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Genetics of Pulmonary Valve Stenosis in Bulldogs

By

SAMANTHA KOVACS
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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in the

OFFICE OF GRADUATE STUDIES

of the

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DAVIS

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Abstract

Pulmonary valve stenosis (PS) is the most common congenital heart defect in dogs. The condition is due to abnormal valve anatomy present at birth that leads to stenosis of the right ventricular outflow tract. The valve stenosis results in pressure overload of the right ventricle. If severe enough, the stenosis leads to right ventricular hypertrophy, arrhythmias, exercise intolerance, and right-sided heart failure. Severity of disease is typically determined based on velocity of blood flow across the lesion via echocardiography. The median survival time for dogs with severe PS is 4-5 years with medical management, while moderately affected cases have a variable prognosis. The prognosis for mild PS is generally good.

The Bulldog, which is the fourth most popular breed according to AKC dog rankings, is a breed highly overrepresented in PS cases**.** Due to this breed predisposition PS is suspected to be inherited. Mildly affected dogs are frequently not diagnosed as traditional auscultation-based tests are insensitive in dogs with profound airway sounds and barrel-shaped chests such as the Bulldog. These subclinical cases make it incredibly difficult to successfully screen and remove affected individuals from the breeding program. Thus, hindering breeding efforts to reduce disease prevalence. Therefore, the development of a genetic screening test represents the most practical approach to identification and reduction of disease frequency in the breed. Additionally, severe and moderately affected PS cases are treated with traditional balloon valvuloplasty, which has good but variable success. The procedure has a significant cost, does not restore normal function, and there is considerable anesthetic risk for the brachycephalic breeds that are predisposed to PS. Additionally, some PS-affected dogs have concurrent coronary anomalies, which are expensive to identify and make balloon valvuloplasty contraindicated, further highlighting the need for genetic screening tests for this condition.

There is limited information about the genetic cause of PS in humans with the majority of mutations involving the RAS-MAPK pathway. Although PS is commonly seen in puppies, no mutation to date has been identified, although literature suggests a possible recessive pattern of inheritance. Through the combined efforts of the clinical cardiology service at the University of California Davis, clinical cardiology service at Colorado State University, and the Translational Cardiac Genetics and Pharmacogenomics Laboratory in the University of California Davis School of Veterinary Medicine we aim to elucidate the genetic mechanisms of PS in Bulldogs.

The goal of this dissertation is to compile and contribute to what is currently known about the genetics of PS in humans and animals. A retrospective study in a large referral hospital population allowed determination of the prevalence of this disease in dogs and confirmed common breed predisposition. A pedigree analysis was performed in the most affected breed, the Bulldog, to elucidate a common mode of inheritance. A genome-wide association study was performed to refine a region of interest in the canine genome that is associated with PS. Whole genome sequencing followed by variant analysis allowed identification of mutations associated with the disease. The most plausible variants underwent validation in a large population of dogs using MassArray and those that continued to segregate were followed up with immunohistochemistry in cardiac tissue. Finally, the results of this project will contribute to the understanding of heart development and congenital heart disease in dogs overall, thus guiding novel prevention and therapeutic strategies for PS.

Acknowledgments

This dissertation was conducted at the University of California Davis, School of Veterinary Medicine in the Stern Translational Cardiac Genetics and Pharmacogenomics Laboratory for completion of a doctoral of philosophy degree in Integrative Genetics and Genomics.

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I dedicate this dissertation to my husband Les Kovacs for his unconditional love and continued support during my PhD. His patience and understanding encouraged me to surmount the obstacles I encountered. His excitement in this project energized deeper exploration and learning.

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Introduction

Pulmonary valve stenosis (PS) is a devastating inherited heart disease in dogs and children. In recent studies, PS is the most common canine congenital heart defect in both tertiary cardiology populations[1] and shelter populations.[2] Bulldogs have a higher predisposition compared to other breeds, but many breeds may be affected with PS.[3,4] PS is the result of congenital pulmonary valve malformations.[3,5,6] A thorough pathological examination of pulmonary valves in Beagles afflicted with varying severity of PS revealed that the disease had a spectrum of phenotypes that ranged between two predominant forms: Type A and Type B.[7] Type A PS is when the pulmonary valve leaflets are fused and dome during systole, but there may be various degrees of valve thickening.[8] Type A is the most common form of valvular PS observed in dogs.[1,3,9] Type B PS is when the three pulmonary valve leaflets are thickened or dysplastic, but the commissures of the leaflets do not fuse. Type B PS is frequently seen with a narrowing of the valve annulus.[1] Although Type A is more common in the overall canine population, Type B is reportedly more common in Bulldogs and Boxers.[3,9]

In addition to traditional PS types seen in all dogs, some breeds are concurrently diagnosed with coronary artery anomalies that contribute to a PS-like condition or limit therapy.[8,10,11] Bulldogs and Boxers are highly predisposed to these coronary anomalies suggesting a genetic cause.[10–12] The coronary anomaly originally termed R2A is an anomalous left main coronary artery which incorrectly originates from the right coronary ostium and encircles the right ventricular outflow tract and pulmonary valve, potentially leading to a stenosis.[10,12,13] These anomalies are diagnosed by CT angiography or conventional angiography. Unfortunately, the presence of the anomalous coronary artery limits treatment options for PS.[8,14]

Regardless of the type of PS, the abnormal anatomy limits right ventricular outflow and results in pressure overload on the right ventricle. The most common clinical signs are manifested as exercise intolerance and shortness of breath, although collapse and fluid accumulation in the abdomen secondary to right heart failure are also encountered.[3] Since PS is a congenital cardiac abnormality present at birth, many dogs are diagnosed at a young age.[3,13,15] Currently, the gold standard for diagnosis of PS is transthoracic echocardiogram and assessment of the right ventricular pressure gradient.[8] Echocardiographically-derived transpulmonary peak pressure gradients >80mmHg are considered severe, 50-80mmHg are moderate, 20-49 mmHg are mild, and <16mmHg are normal.[8,13,15] Survival times are reported at 4-5 years for severely affected dogs without surgical intervention or with β blockade alone.[3,15] Moderately affected dogs have a variable prognosis.[3,6] Mildly affected dogs have a good prognosis and may live a normal life.[3,6,13] However, the ability to pass on this defect to offspring exists for all categories and there is no information available regarding the inheritance of disease severity/expression (ie. mildly affected dogs may produce severely affected progeny).[3] This highlights the importance of understanding the genetics of PS in dogs.

Balloon valvuloplasty is the treatment of choice for severe PS cases and for dogs with clinical signs referable to PS.[6] However, this treatment is palliative because it relieves the clinical signs without addressing the underlying genetic cause or returning the valve to normal function.[16] Type A PS cases have a better success rate post balloon valvuloplasty compared to Type B PS cases.[3,9,17] Balloon valvuloplasty is contraindicated when coronary artery anomalies such as the R2A anomaly are present because when the balloon is inflated there is risk of damaging or rupturing the aberrant coronary artery causing death.[8,12,16,18] CT angiography performed immediately prior to surgery is highly recommended to confirm if an anomalous

coronary artery is present, but this technique increases anesthesia times and elevates procedural cost.[15,16,19] Bulldogs are predisposed to Type B PS, R2A anomalies, and due to their airway conformation have increased anesthetic risk which may limit the success of balloon valvuloplasty in the breed. Therefore, identifying a genetic cause in the breed to reduce the prevalence is warranted.

Many congenital cardiac diseases have a genetic basis in both humans and dogs.[20–22] Pulmonary valve stenosis is inherited in humans with a variable pattern of inheritance.[23,24] Although there is not an identified mutation for the majority of human PS cases, the genes with known mutations are in the RAS-MAPK pathway or transcription factors known to play a role in cardiac development such as GATA4.[24] An extensive genetic study in Beagles afflicted with PS concluded that the disease in that breed is expected to be polygenic and inherited in a recessive manner.[7] However, they were not able to determine the genes that contributed to the defect, likely due to the lack of advanced genetic techniques at the time of the study. Fortunately, the high prevalence of this disease in Bulldogs and advancement of canine genetic tools is an excellent combination to determine the genetic defect responsible for PS.

A common research pathway used previously to discover the genetic cause of disease in dogs is the combination of a genome-wide association study (GWAS) to narrow down a region of interest in the genome that segregates with disease followed by whole genome sequencing (WGS) to find the exact variant responsible for the disease.[21,25] WGS for deleterious mutation identification is when an individual's genome is sequenced, aligned to a reference genome, and variants are filtered by various methodologies until a causative variant is identified. This method is commonly employed in research since the costs of WGS have decreased dramatically with next generation sequencing technologies. This method is beneficial as it can identify a wide

spectrum of variations in both coding and non-coding regions such as single base pair polymorphisms, insertions, deletions, and larger structural variations, many of which would be missed by classical candidate gene sequencing approaches.[26] Additionally, validation of variants using MassArray technologies in a larger population of other dog breeds helps to prune the list of disease associated variants and reduce false positive calls.[27]

The aim of this dissertation is to elucidate the genetic architecture of PS in Bulldogs. It consists of three chapters that have been published in a peer review journal or will be submitted for future publication. The first chapter entitled *Pulmonary valve stenosis: a comparative study in humans and dogs* is a review paper that will be submitted to *Canine Genetics and Epidemiology* as part of a thematic series on canine cardiovascular disease. This chapter will discuss what is currently known about the genetics of PS in humans and dogs and includes findings from this dissertation. The second chapter, entitled *Congenital cardiac outflow tract abnormalities in dogs: prevalence and pattern of inheritance from 2008 to 2017,* was published in *Frontiers Veterinary Science* in 2019.[28] It discusses the prevalence of canine PS in a tertiary veterinary hospital population, the most common affected breeds, and the proposed mode of inheritance in the most common breed. The third chapter entitled *A Large structural variant in ZNF446 is associated with pulmonary valve stenosis in Bulldogs* will be submitted to *PLOS Genetics.* This manuscript utilized a wide range of genetic techniques to identify a large deletion that is associated with a protective effect against PS in Bulldogs.

The addendum of this dissertation includes additional chapters that consist of published studies completed by the author. The fourth chapter entitled *Unraveling the Genetics Behind Equid Cardiac Disease* was published in *Veterinary Clinics: Equine Practice* in 2020.[29] This was a literature review summarizing what is currently known about genetics of cardiovascular disease

in alternate veterinary species. The fifth chapter entitled*, Pedigree analysis of atrial fibrillation in Irish wolfhounds supports a high heritability with a dominant mode of inheritance* was published in *Canine genetics and epidemiology* in 2019.[30] This illustrated the careful collection of phenotypic data and pedigree data to determine mode of inheritance and perform a robust heritability analysis on a cardiovascular disease that exhibits age-dependent penetrance. The sixth chapter entitled, *Varying expression of mu and kappa opioid receptors in cockatiels (Nymphicus hollandicus) and domestic pigeons (Columba livia domestica)* was published in *Frontiers in genetics* in 2020.[31] It utilized qPCR to interrogate gene expression in avian veterinary species.

Not included in this dissertation are the seven additional publications in which the author is a co-author for their role in data collection, data analysis, figure creation, and/or writing of the manuscript. Two papers are associated with novel diagnostics or treatments for PS.[32,33] Three papers are drug studies in either purpose-bred or client-owned cats with hypertrophic cardiomyopathy.[34–36] One is a heritability and pedigree analysis in a colony of *Rhesus macaques* with hypertrophic cardiomyopathy.[37] One is generation of a golden retriever reference interval for the amino acid in taurine.[38]

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Chapter 1. Canine Model of Pulmonary Valve Stenosis with a Genetic Literature Review

This chapter is drafted with intent to submit to submit to Canine medicine and genetics.

Abstract

Pulmonary valve stenosis (PS) naturally-occurs in dogs at a high frequency and is an untapped resource for genetic studies. It is characterized by right ventricular outflow tract obstruction due to an abnormal pulmonary valve. In this review we focus on the pathologic and histologic similarities of PS in humans and dogs. Specifically, how there is a spectrum of valve abnormalities that consist of thickened valve leaflets and possible fusion of valve leaflets. We briefly touch on diagnostics and treatments with specific regard to similarities of the diagnostic work up and standard of care. Then we delve into what is currently known genetically about the disease in both species with highlights of how dogs may be useful research models despite limited published genetic literature.

Introduction

The most common birth defects are those involving the heart with 2.4 million people affected.[1–3] Isolated PS occurs in 7 of every 10,000 live births, which is about 7-9.7% of all congenital heart disease cases.[2,4] However, this disease may be underreported due to mild cases or its combination with a larger defect such as Tetralogy of Fallot.[2] In dogs, PS is one of the most common congenital heart diseases (CHDs), with a prevalence of 20-32% of all CHDs identified depending on the study population.[5,6] Importantly, certain breeds have 4% of their

population affected by naturally-occurring PS.[7] This higher prevalence may allow quicker advancements in research investigating pathogenesis, genetics, and treatments.

Historically and to present day dogs have been used extensively in cardiovascular research.[8,9] Previous reviews have compared the utility of naturally-occurring CHD in canines as a biomedical model for their human counterpart.[10] Many cardiovascular diseases share the same pathophysiology, lesions, and clinical findings between dogs and humans.[8] The first largescale investigation into naturally-occurring cardiovascular diseases in dogs took place from 1948-1964.[11] At that time, PS was one of the most common canine CHDs observed,[11] a trend that continues to present day.[5,12] PS is caused by a narrowing of the right ventricular outflow tract, that may ultimately lead to right-sided congestive heart failure if severe.[13] The current therapy for PS in both species is a percutaneous balloon valvuloplasty; this therapy was developed in dogs and tested in a Bulldog with naturally-occurring PS prior to it being attempted in humans.[14,15] Although balloon valvuloplasty is currently the standard of care, it does not return the valve to normal function, stenosis can reoccur, and this procedure may be contraindicated in some forms of the disease in both dogs and humans.[16,17]

Many human CHDs have a genetic component and the evidence for genetics of congenital cardiovascular disease in dogs is increasing.[18–21] In humans the majority of mutations in PS involve transcription factors related to heart development or cell signaling pathways such as the RAS/MAPK pathway.[22,23] To date in dogs, no genetic mutation has been identified, but the predisposition of certain breeds and pedigree studies support a genetic cause.[7,24,25] If the genetic cause can be identified, it can increase our understanding of the molecular pathogenesis due to the higher frequency and larger litter sizes, which can lead to novel prevention and therapeutic options.

The purpose of this review is to present PS pathogenesis, gross and histologic anatomy, diagnosis, treatments, and genetics in man parallel with dogs. The aim is to support the claim that dogs are a useful naturally-occurring model of valvular PS, which is the most common form of PS in the dog.[26,27] The literature review was conducted by electronically searching Google scholar and PubMed for relevant peer reviewed articles and subsequently retrieving articles contained in those references. When applicable the most recent data was utilized, or a summary of the consolidated literature was presented.

What makes a good animal model?

When research on a disease is impossible or highly unethical in man such as investigating the pathogenesis of a CHD, animal models become a good surrogate.[28] There are many considerations on what makes the ideal animal model. For example, mice are commonly used because they are low cost, easy to genetically manipulate, and easy to raise. However, mice may not accurately represent the human pathophysiology of heart disease because the disease was artificially produced, and mice cardiac anatomy and physiology is different due to their small stature. Therefore, finding an animal in which the disease occurs naturally may be a more accurate alternative to understand the genetic contributions and pathogenesis of human disease.[28] This is best illustrated by a well-developed naturally-occurring canine model of Duchenne Muscular Dystrophy, which has subsequently been used to understand genetic involvement, pathophysiology, and therapeutics for the disease.[29,30]

Dogs are an available naturally-occurring model that has not yet been utilized to its full potential to study CHDs such as PS.[31] Not only is PS highly prevalent in dogs, but dog cardiac anatomy more accurately reflects that of human cardiac anatomy.[8] Additionally, the population

structure in dogs due to their domestication from wolves and subsequent breed formation has led to a genetic makeup that makes it easier to identify disease-causing genes.[28] Furthermore, due to inbreeding certain breeds are more predisposed to diseases than others providing a subpopulation that harbors the genetic mutations, which may yield larger effects than those identified in humans. Although purebreds are a genetically homogenous group, they are not genetically-identical like laboratory animals.[32] This is important because it allows the dissection of genetic interactions. Dogs also share similar environments to humans allowing the gene-environmental interactions to be studied. Finally, the inheritance of these diseases can be elucidated with ease since purebred dogs must register their pedigree in official registries.[28]

Pathophysiology of PS

PS occurs when there is an obstruction of right ventricular outflow tract caused by anatomical defects at the valve, above the valve, or below the valve.[1,27,33–36] This disease arises due to abnormal cardiac development; specifically, the conotruncal endocardial cushions are abnormally fused or have dysgenesis.[1] The stenosis results in an increase in right ventricular systolic pressure that causes pressure overload of the right heart. The high pressures can lead to tricuspid valve regurgitation resulting in increased right atrial pressures, development of right atrial enlargement, and subsequent heart failure.[1] Pressure overload also results in compensatory concentric hypertrophy of the right ventricular wall, interventricular septum, and infundibular region.[13,35] The secondary infundibular thickening causes a dynamic stenosis that worsens the disease resulting in a vicious cycle that if severe enough may lead to right congestive heart failure (**Figure 1**).[35,37] The higher the right ventricular systolic pressure the higher five year mortality.[27] The increased velocity across the valve can lead to turbulence which leads to post-

stenotic dilation of the pulmonary artery that can be observed grossly or with radiography, or echocardiography.[35]

Gross Anatomy of PS

The pulmonary valve consists of three thin, semitransparent leaflets that are usually completely seperated.[38] Each leaflet consists of a hinge, belly, free edge and lunula. The hinge is where each valve leaflet attaches to the wall at the annulus.[39] The annulus is the region where the right ventricle transitions to the pulmonary artery.[38] The belly of a leaflet is the main portion of the leaflet and is a thin, transparent region.[38] The free edge is the coapting surface where the leaflets come together. Each leaflet has a nodule of Arantius which is a fibrous swelling of collagen fibers near the central free edge on the ventricular side.[40–42] It is palpable grossly as a firm nodular structure and is a foci of hyperechogenicity on an echocardiographic image. The lunula is the semilunar thin portion on either side of the nodule of Arantius that comes in contact with lunula of the adjacent leaflet to form a seal during diastole.[43] The commissure is the space between the valve leaflets where they run parallel to each other about 2-3mm above the sinotubular junction.[41,44] The sinus is the pocket on the arterial side formed by the leaflet attaching to the wall.[39] There is an interleaflet triangle that is the wall on the ventricular side under the sinuses. A graphic form of this detailed anatomy may be seen in **Figure 2.**

As mentioned previously, PS can be due to lesions to the valve itself, above the valve, or below the valve. However, valvular PS is the most common form of stenosis in dogs and humans and will be the focus of this review.[34,36,45] Grossly PS appears as an indentation at the level of the valve when looking at it externally.[37] With valvular stenosis, the valve leaflets can have varying degrees of thickening and fusion which may be accompanied by annular hypoplasia.[13] In

humans, the typical or “classical” PS is when the valve commissures are fused, thickened, but still mobile.[38] This causes a windsock appearance in which there is a dome with a small central orifice.[1,34,38,46,47] This is known as Type A or Type 1 PS in dogs.[13,36] This form accounts for 42% of PS cases in humans,[38] and 60-71% of cases in dogs.[5,26,48]

Another type of valve abnormality is atypical or dysplastic PS. This abnormality is when the three valve leaflets are thickened, but not fused.[34,38] This excessive tissue results in mishappen, asymmetric, shortened or hypoplastic rigid leaflets that have limited mobility.[1,37,47] This may occur with annular hypoplasia or pulmonary artery dilation resulting in an hourglass appearance to the right ventricular outflow tract.[34,37,38,46] This is known as Type B or Type 2 PS in dogs.[13,36] However, some veterinarians use dysplasia to include the full spectrum of pulmonic valve changes including variable fusion.[27] Historically, canine pulmonary valve dysplasia was further divided into grade 1 or grade 2 depending on the severity of thickening, presence of concurrent valvular hypoplasia or fusion, and impact on right ventricular outflow tract.[24] Type B accounts for 25% of cases in humans[38] and 21-40% of cases in dogs.[5,26,48] There are several other valve abnormalities such as unicommisural and bicuspid valves in humans,[38] but these are rare in dogs.[36,49]

Another cardiac abnormality worth mentioning is coronary anomalies due to its ability to occur concurrently with PS, contribute to the stenosis, and complicate surgical treatment. Normally, in dogs and humans there are two coronary ostia in the right and left sinus of Valsalva of the aortic valve. These ostia then branch the right coronary artery and left coronary artery respectively.[50] There are several types of abnormal coronary artery arrangements, but the overall incidence varies from 0.3%-1.6% in humans.[4,50] A common type of abnormality in dogs, especially brachycephalic breeds, is R2A in which there is a single right ostia that branches both

the left coronary artery and right coronary artery.[5,51–54] The left coronary artery then encircles the right ventricular outlet. This abnormality may contribute to the stenosis by applying external compression to the abnormal valve.[51] Importantly, if undiagnosed it may lead to deaths during surgical repairs of valvular PS due to rupture of the anomalous coronary artery unless a more conservative surgical approach is utilized.[9,51–53,55] The significant breed predisposition of R2A in brachycephalic dogs supports a genetic cause. If a causative variant can be identified, a genetic test could be developed that predicts R2A presence before a patient undergoes surgery. This would allow better therapeutic planning for PS patients.

Histologic Features

The histology of the pulmonic valve in humans and dogs is similar.[24] The pulmonary valve normally consists of three main layers: the ventricularis, spongiosa, and fibrosa.[24,40] The ventricularis, as the name implies, faces the ventricular side of the valve. It a dense fibrous layer consisting of a tight network of reticular fibers, has a smooth edge and has scant fibroblasts that decrease with age.[40,56] It arises from the right ventricular infundibular endocardium.[38] The spongiosa is the middle portion and consists of loosely arranged reticular fibers with intermittent collagen bundles which function as a shock absorber.[40,56] The fibrosa is on the arterial side, has ridges, and consists of circularly arranged collagen.[47,57] Endothelial cells line the entire surface. Normally, the valve is avascular and gets its nutrition by passive diffusion.[43]

Thickening is a characteristic of both Type A and Type B valves. The valve thickening involves the entire length of the leaflet and is due to an increase in the spongiosa with loose myxomatous connective tissue and sparse elastic fibers.[24,38,47] In both humans and dogs, the spongiosa becomes filled with bands of fusiform cells, stellate cells, basophilic cells and blood

vessels.[24,47] Interestingly this appearance more closely resembles the embryonic tissue indicating that the valve never reorganized during development or that there was an overproduction of normal valve components.[24,47] In dogs, dysplastic stenotic pulmonary valves have increased pale staining intracellular material, hemosiderin-containing macrophages, and cartilaginous metaplasia.[37]

The doming phenotype had thickened valves due to spongiosa and collagen deposition, but the layering was preserved.[38] There was also a decrease in elastin.[38] There may be slight or near complete fusion of the valve margins.[38] The dysplastic phenotype was more variable. In some cases the layering is preserved, while in others it is not.[38] The ventricularis normal anatomy is disrupted and the fibroelastic band may be severely reduced or absent.[24,47] There may also be a variation from a slightly hypoplastic leaflet to a completely absent leaflet[38] If the leaflet is small enough then there will be no sinus of Valsalva.[38] When there is a hypoplastic valve, the layers of the valve are preserved just reduced in size.[38] In Type A the annulus may be absent, while in type B it may be absent, partially present, or the fibrous component is replaced by myxomatous tissue.[38] When absent the valve leaflets arise from the pulmonary trunk instead of the annulus.[38]

Diagnosis

At this point, this review focused on the gross pathology and histological features of PS in human and dogs to illustrate the disease similarities between the two species. However, most PS cases are not diagnosed post-mortem. Instead, it is identified based on auscultation of a left basilar, high frequency, crescendo-decrescendo systolic ejection murmur that prompts a cardiac echocardiographic exam in young children and dogs.[27,37,47] The murmur is due to the blood turbulence caused by high velocity flow across the stenosis.[35] The majority of canines are

diagnosed around one year of age.[48,58,59] If diagnosed later in life it is usually due to clinical signs of right congestive heart failure, screening for breeding purposes, or screening due to diagnosis of a family member.[27,37] Most humans are diagnosed as neonates due to clinical signs or will be diagnosed during a pediatric routine exam.[60]

Clinical signs are dependent upon the severity of the stenosis.[27] The severity of PS is determined by measuring the maximal velocity across pulmonic valve using Doppler and then using the Bernoulli equation to determine the pressure gradient.[4,36] Mild disease is a peak gradient less than 36mmHg (peak velocity <3m/s) in humans and 20-49mmHg (peak velocity 2.25-3.5m/sec) in dogs.[4,36] Mild disease is usually subclinical, both humans and dogs have a normal lifespan, and thus treatment is not warranted.[4,13,17,48,61] Moderate stenosis is a peak gradient from 36-64mmHg (peak velocity 3-4m/s) in humans and from 50-80mmHg (peak velocity 3.5-4.5m/sec)in dogs.[4,36] Moderately effected humans and animals may remain subclinical or may develop clinical signs such as exercise intolerance, arrhythmias, syncope, right sided congestive heart failure, and sudden death.[1,48] If clinical signs develop, dogs may require treatment.[48,62,63] Severe disease is a peak gradient over 64mmHg (peak velocity >4m/s) in humans or over 80mmHg (peak velocity >4.5m/s) in dogs.[4,36] Severely affected dogs are at risk of sudden death or heart failure and therefore intervention with a balloon valvuloplasty is warranted if no other contraindications are identified.[64] In humans, if clinical signs are present or the individual has moderate or severe disease surgical intervention is warranted.[4,17,65]

Extensive reviews into the details of diagnosing PS in humans and dogs are previously published, constantly being updated, and beyond the scope of this review.[13,36,49,66] Briefly, the current standard in humans is a transthoracic echocardiography and a cardiac MRI/cardiac CT to evaluate the lesion.[4] If the lesion is moderate or severe, referral to a congenital heart disease

specialist is warranted.[1] In dogs, a transthoracic echocardiography is performed when a murmur is detected; if balloon valvuloplasty is indicated then an angiogram or contrast CT is recommended to rule out coronary anomalies and for procedural planning.[5,62]

Treatments

Balloon valvuloplasty (BVP) is the treatment of choice in humans and dogs when severe enough disease or clinical signs are present.[4,13,61,66] A BVP is a minimally invasive technique where an inflatable interventional balloon is introduced via a catheter through a vessel into the heart. It is aligned across the stenosis and then inflated to stretch or tear open the stenosis.[62] The goal of this treatment is to reduce the systolic right ventricular pressure overload to reduce clinical signs.[13] However, this treatment is palliative because it relieves the clinical signs without addressing the underlying genetic cause or returning the valve to entirely normal function.[67] This procedure can cause or worsen preexisting pulmonary valve insufficiency. This is generally well tolerated in dogs, yet can cause heart failure in humans who are longer-lived.[4,17,65] Restenosis after balloon valvuloplasty is a common concern in both species.[33]

Type A PS cases have a better success rate post balloon valvuloplasty compared to Type B PS cases.[16,26,48] Balloon valvuloplasty is contraindicated when coronary artery anomalies such as the R2A anomaly are present because when the balloon is inflated there is risk of damaging or avulsing the aberrant coronary artery causing death.[36,53,67,68] CT angiography performed prior to intervention is highly recommended to confirm if an anomalous coronary artery is present, but this technique increases anesthesia times and elevates procedural cost which is problematic in veterinary medicine due to limited use of pet insurance.[64,67,69] In humans an alternative treatment for PS is surgical valve replacement. This is preferred when severe pulmonic regurgitation post

BVP is present, the dysplastic phenotype is present, or the annulus is hypoplastic.[4,33] Unfortunately this is not yet an option in dogs. Instead balloon valvuloplasty may be performed with high pressure balloons, possibly proceeded by a cutting balloon, a repeat BVP, or the dog is medically managed.[63,70,71] In dogs with severe dysplastic PS, non-valved pulmonary stents have been used and may provide durable palliation (unpublished), though long-term follow up is needed to evaluate the tolerance of wide-open pulmonary insufficiency.

Dogs may be used to refine the current surgical treatment options for PS or help lead to the development of novel techniques. They could also be used to test novel medical therapeutics to complement balloon valvuloplasty or provide palliation when interventional or surgical correction is not an option. Since interventional and medical options do not currently return the valve to normal function an alternate approach would be to use the higher prevalence in dogs to understand disease pathogenesis and genetics.

Genetics

PS is defined as an anatomical defect that is present at birth due to alterations in embryonic development that is inherited, de novo, or secondary to teratogen or environmental influences although the last two are uncommon.[13] In humans, the development of novel genetic techniques have allowed about 45% of CHD cases to be explained.[18] A database that curates literature on CHDs known as CHDBase revealed numerous genetic associations with PS.[72] There are currently 137 publications encompassing 57 syndromic-PS genes, 90 non-syndromic-PS genes, and 40 genes associated with both syndromic and non-syndromic cases summarized in **Figure 3**.[72] Some of these are well studied genes while others may represent the first case report of a novel player. Many of these mutations do not cause PS in isolation and instead cause it as

part of a complex heart malformation such as Tetralogy of Fallot, double outlet right ventricle, or transposition of great arteries.[1] One of the challenges of identifying PS genes is the spectrum of phenotypes that a single mutation can present as. Unfortunately to date, no gene for PS has been identified in dogs despite evidence that it is an inherited trait.

The most commonly reference genes associated with PS according to CHDBase as of 2022 are: *PTPN11, GATA4*, and *NKX2-5*. Most genes associated with PS are part of the Ras/MAPK pathway such as *PTPN11*. This is a critical pathway to heart development that controls cell proliferation, differentiation and survival.[73] Another large category of genes associated with PS are transcription factors such as *GATA4* and *NKX2.5*. Cardiac transcription factors are critical for regulating the precise orchestration timing and dosages of proteins during development.[74] Additional mutations are associated with syndromes due to genes that have widespread effects in embryologic development. For example, *JAG1* is part of the NOTCH signaling pathway that plays a role in cell differentiation. There are numerous genes associated with PS and with the exponential advancement of genetic techniques more are being quickly identified.

As mentioned previously, many of the genes associated with PS involve a group of disorders known as RASopathies.[75] RASopathies are caused by defects in the RAS-MAPK pathway that lead to a disease syndrome which may or may not include PS.[76] The RASopathy best associated with PS is Noonan syndrome.[77] PS occurs in 50-70% of Noonan Syndrome cases[76] with 6% of all PS cases in one study being affiliated with a Noonan spectrum disorder.[78] Some of the genes involved with Noonan syndrome and PS include *PTPN11, SHOC2, RIT1,RAF1, BRAF, KRAS,* and *SOS1*.[73,75,76] The *PTPN11* gene tends to cause severe PS, while *SOS1* mutations typically cause milder forms of PS.[75] Additional RASopathies include: Costello

syndrome, cardiofaciocutaneous syndrome, neurofibromatosis type 1 syndrome, LEOPARD syndrome and several “Noonan-like” syndromes.[18,22,73,76] Abnormalities that occur with these syndromes include but are not limited to short stature, abnormal faces, cardiovascular abnormalities, cryptorchidism, and pectus abnormalites.[73,76] The genes involved in RASopathies and Noonan syndrome have been extensively reviewed previously.[73,79] It has been previously hypothesized that brachycephalic breeds such as the Bulldog may have a Noonan-like syndrome.[48] This is also the breed that is highly predisposed to getting PS.[7]

There are additional syndromes where PS may be present. Alagille syndrome is caused by mutations in either the ligand (*JAG1*) or the receptor (*NOTCH2*) associated with the NOTCH pathway.[22,80,81] The NOTCH signaling pathway is used to determine cell fates.[82] Weill-Marchesani Syndrome characterized by short stature, short fingers and toes, and eye abnormalities are caused by several mutations in *ADAMTS10*.[83] Robinow syndrome is caused by mutations in the WNT developmental pathway.[84,85] It is characterized by slow body growth, hypertelorism, low set ears, and ptosis of the eyelids.[84,85] Bulldogs are fixed for a mutation in a gene associated with Robinow syndrome, although its connection to PS in the breed has not been investigated.[85] Although not as thoroughly investigated as some of the other syndromes, Simpson-Golabi-Behmel syndrome contains mutations in *GPC3* which is a regulator of embryonic growth via the hedgehog signaling pathway.[86] Other syndromes that require additional research are *PIGL* responsible for CHIME syndrome[87] and Ellis-van Creveld Syndrome caused by *EVC2* mutations.[88] Although PS in dogs is not associated with a defined syndrome, it is possible that the phenotype selected for in certain breeds can be interpreted as a syndrome.

Transcription factors play a critical role in pathogenesis of PS as well as other complex CHDs.[74] Some of the first mutations identified in familial CHD were transcription factors.[74] This is to be expected since transcription factors help facilitate the precise timing and coordination of developmental events. The transcription factors that have been identified in humans with PS are: *GATA4,*[23,89] *HEY2,*[90] *TBX5,*[91] *NKX2.5,*[92] *HAND2,*[93,94] and *LZTR1*.[73,75] A fascinating paper that investigated the protein-transcription factor interactomes of *GATA4* and *TBX5* identified many genes (*SMARCA4,*[95] *PBX1,*[96] *SALL4,*[97] and *ZFPM2*[98]) that have been associated PS in individual cases reports.[74] This suggests that additional research is necessary in these genes. There is strong evidence that a transcription factor mutation is responsible for PS in Bulldogs (unpublished, Chapter 3).

There are additional genes that hold promise but need to be more intensely studied to understand their contribution to PS especially when there are limited case reports. There are mounting case reports for the *ELN* gene whose loci was associated with the William-Beuren Syndrome's microdeletion at 7q11.23.[99–102] Several papers have identified an association with *NPHS2* and *NPHS1*.[103,104] Whole exome sequencing identified mutations in *CNOT1, PBX1, TMED8,* and *USP34* in patients with PS.[105] As always, investigating genes contained in the same family as previously associated genes may be important such as *GATA6, TBX* genes, *ADAMTS19.*[106] If any mutations in these genes are identified in dogs with PS it could provide stronger evidence of a causative effect since it crosses species.

Microdeletions and microduplications are commonly associated with PS with several eventually having a causative gene identified. For example, *GATA4* was identified due to its localization in a Chr8p23.1 deletion syndrome.[89] However, not all larger structural variants associated with PS have a gene identified yet. Interestingly, the 1q21.1 deletion/duplication had

variable phenotypic presentation including syndromic PS, non-syndromic PS, and clinical variability among patients suggesting environmental, epigenetic, and genetic modifiers influence disease presentation.[107] Smith-Magenis syndrome is associated with a deletion at 17p-, however a gene has yet to be identified.[108] The DiGeorge or 22q11 deletion syndrome is commonly associated with complex heart defects although the transcription factor *LZTR1* mentioned previously is contained in this deletion.[109] These regions serve as avenues for future research to identify the gene(s) responsible. These microdeletions and duplications are not limited to humans. In Bulldogs, a large 22k base pair deletion has been found associated with PS (unpublished, chapter 3).

Despite a gene not yet being identified, PS is suspected to have a genetic predisposition in dogs. This is because certain breeds are more predisposed to this abnormality compared to others and purebreds are more susceptible than mixed-breeds.[5,11,32,37,58,110] Although any dog can get PS, brachycephalic breeds are overrepresented.[7] In Italy in a population of Boxers, the prevalence of PS increased from 2001-2009 indicating a need to identify the genetic basis of the disease to efficiently eliminate it from the breeding program.[111] Interestingly, breed screenings caused the prevalence for PS to increase, while the frequency of severe cases went down suggesting genetic modifiers are involved. Further support of a genetic cause is that PS can arise in dog families where consanguineous mating is present despite the breed having a low incidence overall.[112] The prevalence of PS in dogs is likely higher than that of humans due to the effects of inbreeding to create breeds that inadvertently concentrated mutations that alter cardiac development.[58]

The mode of inheritance of PS in humans is variable depending on the gene involved. However autosomal dominant, autosomal recessive, and haploinsufficiency are common. One of

the only X-linked ones identified was *GCP3* which is associated with Simpson-Golbi-Behmel Syndrome.[86] The most complete inheritance study of PS in dogs occurred in beagles despite this not being a breed commonly affected.[24] In Beagles, recessive inheritance was suspected since *affected X normal* failed to produce affected offspring and litters with the highest percentage of affected puppies had affected and related parents. However, incomplete penetrance was suspected regardless of mode of inheritance.[24] A recessive form of inheritance has been described in other breeds.[7,46,58] Other informative pedigrees include Keeshonds, boxers and bulldogs, where there were several instances of clinically normal dogs producing PS affected offspring.[7,11,111] Although a recessive mode of inheritance is most likely, incomplete penetrance or very mild forms of the disease going undetected in the parents remains a possibility until a definitive mutation is identified. X-linked inheritance is unlikely since in several canine studies, no male or female predisposition was found for PS[12,24,27,67] Some studies have reported a male predisposition reported in certain breeds such as Bulldogs[113,114], Boxers [5,111], and Bullmastiffs[37] and in the species overall. [5,6,26,27,66] However, it is unclear whether the variability in sex predisposition likely has to do with differences in the mutations contained within the population studied or an artifact of sample size.

As mentioned previously there is clinical variability among human patients with the same mutation suggesting environmental, epigenetic, and genetic modifiers influence disease presentation.[107,115] This is likely to be the same case in dogs. Since heart development is a complex process involving many genes, alterations in the dosages at any step may result in abnormal anatomy.[18] Patients with pulmonary valve dysplasia had parents and/or siblings that had the typical form of PS.[47] Similarly, in canines full siblings can have the typical dome-shaped abnormality or the dysplastic thickened valve abnormality.[7,24,46] Not only does the morphology

vary within the same litter, but severity does as well.[7,24,58] Out of the 10 matings between beagles afflicted with PS, 25.7% of the offspring had PS and all had same defect as the parents.[28] Since both types of PS are found in the same family, there may be common genetic or developmental mechanisms underlying the formation of both types of the disease. Future investigations into the possibility of a major effect gene with associated modifier genes, dosage alterations, or environmental influences is warranted. Other genetic investigations into CHDs in dogs have suggested a complex inheritance influenced by multiple genes, incomplete penetrance, and environmental factors.[20] It is likely that PS will be similarly inherited in dogs as it is in humans.

Conclusions

The genetics of PS is expanding rapidly with genes in the RAS/MAPK pathway and transcription factors most associated with PS in humans. Despite these current advancements, there are still large indels and GWAS regions associated with PS whose causative genes still need to be identified in humans. Although dogs have been used historically to test novel treatments for PS, their utilization in understanding genetics has not been fully taken advantage of. Dogs have similar gross and histologic anatomical abnormalities compared to humans for PS with a similar spectrum of phenotypes and severities. Dogs get the disease at a higher prevalence which may be inadvertently caused by artificial selection for specific phenotypic features. These phenotypic features are reminiscent of many of the syndromes associated with human PS. If the mutations in humans are subsequently identified in dogs it may allow quicker advancement in understanding the genetics mechanisms and gene interactions.

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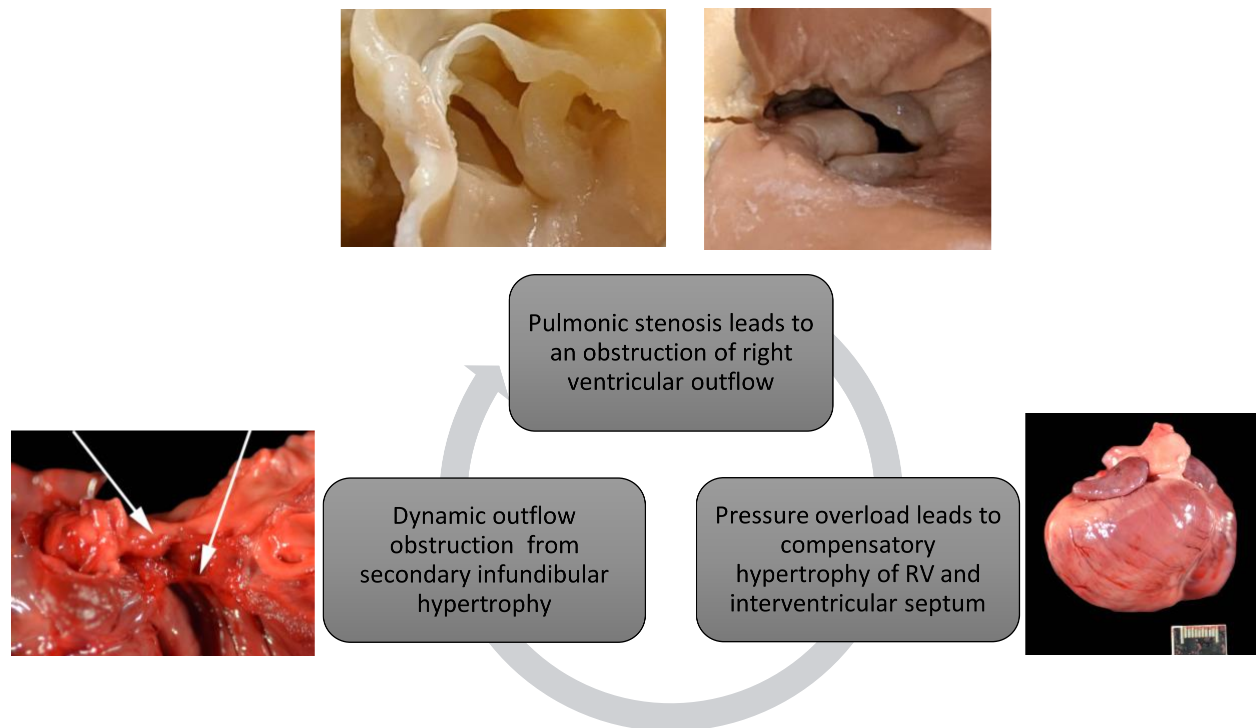

Figure 1.1: PS Pathogenesis

Vicious cycle of PS leading to worsening right ventricular outflow tract obstruction eventually resulting in right sided congestive heart failure. Top: Representative images of thickened and dysplastic canine valves. Doming valve not displayed since typically successfully treated with balloon valvuloplasty. Right image: severe right ventricular hypertrophy. Left image: Top arrow is pointing at thickened pulmonary valves while the bottom arrow is pointing at the infundibular stenosis.

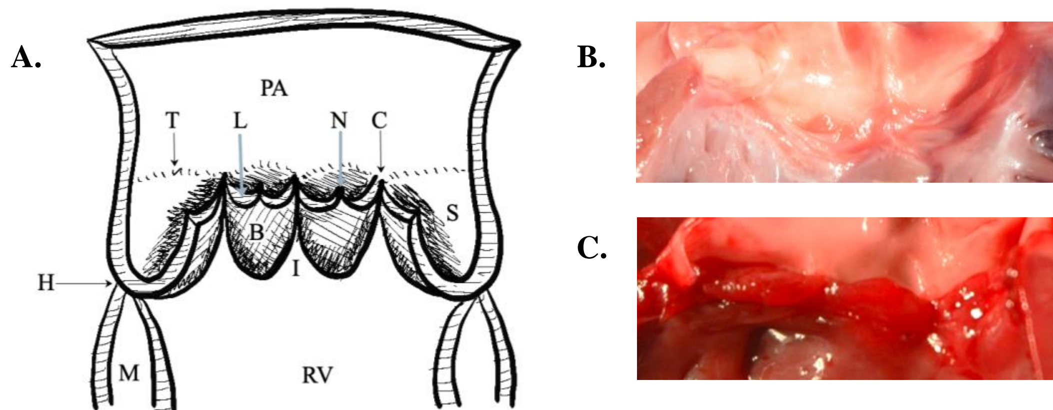

Figure 1.2: Gross anatomy of a pulmonary valve.

A. The right ventricular outflow tract opened longitudinally with one of the three pulmonary valve leaflets cut in half to illustrate internal anatomy. **B.** Normal pulmonary valve with thin, semitransparent leaflets. **C.** Thickened pulmonary valve leaflets in a dog diagnosed with pulmonary valve stenosis. A - annulus. B - belly. C - commissure. H – hinge. I - interleaflet triangle. L - Lunula. M - Myocardium N - Nodule of Arantius. PA - Pulmonary artery. RV - Right ventricle. S - sinus. T - Sinotubular junction.

RASopathies[22,73]

- ***BRAF***
  - Cardiofaciocutaneous (CFC) syndrome[116]
  - Noonan Syndrome
  - Leopard syndrome
- ***HRAS***
  - Costello Syndrome
- ***KRAS***
  - Noonan Syndrome
  - Cardiofaciocutaneous (CFC) Syndrome
- ***MAP2K1/MAP2K2***
  - Cardiofaciocutaneous (CFC) Syndrome
- ***NF1***[76]
  - Neurofibromatosis syndrome
- ***NRAS***[117]
  - Noonan syndrome
- ***PTPNII***[73,75,79,118,119]
  - Noonan syndrome
  - Leopard syndrome
  - Noonan-like multiple giant cell lesion syndrome
- ***RAF1***[4,33]
  - Leopard Syndrome
  - Noonan Syndrome
- ***RIT1***[120]
  - Noonan Syndrome
- ***RASA2***[120]
  - Noonan Syndrome
- ***SHOC2***
  - Noonan Syndrome – like disorder with loose anagen hair
- ***SOS1***[121]
  - Noonan Syndrome
- ***SPRED1***[122]
  - NF1-like syndrome
  - Legius Syndrome

Transcription Factors

- ***GATA4***[23,89,123–125]
  - Deletion of 8p23.1
- ***HAND2/dHAND***[93,94,126]
  - 4q deletion syndrome
  - Non-syndrome
- ***HEY2***[90]
- ***LZTR1***[73,75,109]
  - 22q11 deletion syndrome; DiGeorge Syndrome
- ***NKX2.5***[92,127]
- ***PITX***[128]
- ***TBX5***[91]
  - Holt-Oram syndrome
  - Non-Holt-Oram syndrome
- ***ZIC3***[129]

Syndromes

- Alagille Syndrome[80,115]
  - ***JAG1***
  - ***NOTCH2***
  - ***NOTCH1***
- Andersen Syndrome[130]
  - ***KCNJ2***
- CHIME syndrome[87]
  - ***PIGL***
- Ellis-Van Creveld Syndrome[88]
  - ***EVC***
- Nephrotic Syndrome[103,104]
  - ***NPHS2***
  - ***NPHS1***
- Robinow Syndrome[84]
  - ***DVL3***
  - ***WNT5A***
  - ***DVL1***
- Simpson-Golabi-Behmel syndrome[131]
  - ***GPC3***
- Williams-Beuren[99–102]
  - 7q11.23-q21.11; ***ELN***
- Weill-Marchesani Syndrome[83]
  - ***ADAMTS10***
- Kleefstra/9qSTD Syndrome[132,133]
  - ***EHMT1***

Potential Genes

- ***ADAMST9***[134]
  - **Chr 3p14 deletion**
- ***CNOT1***[105]
- ***GATA6***[135]
- ***PBX1***[96,105]
- ***SALL4***[97]
- ***SMARCA4***[95]
- ***TMED8***[105]
- ***USP34***[105]
- ***ZFPM2***[98]

Potential Regions

- Chr 1q21 deletion/duplication[107]
- Chr 6pter-p24deletion[128]
  - CHARGE syndrome
- Trisomy9p[128]
- Chr 10q26.3 deletion[128]
- Terminal 18q deletion[128]
- Chr 14q23.3 deletion[136]
  - Heart hand Syndrome
- Chr 17p deletion[108]
  - Smith Magenis Syndrome
- Chr 19p13.3-pter deletion[137–139]
  - Mosaicism for ring 19

Figure 1.3 Genes, syndromes, and regions associated with PS.

They are sorted by genes that cause RASopathies, transcription factors, and non-RASopathy syndromes. Also included are lists of additional genes and regions that should be investigated further.

Chapter 2. Congenital Cardiac Outflow Tract Abnormalities in Dogs: Prevalence and Pattern of Inheritance from 2008 to 2017

This chapter was published in 2019 in Frontiers Veterinary Science journal:

Ontiveros ES, Fousse SL, Crofton AE, Hodge TE, Gunther-Harrington CT, Visser LC, et al. Congenital cardiac outflow tract abnormalities in dogs: prevalence and pattern of inheritance from 2008 to 2017. Front Vet Sci. Frontiers; 2019;6: 52.

Ontiveros and I shared first authorship on this paper.

Abstract

Subvalvular aortic stenosis (SAS) and valvular pulmonic stenosis (PS) are two of the most common congenital heart diseases of dogs. The aim of this study was to determine the prevalence and mode of inheritance of these congenital heart diseases in a large veterinary teaching hospital population. Case records of dogs presented to the University of California Davis, Veterinary Medical Teaching Hospital (UCD VMTH) between January 2008 to December 2017 were reviewed retrospectively and pedigree information was obtained when available. There were 259 unique SAS and 336 unique PS cases diagnosed during the study period. The prevalence of SAS was 0.3% of overall hospital admissions and 4.7% for all dogs seen by the cardiology service. The prevalence for PS was 0.41% of overall hospital admissions and 6.1% of dogs seen by the cardiology service. Bullmastiffs and Newfoundlands had the greatest prevalence (6.59% and 4.46% respectively) and odds ratio (52.43 and 34.73 respectively) for SAS. Bulldogs and French Bulldogs had the greatest prevalence (4.8% and 2.7% respectively) and odds ratio (13.32 and 7.52 respectively) for PS. The identified prevalence

of SAS and PS is higher than previously reported. Pedigree analysis in SAS affected Bullmastiffs, Golden Retrievers, and Rottweilers suggested an autosomal recessive pattern of inheritance. The mode of inheritance for PS in the most affected breed, Bulldogs, also appears to be autosomal recessive. The results of this study can be used to inform future selection of breeding pairs and genetic studies aimed at reducing the prevalence of these common congenital heart diseases.

Introduction

Subvalvular aortic stenosis (SAS) and valvular pulmonic stenosis (PS) are two of the most common congenital heart diseases diagnosed in dogs.[1,2] Both diseases involve stenosis of a cardiac outflow tract that results in pressure overload on the respective ventricle and may lead to severe clinical outcomes such as congestive heart failure or sudden cardiac death (Figure 2.1). It was previously reported that certain dog breeds have a higher relative risk for developing congenital heart disease. The breeds with a reported predisposition to SAS include Boxers, German Shepherds, Dogue de Bordeaux, Newfoundlands, Rottweilers, and Golden Retrievers.[2] The breeds with a reported predisposition to PS include Boxers, Bulldogs, and French Bulldogs.[2] Breed specific disease predisposition suggests and underlying genetic etiology for these conditions.[3]

The gold standard for diagnosing SAS is via cardiac necropsy, in which the presence of fibromuscular nodules, a ridge, or ring is observed below the aortic valve.[4] For valvular PS cases, commissural fusion of the valve leaflets, severe thickening of the valve leaflets, and/or annular hypoplasia may be seen at necropsy.[5] For ante-mortem diagnostics, a veterinary cardiologist can perform an echocardiogram exam to access whether a dog is affected with SAS or PS. A standard echocardiogram assessment used to determine SAS or PS disease status

includes 2D, M-mode, color-flow doppler, and continuous-wave doppler modalities which allow determination of severity and morphology of the stenotic lesion.[6]

Prognosis for SAS and PS varies depending on severity and treatment availability. Dogs diagnosed with moderate or severe SAS have an average lifespan of 19 months without medication or intervention.[7] Moderately and severely affected SAS cases can develop cardiac complications that result in sudden death, infective endocarditis, and/or congestive heart failure.[7] Currently, the only medical treatment available for dogs affected with SAS is administration of beta-adrenoceptor blocking drugs that were shown to increase the life expectancy of severely affected cases to 56 months.[8] Although balloon valvuloplasty is an option for SAS affected cases, it was demonstrated that it does not significantly increase their life expectancy any longer when compared to beta-adrenoceptor blocking drug administration.[8]

Of dogs diagnosed with severe PS, 53% die before one year of age without intervention.[9] Available treatments for PS also include administration of beta-blockers but is frequently palliated by the use of percutaneous balloon valvuloplasty.[9] When treated with percutaneous balloon valvuloplasty, only 4% of severe cases die before one year of age.[9] However, not all PS cases can benefit from a percutaneous balloon valvuloplasty. For example, dogs with dysplastic valves and pulmonic annulus hypoplasia have a worse post-valvuloplasty outcome compared to those with fused valves and abnormal pulmonary annulus.[10] Additionally, if an aberrant coronary artery is identified, a balloon valvuloplasty is contraindicated as treatment for PS.[11] The breeds with a reported predisposition to coronary artery anomalies include Bulldogs and Boxers.[12]

Both SAS and PS are heritable congenital heart diseases affecting the cardiac outflow tracts of humans and dogs.[3,13,14] Previous studies have suggested that SAS can have an autosomal dominant with incomplete penetrance or polygenic mode of inheritance in humans and the

Newfoundland breed.[4,14,15] In Golden Retrievers, an inconclusive pedigree analysis suggested either a recessive or polygenic mode of inheritance.[16] To date, the only identified variant associated with canine SAS is an insertion in the *PICALM* gene of Newfoundlands.[14] Although the variant identified is highly penetrant at 80.6% in the Newfoundland breed, the manuscript illustrates that it does not fulfill the criteria of a sole causal variant. Thus, additional genetic studies to identify additional variants are warranted.

In humans, congenital valvular PS presents in syndromic and non-syndromic forms with varying modes of inheritance.[17] Mutations involving the Ras signaling pathway are associated with many of the syndromic forms of PS, while only *GATA4* has been associated with the nonsyndromic form.[17] Although, some genetic risk variants have been associated with human PS, for many children the causative variant remains unknown.[17,18] An extensive genetic study in Beagles afflicted with PS suggested that the disease in that breed is polygenic and inherited in a recessive manner.[5] However, there is no conclusive evidence regarding the inheritance of PS in commonly affected breeds, which hinders breeding efforts to reduce disease prevalence in affected breeds. To date there are no reported variants associated with the development of canine PS.

It has been long understood that specific breeds have increased risk for developing SAS and/or PS, however information on the mode of inheritance in these breeds is limited.[7] The aim of our study is to determine the prevalence of these congenital heart diseases in a large veterinary teaching hospital. Furthermore, we aim to elucidate the mode of inheritance for PS/SAS in breeds when pedigree information on a large number of cases is available. It is essential to assess prevalence and inheritance to aid researchers in developing novel therapeutic approaches or genetic tests that can be used to decrease the prevalence of these conditions.

Materials and methods

Sample population and phenotyping

Case records of dogs presented to the University of California Davis, Veterinary Medical Teaching Hospital (UCD VMTH) between January 2008 to December 2017 were reviewed retrospectively. Only cases diagnosed echocardiographically with SAS or PS by a board-certified cardiologist or cardiology resident under the direct supervision of a cardiologist at the UCD VMTH were included. Hospital records were reviewed to obtain information on the breed, sex, and selected echocardiographic findings used for diagnosis. Additionally, the presence and absence of coronary anomalies diagnosed by coronary angiography in PS dogs was recorded if available. Exclusion criteria included dogs with acquired stenosis or other structural congenital heart defects that could influence accuracy of obtaining outflow tract velocities.

Evaluation of the stenotic lesion with color and continuous wave doppler along with standard 2D and M-mode echocardiographic measurements were completed according to previously published studies.[6,19] Dogs were classified into severity categories (equivocal, mild, moderate, and severe) based on their peak modal recorded aortic outflow velocity (AoV) or peak modal recorded pulmonic outflow velocity (PoV). Dogs affected with SAS were categorized as equivocal (AoV: 2-2.5 m/s), mild (AoV: 2.5-3.5 m/s), moderate (AoV: 3.5-4.5 m/s), or severe (AoV: >4.5 m/s). Similarly, severity of PS was based on PoV and categorized as equivocal (PoV: 1.5-2.5 m/s), mild (PoV: 2.5-3.5 m/s), moderate (PoV: 3.5-4.5 m/s), and severe (PoV: >4.5 m/s). The mild, moderate, and severe categories for both SAS and PS were based off previous literature,[1,6] however a conservative equivocal range was used to reduce misclassification of cases and controls.

Prevalence Calculation

The prevalence of SAS and PS was calculated overall for the hospital's canine population and by breed. Dogs reported to be equivocal for SAS or PS were excluded from this analysis since their phenotype cannot be definitively described. Inclusion of equivocal dogs can result in an overestimation of the prevalence if combined with the affected dogs or an underestimate prevalence if combined with the control dogs.

All statistics were completed using GraphPad Prism Software v7 (La Jolla, CA). Odds ratios, 95% confidence intervals, and chi-square p-values were calculated to determine if a breed or sex predisposition was present for SAS or PS breeds with at least ten reported cases during the study period. If there was not a large enough sample size to perform a chi-square analysis a Fisher's Exact test was used. The following formula was used to calculate the odds ratio:

$$Odds\ Ratio = \frac{(\#\ affected\ for\ breed)(\#\ normal\ mixed\ breed)}{(\#\ normal\ breed)(\#\ affected\ mixed\ breed)}.$$

Pedigree Evaluation

A 3-5 generation pedigree was obtained from owners and breeders for affected and unaffected dogs when possible. This is a routine request for patients diagnosed with congenital heart disease by the UCD VMTH Cardiology service due to the ongoing genetic research initiatives of the unit (IACUC #18106 and 20047 developing genetic library). Disease status was determined based on echocardiogram by a board-certified cardiologist or cardiology resident under the supervision of a cardiologist at UCD VMTH or based on Orthopedic Foundation for Animals reported cardiac screening status. Pedigrees were generated manually using Adobe Illustrator for SAS-affected breeds including the Bullmastiff, Golden Retriever, and Rottweiler as well as PS-affected Bulldogs.

The following defining features were utilized to support proposal of a possible pattern of inheritance:[20]

- *X-linked recessive*: There is a sex predisposition with males being affected more frequently than females. Pedigree analysis highlights females passing down the condition to almost exclusively male offspring. Affected males are unable to pass down the condition to male offspring.
- *X-linked Dominant:* Pedigree analysis reveals no sex predisposition for acquiring the disease. An affected male transmits the disease to all his daughters, but no sons. Every affected individual must have an affected parent resulting in no skipping of generations.
- *Autosomal Dominant*: Pedigree analysis reveals no sex predisposition for acquiring or transmitting the disease. Every affected individual must have an affected parent resulting in no skipping of generations.
- *Autosomal Recessive*: Pedigree analysis reveals no sex predisposition for acquiring or transmitting the disease. An affected individual can have affected or unaffected parents. It may skip generations before showing up in offspring. It is likely to be seen with consanguineous mating.

Results

Prevalence

During the period of January 2008 to December 2017, the UCD VMTH saw a total of 80,943 unique dogs between all services, and the cardiology service saw a total of 5,548 unique dogs. There was a total of 259 unique SAS and 336 unique PS cases diagnosed during this ten-year period (Table 2.1). Furthermore, 16 dogs with PS were concurrently diagnosed with a coronary artery anomaly: 1 mixed breed, 1 Boston Terrier, 1 Pitbull, and 13 Bulldogs. There were 80 reported SAS-affected dogs and 18 PS-affected dogs excluded based on equivocal outflow tract velocities (Supplemental Table 2.1 and 2.2). The breeds with the highest number of

dogs with equivocal AoV were the Boxer (n=15), Golden Retriever (n=14), and mix-breed (n=19) dogs. The breed with the highest number of dogs with equivocal PoV was Bulldogs (n=10). All other PS-affected breeds had either one or no dogs with an equivocal PoV. The prevalence of SAS was 0.3% overall in the population and 4.7% of dogs seen by the cardiology service. The prevalence for PS was 0.41% overall in the population and 6.1% of dogs seen by the cardiology service.

SAS was observed in 47 different breeds and PS was observed in 65 different breeds excluding mix-breed dogs. Overall, we observed an increase in the number of cases diagnosed with SAS at the UCD VMTH during this ten-year period (Figure 2.2A). The number of PS cases decreased steadily until 2014, then began increasing. Breed specific prevalence and odds ratios were calculated for breeds with ≥10 cases (Table 2.2 & 2.3). Bullmastiffs and Newfoundlands had the greatest prevalence (6.59% and 4.46% respectively) and odds ratio (52.43 and 34.73 respectively) for SAS. We observed an increased number of Bullmastiffs, Golden Retrievers, and Pitbull Terriers diagnosed with SAS during this time frame (Figure 2.2B). Bulldogs and French Bulldogs were the breeds with the greatest prevalence (4.8% and 2.7% respectively) and odds ratio (13.32 and 7.52 respectively) for PS. We observed an increased number of Bulldogs, French Bulldogs, and Pit Bull Terriers diagnosed with PS during this time frame (Figure 2.2B).

There was 125 male and 134 female SAS cases overall. When broken down by breed, male Rottweilers had an odds ratio of 5.515 of developing SAS (95% confidence interval: 1.292 to 25.270, p-value=0.018). No other breed that contain ≥10 cases had a male or female predisposition ($p<0.05$) for SAS (Supplemental Table 2.3). There was 192 males and 144 female PS cases overall. Male mixed-breed dogs had an odds ratio of 1.789 of developing PS when compared to females (95% confidence interval: 1.147 to 2.819, p-value=0.013). No other breed

that contain ≥10 cases had a male or female predisposition ($p<0.05$) for PS (Supplemental Table 2.4).

Mode of Inheritance for SAS and PS

Pedigree evaluation was performed for a family of 29 Bullmastiffs that consisted of 15 males and 14 females (Figure 2.3). Two of the males (AoV=2.75 and 2.55 m/s) and one female (AoV=4.39 m/s) were diagnosed as affected with SAS. Additionally, both of the affected males had a direct littermate diagnosed as equivocal for SAS. The male equivocal dog had an AoV=2.25 m/s and the female equivocal dog AoV=2.24 m/s. A total of 11 dogs were considered to be unaffected via echocardiogram and had an AoV ≤ 2 m/s and five dogs were cleared via auscultation by a board-certified cardiologist. We did not have sufficient clinical information for eight dogs in this pedigree. The pedigree illustrates one unaffected-to-unaffected mating producing an affected, equivocal, and unaffected offspring and another unaffected-to-unaffected mating producing one affected female. We did not have additional information for direct littermates of the affected female dog. Furthermore, there was no affected-to-unaffected, equivocal-to-unaffected, or affected-to-equivocal mating in this pedigree. Pedigree analysis results therefore support an autosomal recessive mode of inheritance for Bullmastiffs affected with SAS.

Pedigrees for two families of Golden Retrievers were evaluated (Figure 2.4A and B). The first family contained a total of 20 dogs (10 males and 10 females) and the second family contained a total of 30 dogs (14 males and 16 females). Between both families, there was a total of five Golden Retrievers diagnosed with SAS, two males and three females, with a median AoV of 4.07 m/s (range 2.6 m/s - 6.95 m/s). With regards to first family all 15 unaffected Golden Retrievers were cleared of SAS via auscultation by a board-certified cardiologist. In the second family, three Golden Retrievers were unaffected by echocardiogram (AoV ≤ 2 m/s) and the

remaining 22 dogs were cleared of SAS via auscultation by a board-certified cardiologist. We did not have sufficient cardiac health information for three dogs in family one and two dogs in the second family. Additionally, we did not have sufficient genealogical information and health status information for direct littermates of the affected dogs. Both these pedigrees highlight a total of five unaffected-to-unaffected matings that produced an affected offspring. There was no affected-to-equivocal matings or affected-to-affected matings in this pedigree. Pedigree analysis results for both of these Golden Retriever families support an autosomal recessive mode of inheritance for SAS affected dogs.

Pedigree assessment was also completed for a family of 48 RW that included 22 males and 26 females (Figure 2.5). Four of the RW were diagnosed with SAS, two males (AoV=3 and 3.35 m/s) and two females (AoV= 3 and 4.87). Additionally, one male RW was diagnosed with equivocal SAS (AoV=1.73 m/s). Although the equivocal dog had a normal AoV, 2D echocardiogram assessment indicated mild discrete narrowing of the LVOT below the aortic valve therefore was labeled as equivocally affected. Sixteen dogs were cleared of SAS via echocardiogram (AoV<2 m/s) and 16 dogs were cleared via auscultation by a board-certified veterinary cardiologist. Cardiac health information was unavailable for 11 dogs included in this analysis. This pedigree highlights two unaffected-to-unaffected matings that produced both affected and unaffected offspring. Additionally, an unaffected-to-unknown mating produced affected, equivocal, and unaffected offspring. Furthermore, breeding an affected-to-unaffected RW twice produced unaffected offspring both times. There was no affected-to-affected or equivocal-to-unaffected matings reported in this pedigree. Pedigree analysis results for SAS affected Rottweilers supports an autosomal recessive mode of inheritance.

A pedigree evaluation was performed in a family of 29 Bulldogs that contained 12 males and 17 females (Figure 2.6). There was a total of six Bulldogs diagnosed with PS (5 females and 1 male) and one equivocal female. The PoV of the affected dogs ranged from 3.8m/s to 6.07m/s. The equivocal female had a PoV of 2.0m/s. Eight dogs were cleared of PS via echocardiogram (PoV<1.5m/s) and four dogs were cleared via auscultation. Cardiac health information was unavailable for ten dogs included in this pedigree. There were three matings of unaffected parents which resulted in a litter with one or more PS-affected offspring. There was no affected-to-affected, affected-to-unaffected, or equivocal-to-unaffected matings reported in this pedigree. There was several consanguineous matings present. These findings support an autosomal recessive mode of inheritance for PS in Bulldogs.

Discussion

For this retrospective study, we analyzed the case records from the UCD VMTH to determine the number of dogs diagnosed with either SAS or PS via echocardiogram by a board-certified cardiologist or resident in training under direct supervision of a cardiologist. There was a total of 259 SAS and 336 PS cases diagnosed within 2008-2017. The prevalence of SAS overall was 0.3% and 4.7% and the prevalence of PS overall was 0.41% and 6.1%. These prevalence calculations are higher than what was reported in previous publications.[1,2,21,22] This could be due to the fact that UCD VMTH is a referral hospital, therefore cases are referred here that are identified elsewhere. Another explanation is that the cardiologists at UC Davis offer multiple clinical trials and ongoing genetic studies that may impact the number of dogs referred to this institution and may at least in part account for the increases in SAS cases in 2014 and PS cases in 2015. These types of confounding factors are frequently identified in prevalence studies

from referral institutions which is largely where the current statistics on congenital heart disease prevalence are generated.

SAS was observed in 47 different breeds and PS was observed in 65 breeds excluding mix-breed dogs. Not only are these diseases widespread across dogs, but they also afflict the five most popular breeds according to the 2017 AKC rankings.[1] The breeds identified in this study as being predisposed to SAS, have also been reported previously with the exception of Bullmastiffs and Pitbull Terriers.[2,7,22] Golden Retrievers had the highest number of cases, while Bullmastiffs had the highest odds ratio. The increase in reported SAS cases in Bullmastiffs can be attributed to improved cardiac screening practices that began being recommended by the breed club in 2014. This highlights the importance of echocardiogram clearances for breeds with a high predisposition for SAS, as cardiac auscultation-only clearance can miss mildly affected cases.

The breeds identified in this study as being predisposed to PS have also been reported in the previous literature.[2,7,22] Bulldogs had the most cases of PS and the highest odds ratio. It is well known that Bulldogs are predisposed to PS.[2,9] Bulldogs also had the highest number of cases of coronary anomalies. Interestingly, although French Bulldogs were predisposed to PS, there were no French Bulldogs diagnosed with a coronary anomaly via angiography which is consistent with other studies.[23] Although, Boxers had a high prevalence of PS in previous literature,[2,9] there were very few cases in Boxer dogs seen during the time period studied. A similar temporal trend was demonstrated in Italy following the introduction of stringent screening practices for the Boxer breed.[24] The American Boxer Club implemented strict cardiac

[1] AKC Staff. "Most Popular Dog Breeds - Full Ranking List." American Kennel Club, March 28, 2018, https://www.akc.org/expert-advice/news/most-popular-dog-breeds-full-ranking-list/.

screening practices in 2000,[2] which could explain the reduction in cases since affected dogs have been discouraged from breeding.

In a mixed breed shelter population PS was the most prevalent congenital heart disease, while SAS was the 3rd most common.[1] In the mixed breeds dogs in this study, 30 were affected with SAS and 80 with PS. Interestingly, mixed breeds are commonly mentioned as having high prevalence of PS in many studies,[2,9,25] indicating that PS is not simply a disease of purebred dogs.

The majority of SAS cases were mild or severe. These represent the dogs diagnosed with SAS prior to a surgery or cases referred to the cardiology service after the detection of a left basilar systolic heart murmur by a family veterinarian. The majority of PS cases were moderate or severe. One possible explanation is that many PS dogs are referred to the VMTH for a balloon valvuloplasty surgery when clinical signs are present. The remaining cases were equivocal or mild PS diagnosed after a murmur was detected during a routine physical exam. Many of these mild cases were not displaying clinical signs at the time of diagnosis. This stresses the importance of stringent cardiac screening practices prior to breeding in at risk dog breeds.

Mode of Inheritance

None of the breeds with more than ten cases had a statistically significant difference in the number of males and females except for SAS Rottweilers and PS mixed-breeds. However, the male predisposition identified in the Rottweilers and mixed-breeds is likely not clinically significant. For Rottweilers even though males have a greater odd of developing SAS, X-linked inheritance is unlikely given that unaffected males produced affected female offspring.

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[2] Wallnew, Wendy. “Boxer Cardiomyopathy.” American Boxer Club, 2000, https://americanboxerclub.org/PDF/Wallner_2000.pdf.

A pedigree for SAS affected Bullmastiffs, Golden Retrievers, and Rottweilers was generated to determine the mode of inheritance for each of these breeds. To our knowledge, this is the first reported SAS pedigree evaluation for the Bullmastiff breed. The pedigree suggests that SAS in Bullmastiffs is inherited as an autosomal recessive trait. For the Golden Retrievers, a previous study suggested that SAS can be inherited as an autosomal recessive or polygenic trait.[16] Our pedigree evaluation in this study further supports Stern et al. findings. Results for the Rottweiler pedigree also suggest an autosomal recessive mode of inheritance in this breed. A pedigree for PS affected Bulldogs also suggested an autosomal recessive mode of inheritance. Interestingly, there was variable expressivity for the disease, since litters with multiple affected puppies had different severities. Both the recessive mode of inheritance and variable expressivity supports what was previously found in Beagles.[5] One limitation of these pedigrees is that some control samples were not evaluated via echocardiogram by a board-certified cardiologist and we only had results from their auscultation exams. Therefore, we advise readers to take this into consideration since relatives of PS or SAS affected dogs that were cleared by auscultation, are sometimes diagnosed with either equivocal or mild disease by echocardiogram.

Limitations

One of the limitations in this study is that we sampled from a single hospital population, which may not be representative of the true canine population. Additionally, this was a retrospective study with a lack of follow up evaluations. Although, SAS and PS have a standard echocardiographic diagnosis,[6] these cases were diagnosed by different cardiologists over the ten year period. This can lead to measurement discrepancies between cardiologist. For example, the velocity values used to determine normal, equivocal, and mild PS and SAS cases varies in the literature. This study used a higher velocity cutoff for the equivocal range which would have

reduced the number of cases identified leading to a prevalence underestimation. Furthermore, our prevalence calculations can be underestimated since not all breeds require echocardiogram cardiac clearances. This may lead to a higher number of auscultation clearances which cannot definitively determine whether a dog is cleared of SAS and/or PS.

There are several other reasons why our prevalence estimates may be underestimated. We only included dogs that received an echocardiogram from a board-certified cardiologist. A murmur may be difficult to detect in certain breeds such as those with brachycephaly which may result in those dogs not being referred to a cardiologist. Therefore, some of those cases might be missing from the affected population. Finally, we only included dogs that were diagnosed antemortem by echocardiography. Therefore, prevalence calculations can be underestimated by not including stillborn or neonatal deaths.[26] Despite all these limitations, this retrospective study identified a higher prevalence than previously reported and warrants consideration that these defects may be more frequent than previously thought or perhaps increasing in recent years.

Conclusion

This study demonstrated that PS was at a higher prevalence than SAS in a veterinary hospital population from 2008-2017. Both diseases have increased in this hospital population in recent years when compared to previous literature. For SAS affected Bullmastiffs, Golden Retrievers, and Rottweilers, pedigree analysis suggest an autosomal recessive pattern of inheritance. The mode of inheritance for PS in the most affected breed, Bulldogs, also appears to be autosomal recessive. This has important implications for breeding practices and emphasizes the ultimate need for a reliable genetic screening test.

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Table 2.1 Number of cases from diagnosed with subvalvular aortic stenosis (SAS) or pulmonic stenosis (PS) based on continuous-wave doppler echocardiogram assessment.

Severity of Disease	SAS	PS
Mild	146	35
Moderate	33	54
Severe	80	247
Total	259	336

Table 2.2 Odd Ratio by breed with ≥10 cases for subvalvular aortic stenosis (SAS)

Breed	No. of Cases	Prevalence (%)	Odd Ratio	95% Confidence Interval	P-value
Bullmastiff	17	6.59	52.43	28.53 to 96.34	<0.0001
Newfoundland	10	4.46	34.73	16.77 to 71.95	<0.0001
Boxer	34	2.27	17.23	10.51 to 28.23	<0.0001
Golden Retriever	42	1.42	10.67	6.67 to 17.08	<0.0001
Rottweiler	12	1.17	8.78	4.48 to 17.20	<0.0001
German Shepherd	18	0.64	4.79	2.66 to 8.60	<0.0001
Pitbull Terrier	17	0.61	4.59	2.53 to 8.34	<0.0001
Labrador Retriever	10	0.15	1.09	0.53 to 2.22	0.8204
Mixed Breed	30	0.13	n/a	n/a	n/a

Table 2.2 Odds Ratio by Breed with ≥10 cases for pulmonic stenosis (PS)

Breed	No. of Cases	Prevalence (%)	Odd Ratio	95% Confidence Interval	P-value
Bulldog	56	4.80	13.32	9.42 to 18.84	<0.0001
French Bulldog	23	2.70	7.52	4.71 to 12.02	<0.0001
Pitbull Terrier	35	1.30	3.53	2.37 to 5.26	<0.0001
Chihuahua	17	0.52	1.45	0.86 to 2.45	0.1677
German Shepherd	12	0.43	1.19	0.65 to 2.19	0.5682
Mixed Breed	80	0.38	n/a	n/a	n/a

Supplemental Table 2.1 Number of equivocal dogs affected with SAS

Breed	No. of Equivocal Dogs
Afghan Hound	2
Australian Cattle Dog	1
Australian Shepherd	1
Bouvier des Flandres	1
Boxer	15
Bull Terrier	5
Bulldog	2
Dogo Argentino	1
German Shepherd	1
Golden Retriever	14
Labrador Retriever	2
Miniature Pinscher	2
Mix Breed	19
Newfoundland	4
Pit Bull Terrier	2
Rhodesian Ridgeback	1
Rottweiler	4
Miniature Schnauzer	1
Weimaraner	1
Whippet	1

Supplemental Table 2.2 Number of equivocal dogs affected with PS

Breed	No. of Equivocal Dogs
Boxer	1
Bulldog	10
Bullmastiff	1
Bull Terrier	1
Cavalier King Charles Spaniel	1
Miniature Schnauzer	1
Mixed Breed	1
Newfoundland	1
Shiba Inu	1

Supplemental Table 2.3 Number of males and females for breeds with >10 cases for subvalvular aortic stenosis (SAS).

Odd ratios, 95% confidence intervals, and corresponding p-values are listed.

Breed	Males	Females	Odd Ratio	95% Confidence Interval	P-values
Boxer	18	16	0.996	0.504 to 1.947	>0.999
Bullmastiff	9	8	0.891	0.358 to 2.363	0.808
German Shepherd	12	6	1.671	0.663 to 4.318	0.349
Golden Retrievers	20	22	0.794	0.431 to 1.434	0.534
Labrador Retrievers	2	8	0.234	0.050 to 0.938	0.058
Newfoundland	3	7	0.457	0.126 to 1.705	0.339
Pitbull Terrier	6	11	0.500	0.189 to 1.324	0.223
Rottweiler	10	2	5.515	1.292 to 25.270	0.018
Mixed Breed	10	20	0.480	0.234 to 1.028	0.067

Supplemental Table 2.2 Number of males and females for breeds with >10 cases for pulmonic stenosis (PS).

Odd ratios, 95% confidence intervals, and corresponding p-values are listed.

Breed	Males	Females	Odd Ratio	95% Confidence Interval	P-values
Bulldog	34	22	1.139	0.670 to 1.982	0.680
Chihuahua	4	13	0.335	0.119 to 0.972	0.052
French Bulldog	15	8	1.410	0.617 to 3.179	0.524
German Shepherd	6	6	0.834	0.266 to 2.615	0.779
Pitbull Terrier	19	22	0.792	0.419 to 1.450	0.530
Mixed Breed	52	28	1.789	1.147 to 2.810	0.013

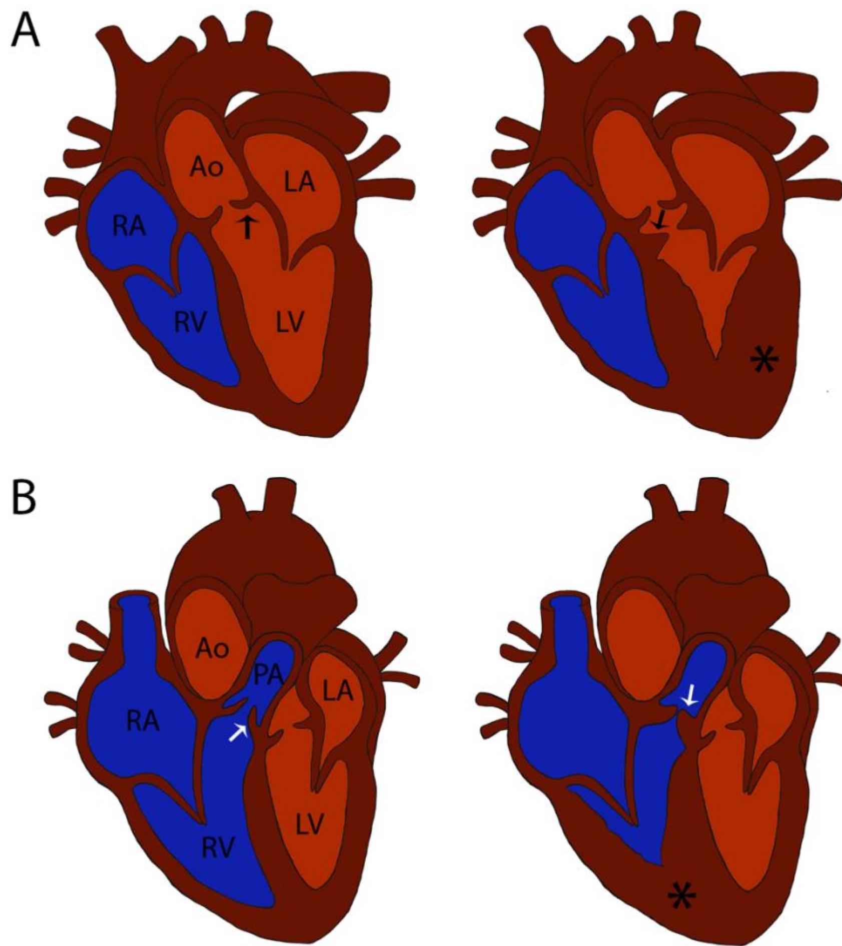

Figure 2.1: Comparison of normal and cardiac outflow tract abnormalities affected hearts. (A) Diagrams of a normal heart and a heart affected with subvalvular aortic stenosis (SAS). Note the stenosis caused by the subvalvular ridge (arrow) and left ventricular hypertrophy (asterisk) in the SAS affected heart compared to the normal heart. (B) Diagrams of a normal heart and a heart affected with pulmonic stenosis (PS). Note the stenosis caused by thickened pulmonic valve leaflets (arrow) and right ventricular hypertrophy (asterisk) in the PS affected heart compared to the normal heart. Structures in the normal heart diagrams are labeled for reference: RV, right ventricle; RA, right atrium; LV, left ventricle; LA, left atrium; Ao, aorta; PA, pulmonary artery; black arrow, aortic valve; white arrow, pulmonic valve.

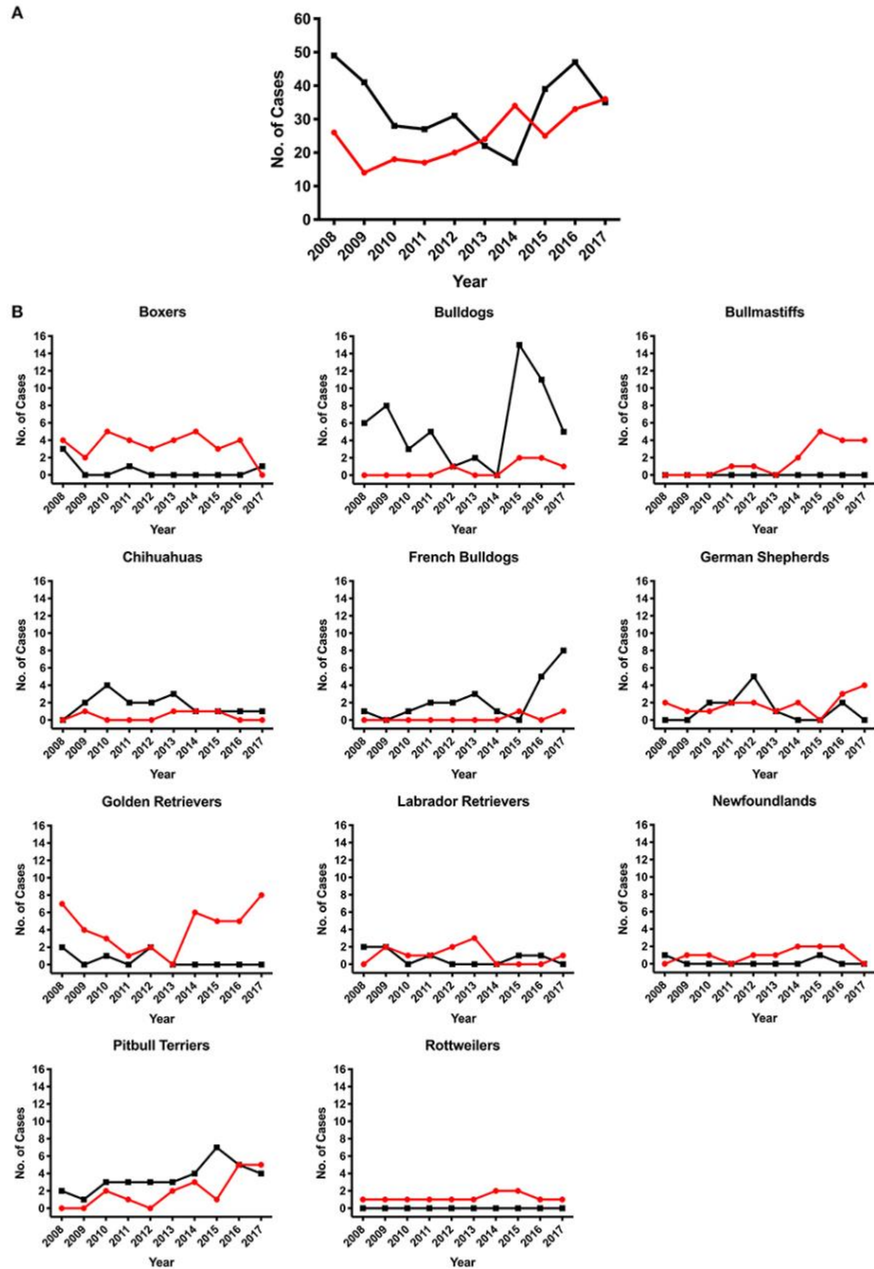

Figure 2.2: Number of subvalvular aortic stenosis (SAS) or pulmonic stenosis (PS) cases diagnosed between January 2008 to December 2017.

Dogs diagnosed with SAS or PS by the cardiology service team at a large university veterinary teaching hospital. A) Overall number of dogs diagnosed with SAS (red line) or PS (black line). B) Breeds that contained ≥ 10 cases of either disease during the study period. The red line illustrates SAS cases and black line illustrates PS cases.

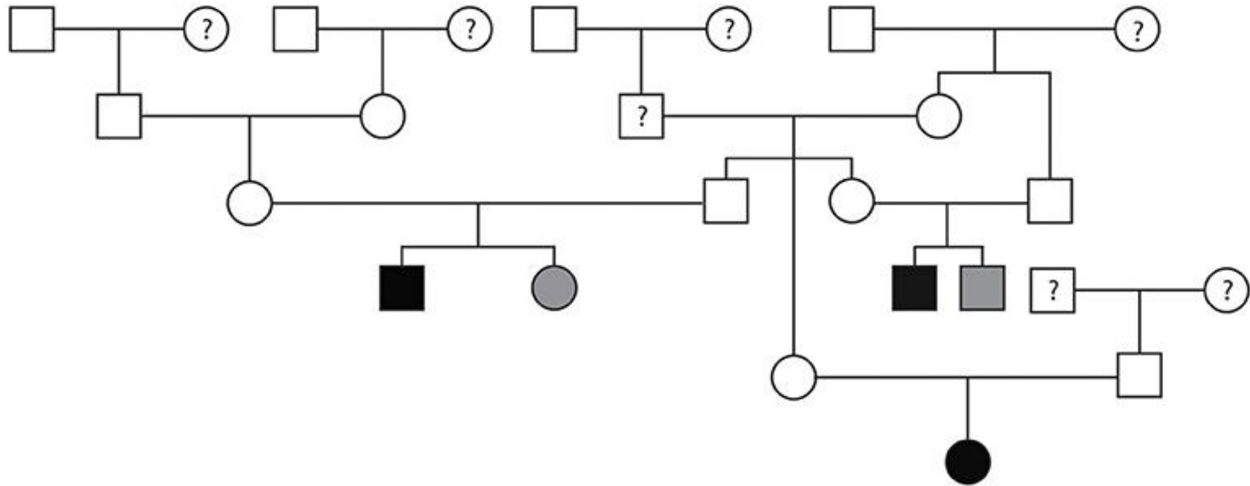

Figure 2.3: Pedigree representing an extended family of 25 Bullmastiffs phenotyped for subvalvular aortic stenosis (SAS).

Affection status is represented as follows: squares represent males, circles represent females, open symbols are SAS unaffected, gray symbols are SAS equivocal, black symbols are SAS affected, and symbols with questions marks have unknown SAS cardiac status.

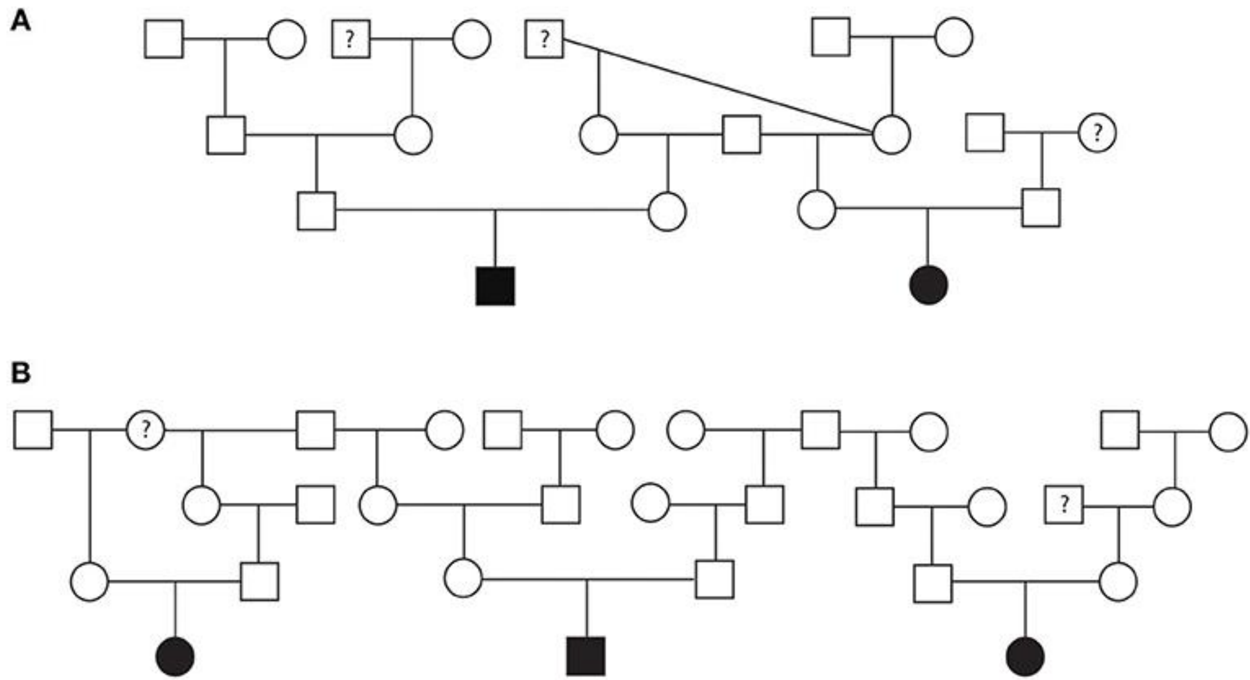

Figure 2.4: Pedigree representing two families of Golden Retrievers that were phenotyped for subvalvular aortic stenosis (SAS).

(A) Represents first family. (B) Represents the second family. Affection status is represented as follows: squares represent males, circles represent females, open symbols are SAS unaffected, gray symbols are SAS equivocal, black symbols are SAS affected, and symbols with questions marks have unknown SAS cardiac status.

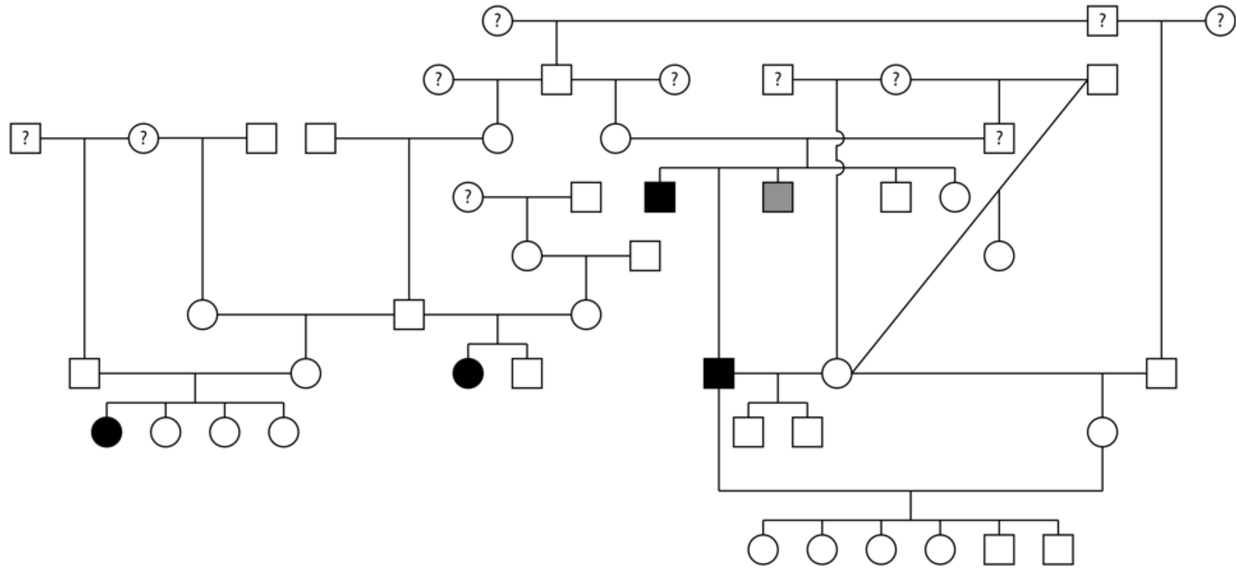

Figure 2.5: Pedigree representing an extended family of 48 Rottweilers phenotyped for subvalvular aortic stenosis (SAS).

Affection status is represented as follows: squares represent males, circles represent females, open symbols are SAS unaffected, gray symbols are SAS equivocal, black symbols are SAS affected, and symbols with questions marks have unknown SAS cardiac status.

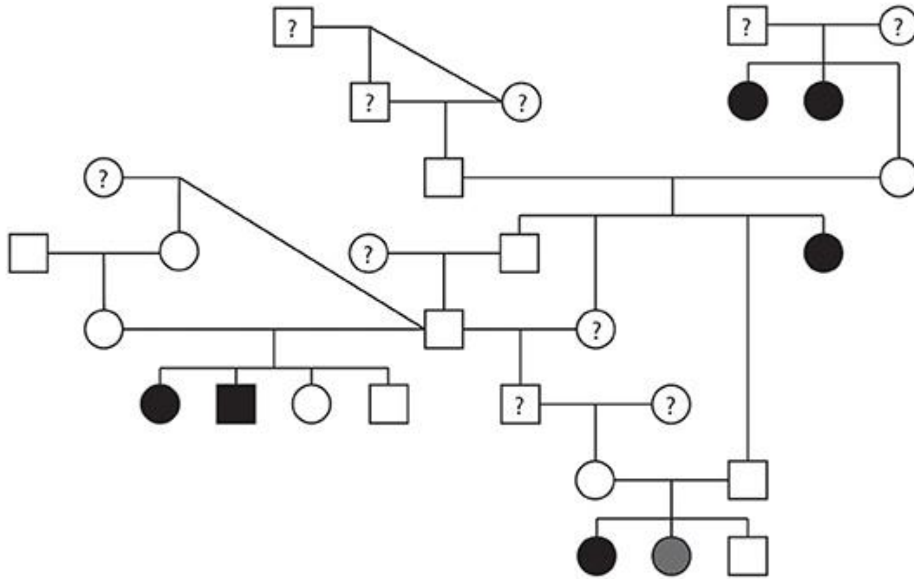

Figure 2.6: Pedigree representing an extended family of 29 Bulldogs phenotyped for pulmonic stenosis (PS).

Affection status is represented as follows: squares represent males, circles represent females, open symbols are PS unaffected, gray symbols are PS equivocal, black symbols are PS affected, and symbols with questions marks have unknown PS cardiac status.

Chapter 3. Large structural variant in ZNF446 is associated with pulmonary valve stenosis in Bulldogs

This chapter is intended for submission to PLOS Genetics:

Kovacs SL, Ontiveros ES, Rivas VN, Scansen BA, Hodge TE, Meurs KM, Gunther-Harrington CT, Kaplan, JL, Cullen JN, Malvick J, Sharpe AN, Li R, and Stern JA. Large structural variant in ZNF446 on canine chromosome one associated with pulmonary valve stenosis in Bulldogs

Abstract

Pulmonary valve stenosis (PS) is one of the most common congenital heart disorders in dogs and Bulldogs have a high predisposition. The purpose of this study was to identify a genetic mutation(s) associated with PS in Bulldogs. A combination of methods were utilized including: genome wide association study, Illumina whole genome sequencing, mass array analysis, and manual structural variant identification using the integrated genome viewer. A large structural variant that segregated between Bulldogs with PS and those without any congenital heart disease was identified on chromosome 1. This variant disrupts *ZNF446*, which is a transcription factor involved in embryonic heart development. Immunofluorescence confirmed it was present in the canine heart valve. This finding should prompt further functional research into the possible role of *ZNF446* and cardiac embryologic development.

Introduction

Pulmonary valve stenosis (PS) is the most common congenital heart defect in dogs and people.[1] PS is the abnormal development of the pulmonic valve leading to a stenosis that limits

ejection of blood into the pulmonary artery. This stenosis can lead to subsequent right ventricular hypertrophy, right-sided congestive heart failure or sudden cardiac death. It is characterized by thickened pulmonary valve leaflets that may be dysplastic or fused with or without a narrowed annulus.[2,3] Prognosis for this disorder varies with severity.[3] Mild cases may be asymptomatic or require minimal medical management to control clinical signs.[3] Severe cases may require interventional palliation via a balloon valvuloplasty.[4]

The genetics of PS is limited in veterinary medicine. There are certain dog breeds such as brachycephalic breeds with a high predisposition suggesting a genetic etiology.[5] Bulldogs are the most commonly affected dog breed and likely have a recessive mode of inheritance for this disorder.[6] PS genetics in humans is frequently is associated with disease syndromes such as Noonan Syndrome.[7] It has been speculated that the Bulldog appearance is reminiscent of the human syndromes and therefore may share a genetic basis.[8] Many identified mutations affect the Ras signaling pathway such as *PTPN11, RAF1, SOS1, and KRAS*.[7,9] Additional variants are in transcription factors which are critical for coordinating cardiac development such as *GATA4.*[10,11] Attempts have been made using a candidate gene approach to identify mutations in transcription factors associated with PS and other congenital heart diseases, but were unsuccessful.[12,13] To the authors' knowledge, no genetic cause has been identified for PS in any veterinary animal including Bulldogs.

The objective of this study was to identify variants associated with PS in Bulldogs. We hypothesize that a variant associated with this disease can be identified using a combination of genetic methods including: genome wide association study, Illumina whole genome sequencing, Mass array, and structural variant analysis with Integrative Genome Viewer (IGV).

Materials and Methods

Phenotyping and sample collection

Full cardiac evaluations including auscultation and 2D, spectral, and color Doppler echocardiograms were performed on each study participant by a board-certified veterinary cardiologist or resident in training under the direct supervision of a board-certified cardiologists. Bulldogs were actively recruited through web advertisements, breed events, and patients at three veterinary academic hospitals. Dogs from other breeds that received echocardiograms at the veterinary academic hospitals that qualified as cases and controls also had DNA collected. Informed consent was obtained (IACUC protocol #18106, #20047).

Evaluation of the right ventricular outflow tract (RVOT) was performed using previously established guidelines.[3] Briefly, dogs with a right parasternal short axis, continuous wave Doppler, right ventricular outflow velocity <1.5m/s, and subjectively normal pulmonic valve morphology were included as control dogs. Dogs with a velocity exceeding 2.5m/s and having obvious pulmonary valve malformations were included as affected dogs (**Figure 3.1**). Pulmonary valve malformations such as thickening, doming, and dysplasia were subjectively determined on the right parasternal short axis view. Valves were considered to have a hypoplastic annulus if the ratio between the aortic annulus diameter to pulmonary annulus diameter was >1.2. The aortic annulus diameter was obtained with the systolic five chamber right parasternal long axis view. The pulmonary annulus diameter was obtained with the systolic basilar right parasternal short axis view. Affected dogs were further stratified into mild, moderate, and severe with 2.5-3.49m/s, 3.5-4.49m/s, and >4.5m/s respectively. Only severe cases were utilized for the initial genetic analysis.

Bulldogs were excluded if they had concurrent congenital or acquired heart disease or had an equivocal disease status. The only congenital heart disease permitted was a patent foramen ovale since this could be a consequence of PS and coronary anomalies since this abnormality is commonly seen concurrently with PS in Bulldogs. Equivocal disease status is defined as dogs with a velocity between 1.5-2.5m/s, a velocity with >2.5 with no valvular changes, or a velocity <1.5m/s with the presence of valvular abnormalities. Dogs with supravalvular or subvalvular PS were excluded. If dogs had a concurrent coronary anomaly that was noted.

A three-generation pedigree was requested from all study participants and used to confirm that no subjects are related within two generations of one another. Venous whole blood (2mL) was collected into an EDTA tube and used to harvest genomic DNA using a commercially available blood DNA extraction kit (Qiagen Puregene, Germantown MD). To ensure adequate DNA samples were present for single nucleotide polymorphism (SNP) genotyping and whole genome sequencing, DNA quality and quantity was determined using a NanoDrop One Spectrophotometer (ThermoFisher, Waltham MA). A 2% agarose gel electrophoresis was performed on each sample to ensure the DNA was not degraded.

Genome Wide Association Study

A total of 48 cases and 45 control bulldogs were submitted for genotype association analysis. An aliquot of 300ng of purified DNA from each dog was submitted to *Neogen's GeneSeek Operations* (Lincoln, NE) for genotyping on the Illumina 230k Canine HD BeadChip array (Illumina, San Diego CA).

Raw genotyping data underwent stringent quality control, filtering, and analysis using Golden Helix SNP and Variation Suite (SVS) software (Golden Helix, Bozeman MT) to identify

SNPS significantly associated with cases compared to controls. To remove poorly genotyped SNPs, SNPs with a call rate of less than 90% were excluded from analysis. To remove poorly genotyped samples, samples with a call rate of less than 95% were excluded from analysis. To remove SNPs that are unlikely to have a significant contribution to the disease, SNPs with a minor allele frequency of less than 0.05 were excluded from analysis. Since the disease is unlikely to be sex-linked based on previous pedigrees and sex distribution, SNPs on the X-chromosome were excluded.

To reduce population stratification only one case and one control from each litter was used if pedigree information was available. To further reduce population stratification, a principle component analysis was performed and any samples >0.1 from the centroid in the PCA were considered outliers and excluded. A QQ-plot was used to visualize the amount of population stratification present, while a genomic inflation factor (λ) was reported to quantitatively evaluate population stratification. Statistical analyses included additive Chi-square association tests, a Bonferroni correction, and 100,000 permutation testing. If the genomic inflation factor was >1.1, a case-control genome wide association was performed using an efficient mixed model association expedited (EMMAX) to correct for cryptic relatedness.[14]

An association plot was generated to illustrate the association between single nucleotide polymorphisms and the disease state of interest. A SNP was considered significant if reached the Bonferroni threshold line is defined as -log (α/No. of tested SNPs) with α=0.05. If the association meets Bonferroni statistical significance, permutation testing (n=100,000) was performed to rule out false positives. If the SNP remained significant, 2 million bases on either side were screened for candidate genes that could plausibly explain the phenotype. To further

narrow the region SNPs were color coded if they followed a recessive mode of inheritance in a separate family not used in the GWAS.

The SNP locations are from the CanFam3.1 (Sep 2011) reference genome. Candidate genes from regions were pulled off Golden Helix from: UCSC RefSeq Genes 57 (03/01/2013), Ensemble Genes 89 (08/02/2017), and NCBI RefSeq Genes 104 (05/23/2016).

Whole genome sequencing, variant prioritization, and mass array analysis

DNA from 12 Bulldog severely affected cases and 6 Bulldog controls submitted for whole genome sequencing at the University of California Davis Genome Sequencing Center. These dogs were selected to be unrelated, represent the extremes of velocities, and followed the genotype segregation found during the GWAS. Paired-end DNA libraries with a fragment size of 150bp were created. The samples were sequenced to attain 30x coverage on the NovaSeq Illumina Sequencer. An established pipeline was used to generate variant call files from the raw Illumina Sequencer raw fastq.gz files.[15] All adapter contamination and low-quality sequences were removed from the raw fastq.gz files using Trimmomatic v0.38 and read quality was assessed with FastQC v0.11.7. Reads were aligned to CanFam3.1 genome using BWA software package v0.7.17. After, duplicate reads were removed using Picard v2.18.11. Variants were called with GATK v4.0 for all Bulldog samples submitted plus an additional 157 samples of previously whole-genome sequenced dogs consisting of various breeds and cardiac phenotypes.

Variant Call Files (VCF) were analyzed with Golden Helix SVS software. Variants underwent quality control and filtering using call rate >85%, minor allele frequency <0.05. A chi-square test was performed in Bulldogs to identify interesting variants. Then a binary chi-square test in a larger population of dogs of other breeds was completed to further narrow down the variants of interest by removing those that are common variants. A separate analysis on the

familial trio identified any variant that segregated with a recessive mode of inheritance within that family, regardless if it segregated with the larger cohort overall. All variants were annotated with Ensembl Variant Effect Predictor (VEP). Variants that were of high or moderate effect were included on the Sequenom Mass Array.

A minimum of 5 ng/ul of DNA for 59 cases and 53 controls was submitted to the University of California Veterinary Genetics Laboratory (Davis CA) for genotyping. Recruitment during the study was ongoing, therefore additional cases and controls were recruited since the GWAS was performed. Custom multiplex assays were designed to validate variants of interest in the WGS sample. Genotype results for each variant were analyzed using Prism v8.4.2 (San Diego CA). Statistical analysis was performed for allelic and genotypic frequencies. If each category in the contingency table was greater than 5, a chi-square test was performed. If the value was less than five, then a Fisher's Exact test was performed. Variants found to be statistically significant (p-value <0.05) between cases and controls using Sequenom Mass Array were reviewed for known RNA/protein expression in the heart and biological function of the variant.

Structural variant identification

Structural variants were identified by manually analyzing BAM files in Integrative Genome Viewer (IGV) 2.8. for 1 million base pairs upstream and downstream of all statistically significant WGS variants. The data was viewed as pairs and sorted by insert size. Tile Data Files (TDF) were generated in IGV to allow identification of read coverage differences up to the chromosomal level. All variants that continued to segregate in the 12 Bulldog cases and 6 Bulldog controls were validated in additional 945 whole genome sequenced dogs of various breeds to analyze segregation in a larger population of dogs. If the variant continued to be

significant then primers were designed for high throughput sequencing in an additional population of dogs.

The following reaction was designed for sequencing the *ZNF446* deletion **(Supplemental Figure 3.1, Supplemental Table 3.1)**. Three primers were utilized: one forward primer outside the deletion, one reverse primer contained in the deleted region, and one primer outside the deleted region. The sequences are available on **Supplemental Table 3.1**. This design would allow the three genotypes to be distinguished. If the genotype is homozygous wild type, one product size of 697bp will be produced by the primer set B3F/B3R. No product will form from the B3F/E3R since the product size would be over 23kbp. If the genotype is homozygous for the deletion, then one product size of 907bp would be produced from the B3F/E3R. No product will be formed from the B3F/B3R since the sequence for B3R would be absent. If the genotype is heterozygous then two products would be produced. In addition, a separate primer set for amplification of 692bp from MC1R was utilized to serve as a control for DNA quality. All PCR products were analyzed on an Applied Biosystems 3730 genetic analyzer.

Tissue immunofluorescence

To determine if the ZNF446 transcription factor is present in the canine heart, pulmonary valve tissue from adult dogs with no known heart disease was obtained from necropsy samples. These samples were obtained from client-owned dogs treated at the Veterinary Medical Teaching Hospital of the University of California, Davis that were euthanized for causes unrelated to this research project. Within 24 hours of euthanasia, the right ventricular outflow tract was opened and a longitudinal slice that encompassed the pulmonary artery, pulmonary valve leaflet, and right ventricular infundibulum was obtained. The tissue was fixed in formalin, paraffin-embedded, sectioned, adhered to a slide, and stored at -20C until further use.

The slide then was deparaffinized with the following washes in a Midas III EMDT machine: xylene (three-minute incubation, three times), 100% ethanol (three-minute incubation, three times), 95% ethanol (three-minute incubation, three times), 70% ethanol (three-minute incubation, three times), and a rinse with deionized water for six minutes. Then the slides were put in TBST for three minutes. The antigens were retrieved by breaking the formalin cross linked bonds. This was achieved by transferring the slides to 1x Ag retrieval solution that was previously heated to 95C. Then the solution and slides were steamed for 20 minutes, incubated at room temperature for 20 minutes, then rinsed for five minutes in TBST three times to remove the Ag retrieval solution.

The slides were triple stained for ZNF446, mesenchymal cells, and nuclei. All steps were performed with the slides protected from light. A slide used as a negative control was prepared using the same steps without adding any antibody. First the slides were blocked with “blocking buffer 1” which is 10% goat serum in TBST (1x, 0.05% Tween) for one hour at 37C on a rocker. Then the slides were incubated with mouse anti-bovine monoclonal Vimentin (Agilent M702001-2) at a dilution of 1:100, and rabbit anti-human polyclonal ZNF446 (Proteintech 16218-1-AP) at a dilution of 1:10. The dilutions were performed with “blocking buffer two” which is 10% goat serum, 0.2% NP-40, and TBST (1x, 0.05% Tween). The slides were incubated overnight at 4C. The slides underwent five washes for five minutes each with TBST (1x, 0.05% Tween). Then the slides were incubated with the secondary antibodies: goat anti-mouse IgG antibody CY5 (Life Technologies A10524) at a 1:50 dilution, goat anti-rabbit IgG AF555 (Invitrogen A21428) at a 1:50 dilution. Both were diluted using blocker buffer 2 and incubated for one hour at 37C on a rocker. The slides underwent five washes for five minutes each with TBST (1x, 0.05% Tween). Then the nuclei were labeled by incubating the slides with

300uM DAPI solution at room temperature for 5minutes. The slides underwent three washes for one minute each with TBST (1x, 0.05% Tween).

Vector TruView was used according to standard protocol to remove unwanted autofluorescence. Slides were washed once for five minutes with PBS. Then the Prolong Gold Antifade reagent was used to mount the final coverslip which helps prevent photobleaching. The slides were incubated for 24 hours at 4C. Immunofluorescence microscopy was performed using an EVOS FL Cell Imaging System (ThermoFisher Scientific, Waltham, MA). To maintain consistency throughout the image-acquisition process, the exposure of each fluorescence channel (DAPI Channel Ex, Texas Red excitation = 357/44 nm, emission = 628/32 nm; green fluorescent protein, excitation = 470/22 nm, emission = 525/50 nm) was kept constant. No statistics were performed since this was used to determine presence or absence of a protein as well as optimize a protocol that could be implemented in the future if case and control Bulldog pulmonic valve samples become available.

Results

Phenotyping and sample collection

There were 164 Bulldogs with echocardiograms screened. Dogs were excluded due to one or a combination of the following: concurrent heart defects (n=16), equivocal velocities (n=12), and direct relatives (n=16). There were 5 mildly affected cases, 14 moderately affected cases, and 48 severely affected cases There were 45 controls. For the GWAS only the 48 severely affected cases and 45 controls were used. There were no statistically significant sex differences between the two groups with cases having 22M/23F and controls having 27M/21F. The age at echocardiographic exam was significantly younger for the cases (1.1+/-1.3yrs) compared to the controls (3.5+/-3.0yrs) ($p<0.0001$). The controls had a normal right ventricular

outflow tract velocity of 1.08+/-0.20m/s. All cases were severely affected with a right ventricular outflow velocity of 5.61+/-0.71m/s. Six cases had a PFO, 12 did not have a PFO, and the remainder the PFO status was unknown. Twelve cases had a coronary anomaly. Twenty-three cases had the dysplastic phenotype, three had the doming phenotype, and 22 had a combination of the two. Twenty-three cases had a hypoplastic annulus. The controls did not have evidence of congenital or acquired heart disease based on echocardiography.

Genome wide association study

There were 48 cases and 45 controls used for the GWAS. No samples failed quality control. A total of 116,457 markers out of 218,885 remained after quality control and exclusion of markers on the X-chromosome. No samples were excluded based on a principal component analysis due to the limited span of the two principal components **(Supplemental Figure 3.2).** The genomic inflation factor (λ) prior to EMMAX correction was 1.36 and after the correction was 0.99. The EMMAX association produced a wide Bonferroni significant peak ($\text{p-value}_{raw}= 2.04x10^{-7}$) on chromosome 1 (**Figure 3.2a**). This peak remained significant after 100,000 permutations ($\text{p-value}_{perm}=1.16x10^{-3}$) (**Figure 3.2b**). This locus ranged from chr1:99456148-109400664 and contained 18 significant SNPS (**Supplemental Table 3.2).** This region contained 79 genes in CanFam3. Refinement of the region using family pedigree analysis and a recessive mode of inheritance reduced the region to chr1:99456148-106073546 **(Figure 3.2c)**.

Whole Genome Sequencing, Variant Prioritization and Mass Array Analysis

There were 12 Bulldog cases and 6 bulldog controls submitted for whole genome sequencing along with a phenotyped familial trio. Variants were called with these dogs and other available whole genome sequencing data for a total of 497 dogs. There was a total of 54,604,655 variants. Only 40,041,169 variants remained after filtering with a call rate of <0.85%. This number

was reduced to 10,173,708 when filtering for a minor allele frequency of 0.05 in the Bulldogs. When performing an association analysis among the 12 Bulldog cases and 6 Bulldog controls there were 64,237 variants that had a p-value of <0.01. This number was reduced to 7063 variants after performing an association of these variants in the large population of dogs to remove common variants. There were 131 variants of high or moderate impact that were elected for Sequenom MassArray analysis. Of those, 49 variants present on chromosome 1 and 26 followed perfect recessive segregation in the Bulldog family. Of the 131 variants, 59 were unsuitable for genotyping on Sequenom Mass Array due to presence in a repetitive region making genotype calls unreliable and were not pursued further. Of the 80 that were successfully genotyped, 34 were statistically significant **(Table 3.1).** However, none of these segregated perfectly between cases and controls. Of the 34 variants, the most significant was a missense variant in *SEMA4D* that had a p-value of 5.37E-07 based on allelic frequencies and 6.8E-05 based on genotypic frequencies.

Structural variant identification

A 22,270bp deletion at position CanFam4, chr1:100196786-100219056 was identified via IGV that segregated in 12 Bulldogs with and 6 Bulldogs without PS **(Figure 3.3).** The break point of the structural variant occurs in the intron between exon 4 and 5 of the gene ZNF446, thus deleting the first four exons. Analysis of 945 canid whole genomes revealed that this variant was mainly present in Bulldogs and Miniature Schnauzers. **(Supplemental Table 3.3).** No individual had a homozygous deletion. Of the 945 genomes analyzed, 45 dogs had the deletion in the heterozygous state: 2/9 Border Collies, 13/39 Bulldogs, 1/3 English Cocker Spaniels, 2/3 Flat coated retrievers, 2/41 Labrador retrievers, 1/10 Miniature poodles, 19/38 Miniature Schnauzers, and 5/67 mixed breeds **(Supplemental Table 3.3).** When Bulldogs with and without the deletion are excluded the prevalence of the deletion in the overall population was 3.5%. Of the 13

bulldogs that had the deletion 10 had a normal cardiac phenotype and in three the heart status was unknown. For the 26 Bulldogs that did not have the deletion, 18 had PS, 2 had equivocal PS, 2 had ARVC, 3 had unknown cardiac phenotypes, and one had a normal cardiac exam.

Primers were designed to allow analysis of a larger cohort of Bulldogs and other breeds with PS. There were 155 Bulldogs submitted for genotyping (**Table 3.3**). These were Bulldogs that were part of the original GWAS study as well as dogs that were newly recruited. One failed genotyping due to poor quality DNA. None were homozygous for the deletion. There were 42 Bulldogs unaffected by PS with the deletion and 45 without the deletion. There were 7 PS affected Bulldogs with the deletion and 60 without the deletion. Of those seven, five had confirmed coronary anomalies, one had suspected coronary anomaly based on echo, and one had normal coronary anatomy. The genotype-phenotype segregation was statistically significant with a p-value of 3.06E-07 The allelic segregation was also significant with a p-value of 6.82E-06.

There were 83 French Bulldogs submitted: 46 with PS and 37 without PS. One failed genotyping due to poor quality DNA and the remainder did not have the deletion. There were 141 non-Bulldog and non-French Bulldog individuals with PS submitted. Two failed genotyping due to poor quality DNA. The remainder did not have the deletion.

Tissue immunofluorescence

Tissue was successfully stained to identify the nuclei (DAPI, blue), mesenchymal cells (vimentin, green), and *ZNF446* (*ZNF446* antibody, red) (**Figure 3.4).** The *ZNF446* transcription factor was perinuclear throughout the valve. However, there was increased cytoplasmic signaling along the ventricularis portion of the valve. No signal was detected on the negative control slide.

Discussion

PS is one of the most common congenital heart disorders in both veterinary medicine and human medicine.[5,16,17] In human medicine, there are several genetic mutations associated with this birth defect.[18] The majority are part of developmental pathways such as the RAS/MAPK pathway and/or are transcription factors.[10,19] This is the first study that has identified a variant associated with PS in veterinary medicine, specifically Bulldogs.

This variant disrupted *ZNF446*, which is a highly conserved transcription factor that is expressed in both embryonic heart tissue and adult heart tissue.[20] The identified variant on chromosome one is a large 22,270 base pair deletion whose break point occurs in the intron between exon 4 and 5. This leads to truncation of the first half of the protein. This transcription factor was first identified from a human embryonic heart library.[20] We confirmed its presence in canine pulmonic valves using immunofluorescence. *ZNF446* is part of the MAPK pathway through the transcriptional repression of both serum response elements (*SRE*) and activating protein 1 (*AP-1*).[20] *SRE* is required for cardiac differentiation and maturation.[21] *SRE* is typically activated by *GATA4* and *NKX2.5*,[21] which are two genes known to cause PS in people when mutated.[7,10,22] *AP-1* plays a large role in heart outflow tract development.[23] Although the exact mechanism of *ZNF446* involvement in cardiac development has not been elucidated yet, there is strong support of its involvement.

Interestingly, this mutation appears to have a protective effect against PS in Bulldogs since it was present in controls instead of cases. Gene dosage alterations are particularly problematic for cardiac development since it is a highly coordinated process.[1] Bulldogs have several mutations that are fixed or at high frequency that alter developmental pathways.[24–28] For example, Bulldogs are fixed for a mutation in *DVL2*.[26] In humans, *DVL1* and *DVL3* causes Robinow syndrome which is occasionally associated with PS.[29] It is possible the mutation in *ZNF446* emerged to

correct for the *DVL2* aberration in cardiac development or other mutated developmental genes. Therefore, the *ZNF446* mutation may have become established to rescue a normal phenotype in the presence of an abnormal genetic background of the Bulldog. However, further research is necessary to investigate this hypothesis.

Although this mutation did not segregate perfectly with Bulldog PS, it did have a surprising genotypic-phenotypic distribution. This deletion was not found in the homozygous state in over 155 Bulldogs analyzed suggesting that embryonic lethality may be occurring. There were 7 PS-affected Bulldogs with the deletion and 60 without the deletion. An unexpectedly high percentage of those dogs with PS and the deletion had coronary anomalies. Coronary anomalies are found at a higher frequency in brachycephalic breeds with PS compared to other breeds with PS.[30] However, it occurs in about a 25% of the Bulldog population[30,31] and not the 71-85% seen with the PS and deletion combination. This could imply that stenosis caused by the coronary anomaly impinging on the outflow tract may not be protected against by this mutation. Bulldogs with coronary anomalies have anecdotally been reported without evidence of PS, strengthening this hypothesis. Since coronary anomalies make the gold-standard interventional treatment for PS risky, understanding the genetic involvement that contributes to coronary anomalies should be explored further.

Finally, there was a high frequency of the deletion in Miniature Schnauzers: 19 with the deletion and 19 without the deletion. The status of these Miniature Schnauzers is unknown, but it is another breed frequently cited in the literature predisposed to PS and shares a haplotype with Bulldogs.[32,33] Therefore, a larger population of Miniature Schnauzers with known cardiac phenotype status should be analyzed to determine how this deletion segregates with disease.

It is possible this deletion is not the true causative mutation for PS but is instead linked to the mutation. There were other variants that were statistically significant such as a *SEMA4D* missense mutation, that could be responsible for or contribute to the phenotype. *SEMA4D* is part of the semaphorin family of genes which play a role in cardiac outflow tract development.[34] *SEMA4D* was more significant than the *ZNF446* based on allelic frequencies, but less significant than *ZNF446* based on genotypic frequencies. This is likely because of the lack of homozygous deletion state for *ZNF446* that was mentioned previously. Future functional studies are necessary to interrogate *ZNF446, SEMA4D* and the other genes identified for their possible role in PS in Bulldogs. Chromosome one is a common region to identify mutations associated with brachycephaly, although this region did not contain any of the currently known Bulldog mutations it may be linked to another unidentified Bulldog trait instead of PS.[25,27,35] The samples for this study came from multiple sources across the country, but it cannot be ruled out that this mutation was specific to the population tested. Therefore, future work would be to test if it is present in a unique population of Bulldogs.

Additional steps would be to interrogate *ZNF446* and other identified genes to determine if over-expression or under-expression in a mouse or zebrafish model alters cardiac development. If samples become available, RNAseq or immunofluorescence should be performed in normal and stenotic Bulldog pulmonary valves to quantify if there are significant differences between the two groups regarding localization and/or expression of *ZNF446*. If there are differences, ChIP-sequencing could be performed to identify the DNA binding sites of the transcription factor which could detect additional genes critical to heart development.

Conclusion

A large structural variant involving the cardiac transcription factor *ZNF446* was found to be associated with a protective effect against PS in Bulldogs. Future functional research is necessary to understand the role that *ZNF446* has in heart development. Additionally, this finding should be verified in an independent population of Bulldogs.

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Table 3.1: Significant variants associated with PS in a larger population of case and control Bulldogs based on Mass Array.

Position	Ref	Alt	Consequence	Impact	Gene	Cardiac RNA/Protein
1:96852165	G	A	Missense	moderate	SEMA4D	RNA, protein
1:100141657	G	A	Missense	moderate	TIM23	RNA
1:100157473	G	C	Missense	moderate	ZFP 345-like	unknown
1:100700638	G	A	Missense	moderate	ZNF 208	RNA
1:101138632	C	T	Missense	moderate	Uncharacterized	unknown
1:101140089	G	A	Missense	moderate	ZFP 660-like	RNA
1:101336715	C	T	Missense	moderate	Olfactory receptor 13H1-like	RNA
1:101358976	C	T	Missense	moderate	ZNF835	RNA, protein
1:101395700	C	T	Missense	moderate	ZFP 26-like	unknown
1:101396796	T	C	Missense	moderate	ZFP 26-like	unknown
1:99526631-99526633	CC	-	Frameshift variant	High	ZNF584	RNA
1:99526638	C	A	Missense	moderate	ZNF584	RNA
1:99557157	C	G	Missense	moderate	RNF225	RNA
1:99576991-99576994	GGT	-	Inframe deletion	moderate	ZNF837	RNA
1:102122751	T	C	Missense	moderate	ZNF865	RNA
1:99526633 1:99497248	C A	A G	Missense Missense	moderate moderate	ZNF584 ZNF132	RNA RNA
9:48734275	C	T	Missense	moderate	MAMDC4	RNA, Protein
1:96830524	G	C	Missense	moderate	SEMA4D	RNA, protein
9:48885356-48885359	GCC	-	Inframe deletion	moderate	EGFL7	RNA, Protein
9:49074942	G	A	Missense	moderate	PMPCA	RNA, Protein
9:49085883	A	G	Missense	moderate	ENTR1	No. Colon, skin, liver, nervous system
22:22966605	A	G	Missense	moderate	LOC106557553	unknown
30:8906795	T	C	Missense	moderate	SPTBN5	RNA
30:8896460	A	G	Missense	moderate	SPTBN5	RNA
1:115296367	T	C	Missense	moderate	ZNF875	No. Nervous system and eye
19:18838933	C	A	Splice donor	High	LOC102156162	unknown
1:115675974	C	T	Missense	moderate	ZNF345	RNA
1:115286739	G	A	Missense	moderate	ZNF875/HKR1	unknown
1:115287152	C	T	Missense	moderate	ZNF875/HKR1	unknown
27:4587654	A	G	Missense	moderate	CERS5	RNA, protein
9:49188301	G	A	Missense	moderate	CCDC187	unknown
1:96868165	G	A	Missense	moderate	SEMA4D	RNA, protein
11:64068313	G	A	Splice donor	High	LOC111098125	unknown
23:37539489	C	T	Stop gained	High	LOC106557652	unknown

Ref= reference allele, Alt= alternate allele, Impact = Functional Impact

Table 3.2: MassArray genotype results

Position	Bulldog Controls			Bulldog Cases			Allelic P-value	Genotypic P-value
	WT	Het	Ho	WT	Het	Ho		
1:96852165	18	5	30	6	0	53	5.37E-07	6.8E-05
1:100141657	26	26	1	54	4	1	8.90E-06	3.37E-07
1:100157473	26	26	1	54	4	1	8.90E-06	3.37E-07
1:100700638	26	26	1	54	4	1	8.90E-06	3.37E-07
1:101138632	26	26	1	54	4	1	8.90E-06	3.37E-07
1:101140089	26	26	1	54	4	1	8.90E-06	3.37E-07
1:101336715	26	26	1	54	4	1	8.90E-06	3.37E-07
1:101358976	1	26	26	1	4	54	8.90E-06	3.37E-07
1:101395700	26	26	1	54	4	1	8.90E-06	3.37E-07
1:101396796	26	25	1	54	4	1	1.28E-05	4.87E-07
1:99526631-99526633	26	26	1	53	5	1	2.49E-05	2.15E-06
1:99526638	26	26	1	53	5	1	2.49E-05	2.15E-06
1:99557157	1	26	26	1	5	53	2.49E-05	2.15E-06
1:99576991-99576994	1	26	26	1	5	53	2.49E-05	2.15E-06
1:102122751	1	26	26	2	3	53	3.15E-05	1.27E-07
1:99526633	26	25	1	53	5	1	3.55E-05	3.30E-06
1:99497248	26	26	1	52	6	1	6.47E-05	6.37E-06
9:48734275	0	10	42	4	25	30	5.56E-04	1.57E-03
1:96830524	1	22	30	2	4	53	7.06E-04	2.12E-05
9:48885356-48885359	0	11	42	4	25	30	9.39E-04	2.40E-03
9:49074942	0	11	42	4	25	30	9.39E-04	2.40E-03
9:49085883	40	12	0	28	27	4	9.73E-04	2.61E-03
22:22966605	35	1	17	50	1	8	9.87E-04	3.72E-02
30:8906795	17	26	10	8	26	25	1.62E-03	9.76E-03
30:8896460	8	23	22	22	24	13	1.83E-03	1.35E-02
1:115296367	8	22	23	0	21	38	2.19E-03	1.90E-03
19:18838933	24	25	4	14	30	15	2.79E-03	1.14E-02
1:115675974	13	25	15	3	29	27	4.58E-03	8.16E-03
1:115286739	13	22	18	2	28	29	4.61E-03	3.61E-03
1:115287152	13	22	18	2	28	29	4.61E-03	3.61E-03
27:4587654	3	18	32	0	11	48	6.12E-03	1.65E-02
9:49188301	0	13	40	6	27	26	1.00E-02	5.12E-04
1:96868165	22	16	4	33	12	0	1.32E-02	3.31E-02
11:64068313	25	22	6	13	35	11	1.35E-02	2.07E-02
23:37539489	4	21	28	9	30	20	3.61E-02	NS

Chr=chromosome, WT= wildtype, Het=heterozygous, Ho=homozygous, NS=not significant

Table 3.3: Genotype results for ZNF446 structural variant in Bulldogs with and without PS along with dogs of varying breeds with PS based on PCR.

Breed	No PS			PS			Allelic P-value	Genotypic P-value
	WT	Het	Ho	WT	Het	Ho		
Bulldog	45	42	0	60	7	0	6.81974E-06	3.06E-07
French Bulldog	46	0	0	37	0	0	NS	NS
Other Breeds	-	-	-	139	0	0	-	-

WT = wild type, no deletion, Het = heterozygous, one copy of the deletion, Ho = homozygous, two copies of the deletion, NS=not significant

Supplemental Table 3.1. Primer Sequences to interrogate a the large ZNF446 structural variant along with the location on the chromosome and PCR product sequence.

<table>
<tr><td>Primer Name</td><td>Primer 5’-3’</td></tr>
<tr><td>B3F</td><td>FAM-CCCTACAACCAGGGCAGTAT</td></tr>
<tr><td>B3R</td><td>FAM-CACCACCCCATTAACACACA</td></tr>
<tr><td>E3R</td><td>FAM-AGGGTGCCTGAAAAATACCC</td></tr>
<tr><td colspan="2"></td></tr>
<tr><td>Wild type Sequence</td><td>CanFam6 not identified by UCSC in silico PCR
CanFam5 chr1:99728820+99752256 23437bp
CanFam4 chr1:100196296+100219751 23456bp
CanFam3 chr1:99442640+99466054 23415bp</td></tr>
<tr><td>Deletion</td><td>CanFam6 not identified by UCSC lift over
CanFam5 chr1:99729310-99751573 22,263bp
CanFam4 chr1:100196786-100219056 22,270bp
CanFam3 chr1:99443130-99465363 22,233bp</td></tr>
<tr><td colspan="2"></td></tr>
<tr><td colspan="2">B3F/E3R Homozygous deletion - 907bp product</td></tr>
<tr><td colspan="2">CCCTACAACCAGGGCAGTATgcagacagtaaaaggggtcatggctggtcctcacccagggagaccaccgtgccgt
atttctccagcattgcatcccagtacaattccttctgtgacgggtccagtagcccccactcctcctgtggggatgcagcccatcactcttggt
gcctgtccactcccccatcctctgccctgccttgcagtgcctacctgcccgtgtccctggttacaatcttactaattacctgagggaacaga
caggttacctaaggctcttctgcttgctaaggatgaagctgagaagggtgtgtgaaggctgggaaatagggcagagaggagctgtcctt
catctcactgttaggacctgtccccctcagaagcccgccccactccatattctagccaggcagctgagaacccctggataggttctgtgtg
gaagtgactgtccctacacccttgccttgaacagtgctgcatcaagaacaa/tggccgctgagcagaagacagagggaggcaatg
ggaatttcagttattccctccttccctctctccctccttccttccctcctccctcccttccttccttccttccttccttccttccttccttccttcc
ttccttccttccttcctcacttcctccctctctcctttccttccttcaagtaggctctatgcccaacatagggcctgaactgacaaccccaatatc
aagagtcacatgctctactgactgagccagccaggtggcccaattatttcatctttatatacataggtctatagatcctggaagctttgcaca
ccccatgcagcatgacaatggagcaggtgagtccctttaaacagactgtgtcccctctgtgaggggactgtgaGGGTATTTTT
CAGGCACCCT</td></tr>
<tr><td colspan="2">B3F/B3R Homozygous wildtype – 697bp</td></tr>
<tr><td colspan="2">CCCTACAACCAGGGCAGTATgcagacagtaaaaggggtcatggctggtcctcacccagggagaccaccgtgccgt
atttctccagcattgcatcccagtacaattccttctgtgacgggtccagtagcccccactcctcctgtggggatgcagcccatcactcttggt
gcctgtccactcccccatcctctgccctgccttgcagtgcctacctgcccgtgtccctggttacaatcttactaattacctgagggaacaga
caggttacctaaggctcttctgcttgctaaggatgaagctgagaagggtgtgtgaaggctgggaaatagggcagagaggagctgtcctt
catctcactgttaggacctgtccccctcagaagcccgccccactccatattctagccaggcagctgagaacccctggataggttctgtgtg
gaagtgactgtccctacacccttgccttgaacagtgctgcatcaagaacaa<u>aaggcctggggacccaagggaacccagccacctctc</u>
<u>cccaagcctctggttacaaaagtaacagagatcctccttggggagctgctgagggctatcagcattcctcgatgtggtgacctggctgca</u>
<u>gagaggttgaaggcagaagggcttacattaggaggtgcagccatctcagggctcagatgaTGTGTGTTAATGGGGTG</u>
<u>GTG</u></td></tr>
</table>

Bold is the flanking region to the deletion Underlined is a portion of the deleted sequence. Uppercase is the primers. The location of the full sequence and full deletion is provided but not listed due to the large size.

Supplemental Table 3.2 Top genome wide association single nucleotide polymorphisms with genotypes for 48 cases and 45 control

Marker	Position	P-value raw	-log10 P-value	Bonf. P-value	Perm. p-value	-log10 Perm. P-value	Minor Allele (D)	Major Allele (d)	DD Case	DD Ctrl	Dd Case	Dd Ctrl	dd Case	dd Ctrl
BICF2G630788655	1:99456148	2.04E-07	6.69	0.02	1.16E-03	2.94	C	T	0	1	4	26	44	18
chr1_99497248	1:99497248	2.04E-07	6.69	0.02	1.16E-03	2.94	G	A	0	1	4	26	44	18
chr1_99586976	1:99586976	2.04E-07	6.69	0.02	1.16E-03	2.94	A	G	0	1	4	26	44	18
chr1_100212305	1:100212305	2.04E-07	6.69	0.02	1.16E-03	2.94	A	G	0	1	4	26	44	18
BICF2S23320544	1:100619743	2.04E-07	6.69	0.02	1.16E-03	2.94	G	A	0	1	4	26	44	18
chr1_100723744	1:100723744	2.04E-07	6.69	0.02	1.16E-03	2.94	A	G	0	1	4	26	44	18
BICF2P180396	1:101249848	2.04E-07	6.69	0.02	1.16E-03	2.94	T	C	0	1	4	26	44	18
BICF2P1270156	1:101333437	2.04E-07	6.69	0.02	1.16E-03	2.94	T	C	0	1	4	26	44	18
BICF2P886164	1:101336715	2.04E-07	6.69	0.02	1.16E-03	2.94	T	C	0	1	4	26	44	18
chr1_101358976	1:101358976	2.04E-07	6.69	0.02	1.16E-03	2.94	T	C	0	1	4	26	44	18
chr1_101549399	1:101549399	2.04E-07	6.69	0.02	1.16E-03	2.94	T	C	0	1	4	26	44	18
BICF2P713788	1:101569290	6.97E-07	6.16	0.08	5.67E-03	2.25	C	T	0	1	5	26	43	18
chr1_101604761	1:101604761	6.97E-07	6.16	0.08	5.67E-03	2.25	T	C	0	1	5	26	43	18
BICF2P397468	1:106073546	2.36E-06	5.63	0.27	0.02	1.63	C	T	1	3	5	26	42	16
BICF2P293961	1:108410809	2.70E-06	5.57	0.31	0.03	1.56	C	T	2	17	24	23	22	5
BICF2S23254580	1:108419160	3.20E-06	5.5	0.37	0.03	1.47	G	A	1	15	22	23	25	7
BICF2S23250181	1:109400664	3.20E-06	5.5	0.37	0.03	1.47	C	A	2	14	20	25	26	6

Bonf = Bonferroni, Perm = Permutation, Ctrl = controls

Supplemental Table 3.3 Genotype results for ZNF446 structural variant in a large population of whole genome sequenced dogs manually with Integrative Genome Viewer

Breeds	No Deletion	Deletion	Total
Afghan Hound	2	0	2
Airedale Terrier	2	0	2
Akita	1	0	1
Alaskan Malamute	2	0	2
American Cocker Spaniel	3	0	3
American Foxhound	1	0	1
American Staffordshire Terrier	1	0	1
Australian Cattle Dog	2	0	2
Australian Shepherd	3	0	3
Basenji	3	0	3
Beagle	2	0	2
Belgian Sheepdog	3	0	3
Belgian Tervuren	3	0	3
Berger Picard	3	0	3
Bernese Mountain Dog	3	0	3
Bichon Frise	3	0	3
Border Collie	7	2	9
Border Terrier	4	0	4
Boston Terrier	7	0	7
Bouvier des Flandres	13	0	13
Boxer	44	0	44
Boykin Spaniel	1	0	1
Brittany	6	0	6
Bull Terrier	2	0	2
Bulldog	26	13	39
Bullmastiff	19	0	19
Cairn Terrier	7	0	7
Cavalier King Charles Spaniel	21	0	21
Chihuahua	2	0	2
Chinese Crested	2	0	2
Chow Chow	1	0	1
Collie	5	0	5

Dachshund	18	0	18
Dalmatian	1	0	1
Doberman Pinscher	10	0	10
English Cocker Spaniel	2	1	3
English Mastiff	1	0	1
English Pointer	2	0	2
English Setter	3	0	3
English Springer Spaniel	2	0	2
Flat-Coated Retriever	1	2	3
French Bulldog	24	0	24
German Shepherd	22	0	22
German Shorthair Pointer	2	0	2
German Wirehair Pointer	2	0	2
Golden Retriever	83	0	83
Gordon Setter	2	0	2
Great Dane	26	0	26
Great Pyrenees	3	0	3
Greater Swiss Mountain Dog	2	0	2
Greyhound	2	0	2
Havanese	2	0	2
Irish Setter	4	0	4
Irish Terrier	1	0	1
Irish Water Spaniel	3	0	3
Irish Wolfhound	20	0	20
Italian Greyhound	2	0	2
Jack Russell Terrier	3	0	3
Kunming Dog	1	0	1
Labrador Retriever	39	2	41
Lhasa Apso	3	0	3
Miniature Poodle	9	1	10
Miniature Schnauzer	19	19	38
New Guinea Singing Dog	1	0	1
Newfoundland	19	0	19
Norwegian Elkhound	2	0	2
Norwegian Lundehund	1	0	1
Nova Scotia Duck Tolling Retriever	17	0	17
Pembroke Welsh Corgi	11	0	11
Pomeranian	13	0	13

Portuguese Water Dog	14	0	14
Pug	9	0	9
Rhodesian Ridgeback	6	0	6
Rottweiler	24	0	24
Saint Bernard	2	0	2
Saluki	4	0	4
Samoyed	2	0	2
Scottish Deerhound	10	0	10
Scottish Terrier	9	0	9
Sheltie	11	0	11
Shiba Inu	2	0	2
Shih Tzu	1	0	1
Shiloh Shepherd	5	0	5
Siberian Husky	17	0	17
Soft Coated Wheaten Terrier	3	0	3
Spanish Greyhound	1	0	1
Standard Poodle	32	0	32
Standard Schnauzer	3	0	3
Tibetan Terrier	2	0	2
Toy Poodle	5	0	5
Weimaraner	5	0	5
Welsh Springer Spaniel	4	0	4
Welsh Terrier	1	0	1
West Highland White Terrier	8	0	8
Whippet	19	0	19
Xoloitzcuintli	1	0	1
Yorkshire Terrier	45	0	45
Mix	62	5	67
Coyote	1	0	1
Grey Wolf	9	0	9
Red Wolf	1	0	1
Total	900	45	945

A.

B.

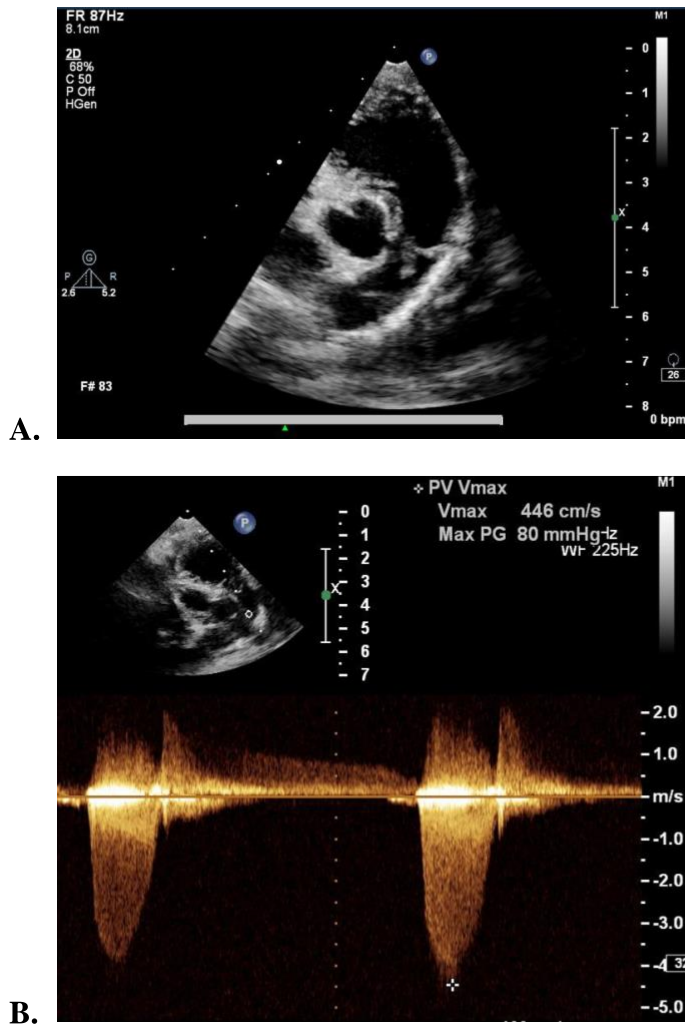


Figure 3.1 Echocardiographic Diagnosis of PS

A. Right parasternal short axis echocardiographic image illustrating thick and fused pulmonary valves. **B.** Color doppler to measure right ventricular outflow tract velocity (m/s) across the stenosis to allow quantification of stenosis severity.

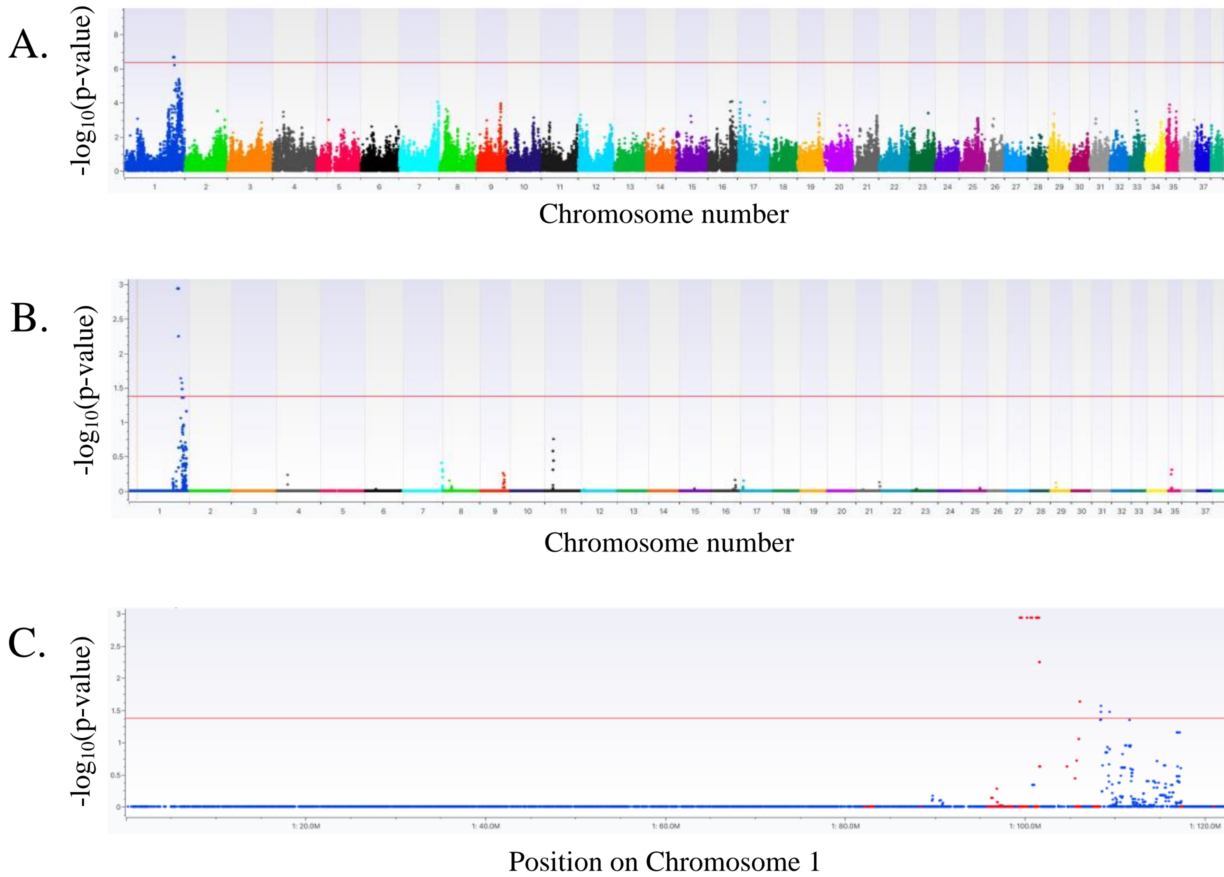


Figure 3.2 Genome-wide association study (GWAS) for PS affected Bulldogs

A) Efficient Mixed-Model eXpedited (EMMAX) Manhattan Plot of –log10(p-values) for SNPs colored-coded by chromosome. Bonferroni significance is indicated by the horizontal red line. B) 100,000 permutation testing to clean up signal C) Chromosome 1 SNPs. Red are SNPs that follow a recessive mode of inheritance in a family which encompasses 2.1MB region containing significant SNPS

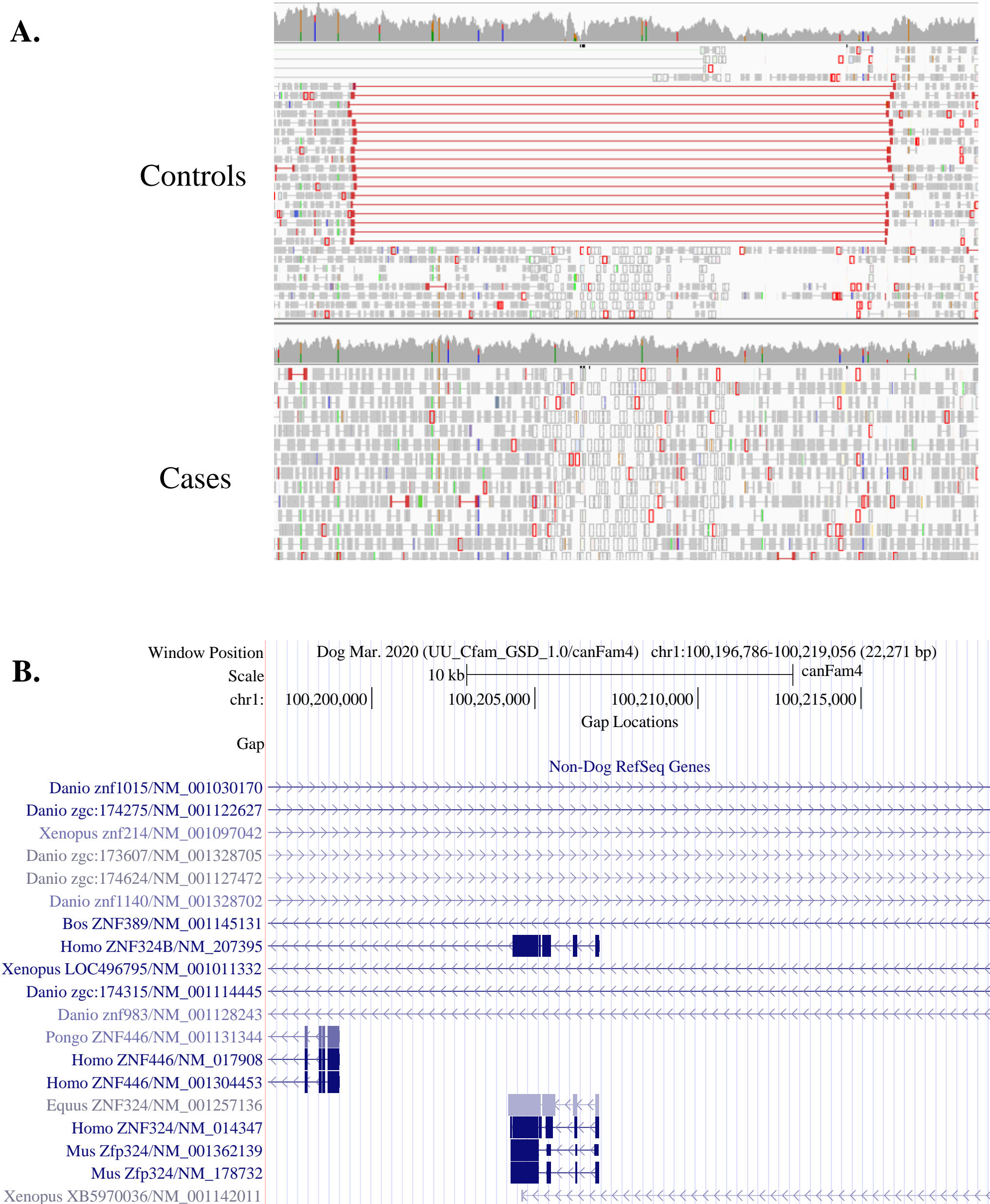


Figure 3.3 The 22,270bp deletion in CanFam4 associated with PS in Bulldogs displayed in A) Integrative Genome Viewer and B) UCSC Genome Browser

Representation of a large structural variant that segregates perfectly between Bulldog controls (top panel in A) and cases (bottom panel in A). This region contains several zinc fingers one of which is ZNF446 with the break point after exon 4.

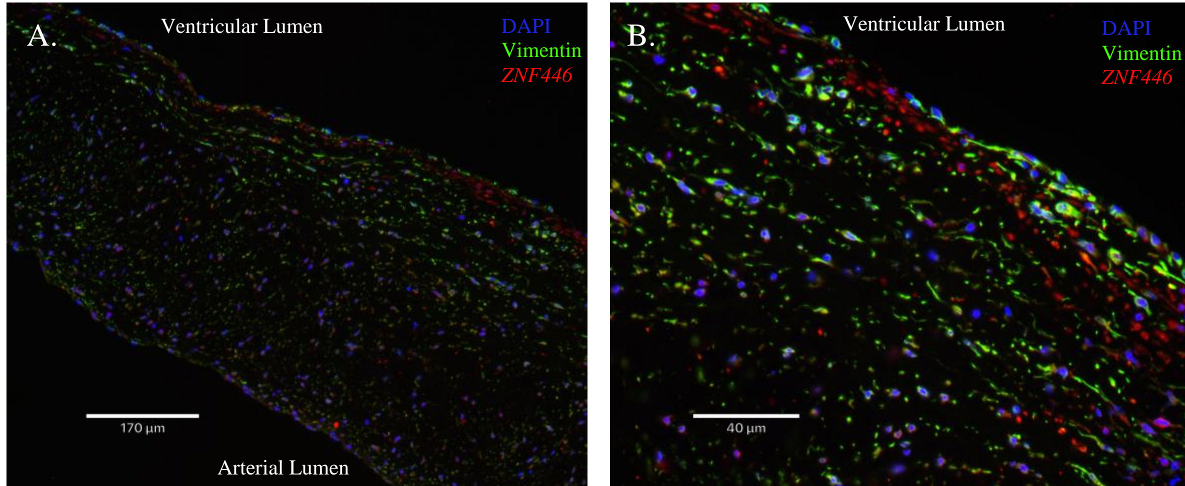
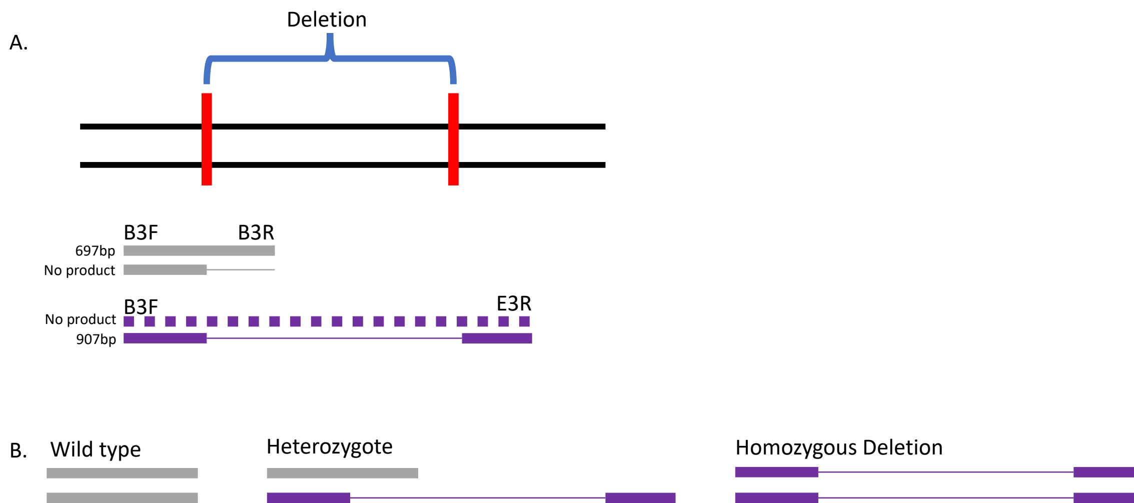
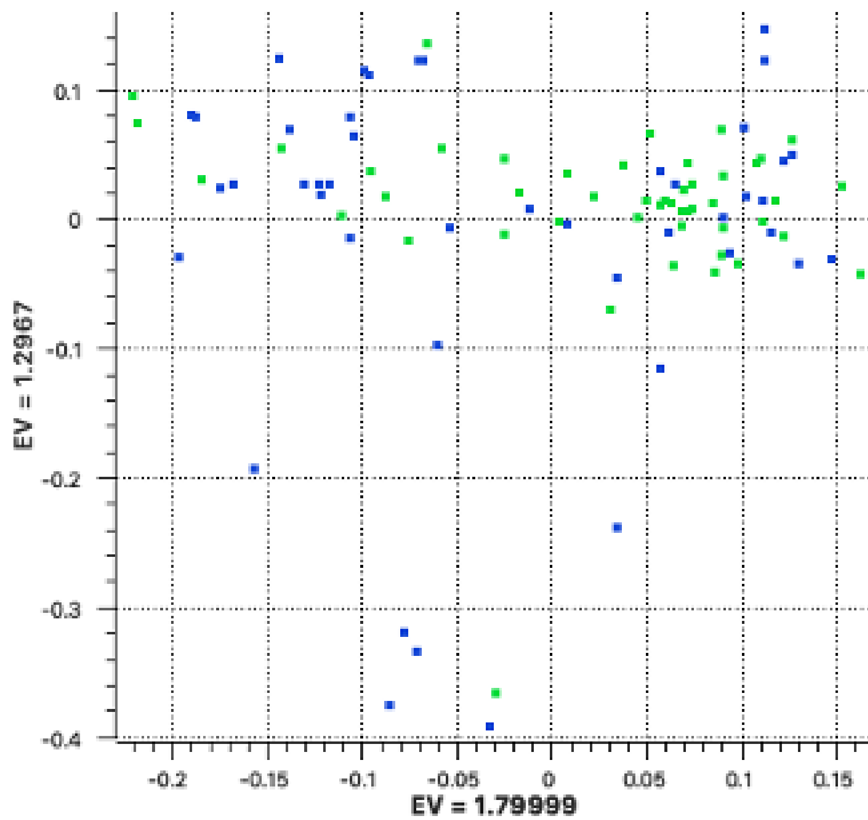


Figure 3.4 Representative immunofluorescence image of a normal canine pulmonary valve. Tissue was blocked and stained to identify the nuclei (DAPI, blue), mesenchymal cells (vimentin, green), and *ZNF446* (*ZNF446* antibody, red). The *ZNF446* transcription factor was perinuclear throughout the valve. However, there was increased cytoplasmic signaling along the ventricularis portion of the valve. A) Original magnification x 20. Scale bar 170um B) Original magnification at 40x. Scale bar 40um.



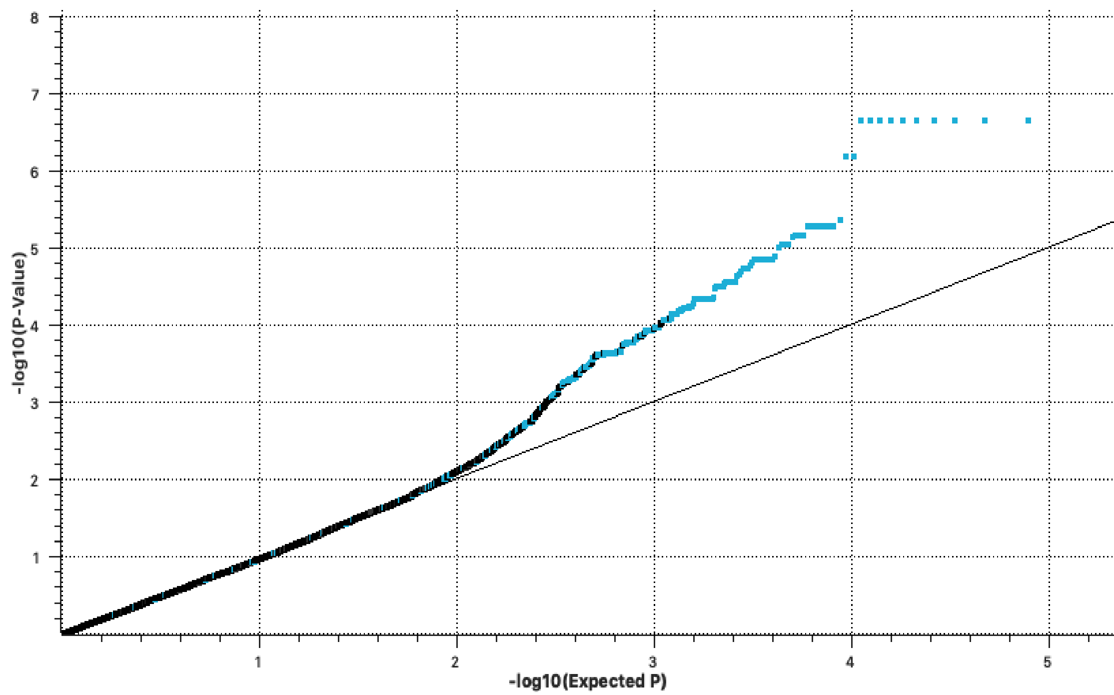
Supplemental Figure 3.1 Design of ZNF446 primer set.

A. Diagram of the products produced by a single forward primer outside the deletion, one reverse primer contained in the deleted region, and one primer outside the deleted region. B. Products expected with various genotypes. If the genotype is homozygous wild type, one product size of 697bp will be produced by the primer set B3F/B3R. No product will form from the B3F/E3R since the product size would be over 23kbp. If the genotype is homozygous for the deletion, then one product size of 907bp would be produced from the B3F/E3R. No product will be formed from the B3F/B3R since the sequence for B3R would be absent. If the genotype is heterozygous then two products would be produced.



Supplemental Figure 3.2 Principal component analysis plot of 48 cases and 45 controls included in the analysis.

Green = Cases, Blue = controls



Supplemental Figure 3.3 EMMAx Quantile-quantile plot.

Blue is SNPs on chromosome 1. Black is all other chromosomes.

Chapter 4 Conclusion and Future Perspectives

This dissertation aimed to understand the genetics surrounding PS in dogs. A literature review was composed to compare the disease in humans and dogs and identify what is currently known about the genetics of PS in both species. A retrospective analysis was performed to identify the prevalence of the disease in veterinary medicine and the most common dog breed affected with PS. A pedigree analysis was performed in that breed to discern the mode of inheritance and identify familial relationships between dogs that could influence downstream genetic analysis. A genome wide association analysis narrowed down a region of interest to be investigated by additional genetic techniques. Whole genome sequencing with subsequent structural variant analysis and a high throughput mass array screening allowed identification of a microdeletion along with several variants of interests. Finally, immunofluorescence localized the main gene of interest to the canine pulmonic valve. This compilation of research helped fill in the knowledge gap regarding PS in dogs and added a few novel genes to be investigated in human PS.

Prior to this dissertation, the genetics surrounding PS in dogs was limited. It has been well established that certain breeds of dogs, especially brachycephalic breeds, are predisposed to PS signifying a genetic cause.[1] There were several papers suggesting a recessive mode of inheritance,[2,3] but these were in breeds that are infrequently affected by PS. Importantly, these papers identified the wide spectrum that PS can present as with single litters having variable valve phenotypes, severities, and suspected incomplete penetrance. This was critical to identify because it alters the confidence at which an “unaffected” animal can serve as a control. No mutation, gene or region had yet to be identified that is associated with canine PS. However,

literature did speculate that the mutation likely involved one of the genes known to cause syndromic PS in humans.[4]

In humans, the genes involved in PS consist of ones affiliated with the RAS/MAPK signaling pathway, transcription factors, and other developmental syndromes.[5,6] These were usually identified by a chromosomal alteration or microdeletion that was investigated further to identify a causal gene of interest.[7] This stresses the genetic heterogeneity of PS, which may indicate that there may be multiple genes responsible for PS in dogs as it is in humans. The mode of inheritance varies depending on mutation, with autosomal recessive, autosomal dominant, and haploinsufficiency being common themes. With haploinsufficiency, if one copy of the gene is altered, then the other wild-type copy is unable to compensate resulting in disease. Thus, illustrating the importance of gene dosage in the genetics of PS. Importantly, known mutations for PS in humans have both incomplete penetrance and variable expressivity which complicate the identification of genes due to inaccurate phenotypic classification.

A retrospective study at the University of California – Davis identified a PS prevalence of 0.41% for all hospital admissions and 6.1% of dogs seen at the cardiology service. This was increased from previous prevalence studies.[1,8] Most cases had severe PS that required surgery. This demonstrates the impact this disease has on veterinary medicine. Bulldogs had the greatest prevalence of PS at 4.8%, suggesting it is the ideal breed to obtain samples from for genetic analysis. This is not surprising since it is well known that Bulldogs are predisposed to PS.[1,4] The other top breeds were the French Bulldog and Pitbull terrier, which are also brachycephalic breeds with numerous anatomical abnormalities that have striking similarity to some of the human syndromes. A pedigree was compiled in Bulldogs which suggested a recessive mode of

inheritance with variable expressivity. This supports what was found in previous Beagle breeding studies.[2]

A genome-wide association study in nearly 100 Bulldogs carefully phenotyped by a board-certified cardiologist allowed identification of genetic loci on Chromosome 1 that passed Bonferroni significance. Refinement of this region using an external family not used in the original analysis and assuming a recessive mode of inheritance narrowed this region to 6.5Mb. The region consisted of 79 different genes. This region did not contain any of the currently known Bulldog mutations, although chromosome one is one of the regions associated with brachycephaly.[9–11] This dissertation was the first to identify a genetic locus associated with PS in any dog breed.

Whole genome sequencing was performed in a subset of Bulldogs to allow identification of variants associated with PS. Variants were prioritized by mode of inheritance, localization to genome association region, segregation between Bulldog cases and controls, predicted effect on protein function, and association with known PS or heart development genes. In a larger cohort of Bulldogs tested by a high throughput mass array, 34 single nucleotide polymorphisms remained statistically significant. The most significant was a missense mutation in *SEMA4D,* a semaphorin expressed in the heart. Other semaphorins are known to play a role in cardiac outflow development.[12] Additional research into *SEMA4D* and the other identified variants are warranted to ascertain their role in cardiovascular development.

Since many human PS mutations are associated with microdeletions,[7] a structural variant analysis surrounding the most significant variants within the genome-wide association was performed. This identified a 22,270bp deletion that truncated the highly conserved ZNF446 transcription factor. Transcription factors have been highly associated with the development of

PS in humans.[13] ZNF446 has been shown to alter expression of proteins involved in the MAPK signaling pathway which is a critical pathway that if altered leads to the development of PS in humans.[14] This transcription factor was first isolated from human embryonic heart cDNA, but is found in the adult human heart as well.[14] Immunofluorescence confirmed its present in normal adult canine pulmonic valves. A future direction to evaluate its distribution in affected and unaffected Bulldogs would be beneficial if tissue becomes available.

Although this mutation did not segregate perfectly with Bulldog PS, it did have a surprising genotypic-phenotypic distribution. This deletion was not found in the homozygous state in over 155 Bulldogs analyzed suggesting that embryonic lethality may be occurring. Another unusual finding is that the deletion occurred in unaffected Bulldogs and thus may be conferring a protective mechanism against PS when it occurs in the genetic background of the Bulldog. It was not found in any French Bulldogs regardless of PS status or 139 PS dogs of other breeds. In a larger population of 945 canine WGS data of unknown cardiac status there was a prevalence of 4.7%. There was a surprising distribution in Miniature Schnauzers, 19 with the deletion and 19 without the deletion. The status of these Miniature Schnauzers is unknown, but it is another breed frequently cited in the literature predisposed to PS and shares a haplotype with Bulldogs.[15,16]

Overall, this dissertation helped to understand the genetic architecture of PS in Bulldogs, but future functional work is necessary to claim causation. Additional steps would be to interrogate ZNF446 and other identified genes to determine if over-expression or under-expression in a mice or zebrafish model alters cardiac development. RNAseq or immunofluorescence should be performed in normal and stenotic Bulldog valves to quantify if there are significant differences in between the two groups regarding localization and/or

expression. If there are differences, ChIP-sequencing could be performed to identify the DNA binding sites of the transcription factor which could detect additional genes critical to heart formation. Since Miniature Schnauzers had a high prevalence of the deletion, that breed should be phenotyped and genotyped to determine if it segregates with that breed as well. It would be interesting to perform a candidate gene approach in Bulldogs to determine if Bulldogs are fixed for any other known PS genes in humans. Finally, future research could look for causative genes in other breeds affected by PS.

One of the goals was to develop a genetic test in Bulldogs to inform breeding practice and reduce the prevalence of the disease. Using this mutation as a genetic test is currently premature and would require validation in a larger, separate population of dogs. However, with the current knowledge about the ZNF446 mutation in the breed, it would be advised to breed carriers with wildtype dogs that do not have PS, but avoid breeding two carriers since that may result in an embryonic lethal. Even if this research does not result in a genetic test, it contributes to the literature about the genetic architecture of the PS since neither of the two main mutations have been associated with PS yet. It also highlights the utility of using dogs as a naturally occurring model for PS to advance genetics.

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Addendum

Chapter 5: Unraveling the Genetics Behind Equid Heart Disease

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Keywords

- Ventricular septal defects
- Congenital heart disease
- Sudden death
- Atrial fibrillation
- Aortic rupture
- Atrioventricular block
- Horse
- Donkey

Key Points

- No genetic analyses have been published for aortic rupture in Friesians or atrioventricular block in donkeys despite strong evidence for a genetic cause.
- Arabians have a high predisposition to congenital heart diseases. A significant genome-wide association locus was found on chromosome 25 for ventricular septal defects in Arabians.

- Although a genome-wide association study for sudden death in racehorses was negative, a genetic cause remains a top theory for partially explaining this condition.
- Standardbred horses have a heritability of 29.6% for atrial fibrillation with a complex mode of inheritance, including polygenicity and environmental factors.

Introduction

The genetics for many cardiac diseases in equids are poorly understood. There are several diseases suspected to have a genetic cause based on increased incidence in a single breed. For example, Friesians are overrepresented for aortic rupture.[1–5] Although a common sire has been implicated for some cases[6] and the Friesian breed has the lowest level of genetic variation among horses due to a severe bottleneck,[7] to date, no genetic studies have been performed to identify the likely genetic cause of aortic rupture in the breed. Similarly, there are several reports for atrioventricular block in donkeys and miniature donkeys.[8,9] It is suspected to be genetic, because of the young age of onset and familial history of similar clinical signs, but not yet proven.[10,11] To date, genetic investigation of equine cardiac disease remains limited to a small number of conditions that are expanded upon in this article.

Ventricular Septal Defects and Other Congenital Heart Defects in Arabians

Ventricular septal defects (VSDs) are the most common congenital heart defects (CHDs) reported in horses.[12–14] VSDs are characterized by an abnormal communication between the left and right ventricles that varies in size, location, and clinical relevance.[15] Even small, high-velocity defects, known as restrictive VSDs, can lead to impaired cardiac function if the cusps of

the aortic valve are drawn into the defect, resulting in aortic insufficiency. When severe, these lesions result in volume overload of the left side of the heart and, ultimately, lead to congestive heart failure.

VSDs are suspected based on auscultation of a loud holosystolic murmur with a point of maximal intensity over the right thorax caused by the shunt. In addition, a holosystolic crescendo-decrescendo murmur most audible over the pulmonary valve region may be heard owing to an increased volume of blood leaving the right ventricle.[16] A diagnosis of VSD is confirmed via either echocardiography or necropsy.[15] With cardiac ultrasound, the defect is visualized as a communication between the two ventricles (Fig. 1). Velocity and direction of flow through the defect are measured to determine if the defect is restrictive (hemodynamically insignificant) or nonrestrictive. The downstream effects of volume overload, such as dilation of the pulmonary artery, dilation of the left atrium, and eccentric hypertrophy of the left ventricle, may be present.

VSDs had a prevalence of 0.35% in a referral population of horses.[14] Warmbloods, standardbreds, and Arabian horses had a higher predisposition to VSDs, whereas thoroughbreds had a lower predisposition.[14,15] Case reports suggest an association between Arabian horses and CHDs, including VSDs; therefore, a genetic basis in the breed is considered likely.[12,17] A familial form of VSDs has been reported in other veterinary species, including pigs and dogs.[18,19] Familial forms of VSDs occur in humans, with a reported heritability of 0.57.[20] The heritability of VSDs in horses has not been reported. A pilot genome-wide association study in 8 VSD-affected Arabians and 30 unaffected Arabians identified a significant region of association with VSD on chromosome 25; however, a gene has yet to be identified.[21] The results suggest that perimembranous VSDs are genetically distinct from complex VSDs that occur as part of

tetralogy of Fallot.[21] In humans, embryologic cardiac genes, such as GATA4, have been associated with VSDs, but further research is required to evaluate if the same genes are involved in horses.[22]

In addition to VSDs, Arabians are predisposed to many other complex CHDs; however, the genetics of other CHDs have not yet been studied. Some of these other defects include isolated or combinations of the following: tricuspid valve dysplasia, mitral valve dysplasia, truncus arteriosus, tetralogy of Fallot, pentalogy of Fallot, patent ductus arteriosus, pulmonic stenosis, tricuspid stenosis, pulmonary atresia, and overriding aorta.12,[23–25] More research is necessary to determine if these defects represent a spectrum of the same genetic mutation or if separate mutations are responsible for each CHD.

Sudden Death in Racehorses

Sudden death is a devastating event that affects the horse-racing industry annually, especially in thoroughbreds.[26–28] Despite a negative result in an early genetic investigation,[28] a genetic cause remains a top theory for explaining this condition. Sudden death represents a rapid and unexpected decline in the condition of an apparently healthy horse.[29] It is the second leading cause of death at racetracks, with the prevalence ranging from 12% to 32% for all postmortem examinations.[28,30,31] Sudden death in horses is suspected to be cardiac related and is often associated with physical activity.[32] One possible cause is a fatal cardiac arrhythmia, because arrhythmias are sometimes associated with exercise in horses.[33–37] Although many horses that die suddenly have negative necropsy findings, common findings include myocardial fibrosis, pulmonary hemorrhage, cardiac lesions, cardiopulmonary failure, and idiopathic blood vessel rupture.[28,32,38,39] Fibrosis could be a contributing factor for promoting an arrhythmogenic

substrate and subsequent sudden death in racing horses, but more research is required to confirm this hypothesis.

The cause of many sudden death cases remains unexplained after systematically excluding other causes, such as toxins, infection, and drug administration.[32] This knowledge, combined with the increased incidence in thoroughbred horses, suggests that genetics may play a role. A genome-wide association study performed with 35 horses that died suddenly from presumed cardiac causes and 34 horses that died of other causes did not yield a significant genetic association.[28] If the disease involves multiple genetic and environmental factors, it is likely that this study was dramatically underpowered. Continued case sampling and repeated genome-wide association studies to investigate this condition are warranted. A candidate gene approach may be warranted in racehorse sudden death to investigate some of the human sudden death genetic associations, including genetic variants within ion channels and occult cardiomyopathic genes within the horse.[40]

Atrial Fibrillation in Racehorses

Atrial fibrillation (AF) is the most common arrhythmia in horses.[14] Horses with AF typically present to the clinic with exercise intolerance or poor performance.[41,42] These clinical signs are due to disorganized electrical activity of the atria leading to an “irregularly irregular” heart rhythm. Diagnosis is confirmed with an electrocardiogram indicating the absence of identifiable P waves with irregular R-R intervals.[41] The prevalence of AF is 0.30% to 2.3% in the horse population, with reduced prevalence in studies when the horse is examined at rest.[14,43–45] Certain breeds of horses are more commonly affected than others, including warmbloods and trotters.[14] Specifically, standardbreds have an increased risk of AF, whereas quarter horses have a

reduced risk of AF.[46] There is strong evidence that a particular line of standardbred sires accounts for a high prevalence of AF cases in the breed.[47]

AF is a complex disease with both environmental and genetic causes. This arrhythmia correlates with body weight, with horses weighing more than 550 kg having an increased risk for AF.[14] The genetic component for AF in standardbreds is supported by the high heritably of AF in the breed. In a Canadian standardbred racehorse population, the heritability was 29.6% 3.9%.[46] When analyzing the effects of sex and gait in the same population, males had a higher heritability than females, both overall and within the pacer population.[48] Although this work supports a partial genetic role for AF in standardbred horses, it is approximately half of the heritability that is reported for AF in humans[49] and Irish wolfhound dogs.[50] This lower heritability found in horses may be due to differences in study design or may indicate that environmental effects play a larger role in equine AF. The mode of inheritance for AF in standardbred horses is proposed to be complex with multiple genetic variants contributing to the disease.[46,47] Future studies may aim to identify genetic loci associated with equine AF and discover novel pathways for therapeutic intervention.

Summary

There have been some strides made in understanding the genetics behind VSDs in Arabians, sudden death in racehorses, and AF in racehorses. Despite these efforts, no genetic mutation has been identified to date for any equid cardiac disease. With the advancement of genetic tools, including cost-effective whole-genome sequencing, the genetic mechanisms for equid cardiac disorders that appear to have a heritable basis will soon be determined.

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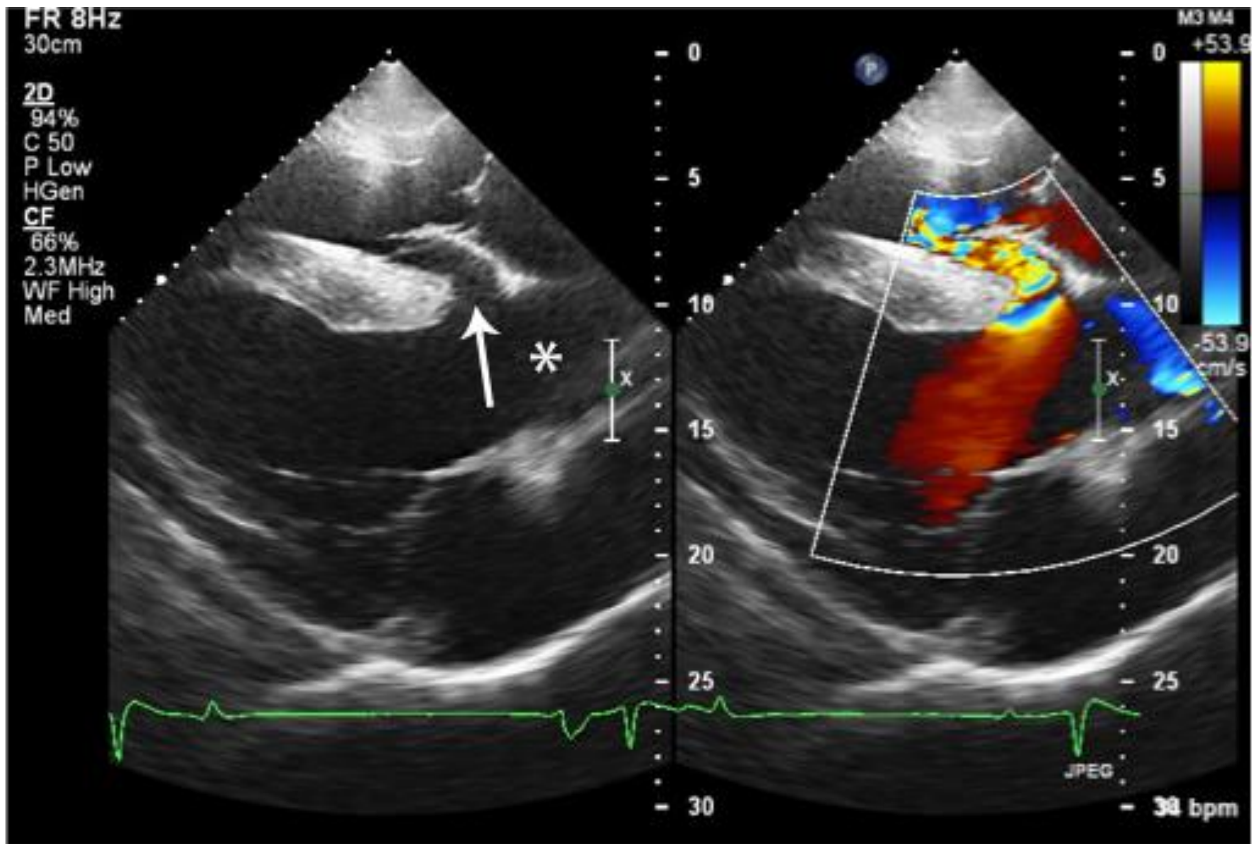

Figure 5.1 A 2-dimensional and color Doppler echocardiogram still image is provided of a 2-year-old Arabian gelding diagnosed with a VSD.

The image is obtained from the right thorax. The VSD (arrow) is visualized with turbulent flow passing into the right ventricle, just below the aortic valve (asterisk).

Chapter 6. Pedigree analysis of atrial fibrillation in Irish wolfhounds supports a high heritability with a dominant mode of inheritance

This chapter was published in 2019 in Canine genetics and epidemiology:

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Abstract

Background: Atrial fibrillation (AF) is the most common arrhythmia in dogs. The Irish Wolfhound breed has a high prevalence of AF making them an ideal breed to investigate possible genetic contributions to this disease. The aim of this study was to perform a heritability analysis in North American Irish Wolfhounds using phenotype data from cardiac screenings performed between 2000 and 2019 in order to determine how much of this disease can be attributed to genetics compared to environmental causes. The second aim was to determine the disease mode of inheritance to help inform prevention and breeding practices.

Results: There were 327 Irish Wolfhounds diagnosed with AF and 136 Irish Wolfhounds over 8 years of age without AF. The estimated mean (95% confidence interval) heritability of AF in Irish Wolfhounds was 0.69 (0.50–0.86). The pedigree was consistent with a dominant mode of inheritance.

Conclusion: Results of this study indicate a strong genetic contribution to AF in Irish Wolfhounds and suggest that future research to identify causative genetic mutations is warranted.

Plain English Summary

Atrial fibrillation (AF) is a common heart rhythm disorder in the dog that may result in clinical signs such as fainting or heart failure. Certain dog breeds, such as the Irish Wolfhound, get AF more often compared to other dog breeds. In humans, AF has high heritability, meaning that if an individual has a relative with AF, they are more likely to get AF due to genetics. This study aimed to determine if genetics contributes to AF in Irish Wolfhounds by looking at the family history of dogs with and without AF from 2000 to 2019. This study determined that in Irish Wolfhounds, AF has a high heritability. Pedigree analysis was consistent with an autosomal dominant mode of inheritance in the Irish Wolfhound meaning that a dog with AF needs to have a parent with AF as well. However, autosomal recessive and polygenic modes of inheritance cannot be definitively excluded. This has important implications for Irish Wolfhound breeding and suggests that additional research to figure out the genetic cause of AF in Irish Wolfhounds is necessary.

Background

Atrial fibrillation (AF) is the most common arrhythmia in dogs.[1] It is characterized by an "irregularly irregular" R-R interval with the absence of identifiable P waves on an electrocardiogram. The irregular frequency of ventricular contraction is due to the disorganized electrical activity of the atria.[2] This may result in reduced cardiac output leading to clinical signs such as exercise intolerance, fainting, and congestive heart failure.[3]

Atrial fibrillation has a prevalence of 0.10–0.43% in dogs seen at a veterinary clinic.[4,5,6] When adjusting the prevalence for dogs with clinical heart disease, the prevalence of AF rises to 6.27–10.5%.[4,5] Certain dog breeds such as the Irish Wolfhound have a high prevalence of AF.[7,8,9] Specifically in North America, the prevalence of AF in Irish Wolfhounds ranges from 8.9–12%

overall.[7,10] AF is typically an age-associated disease; if you adjust the prevalence to only include dogs ≥8 years it increases to 28%.[7] However, this disease can still occur in younger dogs often with more severe clinical signs.[8]

Atrial fibrillation is a complex disease that is influenced by both genetics and the environment.[11,12] In humans, AF is highly heritable.[13] For example, an individual's risk of developing AF increases if their parent or first degree relative has AF.[14,15] Despite AF being described in several veterinary species,[16,17,18,19] the only species with a reported heritability for AF is the Standardbred racehorse.[20]

Atrial fibrillation has been associated with dilated cardiomyopathy (DCM) in Irish Wolfhounds in several studies.[8,10,21,22,23] In humans, AF and DCM can be due to genetically distinct causes or caused by the same mutation.[12,24,25] The same may be true for Irish Wolfhounds because within the breed each disease can occur simultaneously, precede the diagnosis of the other, or occur in isolation.[8,22,23,26] Another possibility is that some diagnoses of DCM may be tachycardia-induced cardiomyopathy due to the increased heart rate from the arrhythmia.[27] Irish Wolfhound hearts with AF, DCM, or both cannot be differentiated based on histopathology suggesting a link between the two diseases.[28] Although AF and DCM may be linked, many of the genetic studies have thus far focused on DCM in Irish Wolfhounds or have combined AF and DCM dogs into a single group instead of examining AF dogs in isolation.[23,29,30] Fortunately, The Irish Wolfhound Foundation has been gathering annual electrocardiograms on a population of Irish Wolfhounds in North America since 2000.[7] This large dataset along with Irish Wolfhounds being overrepresented compared to other dog breeds in the prevalence of AF, is ideal to investigate the contribution of genetics to AF. Therefore, the aim of this study was to perform a heritability analysis in North American Irish Wolfhounds to

determine the genetic contribution to AF in the Irish Wolfhound breed. A second aim was to determine the mode of inheritance for AF in Irish Wolfhounds.

Methods

Historical data was obtained from the Irish Wolfhound Foundation. The Irish Wolfhound Foundation collected this data with owners' consent as part of cardiac screening efforts held primarily at dog show events between the years 2000–2019. Case inclusion criteria was a diagnosis of AF at any age obtained by 6-lead electrocardiograms recorded in conscious, non-sedated, standing Irish Wolfhounds evaluated by a board-certified veterinary cardiologist. Control inclusion criteria was any Irish Wolfhound over 8 years of age that had no identified arrhythmias by 6-lead electrocardiograms recorded in conscious, non-sedated, standing Irish Wolfhounds evaluated by a board-certified veterinary cardiologist. Case and control demographics were recorded and a chi-square test was used to determine if sex had any influence on disease classification.

Dogs were included in the pedigree analysis portion of this study if at least 3 generations of pedigree data was available. Pedigree data was obtained from a free and publicly available database for Irish Wolfhounds.[34] The pedigree was manually generated using Adobe Illustrator. A proposed mode of inheritance was identified using previously established definitions of autosomal, x-linked, recessive and dominant.[35,36]

Heritability analysis

A pedigree containing three generations for each case and each control was used to calculate heritability using MCMCglmm Package in R-software.[37] The heritability analysis was performed using a binary category analysis (i.e. control or affected with AF). This assumes a threshold

model for the liability to disease meaning that the underlying, unobservable risk for AF is assumed to be continuous. However, a diagnosis of AF only occurs when the risk exceeds a threshold of Tao = 0. A generalized mixed model with probit link function was used. A correction for ascertainment bias is not necessary because the dogs were diagnosed as either case or control as part of a breed-wide, annual, and recommended screenings instead of centering around a proband.

Results

There were 327 (153 males, 174 females) Irish Wolfhounds diagnosed with AF. There were 40 affected Irish Wolfhounds that had siblings diagnosed with AF. Specifically, 5 sets of four, 5 sets of three, and 30 sibling pairs with AF. There was not a statistically significant difference between the number of males and females diagnosed with AF ($p = 0.25$). There were 136 (50 males, 86 females) Irish Wolfhounds over 8 years of age that were never diagnosed with AF. There were significantly more females in the control group compared to males ($p = 0.002$).

The estimated mean heritability with a 95% confidence interval for AF in Irish Wolfhounds was 0.69 (0.50–0.86), while the median heritability was 0.70. There were 12 individuals where the phenotypes of both parents were known. There were 95 individuals where the phenotype of at least one parent was known. There were 356 individuals where the phenotype of either parent was unknown. Of the 561 parents of affected Irish Wolfhounds with unknown phenotype status: 9 were not old enough to meet the age restriction of 8 years at the time of writing, 93 died before the age restriction of 8 years, 21 had an arrhythmia that was not AF which disqualified them as a control, and the remainder had unknown cardiac status.

There were 74/327 (22.6%) AF individuals that had at least one parent with AF. There were 13/136 (9.6%) control Irish Wolfhounds that had at least one parent with AF. There were

27 instances of an affected male transmitting AF to a son. No dam had over five AF-affected offspring. There were four sires with over five AF-affected offspring each; two sires had AF and two sires had unknown heart status. The distribution of parental phenotypes for cases and controls is available in Table 1.

A subset of the overall pedigree is displayed in Fig. 1. Figure 1 contains a family of 13 Irish Wolfhounds (8 females, 5 males) spanning 6 generations. An affected individual is identified in every generation. There were 5 affected females and 3 affected males. Note that affected males are siring both affected male and female offspring. There are 4 matings for which both parents are known: three affected/unaffected and one affected/affected combination. There were no unaffected-to-unaffected matings in the pedigree.

X-linked recessive inheritance can be ruled out because there was no statistically significant difference between the number of males and females diagnosed with AF. X-linked dominant inheritance can be ruled out because an affected male transmitted the disease to a son 27 times in the pedigree. A dominant mode of inheritance is supported in a familial cluster because an affected individual is present in every generation when phenotype information is available (Fig. 1). Additionally, of 84 cases with at least one parental phenotype known, 74 (88.1%) have at least one parent diagnosed with AF and no case had two normal parents.

Discussion

This is the first study to characterize the genetic contribution of AF in Irish Wolfhounds. The heritability estimate of AF for Irish Wolfhounds is 69%. This is similar to what was found in a human Danish twin study,[13] but higher than what was found in Standardbred horses.[20] Additionally, this is higher than the previously reported heritability of dilated cardiomyopathy in Irish Wolfhounds, another common cardiac disease in the breed that is frequently associated with

AF.[30] The high heritability estimate obtained in Irish Wolfhounds suggests that genetic effects play an important role in disease expression. This may allow the disease to be carefully selected against to reduce its incidence in the breed.

Findings are consistent with a dominant mode of inheritance for AF in Irish Wolfhounds. This dominant mode of inheritance is similar to what has been found in some human familial forms of AF,[31] but AF in humans is complex with many genes contributing to the development of the disease. The Irish Wolfhound pedigree is suggestive of autosomal dominant because the majority of cases (88.1%) with a known parental phenotype have at least one parent diagnosed with AF. Although based on these numbers it seems unlikely, an autosomal recessive or polygenic mode of inheritance cannot be definitively ruled out as many of the parental phenotypes of the cases and controls were unknown.

Irish Wolfhound DCM is inherited as a major gene model with additional polygenic and sex dependent components.[30] Although the polygenicity of AF cannot be ruled out in this study, X-linked inheritance was ruled out. The polygenicity of DCM in Irish Wolfhounds is supported by a genome-wide association study identifying multiple loci and a study that suggests multiple loci better predicts DCM onset compared to a single loci.[23,29] To the authors' knowledge, no loci have been identified in dogs with AF as the sole criteria for a case. Future genome wide association studies using AF cases compared to controls would be useful to confirm whether AF is polygenic or due to a single gene, as well as determine if AF and DCM share the same genetic loci.

Although a dominant mode of inheritance permits rapid selection against a disease, caution is advised as this approach can dramatically limit genetic diversity, particularly when the disease prevalence is high (as seen with AF in Irish Wolfhounds). Ideally, this insight into

heritability and pattern of inheritance will lead to continued research and discovery of a genetic mutation associated with AF in the Irish Wolfhound breed. To avoid decreasing genetic diversity in the breed, a genetic test would be useful to permit a slower reduction in the incidence of disease by avoiding breeding homozygous-mutant dogs or breeding two heterozygous individuals. A genetic test would also allow the identification of individuals who may develop AF after breeding age or need to be screened more often for AF. Another way to avoid substantially decreasing genetic diversity is by limiting removal of individuals from the breeding pool to only those that are diagnosed at a young age or have an aggressive form of AF that is associated with Irish Wolfhound type cardiomyopathy.[7]

A limitation of this study is that many of the cases and controls did not have parental phenotypes. This may represent a bias in the population due to the way data was collected. The screening events occurred at dog shows with the recommendation that relatives of AF individuals receive follow-up by a local veterinary facility and veterinary cardiologist. Although efforts were made to encourage dogs diagnosed with AF to come to screenings, if a dog is diagnosed with AF by a local veterinarian, those individuals may never attend the dog show screening events and thus be underrepresented in this study.

The lower number of controls compared to cases in the study is likely due to the difficulty of obtaining electrocardiograms on older Irish Wolfhounds. Lowering the age cutoff would allow more parental and litter phenotypes to be known but would also reduce the confidence that the dog would not have developed AF in the future. The median lifespan of an Irish Wolfhound is 7.5 years,[32] however 8 years old was the age used as the control cutoff because a diagnosis of AF is known to increase with age and the investigators considered phenotypic certainty for the unaffected group to be of paramount importance for this study.[7]

Consequently, many dogs passed away before they could be considered a control. Although the Irish Wolfhound Foundation subsidized the screening costs for veteran dogs, senior Irish Wolfhounds are beyond typical breeding age, therefore fewer individuals are likely to receive a cardiac screening unless warranted due to clinical signs.

Another limitation is that only a diagnosis of AF was used for phenotyping and not a diagnosis of AF and DCM. To reduce limiting the number of phenotyped cases and controls, only AF was included in phenotyping for this study because the majority of dogs in the historical dataset did not receive an echocardiographic examination. Only including AF-affected dogs would prevent exclusion of affected individuals that died before developing DCM or those that never received a follow-up heart test. Unfortunately, this means that controls that had DCM but not AF would not have been excluded or cases that only had DCM would not be included.

Many heritability studies compare inbreeding coefficients between cases and controls. However, pedigree-based inbreeding coefficients were not calculated in this study because a previous study found that due to recent population expansion in the Irish Wolfhound breed, the 10-generation inbreeding coefficient is underestimated and inaccurate.[33]

Conclusions

Irish Wolfhounds have a high heritability estimate for atrial fibrillation. A pedigree analysis suggests a dominant mode of inheritance. Future research is necessary to identify the genetic variant(s) driving these high heritability estimates. Once identified, the development of a genetic test is recommended to assist breeding efforts aimed at reducing the incidence of AF in the breed.

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Table 6.1: Distribution of parent phenotypes for Irish Wolfhounds with atrial fibrillation or without atrial fibrillation

Sire Phenotype	Dam Phenotype	Case	Control	Total
Atrial fibrillation	Atrial fibrillation	3	1	4
Atrial fibrillation	Unknown	46	3	49
Unknown	Atrial fibrillation	19	7	26
Atrial fibrillation	Normal	5	1	6
Normal	Atrial fibrillation	1	1	2
Unknown	Normal	8	7	15
Normal	Unknown	2	3	5
Normal	Normal	0	0	0
Unknown	Unknown	243	113	356
Total		327	136	463

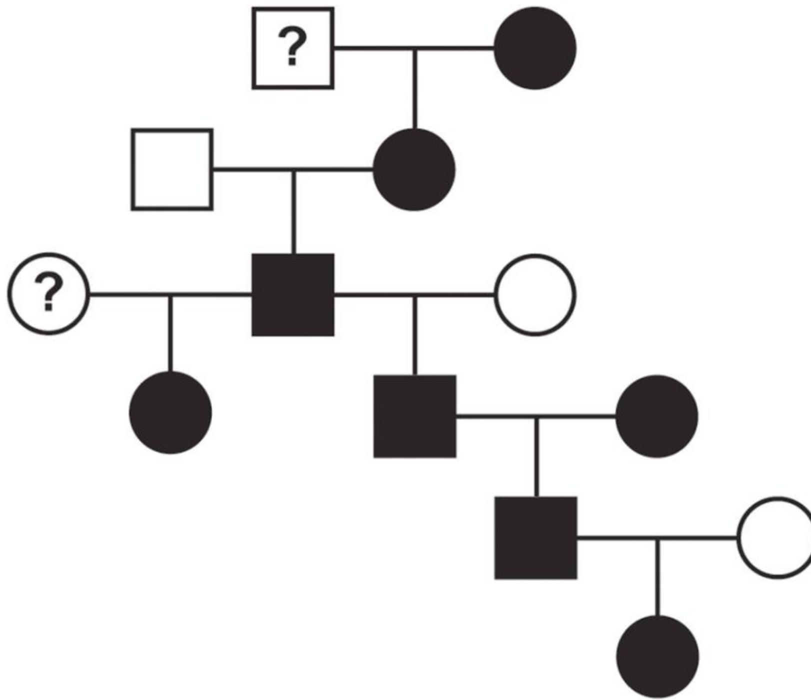

Figure 6.1 An Irish Wolfhound family pedigree that supports a dominant mode of inheritance for atrial fibrillation.

Square = male. Circle = female. White = no atrial fibrillation over eight years of age. Black = diagnosed with atrial fibrillation at any age. Question mark = no cardiac phenotype information available to classify as affected or unaffected

Chapter 7. Varying expression of mu and kappa opioid receptors in cockatiels (Nymphicus hollandicus) and domestic pigeons (Columba livia domestica)

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Fousse SL, Golsen BM, Sanchez-Migallon Guzman D, Paul-Murphy JR, Stern JA. Varying expression of mu and kappa opioid receptors in cockatiels (Nymphicus hollandicus) and domestic pigeons (Columba livia domestica). Frontiers in genetics. 2020 Oct 15;11:1278.

Abstract

Avian species have varying analgesic responses to opioid drugs. Some of this variability could be due to extrinsic factors such as administration route or dose. However, intrinsic factors such as gene expression or polymorphic differences in opioid receptors may be important components.

OBJECTIVES: The objectives of this study were to determine the relative gene expression and polymorphisms present for mu and kappa opioid receptors (*OPRM1* and *OPRK1*) in the cerebrum, brainstem, spinal cord, and footpad of cockatiels and pigeons.

METHODS: Tissue biopsies were obtained from 11 adult cockatiels (6 male and 5 female) and 11 adult pigeons (6 male and 5 female). RNA was extracted and qPCR was performed to determine the level of gene expression for *OPRM1* and *OPRK1* relative to a reference gene phosphoglycerate kinase 1 (*PGK1)* using the ΔΔCt method. Sanger sequencing was performed to identify polymorphisms, if present.

RESULTS: There were higher expression levels of *OPRM1* compared to *OPRK1* in all tissues examined regardless of species ($p < 0.001$, FDR $p < 0.001$) Cockatiels had less *OPRK1* expression in the cerebrum compared to pigeons ($p = 0.005$, FDR $p = 0.004$). Cockatiels had more *OPRM1* expression in the brainstem ($p = 0.045$, FDR $p = 0.029$), but less *OPRM1* expression in the footpad compared to pigeons ($p = 0.029$, FDR $p = 0.021$). No other significant differences in *OPRM1* or *OPRK1* expression were identified across species. Two missense polymorphisms were identified in *OPRK1;* none were found in *OPRM1*.

CONCLUSION: The differential expression of opioid receptors between cockatiels and pigeons could have implications for variability in analgesic response between these two species.

Introduction

Opioids are a diverse group of drugs that modify the transmission and perception of pain in vertebrates. Although three main classes of opioid receptors exist (mu, kappa, and delta), only drugs that act on mu and kappa receptors are frequently used in avian species.[1] The analgesic effects of opioids have a wide range of clinical efficacy depending on the avian species studied. For example, in cockatiels, mu agonists such as buprenorphine or hydromorphone did not yield a significant thermal antinociceptive effect when delivered intramuscularly.[2,3] However, similar doses of hydromorphone that were not effective in cockatiels induced thermal antinociception in another avian species, American kestrels.[4] The current opioids recommended for cockatiels are kappa agonists based on studies in other psittacine species and anecdotal clinical evidence.[5–10]

It is possible that this lack of thermal antinociceptive response to hydromorphone and buprenorphine in cockatiels could be due to external factors such as dose, route of administration, and/or drug metabolism. For instance, orange-winged Amazon parrots did not

have a thermal antinociceptive response to hydromorphone at 0.1 mg/kg, but did at 1 mg/kg.[10] This higher dose has not yet been evaluated in cockatiels.

Another explanation could be genetic differences in opioid receptor structure, distribution, or expression. In humans and other mammals, variable response to opioids can be due to polymorphisms that alter the expression or amino acid sequence of the opioid receptors.[11,12] The same may be true for avian species since genetically different strains of chickens have diverse responses to the same opioid administration.[13] The structure of mRNA, splicing differences, and sequence homology for opioid receptors was previously reported for three diverse avian species.[14] However, the relative tissue expression of mu and kappa receptors in healthy cockatiels has not yet been reported. Fortunately, a set of reliable qPCR reference genes across avian species has previously been developed.[15] These reference genes along with the availability of avian-specific opioid receptor primer sets allow for a robust investigation into opioid receptor expression across species and tissue types.[14,16]

A side-by-side investigation into the expression of mu opioid (*OPRM1*) and kappa opioid (*OPRK1*) receptors in various tissues of two diverse avian species, *Nymphicus hollandicus* and *Columba livia domestica*, is beneficial to determine if opioid expression or polymorphic differences exist that may explain the variability of analgesic response seen in cockatiels. The pigeon represents an ideal species for comparison since it is widely used in opioid research and can discriminate between kappa opioid agonists and mu opioid agonists.[17] This study is limited to mu and kappa opioid receptors since those are the opioids currently used and actively researched in the field of avian analgesia.[1]

Objectives

The objectives of this study were to identify polymorphisms and compare the relative expression levels for *OPRM1* and *OPRK1* mRNA using real-time PCR analysis for tissue biopsies harvested from cerebrum, brainstem, spinal cord and footpad in two avian species. These tissues were selected to represent different regions of the opioid receptor pathway and have been shown to be present in avian tissues previously.[14] Based upon previous clinical and research observations, we hypothesized that cockatiels will have higher *OPRK1* expression compared *OPRM1* expression in various tissues. Additionally, we hypothesized that cockatiels will have higher *OPRK1* expression and lower *OPRM1* expression compared to pigeons.

Materials and Methods

Animals and Ethical Information

This study used 11 (6 male and 5 female) adult domestic pigeons (*Columba livia domestica)* and 11 (6 male and 5 female) adult cockatiels (*Nymphicus hollandicus*). Cockatiels were from the research colony of the Animal Science Department at the University of California – Davis. Pigeons were purchased from an authorized campus vendor.[1] All birds were determined to be healthy based on physical examination prior to euthanasia and tissue collection. Tissue collection occurred on May 12, 2017. Birds were euthanized with intravenous administration of 100 mg/kg IV pentobarbital which adheres to the 2013 American Veterinary Medical Association Guidelines for avian euthanasia.[18] Protocols have been approved by the Institutional Animal Care and Use Committee at the University of California – Davis (IACUC #20331).

Sample Collection and Processing

Biological samples were rapidly dissected immediately after death using a separate sterile 4mm punch biopsy instrument for each sample. Punch biopsies were taken from the caudal cerebrum (telencephalon), brainstem (medulla oblongata), spinal cord (cervical and thoracic region), and center of the footpad with skin. The punch biopsy sample was immediately placed in 3mL of RNA*later*™ RNA stabilization reagent (Invitrogen Ambion) and stored at 4C for 48–72 h, after which the RNA*later*™ solution is decanted and tissue samples are stored at −80°C. Samples remained at −80°C until all samples of a single tissue type could be extracted at the same time. No tissues types were stored for <1 month or >1 year prior to extraction.

RNA Extraction

All steps of the experiment from tissue storage to analysis were performed in the investigator's laboratory (JS). The cerebrum, brainstem, and spinal cord samples were homogenized with the Bullet Blender Tissue Homogenizer (Next Advance, Troy, NY, United States). Tissue biopsy was placed in a tube containing Buffer RLT from the RNeasy® MiniKit (Qiagen, Hilden, Germany) and zirconium oxide beads weighing twice the mass of the tissue sample. The tissue was processed at full speed for 5 min, repeatedly, until completely homogenized.

Due to the fibrous nature of the footpad tissue, the footpad tissue sample was homogenized with the 1600 MiniG® (SPEX Sample Prep, Metuchen, NJ, United States). The footpad tissue biopsy was placed in a 5mL polyethylene tube SPEX Sample Prep, Metuchen, NJ, United States) containing a 3/8″ 440-stainless steel grinding ball (OPS Diagnostics, Lebanon, NJ, United States). This sample was processed at 1,000 rpm for 2 min, refrozen in liquid nitrogen, and processed again at 1,000 rpm for 2 min. The tissue was combined with Buffer RLT from the RNeasy MiniKit and processed at 1,000 rpm for 1 min repeatedly until homogenized.

Lysate from both homogenization procedures was used for RNA extraction using the RNeasy® MiniKit with the exception of spinal cord which was extracted with the RNeasy® Fibrous Tissue Kit (Qiagen, Hilden, Germany). RNA quantity and purity were assessed on the NanoDrop Spectrophotometer (ThermoFisher Scientific). The Quantitech® Reverse Transcription Kit (Qiagen, Hilden, Germany) was used to remove genomic DNA contamination if present and convert 100 ng of RNA to cDNA using the kits random hexamer primers. The cDNA was stored at −20°C until use in qPCR.

qPCR Primers

The primer sets used were previously published for avian species (Table 1).[14–16] The reference genes paired with the original opioid receptor primer set were tested if the primer sequences were available (GAPDH and ACTB).[14] Additionally, a previously published reference gene, PGK1, that was stably expressed, specific, and similar in size to the opioid products was tested.[15] All qPCR primers were validated using a melting curve analysis on pooled cDNA from both pigeons and cockatiels for all tissue types to ensure that a single product was amplified (Supplementary Figure 1). If there was not a single melting curve, those primer sets were excluded from further analysis. The products were sequenced by the UC DNA Sequencing Facility using an ABI 3730 Capillary Electrophoresis Genetic Analyzer with ABI Big Dye Terminator v3.1 Cycle Sequencing chemistry. Sequences were aligned and polymorphisms identified with DNASTAR Navigator SeqManPro software. UCSC genome browser and NCBI Ensembl was used to determine position of polymorphisms in the *Columba livia* rock pigeon assembly (GCF_000337935.1).[19]

qPCR Protocol

The quantitative real-time polymerase chain reaction (qPCR) experiment was performed according to Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines.[20] The Rotor-Gene SYBR green PCR kit (Qiagen, Hilden, Germany) was used in conjunction with the Rotogene Q cycler (Qiagen, Hilden, Germany) to perform qPCR analysis. The protocols for the qPCR primers were optimized by increasing or decreasing forward and reverse primer concentration to ensure that efficiency of each primer set was between 0.9 and 1.1. For *OPRM1* amplification a 10 µL reaction volume was used (5 µL of 2X Buffer Master Mix, 0.5 µL of 5 ng/µL of forward and reverse primer, 3 µL of water, and 1 µL of cDNA). For OPRK1 amplification a 10 µL reaction volume was used (5 µL of 2X Buffer Master Mix, 0.5 µL of 10 ng/µL of forward and reverse primer, 3 µL of water, and 1 µL of cDNA). For *PGK1* amplification a 10 µL reaction volume was used (5 µL of 2X Buffer Master Mix, 0.5 µL of 10 ng/µL of forward and reverse primer, 3 µL of water, and 1 µL of cDNA). Cycling included a hold temp of 95C for 5 min, followed by 40 cycles of 95C for 5 s and 60°C for 10 s. Samples were run in triplicate. A no-template-control and a no reverse-transcription control was run on each plate. The analysis was performed using Rotor-Gene Q Series Software Version 2.1.0.

Statistical Analysis

A sample technical triplicate was excluded if the standard deviation for the cycle threshold (Ct) values was greater than one. The following calculation of relative changes in gene expression was used: $\Delta Ct = Ct_{reference\ gene} - Ct_{opioid\ receptor\ gene}$. The ΔΔCt was calculated as $\Delta\Delta Ct = average\Delta Ct_{pigeons} - average\Delta Ct_{cockatiels}$. Fold change was reported as $2^{\Delta\Delta Ct}$. For species comparisons, a fold change greater than one identifies relatively greater gene expression in pigeons, while a fold change less than one identifies relatively greater gene expression in

cockatiels. For opioid receptor comparisons, a fold change greater than one identifies relatively greater gene expression in OPRM1 compared to OPRK1. The delta-delta CT values and fold change differences were determined using a standard formula worksheet (Mac Microsoft Office 365 Excel Version 16.20).

Statistical analysis was performed using *Prism8* (GraphPad Software, San Diego, CA, United States). A biological sample was excluded if identified as an outlier by the ROUT method (Q = 1%). To determine the influence of sex on gene distribution, a Shapiro-Wilk normality test was used to test for normality for sex in each tissue type. If both sexes had a normal distribution, an unpaired *t*-test was performed. If either sex did not pass normality, a Mann-Whitney test was performed.

To determine the effect of species on gene expression, the D'Augustino-Pearson Omnibus Normality Test was used to determine if the results were parametric. If both the cockatiel and pigeon sample set was parametric for a specific tissue type the ΔCt differences were tested using an unpaired *t*-test with results reported as (mean ± SD). If either the cockatiel and/or pigeon sample set was non-parametric for a specific tissue type the ΔCt differences were tested using a Mann-Whitney Test with results reported as (median ± inter quartile range). A statistically significant difference in ΔCt was set at a *p*-value less than 0.05. To correct for multiple testing, the False Discovery Rate (FDR) correction was utilized.

Results

There were three reference genes tested: *PGK1, GAPDH*, and *ACTB*. *GAPDH* and *ACTB* were non-specific, while *PGK1* was specific (Supplementary Figure 1). The two opioid genes were also specific to a single product (Supplementary Figure 1). Sequencing of the single

products confirmed that the expected gene was amplified (Supplementary File 1). There were 12 polymorphisms identified within the two opioid genes (Supplementary Table 1). None of the polymorphisms were in the primer binding region. Ten were predicted to be synonymous. Two were predicted to be a missense mutation in *OPRK1* resulting in an amino acid change from methionine in pigeons to either valine or isoleucine in cockatiels (Supplementary Figure 2).

The optimized cycle thresholds for *OPRK1*, *OPRM1*, and *PGK1* were 0.0996, 0.0136, and 0.3832, respectively. Kappa and mu opioid receptor mRNA were expressed in all tissues studied (Figure 1). There were no significant sex differences for the opioid receptors within each tissue type (Supplementary Table 2); therefore, the remaining data was analyzed with sexes combined (Supplementary Table 3). Data were normally distributed, and no outliers were detected, consequently all combined data was analyzed with a *t*-test. There was one pigeon and one cockatiel with *OPRK1* expression levels in the footpad below the limit of detection for this assay. There were significantly higher expression levels of *OPRM1* compared to *OPRK1* in all tissues examined regardless of species (Table 2). These differences remained significant after FDR corrections.

There was differential expression between cockatiels and pigeons for the opioid receptors tested (Table 3). The cockatiel cerebrum had 271% less *OPRK1* gene expression compared to pigeons ($p = 0.005$) (Figure 1B). The cockatiel brainstem had 49% more *OPRM1* gene expression compared to pigeon ($p = 0.045$) (Figure 1A). The cockatiel footpad had 141% less *OPRM1* gene expression compared to the pigeon ($p = 0.029$) (Figure 1A). All of these differences withstood FDR corrections (Table 3). No other statistically significant differences in *OPRK1 or OPRM1* expression for the cerebrum, brainstem, spinal cord, or footpad tissues were identified between cockatiels and pigeons (Table 3).

Discussion

This study focused on one specific aspect that could influence drug response, expression of the drug receptor. Variation of opioid receptor expression exists between tissue types within an avian species and across avian species. This is not surprising since variation in opioid receptor expression has been found in other species such as humans and rodents.[21] Similar to humans, both the cockatiel and pigeon tissues in this study had relatively lower *OPRK1* expression levels compared to *OPRM1* in all tissues with expression data.[22] However, the findings documenting the level of expression for each type of receptor were somewhat unexpected when considering clinical experience and the previous antinociception studies in cockatiels.[2,3] Kappa agonistic drugs are the current recommendation for cockatiels based on studies in other psittacine species;[5,6,8] however, there was less *OPRK1* in the cerebrum compared to pigeons and less *OPRK1* compared to *OPRM1* in cockatiel tissues overall. This suggests that other pharmacokinetic or pharmacodynamic factors in addition to opioid receptor expression may be responsible for the varying analgesic response in avian species and should be investigated further.

Only two polymorphisms predicted to alter protein structure were identified. Both affected amino acid 221 in *OPRK1* in birds, corresponding to amino acid 131 or 220 in the *OPRK1* receptor in humans depending on the transcript. A missense variant at this position has been identified in humans (Ensembl rs1195432072), however no phenotype data was available and the effect is predicted to be benign by polyphen. This position is also poorly conserved across vertebrates (Supplementary Figure 2).[19] Polymorphisms in opioid receptors

could alter drug response.[12] Although this variant is unlikely to influence gene expression, verifying its effect could be an avenue of future research.

Interestingly, there was decreased *OPRM1* expression in cockatiel footpad compared to pigeon footpad which supports a possible explanation for the lack of analgesic effect in cockatiels receiving mu-agonists during thermal foot withdrawal antinociceptive studies.[2,3] A large limitation of this study is a potential lack of power due to the small number of animals utilized. It is possible that some differences in opioid receptor distributions were not identified due to the limited sample size. Therefore, additional work with larger samples sizes is necessary to determine if the results found in this study are valid. If they are valid, future studies may aim to evaluate expression levels of *OPRM1* in other peripheral tissues used in antinociception studies.

Another possibility for observed differences in opioid response that was not addressed in this study was the presence of alternate transcripts of opioid receptors. A previous study in birds found that *OPRM1* had several different splicing transcripts that varied within and across species.[14] The reported splicing variants in avian species occurred at the 3′ end of the gene involving exons 3 and 4, but splicing variants at the 5′ end were not investigated.[14] In mice, rats, and humans there are splicing variants that lack exon 1 or exon 2.[23] This study used a single primer set contained within exon 2 to interrogate the expression of *OPRM1*. While this would allow the 3′ end splicing variants to be avoided, if splicing variants involving exon 2 are present in avian species, they would be missed by this study and likely lower the perceived expression levels of the receptor.

It is important to note that although opioid receptors are critical to the endogenous response to pain; they also participate in other normal physiological functions. However, the

expression levels in those other tissues were not the focus of this study; therefore, the observed difference in level of expression for each receptor type does not distinguish its role in other diverse physiological functions.

It is possible that there are more detailed differences in opioid receptor gene expression that were not detected by this study. For example, in a separate study that used autoradiographic receptor binding techniques, there were differences in opioid distributions in specific regions of the pigeon brain.[24] Although efforts were made to take punch biopsies in repeatable, specific locations from each individual, gene expression differences may have been identified if finer dissections of the neurological opioid pathway were performed. For example, interrogating specific regions of the brain known to have a large role in analgesia such as the thalamus may have yielded species difference undetected by this study. Another limitation is that this study only evaluated two opioid receptors, *OPRM1* and *OPRK1*, and two avian species. It is conceivable that some of the variability in antinociceptive response may be due differential expression of other opioid receptors such as delta or differential expression in other species. An important limitation is that this study only looked at mRNA expression levels, not protein levels which could influence drug affinity for the receptors. More work is necessary to investigate the impact that the variation of gene expression has on opioid response.

Conclusion

This preliminary study identified differential expression of mu and kappa opioid receptor genes within tissues of individual avian species and compared between two avian species. However, future research is necessary to determine if the expression differences between these two species occurs at a functional protein level, if these differences occur in other avian species, and if these differences influence analgesic responses.

Endnote

1. ^ Stuart's Farm Fresh

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Table 7.1. Primer sets tested for qPCR

Gene	Species	Location	Size (base pairs)	Primer set F 5′–3′ R 5′–3′	Efficiency
OPRM1 (16)	GenBank: DV580289.2 *Taeniopygia guttata* (Zebra finch)	Exon 2	165	GCAGATGCCCTAGCAACAAG CACGTAGCGATCCACACTCA	1.04
OPRK1 (14)	*Amazona aestiva* (Blue-fronted Amazon Parrot) NCBI:txid12930	Spans intron 3	176	CACCTCTCAAGGCAAAGATAA ACAGATTTTCATGAAGATGTCCC	1.00
PGK1 (15)	*Gallus gallus* Chicken NM_204985	Spans intron 1	167	AAAGTTCAGGATAAGATCCAGCTG GCCATCAGGTCCTTGACAAT	0.98
GAPDH (14)	*Amazona aestiva* (Blue-fronted Amazon Parrot) NCBI:txid12930	Unknown	177	GCCATTCCTCCACCTTTGATG GCTGTGTGTTCGGCTCACTC	Excluded
ACTB (14)	*Amazona aestiva* (Blue-fronted Amazon Parrot) NCBI:txid12930	Unknown	200	CAACTGGGATGACATGGAGA GCACAGCCTGGATGGCCAC	Excluded

Table 7.2. Fold change expression differences between OPRM1 and OPRK1 in cerebrum, brainstem, spinal cord, and foot pad of cockatiels and pigeons.

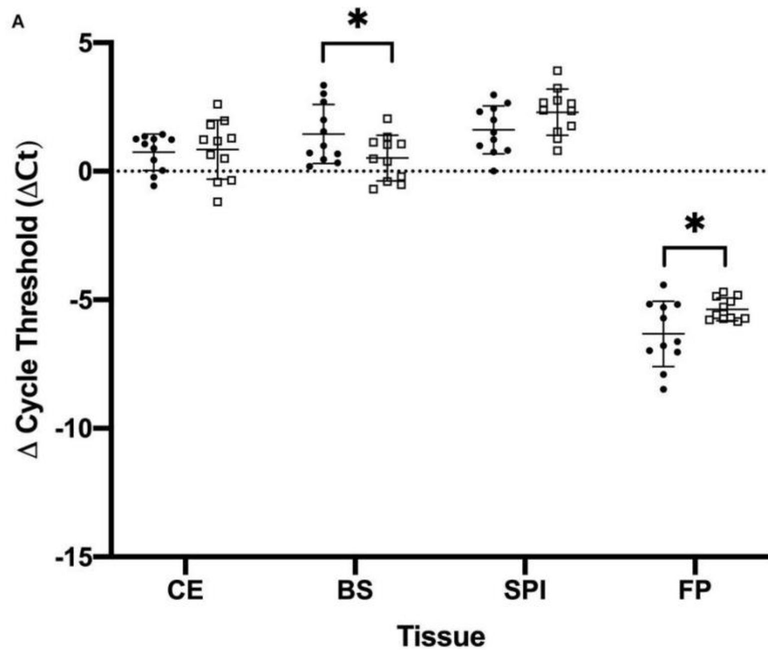
Species	Tissue	Fold-change	*P*-value	*P*-value (FDR)
Cockatiel	Cerebrum	10.25	<0.0001*	<0.0001
Pigeon	Cerebrum	3.49	0.0003*	0.0002
Cockatiel	Brainstem	88.54	<0.0001*	<0.0001
Pigeon	Brainstem	82.78	<0.0001*	<0.0001
Cockatiel	Spinal cord	39.02	<0.0001*	<0.0001
Pigeon	Spinal cord	42.99	<0.0001*	<0.0001
Cockatiel	Foot pad	20.03	<0.0001*	<0.0001
Pigeon	Foot pad	47.61	<0.0001*	<0.0001

A fold change of greater than one identifies relatively greater gene expression in OPRM1 compared to OPRK1. An asterisk indicates statistically significant p-values.

Table 7.3. Fold change expression differences between cockatiels and pigeons for OPRK1 and OPRM1 in the cerebrum, brainstem, spinal cord, and foot pad.

Primer set	Tissue	Fold-change	*P*-value	*P*-value (FDR)
OPRK1	Cerebrum	2.71	0.005*	0.0039*
OPRK1	Brainstem	0.50	0.352	0.1778
OPRK1	Spinal Cord	1.68	0.194	0.1055
OPRK1	Foot Pad	0.72	0.421	0.1984
OPRM1	Cerebrum	1.05	0.804	0.3553
OPRM1	Brainstem	0.49	0.045*	0.0289*
OPRM1	Spinal Cord	1.77	0.094	0.0554
OPRM1	Foot Pad	1.41	0.029*	0.0205*

A fold change greater than one identifies relatively greater gene expression in pigeons, while a fold change less than one identifies relatively greater gene expression in cockatiels. An asterisk indicates statistically significant p-values.



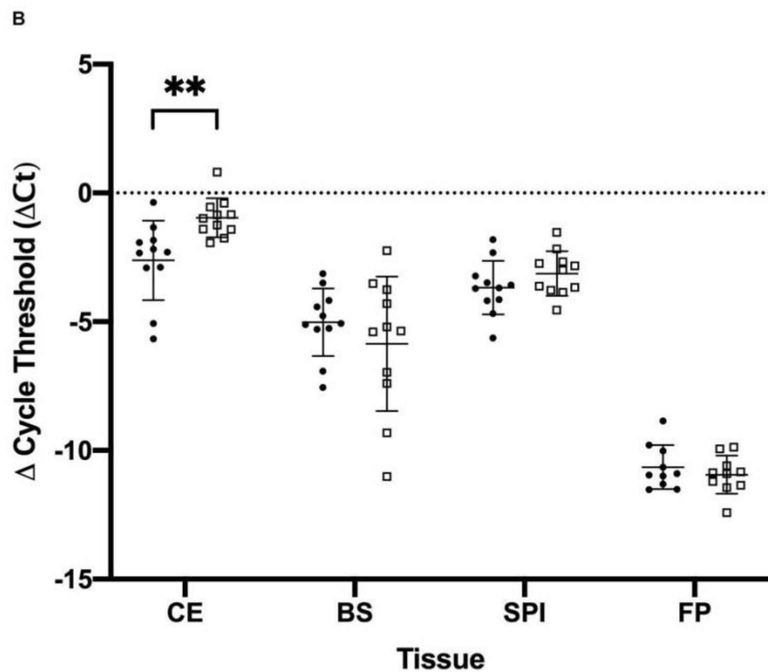


Figure 7.1. Comparison of the amount of mu opioid receptor (OPRM1) and kappa opioid receptor (OPRK1) gene expression in tissues of cockatiels and pigeons normalized to the reference gene phosphoglycerate kinase 1 (PGK1).

(A) OPRM1 (B) OPRK1. Mean and standard deviation are displayed. Parrots are closed circles. Pigeons are open squares. $*p < 0.05$; $**p < 0.01$.

Supplemental Table 7.1. Opioid receptor polymorphisms with respect to the predicted *Columba livia* mRNA sequences

Accession Number	Polymorphism	Individual	Predicted effect
XM_005507657.2 Predicted *Columba livia* *OPRM1* mRNA	c.154 T>C	All cockatiels	Synonymous
XM_005507657.2 Predicted *Columba livia* *OPRM1* mRNA	c. 169 C>T	All cockatiels	Synonymous
XM_005507657.2 Predicted *Columba livia* *OPRM1* mRNA	c. 181 G>A	All cockatiels	Synonymous
XM_005507657.2 Predicted *Columba livia* *OPRM1* mRNA	c.205 A>C	All cockatiels	Synonymous
XM_005507657.2 Predicted *Columba livia* *OPRM1* mRNA	c. 208 C>C/T	Cockatiels 3, 4, 7, and 8	Synonymous
XM_005507657.2 Predicted *Columba livia* *OPRM1* mRNA	c. 220 C>T	All cockatiels	Synonymous
XM_005507657.2 Predicted *Columba livia* *OPRM1* mRNA	c. 238 T>C	All cockatiels	Synonymous
XM_005507657.2 Predicted *Columba livia* *OPRM1* mRNA	c. 265 A>G	All cockatiels	Synonymous
XM_005505451.3 Predicted *Columba livia* *OPRK1* mRNA	c. 1018 T>C	All cockatiels	Synonymous
XM_005505451.3 Predicted *Columba livia* *OPRK1* mRNA	c. 1105 C>T	All cockatiels	Synonymous
XM_005505451.3 Predicted *Columba livia* *OPRK1* mRNA	c. 1133 G>A	All cockatiels	Missense p.Val221Met
XM_005505451.3 Predicted *Columba livia* *OPRK1* mRNA	c. 1135 G>G/T	Cockatiels 3, 4, and 6	Missense p.Val221Ile

Supplemental Table 7.2. Significance of sex effect on gene expression for each tissue type and opioid receptor

	OPRK1 p-values	*OPRM1* p-values
Cockatiel Cerebrum	0.66#	0.60
Pigeon Cerebrum	>0.99#	0.91
Cockatiel Brainstem	0.11	0.62
Pigeon Brainstem	0.62	0.59
Cockatiel Spinal Cord	0.24	0.61
Pigeon Spinal Cord	0.78	0.77
Cockatiel Footpad	0.83	0.33
Pigeon Footpad	0.88	0.59

Samples did not pass normality and a non-parametric Mann-Whitney test was used to determine significance

Supplemental Table 7.3. Delta Ct values for each tissue type, receptor, species, and sex

	Ct Value	Species (Cockatiel=1, Pigeon = 0)	Sex (M=1, F=0)	Receptor (0=OPRK1, 1=OPRM1)	Tissue (0=cerebrum, 1=brainstem, 2=spinal cord, 3=footpad)
Pt 1	-5.07	1	1	0	0
Pt 2	-2.18	1	1	0	0
Pt 3	-2.34	1	1	0	0
Pt 4	-1.34	1	1	0	0
Pt 5	-1.92	1	0	0	0
Pt 6	-2.30	1	0	0	0
Pt 7	-2.91	1	0	0	0
Pt 8	-0.37	1	1	0	0
Pt 9	-2.88	1	1	0	0
Pt 10	-5.67	1	0	0	0
Pt 11	-1.84	1	0	0	0
Pg 1	-1.94	0	0	0	0
Pg 2	-0.99	0	1	0	0
Pg 3	-0.84	0	0	0	0
Pg 4	-1.42	0	1	0	0
Pg 5	-0.85	0	1	0	0
Pg 6	-0.40	0	0	0	0
Pg 7	-1.25	0	1	0	0
Pg 8	0.81	0	1	0	0
Pg 9	-0.54	0	0	0	0
Pg 10	-1.41	0	1	0	0
Pg 11	-1.76	0	0	0	0
Pt 1	0.89	1	1	1	0
Pt 2	1.06	1	1	1	0
Pt 3	0.03	1	1	1	0
Pt 4	1.25	1	1	1	0
Pt 5	-0.23	1	0	1	0
Pt 6	1.23	1	0	1	0
Pt 7	1.36	1	0	1	0
Pt 8	1.43	1	1	1	0
Pt 9	0.44	1	1	1	0
Pt 10	1.25	1	0	1	0
Pt 11	-0.57	1	0	1	0
Pg 1	-1.19	0	0	1	0
Pg 2	1.97	0	1	1	0
Pg 3	2.61	0	0	1	0
Pg 4	1.23	0	1	1	0
Pg 5	1.28	0	1	1	0

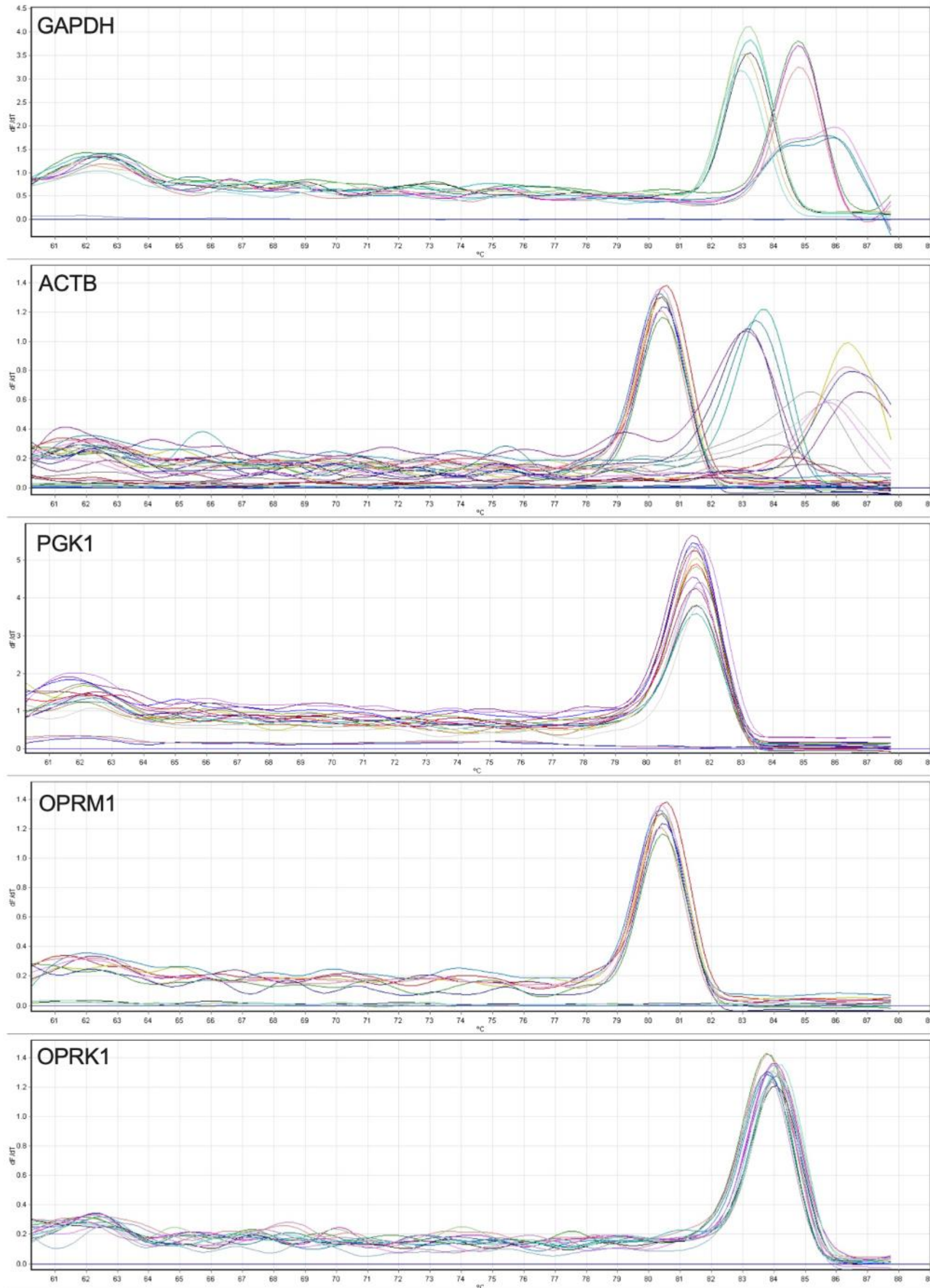
Pg 6	1.17	0	0	1	0
Pg 7	-0.35	0	1	1	0
Pg 8	0.64	0	1	1	0
Pg 9	1.81	0	0	1	0
Pg 10	0.50	0	1	1	0
Pg 11	-0.42	0	0	1	0
Pt 1	-5.26	1	1	0	1
Pt 2	-7.56	1	1	0	1
Pt 3	-6.92	1	1	0	1
Pt 4	-5.29	1	1	0	1
Pt 5	-4.78	1	0	0	1
Pt 6	-3.14	1	0	0	1
Pt 7	-5.11	1	0	0	1
Pt 8	-5.06	1	1	0	1
Pt 9	-3.49	1	1	0	1
Pt 10	-4.42	1	0	0	1
Pt 11	-4.17	1	0	0	1
Pg 1	-7.40	0	0	0	1
Pg 2	-9.31	0	1	0	1
Pg 3	-11.01	0	0	0	1
Pg 4	-6.96	0	1	0	1
Pg 5	-5.40	0	1	0	1
Pg 6	-4.30	0	0	0	1
Pg 7	-5.20	0	1	0	1
Pg 8	-3.76	0	1	0	1
Pg 9	-3.52	0	0	0	1
Pg 10	-2.24	0	1	0	1
Pg 11	-5.36	0	0	0	1
Pt 1	0.19	1	1	1	1
Pt 2	0.32	1	1	1	1
Pt 3	0.46	1	1	1	1
Pt 4	2.70	1	1	1	1
Pt 5	0.71	1	0	1	1
Pt 6	3.35	1	0	1	1
Pt 7	1.55	1	0	1	1
Pt 8	0.99	1	1	1	1
Pt 9	3.01	1	1	1	1
Pt 10	0.67	1	0	1	1
Pt 11	2.00	1	0	1	1
Pg 1	-0.40	0	0	1	1
Pg 2	0.48	0	1	1	1
Pg 3	1.33	0	0	1	1
Pg 4	-0.70	0	1	1	1
Pg 5	1.12	0	1	1	1

Pg 6	1.04	0	0	1	1
Pg 7	-0.52	0	1	1	1
Pg 8	-0.21	0	1	1	1
Pg 9	1.06	0	0	1	1
Pg 10	2.05	0	1	1	1
Pg 11	0.39	0	0	1	1
Pt 1	-1.81	1	1	0	2
Pt 2	-3.48	1	1	0	2
Pt 3	-4.14	1	1	0	2
Pt 4	-3.71	1	1	0	2
Pt 5	-5.64	1	0	0	2
Pt 6	-4.18	1	0	0	2
Pt 7	-2.32	1	0	0	2
Pt 8	-3.23	1	1	0	2
Pt 9	-3.58	1	1	0	2
Pt 10	-3.69	1	0	0	2
Pt 11	-4.68	1	0	0	2
Pg 1	-2.83	0	0	0	2
Pg 2	-1.53	0	1	0	2
Pg 3	-2.67	0	0	0	2
Pg 4	-3.77	0	1	0	2
Pg 5	-2.74	0	1	0	2
Pg 6	-2.17	0	0	0	2
Pg 7	-3.67	0	1	0	2
Pg 8	-3.62	0	1	0	2
Pg 9	-4.55	0	0	0	2
Pg 10	-3.00	0	1	0	2
Pg 11	-3.86	0	0	0	2
Pt 1	2.32	1	1	1	2
Pt 2	1.52	1	1	1	2
Pt 3	1.23	1	1	1	2
Pt 4	0.99	1	1	1	2
Pt 5	0.82	1	0	1	2
Pt 6	0.75	1	0	1	2
Pt 7	0.00	1	0	1	2
Pt 8	2.00	1	1	1	2
Pt 9	2.44	1	1	1	2
Pt 10	2.65	1	0	1	2
Pt 11	2.97	1	0	1	2
Pg 1	0.80	0	0	1	2
Pg 2	2.64	0	1	1	2
Pg 3	1.27	0	0	1	2
Pg 4	1.53	0	1	1	2
Pg 5	3.23	0	1	1	2

Pg 6	2.38	0	0	1	2
Pg 7	2.35	0	1	1	2
Pg 8	1.76	0	1	1	2
Pg 9	2.66	0	0	1	2
Pg 10	2.76	0	1	1	2
Pg 11	3.91	0	0	1	2
Pt 1	-8.86	1	1	0	3
Pt 2	-11.31	1	1	0	3
Pt 3	-10.65	1	1	0	3
Pt 4	-10.91	1	1	0	3
Pt 5	-9.80	1	0	0	3
Pt 6		1	0	0	3
Pt 7	-10.02	1	0	0	3
Pt 8	-11.51	1	1	0	3
Pt 9	-10.99	1	1	0	3
Pt 10	-11.52	1	0	0	3
Pt 11	-10.96	1	0	0	3
Pg 1		0	0	0	3
Pg 2	-10.83	0	1	0	3
Pg 3	-11.36	0	0	0	3
Pg 4	-11.44	0	1	0	3
Pg 5	-11.21	0	1	0	3
Pg 6	-9.88	0	0	0	3
Pg 7	-10.90	0	1	0	3
Pg 8	-10.87	0	1	0	3
Pg 9	-9.95	0	0	0	3
Pg 10	-10.60	0	1	0	3
Pg 11	-12.42	0	0	0	3
Pt 1	-4.42	1	1	1	3
Pt 2	-6.98	1	1	1	3
Pt 3	-8.48	1	1	1	3
Pt 4	-7.91	1	1	1	3
Pt 5	-5.17	1	0	1	3
Pt 6	-5.18	1	0	1	3
Pt 7	-6.79	1	0	1	3
Pt 8	-5.29	1	1	1	3

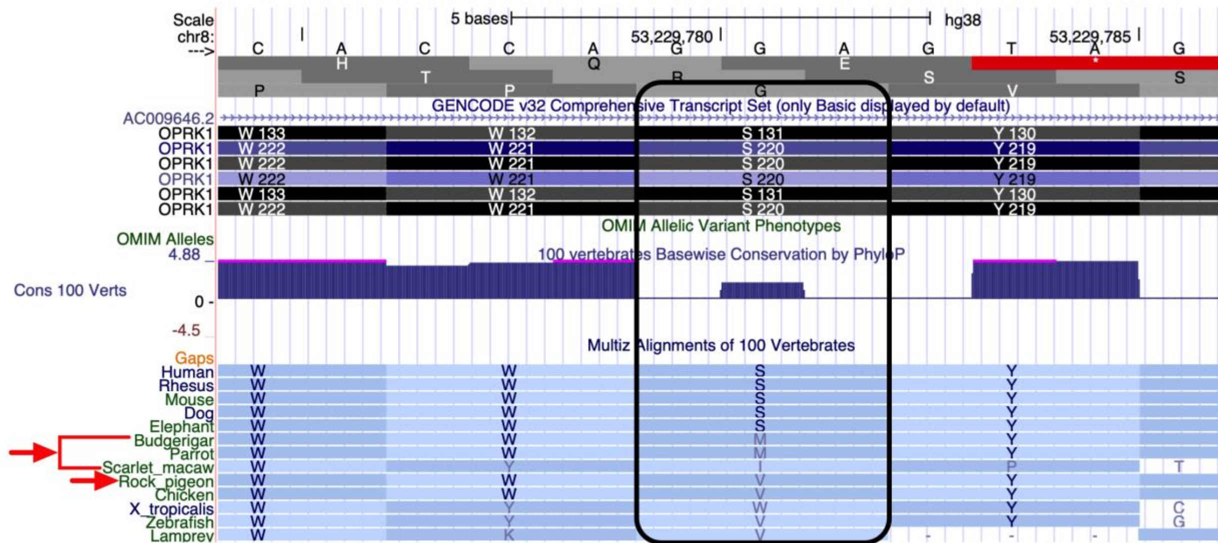
Supplemental Table 7.4 Supplemental Material Data Sheet 1 FASTA

>OPRK1 Nymphicus hollandicus 176 bp
CACCTCTCAAGGCAAAGATAATCAACATCTGTATCTGGCTATTGTCCTCATCTGTT GGTATATCTGCAATAGTTCTTGGAGGTACCAAAGTCAGGGAAGATACTGCTAGCA CTGAATGTTCCTTGCAGTTTCCAGACAGAGATTACATGTGGTGGGACATCTTCATG AAAATCTGT
>OPRK1 Columba livia domestica 176 bp
CACCTCTCAAGGCAAAGATAATCAACATCTGCATCTGGCTATTGTCCTCATCTGTT GGTATATCTGCAATAGTTCTTGGAGGTACCAAAGTCAGGGAAGATACTGCTAGCA CTGAATGCTCCTTGCAGTTTCCAGACAGAGATTACGTGTGGTGGGACATCTTCATG AAAATCTGT
>OPRM1 Nymphicus hollandicus 165 bp
GCAGATGCCCTAGCAACAAGTACCCTGCCATTCCAGAGTGTGAATTACTTAATGG GAACATGGCCATTTGGTACCATCCTTTGTAAGATTGTTATATCCATAGACTATTAC AATATGTTCACCAGTATCTTTACGCTCTGCACCATGAGTGTGGATCGCTACGTG
>OPRM1 Columba livia domestica 165 bp
GCAGATGCCCTAGCAACAAGTACTCTGCCATTCCAGAGCGTGAATTACTTGATGG GAACATGGCCATTTGGTACAATCCTTTGTAAGATCGTTATATCCATAGACTACTAC AATATGTTCACCAGTATCTTTACACTCTGCACCATGAGTGTGGATCGCTACGTG



Supplemental Figure 7.1. Specificity of primers in pigeons and cockatiels.

Melting curves of five dilutions of pooled tissue samples from all individuals for each species run in triplicate. Gene names are in the panel.



Supplemental Figure 7.2. Basewise conservation of OPRK1 missense variant illustrating poor conservation of the amino acid across vertebrates.

Image obtained from http://genome.ucsc.edu using the Human Dec 2013 (GRCh38/hg38) assembly.