GENOMIC ANALYSES OF INDUCED HYPERCHOLESTEROLEMIA AND ATHEROSCLEROSIS IN A MIXED BREED COLONY OF DOGS AND DEVELOPMENTAL ABNORMALITIES IN THE HAVANESE

A Dissertation

by

ALISON NICOLE STARR

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

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Genetics

GENOMIC ANALYSES OF INDUCED HYPERCHOLESTEROLEMIA AND ATHEROSCLEROSIS IN A MIXED BREED COLONY OF DOGS AND DEVELOPMENTAL ABNORMALITIES IN THE HAVANESE

A Dissertation

by

ALISON NICOLE STARR

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

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Keith E. Murphy
Committee Members,	Sharon C. Kerwin
	Ann B. Kier
	Charles R. Long
Chair of Interdisciplinary Faculty,	James R. Wild

December 2007

Major Subject: Genetics

ABSTRACT

Genomic Analyses of Induced Hypercholesterolemia and Atherosclerosis in a Mixed Breed Colony of Dogs and Developmental Abnormalities in the Havanese.

(December 2007)

Alison Nicole Starr, B.S. Clemson University

Chair of Advisory Committee: Dr. Keith E. Murphy

The domestic dog, *Canis lupus familiaris*, is a unique model system for the dissection of hereditary diseases. Selective breeding practices have created more than 300 distinct breeds of dogs, born from a desire to create specific physical and behavioral characteristics. Breeds represent closed breeding populations and the extensive records maintained for members of each breed (e.g., multi-generational pedigrees, veterinary medical records) present an incredible tool for genetic research. Two closed populations were used in the work presented here: a colony of mixed-breed dogs segregating resistance and sensitivity to cholesterol feeding, and a purebred pet population of Havanese experiencing a high frequency of developmental abnormalities.

Estimates of heritability were calculated for each disease to evaluate the degree of phenotypic variation attributable to genetics among dogs in the populations used. A heritability of 0.55 (± 0.16) was identified for cholesterol resistance and sensitivity in the mixed-breed colony. The small sample size prevented the use of complex segregation analyses to examine mode of transmission. A heritability of 0.36 (± 0.26) was calculated

for the composite phenotype in the Havanese, encompassing the spectrum of abnormalities in the breed. Polygenic inheritance was identified for the composite phenotype, but the action of a major gene was identified by complex segregation analyses in the Havanese.

Complex diseases preclude the use of a candidate gene approach, owing to the multitude of genes involved in the disease process. Whole genome screens provide a practical approach to the identification of chromosomal region(s) associated with a disease phenotype by narrowing the search for candidate gene(s). The Minimal Screening Set – 2 (MSS-2) was used in the present studies to evaluate the segregation of microsatellite markers in pedigrees for both the mixed-breed colony and the Havanese. No significant LOD scores were identified, though suggestive LOD scores were obtained in both analyses.

A canine-specific oligonucleotide microarray was used to create gene expression profiles for developmental abnormalities in the Havanese and for cholesterol sensitivity in the mixed-breed colony dogs. Distinct expression profiles were generated for each group, and several genes of interest were identified as being both differentially expressed ($>\pm 2$-fold change) and statistically significant ($p\text{-value}<0.05$).

DEDICATION

For my family

For Tessa

ACKNOWLEDGEMENTS

I would first like to thank my committee chair, Dr. Keith Murphy, for taking a chance on a sight-unseen Clemson Tiger. He has provided me every opportunity to succeed and I will be forever grateful for his advice and mentorship over the course of my graduate career. I would also like to thank my committee members, Drs. Sharon Kerwin, Ann Kier, and Charles Long, for their guidance, encouragement, and expertise.

I want to thank my many colleagues in the Canine Genetics Laboratory for their help and support during the course of my graduate career. Two particular members, Dr. Leigh Anne Clark and Dr. Kate Tsai, have been invaluable to me. Their instruction, advice, and endless edits in the lab, and more importantly, their friendship out of the lab have been and continue to be deeply treasured.

I have several collaborators whose contributions to this work were indispensable. I would like to thank: Dr. James T. Willerson and Janice McNatt from the University of Texas Health Science Center in Houston for allowing me to participate in the research of their extraordinary atherosclerosis colony; Dr. Thomas Famula from the University of California-Davis for carrying out the statistical analyses and helping me overcome my mathematical and statistical ineptitude; Dr. Nathan Markward from the Pennington Biomedical Research Center, Louisiana State University System, for his endless explanations of Rasch models of measurement.

I want to extend a special thanks to the many Havanese breeders and owners who supported the research by contributing samples and pedigrees from their dogs, most

notably, Mrs. Diane Klumb, Dr. Joanne Baldwin, Ms. Karon Fowler, and Ms. Nancy Simpson for their time, effort, and dedication to the betterment of the breed.

Additionally, I wish to thank Dr. John Diehl, my undergraduate advisor at Clemson, for recognizing in me an aptitude for research that I did not know existed. He encouraged me to pursue a career in science, thereby introducing me to the joys and frustrations that accompany the single-minded pursuit of a puzzle in nature, and for his recommendation of a graduate degree under the direction of Dr. Murphy. To him, I will be eternally grateful as well.

Finally, I wish to thank my mother and father for their unending love, support, and encouragement through every step of my academic career, and to my brother, for being my biggest fan.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION: THE DOG AS A MODEL

> *All knowledge, the totality of all questions and all answers, is contained in the dog*. ~Franz Kafka

Canine genetics

Among mammals, the domestic dog (*Canis lupis familiaris*) is the most diverse species, unequaled with respect to its varying morphology, coat color and behavior. Fossil records indicate the wolf was domesticated approximately 15,000 years ago (Savolainen et al. 2002), and the present-day dog population diverged exclusively from these early domesticated wolves (Vilà et al. 1997). Selective breeding practices have produced more than 300 breeds of dog (FCI 2007). These breeds were borne from a desire to create companion animals with specific behavioral and physical characteristics. Each breed is a closed population, and as such, has limited genotypic and phenotypic heterogeneity caused by founder events, population bottlenecks, and the use of popular sires. This unique population structure, along with detailed pedigrees, short gestation time, large litter size, and superior medical surveillance, makes the dog a valuable tool for the dissection of genetic traits.

Over 480 hereditary diseases have been described in the dog and more than 200 of these have decidedly similar clinical presentations to corresponding diseases of the human (OMIA 2007). The aforementioned breeding practice designed to produce dogs

---

This dissertation follows the style of Journal of Heredity.

with specific traits has had an undesired consequence: the propagation of autosomal recessive diseases. The presence of deleterious alleles, when combined with the common practice of inbreeding, results in the mating of asymptomatic carriers. This obviously increases the number of autosomal recessive diseases (Ostrander et al. 2000) Almost half (46%) of the hereditary diseases in the dog occur largely in one or a few breeds (Patterson 2000, Sargan 2004). The natural occurrence of these conditions makes the dog a more valuable model than the mouse, whose widely studied disease models are primarily induced. The dog also has a longer life span and a more comparative body/tissue size with the human than does the mouse.

Canine genomics

Many recent advances in canine genomics have accelerated research of canine hereditary diseases. Most notably, the completed 7.6X sequence of the canine genome (Lindblad-Toh at al. 2005) has provided the ultimate tool for genetic research and has aided the development of canine-specific oligonucleotide arrays and single nucleotide polymorphism (SNP) arrays for large-scale genomic studies. These new technologies can be used to create disease profiles and identify candidate genes or pathways responsible for the disease, or for disease progression.

The canine genome is comprised of 38 pairs of autosomes, plus the X and Y sex chromosomes. From sequence analysis, the genome has been determined to be 2.4 gigabases (10^9 bases) in size (Lindblad-Toh et al. 2005). The most recent estimate of the number of canine genes is 19,000; approximately 75% of these are orthologous among dog, mouse, and human (Parker and Ostrander 2005).

Cytogenetic studies, including a standardized karyotype (Breen et al. 1999) and radiation hybrid (RH) maps (Priat et al 1998, Vignaux et al 1999, Breen et al 2001), have facilitated the development of maps of the dog that cane be compared to various species (Sargan et al. 2000). The identification and localization of both markers (i.e., microsatellites, SNPs) and specific genes has yielded insight into genome conservation and rearrangement. From these studies, it is clear that the canine genome is remarkably similar to the human genome; e.g., from a comparative standpoint there are low levels of chromosomal rearrangements in the two genomes. The numbering of canine chromosomes, mapping of markers and genes, has been indispensable to chromosome-specific approaches to genome screens.

Linkage analyses

Linkage maps (Mellersh et al. 1997, Breen et al 2001) and multiplexed microsatellite screening sets (Cargill et al. 2002, Clark et al. 2004) have streamlined studies designed to identify regions of the genome that harbor causative genes or quantitative trait loci (QTLs). The complexity of many diseases - with many genes and pathways known to play a role in disease initiation and progression - prohibits the use of a candidate gene approach, thus necessitating the use of linkage analyses, either classical linkage or linkage disequilibrium (LD). Classical linkage analyses trace allele identity-by-descent using multi-generational pedigrees (minimum of three generations). This approach is based on the number of alleles shared among family members at specific chromosomal loci, and the associated phenotypic similarity between these members.

Classical linkage is informative for well-described families in which the segregation of disease over several generations can be observed, and every family member can be accounted for. The limitations of classical linkage are that it requires detailed, informative pedigrees and samples from all members of the family. These constraints make the LD approach to association studies more attractive. LD uses unrelated affected and unaffected dogs in screens of the genome for alleles that are conserved among members of the groups. Recent advances in association studies (Sutter et al. 2004, Parker et al. 2004) highlight the use of LD for mapping disease traits of the dog. Linkage analyses, classical or LD, do not detect specific gene(s). Rather, regions of the genome within which candidate genes reside are identified by linkage analysis.

Population structures for research

Atherosclerosis and physiologic development are two examples of conditions under complex genetic control. Many pathways and genes are known to contribute to the development of both atherosclerosis and embryonic patterning, complicating the study of these conditions. Two distinct population structures were used to investigate these complex traits: a cross-bred, Labrador-hound (Lab-mix) population segregating extreme phenotypes, and a purebred population with a variable, syndrome-like phenotype.

The Lab-mix population demonstrates a cross-bred pedigree segregating extreme phenotypes in response to cholesterol feeding: cholesterol sensitivity leading to the formation of atherosclerotic lesions, and cholesterol resistance with no associated lesions. The dogs were initially bred for use as surgical models for human angioplasty, but when the breeding colony began segregating different phenotypic responses to

cholesterol feeding, the investigators became interested in the genetics underlying the phenotypic differences, and whether the process of atherosclerosis in the dog would mimic human atherosclerosis. Atherosclerosis is known to be a complex disease with multiple environmental and genetic factors contributing to the disease presentation. It has been suggested that for the study of complex diseases, a restricted population of only those family members segregating the most extreme phenotypes should be used to increase the power of linkage studies (Risch and Zhang 1995). In doing so, the amount of genetic variation among family members sharing the same phenotype is decreased, and between the extremes, is maximized. In short, the high degree of inbreeding in closed populations facilitates the search for disease-causing genes by decreasing the polymorphisms that exist in open populations.

The purebred domestic dog provides researchers with perfect tool for the study of hereditary diseases. That is, the closed breeding populations of purebred dogs contain individuals who are largely homogeneous at the phenotypic and genotypic levels. The Havanese breed originated in Cuba, with descriptions dating back to the 1700's. In the 1970's, Havanese were introduced in the United States (US) by eight founding dogs. Havanese kindred from Hungary, Costa Rica, and Cuba have been added in the last twenty years to the US Havanese population (D. Klumb, personal communication).

Atherosclerosis

Hypercholesterolemia is directly associated with the development of atherosclerosis, coronary heart disease (CHD), and stroke. Prolonged hypercholesterolemia often leads to the development of atherosclerosis. The clinical

complications of atherosclerosis include myocardial infarction (MI), CHD, and stroke. Over 35 million Americans have cholesterol levels in the range that places them at risk (greater than 240 mg/dL) for developing CHD or stroke (American Heart Association (AHA) 2007). CHD is the leading cause of death in the United States, and stroke is the third leading cause of death (AHA 2007). For these reasons, research into risk factors, disease progression, and treatment protocols for atherosclerosis is important.

In humans, an excess of low-density lipoprotein (LDL) circulating in the plasma causes macrophages to express scavenger receptors that bind the free LDL. Macrophages lack a feedback mechanism to regulate intracellular cholesterol levels, resulting in an accumulation of cholesterol in the cytoplasm. The accumulation of cholesterol-filled macrophages (foam cells) in the subendothelium leads to the development of fatty streak lesions – the first visible abnormality of the vascular wall. An inflammatory reaction is induced by the disruption of the vascular wall integrity. Smooth muscle cells (SMCs) immigrate to the fatty streak from the medial layer, producing an early atheroma with a fibrous cap. In advanced lesions, calcification, ulceration, and hemorrhage of small vessels can disrupt the lesion stability. The most serious health threat in atherosclerosis is that of arterial occlusion, either resulting from a chronic or acute condition. Chronic conditions are caused by large, advanced lesions that block the blood flow in a vessel. Acute occlusion results from the rupture of a plaque, releasing necrotic material from the center of the plaque. When this material reacts with the blood components, a thrombus forms, and it may become lodged in small arteries

thereby causing myocardial infarction or stroke, depending in which tissue the occlusive thrombus occurs.

Because atherosclerosis is known to involve both genetic and environmental factors, having a controlled, uniform environment for all study participants minimizes external factors which may complicate human studies of atherosclerosis. For this reason, the colony of Lab-mix dogs is a particularly powerful tool as a large animal model of atherosclerosis.

Developmental abnormalities

A multitude of abnormalities arising from aberrant embryonic development have been described in virtually every species. Embryonic development is a highly regulated process requiring the precisely coordinated actions of many genes, and the disruption of these genes and tightly-controlled pathways are responsible for many birth defects. One such pathway is that of cholesterol biosynthesis. The disruption of any gene involved in cholesterol biosynthesis results in a syndrome of developmental abnormalities; including cleft palate, open fontanel, facial dysmorphes, toe syndactyly, and chondrodysplasia (Nwokoro et al 2001). Cholesterol is an essential component of development and it acts as the covalent adduct for the hedgehog proteins (Porter et al 1996).

The hedgehog (Hh) proteins are a family of signaling molecules involved in embryonic patterning (Porter et al 1996). Disruption of the cholesterol biosynthesis pathway prevents endogenous production of cholesterol, directly affecting the ability of Hh proteins to function in enbryogenesis (Cooper et al 2003). Members of the Hh family

include sonic hedgehog (*Shh*), desert hedgehog (*Dhh*), and Indian hedgehog (*Ihh*). *Shh* dysfunction results in abnormal development of the brain, skeleton, and gonads.

The Havanese breed has presented with open fontanels, cleft palates, hydrocephalus, cryptorchidism, osteochondrosysplasia, luxating patellas, elbow dysplasias, Legg-Calve-Perthes, liver shunts and hepatic microvascular dysplasias, heart murmurs, atrial septal defects, cataracts, deafness, and missing incisors. The incidences of each of these conditions within the breed are unknown, but a number of the abnormalities are strikingly similar to abnormalities produced in cholesterol biosynthesis disorders.

Heritability and CSA

Heritability is a measure used to estimate the proportion of phenotypic variation which can be attributed to genetic variance in a sample population. This estimate is calculated for non-Mendelian traits to predict the response to selective breeding. Heritability is measured from a scale of 0 to 1, such that a score of 0 indicates no genetic contribution, and a score of 1 indicates total genetic control. Various studies of atherosclerosis in the human have estimated heritabilities between 0.21 and 0.92, depending on the method of measurement of atherosclerosis. The heritability of atherosclerosis in the dog has not previously been reported. The abnormalities in the Havanese have not been investigated prior to the present study, therefore no heritability estimates exist.

Complex segregation analyses (CSA) are used to evaluate various modes of disease transmission in a defined pedigree to identify the pattern of inheritance which

has the best statistical fit. This is accomplished through use of computer modeling programs, CSA characterizes a trait as exhibiting either Mendelian inheritance (*i.e.*, autosomal recessive, autosomal dominant, X-linked) or a non-Mendelian inheritance (*i.e.*, polygenic, multifactoral).

Complex diseases result from interplay of genetic and environmental factors. One consequence of such interactions of multiple genes is that there may be one which exerts a greater influence in phenotypic expression; this is designated a major gene. Complex diseases are precluded from having a simple inheritance pattern; however, the action of a major locus can be detected using CSA.

Rasch models

Rasch modeling is a frequently employed approach in psychology, sociology and education to measure such traits as abilities and attitude. This approach is particularly used in psychometrics, or those fields concerned with the psychological or educational measurement. In such fields, behaviors and abilities are analyzed. These traits, in essence, are complex traits. Both environment and genetics play a role in the development of specific behavior or ability. Thus, this approach in education and psychology may very well be directly applicable to the study of complex disease in the biomedical research and may provide powerful insight into the phenotype of interest by measuring many traits for simultaneous analysis. Rasch models have not previously been used in canine genetics, and the inclusion herein provides the first evidence of how conventional statistical approaches (*e.g.*, heritability, CSA) can be integrated with novel approaches to measurement of traits.

Objectives

The specific objective of this work was to study the genetics of two different population structures of dogs segregating complex diseases. To accomplish this objective, it was necessary to (1) assemble multigenerational pedigrees for both the Lab-mix and Havanese samples, (2) complete estimates of heritability and complex segregation analyses for Lab-mix and Havanese pedigrees, (3) perform linkage analysis on Lab-mix and Havanese kindred for atherosclerosis susceptibility loci and loci contributing to developmental abnormalities, respectively, and (4) develop gene expression profiles for cholesterol-resistant and sensitive dogs, as well as affected Havanese, using an oligonucleotide array.

CHAPTER II

HERITABILITY AND GENETIC EVALUATION OF INDUCED HYPERCHOLESTEROLEMIA AND ATHEROSCLEROSIS IN THE DOMESTIC DOG

Overview

Hypercholesterolemia and atherosclerosis are major health concerns and, to date, research has been focused on genetic and environmental contributing factors that contribute to these diseases as well as on treatment. Familial associations exist in humans, and studies have confirmed a polygenic mode of transmission. Several models of hypercholesterolemia and arteriosclerosis exist. For example, an induced model of atherosclerosis has been developed in a colony of mixed breed dogs. Described herein are the heritability studies, linkage analyses, and global gene expression data generated as part of the investigation of induced hypercholesterolemia and atherosclerosis in the domestic dog.

Introduction

Hypercholesterolemia is directly associated with the development of atherosclerosis, coronary heart disease (CHD), and stroke. Over 35 million Americans have cholesterol levels in the range that places them at risk (greater than 240 mg/dL) for developing CHD or stroke (American heart assoc 2007). CHD is the leading cause of death in the United States, and stroke is the third leading cause of death (AHA 2007).

For these reasons, research into risk factors, disease progression, and treatment protocols for hypercholesterolemia and atherosclerosis is critical.

Both large and small animal models of atherosclerosis exist for the study of disease susceptibility, initiation, progression, and treatment. Animal models of atherosclerosis include primates, the pig, dog, rat, mouse, and rabbit. A brief summary of these models of atherosclerosis compared to human atherosclerosis is outlined in Table 1 (Russell and Proctor 2006, Blanton and Peeters 1976, Yanni 2004). No single model of atherosclerosis is without shortcomings, and the data produced from studies of model animals are not easily extrapolated to the human.

Table 1: Comparison of the animal models of atherosclerosis to the natural atherosclerosis in humans.

Model	*Phylogenetically close to human*	*Metabolism*	*Diet*	*Atherosclerosis development*
Primate	+++	Similar	Omnivore	Natural and Induced
Pig	++	Similar	Omnivore	Induced
Dog	++	Dissimilar	Carnivore	Induced
Rabbit	+	Dissimilar	Herbivore	Induced
Rat	+	Similar	Omnivore	Induced
Mouse	+	Similar	Herbivore	Induced

The dog has been used as a model for many human hereditary diseases, both with single gene and complex inheritance patterns (for reviews see: Ostrander et al. 2000, Sutter and Ostrander 2004, Switonski et al. 2004, Tsai et al. 2007). Spontaneous cases of atherosclerosis in the pet population are predominantly secondary to another disturbance,

e.g., hypothyroidisim, diabetes mellitus, or microorganism infection (Sottiaux 1999, Sako et al. 2002, Hess et al. 2003). Accordingly, canine atherosclerosis in the research setting results from manipulation of thyroid function (Rawitscher et al. 1973, Sabiston et al. 1961, Steiner et al. 1949) or surgical intervention (Pertsemlidis et al. 1973, Rapold et al. 1992, Deng et al. 1994). Experimental models of atherosclerosis in the dog, when compared to other models of atherosclerosis, show a similar distribution of lesions as related to age to the human (Weinberg 2004).

A colony of Lab-mix dogs which segregates cholesterol-sensitive and cholesterol–resistant phenotypes has been developed. These dogs are suitable for long-term studies of cholesterol-supplemented diets, in contrast to the rabbit, in which hepatotoxicity develops from long term cholesterol-feeding (Yanni 2004). Dogs have been successfully maintained on the high-cholesterol diet for up to one year. The dogs, when intermittently challenged with cholesterol, have a lifespan of 9 - 12 years, comparable to a Labrador-mix breed pet lifespan of 10 and 14 years.

Many dogs challenged with the cholesterol diet exhibit dermatologic abnormalities (dry, flaky skin) and otitis externa. These abnormalities occur predominantly in the sensitive dogs and are attributed to impaired circulation. A diet change to regular dog chow resolves these abnormalities.

The Lab-mix model of atherosclerosis was developed by selective breeding for sensitive and resistant dogs. The normal cholesterol level in the dog is 116-254 mg/dL. Upon the challenge of dietary cholesterol, a continuum of cholesterol levels is observed. Dogs are classified as sensitive, resistant, or average based on the response to the dietary

challenge. Only the most sensitive (cholesterol >900 mg/dL) and resistant dogs (cholesterol <400) were used to propagate the colony. Dogs exhibiting an average response (~600-890 mg/dL) to the cholesterol challenge were not included in this study.

The dog has traditionally been thought of as a naturally cholesterol-resistant species (Abell et al. 1956, Pertsemlidis et al. 1973). Dogs are extremely efficient at maintaining cholesterol homeostasis with their ability to increase or decrease cholesterol excretion and/or cholesterol biosynthesis in response to dietary cholesterol intake (Pertsemlidis et al 1973). Dogs also have a protective mechanism against myocardial infarction because of the development of collateral myocardial arteries (Helisch and Schaper 2003). The main transporter of cholesterol in the dog is HDL. Nearly 87% of lipoprotein content in the dog is HDL, with ~11% lipoprotein as LDL, in direct inverse to man (~11% and 86%, respectively) (Maldonado et al. 2001). While this inverse correlation between dogs and the human with respect to lipid metabolism may appear to pose a limitation of the canine model, it should be noted that lipid metabolism in the mouse and rat are also based on HDL (Russell and Proctor 2006).

Presented here are the genetic analyses of induced hypercholesterolemia and atherosclerosis in a colony of mixed-breed dogs.

Materials and methods

Dogs

The Lab-mix dogs in this study were fed an atherogenic test diet (Harlan Teklad Laboratory Diets, Madison, WI) comprised of 5% cholesterol by weight. The dogs used in this study weigh between 30 and 40 kgs. Twenty-seven dogs were given 2.8 kg of diet

per day *ad libitum.* The dogs were maintained on the diet for 12 months. Each dog had venous blood drawn monthly to monitor cholesterol (Cholestech LDX, Cholestech Corp., Hayward, CA), serum chemistries (VetTest, IDEXX, Westbrook, ME) and complete blood counts (CBC) (Forcyte, Oxford Science, Oxford, CT). Tissues were collected from dogs at the time of necropsy.

A five-generation pedigree was assembled for the Lab-mix dogs. The pedigree contained 84 dogs: 38 females and 46 males. These dogs include the 27 fed the cholesterol diet and 56 additional dogs, completing sibling and parent relationships (Fig 1).

Linkage

Twenty-eight dogs were selected for a whole genome screen using the MSS-2, a multiplexed, canine-specific collection of microsatellite markers (Clark et al. 2004). Whole blood was collected from each dog and DNA was isolated using the Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN).

Four fluorescent dyes were used to label the forward primers, and the PCR protocol described by Clark et al. (2004) was modified for the use of robotics (Bio-Tek Precision 2000, Winooski, VT). Stocks of DNA were maintained at 12.5 ng/µl and 4 µl of DNA was added for a total of 50ng of DNA to each reaction. As a result, no ddH2O was added to the reaction. PCR products were resolved on an ABI 3730 sequencer (Applied Biosystems, Foster City, CA) with an internal size standard (Gene Scan 500 LIZ, Applied Biosystems). Genotypes were assigned using Genemapper® software v3.7 (Applied Biosystems).

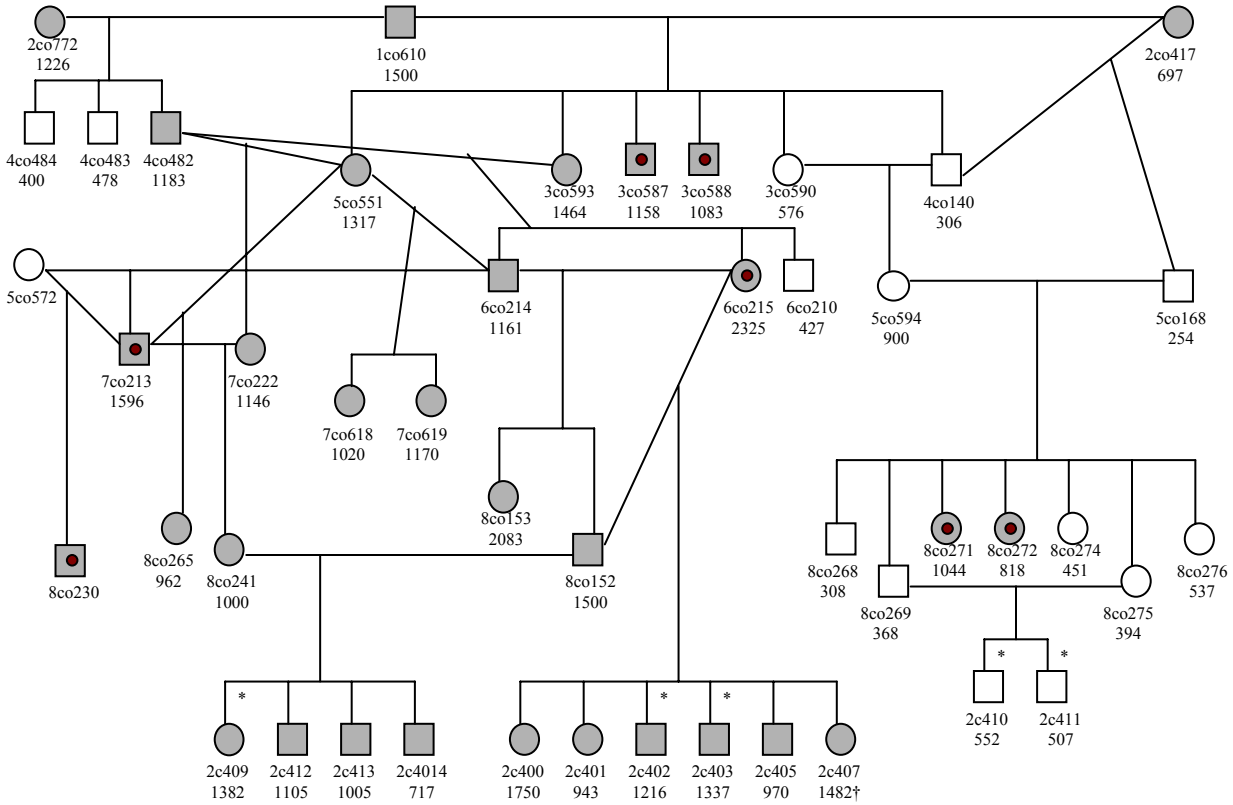

Figure 1: Lab-mix kindred for the sensitive (grey) and resistant (white) dogs. Sensitive dogs marked with a maroon circle experienced a stroke. The five dogs selected for the gene expression studies are indicated with (*). Average cholesterol levels (mg/dL) for each dog over the course of the cholesterol challenge are listed below each individual. (†) Dog died suddenly in the middle of the study, so this number is the average of months measured.

Two-point LOD scores were calculated using the SOLAR software package version 2.1.5 (Almasy and Blangero 1998). Each dog was assigned two phenotypes: either cholesterol setsitive (1) or cholesterol resistant (0), and a continuous cholesterol level for evaluation.

Global gene expression

Gene expression in the Lab-mix was assessed using the Affymetrix canine genome 2.0 array (Santa Clara, CA). The array contains 42,929 total probe sets (controls included). Eighteen thousand transcripts are canine-specific and more than 20,000 predicted gene sequences are included based on homology with known genes in other species.

Two resistant and three sensitive dogs were selected for the expression study. The sensitive and resistant dogs can trace common ancestry through a male and female four generations previous. The two resistant dogs were full-siblings. Two of three sensitive dogs were full-siblings; the third was a half-sibling. (Figure 1). All five dogs were age-matched.

Samples from the heart, liver, and brain were collected from the five dogs at the time of euthanasia and necropsy. Tissue was stored in RNAlater® (Ambion, Austin, TX). RNA isolated from the tissues was prepared by Viagen Inc. for hybridization to the Affymetrix canine 2.0 array using the classic protocol (Chomczynski and Sacchi 1987). Ambion's linear amplification kit, MessageAmp^{TM} aRNA Kit (Ambion Inc.), was used for first- and second-strand cDNA, and biotin-labeled cRNA synthesis. Two micrograms of total RNA were used to start the single round of amplification. All samples were run

in duplicate. Fragmentation of the labeled cRNA was performed by incubating the samples at 95˚C for 35 minutes in a solution containing 40mM Tris-acetate (pH 8.1), 100mM KOAc, and 30mM MgOAc. Forty micrograms of the fragmented, labeled cRNA was then hybridized to each of the GeneChip® (Affymetrix) oligonucleotide arrays. These arrays were washed, stained, and scanned in accordance with a previously published protocol (Ji et al. 2004).

High-resolution GeneChip images were collected on the GeneChip Scanner 3000. Affymetrix GeneChip operating software was used to quantify image data and calculate gene expression values.

GeneSpring software (Silicon Genetics, Redwood City, CA) was used to perform gene clustering, Students' *t*-test, and Bonferroni multiple testing corrections. Samples were grouped separately to identify differentially expressed genes.

Results

Dog response to diet

Sensitive dogs fed an atherogenic diet longer than 3 months had dramatic increases in serum cholesterol levels and subsequent development of atherosclerotic lesions in small arteries (i.e., femoral arteries, iliac a., coronary a., circle of Willis). Resistant dogs fed the high-cholesterol diet responded with small to moderate increases in serum cholesterol but developed no lesions, despite the one-year feeding period. The average cholesterol level of sensitive dogs was 1237 mg/dL, compared to the average 424 mg/dL in resistant dogs, when challenged with the atherogenic diet.

Estimate of heritability

Table 2 presents evidence showing that atherosclerosis, as measured by cholesterol levels, is an inherited disorder in the Lab-mix. The estimate of heritability of 0.550 (± 0.16) supports a significant heritable component for this illness. Additionally, a binary scoring system of 0 (resistant) and 1 (sensitive) was evaluated for heritability. The estimates for this trait were 0.582 (± 0.21).

Table 2: Estimate of heritability in a threshold model for the continuous cholesterol levels and the binary score of atherosclerosis.

	Estimate	**Standard Error**
Cholesterol level	0.550	0.16
Binary score	0.582	0.21

Linkage

Two-hundred and five microsatellite markers were genotyped and analyzed for linkage with the cholesterol phenotype. The maximum LOD score attainable from this pedigree was determined to be 1.8, based on a perfectly segregating autosomal recessive trait. Using the cholesterol trait, four markers had LOD scores which suggested further investigation: CPH14 (CFA05) 1.06, REN162C04 (CFA07) 0.96, AHT137 (CFA11) 0.89 and C11.873 (CFA11) 1.24. The binary trait had only two markers with LOD scores > 0.80 and both were located on CFA11: C11.873 (0.85) and FH2004 (0.89).

Global gene expression

The second generation canine Affymetrix oligonucleotide array was used to assess gene expression in resistant and sensitive tissues. A disease profile for atherosclerosis in the heart, liver, and brain tissues was created. The sensitive and resistant dogs had significantly different expression profiles that allowed the software to cluster the dogs in the two respective groups. Using the Bonferroni multiple testing methods, 49 genes in the liver, 13 genes in heart, and 477 genes in brain were identified as being both statistically different (p-value <0.05) and differentially expressed (greater than ± 2-fold) . Of these, 48 were up-regulated and 1 down-regulated in the liver; 6 were up-regulated and 7 down-regulated in the heart; 326 were up-regulated and 77 down-regulated in the brain. An abbreviated list of results is listed in Table 3.

Discussion

Atherosclerosis has an established polygenic inheritance with environmental risk factors playing a large role in the disease progression in humans (Lusis et al. 2004). One advantage of the canine model of atherosclerosis is the normalization of environmental factors among dogs in the study. Each dog in the study is maintained in the same environment, exposed to the same external influences, and fed the same diet – all of which are variables contributing to variation of mRNA expression. Thus, differences observed between the resistant and sensitive dogs should primarily be due to genetic control.

Complex segregation analysis, developed by Bonney (1986), can clarify the mode of transmission by evaluating both single locus transmission and polygenic

Table 3: List of differentially expressed genes in pathways known to contribute to atherosclerosis development in the liver. Cells containing multiple values represent fold changes calculated for several probes from a specific gene. (*) indicates a significant *P*-values (<0.05).

Pathways and Genes	Fold change
Bile Acid Regulation	
Organic anion transporting polypeptide A	5.89
Organic anion transporting polypeptide C	4.79
Endothelial Dysfunction	
Endothelial nitric oxide synthase	1.13
Angiotensinogen	105.0; 17.4
Angiotensin converting enzyme 2 precursor	6.76
Cholesterol Biosynthesis	
Mevalonate kinase	2.68; 4.50; 9.15
Squalene monooxygenase (epoxidase)	0.21; 0.21; 0.26
Reverse Cholesterol Transport	
ATP-binding cassette protein A1	0.43; 2.78; 8.35
Scavenger receptor class B type 1	1.41; 16.36
Nuclear Receptors/Cholesterol Efflux	
Retinoid X receptor beta	6.34
Thyroid receptor interacting 3	1.10
Lipoproteins	
Apolipoprotein AV	4.64
Apolipoprotein L6	10.27
Apolipoprotein B	0.93
Apolipoprotein AIII	0.65
Bile Acid Synthesis	
Cholesterol 7α-hydroxylase	5.42
Sterol 12 α-hydroxylase	36.95
CEL and CEL regulation	
Carboxyl ester lipase	0.149*
ASM-like phosphodiesterase	3.60
Phospholipase A2 group 1B	2.50
Cellular Lipid Synthesis and Fatty Acid Degradation	
Acyl-CoA synthetase long-chain family member 1	48.12*
Acyl-CoA synthetase long-chain family member 5	143.5*

inheritance. The sample size used in the present study precluded the use of this approach. More dogs and the associated clinical data from the colony will need to be collected to determine the inheritance of hypercholesterolemia and atherosclerosis in the Lab-mix model.

Limitations to the use of the threshold model in evaluations of heritability exist, and the addition of other dogs or kindred to the studies may alter the results presented here. The population structure described herein, however, meet the minimum requirements of the threshold model. The heritability identified for canine atherosclerosis in this study (0.55-0.58) is higher than that estimated for the human (~0.40). This estimate indicates a significant contribution of genetic factors accounting for the phenotypic variation in the population of Lab-mix dogs.

Because the Lab-mix colony is a closed breeding population with few founders and significant inbreeding, the dogs are largely genetically homogeneous. Very few differences in gene expression were observed between the two groups in both the liver and the heart, two of the most important organs in atherosclerosis. These differences, in theory, may be directly related to disease. The statistical approach used in this analysis examined only those genes with a statistically significant (<0.05) p-value among dogs in the two classes and differentially regulated (>±2-fold change) between the two groups. Recent studies evaluating canine gene expression using microarrays have used a lower (1.5; 1.6) fold change (Lindberg et al 2007, Maccoux et al. 2007) or only statistically significant genes (Clements et al. 2007). Reducing the stringency employed here may have allowed the identification of more known atherosclerosis-related genes.

Several intriguing genes identified by the oligonucleotide array in sensitive dogs include: *ACSL5* (143.5-fold up-regulated), *ACSL1* (48-fold up-regulated), and *CEL* (6.7-fold down-regulated) in the liver, and *MMP28* (2.7-fold down-regulated) in the heart. *CEL* (carboxyl ester lipase) is located on CFA09 (HSA9q34) and has both cholesterol binding and transfer activites (Hui and Howles 2002). Li and colleagues (1996) describe CEL-facilitated reverse cholesterol transport by the delivery of cholesterol from HDL to the liver. Additionally, CEL in the vessel walls appears to provide a protective mechanism against atherosclerosis development (Hui and Howles 2002).

ACSL1 is located on CFA16 (HSA4q34-35) and *ACSL5* is located on CFA28 (HSA10q25.1-q25.2). These are involved in the fatty acid metabolism and peroxisome proliferator-activated receptor (PPAR) pathways (Rebhan et al. 1997). The identification of two differentially regulated paralogs in a single tissue suggests further research in the fatty acid and PPAR pathways should be done. MMP28 (CFA09; HSA17q11-q21.1) is involved in remodeling and repair of tissues (Rebhan et al. 1997). The differential expression of this gene is most likely as a responsive reaction to the vascular injury occurring in sensitive dogs, and the inflammatory processes induced by atherosclerosis development.

The linkage analysis did not yield any statistically significant LOD scores (LOD<3). This may be due in part to the small sample size and also to a polygenic mode of inheritance. Several linkage studies in murine models have identified QTLs for atherosclerosis (Allayee et al. 2003, Smith 2003, Dweyer et al. 2004), and the homologous regions in the canine genome were therefore examined. One marker with a

suggestive LOD score (1.24), C11.873, is located on CFA11 in a region sharing homology with HSA09 and MMU04. Chromosome 4 in the mouse has an atherosclerosis-susceptibility QTL (Wang et al 2003, Korstanje et al. 2004). The homologous region in the human is HSA9q31.1. One intriguing gene located in this region is *ABCA1*, a mediator of cholesterol removal from cells by HDL (Lusis et al. 2004). Another candidate gene identified in this region of the mouse is *TLR4* (Wang et al 2005). TLR4 is an activator of inflammatory cells and regulates both innate and adaptive immune responses (Li and Sun 2007). The initiation and progression of atherosclerosis is due to inflammation and immune responses (Libby 2002). *TLR4* expression has been detected in atherosclerotic lesions and is associated with disease progression (Li and Sun 2007), but the mechanisms by which this occurs is not fully understood.

A second marker, on CFA05 (CHP14), is located in a region sharing homology with HSA16q22.1-21 and MMU08. *LCAT* encodes a reverse cholesterol transport product, and is located at HSA16q22.1-q21. Murine autosome 8 also contains a QTL for atherosclerosis corresponding to HSA16q22-24 (Wang et al 2005).

Both *ABCA1* and *LCAT* were identified as candidate genes from the QTL regions of the mouse (Wang et al 2005). The identification of regions harboring these two genes suggests the involvement of reverse cholesterol transport (HDL) in the onset of atherosclerosis in the Lab-mix. Because HDL is the predominant cholesterol transporter in the dog, a defect of the reverse cholesterol transport process (removal of cholesterol from tissue to the liver) would prevent cholesterol removal and thus allow the build-up of cholesterol in tissues, leading to the development of atheromas.

Another explanation for reverse transport-related genes could include excess cholesterol accumulation in HDL, leading to atheromas comprised of HDL molecules. HDL itself has been shown to be sufficient to induce atherosclerosis when oxidatively modified (Carter et al 1987). Histochemical studies to evaluate the composition of atherosclerotic plaques would distinguish between the two potential explanations.

The results from this work suggest that an error in reverse cholesterol transport causes cholesterol-sensitivity in the atheromatous dogs, and *CEL*, *ABCA1*, and *LCAT*, represent plausible candidates. Even so, it seems quite likely that many false negatives were encountered in the linkage analyses due to the high degree of homogeneity in the small population. Future studies will include investigation of the three aforementioned genes, as well as the addition of more dogs from the colony in order to increase the power of the pedigree. Additionally, histopathological studies are planned for the comparison of canine atherosclerotic lesions to those found in the human.

In conclusion, multiple approaches were taken to evaluate the genetics of atherosclerosis in the domestic dog. Estimates of heritability, linkage analyses, and gene expression analyses were completed for a colony of mixed breed dogs segregating atherosclerosis. Atherosclerosis was determined to be a highly heritable trait in the dog. The highest LOD scores produced in the linkage analyses, though not statistically significant, were found for genomic regions sharing homology with murine QTLs for atherosclerosis. The global gene expression profiles revealed a number of genes; some have previously described associations with atherosclerosis, and others have yet to be studied.

CHAPTER III

HEREDITARY EVALUATION OF MULTIPLE DEVELOPMENTAL ABNORMALITIES IN THE HAVANESE DOG BREED*

Overview

The Havanese is a toy breed that presents with a wide range of developmental abnormalities. Skeletal defects, particularly osteochondrodysplasia (CD), are the most frequently observed anomalies. Cataracts, liver shunts, heart murmurs, and missing incisors are also common in this breed. Estimates of heredity and complex segregation analyses were carried out to evaluate modes of transmission for these abnormalities. A moderate heritability was identified, and evidence for a single major locus was found. Novel statistical analysis methods have identified four traits which co-segregate: cataracts, hepatic abnormalities, CD, and cardiac abnormalities. A canine-specific microarray was used to identify changes in gene expression in the liver that accompany the aforementioned developmental problems. One hundred thirteen genes were found to be differentially regulated in the Havanese.

Introduction

The Havanese is a toy dog that presents with multiple developmental abnormalities. The breed originated in Cuba as an aristocratic pet. In the 1970's, the

---

*Reprinted with permission from Starr AN, Famula TR, Markward NJ, Baldwin JV, Fowler KD, Klumb DE, Simpson NL, Murphy KE, 2007. Hereditary evaluation of multiple developmental abnormalities in the Havanese dog breed. J Hered. 98(5):510-517.

breed was introduced into the United States and the current US population is reported to have developed from four kindred from Cuba, Costa Rica, Russia, and the US. Cataracts, retinal detachment, cancer, CD, and premature death were described in the founding population.

Breeders of Havanese are concerned with the extensive abnormalities afflicting the breed and began assembling phenotypic data for use in future genetic studies. In 2004, members of the Havanese Club of America (HCA) created an anonymous owner survey, allowing them to quantify the frequency of each abnormality within the breed as a whole (J Ruthford, unpublished data). Abnormalities of the forelegs occurred most frequently with 44% of the population having bowed, shortened, or asymmetric forelegs. Additionally, 7% of dogs had abnormal hips, 5% had luxating patellas (lateral and bilateral), and 1% had Legg-Calve-Perthes. Eleven percent of dogs were found to have atypical lenses (cataracts of all types and sizes) in the HCA survey, and similarly, a retrospective case study of canine cataracts identified the Havanese as having the second highest prevalence of cataract (11.57%) among breeds reported in the Veterinary Medical Data Base (VMDB) (Gelatt and MacKay 2005). Six percent of Havanese were diagnosed with heart problems; the most common is cardiac murmur (82%), followed by mitral valve insufficiency (31%). In an additional case study, Havanese were found to have the highest proportion of liver shunts among breeds reported in the VMBD (3.2%) (Tobias and Rohrbach 2003). Cryptorchidism occurs in 5% of male dogs. Eighteen percent of the dogs in the survey presented with some deviation from normal dentition.

Many types of cancer have been described in the breed but estimates of incidences are not available.

In an effort to identify heritable risk factors, we collected detailed phenotypes and pedigree information on 253 Havanese. A subset of 122 dogs was chosen to generate initial estimates of heritability and to assess disease transmission using complex segregation analysis. In addition, the co-occurrence of the diverse abnormalities observed in the Havanese was tested to determine whether the spectrum could be analyzed as a composite phenotype, drawing on the Rasch family of measurement models to construct a syndrome scale (Rasch 1960).

While statistical approaches are necessary, it is also important to assess the underlying genetic changes that either cause the purported syndrome or are in response to various clinical abnormalities. Thus, a canine-specific oligonucleotide array was used to generate a gene expression profile for affected Havanese and 113 genes were found to be differentially regulated. Genes involved in DNA repair, methylation, various metabolic, catabolic, and biosynthetic processes, ion transport, cholesterol absorption and transport, and skeletal development were among those found to be differentially regulated. Described herein are statistical and genetic analyses of anomalies of the Havanese.

Materials and methods

Assessment of phenotype and analysis

The initial screen involved the collection of detailed phenotypic data on 122 Havanese. Pedigree information for an additional 60 animals, including parents and

grandparents, was also incorporated, although no phenotypic data were available for these dogs. The 182 dogs were assembled into 4 kindred, with 12 additional small, unrelated parent-offspring trios. Eleven phenotypes were collected and used to evaluate overall disease severity: skeletal (CD), ophthalmic (cataracts), cardiac (murmurs), hepatic (liver shunts), pre- and post-prandial bile acid levels, alanine aminotransferase (ALT), cholesterol, taurine, height, and weight. Only skeletal, ophthalmic, cardiac, and hepatic data were used to evaluate overall clinical status. The only additional variable included in the analysis was gender.

Due to the complex nature of phenotypic variability in the Havanese, two systems of overall disease scoring, binary (dichotomous) and multi-categorical (polytomous), were implemented to evaluate the transmission of clinically defined traits. The dichotomous system categorized dogs as affected if one abnormality was present, regardless of severity. This approach yielded two classes of dogs, unaffected and affected. The polytomous variable rated dogs based on the severity of clinical abnormalities. A distribution of scores from 0 (unaffected) to 3 (severely affected) was used to rate the overall health of individual dogs.

The dichotomous approach allowed the analysis of 25 unaffected and 22 affected male dogs and 34 unaffected and 36 affected female dogs with complete data. When using the polytomous variable, 25 males were scored as unaffected (score 0), 12 were scored in category 1, 6 in category 2, and 4 in category 3. Thirty-four females were scored as unaffected (score 0), 17 were scored in category 1, 5 in category 2, and 14 in category 3. Thirty-one males and 32 females had an unknown disease status. These

unknown dogs are either deceased or not active in the study, preventing any phenotypic data from being collected. Seventy-eight male dogs and 102 female dogs comprised the Havanese sample in the statistical analyses.

These more conventional approaches to phenotype development--yielding the dichotomous and polytomous variables described above--were complemented by an additional step that invoked a Rasch model (Rasch 1960) to construct a Havanese "syndrome" scale that simultaneously captures information about the primary phenotype of interest and other ordinal (CD, cataract, murmur, liver shunt) and continuous (pre- and post-prandial bile acid levels, ALT, cholesterol, taurine, height, weight) traits that may be relevant to disease diagnosis and prevention. Rasch models (Andrich 1978, Masters 1982, Müller 1987, Rasch 1960, Wright 1999, Wright and Masters 1982, Wright and Stone 1979) are frequently encountered in education, psychology, and sociology where they are used to develop unidimensional scales of reading ability, psychopathology, disability, and other latent traits that are measured using multi-item test and survey instruments. Analytical approaches outlined in Bezruczko (2005) were drawn on to implement the Rasch partial credit model (Masters 1982) (1) to explore the process whereby a composite Havanese phenotype--characterized by a linear, additive, and equal-interval metric--can be defined and validated from heterogeneous data structures and (2) to demonstrate how Rasch measures, estimated at the level of individual dogs, can be used as sufficient statistics in the context of quantitative and population genetics.

To accomplish these goals, we parameterized the Rasch model in the following manner. Given a matrix of N dogs (rows) who were measured on L discrete phenotypes (columns), the stochastic relationship between the "syndrome severity" of dog n and the "clinical sensitivity" of phenotype i is captured by the equation

$$ln[(P_{ni})/(1 - P_{ni})] = B_n - D_i,$$

where

$$B_n = ln[(P_n)/(1 - P_n)]$$

is the syndrome severity "measure" estimated for dog n,

$$D_i = ln[(P_i)/(1 - P_i)]$$

is the clinical sensitivity "calibration" estimated for phenotype i, and $-\infty < B_n, D_i < +\infty$, respectively. In the above model, P_n is the relative frequency of disease for dog n observed across all L phenotypes, P_i is the relative frequency of disease status recorded for phenotype i across all N dogs, and

$$P_{ni} = exp(B_n - D_i)/[1 + exp(B_n - D_i)],$$

is dog n's expected probability of being affected on phenotype i. The Rasch partial credit model (Masters 1982)

$$B_n - D_{ik}$$

expands this elementary framework by permitting each phenotype i to have a unique

number of severity levels or "steps" *k*. The model accommodates these differences and assures that estimates of dog measures and phenotype calibrations are unbiased by heterogeneous unit scales observed in our database.

Phenotype scores were assigned using the approach described by Perkins and Wright (2005) in their study of gout risk factors. Each dichotomous or polytomous phenotype was converted to a *k*-level rating scale (0-*k*) whose "steps" reflected the number of ordinal levels observed in the raw data. In like manner, each continuous phenotype was converted to a 10-level rating scale (0-9) whose "steps" were collinear with unit increases in the variable's original metric. The rescored data were then submitted to Winsteps® (Linacre 2006), a popular Rasch measurement software program, and the syndrome scale (Figure 2) was constructed using the Rasch partial credit model and a joint maximum likelihood estimation (JMLE) procedure (Linacre 2006).

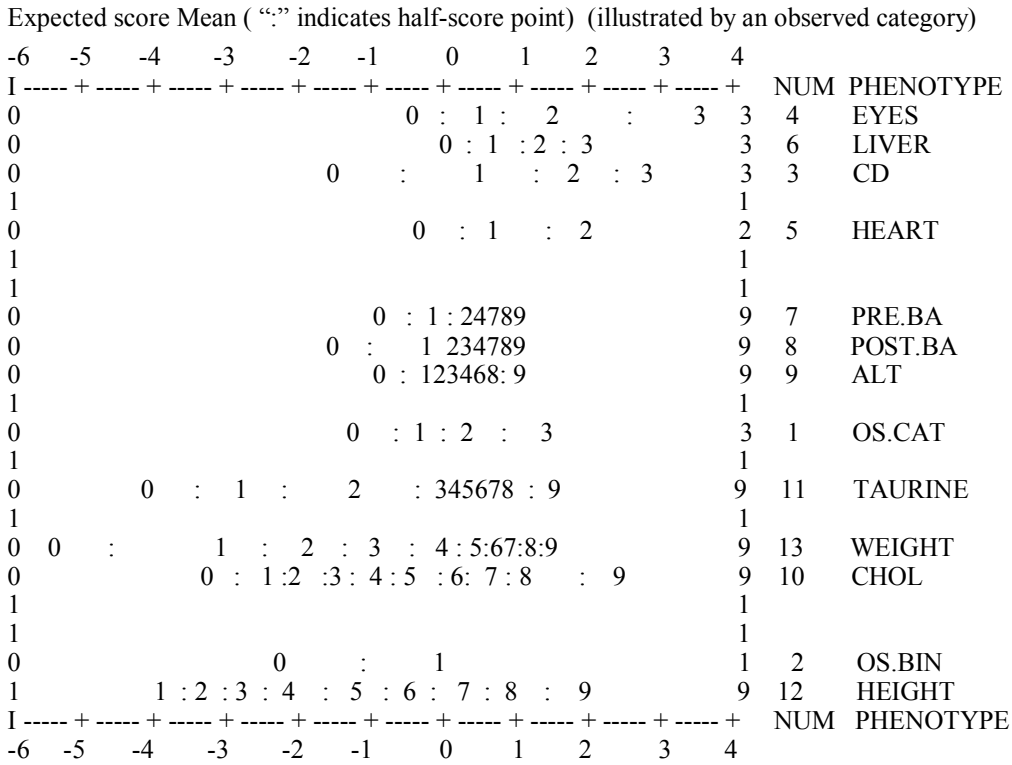

Figure 2: Wright map of dog measures and phenotype calibrations. The left side of the figure maps the distribution of dogs where (.) represents one dog and (#) represents two dogs. The right side of the figure illustrates the distribution of phenotypes. Figure indicates fewer severely affected dogs are observed than mildly affected dogs, but severely affected dogs are more likely to have rare (severe) clinical signs. Phenotype abbreviations: ALT – alanine aminotransferase; CARD – heart abnormalities; CHOL – cholesterol; DICH – dichotomous score; HEPAT – hepatic abnormalities; OPHTH – ophthalmologic abnormalities; POLY – polytomous score; POST.BA – postprandial bile acids; PRE.BA – fasted bile acids. Along vertical axis, T = two standard deviations, S = one standard deviation, M = mean.

Estimation of heritability

Our analysis of disease in the Havanese follows two distinct paths, given the nature of the observations recorded. One of the traits was measured as a binary condition (unaffected, affected). In addition, a rating scale (0, 1, 2, 3) was utilized to indicate disease severity. Although such scores are not guaranteed to follow a normal distribution, simulation studies have shown that analyses based on nonlinear threshold models provide little improvement in precision over models built upon normally distributed residuals when four or more categories are used (Meijering and Gianola 1985). For this reason, along with the simplicity associated with employing linear models for heritability estimation and complex segregation analysis, software that evaluates (1) the binary trait in a binary threshold model, and (2) the four-category trait as if it has normally distributed residuals was utilized.

A threshold model for the liability to disease was implemented to estimate the heritability of the binary phenotype. The strategy employed is similar to that used in the evaluation of Addison's disease by Oberbauer et al. (2006). Calculations were carried out using SOLAR (Almasy and Blangero 1998, Blangero et al. 2005), making use of the approach documented by Duggirala et al. (1997).

The analysis of the polytomous trait followed a similar trajectory without the assumptions of an underlying continuous variable and an unobservable set of thresholds. This phenotype was treated as a continuous trait, recognizing that the phenotypes of related animals would be correlated based on the degree of relatedness and the magnitude of the genetic variance. The exact representation of this model can be found

in Almasy and Blangero (1998). Although the phenotype is not continuous, the simulation work of Meijering and Gianola (1985) suggested that linear models can be used to analyze polytomies of 4 or more categories without substantial loss of efficiency. SOLAR (Almasy and Blangero 1998, Blangero et. al. 2005) was again employed to derive heritability estimates, using a multivariate normal to maximize the likelihood (NOTE: SOLAR is capable of accommodating only binary or normally distributed phenotypes).

It should be noted that because the data represent owner submissions, the data were collected in a non-random fashion. Moreover, this is a study of inheritance, so the data were constructed around probands. Such data typically require some adjustment for ascertainment bias. However, the mixed linear models utilized in this study accommodate non-randomly sampled data (Henderson 1984) as long as the dogs added into the study to complete the pedigree associations can be considered a random sample of Havanese. In addition, a test of the effect of gender on the predisposition to disease was tested using a likelihood ratio test.

Complex segregation analysis

The possibility that the dichotomous disease or the polytomous disease in Havanese is influenced by the action of a single segregating locus of large effect can also be examined. Complex segregation analysis, developed by Bonney (1986), is intended to integrate Mendelian transmission genetics at a single locus with the patterns of covariance expected in polygenic inheritance. Lynch and Walsh (1998) provide a more complete description of complex segregation analysis and the methods used in this

investigation follows that employed in Oberbauer et al. (2006). Elston et al. (1975) outlined the criteria that must be satisfied before acceptance of the single major locus model so as to reduce the risk of false positive declarations of a major locus model. Evaluation of the models necessary for complex segregation analysis was conducted with the Bayesian software package iBay (2006, version 1.0). The iBay software is an extension of MaGGic (Janss 1998) rewritten to accommodate complex segregation analysis in binary traits, as well as normally distributed phenotypes, for pedigrees that include inbreeding.

Note also that the iBay software models the unobservable scale of the binary threshold trait such that the residual variance is fixed at 1.0 (i.e., $\sigma e2 = 1$). In the case of the ordered categorical trait, the iBay software evaluates phenotypes under a normal distribution (iBay, as does SOLAR, evaluates binary or normally distributed phenotypes).

Creation of the Gibbs sample requires several key assumptions about the behavior of these unknown parameters. Though a variety of models can be considered, all are some variant of the following: gender as a fixed effect with a flat (i.e., uniform) prior density, the polygenic variance component with a flat prior density, as well as flat prior densities for the additive, dominance, and allele frequency parameters. A Gibbs sample of 5,000 was generated, beginning with the creation of 300,000 total samples, a "burn-in" of 50,000 and a sampling rate of every 100-th Gibbs value. This process was repeated to create two replicate chains. From the 5,000 Gibbs samples, the mean, standard deviation, mode and the upper and lower limits of a 95% highest density region

(HDR) was computed for each of the unknown parameters. For the binary trait, iBay fixes the residual variance at 1, and allows the polygenic variance to be generated as part of the Gibbs sampling process. For the ordered categorical trait, where the phenotype is assumed to follow a normal distribution, the residual and polygenic variances are generated in the Gibbs sampling process.

Global gene expression

The Affymetrix canine genome 2.0 array (Santa Clara, CA) was used to assess gene expression in the Havanese. The array contains 42,929 total probe sets (including all controls) and includes 18,000 canine specific transcripts and over 20,000 predicted gene sequences based on sequence similarity with known genes in other species.

Three Havanese samples were selected for this study. Two dogs were half-siblings approximately one year apart in age. The third dog was a puppy selected for her medical history. All dogs were affected based on the Havanese grading criteria. Liver samples were collected at the time of euthanasia. One dog suffered from metastatic lung disease, mitral valve insufficiency, and CD, another suffered from severe CD, and the third, the puppy, had a suspected liver shunt and lung abnormalities noted in the necropsy. These dogs were euthanized by their respective veterinarians. Tissue was stored in RNAlater® (Ambion, Austin, TX). Because these are client-owned dogs, age-matched normal Havanese were not available. Tissues from three clinically normal mixed-breed dogs were used as normal controls. These dogs displayed none of the abnormalities appearing in the Havanese and were determined to be clinically normal by standard blood chemistry panels and detailed physical exams.

RNA isolated from the liver was prepared by Viagen Inc. for hybridization to the Affymetrix canine 2.0 array using the classic protocol (Chomczynski and Sacchi 1987). Ambion's linear amplification kit, MessageAmp^{TM} aRNA Kit (Ambion Inc.), was used for first-strand cDNA, second-strand cDNA, and biotin labeled cRNA synthesis. Two micrograms of total RNA were used to start the single round of amplification. Duplicate experiments were performed for all samples. Fragmentation of the labeled cRNA was performed as follows: incubation at 95˚C for 35 minutes in a solution containing 40mM Tris-acetate (pH 8.1), 100mM KOAc, and 30mM MgOAc. Forty micrograms of the fragmented, labeled cRNA was then hybridized to each of the GeneChip® (Affymetrix) oligonucleotide arrays. These arrays were washed, stained, and scanned in accordance with a previously published protocol (Ji et al. 2004).

High-resolution GeneChip images were collected on the GeneChip Scanner 3000. Affymetrix GeneChip operating software was used to quantify image data and calculate gene expression values.

GeneSpring software (Silicon Genetics, Redwood City, CA) was used to perform gene clustering, Students' *t*-test, and Bonferroni multiple testing corrections. Samples were grouped separately to identify differentially expressed genes. Gene expression ratios were calculated between the two groups.

Results

Estimation of heritability

Estimates of heritability of 0.36 (± 0.27) for the dichotomous disease and 0.36 (± 0.16) for the polytomous disease support a moderate hereditary component and suggest

the clinical disease described herein may have a hereditary component (Table 4). No difference in risk between gender was observed (data not presented).

Table 4: Estimate of heritability in a threshold model for the dichotomous and polytomous disease.

	Estimate	Standard Error	p-value
Dichotomous	0.359	0.27	0.026
Polytomous	0.358	0.16	0.0016

Complex segregation analysis

The results of the complex segregation analysis provide no conclusive evidence for the action of a single major locus influencing the dichotomous disease trait (data not presented). However, the results of the complex segregation analysis for the polytomous disease trait do provide evidence for the action of a single major locus (Table 5). For all parameters of interest, the 95% highest density region (HDR) does include zero, a clear demonstration that the actions of a major locus for this disease in the Havanese are supported. Not presented is the equivalent analysis accommodating non-Mendelian transmission of the putative major allele. Such an analysis is one of the criteria established by Elston et al. (1975) when considering the action of a major locus. When evaluated through the 95% HDR, overlap with Mendelian transmission estimates were demonstrated, indicating the Mendelian model provides the best fit to the data.

Table 5: Marginal posterior means, modes, standard deviations and limits to the 95% highest density regions of model parameters for the polytomous score in Havanese in a Bayesian mixed-inheritance model with a completely recessive major locus.

	Residual Variance	Polygenic Variance	Major Locus Variance	Additive Effect (a)	Dominance Deviation (d)	Frequency (q)
Mean	0.29	0.06	0.82	1.10	-1.10	0.46
Mode	0.28	0.01	1.65	1.07	-1.15	0.42
SD	0.06	0.06	0.22	0.08	0.08	0.07
HDR 95% Low	0.08	0.00	0.31	0.88	-1.34	0.22
HDR 95% High	0.59	0.61	1.74	1.39	-0.83	0.73

The Havanese syndrome scale

The Rasch analysis yielded linear measures, standard errors, and model fit statistics for each dog and phenotype included in the analysis (not presented). Average *Outfit* and *Infit* mean-square statistics (Bond and Fox 2001, Wright 1984, Wright and Masters 1981) were satisfactory for dogs (*Infit* = 0.99, *Outfit* = 0.90) and phenotypes (*Infit* = 1.00, *Outfit* = 0.96), lending provisional support of the scale's specific (local) objectivity and validity as a unidimensional "yardstick" of Havanese trait variation. Figure 2 illustrates the relative distributions of dog measures (left) and phenotype calibrations (right) along the range of measurement.

The common unit of the scale allows interpretation of the relationship between dogs and phenotypes with ease and clarity: dogs with higher measures are more likely to be affected with the syndrome than dogs with lower measures, and phenotypes with higher calibrations are less sensitive to clinical detection, generally speaking, than phenotypes with lower calibrations. The Rasch model unifies these two distances in the following manner: Dogs with higher measures are 1) more likely to be affected on phenotypes whose sensitivity calibrations reside below his or her position on the scale; 2) less likely to be affected on phenotypes whose calibrations reside above his or her position on the scale; and 3) equally likely to be affected on phenotypes whose calibrations reside at his or her position on the scale. The mean (M) and standard deviation (S = 1 SD, T = 2 SD) of dog measures are indicated by "TSMST," respectively.

Figure 3 depicts how a breeder might implement these principles in practice. The logit range at the top and bottom of the graphic is the measurement continuum. Phenotypes are documented to the far right with the same abbreviations listed in Figure 2, and the numbers in the body of the table are the phenotype categories described previously. The colon (:) between each pair of adjacent categories indicates the point on the measurement scale at which a dog whose estimated measure falls at the location has an equal (50/50) probability of being classified in the lower or upper category. For example, the expected probability of disease (DICH) for a dog with a measure of -1.00 is 0.50.

Global gene expression using microarray

A canine-specific oligonucleotide array was used to create a disease expression profile. The profiles displayed significantly different expression patterns such that normal and affected dogs clustered into their respective groups. Under the most stringent statistical parameters, 113 genes were identified as being differentially expressed in liver, with a greater than two-fold difference in expression and a p-value ≤ 0.05. Of these, 83 were down-regulated and 30 were up-regulated (abbreviated results in Table 6).

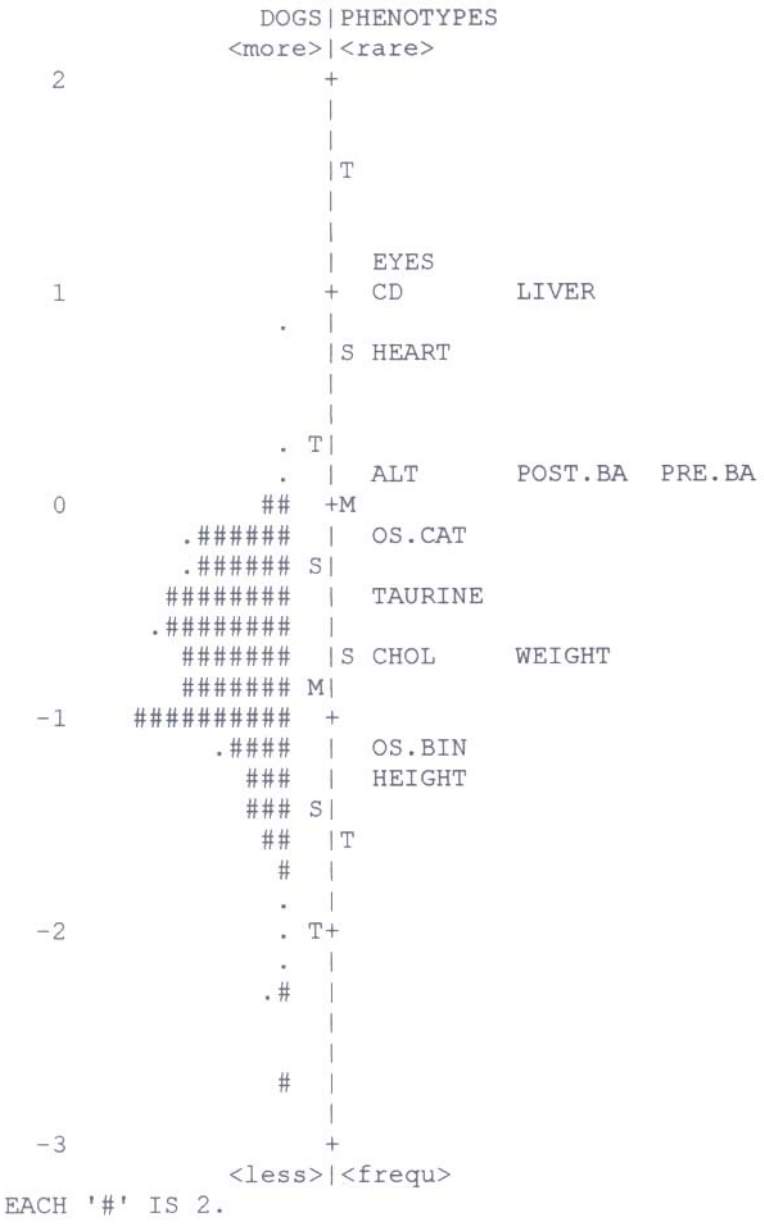

Figure 3: Illustration of the expected score means. Each trait has the categorical score distribution along the horizontal axis. A (:) indicates a half-score point for an observed category. Abbreviations used are the same as in Figure 2.

Table 6: List of differentially regulated genes identified by the gene expression assay. Affymetrix probe names were searched in the NetAffx analysis center to identify the canine locus name (Dog LOC). A search in Gene Cards (http://www.genecards.org/) using Dog LOC identified the orthologous human loci or genes. The list presented includes only those probes that identified gene orthologs. (Adapted from Starr et al. 2007)

Gene	Dog LOC	Fold Change	P-Value	Location	Probe Name
HELLS	LOC486809	0.143	0.047	28	CfaAffx.12909.1.S1_at
MGAM	LOC475523	0.168	0.0117	16	CfaAffx.6714.1.S1_at
NQO1	LOC610935	0.186	0.00109	5	CfaAffx.31023.1.S1_at
PKP4	LOC478759	0.191	0.0102	36	Cfa.3365.1.A1_at
OXCT1	LOC479347	0.194	0.00597	4	Cfa.10153.1.S1_at
ABCG8	LOC474571	0.219	0.00299	10	Cfa.7435.1.A1_at
EPHX1	LOC480113	0.234	0.00133	7	CfaAffx.24797.1.S1_at
EPHX1	LOC480113	0.241	0.00925	7	Cfa.4088.1.A1_at
C5orf4	LOC489163	0.244	0.000264	4	Cfa.12428.1.A1_at
CXCL12	CXCL12	0.251	0.0156		Cfa.20779.1.S1_at
PHKA2	LOC480857	0.251	0.00396	X	Cfa.5653.1.A1_at
EPHX1	LOC480113	0.256	0.00471	7	Cfa.4088.1.A1_s_at
PKLR	PKLR	0.257	0.0467	7	Cfa.8012.1.A1_at
C10orf59	LOC610448	0.258	0.00505	26	Cfa.12560.1.A1_at
NQO1	LOC610935	0.278	0.000742	5	Cfa.16827.1.A1_at
PKP4	LOC478759	0.281	0.00054	36	Cfa.3365.1.A1_s_at
KCNN2	LOC474640	0.289	0.00289	11	Cfa.5095.1.A1_s_at
C1orf32	LOC490364	0.291	0.0176	7	Cfa.9794.1.A1_at
LTB4DH	LOC474802	0.297	0.0332	11	Cfa.12192.1.A1_s_at
SPHK2	LOC484401	0.313	0.00947	1	Cfa.1378.1.A1_at
HTATIP2	LOC476886	0.315	0.00544	21	Cfa.3269.1.S1_at
ALDH7A1	LOC481486	0.342	0.0478	11	Cfa.15706.1.A1_at
CRYL1	LOC486050	0.344	0.0243	25	Cfa.4354.1.S1_a_at
MFAP3L	LOC477356	0.346	0.00988	25	Cfa.21163.1.S1_s_at
SMARCA1	LOC481046	0.347	0.0231	X	Cfa.1931.1.A1_at
ESD	LOC607116	0.349	0.00273	22	Cfa.15993.1.S1_at
NDUFV1	LOC476004	0.384	0.0348	18	CfaAffx.17348.1.S1_at
LOC341392	LOC479821	0.398	0.0111	6	Cfa.13263.1.A1_s_at
NRXN1	LOC474589	0.400	0.00133	10	Cfa.7236.1.A1_s_at
SMARCA2	LOC476335	0.401	0.0126	1	CfaAffx.3959.1.S1_s_at
MFAP3L	LOC477356	0.401	0.0141	25	CfaAffx.12367.1.S1_at
LOC341392	LOC479821	0.408	0.00963	6	CfaAffx.27532.1.S1_at
ABCG5	LOC481354	0.414	0.00491	10	CfaAffx.4636.1.S1_at
GCLM	LOC612283	0.422	0.0287	6	CfaAffx.30811.1.S1_s_at
EXOC2	LOC478696	0.422	0.00209	35	CfaAffx.14491.1.S1_at
DST	DST	0.424	0.00148		CfaAffx.4509.1.S1_s_at
CD59	LOC475945	0.429	0.00901	18	Cfa.838.1.A1_at
TMEM135	LOC607444	0.431	0.0112	21	Cfa.19775.1.S1_at

Table 6 continued

Gene	Dog LOC	Fold Change	P-Value	Location	Probe Name
FYCO1	LOC476649	0.440	0.0434	20	Cfa.10325.1.A1_at
C6orf192	LOC611318	0.444	0.0173	1	Cfa.2541.1.A1_at
DST	DST	0.444	0.00938		Cfa.557.1.A2_at
AGXT2L2	LOC481448	0.448	0.047	11	Cfa.2854.1.S1_at
TYMS	LOC476100	0.463	0.00166	19	Cfa.9380.1.A1_at
THEM2	LOC610883	0.463	0.00276	35	Cfa.15345.1.S1_at
AHCYL1	LOC611790	0.472	0.00118	6	Cfa.21135.1.S1_at
EPB41L4B	LOC474797	0.473	0.0221	11	Cfa.14183.1.A1_at
HEBP1	LOC477690	0.494	0.0284	27	Cfa.12157.1.A1_at
RETSAT	LOC483083	0.499	0.00395	17	Cfa.8888.1.A1_at
ADRBK2	LOC486327	0.500	0.0202	26	Cfa.8027.1.A1_at
BCL2A1	LOC488770	**2.029**	0.00796	3	Cfa.21056.1.S1_at
RABGEF1	LOC479706	**2.287**	0.0252	6	CfaAffx.16844.1.S1_at
RPS6KA3	LOC491768	**2.578**	0.0375	X	Cfa.19022.1.S1_s_at
SSR1	SSR1	**2.760**	0.000788	35	CfaAffx.14997.1.S1_s_at
FGG	LOC475474	**2.780**	0.00583	15	Cfa.13273.1.A1_x_at
TM6SF2	LOC609715	**2.883**	0.025	20	CfaAffx.22123.1.S1_s_at
TM9SF1	LOC480261	**3.108**	0.0207	8	Cfa.18439.1.S1_s_at
SEPX1	LOC611032	**3.150**	0.0368	6	Cfa.12212.1.A1_at
DDX3X	LOC480886	**3.181**	0.0315	X	CfaAffx.22006.1.S1_at
C12orf23	LOC610769	**3.863**	0.0224	10	CfaAffx.3602.1.S1_at
FCN1	LOC608247	**3.996**	0.0374	9	Cfa.13207.1.A1_s_at
MAN1A1	LOC476275	**4.014**	0.0187	1	CfaAffx.2332.1.S1_s_at
STAT3	LOC490967	**4.061**	0.0154	9	Cfa.5199.1.A1_at
FAM107A	LOC607380	**4.067**	0.00242	20	Cfa.18258.1.S1_at
HN1	HN1	**4.358**	0.00138	9	CfaAffx.8018.1.S1_s_at
PGM3	LOC474981	**5.628**	0.0227	12	Cfa.20949.1.S1_s_at
MUT	LOC474930	**5.650**	0.00145	12	Cfa.19060.1.S1_s_at
ALPL	ALPL	**6.756**	0.0179	2	CfaAffx.22870.1.S1_s_at
DNAJC3	LOC476966	**7.770**	0.0256	22	Cfa.7822.1.A1_s_at
SPINK2	LOC611439	**7.796**	0.0124	13	Cfa.11889.1.A1_at
SLC16A7	LOC481138	**7.861**	0.0376	10	CfaAffx.1420.1.S1_at

Discussion

Havanese are afflicted with multiple developmental abnormalities, most frequently CD, cataracts, heart murmurs, and missing incisors. This initial investigation of the breed has yielded intriguing results. Estimates of heritability calculated for the data set support the possibility that this collection of multi-organ abnormalities is inherited as a single disease. Complex segregation analyses support a major locus model under the polytomous scoring method.

Affected Havanese present with variable phenotypes. Initial phenotype scoring utilized a binary system which yielded an estimate of heritability of 0.36, supporting the hypothesis that this cluster of phenotypes has a hereditary basis. Segregation analysis did not elucidate a mode of transmission or even provide evidence for a major locus. When dogs were re-evaluated using a multi-categorical scoring system, we obtained the same estimate of heritability and evidence to support the existence of a major locus. Penetrance and expressivity could provide a plausible explanation for the inconsistent disease presentation.

The heritability value of 0.36 indicates a moderate genetic contribution to disease variability. Low levels of genetic variance or an inability to detect all genetic variance may contribute to this modest estimate. The introduction of unrelated dogs into the kindred of Havanese from this study may increase the genetic variance in the population and therefore increase the heritability. In a small population such as the Havanese, which ranked 38th in American Kennel Club registration in 2006 (American Kennel Club

2007), low genetic variance is expected due to the small number of founders and popular sires.

The identification of CD as a variable that co-segregates with the hypothesized syndrome, coupled with the alignment of the CD and DICH thresholds in Figure 3, suggests the benefit of using CD as a physiological marker for disease. Dogs categorized as CD are more likely to have other abnormalities; therefore, the CD phenotype may be useful in predicting the overall health of an individual for breeding purposes. A standardized evaluation of antebrachial conformation could provide objective measures of forelimb shortening, bowing, or asymmetry.

The Rasch analysis provided a first, imperfect glimpse of how these tasks might be accomplished in practice. The measurement properties and clinical validity/utility of the syndrome scale have yet to be assessed in a truly rigorous manner; however, this analytical approach is useful for integrating heterogeneous clinical data and may facilitate the development of a robust phenotype that better serves researchers and breeders. Future studies will be concerned with these and related questions, as well as the potential of incorporating genetic information in the scale construction process (Markward 2004, Markward and Fisher 2004).

The clinical signs described in the Havanese are similar to signs characteristic of many human syndromes. This prohibits the use of a candidate gene approach to dissect the underlying genetics. While there are similarities between various human diseases and the signs in the Havanese, there are not any human syndromes that capture the complete spectrum of Havanese abnormalities. Thus, in order to develop a genetic profile for the

Havanese, an oligonucleotide array was used to identify gene expression differences between affected and normal Havanese. Hepatic tissues were used in this experiment with the goal being to delineate genetic biomarkers important in the disease process. The liver was chosen because of the inclusion of hepatic abnormalities in the overall syndrome. Future studies may include use of other tissues to create a better spectrum of gene expression profiles across affected tissues. The differentially regulated genes identified are involved in DNA repair, methylation, various metabolic, catabolic, and biosynthetic processes, ion transport, cholesterol absorption and transport, and skeletal development, among other processes (Rebhan et al. 1997). Genes of particular interest include *ABCG8* (4.5-fold down-regulated), *ABCG5* (2.4-fold down-regulated), and *ALPL* (6.8-fold up-regulated) (Table S1).

ABCG5 and *ABCG8* encode a heterodimer (Berge et al. 2000) instrumental in the absorption and transport of dietary cholesterol, as well as in sterol excretion into bile (Rebhan et al. 1997, Klett et al. 2004, Oram and Vaughan 2006). Both genes are members of the ATP-binding cassette family, sub-family G (Rebhan et al. 1997). In the human, defects in either of these genes cause sitosterolemia (MIM 210250), an autosomal recessive disorder characterized by increased absorption of all sterols (Bhattacharyya and Connor 1974, Beaty et al. 1986). The increased absorption results in hypercholesterolemia and high plasma levels of plant sterols, which leads to atherosclerosis and coronary artery disease in man (Berge et al. 2000). The role these two genes play in canine hepatocytes has not been directly investigated.

Reduced expression of *ABCG5* and *ABCG8* in affected Havanese may indicate a coordinated response to reduce absorption of sterols by enterocytes as well as increase hepatobiliary secretion of cholesterol. Alternatively, expression levels of *ABCG5* and *ABCG8* may be a reflection of a mutation affecting the normal function of the heterodimer. It is plausible that both *ABCG5* and *ABCG8* are down-regulated in response to some abnormality in the cholesterol biosynthesis or metabolism pathways. The dog has long been recognized as a species extremely efficient at regulating cholesterol levels (Abell et al. 1956, Pertsemlidis et al. 1973): dogs compensate for increased dietary sterol absorption by increasing excreted cholesterol and bile acids and/or by inhibiting cholesterol biosynthesis. Thus, it would follow that a decrease in sterol absorption would increase cholesterol biosynthesis or decrease excreted cholesterol and bile acids.

ALPL presents an interesting potential candidate gene for the disease in the Havanese. Mutations in *ALPL* cause hypophosphatasia (MIM 146300, 241500, 241510), an inherited condition with variable clinical expression affecting skeletal ossification and mineralization (Whyte 1994). The skeletal abnormalities of hypophosphatasia result from impaired activity of alkaline phosphatase liver/bone/kidney (Weiss et al. 1988, Henthorn et al. 1992). There are five forms of hypophosphatasia in humans: perinatal (lethal), infantile, child, adult, and odontohypophosphatasia (reviewed in Whyte 1994 and Mornet 2000). The transmission of hypophosphatasia is generally reported as autosomal recessive, though isolated families have an autosomal dominant form (Danovitch et al. 1968, Bixler et al. 1974, Moore et al. 1999, Hu et al. 2000). Improperly ossified or mineralized bones, specifically the weight-bearing bones in the limbs, can

result in a bowed morphology (Sergi et al. 2001). The clinical signs of odontohypophosphatasia mimic the primary signs in the Havanese: skeletal defects and abnormal dentition. Secondary genes would be expected to contribute to the organ abnormalities since mutations in *ALPL* have clinical manifestations limited to skeletal and dental anomalies.

In addition to its role in skeletal development, *ALPL* is also involved in folate biosynthesis (Rebhan et al. 1997). Folate is a naturally occurring B-vitamin important in many biological processes, including DNA synthesis and repair and neural tube formation (Kelemen 2006, Blom et al. 2006). Down-regulation of another gene involved in folate biosynthesis, *SMARCA2* (2.5-fold) (Table S1) suggests that further research into folate and folic acid (synthetic form of folate) is prudent.

In summary, a bipartite approach of statistical evaluation and global gene expression profiling was completed to study the complex developmental disease in the Havanese breed. The data presented herein establish the disease in the Havanese as an inherited condition. Future studies include (1) creating an objective antebrachial conformation measure for CD to identify dogs at risk of disease, (2) collecting and analyzing serological and hematological data for each dog in the study to look for segregation with the diseased phenotype, and (3) evaluating candidate genes identified by this study through sequencing and linkage analyses.

CHAPTER IV

CHROMOSOME-SPECIFIC LINKAGE ANALYSES OF DEVELOPMENTAL ABNORMALITIES IN THE HAVANESE DOG BREED

Overview

The Havanese is a toy breed that experiences a high incidence of developmental abnormalities including osteochondrodysplasia (CD), cataracts, heart murmurs, and liver shunts. Affected dogs present with a variable phenotype. In a previous study, complex segregation analysis suggested an autosomal recessive mode of inheritance for the collection of abnormalities. Because of the similarity of the Havanese abnormalities with human syndromes of disrupted cholesterol biosynthesis, a partial genome screen was carried out to examine regions of the genome known to harbor cholesterol biosynthesis-related genes. No evidence for linkage was obtained in this partial screen, thus future work will include completion of a whole genome screen in an effort to identify the major locus.

Introduction

The Havanese, the national dog of Cuba and part of the Bichon family, is a toy breed recognized by the American Kennel Club (AKC) in 1999. A complete and thorough account of the history of the breed is not available, but descriptions of the breed date back to the 1700s - known then as the Blanquito de la Cubano (White Cuban), the Dog of Havana, or the Havana Silk Dog (Klumb and Baldwin 2005).

Four primary populations existed in the late twentieth century: Cuban, Costa Rican, American, and Russian/Western European. A large percentage of the modern American population of Havanese is descended from 8 foundation dogs. These dogs were the founders of the AKC stud book and the offspring of nine pedigreed dogs obtained by the first Havanese breeder in the United States. Since then, European Havanese have been imported to increase genetic diversity of the breed.

The collection of abnormalities present in the Havanese is thought to be inherited in a syndrome-like fashion, in which four abnormalities co-segregate: CD, heart and eye abnormalities, and liver shunts (Starr et al. 2007). Starr et al. carried out complex segregation analyses which suggested an autosomal recessive mode of inheritance and provided evidence for the action of a major locus for the syndrome-like phenotype.

The involvement of the cholesterol biosynthetic pathway in the Havanese abnormalities presents an intriguing possibility. Inborn errors of cholesterol biosynthesis result in abnormalities affecting multiple organ systems – primarily skeletal, ocular, cerebral, oral, integumentary, cardiac, renal, and genital defects (reviewed in Nowaczyk and Waye 2001, Andersson 2002, Herman 2003). Cholesterol biosynthesis disorders include Smith-Lemli-Opitz syndrome (SLOS, MIM#270400), desmosterolosis (MIM#602398), X-linked chondrodysplasia punctata (CDPX2, MIM#302960), congenital hemidysplasia icthyosiform erythroderma limb defects syndrome (CHILD syndrome, MIM#308050), lathosterolosis (MIM#607330), and hydrops-ectopic calcification-moth-eaten skeletal dysplasia (HEM dysplasia, MIM#215140). These

syndromes have been extensively studied, and are disorders characterized by various malformations.

Genome screens are widely utilized to narrow the search for candidate genes (Acland et al.1999, Clark et al. 2005a, Clark et al. 2005b). The minimal screening set 2 (MSS-2) is a comprehensive set of 327 microsatellite markers which offer an average 9 Mb coverage of each canine chromosome. The MSS-2 is well-suited for completing partial genome screens because the markers were multiplexed in chromosome-specific fashion (Clark et al. 2004). A multiplex-specific approach was used to evaluate regions harboring genes of interest which were identified by the oligonucleotide array (see Chapter III), developmental genes, and genes involved in cholesterol biosynthesis.

From a gene expression profile of the Havanese disease using an oligonucleotide array, three genes of interest were identified: *ALPL*, *ABCG5*, and *ABCG8*. Candidate genes in similar diseases (which do not encompass the entire spectrum of Havanese abnormalities) include *COL2A1* (Ocular-skeletal dysplasia) (Du et al. 2000, Meredith et al. 2007), *COL10A1* (chondrodysplasia) (Young et al. 2006), *FGFR1*, *FGFR2*, *FGFR3* (multiple limb malformations) (Wilkie et al 2002). No obvious candidate genes related to cardiac and hepatic abnormalities exist, due to the numerous pathways contributing to development of organs and the entire cardiovascular system. Unfortunately, hematology and serum chemistry panels have been examined in only a few studies of canine skeletal dysplasias (Fletch et al. 1973, Terpin and Roach 1981) to determine whether biological markers were associated with disease.

Materials and methods

Phenotypes

Dogs included in the study were examined by their respective veterinarians; blood was collected for a serum liver chemistry panel (SA320) and a complete blood count (CBC) (Antech Diagnostics, Lake Success, NY). Height and weight measurements were collected from all dogs. One overall score was used to evaluate the collection of signs and was designated 0, 1, 2, or 3 (unaffected, mild, moderate, or severe), using the scoring system developed by Starr et al. (2007). The possibility that additional phenotypes were segregating independently from the overall phenotype was evaluated using a binary score of affected or unaffected for CD and heart abnormalities.

Hematology and serum chemistry panels

Venous blood samples were collected for 97 dogs by each animal's attending veterinarian. Whole, serum-separated, and pre- (p-) and post-prandial (pp-) blood samples were submitted to Antech Diagnostics according to the diagnostic center's protocol (SA320, Antech Diagnostics) for determination of CBC, serum chemistries, and p- and pp-bile acid levels.

Dogs were grouped by forelimb status (normal or CD) or by overall disease status. For each group, 17 biomarkers were evaluated: glucose (GLU), blood urea nitrogen (BUN), total protein (TP), albumin (ALB), bilirubin (BILI), alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), globulin (GLOB), gamma glutamyltransferase (GGT), hemoglobin (Hgb), hematocrit (Hct), white blood cell (WBC), red blood cell (RBC), mean corpuscular

volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Chemistry and hematology results were analyzed in two complementary ways. Firstly, analysis-of-variance (ANOVA) and F-tests were implemented to compare the raw (continuous) biomarker values of affected and unaffected dogs. Secondly, chi-squared tests were conducted to test for association between disease status and each biomarker after it had been classified into two-category (normal, abnormal) and three-category (low, normal, high) classification variables using the canine reference ranges described in The Merck Veterinary Manual (8th Ed.).

Linkage

Buccal epithelial cells were collected from 122 dogs for DNA extraction using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN).

The forward primers were labeled with one of four fluorescent dyes and PCR was completed as described in Clark et al. (2004), modified for the use of robotics (Bio-Tek Precision 2000, Winooski, VT): Stocks of DNA were maintained at 12.5 ng/µl, 4 µl of DNA was added for a total of 50ng of DNA to each reaction, and no ddH2O was added to the reaction. PCR products were resolved on an ABI 3130 sequencer (Applied Biosystems, Foster City, CA) with an internal size standard (Gene Scan 500 LIZ, Applied Biosystems). Genotypes for all dogs were assigned using Genemapper® software v3.7 (Applied Biosystems). Data were collected for 20 chromosomes which harbored candidate genes (Table 7).

Table 7: List of candidate genes investigated in the Havanese study. (*) indicates genes identified by microarray analysis (Starr et al. 2007).

Candidate Genes	Canine Chromosome	Disorder/Condition (reference or MIM#)
*SMARCA2**	1	--
*ALPL**	2	Hypophosphatasia (146300,241500, 241510)
FGFR3	3	Achondroplasia (100800), hypochondroplasia (146000)
FGF10	4	lacrimo-auriculo-dento-digital syndrome (LADD syndrome)(149730)
DHCR24	5	Desmosterolosis (602398)
SC5DL	5	Lathosterolosis (607330)
*ABCG5**	10	Sitosterolemia (210250)
*ABCG8**	10	Sitosterolemia (210250)
SC4MOL	15	Cholesterol biosynthesis
IGF1	15	Height (Sutter et al. 2007)
SHH	16	Embryonic patterning (600725)
FGFR1	16	Kallmann syndrome (147950)
DHCR7	18	SLOS (270400)
FDFT1	25	Cholesterol-related cataracts (Mori et al. 2006)
DHH	27	Embryonic patterning (605423)
COL2A1	27	Stickler syndrome, type 1 (108300)
FGFR2	28	Limb growth (Arman et al 1999)
LSS	31	Cholesterol-related cataracts (Mori et al. 2006)
IHH	37	Embryonic skeletal patterning (600726)
HSD17B7	38	Cholesterol biosynthesis

Two-point LOD scores were calculated using the Sequential Oligogenic Linkage Analysis Routines (SOLAR) software package version 2.1.5 (Almasy and Blangero 1998). For analysis, each pedigree member was assigned a score for each trait as described in the above section. The traits used in the analysis were: composite score, CD, Heart, Cholesterol, Height, and Weight.

Results

The Havanese kindred had an average F value of 0.011 (n=122). The average values for serum chemistries and CBCs in normal and CD Havanese are listed in Table 8. No significant p-values were obtained in the comparison of mean values between normal and CD dogs.

Table 8: The average levels from hematology and serum chemistries, and calculated p-values between normal and CD Havanese.

Parameter	*Normal dogs*	*CD dogs*	***p-value***
Glu (mg/dL)	94.28 ± 19.33	91.97 ± 21.48	0.606
BUN (mg/dL)	16.23 ± 4.53	15.00 ± 3.31	0.135
TP (g/dL)	6.26 ± 0.53	6.26 ± 0.45	0.998
Alb (g/dL)	3.52 ± 0.38	3.54 ± 0.34	0.713
Tbili (mg/dL)	0.14 ± 0.08	0.15 ± 0.08	0.375
AlkPhos (u/L)	49.50 ± 59.42	34.79 ± 24.34	0.119
ALT (u/L)	67.08 ± 67.91	58.38 ± 58.56	0.516
AST (u/L)	29.19 ± 12.68	33.47 ± 26.98	0.389
Glob (g/dL)	2.74 ± 0.53	2.72 ± 0.33	0.790
GGT (u/L)	6.02 ± 2.42	6.46 ± 3.28	0.506
Hgb (g/dL)	15.93 ± 1.86	16.47 ± 1.39	0.176
Hct (%)	46.93 ± 7.70	49.62 ± 4.15	0.106
WBC (10^3/uL)	11.43 ± 9.48	9.71 ± 2.58	0.206
RBC (10^6/uL)	6.93 ± 2.47	6.80 ± 0.57	0.754
MCV	71.58 ± 6.59	72.92 ± 4.04	0.284
MCH	24.35 ± 0.96	24.21 ± 0.84	0.507
MCHC	33.78 ± 1.88	33.32 ± 1.99	0.347

Tables 9 and 10 provide the data for two Chi-square analyses of the overall disease score using classified continuous data: normal vs. abnormal and low/normal/high, based on the reference ranges identified by The Merck Veterinary Manual. Not presented is the analysis of overall disease using continuous data because no significant p-values were obtained.

Table 9: Chi-square analysis of 17 parameters in overall disease phenotype, using the low/normal/high scoring system. (*) indicates p-value<0.10, (**) indicates p-value<0.05.

Phenotype	*Value*	*DF1*	**p-value**
GLU	1.4536	3	0.693
BUN	2.2989	3	0.5127
TP	12.5507	6	0.0508*
ALB	1.5024	6	0.9593
BILI	N/A	N/A	N/A
ALKP	5.4905	6	0.4826
ALT	4.1797	6	0.6524
AST	9.8017	6	0.1333
GLOB	2.8722	6	0.8247
GGT	2.3501	3	0.503
Hgb	2.7987	6	0.8337
Hct	11.325	6	0.0788*
WBC	2.0411	6	0.9159
RBC	3.1124	3	0.3746
MCV	8.6250	6	0.1958
MCH	4.7981	3	0.1872
MCHC	14.5756	6	0.0238**

Table 10: Chi-square analysis of 17 parameters in overall disease phenotype, using the normal/abnormal scoring system. (*) indicates p-value<0.10, (**) indicates p-value<0.05.

Phenotype	*Value*	*DF1*	*p-value*
GLU	6.8593	3	0.0765*
BUN	1.7050	3	0.6358
TP	1.6661	3	0.6445
ALB	1.1580	3	0.7631
BILI	2.8934	3	0.4084
ALKP	3.3820	3	0.3364
ALT	2.5430	3	0.4676
AST	1.9099	3	0.5913
GLOB	2.2955	3	0.5134
GGT	3.1643	3	0.3670
Hgb	2.1127	3	0.5494
Hct	3.3011	3	0.3475
WBC	3.3989	3	0.3341
RBC	1.0681	3	0.7848
MCV	6.6964	3	0.0822*
MCH	N/A	N/A	N/A
MCHC	0.5172	3	0.9151

One hundred twenty-one Havanese were genotyped for 114 microsatellite markers located on 21 chromosomes. Maximum LOD scores for the composite (overall) score, CD, Heart, and Height traits are listed in Table 11. The maximum LOD scores for the Cholesterol and Weight traits were 0.38 and 0.62, for marker FH2324 (CFA25) and C03.629 (CFA03), respectively (data not shown).

Table 11: List of markers identified as having LOD scores >0.90 for the traits composite phenotype, CD, heart, and height.

Trait	LOD	Marker	Chromosome
Composite phenotype	0.98	FH2613	CFA02
	1.34	FH3210	CFA02
	1.25	FH3115	CFA03
	1.66	FH3320	CFA05
	1.09	C10.16	CFA10
	1.10	FH2155	CFA16
	1.60	FH2441	CFA21
	0.98	REN02C20	CFA38
CD	1.33	REN72K15	CFA27
	1.49	REN118L14	CFA27
	1.36	FH2952	CFA29
	1.46	FH2377	CFA34
Heart	1.11	FH2274	CFA02
	1.13	REN70M14	CFA02
	1.85	FH2132	CFA02
	1.91	FH2613	CFA02
	1.32	FH2145	CFA03
	0.91	FH3815	CFA18
	1.80	FH4060	CFA18
	1.23	AHT125	CFA24
	1.06	FH2324	CFA25
	1.06	FH2141	CFA25
	1.13	FH3627	CFA25
Height	1.07	REN303C04	CFA04
	1.45	FH2360	CFA15

Discussion

A chromosome-specific genome screen was completed for Havanese kindred in an effort to identify regions of the genome that harbor the major gene identified by the complex segregation analysis in Chapter III (Starr et al. 2007). The lack of obvious candidate genes prohibited a candidate gene approach; therefore, linkage analysis was employed as an alternative in order to narrow the search for causative genes. The lack of statistically significant LOD scores necessitates the collection of marker data for the remaining chromosomes for the Havanese kindred. Insignificant LOD scores may be the result of uninformative markers, markers chosen which were not located close enough to candidate genes, or poor candidate gene choices. Intriguing regions identified by the screen (Table 10) will be further explored by the addition of more markers. The identification of four markers as having LOD scores over 1.0 for the heart trait on CFA02, and three markers on CFA25 suggests that these autosomes may harbor cardiac-related QTLs or genes.

CD was identified as being the best indicator of the overall disease phenotype (Starr et al. 2007) in the Havanese. Therefore, serum chemistries and CBCs for CD dogs and normal dogs were evaluated for additional biomarkers of disease. The hematology and serum chemistry results from Terpin and Roach's evaluation of chondrodysplastic Alaskan Malamutes (1981) were compared to those of the Havanese to determine whether a similar pathological condition exists in both dog breeds. CD Alaskan malamutes were diagnosed with hemolytic anemia, accompanied by low Hb, and BUN,

CREA, TP, and ALB and increased Phosphorous, UA, and GLU, as compared to normal Alaskan malamutes (Terpin and Roach 1981).

The trends for AST, MCV, BUN, ALT, and MCHC were similar among affected dogs of the two breeds (former two- low, latter three- high), but the analyses of continuous data did not yield significant p-values between normal and CD Havanese. Fletch et al. also described hemolytic anemia in a colony of chondrodysplastic Alaskan malamutes by observing high (>3:1) Hct : Hgb ratios (1973). Such ratios were not observed in the Havanese population, and the data described herein do not support the theory that CD-affected Havanese have hemolytic anemia. Serum chemistry and CBCs have been completed for CD-affected Scottish deerhounds (Breur et al. 1989) and chondrodysplastic dwarf Samoyeds (Meyers et al. 1983) for which no differences between CD-affected and normal dogs were observed in the CBC or serum chemistries.

The failure to identify any biochemical parameters that are statistically different between normal and CD-affected dogs necessitated the classification of serum chemistry and CBC values using a standard reference range (Aiello 1998). Using the two-category classification scheme (normal/abnormal), GGT was identified as a differential biomarker between the two groups (p-value 0.0195, 1DF). GGT is a liver enzyme that is widely used as a measure of hepatic and biliary disorders in medicine. A recent study by Hiramatsu et al (2007) identified GGT as having cytokine activity that stimulates osteoclastogenesis independent of its enzymatic activity. Osteoclasts are multinucleate cells originating from hematopoietic cells, and are responsible for bone resorption and calcium homeostasis. Retarded growth, cataracts, and osteoporosis have been identified

in GGT-deficient mice (Lieberman et al 1996, Levasseur et al 2003). Conversely, increased GGT levels are also associated with osteoporosis (Hiramatsu et al 2007), indicating the importance of maintaining normal GGT levels for optimal skeletal integrity. Radiological evaluation of Havanese across various age ranges has not been completed; such a study would be a prudent undertaking to determine whether the GGT cytokine activity plays a role in the pathogenesis of the Havanese skeletal abnormalities.

The role that TP, Hct, and MCHC (3-category scoring) and GLU and MCV (2-category) play in the overall phenotype are not understood. None of the parameters are singularly diagnostic, and the lack of additional significant parameters precludes any insight into the pathophysiologic process in the Havanese abnormalities.

In summary, a multi-trait, chromosome-specific linkage analysis was completed for 20 chromosomes in Havanese kindred to narrow the search for the major causative gene. Several areas of interest were identified, but no significant LOD scores were obtained. A whole genome screen will be subsequently completed. Clinical biochemical parameters were evaluated for CD and normal dogs, and GGT was identified as statistically different. Several biochemical differences were observed in the analyses of the normal, and mildly, moderately, or severely affected Havanese, but did not provide insight as to the underlying pathology of the collection of abnormalities.

CHAPTER V

CONCLUSION

An increased understanding of canine genetics has direct applications to the improvement of both canine and human health. The objectives of this work were to characterize the genetics of two complex diseases: induced atherosclerosis in a colony of mixed breed dogs, and a developmental disease in the Havanese breed.

Atherosclerosis is a common condition in humans, leading to severe health risks including myocardial infarction and stroke. Much is known about atherosclerosis in the human and mouse, aiding the work described in Chapter II. The linkage analyses completed for sensitive and resistant dogs failed to yield statistically significant LOD scores. Regions which harbored the highest LOD scores, however, were homologous to atherosclerosis QTLs in the mouse. Two such QTLs in the mouse (MMU04 and MMU08) contain several candidate genes and these will be examined further in future studies of atherosclerosis in the Lab-mix colony.

An expression profile was generated for three tissues in the Lab-mix colony. The expression patterns and differentially regulated genes varied among the tissues. Further investigation of the disease profiles may yield insight into the genome-wide response factors and mechanisms associated with atherosclerosis.

Chapter III describes the developmental abnormalities in the Havanese. The Havanese breed experiences a problematic collection of abnormalities. Because of the vast phenotypic array, and the number of anomalies present in the breed, it was

reasonable to think that the overall phenotype may be governed by multiple genes. The complex segregation analyses, while indicating a polygenic mode of inheritance, did identify the action of a major locus segregating in an autosomal recessive mode of inheritance.

Autosomal recessive diseases are particularly difficult for breeders to eradicate from a population because of the inability to detect carriers. Approximately two-thirds of canine hereditary diseases are inherited in an autosomal recessive fashion (Ostrander and Kruglyak 2000), owing to the propagation of recessive alleles in small, closed, inbred populations, and the inability to effectively select against carriers without genetic tests for such diseases.

The segregation of a major locus indicated that a genome screen to identify chromosomal regions which may harbor causative gene(s) should be performed. Chapter IV describes the multiplex-specific approach to linkage analyses for the collected Havanese kindred. Several regions of interest were identified, but no significant LOD scores were obtained. Future research will include the analysis of the remaining multiplexes in the MSS-2 and further work in identifying biomarkers for the Havanese developmental disease.

An oligonucleotide microarray creates a comprehensive disease profile and provides a “snapshot” of biological processes involved in the disease. Biomarkers for early diagnosis of diseases may be identified during the course of these studies. The use of microarrays is a relatively recent advance. Therefore, there is no “gold standard” for analysis of data. The stringent statistical parameters employed in these studies may have

resulted in a high number of false negatives. An additional complication is the incomplete annotation of the Canine_2 Affymetrix GeneChip. As the chip continues to be annotated and the presently unknown genes are identified, further insight may be gained into these two diseases.

Interestingly, while both populations and diseases studied are dramatically different, analyses in each population identified cholesterol-related genes. The heterodimer ABCG5 and ABCG8 identified by the oligonucleotide array in the Havanese indicated a potential cholesterol absorption and transport deficiency. Several chromosomes known to harbor genes involved in cholesterol biosynthesis and regulation have intriguing LOD scores (Chapter IV). Because only a partial genome screen was completed, the remaining MSS-2 markers must be analyzed to fully interpret the results of the screen. The identification of reverse cholesterol transport genes in both QTLs indicated by linkage analyses and the microarray analyses (Chapter II) provide an interesting pathway upon which further research in atherosclerosis in the dog can be based.

In conclusion, multiple approaches were taken to identify the underlying genetics of atherosclerosis in a colony of mixed-breed dogs, and developmental abnormalities in the Havanese breed. Estimates of heritability, linkage analyses, and disease profiling using oligogenic nucleotide arrays were completed to better characterize the hereditary diseases segregating in these populations.

REFERENCES

Abell LL, Mosbach EH, Kendall FE, 1956. Cholesterol metabolism in the dog. J Biol Chem. 220:527-536.

Acland GM, Ray K, Mellersh CS, Langston AA, Rine J, Ostrander EA, Aguirre GD, 1999. A novel retinal degeneration locus identified by linkage and comparative mapping of canine early retinal degeneration. Genomics. 59:134-142.

Aiello SE, 1998. Merck Veterinary Manual. Whitehouse Station, NJ: Merck & Co, Inc.

Allayee H, Ghazalpour A, Lusis AJ, 2003. Using mice to dissect genetic factors in atherosclerosis. Arterioscler Thromb Vasc Biol. 23:1501-1509.

Almasy L, Blangero J, 1998. Multipoint quantitative-trait linkage analysis in general pedigrees. Am J Hum Genet. 62:1198-1211.

American Heart Association (AHA), 2007. Know the Facts, Get the Stats. [cited 2007 June 1]. Available from: http://www.americanheart.org/presenter.jhtml?identifier=3000996.

American Kennel Club, 2007. AKC Dog Registration Statistics. [cited 2007 May 3]. Available from: http://www.akc.org/reg/dogreg_stats.cfm.

Andersson HC, 2002. Disorders of post-squalene cholesterol biosynthesis leading to human dysmorphogenesis. Cell Mol Biol. 48:173-177.

Andrich D, 1978. A rating scale formulation for ordered response categories. Psychometrika. 43:561-573.

Arman E, Haffner-Krausz R, Gorivodsky M, Lonai P, 1999. Fgfr2 is required for limb outgrowth and lung-branching morphogenesis. Proc Natl Acad Sci USA. 96:11895-11899.

Beaty TH, Kwiterovich PO Jr., Khoury MJ, White S, Bachorik PS, Smith HH, Teng B, Sniderman A, 1986. Genetic analysis of plasma sitosterol, apoprotein B, and lipoproteins in a large Amish pedigree with sitosterolemia. Am J Hum Genet. 38:492-504.

Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, Shan B, Barnes R, Hobbs HH, 2000. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science. 290:1771-1775.

Bezruczko N, 2005. Rasch measurement in the health sciences. Pine Grove, MN: JAM Press.

Bhattacharyya AK, Connor WE, 1974. Beta-sitosterolemia and xanthomatosis: a newly described lipid storage disease in two sisters. J Clin Invest. 53:1033-1043.

Bixler D, Poland C III, Brandt IK, Nicholas NJ, 1974. Autosomal dominant hypophosphatasia without skeletal disease. Am J Hum Genet. 26:14A.

Blangero J, Almasy L, Goring H, Williams J, Dyer T, Peterson C, 2005. Sequential oligogenic linkage analysis routines (SOLAR). 3.0.4 software edition.

Blanton V, Peeters H, 1976. The nonhuman primates as models for studying human atherosclerosis: studies on the chimpanzee, the baboon, and the rhesus macacus. Adv Exp Med Biol. 67:33-64.

Blom HJ, Shaw GM, den Heijer M, Finnell RH, 2006. Neural tube defects and folate: case far from closed. Nat Rev Neurosci. 7:724-731.

Bond TG, Fox CM, 2001. Applying the Rasch model: fundamental measurement in the human sciences. Mahwah, NJ: Lawrence Erlbaum Associates.

Bonney GE, 1986. Regressive logistic models for familial disease and other binary traits. Biometrics. 42:611-625.

Breen M, Langford CF, Carter NP, Holmes NG, Dickens HF, Thomas R, Suter N, Ryder EJ, Pope M, Binns MM, 1999. FISH mapping and identification of canine chromosomes. J Hered. 90:27-30.

Breen M, Jouquand S, Renier C, Mellersh CS, Hitte C, Holmes NG, Chéron A, Suter N, Vignaux F, Bristow AE, Priat C, McCann E, André C, Boundy S, Gitsham P, Thomas R, Bridge WL, Spriggs HF, Ryder EJ, Curson A, Sampson J, Ostrander EA, Binns MM, Galibert F, 2001. Chromosome-specific single-locus FISH probes allow anchorage of an 1800-marker integrated radiation-hybrid/linkage map of the domestic dog genome to all chromosomes. Genome Res. 11:1784-1795.

Breur GJ, Zerbe CA, Slocombe RF, Padgett GA, Braden TD, 1989. Clinical, radiographic, pathologic, and genetic features of osteochondrodysplasia in Scottish deerhounds. J Am Vet Med Assoc. 195:606-612.

Cargill EJ, Clark LA, Steiner JM, Murphy KE, 2002. Multiplexing of canine microsatellite markers for whole-genome screens.
Genomics. 80:250-253.

Carter RS, Siegel RJ, Chai AU, Fishbein MC, 1987. Immunohistochemical localization of apolipoproteins A-I and B in human carotid arteries. J Pathol 153**:**31-36.

Chomczynski P, Sacchi N, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162:156-159.

Clark LA, Tsai KL, Steiner JM, Williams DA, Guerra T, Ostrander EA, Galibert F, Murphy KE, 2004. Chromosome-specific microsatellite multiplex sets for linkage studies in the domestic dog. Genomics. 84:550-554.

Clark LA, Wahl JM, Steiner JM, Zhou W, Ji W, Famula TR, Williams DA, Murphy KE, 2005a. Linkage analysis and gene expression profile of pancreatic acinar atrophy in the German Shepherd Dog. Mamm Genome. 16:955-962.

Clark LA, Credille KM, Murphy KE, Rees CA, 2005b. Linkage of dermatomyositis in the Shetland Sheepdog to chromosome 35. Vet Dermatol. 16:392-394.

Clements DN, Carter SD, Innes JF, Ollier WE, Day PJ, 2007. Gene expression profiling of normal and ruptured canine anterior cruciate ligaments. Osteoarthritis Cartilage. Epub ahead of print. doi:10.1016/j.joca.2007.06.013.

Cooper MK, Wassif CA, Krakowiak PA, Taipale J, Gong R, Kelley RI, Porter FD, Beachy PA, 2003. A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis. Nat Genet. 33:508-13.

Danovitch SH, Baer PN, Laster L, 1968. Intestinal alkaline phosphatase activity in familial hypophosphatasia. New Eng J Med. 278:1253-1260.

Deng X, Marois Y, King MW, Guidoin R, 1994. Uptake of 3H-7-cholesterol along the arterial wall at an area of stenosis. ASAIO J. 40:186-191.

Du F, Acland GM, Ray J, 2000. Cloning and expression of type II collagen mRNA: evaluation as a candidate for canine oculo-skeletal dysplasia. Gene. 255:307-16.

Duggirala R, Williams JT, Williams-Blangero S, Blangero J, 1997. A variance component approach to dichotomous trait linkage analysis using a threshold model. Genet Epidemiol. 14:987-992.

Dweyer JH, Allayee H, Dweyer KM, Fan J, Wu H, Mar R, Lusis AJ, Mehrabian M, 2004. Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. N Engl J Med. 350:29-37.

Elston RC, Nasmboodrii KK, Glueck CJ, Fallat R, Tsang R, Leuba V, 1975. Studies of the genetic transmission of hypercholesterolemia and hypertriglyceridemia in 195 member kindred. Ann Hum Genet. 67-87.

FCI (Fédération Cynologique Internationale). [cited 2007 September]. Available from: http://www.fci.be/home.asp?lang=en.

Fletch SM, Smart ME, Pennock PW, Subden RE, 1973. Clinical and pathologic features of chondrodysplasia (dwarfism) in the Alaskan malamute. J Am Vet Med Assoc. 162:357-361.

Gelatt KN, MacKay EO, 2005. Prevalence of primary breed-related cataracts in the dog in North America. Vet Ophthalmol. 8:101-111.

Helisch A, Schaper W, 2003. Arteriogenesis: the development and growth of collateral arteries. Microcirc. 10:83-97.

Henderson CR, 1984. Animal additive genetic model. In: Applications of linear models in animal breeding. University of Guelph**,** Guelph, Canada; p.335-339.

Henthorn PS, Raducha M, Fedde KN, Lafferty MA, Whyte MP, 1992. Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia. Proc Natl Acad Sci USA. 89:9924-9928.

Herman GE, 2003. Disorders of cholesterol biosynthesis: prototypic metabolic malformation syndromes. Hum Mol Genet. 12 Spec No 1:R75-R88.

Hess RS, Kass PH, Van Winkle TJ, 2003. Association between diabetes mellitus, hypothyroidism or hyperadrenocorticism, and atherosclerosis in dogs. J Vet Intern Med. 17:489-494.

Hiramatsu K, Asaba Y, Takeshita S, Nimura Y, Tatsumi S, Katagiri N, Niida S, Nakajima T, Tanaka S, Ito M, Karsenty G, Ikeda K, 2007. Overexpression of gamma-glutamyltransferase in transgenic mice accelerates bone resorption and causes osteoporosis. Endocrinology. 148:2708-2715.

Hu JC-C, Plaetke R, Mornet E, Zhang C, Sun X, Thomas HF, Simmer JP, 2000. Characterization of a family with dominant hypophosphatasia. Eur J Oral Sci. 108:189-194.

Hui DY, Howles PN, 2002. Carboxyl ester lipase: structure-function relationship and physiological role in lipoprotein metabolism and atherosclerosis. J Lipid Res. 43:2017 - 2030.

Janss LLG, 1998. MAGGIC: A package of subroutines for genetic analyses with Gibbs sampling. In: Proceedings of the 6th World Congress on Genetics Applied to Livestock Production: vol 27. Organizing Committee on 6th WCGLAP, Armidale, Australia. p. 459-460

Ji W, Zhou W, Gregg K, Yu N, Davis S, Davis S, 2004. A method for cross-species gene expression analysis with high-density oligonucleotide arrays. Nucleic Acids Res. 32:e93.

Kelemen LE, 2006. The role of folate receptor alpha in cancer development, progression and treatment: cause, consequence or innocent bystander? Int J Cancer. 119:243-250.

Klett EL, Lu K, Kosters A, Vink E, Lee MH, Altenburg M, Shefer S, Batta AK, Yu H, Chen J, Klein R, Looije N, Oude-Elferink R, Groen AK, Maeda N, Salen G, Patel SB, 2004. A mouse model of sitosterolemia: absence of Abcg8/sterolin-2 results in failure to secrete biliary cholesterol. BMC Med. 2:5.

Klumb D, Baldwin J, 2005. The Havanese. Charleston, SC. BookSurge Publishing.

Korstanje R, Eriksson P, Samnegard A, Olsson PG, Forsman-Semb K, Sen S, Churchill GA, Rollins J, Harris S, Hamsten A, Paigen B, 2004. Locating Ath8, a locus for murine atherosclerosis susceptibility and testing several of its candidate genes in mice and humans. Atherosclerosis. 177:443-450.

Levasseur R, Barrios R, Elefteriou F, Glass DA 2nd, Lieberman MW, Karsenty G, 2003. Reversible skeletal abnormalities in gamma-glutamyl transpeptidase-deficient mice. Endocrinology. 144:2761-2764

Li F, Huang Y, Hui DY, 1996. Bile salt stimulated cholesterol esterase increases uptake of high density lipoprotein-associated cholesteryl esters by HepG2 cells. Biochemistry. 35:6657-6663.

Li H, Sun B, 2007. Toll-like receptor 4 in atherosclerosis. J Cell Mol Med. 11:88-95.

Libby P, 2002. Inflammation in atherosclerosis. Nature. 420:868-874.

Lieberman MW, Wiseman AL, Shi ZZ, Carter BZ, Barrios R, Ou CN, Chevez-Barrios P, Wang Y, Habib GM, Goodman JC, Huang SL, Lebovitz RM, Matzuk MM,1996. Growth retardation and cysteine deficiency in gamma-glutamyl transpeptidase-deficient mice. Proc Natl Acad Sci USA. 93:7923-7926.

Linacre JM, 2006. WINSTEPS Rasch measurement computer program. Chicago: Winsteps.com.

Lindberg J, Saetre P, Nishino S, Mignot E, Jazin E, 2007. Reduced expression of TAC1, PENK and SOCS2 in Hcrtr-2 mutated narcoleptic dog brain. BMC Neurosci. 8:34.

Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas EJ 3rd, Zody MC, et al., 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature. 438:803-819.

Lusis AJ, Mar R, Pajukanta. 2004. Genetics of atherosclerosis. Annu Rev Genomics Hum Genet. 5:189-218.

Lynch M, Walsh B, 1998. Genetics and analysis of quantitative traits. Sunderland (MA): Sinauer Associates.

Maccoux LJ, Clements DN, Salway F, Day PJR, 2007. Identification of new reference genes for the normalisation of canine osteoarthritic joint tissue transcripts from microarray data. BMC Mol Biol. 8:62.

Maldonado EN, Romero JR, Ochoa B, Aveldaño MI, 2001. Lipid and fatty acid composition of canine lipoproteins.Comp Biochem Physiol B Biochem Mol Biol. 128:719-29.

Markward NJ, 2004. Establishing mathematical laws of genomic variation. J Appl Meas. 5:1-14.

Markward NJ, Fisher WP Jr., 2004. Calibrating the genome. J Appl Meas. 5:129-141.
Masters GN, 1982. A Rasch model for partial credit scoring. Psychometrika. 47:149-174.

Masters GN, 1982. A Rasch model for partial credit scoring. Psychometrika. 47:149-174.

Meijering A, Gianola D, 1985. Linear vs. nonlinear methods of sire evaluation for categorical traits: a simulation study. Genet Sel Evol. 17:115-132.

Mellersh CS, Langston AA, Acland GM, Fleming MA, Ray K, Wiegand NA, Francisco LV, Gibbs M, Aguirre GD, Ostrander EA.1997. A linkage map of the canine genome. Genomics. 46:326-336.

Meredith SP, Richards AJ, Bearcroft P, Pouson AV, Snead MP, 2007. Significant ocular findings are a feature of heritable bone dysplasias resulting from defects in type II collagen. Br J Ophthalmol. 91:1148-1151.

Meyers VN, Jezyk PF, Aguirre GD, Patterson DF, 1983. Short-limbed dwarfism and ocular defects in the Samoyed dog. J Am Vet Med Assoc. 183:975-979.

Moore CA, Curry CJR, Henthorn PS, Smith JA, Smith JC, O'Lague P, Coburn SP, Weaver DD, Whyte MP, 1999. Mild autosomal dominant hypophosphatasia: in utero presentation in two families. Am J Med Genet. 86:410-415.

Mori M, Li G, Abe I, Nakayama J, Guo Z, Sawashita J, Ugawa T, Nishizono S, Serikawa T, Higuchi K, Shumiya S.2006. Lanosterol synthase mutations cause cholesterol deficiency-associated cataracts in the Shumiya cataract rat. J Clin Invest. 116:395-404.

Mornet E, 2000. Hypophospatasia: The mutations in the tissue-nonspecific alkaline phosphatase gene. Hum Mutat. 15:309-315.

Müller H, 1987. A Rasch model for continuous traits. Psychometrika. 52:165-181.

Nowaczyk MJM, Waye JS, 2001. The Smith-Lemli-Opitz syndrome: a novel metabolic way of understanding developmental biology, enbryogenesis, and dysmorphology. Clin Genet. 59:375-386.

Nwokoro NA, Wassif CA, Porter FD, 2001. Genetic disorders of cholesterol biosynthesis in mice and humans. Mol Genet Metab. 74:105-119.

Oberbauer AM, Bell JS, Belanger JM, Famula TR, 2006. Genetic evaluation of Addison's disease in the Portuguese Water Dog. BMC Vet Res. 2:15-21.

OMIA (Online Mendelian Inheritance in Animals). [cited 2007 September]. Available from: http://omia.angis.org.au/.

Oram JF, Vaughan AM, 2006. ATP-Binding cassette cholesterol transporters and cardiovascular disease. Circ Res. 99:1031-1043.

Ostrander EA, Galibert F, Patterson DF, 2000. Canine genetics comes of age. Trends Genet. 16:117-24.

Ostrander EA, Kruglyak L, 2000. Unleashing the canine genome. Genome Res. 10:1271-1274.

Parker HG, Kim LV, Sutter NB, Carlson S, Lorentzen TD, Malek TB, Johnson GS, DeFrance HB, Ostrander EA, Kruglyak L, 2004. Genetic structure of the purebred dog. Science. 304:1160-1164.

Parker HG, Ostrander EA, 2005. Canine genomics and genetics: running with the pack. PLoS Genet. 1:e58.

Patterson DF, 2000. Companion animal medicine in the age of medical genetics. J Vet Intern Med. 14:1-9.

Perkins K, Wright BD, 2005. Using Rasch measurement with medical data. In: Bezruczko N, editor. Rasch measurement in the health sciences. Pine Grove, MN: JAM Press.

Pertsemlidis D, Kirchman EH, Ahrens EH Jr, 1973. Regulation of cholesterol metabolism in the dog. I. Effects of complete bile diversion and of cholesterol feeding on absorption, synthesis, accumulation, and excretion rates measured during life. J Clin Invest. 52:2353-2367.

Porter JA, Young KE, Beachy PA,1996. Cholesterol modification of hedgehog signaling proteins in animal development. Science. 274:255-259.

Priat C, Hitte C, Vignaux F, Renier C, Jiang Z, Jouquand S, Chéron A, André C, Galibert F, 1998. A whole-genome radiation hybrid map of the dog genome. Genomics. 54:361-378.

Rapold HJ, Stassen T, Van de Werf F, Collen D, 1992. Comparative copper coil-induced thrombogenicity of the internal mammary, left anterior descending coronary, and popliteal arteries in dogs. Arterioscler Thromb. 12:639-644.

Rasch G, 1960. Probabilistic models for some intelligence and attainment tests. Copenhagen: Danish Institute of Educational Research. Reprint: Rasch G. 1980. Chicago: University of Chicago Press.

Rawitscher RE, Smith GW, Muller WH Jr, 1973. Experimental canine coronary atherosclerosis. Ann Surg. 177:357–361.

Rebhan M, Chalifa-Caspi V, Prilusky J, Lancet D, 1997. GeneCards: encyclopedia for genes, proteins and diseases. Weizmann Institute of Science, Bioinformatics Unit and Genome Center (Rehovot, Israel). Available from: http://www.genecards.org/.

Risch N, Zhang H, 1995. Extreme discordant sib pairs for mapping quantitativetrait loci in humans. Science. 268:1584 –1589.

Russell JC, Proctor SD, 2006. Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. Cardiovasc Pathol. 15:318-330.

Ruthford J. Data Report: HCA Havanese Health 2004 Survey. Unpublished data. Mrs. Ruthford may be contacted via email at jruthford@comcast.net.

Sabiston DC, Smith GW, Talbert JL, Gutelius J, Vasko JS, 1961. Experimental production of canine coronary atherosclerosis. Ann Surg. 153:13-22.

Sako T, Takahashi T, Takehana K, Uchida E, Nakade T, Umemura T, Taniyama H, 2002. Chlamydial infection in canine atherosclerotic lesions. Atherosclerosis. 162:253-259.

Sargan DR, Yang F, Squire M, Milne BS, O'Brien PC, Ferguson-Smith MA., 2000. Use of flow-sorted canine chromosomes in the assignment of canine linkage, radiation hybrid, and syntenic groups to chromosomes: refinement and verification of the comparative chromosome map for dog and human. Genomics. 69(2):182-195.

Sargan DR, 2004. IDID: inherited diseases in dogs: web-based information for canine inherited disease genetics. Mamm Genome. 15:503-506.

Savolainen P, Zhang Y, Luo J, Lundeburg J, Leitner T, 2002. Genetic evidence for an east Asian origin of domestic dogs. Science. 298:1610-1613.

Sergi C, Marnet E, Troeger J, VoigtlaenderT, 2001. Perinatal hypophosphatasia: radiology, pathology and molecular biology studies in a family harboring a splicing mutation (648+1A) and a novel missense mutation (N400S) in the tissue-nonspecific alkaline phosphatase (TNSALP) gene. Am J Med Genet. 103:235-240.

Smith J, 2003. Quantitative trait locus mapping for atherosclerosis susceptibility. Curr Opin Lipidol. 14:499-504.

Sottiaux J, 1999. Atherosclerosis in a dog with diabetes mellitus. J Small Anim Pract. 40:581-584.

Starr AN, Famula TR, Markward NJ, Baldwin JV, Fowler KD, Klumb DE, Simpson NL, Murphy KE, 2007. Hereditary evaluation of multiple developmental abnormalities in the Havanese dog breed. J Hered. 98:510-517.

Steiner A, Kendall FE and Bevans M. 1949. Production of arteriosclerosis in dogs by cholesterol and thiouracil feeding. Amer Heart J. 38:34

Sutter NB, Ostrander EA, 2004. Dog star rising: the canine genetic system. Nat Rev Genet. 5:900-910.

Sutter NB, Eberle MA, Parker HG, Pullar BJ, Kirkness EF, Kruglyak L, Ostrander EA, 2004. Extensive and breed-specific linkage disequilibrium in Canis familiaris. Genome Res. 14:2388-96.

Sutter NB, Bustamante CD, Chase K, Gray MM, Zhao K, Zhu L, Padhukasahasram B, Karlins E, Davis S, Jones PG, Quignon P, Johnson GS, Parker HG, Fretwell N, Mosher DS, Lawler DF, Satyaraj E, Nordborg M, Lark KG, Wayne RK, Ostrander EA. 2007. A single IGF1 allele is a major determinant of small size in dogs. Science. 316:112-115.

Switonski M, Szczerbal I, Nowacka J, 2004. The dog genome map and its use in mammalian comparative genomics. J Appl Genet. 45:195-214.

Terpin T, Roach MR, 1981. Chondrodysplasia in the Alaskan Malamute: involvement of arteries, as well as bone and blood. Am J Vet Res. 42:1865-1873.

Tobias KM, Rohrbach BW, 2003. Association of breed with the diagnosis of congenital portosystemic shunts in dogs: 2,400 cases (1980-2002). J Am Vet Med Assoc. 223:1636-1639.

Tsai K, Clark LA, Murphy KE, 2007. Understanding hereditary diseases using the dog and human as companion model systems. Mamm Genome. 18:444-451.

Vigneaux F, Hitte C, Priat C, Chuat JC, Andre C, Galibert F, 1999. Construction and optimization of a dog whole-genome radiation hybrid panel. Mamm Genome. 10:888-894.

Vilà C, Savolainen P, Maldonado JE, Amorim IR, Rice JE, Honeycutt RL, Crandall KA, Lundeberg J, Wayne RK, 1997. Multiple and ancient origins of the domestic dog. Science. 276:1687-1689.

Wang S, Shi W, Wang X, Lusis AJ. 2003. Identification of a locus in mice that influences aortic aneurism formation. Circulation. 108:IV-177.

Wang X, Ishimori N, Korstanje R, Rollins J, Paigen B, 2005. Identifying novel genes for atherosclerosis through mouse-human comparative genetics. Am J Hum Genet. 77:1-16.

Weinberg PD, 2004. Rate-limiting steps in the development of atherosclerosis: the response-to-influx theory. J Vasc Res. 41:1-17.

Weiss MJ, Cole DE, Ray K, Whyte MP, Lafferty MA, Mulivor RA, Harris H, 1988. A missense mutation in the human liver/bone/kidney alkaline phosphatase gene causing a lethal form of hypophosphatasia. Proc Natl Acad Sci USA. 85:7666-7669.

Whyte MP, 1994. Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocr Rev. 15:439-461.

Wilkie AO, Patey SJ, Kan SH, van den Ouweland AM, Hamel BC, 2002. FGFs, their receptors, and human limb malformations: clinical and molecular correlations. Am J Med Genet. 112:266-278.

Wright BD, 1984. Despair and hope for educational measurement. Contemp Ed Rev. 1:281-288.

Wright BD, 1999. Fundamental measurement for psychology. In: Embretson SE, Hershberger SL, editors. The new rules of measurement: what every educator and psychologist should know. Hillsdale, NJ: Lawrence Erlbaum Associates.

Wright BD, Masters GN, 1981. The measurement of knowledge and attitude (Research memorandum no. 30) Chicago: Statistical Laboratory, Department of Education, University of Chicago.

Wright BD, Masters GN, 1982. Rating scale analysis: Rasch measurement. Chicago: MESA Press.

Wright BD, Stone MH, 1979. Best test design: Rasch measurement. Chicago: MESA Press.

Yanni AE, 2004. The laboratory rabbit: an animal model of atherosclerosis research. Lab Anim. 38:246-256.

Young AE, Ryun JR, Bannasch DL, 2006. Deletions in the COL10A1 gene are not associated with skeletal changes in dogs. Mamm Genome. 17:761-768.

VITA

Name:	Alison Nicole Starr
Address:	Texas A&M University College of Veterinary Medicine Murphy Laboratory MS 4467 College Station, TX 77843
Email Address:	astarr@cvm.tamu.edu
Education:	B.S., Animal and Veterinary Sciences, Clemson University, 2003