph. The red box indicates the substitution of the nucleotide that will change the codon present at that location. Letters correspond to the nucleotide. A = adenine, T = thymine, C = cytosine, G = guanine
Figure 5: Missense mutation with a non-conservative substitution. N = asparagine, K = lysine
Figure 6: Missense mutation with a non-conservative substitution at the second locus on the \textit{SPINK1} gene. N = asparagine, T = threonine
**Figure 7:** Sequencing results of the intron/exon boundary between the third intron and the third exon of the *SPINK1* gene in Miniature Schnauzers with evidence of pancreatitis. The bold red line indicates the boundary between exon and intron with the blue arrow pointing upstream toward exon 3 and the green arrow pointing downstream toward intron 3. The red box indicates the set of nucleotides that is reduplicated further downstream following a variable poly t insertion in the blue box.
product.

The aim of this study was to further investigate the effects of the intron-exon boundary mutation by evaluation of the cDNA of Miniature Schnauzers that are homozygous, heterozygous, or wild type for the three SPINK1 variants.

Materials and methods

Breeders of pedigreed Miniature Schnauzers were solicited throughout Texas by contacting the Miniature Schnauzer breed association. Breeders were telephoned or emailed to recruit female, sexually intact Miniature Schnauzers bitches that were ready to be retired and adopted to new homes. These dogs had to be clinically healthy on physical examination and have unremarkable full bloodwork, including a complete blood count, chemistry panel, and a urinalysis. In addition, blood samples were collected for genotyping each dog for the three known SPINK1 variants. DNA was extracted and isolated from each dog using a commercially available kit. Each dog’s genotype for the three variants was determined using PCR. Briefly, exon 2 and the boundary of the intron 3 and exon 4 were amplified with SPINK1 specific primers using a PCR as previously reported. For exon 2, the 25 µl reaction mixture contained approximately 50 ng of genomic DNA, 10X Buffer (15 mM Tris HCl, 50 mM KCl (pH 8.0)), 2.5 mM MgCl₂, 10 µM of each dNTP, 0.4 µM of each sense and antisense primer, and 2 units of polymerase. For the fourth exon, the MgCl₂ concentration was greater at 3.5 mM. PCR cycling conditions were as previously described. The PCR product was directly sequenced in both directions using the terminator sequencing mix and the products were
analyzed and separated on a DNA sequencer to determine the genotype of each dog. Dogs that were found to have the variants as well as a single dog that was wild type for the variates were subsequently purchased from the breeders and housed at the Texas A&M University College of Veterinary Medicine Teaching Hospital.

Once each dog was settled into the hospital, an additional examination was performed by a veterinarian and another blood sample was taken to evaluate each dog’s serum Spec cPL concentration in order to assure pancreatitis was not present at the time of surgery. Each dog was fasted overnight and taken to surgery under general anesthesia, which was performed by a board certified veterinary surgeon. Prior to surgery each dog was given a morphine epidural and was induced with propofol after hydromorphone and glycopyrrolate premedication. Monitoring of anesthesia was performed by a board certified anesthesiologist. Crystalloids were administered intravenously at a rate 10 ml/kg/hour to maintain perfusion and normotension. The first procedure performed was an ovariohysterectomy. Briefly, the ventral abdomen was clipped and surgically prepped from the area of the xiphoid to the pubis. A surgical incision was made just caudal to the umbilicus extending 4 – 8 cm caudal through the skin and subcutaneous tissue to expose the linea alba. The linea alba was grasped with forceps and a stab incision into the abdominal cavity was made. This incision was extended with Mayo scissors. The reproductive tract was then identified by confirming that the uterine horn extends to an ovary cranial and the uterine bifurcation and cervix caudally. The suspensory ligament was identified at its attachment to the ovarian pedicle. This ligament was stretched and torn to allow exteriorization of the ovary. A
hole was made in the broad ligament caudal to the ovarian pedicle and one to two
Rochester-Carmalt forceps were placed across the ovarian pedicle proximal to the ovary
and one additional forceps across the proper ligament of the ovary. Using absorbable
suture, the pedicle was ligated with circumferential and transfixing ligatures following
forceps removal. After ligation, the ovarian pedicle was transected between the sutures
and the ovary. The remaining stump was inspected for bleeding and knot security and
then released back into the abdominal cavity. This process was repeated for the other
ovary. To ligate the uterus, a figure-eight suture was placed through the uterine body
near the cervix, then a second circumferential ligature was placed closer to the cervix. A
Carmalt forceps were placed proximal to the ligatures and the uterine body was
transected with a scalpel between the forceps and the ligature. Upon ensuring the uterine
stump was not hemorrhaging, it was released back into the abdominal cavity.

After completing the ovariohysterectomy, the skin, subcutaneous, and linea alba
incision was extended cranial to the xiphoid as needed to visualize the pancreas. The
pancreas was identified by cranial retraction of the free portion of the greater omentum
and grossly evaluated for any pancreatic pathology. A small portion of the distal aspect
of the right lobe of the pancreas was incised by using individual lobule dissection. To
perform this procedure, an incision was made in the mesoduodenum and a portion of the
distal right limb of the pancreas was exteriorized. Using a small pair of Metzenbaum
scissors, the lobules were gently teased and removed from the body of the pancreas.
Attempts were made to preserve pancreatic ducts and hemorrhage was controlled by
using small absorbable sutures as needed. The pancreas was then observed for gross
hemorrhage prior to body wall closure. Closure of the linea alba was performed by using absorbable suture material in a simple continuous pattern, followed by closure of the subcutaneous and intradermal tissues in a similar fashion. The skin incision was then covered by a bandage. After surgery, each dog was recovered in the intensive care unit of the Texas A&M University College of Veterinary Medicine’s Teaching Hospital under the care of a veterinarian. Each dog was administered IV lactated Ringers solution at rate of 120 ml/kg/day and given injectable buprenorphine at 0.01 mg/kg intravenously every 8 hours for pain control and then transitioned to oral meloxicam at a dose of 0.1 mg/kg orally every 24 hours. Blood samples were collected at approximately 12, 36, and 60 hours after surgery to monitor for possible post-operative pancreatitis.

At the time of surgery, the pancreatic biopsy was immediately placed into liquid nitrogen. Once completely frozen, the tissue sample was extracted from the liquid nitrogen and immediately stored at -80°C. In order to extract mRNA from the pancreas, a commercially available purification kit was used in a modified manner. Each tissue sample was retrieved from the freezer, quickly weighed, and while still frozen was placed in a vial containing 1 ml of TRIzol per 100 mg of pancreatic tissue. The sample was immediately homogenized using a tissue homogenizer. To extract the mRNA, after a 5 minute incubation, 0.2 mLs of chloroform was added to the sample per every 1 ml of TRIzol that had been previously added. The sample was incubated for 3 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C. After separation, the upper phase was transferred to a clean tube. This upper phase was then mixed with an equal volume of 70% ethanol and transferred to a spin column and centrifuged for 15 seconds at
12,000 x g to bind the sample to the spin cartridge. The sample was then washed twice with a commercially available wash buffer. The final purified mRNA was extracted from the column with RNase free water. The samples then underwent spectrophotometry to evaluate their purity and concentration. Samples were stored at -80°C prior to reverse transcriptase polymerase chain reaction (RT-PCR).

Primers were constructed to amplify the entire cDNA of the *SPINK1* gene. The forward primer was 5’ TGGCCCTGTTGAGCTTATCTGGTA and the reverse primer was 5’TCAGGCCCACTAGGCATTGTAGTA. Using a commercially available RT-PCR kit, 1 µg of RNA was combined with 11.4 µl of RNAase free water, 10 µl of 5x RT-PCR buffer (2.5 mM Mg²⁺), 400 µM of each dNTP, 0.6 µM of each primer, and 2 µl of reverse transcriptases. RT-PCR was performed in a thermocycler according to the manufacture’s recommendations. cDNA from each dog was sequenced in the sense and anti-sense directions as described previously above. cDNA sequences were compared to the known *SPINK1* cDNA sequence from the canine genome assembly CanFam 2.0.

After surgery each dog was fully recovered and these dogs were adopted out to new homes. This project was approved under the Texas A&M University animal use protocol 2010-242.

**Results**

In total, 15 Miniature Schnauzers were screened for inclusion into the study. However only 4 Miniature Schnauzers were able to be obtained and meet full requirements for surgery. Each dog was sequenced to determine whether they contained
the previously identified exon 2 missense and the intron/exon boundary mutations. One dog was wild type for all three variants, two were heterozygous for each of the variants and one dog was homozygous for all three variants.

All four dogs recovered from surgery and were successfully adopted. Spec cPL serum concentrations remained within the reference interval at 12, 36, and 60 hours post op for all dogs.

cDNA was successfully isolated from each of the dogs’ pancreatic biopsy using the technique described. RT-PCR was also successfully performed on each sample. The subsequent amino acid sequences (as would be coded) were aligned however there were no differences noted in the length or any other abnormalities with the exception of the exon 2 variants (Figure 8).

Discussion

The SPINK1 gene encodes for the protein pancreatic secretory trypsin inhibitor, which is thought to act as one of the pancreas fail-safe mechanisms to prevent auto-digestion.9,16,128 The protein is expressed within the acinar cell of the pancreas and has been shown to bind to prematurely activated trypsin, thus preventing or limiting the co-localization of zymogens with lysosomes.5,9,17

Homozygosity for the N34S SPINK1 gene mutations in humans is now considered causative for idiopathic pancreatitis.21,36,40,45 Heterozygous individuals are not considered causative unless no other mutations are identified in other genes known to cause pancreatitis such as the PRSSI or CFTR.21,45 In humans, an intron mutation
Figure 8: Amino acid sequence of Miniature Schnauzers with SPINK1 variants. WILD = wild type sequence, HETZ = Heterozygous for variants, HOMO = Homozygous for the variants. Letter code for individual amino acids. X = indicates that the amino acid cannot be determined due to heterozygosity. Red and blue are the sites of the exon 2 variants.
was identified and was found to cause the skipping of exon 3, which is the location of the trypsin binding site and transcription expression was decreased to 62% of wild-type SPINK1 in heterozygous patients.\textsuperscript{135}

This paper likely describes the first successful attempt to purify mRNA directly from the pancreas of dogs. mRNA extraction from the pancreas has been difficult due to extremely high number of proteases and other degradation enzymes present within the pancreas.\textsuperscript{145} cDNA was successfully transcribed in a wild type dog, two heterozygous dogs, and a homozygous dog with the SPINK1 variants previously described to be associated with the phenotype of clinical signs of pancreatitis and increased Spec cPL concentrations.

A previous study suggested that the intron/exon boundary variant is unique to Miniature Schnauzers, however cDNA analysis does not appear to alter the final transcript in length and all 4 exons remain in place.\textsuperscript{124} While the cDNA does not alter the transcript, other than the two exon variants, this does not mean that any of the 3 variants are not causative. Indeed, many mutations have been found to be coded correctly but \textit{in vitro} experimentation revealed decreased levels of mRNA expression or dysfunction of the subsequent protein.\textsuperscript{21}

Further studies using cell culture where the SPINK1 mutated protein is expressed may clarify any effects on protein expression levels. Additionally, further studies documenting the mutated protein’s ability to bind trypsin are also warranted. Finally, x-ray crystallography analysis may demonstrate any changes in protein folding that may open or close binding sites necessary for protein function.
Even though this study was extremely small (n=4), it confirms previous studies that pancreatic biopsy, when collected with caution, is a safe procedure. All dogs recovered well from surgery with no clinical signs of pancreatitis and their Spec cPL concentrations remained within the reference interval for the 60 hour post-operative period.
Footnotes

a PUREGENE® DNA Purification Kit, Gentra Systems, Inc., Minneapolis, MN

b ABI BigDye Terminator Sequencing Mix, Applied Biosystems, Foster City, CA

c ABI PRISM 337 DNA Sequencer, Applied Biosystems, Foster City, CA

d Spec cPL®, IDEXX Laboratories, Inc., Westbrook, ME

e Tegaderm™, 3M, St. Paul, MN

f RNeasy Plus Micro Kit, Qiagen, Hilden, Germany

g TRIzol® RNA Isolation Reagents, ThermoFisher Scientific, Waltham MA

h QIAprep Spin Miniprep, Qiagen, Hilden, Germany

i Buffer RW1, Qiagen, Hilden, Germany

j RNase Free Water, Qiagen, Hilden, Germany

k NanoDrop 1000. NanoDrop Products, Wilmington, DE


m OneStep RT-PCR, Qiagen, Hilden, Germany

n MasterCycler Gradient Thermocycler, Eppendorf, Hamburg, Germany

o Canis lupus familiaris (dog) genome
CHAPTER VI
SUMMARY AND CONCLUSIONS

The Miniature Schnauzer has long been suspected to have a genetic predisposition to pancreatitis. However, few studies have been attempted to prove this association. The primary roadblock to this success was the absence of a non-invasive marker for diagnosing the condition. With the advent of cPLI, further study of such an association became feasible.

In humans, several genetic mutations have been discovered that are now considered to play an important role in the etiology of chronic and idiopathic pancreatitis. Previous studies have investigated several candidate genes in Miniature Schnauzers (i.e., cationic, anionic, and lipoprotein lipase genes) with no success. The hypotheses of this study were that 1) Miniature Schnauzers are predisposed to the development of pancreatitis based on a phenotype of an increased serum cPLI concentration and clinical signs, 2) the SPINK1 gene would harbor potential genetic mutations, and 3) using SNP analysis, we would be able to identify gene(s) or regions that are associated with the phenotype of pancreatitis. Thus, the objective of this study was 1) to prove an association with the clinical condition in the Miniature Schnauzer breed, 2) to investigate other possible candidate genes, and 3) to explore the genome for other potential targets.
In the first part of this study, a large scale retrospective study was constructed to look at the breed prevalence of increased serum Spec cPL concentrations in a large population of clinical samples submitted for analysis. A large sample size was obtained and the numbers of dogs within a breed and the percentage of those with an increased Spec cPL concentration were compared to the frequency of the population as a whole minus the breed of interest. This study was able to identify 13 breeds with a statistically significant odds ratio for having an increased Spec cPL concentration. The top dog breeds were the Miniature Pinscher (OR: 3.64), the Boxer (OR: 3.49), the Keeshond (OR: 3.24), the Pomeranian (OR: 2.53), and the Alaskan Eskimo (OR: 2.02). Miniature Schnauzers, although not showing the highest association, were also significantly associated (OR: 1.23). While proving the association for Miniature Schnauzers was the primary aim of the study, this project was able to identify three closely related dog breed, (i.e., Keeshonds, Pomeranians, and Eskimos), which all originally descended from Germany. Further investigation into this group of dogs is warranted.

This study was also able confirm early reports that dogs with pancreatitis are more likely to be older (median 9 years vs. 6 years). However this is the first report to demonstrate a statistically significantly increased prevalence in female dogs (53.4% vs. 46.6%; P <0.0001). Earlier studies demonstrated that neutering increased the risk, however sexual status was not investigated in this study due to the very small number of intact animals in this large population of dogs.

The second part of the study investigated the candidate gene SPINK1. This gene encodes for the pancreatic secretory trypsin inhibitor, which acts to bind prematurely
activated trypsin within the acinar cell of the pancreas. Earlier studies in humans pointed to mutations of this gene being causative for idiopathic chronic pancreatitis.\textsuperscript{43,44} Later studies, similar to what was found in this study, demonstrated a high prevalence of the variants in the general population.\textsuperscript{43} However, a recent meta-analysis was able to demonstrate that \textit{SPINK1} mutations can indeed be causative.\textsuperscript{45} Currently, homozygosity of the most common \textit{SPINK1} mutation is considered the etiology of pancreatitis when present. Heterozygosity for the same mutation is also considered causative if the individual does not have any mutations in the \textit{PRSS1, CFTR} and other known genes.\textsuperscript{21}

In this study, three variants in the \textit{SPINK1} gene were identified. Two of the variants were located in exon 2 and changed the coding of the three nucleotide codon. The other variant that was found, was unique to only Miniature Schnauzers and was found at the boundary of exon 3 with intron 3. It was found in the study that all three variants were always linked in the Miniature Schnauzers, and those that were homozygous for all three variants were 23 times more likely to have the phenotype of pancreatitis. The study does open up the question as to why, although at a much lower prevalence, the exon variants are common in the general population. These variants may be SNP’s that are in linkage with the intron/exon boundary mutation or perhaps being only homozygous makes this important. Miniature Schnauzers have also been demonstrated to be prone to familiar hyperlipidemia and this may a play a role in the phenotype. This study posed the question if other genes may also be involved in the etiology of pancreatitis in the Miniature Schnauzer. It also posed the question as to whether the variants of the \textit{SPINK1} gene lead to an altered final transcript.
The third component of this study was designed to answer questions raised during the second part of the study. It was clear that not all Miniature Schnauzers with pancreatitis were homozygous for all three variants although there are other known risk factors for the development of pancreatitis. The goal of the second part of this study was to conduct a genome wide investigation into other possible targets as well as to determine if the region of the \textit{SPINK1} gene would be identified as a candidate gene were the aims of this study. The genome wide scan was able to point at a small region on chromosome 2 that was associated with the phenotype established. Interestingly, the \textit{SPINK1} gene was in this region. While there were many other genes in this area, none others have been identified in human studies as being potential candidate genes. This finding begs the question as to whether \textit{SPINK1} variants alone are the genetic component or if there is some other environmental factor (dietary indiscretion) that is also needed. However, it is possible that other genes in this area could also be associated and more refined SNP mapping using a large population of dogs would be needed to further locate genes that may be worth sequencing.

The final section of this study was to investigate the missense variants that were identified in the second part of this study. In order to achieve this, a protocol was developed for the extraction of the mRNA from the pancreas of live patients undergoing surgery for other reasons. Indeed, collection mRNA from deceased patients is almost impossible due to the large amounts of proteases and digestive enzymes present in the pancreas.\textsuperscript{145} Dogs that were to be sexually altered were obtained from breeders and placed in new homes after completion of the study. During their elective surgery,
pancreatic biopsies were collected for mRNA extraction. cDNA primers of the \textit{SPINK1} gene were constructed to amplify the entire cDNA of the canine gene. Using RT-PCR, cDNA was successfully transcribed and dogs that were homozygous, heterozygous, and wild type for the variants were compared. While the exon variants did change the coding sequence as was predicted, the intron/exon boundary variant did not alter the final form of canine \textit{SPINK1} cDNA. However, mutations can affect protein production in a variety of ways with exon skipping or truncation being only one mechanism. The exon variants could also affect the structure and function of the subsequent protein or any of the variants may alter the expression of the protein \textit{in vivo}. This study warrants further investigation using cell culture to analyze expression and \textit{in vitro} trypsin binding.

In summary, the main conclusions of this study were:

1) Miniature Schnauzers for which samples have been submitted for pancreatic function testing are more likely to be positive for pancreatitis than the population as a whole.
2) Keeshonds, Pomeranians, and Eskimos are a closely related group of dogs that, based on this study, are predisposed to having an increased serum Spec cPL concentration.
3) Dogs with an increased Spec cPL concentration are more likely to be female and of older age than their cohorts.
4) Miniature Schnauzers have three missense variants located in their \textit{SPINK1} gene. These variants were always in linkage in this study.
5) Homozygosity for these three variants in the Miniature Schnauzers is significantly associated with clinical signs of pancreatitis and an increased serum cPLI concentration.
6) The exon variants identified are not unique to Miniature Schnauzers. However, they occur at a lower prevalence in other breeds than in the Miniature Schnauzers.

7) The intron/exon boundary variant is unique to Miniature Schnauzers.

8) The region between 40,339,715 to 44,189,359 on canine chromosome 2 is associated with having clinical signs of pancreatitis and an increased serum Spec cPL concentration. The *SPINK1* gene is also located in this region.

9) mRNA can be successfully extracted from the pancreas of live dogs in excellent quality and usability for downstream application.

10) The intron/exon variant of the *SPINK1* gene does not alter the final cDNA product.
REFERENCES


