

Hereditary Evaluation of Multiple Developmental Abnormalities in the Havanaese Dog Breed

ALISON N. STARR, THOMAS R. FAMULA, NATHAN J. MARKWARD, JOANNE V. BALDWIN, KARON D. FOWLER, DIANE E. KLUMB, NANCY L. SIMPSON, AND KEITH E. MURPHY

From the Department of Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843-4467 (Starr and Murphy); the Department of Animal Science, University of California, Davis, CA 95616-8521 (Famula); the Department of Genetic Epidemiology, Pennington Biomedical Research Center, Baton Rouge, LA 70808 (Markward); and H.E.A.R.T. Goochland, VA 23063 (Baldwin, Fowler, Klumb, and Simpson).

Address correspondence to K. E. Murphy at the address above, or e-mail: kmurphy@cvm.tamu.edu.

Abstract

The Havanaese is a toy breed that presents with a wide range of developmental abnormalities. Skeletal defects, particularly osteochondrodysplasia (OCD), are the most frequently observed anomalies. Cataracts, liver shunts, heart murmurs, and missing incisors are also common in this breed. Estimates of heritability 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 were used to identify four traits that co-segregate: cataracts, hepatic abnormalities, OCD, 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 and thirteen genes were found to be differentially regulated in the Havanaese.

The Havanaese is a toy dog that presents with multiple developmental abnormalities. The breed originated in Cuba as an aristocratic pet. In the 1970s, the breed was introduced into the United States of America and the current U.S. population is reported to have developed from 4 kindred originating in Cuba, Costa Rica, Russia, and the U.S. Cataracts, retinal detachment, cancer, osteochondrodysplasia (OCD), and premature death were described in the founding population.

Havanaese breeders concerned with the extensive abnormalities afflicting the breed began assembling phenotypic data for use in genetic studies. In 2004, members of the Havanaese Club of America (HCA) created an anonymous owner survey, allowing them to quantify the frequency of each abnormality within the breed as a whole (Ruthford J, 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. Similarly, a retrospective case study of canine cataracts identified the Havanaese 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 Havanaese were diagnosed with cardiac problems; the most common is heart murmur (82%), followed by mitral valve insufficiency (31%). In an additional case study, Havanaese were found to have the highest proportion of liver shunts among breeds reported in the VMDB (3.2%) (Tobias and Rohrbach 2003). Cryptorchidism occurs in 5% of 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 Havanaese. 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 Havanaese was tested to determine whether the spectrum could be analyzed as a composite phenotype, drawing on

the Rasch (1960) family of measurement models to construct a syndrome scale.

Although 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 the 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, comprised of 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 (OCD), ophthalmic (cataracts), cardiac (murmurs), hepatic (liver shunts), pre- and postprandial 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, 2 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 2 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 78 males (25 unaffected and 22 affected dogs) and 103 females (34 unaffected and 36 affected) 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.

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 (1960) model to construct a Havanese “syndrome” scale that simultaneously captures information about the primary phenotype of interest and other ordinal (OCD, cataract, murmur, liver shunt) and continuous (pre- and postprandial bile acid levels, ALT, cholesterol, taurine, height, weight) traits that may be relevant to disease diagnosis and prevention. Rasch (1960) models (Andrich 1978; Wright and Stone 1979; Masters 1982; Wright and Masters 1982; Müller 1987; Wright 1999) 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) that 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

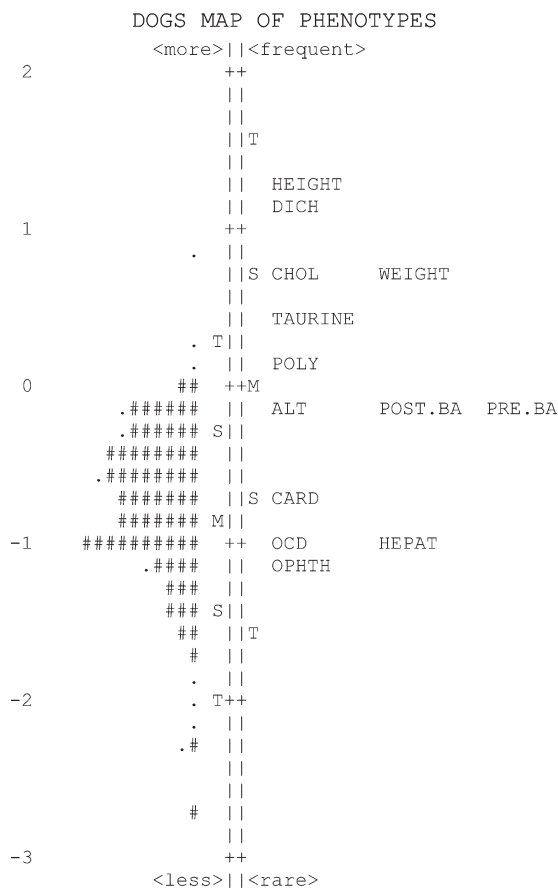


Figure 1. Wright map of dog measures and phenotype calibrations. The left side of the figure maps the distribution of dogs where (.) represents one dog and (#) represents 2 dogs. The right side of the figure illustrates the distribution of phenotypes. Figure indicates that fewer severely affected dogs are observed than mildly affected dogs, but severely affected dogs are more likely to have rare (severe) clinical signs. Phenotype abbreviations: CARD, cardiac abnormalities; CHOL, cholesterol; DICH, dichotomous score; HEPAT, hepatic abnormalities; OPTH, ophthalmologic abnormalities; POLY, polytomous score; POST.BA, postprandial bile acids; PRE.BA, fasted bile acids. Along vertical axis, T = 2 standard deviations, S = 1 standard deviation, M = mean.

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 1) was constructed using the Rasch partial credit model and a joint maximum likelihood estimation procedure (Linacre 2006).

Estimation of Heritability

Our analysis of disease in the Havanese follows 2 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 on normally distributed residuals when 4 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 4-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), 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) was again employed to derive heritability estimates, using a multivariate normal to maximize the likelihood (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 nonrandom 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 nonrandomly 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 follow 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 1995) 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., $\sigma_e^2 = 1$). 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 5000 was generated, beginning with the creation of 300 000 total samples, a “burn-in” of 50 000, and a sampling rate of every 100th Gibbs value. This process was repeated to create 2 replicate chains. From the 5000 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 more than 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 1 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 OCD, another suffered from severe OCD, 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 Inc, Austin, TX). Because these are client-owned dogs, age-matched normal Havanese were not available. Tissues from 3 clinically normal mixed-breed dogs were used as normal controls. These dogs dis-

Table 1. 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

played 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™ 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 min in a solution containing 40 mM Tris-acetate (pH 8.1), 100 mM KOAc, and 30 mM 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, Student's *t*-test, and Bonferroni multiple testing corrections. Samples were grouped separately to identify differentially expressed genes. Gene expression ratios were calculated between the 2 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 that the clinical disease described herein may have a hereditary component (Table 1). No difference in risk between gender was observed (data not presented).

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 2). For all parameters of interest, the 95% HDR does include zero, a clear

Table 2. Marginal posterior means, modes, standard deviations, and limits to the 95% HDRs 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

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 that the Mendelian model provides the best fit to the data.

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 (Wright and Masters 1981; Wright 1984; Bond and Fox 2001) 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 1 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 2 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 ($S = 1SD$, $T = 2SD$) of dog measures are indicated by “TSMST.”.

Figure 2 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 1, 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 2-fold difference in expression and a P value ≤ 0.05 . Of these, 83 were down regulated and 30 were up regulated (abbreviated results in Supplementary Table S1).

Discussion

Havanese are afflicted with multiple developmental abnormalities, most frequently OCD, 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 that 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 reevaluated 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

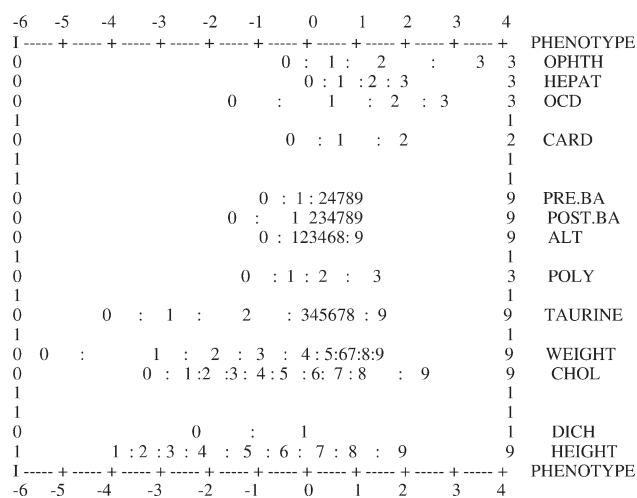


Figure 2. Illustration of the expected score means. Each trait has the categorical score distribution along the horizontal axis. A (:) indicates a half-score point for an observed category. Abbreviations used are the same as in Figure 1.

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 OCD as a variable that co-segregates with the hypothesized syndrome, coupled with the alignment of the OCD and DICH thresholds in Figure 2, suggests the benefit of using OCD as a physiological marker for disease. Dogs categorized as OCD are more likely to have other abnormalities; therefore, the OCD 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. Although there are similarities between various human diseases and the signs in the Havanese, there are no 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) (Supplementary 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 adenosine triphosphate-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; Beatty 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 2 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; Pertsemliadis 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 5 forms of hypophosphatasia in humans: perinatal (lethal), infantile, child, adult, and odontohypophosphatasia (reviewed in Whyte 1994; 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 because 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 (Blom et al. 2006; Kelemen 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 OCD 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.

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Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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