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Structural and molecular pathology of the atrium in boxer arrhythmogenic cardiomyopathy

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STRUCTURAL AND MOLECULAR PATHOLOGY OF THE ATRIUM IN BOXER ARRHYTHMOGENIC CARDIOMYOPATHY

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List of Abbreviations

AC	arrhythmogenic cardiomyopathy
AF	atrial fibrillation
APC	atrial premature complexes
ARVC	arrhythmogenic right ventricular cardiomyopathy
CAC	cacodylate buffer
Cad	cadherin
Cx40	connexin 40
Cx43	connexin 43
Cx45	connexin 45
DAPI	4',6-diamidino-2-phenylindole
DP	desmoplakin
ECG	electrocardiogram
FS	fractional shortening
ICD	implantable cardioverter defibrillator
JUP	plakoglobin
L	lethargy
La	left atrium
LA/Ao	left atrial/aortic root ratio
LV	left ventricle
LVDD	left ventricular diastolic diameter
LVDS	left ventricular systolic diameter
N	Absence of clinical signs
PBS	phosphate buffered saline
PE	pulmonary edema
PKP2	plakophilin-2
Ra	right atrium

RYR2	ryanodine receptor
RV	right ventricle
S	syncope
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SVE	supraventricular ectopy
SVT	supraventricular tachycardia
TEM	transmission electron microscopy
TMEM43	transmembrane protein 43
VPC	ventricular premature complexes
VT	ventricular tachycardia
WB	western blot

Abstract

Arrhythmogenic cardiomyopathy (AC), also known as arrhythmogenic right ventricular cardiomyopathy (ARVC), is a disease characterized by fatty or fibro-fatty myocardial replacement, predominantly in the right ventricle and to a lower extent the left ventricle. It is recognized as a disease affecting the cardiac intercalated disc. Clinically, it is associated with ventricular arrhythmias, although atrial arrhythmias and atrial histopathological changes characteristic of AC have occasionally been reported. The full extent of atrial involvement in AC has not been investigated. Therefore, we aimed to apply histopathology, immunochemical detection, immunolocalization and transmission electron microscopy (TEM) techniques to characterize the distribution of desmosomal and gap junction proteins at the intercalated disc in the atria of boxers with AC. We hypothesized that histological changes consistent with AC and alterations to the intercalated disc proteins are present in the atria of boxer dogs with AC. The hearts from 14 control and 13 boxers with confirmed AC were studied. Right and left atrial sections from 11 boxers were examined by immunofluorescence. Samples from 10 boxers were used for Western blot analysis. The intercalated disc proteins investigated were connexin 43 (Cx43), connexin 45, connexin 40, plakoglobin, plakophilin-2, desmoplakin, and cadherin. Transmission electron microscopy was performed on the right and left atrial sections of 2 boxers and 2 controls. Western blot band relative density indicated a significant decrease of Cx43 in the right atrium of affected boxers compared to controls. There was no difference between controls and boxers for the other proteins investigated. Immunofluorescence analysis showed that the number of Cx43 signals and the signal intensity for plakoglobin was decreased in the left and right atrium of affected boxers. Transmission electron microscopy suggested disruption of the intercalated disc in affected boxers. In conclusion, these results indicate the alteration of intercalated disc proteins in the atrial myocardium of boxers, showing atrial involvement in

addition to the ventricles. These findings support the use of the broader term of AC rather than ARVC to describe this disease. The decrease in the amount of Cx43 in conjunction with the histological changes could represent the substrate for the atrial arrhythmias associated with AC.

Chapter 1

Introduction

Arrhythmogenic cardiomyopathy (AC) is a myocardial disease characterized by ventricular arrhythmias, syncopal episodes and sudden death in human patients (Corrado, Basso et al. 2000). The disease was originally described as a developmental condition affecting the right ventricle, and was therefore termed arrhythmogenic right ventricular dysplasia (Marcus, Fontaine et al. 1982). However, as the understanding of the disease has changed, so has the name of the disease. The use of the term arrhythmogenic right ventricular cardiomyopathy (ARVC) became more common when it was recognized as a progressive cardiomyopathy, characterized by fatty or fibro-fatty replacement of the right ventricular myocardium, suggestive of an injury and repair process (Basso, Thiene et al. 1996). Most recently, it has been shown that although the disease affects primarily the right ventricle, there is significant involvement of the left ventricular myocardium (Norman, Simpson et al. 2005). Currently, it is widely accepted that both ventricles can be affected by the disease, which has led to an expert consensus and the suggestion to change the nomenclature of the disease from arrhythmogenic right ventricular cardiomyopathy to the broader term of arrhythmogenic cardiomyopathy (Basso, Corrado et al. 2009; Sen-Chowdhry, Morgan et al. 2010). The disease has also been recognized in dogs, with boxer dogs being more commonly affected (Basso, Fox et al. 2004).

In humans, arrhythmogenic cardiomyopathy has been linked to mutations of genes coding for proteins of the desmosomes in the intercalated disc (Marcus, McKenna et al. 2010; Corrado, Basso et al. 2011). The intercalated disc is responsible for cell adhesion between cardiomyocytes and is formed by three types of intercellular connections: desmosomes, adherens junctions and gap junctions. The desmosomes and adherens junctions are responsible for the

mechanical coupling between cardiac myocytes; the gap junctions facilitate cell-to-cell propagation of the electrical impulse. Mutations in the genes coding for plakoglobin (JUP), desmoplakin (DP), plakophilin-2 (PKP2), desmoglein-2 and desmocollin-2, all proteins of the desmosomes, have been linked to AC in humans (Rampazzo, Nava et al. 2002; Gerull, Heuser et al. 2004; Heuser, Plovie et al. 2006; Pilichou, Nava et al. 2006). Immunofluorescence of ventricular tissue in humans and boxers with AC has shown a decrease in the signal of various desmosomal proteins at the level of the intercalated disc, which is more pronounced for plakoglobin (Oxford, Everitt et al. 2007; Asimaki, Tandri et al. 2009). Moreover, transmission electron microscopy (TEM) has shown a decrease in the number of desmosomes in the right ventricle of both humans and boxers with AC (Basso, Czarnowska et al. 2006; Oxford, Danko et al. 2011). In addition to the desmosomes, gap junctions are also affected by AC. Decreased expression and altered localization of connexin 43 (Cx43), a protein of the gap junctions, has been identified via immunofluorescence of ventricular tissue in humans and boxers with AC (Kaplan, Gard et al. 2004; Oxford, Everitt et al. 2007). Transmission electron microscopy of left and right ventricular samples of boxers with AC confirmed a decrease in the number of gap junctions at the level of the intercalated disc (Oxford, Danko et al. 2011). Alterations of gap junctions have been linked to arrhythmogenesis and could contribute to the arrhythmias seen with AC (Severs, Bruce et al. 2008; Basso, Corrado et al. 2009)

During evaluation of boxers presented to the cardiology services of Louisiana State and Cornell University, it was noticed that, in addition to the ventricular arrhythmias, some boxers with AC also have atrial arrhythmias. Atrial arrhythmias and sinus node dysfunction have occasionally been reported in humans and boxers with AC (Harpster 1983; Nogami, Adachi et al. 1990; Takemura, Kono et al. 2008; Thomason, Kraus et al. 2008; Chu, Zado et al. 2010). It

is possible that these atrial arrhythmias could be the cause of syncopal episodes in some boxers with AC. Syncopal episodes as a result of bradyarrhythmias and sinus node dysfunction have been reported previously in boxers with ventricular tachycardia (Thomason, Kraus et al. 2008). Atrial histopathological changes consistent with AC have been reported before in both humans and boxer dogs (Nogami, Adachi et al. 1990; Basso, Fox et al. 2004). However, careful evaluation of the atrial tissue in AC has not been performed. Therefore, the degree to which AC can affect the atrial myocardium is unknown.

We aimed to investigate if the atrial myocardium of boxers with AC is affected by the disease and whether alterations in the desmosomal and gap junction proteins at the level of the intercalated disc are present in the atrial tissue of boxers with AC. We explored this question by using histopathology, immunochemical detection, immunolocalization, and TEM to characterize the distribution of desmosomal and gap junction proteins at the level of the intercalated disc in boxer dogs. We hypothesized that histological changes consistent with AC and alterations of the proteins of the intercalated disc are present in the atria of boxer dogs with AC. If present, these changes could be responsible for the atrial arrhythmias seen with AC.

Chapter 2

Literature Review

Historical perspective on arrhythmogenic cardiomyopathy

In people, arrhythmogenic cardiomyopathy is an inherited myocardial disease characterized by fatty or fibro-fatty replacement of the right ventricular myocardium and is associated with ventricular arrhythmias and sudden death (McKenna, Thiene et al. 1994; Richardson, McKenna et al. 1996; Fontaine, Fontaliran et al. 1998). Early pathological changes may be limited to an area demarcated by the free wall of the right ventricular inflow tract, outflow tract and apex, known as the "triangle of dysplasia" (Marcus, Fontaine et al. 1982; Marcus, McKenna et al. 2010). Progression of the disease leads to broader right ventricular involvement and in many cases, left ventricular involvement as well (Corrado, Basso et al. 1997). In 1982, Marcus et al. reported 24 cases of right ventricular dysplasia with fatty replacement of the right ventricle (Marcus, Fontaine et al. 1982). At the time, the disease was believed to be a developmental disorder, hence its designation as ventricular dysplasia (Basso, Thiene et al. 1996; Azaouagh, Churzidse et al. 2011). Thiene et al. described the autosomal pattern of inheritance of AC in a group of people from Veneto, a northeast region of Italy, in 1988 (Thiene, Nava et al. 1988). These affected individuals suffered from a right ventricular cardiomyopathy with ventricular arrhythmias leading to palpitations or syncopal episodes. However, in many of these individuals, sudden death was the first sign of disease. Human disease prevalence varies based on geographical location but it has been estimated to range from 1:1000 to 1:5000 individuals (Basso, Corrado et al. 2009; Marcus, McKenna et al. 2010).

In veterinary medicine, AC is mainly thought of as a canine disease, although the first veterinary report of AC was made in a group of cats in 2000 (Fox, Maron et al. 2000). However

before this report, a primary myocardial disease, characterized by ventricular arrhythmias, syncope episodes and sudden death had long been described in boxer dogs and termed "boxer cardiomyopathy" (Harpster 1983; Harpster 1991). Pedigree analysis of boxers affected by ventricular arrhythmias has shown that these ventricular arrhythmias are inherited as an autosomal dominant trait in certain boxers (Meurs, Spier et al. 1999). It is likely that these early reports of "boxer cardiomyopathy" and inherited ventricular arrhythmias corresponded to a manifestation of AC. In 2004, Basso et al. first suggested that "boxer cardiomyopathy" may be a possible animal model of AC (Basso, Fox et al. 2004). They described the spontaneously occurring inherited "boxer cardiomyopathy" as being characterized by fibro-fatty infiltration of the right ventricular myocardium, and to a lesser extent the left ventricular and atrial myocardium. Clinically, the disease was characterized by ventricular tachycardia, right ventricular enlargement, syncope and sudden death, resembling the manifestation of AC in humans. AC has also been documented, although less frequently, in other breeds including English bulldogs (Santilli, Bontempi et al. 2009; Santilli, Bontempi et al. 2011).

Clinical perspective on arrhythmogenic cardiomyopathy

The clinical presentation of AC is similar in both humans and animals. There are three stages to the disease that are usually observed in succession as AC worsens: 1) Initially, the patient is asymptomatic and ventricular arrhythmias are an incidental finding during physical examination for an unrelated condition. 2) The patient presents for signs associated with ventricular arrhythmias, including syncope, episodic weakness or palpitations (in humans). 3) The patient presents with signs of congestive heart failure secondary to systolic dysfunction and frequently a history of weakness or syncope related to the arrhythmias (Harpster 1983; Harpster 1991; Meurs 2004; Marcus, McKenna et al. 2010). However, as previously mentioned, in some

cases sudden death is the first and only sign of the disease. In this disease, arrhythmias seem to be triggered by strenuous activity (Azaouagh, Churzidse et al. 2011). Therefore, people diagnosed with AC are encouraged not to participate in sports.

The most common physical examination finding reported, in both boxers and humans, is the detection of an arrhythmia; this can be either single premature beats or paroxysms of tachycardia. On occasion, the arrhythmia is permanent when atrial fibrillation is present. Another physical examination abnormality common with AC is the presence of a systolic murmur on auscultation. Apical systolic murmurs result from mitral or tricuspid insufficiency from the annular dilation that accompanies cardiomegaly. Less frequently, a cardiac gallop is detected (Harpster 1983; Harpster 1991; Meurs 2004). Left basilar systolic murmurs may be noted in some boxers, but these should not be linked to AC as they are usually physiologic or secondary to subaortic stenosis (Meurs 2004; Hariau and Carpenter 2010).

Electrocardiography (ECG) plays an integral part in the clinical diagnosis of AC. The most common electrocardiographic change noted is ventricular ectopy, in the form of ventricular premature complexes (singles, couplets, triplets or bigeminy) or ventricular tachycardia. The ventricular ectopic beats tend to arise from the right ventricular myocardium, and are classically described as having a left bundle branch block pattern morphology with a predominantly positive deflection of the QRS complex in leads II, III and aVF (Kraus, Moïse et al. 2002; Basso, Fox et al. 2004; Meurs 2004; Cox, van der Smagt et al. 2009). If severe, the ventricular tachycardia can lead to syncopal episodes and potentially sudden death. The presence of conduction abnormalities manifested as abnormal late potentials measured by signal averaged electrocardiography has also been associated with AC in both humans and boxers (Spier and Meurs 2004; Marcus, McKenna et al. 2010). Humans with AC can also have prolonged P-wave

duration and abnormalities in P-wave morphology, suggesting that the conduction abnormalities are not limited to the ventricles and that the atria may also be affected (Platonov, Christensen et al. 2011). In both humans and boxers with AC, atrial arrhythmias such as supraventricular tachycardia and atrial fibrillation have been reported to occur occasionally; atrioventricular block and sinus node dysfunction have been observed infrequently (Harpster 1983; Nogami, Adachi et al. 1990; Harpster 1991; Baumwart, Meurs et al. 2005; Takemura, Kono et al. 2008; Thomason, Kraus et al. 2008; Chu, Zado et al. 2010). If severe enough, these atrial arrhythmias could also lead to syncope or contribute to the progression to heart failure in human and veterinary patients afflicted with AC. Because arrhythmias can occur intermittently, AC should not be ruled out if they are absent on a screening electrocardiogram. The frequency of the ventricular beats can have as much as an 80% day to day variation with AC (Spier and Meurs 2004). To increase the possibility of identifying these arrhythmias, 24-hour Holter monitoring is recommended if there is evidence of arrhythmia on auscultation or a history of clinical signs such as syncope (Meurs 2004). On occasion, it may be necessary to perform additional Holter recordings or use an event monitor to increase the likelihood of arrhythmia detection (Meurs and Spier 2009). In humans, the presence of >500 ventricular beats in 24 hours is considered in the criteria for the diagnosis of AC (Corrado, Basso et al. 2011). In boxers the presence of >100 ventricular beats in 24 hours is suggestive of AC, especially if these ectopic beats are present in the form of couplets, triplets, bigeminy or ventricular tachycardia (Meurs and Spier 2009; Stern, Meurs et al. 2010). A Holter monitoring screening scheme has been suggested by Meurs et al., based on the number of ventricular premature complexes (VPCs) per 24 hours of 600 asymptomatic boxer dogs. It separates the affected animals into 5 groups: 1) 0-50 VPCs; within normal limits 2) 51-100 VPCs; indeterminate 3) 100-300 VPCs; suspicious 4) 100-300 VPCs with increased complexity

(couplets, triplets or ventricular tachycardia) or 300-1000 VPCs; likely affected 5) Greater than 1000 VPCs; affected, consider treatment (Meurs and Spier 2009). This classification should not be used alone but instead combined with all the other diagnostic findings before making recommendations. It should be noted that neither the number of VPCs nor the complexity of the arrhythmias can be used as a sole factor to predict the risk of an animal to become symptomatic for the disease (Meurs and Spier 2009).

Echocardiographic findings of AC can be highly variable. Although AC is a primary myocardial disease, structural changes may only be present on histopathology and not be evident on echocardiography (Meurs 2004). The expected findings on echocardiography vary depending on the stage of disease progression. The majority of asymptomatic boxers, as well as most dogs suffering from syncopal episodes, have normal echocardiograms, but signs of depressed ventricular function and chamber dilation are reported on occasion. Atrial enlargement also develops as a result of ventricular dysfunction, and is more pronounced in dogs with congestive heart failure. Although the complex shape and mechanical properties of the right ventricle prevents accurate and systematic evaluation via echocardiography (Baumwart, Meurs et al. 2009), careful examination of the right side of the heart of affected dogs may reveal localized right ventricular dilation and dysfunction (Santilli, Bontempi et al. 2011). Increases in right ventricular inflow and right ventricular outflow tract dimensions are typically noted during echocardiography in humans with AC, with approximately 62% of the patients having abnormal right ventricular function (Yoerger, Marcus et al. 2005). Right ventricular outflow tract dilation in combination with regional right ventricular akinesia, dyskinesia, or aneurysm are part of the echocardiographic diagnosis criteria of AC in humans (Marcus, McKenna et al. 2010).

Decreased right ventricular ejection fraction has been shown in AC boxers when measured via ECG- gated magnetic resonance imaging (Baumwart, Meurs et al. 2009). Ultimately, disease progression may lead to biventricular failure, making it difficult to distinguish AC from idiopathic dilated cardiomyopathy (Corrado, Basso et al. 1997; Corrado, Basso et al. 2000; Baumwart, Meurs et al. 2005; Marcus, McKenna et al. 2010).

Because of the difficulty in diagnosing AC, as well as its multiple possible manifestations, a list of diagnostic criteria was created for AC in humans in 1994. The "Task Force Criteria" combine data collected from multiple diagnostic tools, including ECG, echocardiography, genetic testing, and histopathology to establish a clinical diagnosis of AC (McKenna, Thiene et al. 1994; Corrado, Basso et al. 2011). The findings from these tests are divided into a set of major and minor criteria for the diagnosis of AC (Table 1.1). A positive diagnosis of AC based on the list of "Task Force Criteria" necessitates the presence of two major criteria, one major plus two minor criteria or four minor criteria (McKenna, Thiene et al. 1994).

The criteria were recently updated to incorporate recent advances, thereby increasing their diagnostic potential while maintaining their specificity for the disease (Marcus, McKenna et al. 2010). The modified list gives more emphasis to the family history of the patient. Indeed, a family history of confirmed disease increases the likelihood of disease in an individual from 1:1000-1:5000 to 1:2 (Marcus, McKenna et al. 2010). Because the "Task Force Criteria" were designed for the diagnosis of AC in humans, there are a number of factors that cannot be easily applied to boxers with AC. However, others are shared between humans and boxers, which makes these guidelines useful for the diagnosis of AC in boxers.

Table 1.1. Original Task Force Criteria

Major	Minor
Global or regional dysfunction and structural alteration	
• Severe dilation and reduction of RV ejection fraction with no (or only mild) LV impairment • Localized RV aneurysms (akinetic or dyskinetic areas with diastolic bulging) • Severe segmental dilation of the RV	• Mild global RV dilation and/or ejection fraction reduction with normal LV • Mild segmental dilation of the RV • Regional RV hypokinesia
Tissue characterization of wall	
• Fibrofatty replacement of myocardium on endomyocardial biopsy	
Repolarization abnormalities	
	• Inverted T waves in right precordial leads (V_2 and V_3) (people age > 12 years, in the absence of right bundle-branch block)
Depolarization/conduction abnormalities	
• Epsilon Waves or localized prolongation (>110 ms) of the QRS complex in right precordial leads (V_1 to V_3)	• Late potentials (signal averaged ECG)
Arrhythmias	
	• Left bundle-branch block-type ventricular tachycardia (sustained and nonsustained) (ECG, Holter, exercise) • Frequent ventricular extrasystoles (>1000 per 24 hours) (Holter)
Family history	
• Familial disease confirmed at necropsy or surgery	• Family history of premature sudden death (<35 years of age) due to suspected AC • Familial history (clinical diagnosis based on present criteria)

Criteria of particular interest for the diagnosis of AC in boxers include the presence of sustained or nonsustained ventricular tachycardia with left bundle branch block morphology, the number of ventricular beats in a 24-hour period, the presence of a family history of the disease, the finding of regional structural or functional changes via imaging (echocardiography, MRI), and histopathology showing fatty or fibro-fatty replacement of the ventricular myocardium. Among all the different factors evaluated by the group forming the task force, histopathological evaluation of the myocardium remained the gold standard for a definitive diagnosis of AC (McKenna, Thiene et al. 1994; Marcus, McKenna et al. 2010).

Treatment of AC aims at alleviating the clinical signs, as there is no specific treatment for the disease. In the absence of clinical signs and if the arrhythmia burden is mild, no treatment may be necessary. However, if the arrhythmia is of clinical concern or if there are collapsing episodes, antiarrhythmic therapy is implemented. In dogs, common protocols for the treatment of ventricular arrhythmias include the use of sotalol monotherapy or a combination of mexiletine and a β-blocker (Meurs and Spier 2009). The response to antiarrhythmic therapy should be assessed via 24-hour Holter monitoring, keeping in mind that there could be as much as an 80% daily variation in the number of ventricular ectopies in dogs with AC (Spier and Meurs 2004). In cases where congestive heart failure is present, standard heart failure therapy is initiated with the need for addition of antiarrhythmic therapy determined by the patients underlying rhythm. The administration of antiarrhythmic drugs can lead to a reduction in clinical signs and therefore improve quality of life but the risk for sudden death from AC is not decreased by these medications. In boxers, the use of antiarrhythmic medications as the sole therapy for controlling these malignant arrhythmias is the current standard of care. However, in humans the use of implantable cardioverter defibrillators (ICDs) in combination with antiarrhythmic medications is

recommended in individuals with syncope or hemodynamically unstable ventricular tachycardia to reduce the clinical signs and the risk of sudden death associated with AC (Azaouagh, Churzidse et al. 2011; Corrado, Basso et al. 2011). Implantable cardioverter defibrillator (ICD) therapy is not widely available in dogs and its clinical use is limited to a few case reports (Nelson, Lahmers et al. 2006; Pariaut, Saelinger et al. 2011). A recent study, evaluating the use of ICDs in 9 healthy dogs showed that ICDs can be safely implanted in dogs with adequate safety margins allowing successful defibrillation therapy (Pariaut, Saelinger et al. 2012). However, additional research is needed to improve ICD therapy in dogs. With continued research, ICD therapy may become more available in veterinary medicine, and it may become an important tool in the treatment of AC.

Histopathological perspective on arrhythmogenic cardiomyopathy

The typical histopathological changes associated with AC are fatty or fibro-fatty replacement of the right ventricular myocardium, which exhibits features of myocyte degeneration and death suggesting an injury and repair process (Basso, Thiene et al. 1996; Basso and Thiene 2005). The fatty or fibro-fatty replacement of the myocardium tends to start in the subepicardium, extending to the midmyocardium and eventually becoming transmural as the disease progresses (Basso, Corrado et al. 2009). Fatty infiltration of the right ventricle alone should not be considered as a sole diagnostic factor of AC as some intramyocardial fat can be a normal finding in the right ventricle (Azaouagh, Churzidse et al. 2011). Also, in both the fatty and fibro-fatty form of AC, there will be some degree of degenerative changes of the myocytes as well as interstitial fibrosis histologically (Basso and Thiene 2005). What has been viewed as "the classic" form of AC, is a disease process that would affect primarily the right ventricle. However, involvement of the left ventricular myocardium has been well documented in both 76

% of humans and 48 % of boxers with AC (Corrado, Basso et al. 1997; Basso, Fox et al. 2004). Fatty or fibro-fatty changes in the left and right atrial tissue have also been reported in AC with 8 out of 23 boxers (35%) having atrial changes in the first report of AC in this breed (Basso, Fox et al. 2004). Atrial changes have been documented in humans case reports; however, research studies of atrial tissues are not available probably due to inadequate examination of atrial tissues (Nogami, Adachi et al. 1990; Corrado, Basso et al. 1997). In addition to the typical fatty or fibro-fatty replacement, myocarditis associated with apoptosis has been commonly documented in humans and boxers with AC, with the fibro-fatty form of the disease showing a higher occurrence of myocarditis (Basso, Thiene et al. 1996; Basso, Fox et al. 2004). In the first report of AC in boxers, focal or multifocal lymphocytic infiltrates characteristic of myocarditis were reported in the right ventricle (61%), left ventricle (70%), and atria (17%) of AC boxers (Basso, Fox et al. 2004). At this time, it has not been determined whether myocarditis is a reaction to cell death or contributes to it (Basso, Thiene et al. 1996; Corrado, Basso et al. 2011). However, it is believed that myocarditis in AC patients could be a factor in the development of life-threatening arrhythmias (Basso, Fox et al. 2004; Corrado, Basso et al. 2011). Despite the importance of histopathology for a final diagnosis of AC, the clinical diagnosis of AC should be based on the combination of the clinical criteria described by the "Task force". Histopathological evaluation requires an invasive procedure, such as endomyocardial biopsy, that may not be feasible in all patients, especially in veterinary medicine. In addition, although diagnosis is definitive when fatty or fibro-fatty myocardial replacement is present, segmental involvement of the myocardium could lead to false negative diagnosis considering the small size of the biopsy samples (Marcus, McKenna et al. 2010; Azaouagh, Churzidse et al. 2011).

Molecular perspective on arrhythmogenic cardiomyopathy

In humans, AC is usually found to be an autosomal dominant inherited disease with incomplete penetrance. In addition, there are some recessive forms, Naxos disease and Carvajal syndrome, that are associated with skin lesions (Kaplan, Gard et al. 2004; Marcus, McKenna et al. 2010). In 1998, Naxos disease was mapped to chromosome 17 (Coonar, Protonotarios et al. 1998). The external phenotype of the disease made it ideal for the study of AC as cardiac disease could be assertively assigned by the presence of the skin and hair changes (McKoy, Protonotarios et al. 2000). As a matter of fact, it was the study of this disease that introduced the idea that AC could be a cell junction disease. In 2000, the first AC gene mutation was identified, when McKoy et al. discovered that a mutation in the Plakoglobin gene, affecting the C-terminal of the protein, was responsible for Naxos disease (McKoy, Protonotarios et al. 2000). Plakoglobin is an important protein for the formation of cell-to-cell connections in many tissues. The fact that the skin and the heart share similar cell-to-cell connections explains how a mutation in a single gene, corresponding to a protein of the desmosome and adherens junctions, can cause clinical signs associated with both organs. Currently, it is widely accepted that AC is a disease of the intercalated disc and more specifically of the desmosomes that compose it. The intercalated discs are the end-to-end connection of cardiomyocytes and are formed by three types of intercellular junctions: desmosomes, adherens junctions and gap junctions. The desmosomes and adherens junctions are primarily responsible for the mechanical coupling between cardiomyocytes, whereas the gap junctions facilitate cell-to-cell electrical impulse propagation. In recent years, mutations in many more genes that code for proteins of the desmosomes and adherens junctions have been shown to be the cause of AC. For example, a mutation in desmoplakin (a desmosomal protein) has been identified as the cause for Carvajal syndrome

(Norgett, Hatsell et al. 2000). Autosomal dominant mutations in plakoglobin (JUP), desmoplakin (DP), plakophilin-2 (PKP2), desmoglein-2 and desmocollin-2, all proteins of the desmosome, have subsequently been linked to AC in humans (Rampazzo, Nava et al. 2002; Gerull, Heuser et al. 2004; Heuser, Plovie et al. 2006; Pilichou, Nava et al. 2006). In 2005, Norman et al. described a dominant mutation in desmoplakin that produces a primarily left-sided form of AC (Norman, Simpson et al. 2005). In addition to the mutations of desmosomal proteins, mutations in the genes coding for the ryanodine receptor (RYR2) and transforming growth factor beta-3 have also been reported to be associated with AC (Tiso, Stephan et al. 2001; Beffagna, Occhi et al. 2005). The latest gene to be identified as a cause of AC was the transmembrane protein 43 (TMEM43) gene; little is known about this gene at this time but it is suspected to play a role in an adipogenic pathway (Merner, Hodgkinson et al. 2008; Corrado, Basso et al. 2011). Despite the discovery of many of the genes that can lead to AC, currently over 50% of patients with AC still do not have an identifiable genetic mutation (Saffitz 2009). In boxers with AC, a reduction in the right and left ventricular expression of the ryanodine receptor and the left ventricular levels of calstabin-2, a protein that modulates the RYR2 receptor function, have been demonstrated (Meurs, Lacombe et al. 2006; Oyama, Reiken et al. 2008). However, only one genetic mutation has been associated with AC to date, the striatin gene that codes for a protein that colocalizes with JUP, PKP2, and DP in the intercalated disc (Meurs, Mauceli et al. 2010). In this study, boxers positive for the striatin mutation had a higher number of VPCs on 24 hour Holters, with homozygous dogs being more affected than dogs that are heterozygous for the mutation. Immunofluorescence of ventricular tissue in AC boxers has shown decreased signal for PKP2, DP, and a complete lack of JUP in the intercalated disc (Oxford, Everitt et al. 2007). Immunofluorescence has gained interest as a reliable way to show

the altered desmosomal proteins, helping in the diagnosis of AC. In fact, in humans, immunofluorescence for JUP has been shown to have 91% sensitivity and 82% specificity as a diagnostic test for AC (Asimaki, Tandri et al. 2009). It has been suggested that immunofluorescence for desmosomal proteins, like JUP, could be incorporated into the diagnostic criteria in the future. Transmission electron microscopy has also been used to evaluate the intercalated disc area in both boxers and humans, showing a significantly lower number of desmosomes the right ventricle of AC affected individuals compared to controls (Basso, Czarnowska et al. 2006; Oxford, Danko et al. 2011). The fact that over 50% of humans do not have an identifiable genetic mutation and that boxers have decreased immunofluorescence signal for some desmosomal proteins, similar to human AC patients with confirmed genetic mutations, suggests that the desmosomal proteins in boxers with AC are also defective, even if a specific genetic mutation in these proteins has not been discovered at this time.

In humans, not all individuals in which a genetic mutation linked to AC is present will develop the phenotypical changes associated with the disease given the incomplete penetrance and variable expression of these genes (Corrado, Basso et al. 2011). Despite the recognition of AC as a disease of the cardiac desmosomes, the specific mechanism of how disruption of these desmosomal proteins leads to AC is still unknown. The most accepted hypothesis is that the integration of defective desmosomal proteins into the intercalated disc facilitates the detachment of myocytes at this location, especially during periods of mechanical stress, leading to the degeneration and apoptosis of cardiomyocytes with development of fibro-fatty replacement as a result of the repair process (Corrado, Basso et al. 2011). Because the desmosomes and adherens junctions provide the mechanical connection between myocytes, it seems logical that disruption to their integrity would lead to myocyte detachment and damage. However, to view this as a

simple mechanical cause and effect scenario could be oversimplifying what actually occurs, as it has also been shown that intercalated disc proteins have a complex interaction with each other and can also play a part in signaling pathways. Desmosomes have been proposed to regulate transcription genes involved in adipogenesis and apoptosis. For example, it has been suggested that nuclear translocation of plakoglobin, in desmoplakin deficient mice, mediates changes in the Wnt pathway causing the differentiation of cardiomyocytes into the adipocyte lineage (Garcia-Gras, Lombardi et al. 2006; Delmar and McKenna 2010). Decreases in Ankaryn-G, a cytoskeletal adaptor protein that is associated with the voltage-gated sodium channels at the intercalated disc, in cultured myocytes has been shown to lead to decreased PKP-2 expression and decreased intercellular adhesion strength (Sato, Coombs et al. 2011). Interestingly, a reciprocal relationship was also noted with a decrease in PKP-2 leading to a decrease in Ankaryn-G expression and decreased intercellular adhesion strength (Sato, Coombs et al. 2011). This serves to illustrate the complex and yet incompletely understood interaction between intercalated disc proteins that were previously viewed as independent complexes located within the same general area (Delmar 2012). Therefore, it is likely that the combination of the mechanical forces, the interaction of the mutated desmosomal proteins with other intercalated disc components and signaling pathways lead to the altered intercalated disc integrity rather than any one mechanism on its own.

Because of the previously discussed interaction between the intercalated disc components, genetic mutations affecting the desmosomal proteins do not only alter the integrity of the desmosomes and adherens junctions but also lead to altered gap junctions as these connections are usually found adjacent to the desmosomes in the intercalated disc. Gap junctions are composed of a collection of intercellular channels. Each intercellular channel is composed of

a pair of connexons on each side of the plasma membrane. Each connexon is composed of six connexin molecules. There are 3 main connexin proteins expressed in cardiac tissue: connexin 40 (Cx40), connexin 43 (Cx43) and connexin 45 (Cx45) (Severs, Bruce et al. 2008). Connexin 43 is the main connexin found in the gap junctions of ventricular tissue (Davis, Kanter et al. 1994). Immunofluorescence analysis of ventricular tissue in both affected humans and boxers has shown decreased Cx43 expression and altered localization, to the lateral aspect of the myocytes instead of forming part of the end to end connections (Kaplan, Gard et al. 2004; Oxford, Everitt et al. 2007). These findings have also been confirmed via transmission electron microscopy with AC affected boxers having a decrease in the number of gap junctions at the level of the intercalated disc in both the right and left ventricle (Oxford, Danko et al. 2011). Gap junctions only provide the electrical connection between cells and have been described as being particularly susceptible to shear stress (Saffitz, Hames et al. 2007). Therefore, once the mechanical connection provided by desmosomes and adherens junctions has been disrupted, the gap junctions are not able to sustain the remaining forces. Another theory on how damage to the desmosome affects the gap junction integrity is that the gap junction and desmosome are not two independent components of the intercalated disc but that on the other hand they are interconnected with molecular changes to one affecting the other. An example of this can be seen by the fact that decreased expression of PKP-2 in cultured myocytes leads to decreased Cx43 expression, suggesting that increased shear stress is not the only factor altering gap junction expression in AC (Oxford, Musa et al. 2007). Gap junction remodeling is not limited to the disease process in AC, but can occur as a consequence of disruption to the intercalated disc or cardiomyocyte integrity, as decreases and lateralization of gap junctions have also been shown to occur secondary to CHF in other cardiovascular diseases (Severs, Bruce et al. 2008).

Therefore, the alterations in gap junctions could be directly related to the pathogenesis of AC or a result of end stage disease leading to a "final common pathway" of disease regardless of the cause of cardiomyopathy (Vatta, Marcus et al. 2007). However, alterations to the gap junctions have been shown to occur in AC before the presence of cardiac pathology (Kaplan, Gard et al. 2004), suggesting that they are part of the initial pathological process in AC even if they also occur in the end stages of other cardiac diseases. Irrespective of the reason for gap junction alteration, it has been shown that alterations in their function and location can lead to arrhythmogenesis (Severs, Bruce et al. 2008). For example, in a chimeric mouse model, heterogeneous Cx43 expression in the myocardium was linked to conduction abnormalities, contractile dysfunction, and occasional ventricular tachycardia (Gutstein, Morley et al. 2001). Therefore, it is likely that altered gap junction expression and localization contribute to the arrhythmogenic substrate created by the fatty or fibro-fatty infiltration of the myocardium in AC.

In conclusion, AC is a disease dominated by ventricular arrhythmias. The substrate for these arrhythmias is likely a combination of the diffuse fatty or fibro-fatty myocardial replacement and an alteration in gap junction number and location at the molecular level. While most of the attention has been directed to the life-threatening ventricular arrhythmias, one cannot ignore the occurrence of atrial arrhythmias with AC. Therefore, we aim to investigate if the atrial myocardium of AC boxers is affected by the disease and whether alterations in desmosomal proteins and gap junctions can be detected in this tissue, potentially representing the anatomic substrate for the atrial arrhythmias.

Chapter 3

Materials and Methods

Samples

Tissues were collected from 13 boxer dogs with a clinical diagnosis of AC at 3 institutions, Louisiana State University, Cornell University and the University of Pennsylvania. Sample collection took place less than one hour after sudden death or euthanasia at the owner's request because of concurrent disease or heart disease for which the prognosis was poor. Control samples from 14 mongrel dogs without cardiac disease, based on physical examination, heart weight to body weight ratio and gross examination of the hearts were obtained after euthanasia for a separate study. Ten boxers had echocardiograms and 10 boxers had 24-Holter recordings available for a detailed analysis and quantification of arrhythmias. Genetic testing was performed as part of the clinical evaluation in 5 boxers. Full thickness right ventricular, left and right atrial samples were obtained. The samples were flash-frozen and saved at -80 ºC for western blot; fixed in 10% phosphate-buffered formalin and embedded in paraffin for histopathology and immunofluorescence; fixed in 1.25% gluteraldehyde and 2% formaldehyde in Cacodylate buffer (CAC) for transmission electron microscopy.

Pathology

Sections were cut in 5-µm thick slices. The left and right atria from all dogs were stained with hematoxylin and eosin for routine histopathologic evaluation and Masson trichrome to better characterize fibro-fatty infiltrates. The right ventricle of boxer dogs was also stained in a similar fashion to corroborate the diagnosis of AC. All the samples were classified as normal or as having fibro or fibro-fatty replacement by a blinded single investigator with expertise in cardiac pathology who was not aware of the group status.

Localization of intercalated disc proteins by immunofluorescence

Immunofluorescence was performed on full thickness left and right atrial samples of 14 control and 11 boxer dogs. The samples were fixed in 10% buffered formalin, embedded in paraffin and sectioned into 5-µm thick slices. Sections were deparaffinized and antigen retrieval performed with citrate buffer. Sections were blocked in 10% normal goat serum (Invitrogen, Carlsbad, CA) or 5% bovine serum albumin, 0.1% triton buffer for 90 minutes, and then incubated overnight at 4°C with the appropriate primary antibodies. After incubation, the samples were washed in phosphate-buffered saline (PBS) and incubated with the appropriate secondary antibodies for 1 hour at room temperature. The samples were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA) and examined with an Olympus IX71 inverted microscope. The secondary antibodies used were Alexafluor 484 goat anti-rabbit and Alexafluor 598 goat anti-mouse (Molecular Probes, Division of Invitrogen, Carlsbad, CA). During immunofluorescence analysis, both the distribution and the intensity of the immunoreactive signal were evaluated. Five 20X fields from each sample were evaluated and selected for analysis. A total of 10 intercalated discs were selected and the immunoreactive signal intensity of the protein tested was assessed with the software ImageJ (National Institutes of Health, Bethesda, MD), as previously described (Gavet and Pines 2010). The intensity per pixel of each intercalated disc was measured and the intensity per pixel of an equally sized area of background adjacent to the intercalated disc was subtracted.

The following primary antibodies were tested: polyclonal rabbit anti-cadherin (Sigma Aldrich, St. Louis, MO), polyclonal rabbit anti-desmoplakin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal mouse anti-plakoglobin (Sigma Aldrich, St. Louis, MO), polyclonal rabbit anti-connexin 43 (EMD Millipore, Billerica, MA), polyclonal rabbit anti-

connexin 40 (Invitrogen, Carlsbad, CA), polyclonal rabbit anti-connexin 45 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and monoclonal mouse anti-plakophilin 2a and 2b (Meridian Life Science, Inc., Memphis, TN).

Immunochemical detection of intercalated disc proteins

Western blot was performed on left and right atrial samples of 14 control and 10 boxer dogs. Frozen tissue samples were homogenized in Laemmli buffer and protein amount measured by RC DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% to 15% precast polyacrylamide gradient gel (Bio-Rad Laboratories, Inc., Hercules, CA), transferred onto nitrocellulose, blocked for 1 hour at room temperature with 5% non-fat milk in PBS-Tween (0.2%), and then incubated overnight at 4°C with the appropriate primary antibodies. To ensure equal loading, blots were stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Inc., Rockford, IL), blocked once more with 5% non-fat milk in PBS-Tween for 1 hour at room temperature, and then incubated with anti-beta Actin antibody (Abcam, Cambridge, MA). Western blot band relative density was calculated for all the protein tested using the software ImageJ (National Institutes of Health, Bethesda, MD), as outlined in the ImageJ documentation (Schneider, Rasband et al. 2012). Student's t test was used to compare the left and right atrial western blot band relative density, of each protein tested, of control dogs to that of boxer dogs.

The following primary antibodies were used: polyclonal rabbit anti-cadherin (Sigma Aldrich, St. Louis, MO), monoclonal mouse anti-desmoplakin (AbD Serotec, Kidlington, UK), monoclonal mouse anti-plakoglobin (Sigma Aldrich, St. Louis, MO), polyclonal rabbit anti-connexin 43 (EMD Millipore, Billerica, MA), polyclonal rabbit anti-connexin 40 (Abcam,

Cambridge, MA), polyclonal rabbit anti-connexin 45 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and monoclonal mouse anti-plakophilin 2a and 2b (Meridian Life Science, Inc., Memphis, TN).

Transmission electron microscopy

Transmission electron microscopy was performed on left and right atrial samples of 2 control and 2 Boxer dogs. The samples were fixed for 1 hour with 1.25% glutaraldehyde and 2% formaldehyde in 0.1M sodium CAC buffer, pH 7.4. After fixation, the samples were washed overnight in 0.1M CAC buffer (pH 7.4) with 5% sucrose. They were then treated with osmium tetroxide, dehydrated, infiltrated using increasing concentrations of epoxy resin and embedded in epoxy resin. The tissue samples were sectioned to 75 nm thickness and stained with uranyl acetate followed by Reynold's lead citrate and examined using a JEOL JEM-1011 transmission electron microscope equipped with AMT image capture engine. For each section, 3-5 different fields were evaluated at 5,000X magnification. The fields were selected based on the quality of the sectioning, lack of adipocyte infiltration (in AC dogs), and the amount of intercalated discs. Images were obtained at 15,000X magnification following the course of the intercalated discs.

Statistical analysis

When indicated, results are expressed as mean ± SD or as median and range. D'Agostino and Pearson normality test was used to test for Gaussian distribution. The western blot and immunofluorescence data was compared using the Student's t test when the data was normally distributed data or the Mann-Whitney U test if the data was not normally distributed. All calculations were performed using the statistical analysis software, GraphPad Prism version 5.0 (GraphPad Software, San Diego CA). A value of $P<0.05$ was considered statistically significant.

Chapter 4

Results

Animals

The demographic data of the control and AC boxers is summarized in table 4.1. Hearts were obtained from 14 controls with a median age of 5 months (range 5-7 months), mean weight of 18.1 ± 2.7 kg, and 13 boxer dogs with a median age of 8 years (range 2-11 years), and a mean weight of 31.0 ± 3.6 kg. The control dogs had a mean heart weight (g) to body weight (kg) ratio of 6.99 ± 0.52. For the AC boxers, this ratio could be calculated in 3 dogs; it was 9 (dog #1), 8.37 (dog #5), and 9.21 (dog #11). Seven boxers had echocardiographic evidence of myocardial failure, with increased left ventricular end systolic diameter and decreased fractional shortening. Six boxers had atrial dilation determined by a left atrium to aortic root ratio of >1.8 (Table 4.2). Nine boxers had supraventricular ectopy (SVE) noted via electrocardiography or 24-hour Holter recording at the time of the first evaluation before the administration of antiarrhythmic medication. The SVE were most commonly atrial premature complexes, couplets or triplets (6 boxers), followed by supraventricular tachycardia (2 boxers) and atrial fibrillation (1 boxer). The number of supraventricular ectopic beats, in 24 hours, for the 6 boxers with atrial premature complexes are reported in table 4.2. Ventricular ectopy with a wide positive R wave in leads II, III and aVF was the predominant arrhythmia in all the boxers with the exception of two boxers who had primarily

Table 4.1. Demographic Data of Controls and AC Boxers

	Control	AC Boxer
Number of dogs	14	13
Age	5 months (5-7 months)	8 years (2-11 years)
Body weight (kg)	18.1 ± 2.7	31.0 ± 3.6
Sex	14 males 0 females	6 males 7 females
Intact	1/14	5/13

SVE (dog #3 with supraventricular tachycardia and dog #11 with atrial fibrillation). Six boxers had a history of syncopal episodes. The results of genetic testing for the striatin mutation were available in 5 boxers. Out of these 5 boxers, 2 were heterozygous (dog #6 and dog #13), 1 was homozygous (dog #4) and 2 were negative (dog # 8 and dog #12) for the striatin mutation.

Table 4.2. Summary of Clinical Findings in 13 AC Boxers

			Echocardiography			
Dog #	**Rhythm**	**Clinical signs**	**LVDD (cm)**	**LVSD (cm)**	**FS (%)**	**LA/Ao**
1	SVT/VPC	S,PE	6.2	5.1	18	2.5
2	VT	L	4.7	4.3	8.6	2.3
3	SVT/VPC	N	5.1	3.8	25.5	1.5
4	VT/APC (3)	S	5.2	4.2	19	1.8
5	VT/APC (18)	S,L	5.1	4.5	12	2.3
6	VT/APC (31)	S	4.2	3.1	26	1.8
7	VT	N	-	-	-	-
8	VT/APC (17)	N	4.3	3	30	2.1
9	VT	N	4	2.9	27.5	2
10	VT	N	-	-	-	-
11	AF	S	5	4.9	2	2.7
12	VPC/APC (1)	N	4	2.2	45	1.5
13	VT/APC (60)	S	-	-	-	-

APC = atrial premature complexes; FS = percent fractional shortening; L = lethargy; LA/Ao = left atrial/aortic root ratio; LVDD = left ventricular diastolic diameter; LVDS = left ventricular systolic diameter; N = no clinical signs; PE = pulmonary edema; S = syncope; SVT = supraventricular tachycardia; VPC = ventricular premature complexes; VT = ventricular tachycardia. Number in parenthesis represents the number of supraventricular ectopic beats, in 24 hours.

Pathological findings

Upon histopathological evaluation, the tissues were classified as normal or as having fatty or fibro-fatty myocardial replacement by a single investigator who was not aware of the group status. All 13 boxers showed the characteristic histopathological lesions of AC, with fatty (8/13) or fibro-fatty (5/13) replacement of the right ventricular myocardium. Left atrial lesions were seen in 9 out of 12 boxers, with 8 classified as fatty and 1 classified as fibro-fatty myocardial replacement (Figure 4.1 A and B). The left atrium was not available for evaluation in

one boxer. Right atrial lesions were identified in 12 out of 13 boxers, with 7 classified as fatty and 5 classified as fibro-fatty myocardial replacement (Figure 4.1 C-E). Two boxers had a normal left atrium and right atrial changes (dog #1 and dog #3) and 1 boxer (dog #10) had a normal left and right atrium. Inflammatory infiltrates suggestive of myocarditis were identified in the atria of 5 out of 13 AC boxers; infiltrates were seen in the right atrium alone in 4 boxers and in both the left and right atrium in 1 boxer. All the control dogs were classified as having normal atrial and right ventricular myocardium.

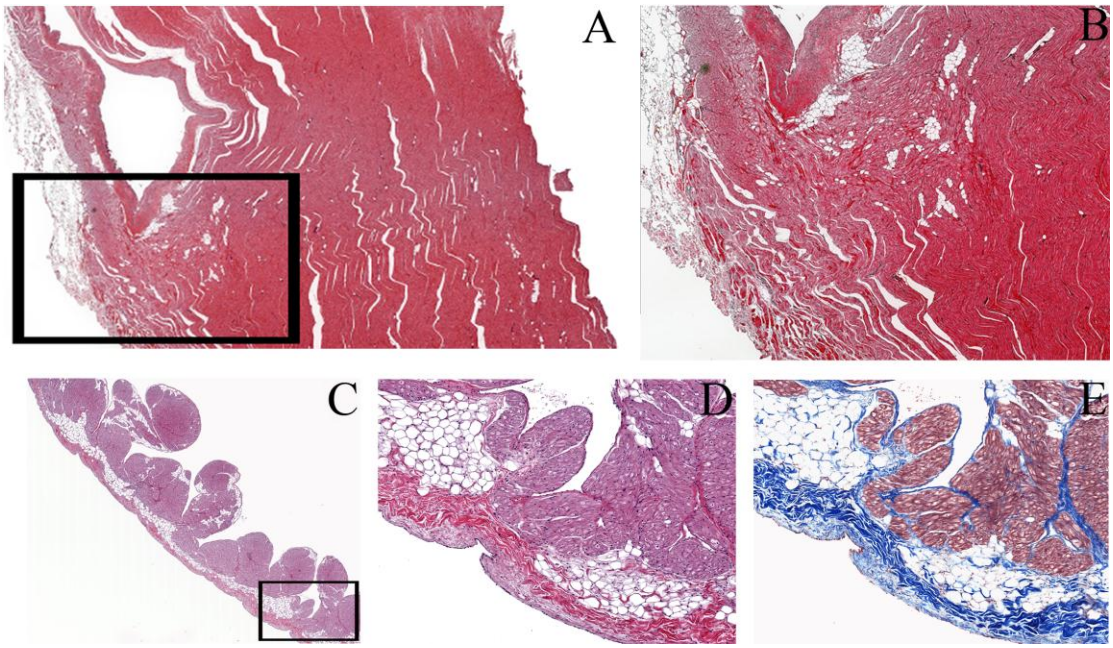

Figure 4.1. Histopathology of the atrial myocardium from an AC boxer. (A) Left atrium (10X) showing relatively heterogeneous atrial myocardium. Box highlights section depicted in (B). (B) Higher magnification (30X) through the epicardium towards the mid mural region. Small, scattered clusters of adipocytes are present. There is some vertical tissue separation caused by processing artifact. (C) Right atrium showing generalized subepicardial replacement of myocytes with adipocytes, and focal regions of atrial tissue with generalized fatty infiltrate. Box contains the section highlighted to right. H&E, 10X. (D) Magnified section from (C) showing fatty replacement of myocytes. Occasional small, atrophic myocytes can be observed, surrounded by adipocytes. Mild interstitial fibrosis is present. H&E, 70X. (E) Same section as (D) stained with Masson trichrome stain to illustrate collagen (blue) located sub-epicardially and interstitially. 70X

Localization of intercalated disc proteins by immunofluorescence

Immunofluorescence analysis was conducted on the left and right atrial sections for the following proteins: cadherin, PKP-2, DP, JUP (adherens junctions and desmosomes), Cx40, Cx43, and Cx45.

Based on our samples we could not see a difference in the distribution of the immunoreactive signal for the adherens junctions and desmosomal proteins evaluated; nor was there a difference for the gap junction protein Cx40. No immunoreactive signal for Cx45 was detected in the samples collected. A decrease in the number of Cx43 signals per 20X field was noted in the left and right atrium of AC boxers compared to the controls (Figure 4.2). However, the immunoreactive signal intensity for Cx43 was not different between the groups (Figure 4.2 and 4.3). Likewise, there was no difference in the immunoreactive signal for cadherin, Cx40, desmoplakin, and PKP-2 (Figure 4.3).

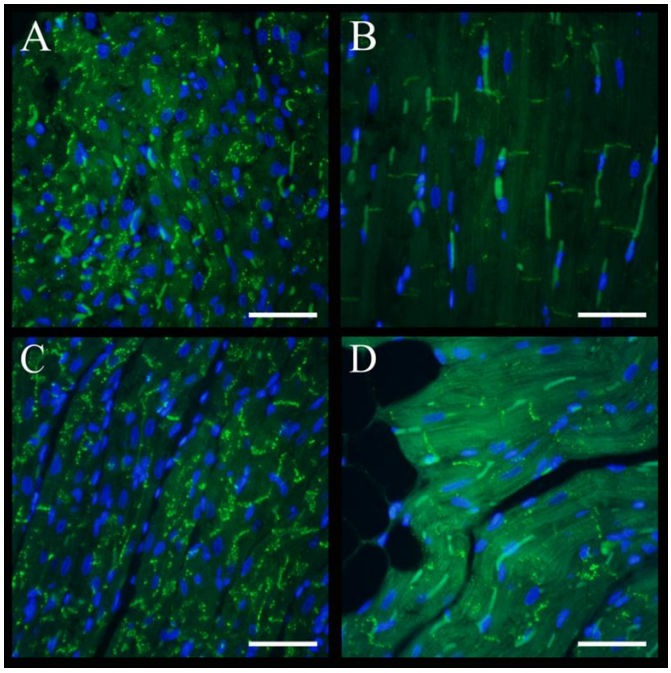

Figure 4.2. Immunofluorescence of Connexin 43 showing decreased density of Cx43 in the AC boxers. The bright green fluorescence represents Cx43 and the blue fluorescence is DAPI staining the nucleus. (A) left atrium control dog, (B) left atrium AC boxer, (C) right atrium control dog, (D) right atrium boxer. Scale bars denote 50 μm.

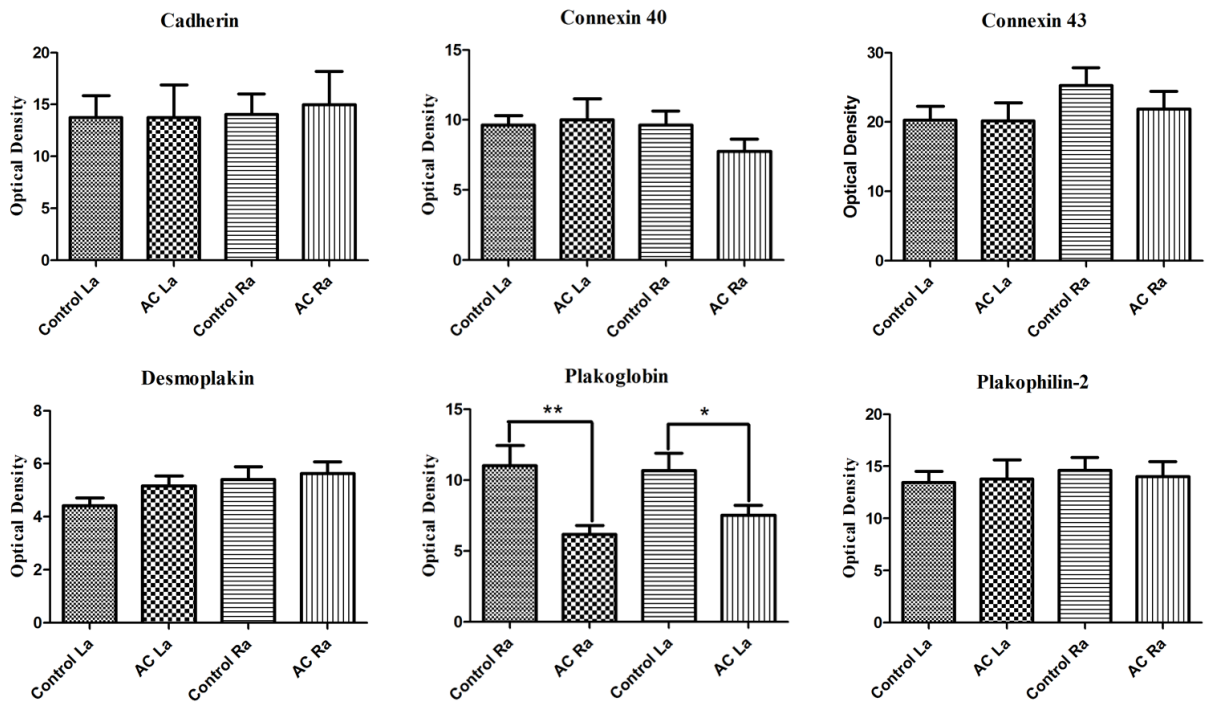

Figure 4.3. Optical density calculations of the immunoreactive signal from cadherin, Cx40, Cx43, DP, JUP, and PKP-2. The immunoreactive signal for JUP is decreased in the left and right atrium of the AC boxers. (*=P<0.05 and **=P<0.01)

The JUP immunoreactive signal intensity was significantly decreased in the right atrium of AC boxers when compared to the right atrium of the control dogs (optical density 6.19 ± 2.06 versus 11.05 ± 5.31, P=0.009) (Figure 4.3 and 4.4). Similarly, the immunoreactive signal intensity for JUP was decreased in the left atrium of boxers when compared to the left atrium of the control dogs (optical density 7.53 ± 2.21 versus 10.15 ± 2.89, P=0.032) (Figure 4.3 and 4.4). Two boxers (dog #9 and #10) showed almost no detectable signal. Overall, the immunofluorescence results suggest the presence of altered desmosomal proteins and gap junction remodeling in the atrial myocardium of AC afflicted boxers.

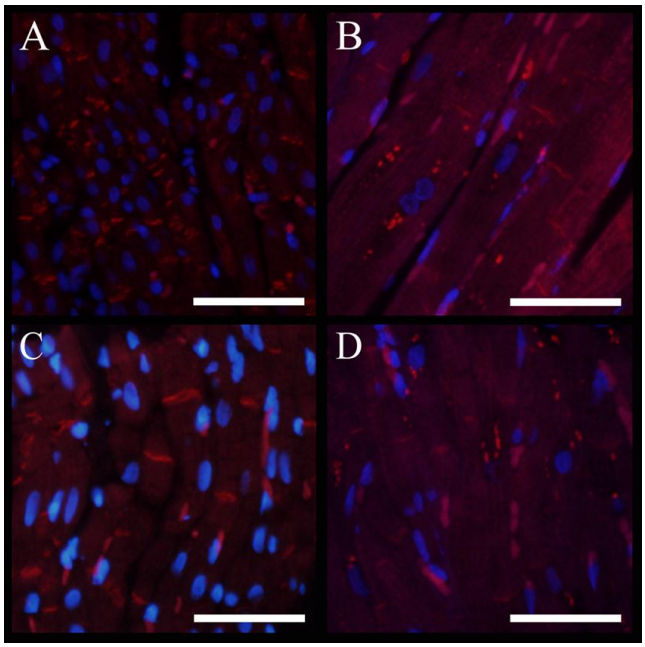

Figure 4.4. Immunofluorescence for Plakoglobin showing decreased JUP immunoreactive signal in the AC boxers. The bright red fluorescence represents JUP and the blue fluorescence is DAPI staining the nucleus. (A) left atrium control dog, (B) left atrium AC boxer, (C) right atrium control dog, (D) right atrium boxer. Scale bars denote 50 μm.

Immunochemical detection of intercalated disc proteins

Western blot was performed from the left and right atrial tissue lysates for the same proteins evaluated via immunofluorescence. There was no difference in the western blot band density for the desmosomal proteins: JUP, PKP2, DP or Cadherin (Figure 4.5).

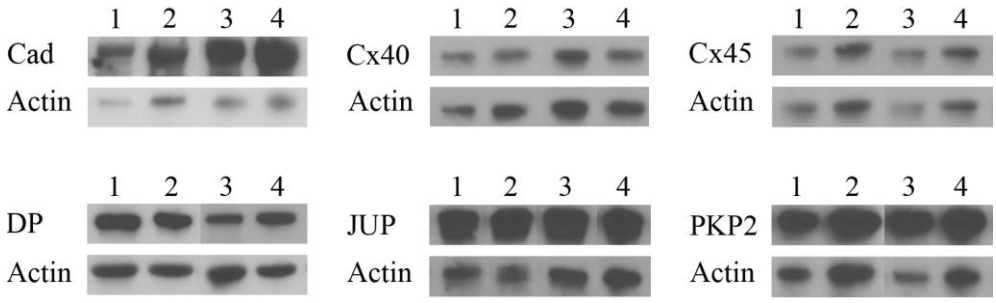

Figure 4.5. Representative western blots for Cadherin (Cad), Connexin 40 (Cx40), Connexin 45 (Cx45), Desmoplakin (DP), Plakoglobin (JUP), Plakophilin-2 (PKP2) and actin (loading control). (1) left atrium control, (2) left atrium AC boxer, (3) right atrium control, (4) right atrium boxer.

Similarly there was no difference in the band density for the gap junction proteins Cx40 and Cx45 (Figure 4.5). However, the band density for connexin 43 was significantly decreased in the right atrium of AC boxers compared to the controls (band density Cx43/Actin 0.68 +/- 0.23 versus 1.115 +/- 0.3, P=0.004) (Figure 4.6). Connexin 43 also appeared to be decreased in the left atrium; however this did not reach statistical significance, P=0.18. Overall, these results suggest a reduction in the total protein content of connexin 43 in the atria of AC afflicted boxers.

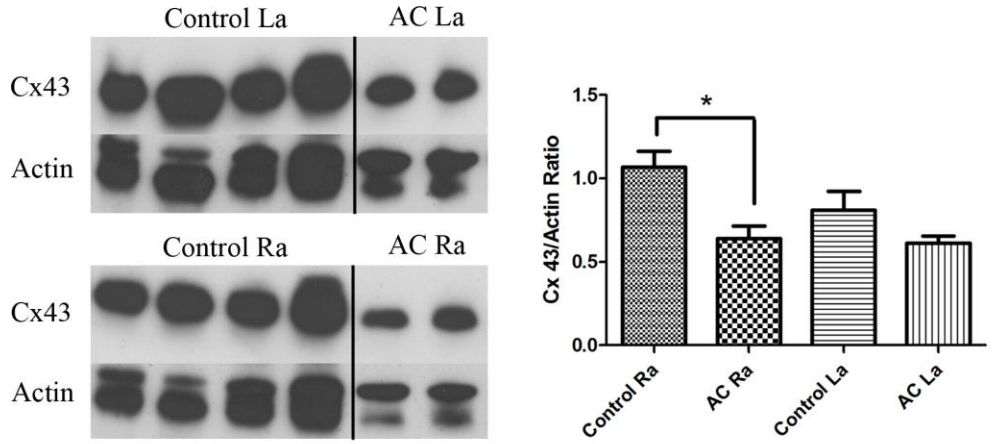

Figure 4.6. Representative western blot for Cx43 and actin (loading control) showing decreased Cx43 in the right atrium of the AC boxers. The left atrium also appeared to have decreased Cx43 however this did not reach statistical significance. (*****= P<0.05)

Transmission electron microscopy

Transmission electron microscopy was performed in the left and right atrial samples of 2 boxers and 2 controls. Adipocytes were identified in between the cardiomyocytes in the boxers but not in the controls (Figure 4.7). In the AC boxers, some areas of the intercalated disc appeared widened and altered in structure with the presence internalized gap junctions (Figure 4.7), potentially indicating an increased remodeling of the intercalated disc of boxers with AC. The TEM results suggest that boxers afflicted with AC have disruption of the intercalated disc in the atrial myocardium.

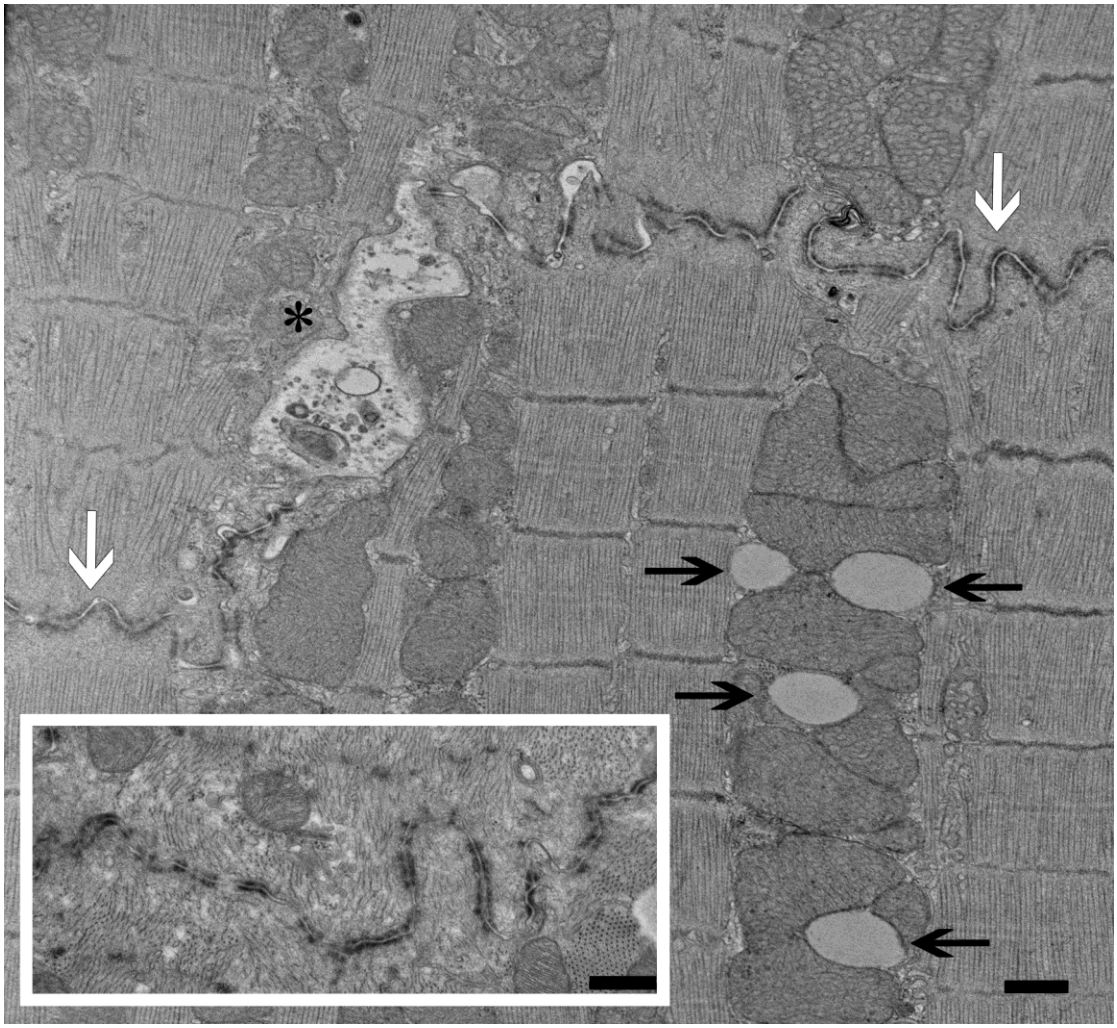

Figure 4.7. Electron micrograph of the intercalated disc (white arrows) showing the presence of adipocyte infiltration (black arrows) and disruption of the intercalated disc (black asterisk) in an AC boxers. Area inside the box shows the intercalated disc in a control dog. Scale bar denotes 500 nm

Chapter 5

Discussion and Conclusion

Arrhythmogenic cardiomyopathy is also known as arrhythmogenic right ventricular cardiomyopathy as it was previously described as a disease affecting solely the right ventricular myocardium; now it is widely accepted that the disease can also affect the left ventricular myocardium (Asimaki and Saffitz 2012). However, it is unknown to what degree the atrial myocardium is affected by AC. The present study was designed to determine if the atrial myocardium of boxers with AC is also affected by the disease and whether alterations to the intercalated disc proteins are present in the atrial tissue of boxers with AC. The major findings were: 1) the presence of supraventricular arrhythmias in the AC boxers; 2) the atrial myocardium of the AC boxers has histopathological changes consistent with AC; 3) decreased JUP immunoreactive signal intensity without a change in the total western blot protein concentration; 4) decreased number of Cx43 immunoreactive signals with a decreased in the western blot protein concentration.

The Holter monitor data shows that even though AC is a disease dominated by ventricular arrhythmias, supraventricular ectopy was noted in 9 out of 13 boxers in the current study. In the majority of the boxers these arrhythmias were mild and of no clinical concern. However, in two boxers (dog # 3 and dog #11) supraventricular arrhythmia was the predominant rhythm. While most of the attention has been directed to the life-threatening ventricular arrhythmias it is important that when present the atrial arrhythmias are not ignored. If severe enough, these arrhythmias could also lead to syncope or contribute to the progression to heart failure (Nogami, Adachi et al. 1990; Shinbane, Wood et al. 1997). Atrial arrhythmias can be the result of atrial dilation and fibrosis secondary to heart failure (Burstein, Comtois et al. 2009). It is

possible that the arrhythmias seen in our study population were caused by atrial dilation and fibrosis secondary to heart failure, as the majority (60%) of the boxers had atrial dilation. For example the boxer with atrial fibrillation (dog #11) had the largest left atrium to aortic root ratio in our study population. Interestingly, the boxer with primarily SVT (dog #3) had no atrial dilation, suggesting that AC may contribute to the atrial arrhythmias independently of atrial dilation.

Blinded histopathological assessment showed changes characteristic of AC in the atria of 12 out of the 13 boxers and in none of the controls. The quality of the atrial sections for the boxer described to have no changes consistent with AC was poor, potentially contributing to an incorrect histopathological classification. The fatty or fibro-fatty myocardial replacement was present in the atria of boxer regardless of the presence of SVE. Atrial histopathological changes characteristic of AC have been reported previously in 8 out of 23 boxers in the first report of AC in this breed (Basso, Fox et al. 2004). Inflammatory infiltrates suggestive of myocarditis were identified in the atria of 38% of the AC boxers in our study population. Inflammatory infiltrates characteristic of myocarditis have been reported in the right ventricle (61%), left ventricle (70%), and atria (17%) of AC boxers (Basso, Fox et al. 2004). These results indicate that even though AC has been reported to primarily affect the right and less frequently the left ventricular myocardium, the atrial myocardium is also affected by the disease. The fatty or fibro-fatty replacement of the atrial myocardium could represent the substrate for the atrial arrhythmias in AC boxers. However, the present study does not provide a direct link between the atrial arrhythmias and AC.

In order to further characterize the extent to which AC affects the atrial myocardium, we investigated whether alterations in the desmosomal proteins and gap junctions were present in

these boxers. Immunofluorescence analysis of the tissue showed that the cadherin immunoreactive signal intensity was similar to that of the controls, a finding that is consistent with other reports (Kaplan, Gard et al. 2004; Oxford, Everitt et al. 2007; Asimaki, Tandri et al. 2009). We failed to identify a difference in the immunoreactive signal intensity level for PKP2 and DP. In human AC, it has been shown that a decrease in the ventricular immunoreactive signal intensity for these proteins is variable and appears to be related to the type of genetic mutation (Asimaki, Tandri et al. 2009). A similar finding has been shown in boxers, with the ventricular immunoreactive signal intensity for PKP2 and DP having more variability in affected patients (Oxford, Everitt et al. 2007). On the other hand, a decrease in the ventricular immunoreactive signal intensity for JUP appears to be a more consistent finding in boxers, something that has also been shown in humans with AC regardless of the kind of genetic mutation (Oxford, Everitt et al. 2007; Asimaki, Tandri et al. 2009). This decrease in ventricular immunoreactive signal intensity for JUP has been shown to be 91% sensitive and 82% specific for the diagnosis of AC in humans (Asimaki, Tandri et al. 2009). In our population we found loss of the atrial immunoreactive signal intensity for JUP in the intercalated disc of AC boxers. Yet, western blot analysis demonstrated normal expression of JUP. Western blot has previously failed to detect a difference in the expression of desmosomal proteins (JUP, PKP2, DP) in the ventricular tissue of both humans and boxers with AC, suggesting that the loss of immunoreactive signal intensity is not due to loss of the total protein content in the myocyte, but likely results from failure of the altered proteins to appropriately incorporate in the desmosome preventing the formation of functional desmosomal units at the level of the intercalated disc (Kaplan, Gard et al. 2004; Oxford, Everitt et al. 2007; Asimaki, Tandri et al. 2009). The decrease in the immunoreactive signal intensity for JUP serves as an additional indication that

alterations to the atrial intercalated disc are present in boxer AC. However, a specific link between the alterations to the intercalated disc proteins such as JUP and the fatty or fibro-fatty myocardial replacement that characterizes AC has not been established. It has been suggested that the incorporation of abnormal desmosomal proteins into the intercalated disc leads to disruption of the mechanical coupling provided by the desmosome and that it facilitates myocyte detachment leading to cell injury, apoptosis, and the development of fatty or fibro-fatty myocardial replacement (Corrado, Basso et al. 2011; Asimaki and Saffitz 2012). This hypothesis appears to be a simplistic view of the disease process in AC, as it has been shown that desmosomal proteins also regulate changes in signaling pathways. Importantly, nuclear translocation of plakoglobin, in desmoplakin deficient mice, mediates changes in the Wnt pathway resulting in fibrosis, fatty replacement of the myocardium and ventricular arrhythmias (Garcia-Gras, Lombardi et al. 2006; Delmar and McKenna 2010). It is likely that both the altered mechanical load and the interaction of the abnormal desmosomal proteins with signaling pathways contribute to the disease process in AC.

Previous studies evaluating Cx43 in the ventricular tissue of both humans and boxers affected with AC have shown decreased Cx43 immunoreactive signal intensity and altered localization, to the lateral aspect of the myocytes instead of forming part of the end to end connections (Kaplan, Gard et al. 2004; Oxford, Everitt et al. 2007). In the present study, the "lateralization" of Cx43 could not be examined as the atrial myocardium normally expresses gap junctions at the site of end to end cell apposition and on the lateral aspect of the myocytes (Severs, Bruce et al. 2008). In the present study we did not see a decrease in the immunoreactive signal intensity for Cx43. However, a decrease in the number of Cx43 signals was noted via immunofluorescence in the AC boxers. The decrease in the amount of Cx43 was confirmed by

western blot analysis in the right atrium. Connexin 43 also appeared be decreased in the left atrium; however this did not reach statistical significance. We also failed to identify a difference between the control and AC boxers for Cx40 and Cx45. These proteins have been shown to be expressed in the atrial myocardium of dogs as part of the gap junction; however they have not been previously evaluated in AC affected animals (Davis, Kanter et al. 1994). No immunoreactive signal for Cx45 was noted in the samples collected, yet weak Cx45 bands were detected via western blot. It is possible that the anti-connexin 45 antibody did not yield an adequate signal with the immunofluorescence protocol used, or more likely there was minimal expression in the atrial samples collected, as Cx45 is primarily found in the sinus and atrioventricular node (Giovannone, Remo et al. 2012). The amount of Cx45 present is these samples was likely below our level of detection via immunofluorescence but still identifiable with western blot (Oxford, Everitt et al. 2007). Overall the changes seen via immunofluorescence and western blot suggest the presence of gap junction remodeling in the atria of AC affected boxers. Although gap junctions have not been shown to be directly affected by AC, their remodeling has been shown to occur in the ventricular tissue of both humans and boxers with AC (Kaplan, Gard et al. 2004; Kaplan, Gard et al. 2004; Oxford, Everitt et al. 2007). It is believed that normal gap junction function is dependent on the mechanical coupling provided by the desmosomes (Asimaki and Saffitz 2012). Therefore, once the mechanical connection provided by desmosomes has been disrupted, the gap junctions cannot sustain the remaining forces and remodeling occurs. This mechanism could explain why transmission electron microscopy of the atrial myocardium in the AC boxers showed internalized gap junctions near the widened altered structure sections of the intercalated disc. Signaling pathways triggered by the altered mechanical load, interaction with abnormal desmosomal proteins and

cytokine production are also suspected to play a role in gap junction remodeling (Asimaki and Saffitz 2012). For example, decreased expression of PKP-2 in cultured myocytes leads to decreased Cx43 expression, suggesting that increase mechanical load is not the only factor leading to gap junction remodeling in AC (Oxford, Musa et al. 2007). Alterations to gap junction structure and function may lead to arrhythmogenesis (Severs, Bruce et al. 2008). For example, heterogeneous Cx43 expression in the myocardium of chimeric mice was linked to conduction abnormalities and occasional ventricular tachycardia (Gutstein, Morley et al. 2001). Our results are consistent with the notion that gap junction remodeling may contribute to the arrhythmogenic substrate created by the fatty or fibro-fatty myocardial replacement in AC. However, it should be noted that functional measurements evaluating this hypothesis have not been performed, as the majority of the studies evaluating gap junction remodeling in AC have been based on immunohistochemistry, which does not provide information about function or conduction changes (Asimaki and Saffitz 2012; Duffy 2012).

Transmission electron microscopy showed adipocyte infiltration and areas of widened, altered structure of the intercalated disc in the AC boxers. Previous TEM studies in boxers and humans have shown adipocyte infiltration and a significantly lower number of desmosomes in the right ventricle of AC affected individuals compared to controls (Basso, Czarnowska et al. 2006; Oxford, Danko et al. 2011). Quantification of the numbers of desmosomes was not possible in the present study due the small number of dogs evaluated with TEM.

There are some limitations to in the interpretation of our results. Our results are based on a small number of boxers with AC and non-age matched healthy control. Ideally age matched boxers without AC would serve as controls. However, there is no test to ascertain that a healthy boxer is not an asymptomatic carrier of the disease. The TEM results are based on the

assessment of only 2 boxers, therefore additional samples are needed for evaluation and analysis of the ultrastructural changes in atrial myocytes of boxers with AC. Another limitation is that postmortem changes to the tissues that may alter our molecular results, as loss of intercalated disk integrity and gap junction remodeling, can occur soon after death. To minimize this risk, cardiac tissues were collected no longer than 1 hour after euthanasia, and Cx43 has been shown to be stable for 2 hours (Oxford, Everitt et al. 2007). We did not evaluate the location or expression of the striatin protein via immunofluorescence or western blot in these boxers. Genetic testing was performed in 5 out of the 13 boxers as part of their clinical evaluation; however, some of the boxer tissue was collected prior to the discovery of the striatin mutation and therefore DNA was not stored for genetic testing. The majority of the boxers in the study were euthanized because of end stage AC. Therefore, it is possible that the molecular changes seen in this study are due to the end stage cardiac disease and heart failure rather than changes secondary to AC (Vatta, Marcus et al. 2007). Lateralization and decreased Cx43 signal has been shown to occur secondary to myocardial infarction and congestive heart failure (Severs, Bruce et al. 2008; Kieken, Mutsaers et al. 2009). However, decreased expression of Cx43 and JUP has been shown to occur in a patient with Naxos disease, a recessive form of AC with cutaneous phenotype, before the presence of cardiac pathology (Kaplan, Gard et al. 2004). This suggests that the alterations in JUP and Cx43 do occur secondary to AC instead of being the result of congestive heart failure and end stage disease. Additional studies evaluating the expression of Cx43 and JUP in dogs with congestive heart failure due to diseases other than AC and in boxers with early AC would be needed for confirmation.

The present study shows that the atrial myocardium of boxers with AC has histologic changes consistent with AC, decrease connexin 43 seen via immunofluorescence and western

blot, and weaker immunoreactive signal intensity for plakoglobin. Together, the results indicate that in addition to the ventricular myocardium, the atrial myocardium is affected in boxers with AC. These findings support the use of the broader term of arrhythmogenic cardiomyopathy rather than arrhythmogenic right ventricular cardiomyopathy to describe this disease. The decrease in the amount of Cx43 in conjunction with the histological changes could represent the substrate for the atrial arrhythmias associated with AC.

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Vita

Jorge Luis Vila was born in 1982 in Mayaguez, Puerto Rico to Luis Vila and Wanda Colon. He has two younger siblings, Lismary Vila and Joel Vila. He attended Pomayuan Private School, graduating with high honors in May 2000. After high school, he left Puerto Rico to attend Iowa State University to pursue a degree in Animal Science and pre-veterinary medicine. In 2003, he was admitted into the Louisiana State University School of Veterinary Medicine. During his time in veterinary school, he developed an interest in cardiology and decided to pursue specialization in this area. After receiving the degree of Doctor of Veterinary Medicine in May, 2007, he went to Michigan State University to complete a small animal rotating internship. Unfortunately, after completion of the internship he was not among the candidates selected to continue training in a cardiology residency. He moved to Richmond, Virginia where he worked as an emergency veterinarian for a specialty and referral center. During this time he began to date Dr. Stephenie Abbott, a veterinary classmate who still lived in Louisiana. After 2 years in Virginia, he decided to return to Louisiana to be closer to Stephenie and to pursue a Master degree, with the hope of becoming a more competitive candidate for a cardiology residency. He enrolled in Louisiana State University in August 2010 and began work on his Master degree, originally in the cardiovascular pathophysiology lab under the guidance of Associate Professor Joseph Francis, B.V.Sc & A.H., M.V.Sc., Ph. D. A year into the Master program, the opportunity to work with the Cardiology Service of Louisiana State University, in addition to the cardiovascular pathophysiology lab, presented itself. At this time, he began to study under Assistant Professor of Veterinary Cardiology Romain Pariaut, D.V.M., Diplomate ACVIM-CA, as his major professor. During this time, he married Dr. Stephenie Abbott in May 2012. In July 2012, he began a cardiology residency at Louisiana State University School of

Veterinary Medicine under the guidance of his major professor Dr. Pariaut and Assistant Professor of Veterinary Cardiology Caryn Reynolds, D.V.M., Diplomate ACVIM-CA.