CLINICAL UTILITY OF EXERCISE TESTING AND HIGH-SENSITIVITY CARDIAC TROPONIN I IN EARLY DIAGNOSIS OF ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY IN APPARENTLY HEALTHY BOXER DOGS

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Department of Companion Animals
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ABSTRACT

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a primary myocardial disease recognized in dogs and humans. It is characterized by fibrofatty replacement of right ventricular myocardium, and less frequently, of the interventricular septum, left ventricular and atrial myocardium. Boxer dogs have a genetic predisposition for ARVC. Family history of the disease, documentation of ventricular arrhythmias (premature ventricular complexes, PVCs), history of syncope, or exercise intolerance are important clinical data to guide the diagnosis in affected dogs. In dogs with a lack of clinical signs (apparently healthy dogs), the electrocardiographic finding of ventricular arrhythmias is a major means of suspecting a diagnosis of ARVC antemortem. Of concern, however, is that the earliest latent stage of ARVC is characterized by normal electrocardiographic findings with undetectable structural heart disease but a risk for sudden cardiac death. The identification of this subclinical population of Boxer dogs having latent disease is challenging but important for prognosis and monitoring recommendations and in selection of breeding animals. Therefore, testing that can unmask ventricular arrhythmias or increase blood levels of biomarkers of myocardial injury in subclinical Boxer dogs may be beneficial in guiding monitoring and treatment recommendations. In the present study, we 1) investigated the effects of exercise testing (ExT) on the occurrence of ventricular arrhythmias in apparently healthy Boxer dogs; 2) performed an analytical and clinical comparison between point-of-care (POC) cardiac troponin I (cTnI) and high-sensitivity cTnI (hs-cTnI) assays; and 3) investigated whether an association could be demonstrated between ventricular arrhythmias, cTnI

concentration ([cTnI]), and genetic mutation status in these Boxer dogs. One hundred and fifty-eight client-owned Boxer dogs were screened to select a population of apparently healthy dogs. Of these, 30 dogs completed the study.

This study showed that the effects of ExT on number and complexity of ventricular arrhythmias are inconsistent in apparently healthy Boxer dogs. However, in many dogs, PVCs were clustered around exercising (median, 33.3% of total daily PVCs occurring in the peri-exercise period). The results of this study demonstrated that the two cTnI assays cannot be used interchangeably, the hs-cTnI assay may be a more sensitive and specific test that the POC assay, and [cTnI] measured by either assay does not differ in dogs with and without the striatin mutation that has been associated with ARVC in some Boxer dogs. Furthermore, the results of this study suggest that the exercise-associated increase in [cTnI] and number of PVCs may be a manifestation of increased propensity to the development of the ARVC phenotype in Boxer dogs. Dogs' recent exercise history should be considered when measuring [cTnI] and when quantitating PVCs via 24 h at-home electrocardiogram (Holter monitor). Long-term follow up of these Boxer dogs may provide further evidence in support of our hypotheses and overall conclusion, which could be a focus for future studies.

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[cTnI]	cardiac troponin I concentration(s)
[hs-cTnI]	high-sensitivity cardiac troponin I concentration(s)
[K^+]	plasma potassium concentration(s)
2D	two dimensional
ACM	arrhythmogenic cardiomyopathy
ARVC	arrhythmogenic right ventricular cardiomyopathy
AVCDL	Atlantic Veterinary College Diagnostic Laboratory
BP	blood pressure
CBC	complete blood count
CHF	congestive heart failure
CI	confidence interval
CMR	cardiac magnetic resonance imaging
cTnC	cardiac troponin C
cTnI	cardiac troponin I
cTnT	cardiac troponin T
DCM	dilated cardiomyopathy
ECG	electrocardiogram
ExT	exercise testing
FAC	fractional area change
H	hour
hs-cTnI	high-sensitivity cardiac troponin I
IQR	interquartile range
K_3-EDTA	potassium ethylenediaminetetraacetic acid
LA	left atrium
LA/Ao	left atrial-to-aortic root dimension ratio
LBBB	left bundle branch block
LV	left ventricle, left ventricular
LVEDV	left ventricular volume measured at end-diastole
LVEF	left ventricular ejection fraction
LVESV	left ventricular volume measured at end-systole
LVESVI	left ventricular end-systolic volume indexed to body surface area
LVFS	left ventricular fractional shortening
LVIDd	left ventricular internal dimension measured at end-diastole
LVIDdN	left ventricular internal dimension measured at end-diastole, normalized to body weight
LVIDs	left ventricular internal dimension measured at end-systole
LVIDsN	left ventricular internal dimension measured at end-systole, normalized to body weight
MST	median survival time
POC	point-of-care
PVCs	premature ventricular complexes
RBBB	right bundle branch block
RV	right ventricle, right ventricular

RVAd	right ventricular area measured at end-diastole
RVAs	right ventricular area measured at end-systole
S’	systolic myocardial velocity of the lateral tricuspid annulus
SCD	sudden cardiac death
TAPSE	tricuspid annular plane systolic excursion

1. BACKGROUND AND LITERATURE REVIEW

Arrhythmogenic right ventricular cardiomyopathy (ARVC), previously termed Boxer cardiomyopathy, is an adult-onset primary myocardial disease characterized by ventricular arrhythmias.[1–4] Histologically, it is defined by progressive replacement of normal right ventricular (RV) myocardium with fatty or fibrofatty tissue.[3,4] The residual myocytes interspersed among adipocytes and fibrous tissue provide an ideal substrate for life-threatening ventricular arrhythmias.[1–4] This condition in Boxer dogs shares these phenotypic features with human ARVC. Additionally, ARVC has been recognized as an important cause of sudden cardiac death (SCD) in both apparently healthy Boxer dogs,[5,6] and humans (especially young people and athletes).[1,2] Although histopathological abnormalities predominantly occur in the RV, they may exist in the left ventricle (LV) and occasionally, the interventricular septum.[7,8] Because histopathological changes and clinical manifestations of the disease are not limited to the RV, the broader term arrhythmogenic cardiomyopathy (ACM) recently has been adopted in human medicine.[8] This term has not yet been adopted in veterinary medicine, but may become the preferred term for dogs in future.

Research in human medicine has shown a favorable clinical outcome (decreased mortality rate) in a large cohort of patients diagnosed with ARVC who have undergone close follow-up and treatment.[9] These findings underscore the importance of an early diagnosis of ARVC to establish a focused prevention strategy based on activity restriction, clinical follow-up (identification of alarming symptoms, echocardiographic

abnormalities, and ventricular arrhythmias), and prophylactic therapy to prevent sudden death.[10] Although large-scale longitudinal studies evaluating clinical outcomes in Boxer dogs with ARVC have not been performed, early diagnosis, particularly in dogs with the occult (preclinical, i.e., no externally observable manifestations) form of the disease, remains a major challenge. This is primarily due to the broad spectrum of phenotypic variation (SCD often being the first clinical sign) and lack of a definitive antemortem diagnostic test.

1.1. Historical perspective and epidemiology

Arrhythmogenic right ventricular cardiomyopathy was first described in Boxer dogs in 1983.[4] Unlike cardiomyopathies in other dog breeds, this condition in Boxer dogs was distinguished by extensive fibrofatty replacement of myocardium, lack of marked ventricular dilation, and infrequent occurrence of atrial fibrillation.[4] Due to unique features and a high prevalence in Boxer dogs, this condition formerly was termed "Boxer cardiomyopathy." It is now referred to as ARVC due to similarities between the disease in Boxer dogs and ARVC in humans, including presentation, genetic basis, and histopathology,[5,7] and its recognition in non-Boxer breeds.

Arrhythmogenic right ventricular cardiomyopathy is estimated to affect 1:5000 to 1:2000 people in the general population.[11,12] Evaluation of the first- and second- degree relatives of probands with ARVC suggests that up to 50% of cases are familial.[13] The prevalence of this disease in the Boxer dog breed has not been defined. Isolated cases of

ARVC have also been reported in other dog breeds such as the Siberian husky,[14] Labrador retriever,[15] Bull mastiff,[16] Dachshund,[17] English Bulldog,[18] and in cats.[19]

1.2. Pathology

Arrhythmogenic right ventricular cardiomyopathy is associated with widespread myocardial atrophy with large multifocal areas of myocardial fibrosis and fatty infiltration, myocytolysis, myocyte vacuolization, evidence of myocarditis, and necrosis.[6] The distinctive histopathological feature of ARVC is the replacement of normal RV myocardial tissue by adipose or fibrous tissue.[3,7] In a histological study, the fatty form was observed in 65% and the fibrofatty form in 35% of Boxer dogs affected with ARVC.[7] The fatty form is characterized by diffusely distributed, multifocal regions of adipose cell replacement within the RV wall and trabeculae, extending from epicardium toward endocardium, often in association with mild interstitial fibrosis.[7] The fibrofatty form is characterized by multifocal or diffuse adipose cell replacement associated with areas of replacement fibrosis.[7] Structural changes of the myocardium may also be present in the LV, interventricular septum, and atria, but may be less severe than in the RV.[20,21] Lesions consistent with myocarditis are noted in the majority of affected dogs with RV, LV and left atrium (LA) involvement seen in 61%, 70%, and 17% of Boxer dogs, respectively, and myocardial apoptosis are noted in 39% of these dogs.[7] Whether the inflammation is a primary event or occurs secondary to myocyte cell death is unresolved.[20] However, these processes have been thought to modulate disease progression.[7] It is possible that fatty and fibrofatty patterns represent a continuum of

disease, mediated by myocarditis, in which the fatty form is an early feature and fibrofatty repair results from myocarditis-induced-injury.[7]

In humans, ARVC is considered to be a disease of the desmosome, a mechanical junction within the intercalated disc anchoring cells to each other via the intermediate filaments.[22] The intercalated disc is comprised of 2 additional macromolecular components resolvable by electron microscopy: gap junctions provide a pathway for electrical and metabolic signaling between cells, and adherens junctions provide mechanical anchoring of actin filaments via cadherin molecules.[23] Immunofluorescence analysis of human ARVC affected tissue reveals a characteristic histopathological profile that includes loss of immunoreactive plakoglobin (a desmosomal and adherens junction molecule) and connexin 43 (the cardiac gap junction protein of the adult ventricle) at the site of the intercalated disc.[24] Similar findings have been reported in the ARVC affected Boxer dogs.[25] Quantitative transmission electron microscopy also shows a decrease in the number of desmosomes, gap junctions, and adherens junctions in the LV and RV of Boxer dogs with ARVC compared to those without ARVC.[26]

1.3. Genetic basis

Arrhythmogenic right ventricular cardiomyopathy in Boxer dogs appears to be inherited as an autosomal dominant trait, at least in some Boxer families.[5] Molecular genetic studies in humans have shown that ARVC is a desmosomal disease resulting from mutation in cell adhesion proteins such as plakoglobin, desmoplakin, plakophilin-2, and desmoglein-2.[27–29] Based on these and several other studies, it has generally been

accepted that ARVC results in loss of desmosomal integrity, impaired cell-to-cell adhesion, myocyte detachment, and cell death.[30] Subsequently, these changes may lead to increased susceptibility to mechanical stress and pressure.[22] In some humans with ARVC, mutations of the cardiac ryanodine receptor (RyR2) and their effect on the associated stabilizing protein calstabin-2 have been demonstrated.[31] Dysfunction of this protein leads to disruption of calcium homeostasis, which is critical to normal cardiac contractile function and rhythm.

Despite many similarities between ARVC in humans and in dogs, molecular evaluation of the most common desmosomal ARVC candidate genes for the human disease did not identify a causative mutation in the splice site or exonic regions of these genes in the dog.[32] Boxer dogs with ARVC have decreased RyR2 protein expression[33] and a calstabin deficiency in the LV[34] compared with healthy control dogs, although a causal relationship is not proven. However, in a genome-wide association study, Meurs and others identified a deletion mutation in an untranslated region of the striatin gene, located on chromosome 17, which was observed to be associated with Boxer ARVC.[35] Although the role of striatin in the heart has not been well described, expression in cardiac muscle has been reported.[36] In dogs, striatin localizes to the cardiac intercalated disc and co-localizes to desmosomal proteins previously documented as being involved in the pathogenesis of human ARVC including plakophilin-2, desmoplakin, and plakoglobin.[35] In dogs that are homozygous for the striatin mutation, striatin RNA and protein levels are decreased compared to normal controls.[35] It has been suggested that decreased amounts of this desmosomal protein could lead to a reduction in myocardial

desmosomal integrity in Boxer dogs with ARVC.[35,37] It is noteworthy that a proportion of Boxer dogs diagnosed with ARVC do not carry the striatin deletion mutation and a proportion of dogs without clinical ARVC have the mutation.[35,38] The presence of the striatin mutation in healthy Boxer dogs could be attributed to the phenomenon of incomplete penetrance (i.e. some individuals who carry the pathogenic variant express the associated phenotypic trait while others do not). Based on clinical studies in humans, it has been suggested that several genetic and environmental factors influence the phenotypic expression of the disease. Although an association between the striatin mutation and disease severity has been suggested,[39,40] the existence of ARVC cases without the striatin mutation indicate that there must be other genetic mutations or causative factors responsible for ARVC in some Boxer dogs. A recent pedigree-based genetic analysis suggests that the striatin mutation is not responsible for the disease in Boxer dogs but it is linked with the gene responsible on the same chromosome.[39] This study also suggests that there is an interaction between the known effects of the striatin mutation on the cardiomyocyte and ARVC, because homozygotes for the striatin mutation tend to be severely affected at early ages.[39] However, apart from striatin, no additional genetic or environmental causative factors associated with Boxer ARVC have been identified so far.[39] Currently, there is one commercially available genetic test to identify dogs that have the deletion in the striatin gene. It is important to recognize that molecular genetic testing (currently available or the ones that will be discovered) may identify dogs at risk for developing ARVC but cannot make a clinical diagnosis of ARVC itself. This is because mutation carriers may have no disease phenotype (incomplete penetrance) or may present with various degrees of clinical manifestations, ranging from

absence of overt clinical signs with concealed RV structural abnormalities and no arrhythmias to patients experiencing sudden cardiac death or presenting with signs of congestive heart failure.

1.4. Clinical presentation and natural history

As originally proposed by Harpster, ARVC in Boxer dogs can be grouped into 3 clinical categories.[4,6] The first category (concealed) includes dogs that are subclinical and have premature ventricular complexes (PVCs). The second category (overt) includes dogs with ventricular tachyarrhythmias and syncope or exercise intolerance. The third category (myocardial dysfunction form) includes dogs with myocardial systolic dysfunction and ventricular dilation, sometimes with evidence of congestive heart failure (CHF). Although it has been hypothesized that the three forms or categories represent a continuum of the disease, it has not been objectively proven. The myocardial dysfunction form is less common[40] and whether this represents an end-stage ARVC or idiopathic dilated cardiomyopathy (DCM) is not clear.[41]

Affected dogs may have syncope, weakness, or exercise intolerance. A small percentage of dogs presents with clinical signs associated with CHF such as cough, tachypnea, dyspnea, or abdominal distension. For many dogs, sudden death may be the first clinical manifestation of the disease.[6,40,42] Syncope has been reported in 52-68% of Boxer dogs with ARVC[7,43] and exercise intolerance in 24% of affected dogs.[43] In a study involving 23 Boxer dogs with a confirmed diagnosis of ARVC (based on postmortem examination), 39% (9/23) had experienced sudden death: 3 during exercise, 4 while

walking slowly and 2 while sleeping.[7] Another 13% were euthanized for refractory right-sided CHF. The median survival time (MST) was 365 days (range, 7 - 1971 days).[43] Conversely, in another study, the majority of dogs were still alive at 9 years of age and did not have a shorter survival time than the control dogs.[38] Age at the time of diagnosis, presence of clinical signs, presence of a greater number of PVCs, presence of the striatin mutation, and myocardial dysfunction are risk factors for a poor prognosis.[39,43,44] In a retrospective study involving 62 Boxer dogs with ARVC, shorter survival was observed in dogs with syncope (365 days versus 693 days in dogs without syncope, p=0.012) and the probability of death within a year (odds ratio) was 4.8 times greater in dogs with syncope than in dogs without syncope (p=0.013). Younger dogs tended to have a better prognosis, with a MST of 292 days for dogs older than 8 years at the initiation of treatment, 620 days in dogs between 4 and 8 years, and 1460 days in dogs younger than 4 years.[43] A significant association between MST and the severity of ventricular arrhythmias has been demonstrated, with shorter survival in dogs with frequent and complex arrhythmias (>1000 PVCs/24 hour {24 h}, couplets, triplets, ventricular tachycardia) compared to dogs with >1000 PVCs/24 h but without couplets/triplets/ventricular tachycardia (p=0.03).[43] Survival times does not appear to differ in dogs receiving different antiarrhythmic medications.[43]

Arrhythmogenic right ventricular cardiomyopathy in Boxer dogs has been regarded as an adult-onset myocardial disease. Despite evidence of a genetic basis and transmission from parents to offspring, most affected Boxer dogs do not develop the disease phenotype until middle age.[38] In some of the earliest descriptions of the disease,

affected dogs were of a wide age range (1-15 years) with a mean of 6.9-8.2 years and a median of 7-8.5 years at the time of initial diagnosis.[4,6] This observation is consistent with the age-related penetrance of gene mutations that causes ARVC observed in humans, where it has been suggested that age-related penetrance may be because of progressive exposure of the abnormal myocardium to mechanical stress. In humans, clinical manifestations of ARVC usually develop during adolescence or young adulthood.[12] Young age has been described as the most powerful independent predictor of ventricular fibrillation in human patients with ARVC.[45]

1.5. Diagnosis

Establishing a clinical diagnosis of ARVC is often difficult because of the nonspecific nature of disease features and the broad spectrum of phenotypic manifestations described above. Despite the presence of histopathologic myocardial lesions, most affected dogs lack detectable changes in cardiac function as assessed by routinely available non-invasive diagnostic tests such as echocardiography.[40] The clinical diagnosis in humans is based on criteria defined by the European Society of Cardiology Task Force in 1994,[46] and revised in 2010.[47] The criteria include results from echocardiography, cardiac magnetic resonance imaging (CMR), histopathology, characteristic electrocardiographic changes, documentation of arrhythmias, and family history.[47] Such diagnostic criteria have not yet been established in veterinary medicine, and access to some forms of diagnostic testing may be limited and impractical. As such, there is no consensus on diagnostic criteria for ARVC in Boxer dogs.

Many veterinary cardiologists rely on the combination of signalment (adult Boxer dog) and detection of an increased number of PVCs in the absence of other underlying causes for the ventricular arrhythmia. Although ARVC has been considered a primary electrical disorder,[5] not all affected dogs manifest arrhythmias during an outpatient evaluation at the veterinary hospital. Therefore, a 24 h ambulatory electrocardiogram (Holter recording) is currently the most used screening tool to identify dogs with an occult form of the disease. However, this test is not readily available to all clinicians and there is 46-100% spontaneous day-to-day variability in the number of PVCs documented on Holter from the same dog.[48] Due to incomplete penetrance associated with the striatin mutation and lack of enough evidence to support the role of the striatin mutation in the causation of ARVC in Boxer dogs, genetic testing alone cannot be used for reliably identifying affected dogs. As such, considerable interest exists in the early diagnosis of ARVC through alternative noninvasive techniques.

1.5.1. Arrhythmia: in-hospital electrocardiogram (ECG) and 24 h at-home Holter monitoring

Ventricular arrhythmia with a left bundle branch block (LBBB) morphology on ECG suggests that the arrhythmia originates from the RV and is the hallmark of ARVC in Boxer dogs.[6,49] These arrhythmias are characterized by wide QRS complexes with a predominantly positive deflection in the inferior leads (II, III, and aVF) on ECG.[6] The antemortem diagnosis relies on identification of an increased number of PVCs with this characteristic morphology in the absence of other underlying causes for the arrhythmia.

The PVCs may be present singly, in pairs (couplets), in groups of three (triplets), and in runs of paroxysmal or sustained ventricular tachycardia. In a study by Basso and others, PVCs with LBBB morphology were documented by 24 h Holter recording in 83% of the 23 Boxer dogs with a histopathologically confirmed diagnosis of ARVC. Some of these dogs also had PVCs of right bundle branch block (RBBB) morphology with predominantly negative QRS complexes in the inferior leads.[7] The remaining 17% did not have documented arrhythmia. Although less common, supraventricular tachyarrhythmias have also been reported.[6]

Due to the intermittent nature of the arrhythmias, it is not unusual for affected dogs to have a 2-to-5 minute-long in-hospital ECG without any PVCs.[40] Therefore, 24 h Holter monitoring is an important part of the diagnosis, screening, and assessment of the severity of the arrhythmia, and it serves as an important guide for monitoring treatment and prognosis of Boxer dogs with ARVC. There are several challenges in diagnosing ARVC solely based on ventricular arrhythmias, however. Firstly, since PVCs occur in healthy humans[50] and dogs,[51,52] defining the minimum number of PVCs in apparently healthy Boxer dogs (preclinical) that will constitute a dog as 'affected' or 'having the disease' remains a challenge. Cutoff values have been proposed for the number of PVCs/24 h to differentiate between affected and healthy Boxer dogs: 50,[5] 100,[53,54] 300,[38] and 1000.[48,55,56] Based on the observation that healthy, large-breed non-Boxer dogs have <24 PVCs/24 h[51] and apparently healthy adult Boxer dogs have <91 PVCs/24 h,[53] it has been proposed that identification of >100 PVCs/24 h in an adult Boxer dog is strongly suggestive of a diagnosis of ARVC.[40] In the human general population, the presence of

≥1 PVC during a standard ECG recording or ≥30 PVCs over a 1-hour recording is associated with increased cardiovascular risk and increased mortality.[57] Similarly, Boxer dogs with increased numbers of PVCs using any of the 3 previously suggested cutoff values (50, 100, and 1000 PVCs/24 h) have significantly shorter survival times.[44] Specifically, Boxer dogs having >50 PVCs/24 h had significantly shorter MST (1393 days) than those having <50 PVCs/24 h (>2083 days; p=0.003),[44] confirming that Boxer dogs with <100 PVCs/24 h still could have ARVC.[7] In a study involving 23 Boxer dogs with ARVC that died or were euthanized due to cardiac or non-cardiac causes 35% (8/23) of them had 0 to 999 PVCs in a 24 h period,[7] suggesting that a lower number of PVCs does not definitively exclude affected dogs. Furthermore, the number of PVCs may be associated with the striatin mutation. Boxer dogs that are homozygous for the striatin mutation had 1091–32 000 PVCs/24 h (median, 5102 PVCs/24 h) and dogs that are heterozygous for the mutation had 109–19 000 PVCs/24 h (median, 2515 PVCs/24 h; p=0.001).[35] Interestingly, however, 11 of the 35 dogs classified as controls (<100 PVCs/24 h) were heterozygous for the striatin mutation in the same study. This provides additional evidence that the striatin mutation may not be the sole explanation for the severity of arrhythmias and disease. For these reasons, using any single cutoff value for the number of PVCs/24 h does not provide a complete assessment of the presence or absence of ARVC in Boxer dogs. Additional diagnostic tests may offer greater insights and may be helpful in identification of dogs with ARVC particularly if used in combination with 24 h Holter recordings.

Secondly, the onset of detectable ventricular arrhythmias in Boxer ARVC may be abrupt. In one study, affected dogs progressed from having a median of 41 PVCs/24 h, which generally would be interpreted as normal in this breed, to a median of 1823 PVCs/24 h, within a year. [38] In addition to annual variability, there is large (46-100%) spontaneous day-to-day variability in the number of PVCs documented on Holter recording (calculated as the percentage difference between the maximum and the minimum daily number of PVCs).[48] These findings emphasize the fact that a single Holter monitoring result may not be a reliable test to include or exclude a diagnosis of ARVC in dogs. Therefore, annual Holter monitoring has been proposed to identify affected dogs.[40]

Finally, the number and severity of ventricular arrhythmias are not predictably correlated with the clinical outcome in any individual patient. In other words, some affected dogs have a large number of PVCs but do not show any clinical signs,[52] whereas some affected dogs with the same number and complexity of ventricular arrhythmias show clinical signs and the disease can progress in severity as they mature. The factors that determine which dogs will eventually show clinical signs for the disease are poorly understood.[40]

In addition to the number of PVCs, the complexity of ventricular arrhythmias (defined as the presence of polymorphic PVCs, bigeminy, trigeminy, couplets, triplets, ventricular tachycardia) is considered a negative prognostic factor in humans with ARVC.[58] Similarly in dogs, complex ventricular arrhythmias are associated with reduced

survival independent of the total number of PVCs.[44,55] In 122 Boxer dogs, in the subgroup of dogs with normal left ventricular systolic function, the presence of polymorphic PVCs (1,660 vs 2,083 days; p=0.019) and ventricular tachycardia (1,244 vs >2,083 days; p=0.002) were significantly associated with shorter MSTs.[44]

The occurrence of ventricular arrhythmia in Boxer dogs with ARVC may offer insights beyond those obtained histopathologically. Myocarditis and fibrofatty myocardial injury and repair are characteristic features of Boxer dogs with ARVC that die suddenly, yet there were no significant differences in the extent of RV fat replacement between Boxer dogs that died of ARVC and those that died of other causes.[7] However, 48% of dogs with ARVC that died suddenly or were euthanized due to cardiac or non-cardiac causes had ventricular tachycardia on 24 h Holter recordings,[7] suggesting arrhythmia severity may be more strongly associated with a poor prognosis than lesion severity in dogs with ARVC. A significantly shorter MST has been reported in Boxer dogs with a higher number of PVCs/24 h and complex ventricular arrhythmias (ventricular couplets, triplets, and ventricular tachycardia).[43]

1.5.2. Echocardiography

Although echocardiography is currently the first-line imaging modality when evaluating human patients for ARVC, diagnosing ARVC by echocardiography is considered challenging in human as well as veterinary medicine. Despite histopathological RV myocardial abnormalities associated with ARVC, there are often no echocardiographically identifiable abnormalities except in humans and dogs with long-

standing disease.[40,59,60] One of the reasons for lack of echocardiographic abnormalities in affected individuals or dogs may be because quantitative assessment of RV function is difficult owing to its complex geometry, separate inflow and outflow regions, prominent endocardial trabeculations, ventricular interdependence, and the marked load-dependence of most indices of RV function.[61] Additionally, a discrepancy between findings on echocardiography and CMR has been observed with a recent human study showing that only 50% of patients satisfying CMR criteria for ARVC also fulfilled the established echocardiographic criteria.[62] Despite these challenges, the echocardiographic criteria recommended for assessing humans for ARVC include evaluation of RV akinesia, dyskinesia, or aneurysms together with measurements of RV outflow tract diameter and RV fractional area change.[11] Although the utility of these criteria in the identification of Boxer ARVC has not been well studied, a recent interest in echocardiographic assessment of RV systolic function in dogs with ARVC has highlighted two, easily obtainable indices of myocardial performance: tricuspid annular plane systolic excursion (TAPSE) and pulsed wave tissue Doppler imaging-derived systolic myocardial velocity of the lateral tricuspid annulus (S').

Tricuspid annular plane systolic excursion is measured using M-mode echocardiography. It is a quantitative estimate of longitudinal RV shortening. Reduction in TAPSE correlates with other measures of decreased RV systolic function such as low RV ejection fraction in humans.[63,64] For this reason and because it is easily obtained and repeatable,[65] TAPSE may be a practical and clinically useful indicator of RV dysfunction in dogs. While TAPSE may serve as a marker of RV systolic function, changes in

TAPSE are not specific to ARVC. Reduced TAPSE is associated with a worse outcome in people with heart diseases other than ARVC, such as congenital heart disease, pulmonary hypertension, acute symptomatic pulmonary embolism, hypertrophic cardiomyopathy, and LV systolic function.[64,66,67] In veterinary medicine, reduced TAPSE has been reported in dogs with pulmonary hypertension[68] and Boxer dogs with ventricular arrhythmia.[60,65] Reduced TAPSE is associated with shorter cardiac survival time in Boxer dogs with ventricular arrhythmias.[65]

Pulsed wave tissue Doppler imaging-derived systolic myocardial velocity of the lateral tricuspid annulus (S') is another index that measures the longitudinal systolic function of a portion of the RV free wall. Like TAPSE, S' has shown good correlation with other measures of global RV systolic function in people, including RV fractional area change and CMR- and radionucleotide-derived RV ejection fraction.[60,69] In veterinary medicine, a reduction in S' exists in Boxer dogs with ARVC (defined as having >100 PVCs/24 h) with and without LV systolic dysfunction.[60]

Although ARVC has been regarded as a disease of the RV, the LV can also be affected, resulting in biventricular dilation and systolic dysfunction.[6,59] Whether these LV changes represent a late stage of disease or a separate entity cannot be determined with only one echocardiogram. In a multicenter human study involving 42 patients with a pathologic diagnosis of ARVC, LV involvement was observed in 76% of hearts.[59] In Boxer dogs with ARVC, LV involvement is a common occurrence and LV lesions consisting of focal, fibrofatty tissue replacement occurred in 48% cases in one study.[7]

Echocardiographically identifiable LV changes in Boxer dogs are uncommon. In one study of 49 Boxer dogs with ARVC, only 2 dogs had LV myocardial dysfunction as defined by decreased LV fractional shortening (11 and 17%, respectively) without LV dilation, and 4 dogs had LV dilation with normal fractional shortening (≥ 25%).[38] When present, LV systolic dysfunction is associated with adverse outcomes, including shorter survival time, in both humans[70] and Boxer dogs.[38,44,55] There may be an association with the myocardial dysfunction form (late-stage) of ARVC and a homozygous genotype for the striatin mutation in dogs. However, this suggestion is based on only one study showing that 30/33 dogs with a DCM phenotype had the striatin mutation and the homozygous genotype were strongly associated with the DCM phenotype ($p=0.005$).[37] Considering that there can be several causes of a DCM phenotype other than ARVC in Boxer dogs, further studies are necessary to make any meaningful conclusion about the relationship between the striatin mutation and the myocardial dysfunction form of ARVC in Boxer dogs.

1.5.3. Cardiac magnetic resonance imaging (CMR)

During the past two decades, CMR has evolved as a noninvasive method to evaluate morphological, functional, and structural abnormalities of the RV in humans.[71,72] Several studies in humans have demonstrated a significant correlation between RV morphological abnormalities identified by CMR and ventricular arrhythmias.[71,73,74] However, no CMR finding is pathognomonic for ARVC because similar morphological abnormalities have also been identified in patients with idiopathic RV outflow tract tachycardia and healthy volunteers.[75] A composite score of CMR findings may be more

sensitive and specific for ARVC.[71] Major CMR criteria (fatty infiltration of RV myocardium, localized RV aneurysm, and severe dilatation and reduction in RV ejection fraction with no/mild LV impairment) are identified more frequently in patients with ARVC than in those without ARVC. Some minor criteria (regional RV hypokinesia, mild segmental RV dilatation, and prominent trabeculae) also are more frequent in ARVC patients, while mild global RV dilatation occurs more frequently in individuals without ARVC. RV aneurysm achieves the highest importance in making a diagnosis of ARVC on CMR (predictive accuracy 76.8%).[71] A sensitivity 93.3% and specificity 89.5% are achieved with a CMR score ≥4: two major criteria, one major and two minor, or four minor criteria.[71]

Comparatively, it is only recently that CMR is being utilized in the evaluation of cardiac diseases in veterinary medicine. The utility of CMR in the evaluation of Boxer ARVC is limited owing to lack of availability, the absence of a standardized protocol, insufficient experience with the modality, and the requirement of specialized training in interpretation. Reported CMR findings in Boxer dogs with ARVC include decreased RV ejection fraction (ARVC 34 ± 11% vs control 53 ± 10%, $p<0.01$)[76] and bright signals within the RV consistent with fatty infiltrates.[7] Abnormal global RV systolic function estimated by CMR may not always be associated with myocardial gross fatty changes, raising the possibility that arrhythmias and myocardial dysfunction may precede the development of morphological abnormalities in dogs with ARVC.[76] However, identification of RV systolic dysfunction could be influenced by small sample size (n=5

per group), use of non-Boxers as controls, and the negative inotropic effects of anti-arrhythmic medications.[76]

1.5.4. Histology

Demonstration of fibrofatty replacement of RV myocardium is considered the definitive diagnostic result that confirms ARVC in humans and dogs. The RV free wall is affected earliest and most severely, but all portions of the atria and ventricles can be involved to some degree.[6,21] Since histological characterization requires tissue sampling, it has very limited antemortem clinical utility. In humans, invasive tissue characterization is usually reserved for patients in whom the diagnosis of ARVC is suspected but cannot be proven by non-invasive testing alone.[11] In veterinary medicine, the histological characterization of ARVC is limited to postmortem specimens due to the invasiveness of myocardial biopsy, need for general anesthesia, cost, possibility of associated life-threatening complications and above all, the limited sensitivity expected due to the patchy or unpredictable, epicardially-predominant distribution of lesions within the RV.

1.5.5. Clinical utility of serum biomarkers: cardiac troponin I

A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[77] Serum biomarker testing is attractive due to its affordability, wide availability, minimal invasiveness, ease of use by general practitioners, and potential utility in screening, prognosticating and monitoring

therapeutic management of heart disease in people as well as in dogs. Among several serum biomarkers investigated in veterinary and human cardiovascular medicine so far, cardiac troponins are viewed as the most sensitive and specific markers of myocardial injury and cellular necrosis.[78] Cardiac troponin (cTn) is a regulatory protein consisting of 3 subunits (cTnI, cTnT, and cTnC) which together function as the molecular switch of cardiomyocyte contraction. Both cardiac and skeletal muscles utilize a troponin complex to mediate sarcomeric contraction, but the 2 isoforms, cardiac and skeletal, are antigenically distinct from each other.[79] Troponin I and T subunits have tissue-specific isoforms for cardiac and skeletal muscle.[80] For troponin C, the cardiac and skeletal isoforms are completely homologous,[80] making the subunit unfit to be used as a cardiac marker. Cardiac troponin T (cTnT) isoforms share more than 50% homology with skeletal isoforms.[81] However, its fetal cardiac isoforms are sometimes expressed in diseased or injured skeletal muscle and could compromise the cardiac specificity of cTnT.[82] Cardiac troponin I (cTnI) is a 24 kDa protein,[80] which shares <50% homology with skeletal isoforms and contains a unique N-terminal peptide. It is not expressed in skeletal muscle during disease states.[83] Cardiac troponin I has many characteristics of an ideal cardiac biomarker: cardiac specificity, high sensitivity for injury (high myocardial tissue content and early release after a cardiac insult), negligible presence in the circulation of healthy individuals, persistence in circulation for days post-injury, and correlation with severity of injury.[84] The full gene sequence of cTnI in dogs and cats has been determined, and the homology of nucleotide and amino acid sequences between canine and feline cTnI is >95 and >96%, respectively.[85] The homology of the nucleotide and amino acid sequences between canine and human cTnI is 91 and 94.3%,

respectively.[85] The close homology of cardiac isoforms among mammalian species permits rapid and accurate measurement of canine cTnI concentrations ([cTnI]) using immunoassays developed for humans.[78]

Cardiac troponin assays are an important part of the standard of care in human subjects suspected to have acute myocardial infarction.[86,87] Based on a large international multicenter study, cTnI is a valuable tool for diagnosis and risk stratification of syncope in human patients.[88] In several human studies, even a small increase in serum [cTnI] (below the 99th percentile) predicts increased mortality and cardiovascular disease morbidity in the general[89] and patient populations.[90] There is increasing evidence in humans that underlying genetic abnormalities associated with cardiomyopathy may predispose patients to myocarditis. A recent study showed elevated troponin levels (in addition to chest pain and CMR findings of myocarditis) in a subset of humans with ARVC and suggested that ARVC may initially present as myocarditis.[91] In dogs, an experimental model of myocardial injury showed that circulating [cTnI] increases in proportion to the extent of cardiac injury.[92] Circulating cardiac troponin concentrations are increased in dogs with naturally-occurring mitral valve disease,[78,93] DCM,[78,93,94] subaortic stenosis,[78] pericardial effusion,[93,95] suspected cardiac contusions,[96] gastric dilation and volvulus,[97] babesiosis,[98] and with doxorubicin administration.[99] Collectively, these studies indicate that circulating [cTnI] is high in patients with cardiac injury.

There are only a few published studies evaluating serum [cTnI] in Boxer dogs specifically. Among these, only one study was designed to evaluate the [cTnI] in Boxer

dogs with ARVC,[42] whereas, other studies had 4 or fewer Boxer dogs as a part of a population of dogs comprised of various breeds.[78,100,101] Boxer dogs with ARVC (≥1000 PVCs/24 h) had higher mean serum [cTnI] (0.142 ± 0.05 ng/mL) compared to control Boxer dogs (<5 PVCs/24 h; 0.079 ± 0.03 ng/mL).[42] A statistically significant yet modest (r = 0.78) positive correlation between serum [cTnI] and severity of ventricular arrhythmia was observed although a large overlap in serum [cTnI] between the apparently unaffected and affected dogs was present.[42] This study requires careful interpretation of the data due to small sample size (n=10 in each group), dogs being on antiarrhythmic medications, and use of a conventional assay with a detection level of 0.01 ng/mL. Furthermore, ventricular arrhythmias (especially as identified by a single Holter recording) alone may not be a true indicator of disease severity for reasons described above. Therefore, the clinical utility of serum [cTnI] in Boxer dogs to evaluate the diagnosis and progression of ARVC remains unclear and warrants further study.

For cTnI measurement, multiple assays have been developed by various manufacturers. Recent advances in cTnI-assay technology have led to the development of assays with higher sensitivity than the so-called conventional assays. These assays provide increased diagnostic accuracy and sensitivity for the identification of cardiomyocyte injury in humans.[102] These improved assays are labeled ‘sensitive’ when able to detect cTnI in ~20-50% of healthy individuals and ‘high-sensitivity’ if they detect a cTnI level in >50% of reference (apparently healthy) subjects and they have a coefficient of variation of <10% at the 99th percentile upper reference limit of the assay.[103] High-sensitivity assays allow the detection of lower [cTnI] compared to

conventional assays. Therefore, the detection of mild myocardial injury, as might be expected in the early stages of myocardial diseases is now possible using these assays. Among cTnI assays that are currently available, three have been validated for use in dogs, showing acceptable analytical and overlap performance.[104–106] Among these assays, the Siemens ADVIA Centaur CP® TnI-ultra is the only assay that meets the criteria for a high-sensitivity assay in dogs as it detects cTnI in >95% of healthy dogs examined and has a low imprecision at both high and low concentrations.[105,107]

Despite availability and validation, only a few studies have evaluated the suitability of high-sensitivity cardiac troponin I (hs-cTnI) assays for detection of early myocardial damage in animals.[105,108–111] In dogs, published reports are either validation studies involving a group of healthy dogs and dogs with various cardiac diseases or arrhythmia,[105,109] an analytical study involving healthy and experimental animal models of myocardial injury (rats, beagle dogs, monkeys),[110] or clinical and drug-safety studies utilizing different species (dog, cat, horse, cattle, rat, and rabbit).[108] In clinical settings, there are two studies evaluating hs-cTnI assay in Doberman pinschers with and without DCM, one of which is published as an abstract[112] and other as a full-text article.[113] In this study comparing the conventional and hs-cTnI assays in Doberman pinschers with various stages of DCM with and without ventricular arrhythmias, the hs-cTnI assay identified more dogs with early DCM (21/29 dogs, 72%) compared to the conventional cTnI assay (18/29 dogs, 62%), suggesting hs-cTnI has the potential to identify early myocardial disease.[113] The use of a hs-cTnI assay has not yet been reported in healthy Boxer dogs or Boxer dogs with ARVC. Since the clinical assessment, electrocardiogram,

and echocardiogram are not sufficient to diagnose or exclude ARVC in most patients with preclinical disease, the addition of a high-sensitivity cTnI assay could help detect early myocardial injury, leading to earlier diagnoses of ARVC.

1.5.6. Clinical utility of exercise stress testing

According to current guidelines in human medicine, patients with ARVC should not participate in competitive sports or moderate to extreme recreational physical activities.[114,115] This recommendation is primarily based on the fact that adrenergic (catecholaminergic) stimulation plays a major role in the induction of ventricular arrhythmias in ARVC,[116] and exercise increases age-related penetrance and arrhythmic risk in ARVC patients.[117] Participation in sports and vigorous exercise increases the risk of development of clinically significant ARVC, earlier age of onset, a progression of the disease, and ventricular arrhythmia.[118] Therefore, the question of whether exercise testing (ExT) could aid in the early identification of ARVC by unmasking ventricular arrhythmias is an intriguing one.

Although ExT has routinely been used as a diagnostic tool in humans with heart disease, its application in ARVC has been limited. This is thought to be due to the low prevalence of the disease compared to other human cardiovascular diseases (e.g., coronary atherosclerosis), perceived arrhythmic risk during exercise, and limited understanding of the application of ExT in RV-predominant disease states.[91] Several studies have evaluated the arrhythmogenic response to ExT in human patients with established ARVC[9,13,119–123] and the safety and prognostic utility of ExT in human

patients with ARVC recently was demonstrated.[91] In veterinary medicine, there are no reports of the effects of ExT in dogs (of any breed type) with ARVC, nor in healthy Boxer dogs.

Apart from arrhythmia, cTnI is one of the cardiovascular parameters that is shown to be influenced by exercise in multiple human studies and a few veterinary studies. Exercise acutely increases circulating troponin concentrations after a brief or long exercise duration, performed at either moderate or high-intensity, in humans [124,125] and in healthy sled dogs.[126,127] Such exercise-induced increases in troponin concentrations are usually considered benign because they occur frequently, are present in apparently healthy individuals, and are not accompanied by clinical signs. In healthy sled dogs, a mild yet significant rise in median plasma point of care [cTnI] was noted within 1 hour after racing (16 miles each day, completing racing within 52-60 minutes, considered moderate-intensity, short-duration) on 2 consecutive days (day 1 – median, 0.06 and range, 0.02–0.2 ng/mL; day 2 - median, 0.07, range, 0.02–0.21 ng/mL).[126] In this study, although the median value for post-exercise [cTnI] was described to be within the reference interval (<0.11 ng/mL), the range of values suggests that there were at least a few dogs that had concentrations higher than the reference interval. Until a longitudinal study is performed comparing long-term cardiovascular outcomes between those who have post-exercise increase compared to those who do not, it cannot be concluded that an exercise-induced increase in circulating [cTnI] is truly a benign process. In fact, in a recent study involving a cohort of older long-distance walkers, exercise-induced [cTnI] elevations after 30-55 km walk in a day with self-selected pace and rest times

independently predicted higher mortality and cardiovascular events.[128] Approximately 10 minutes after participants had finished the walk, cTnI concentrations were above the 99th percentile in 9% of the participants, and 27% of those participants died or had major adverse cardiovascular events during follow-up compared to 7% of participants with post-exercise cTnI below the 99th percentile.[128] The hazard ratio was 2.48 (95% CI, 1.29–4.78) after adjusting for age, sex, cardiovascular risk factors such as hypertension, hypercholesterolemia or diabetes mellitus, cardiovascular diseases such as myocardial infarction, stroke, or heart failure, and baseline [cTnI].[128] This study suggests that exercise-induced increase in [cTnI] may be an early marker for future mortality and cardiovascular events. Higher post-exercise troponins concentrations may represent myocardial injury because of underlying, subclinical cardiac lesions. In another study involving conditioned Alaskan sled dogs, exercise was associated with high serum [cTnI] in all dogs (0.2-1.2 ng/mL; measured within one hour after intense exercise of 100 miles a day).[127] In this study, the baseline serum [cTnI] in all dogs was less than the limit of detection of the immunoassay (0.1 ng/mL), making it impossible to determine the magnitude of increase in [cTnI] in any individual dog.[127] This may lead to incorrect interpretation of it being a benign process. These findings illustrate the possible value of investigating the effects of exercise stress testing on the occurrence of ventricular arrhythmias and circulating [cTnI] as measured by high-sensitivity assays in apparently healthy Boxer dogs.

1.6. Objectives and hypotheses

The objective of this study was to explore the use of ExT and a hs-cTnI assay in identification of apparently healthy Boxer dogs with ventricular arrhythmias and to investigate whether a correlation can be identified between [hs-cTnI] and the striatin mutation associated with ARVC in Boxer dogs.

Aim 1: Investigate whether ExT in apparently healthy Boxer dogs can reveal ventricular arrhythmias that are not apparent on a routine cardiovascular evaluation. I hypothesized that ExT would reveal ventricular arrhythmias in some apparently healthy Boxer dogs.

Aim 2: Explore whether a relationship exists between [hs-cTnI] and ventricular arrhythmias in apparently healthy Boxer dogs. I hypothesized that a) hs-cTnI is more sensitive but less specific than cage-side cTnI assay for identifying Boxer dogs with ventricular arrhythmias; b) [hs-cTnI] increases with the severity of the arrhythmias.

Aim 3: Investigate whether [hs-cTnI] differ between dogs with and without the striatin mutation associated with ARVC in Boxer dogs. I hypothesized that [hs-cTnI] would be higher in dogs with the striatin mutation in comparison to those without this mutation.

2. MATERIALS AND METHODS

The study protocol was approved by the Institutional Animal Care Committee (#19-005) at the Atlantic Veterinary College, University of Prince Edward Island, Canada. Informed, signed consent was obtained from the owner of each dog enrolled in the study.

2.1. Animals

Client-owned Boxer dogs >1 year of age were prospectively recruited for participation in the study. Dogs were selected for inclusion if they were purebred Boxer dogs with no clinical signs of cardiac disease, and not receiving cardiac medications, including antiarrhythmic medications. Dogs were excluded if physical examination revealed evidence of systemic disease or orthopedic disease (e.g., lameness, renal, respiratory diseases) which, in the opinion of the investigator, could affect exercise performance, influence biomarker concentration, or affect arrhythmia interpretation. Dogs were excluded if they were diagnosed with ARVC (syncope, collapse, CHF, exercise intolerance) at any time before being enrolled in the study. Dogs were also excluded if they had echocardiographic or clinical evidence of congenital heart disease (including clinically significant subaortic stenosis defined as subcostal peak transvalvular aortic velocity >3.0 m/s measured by spectral Doppler echocardiography), acquired valvular disease, pericardial disease, dilated cardiomyopathy, or other myocardial diseases that, in the opinion of the investigator, could confound arrhythmia interpretation.

2.2. Study design

The study design is summarized in Figure 1. For each prospective participant, a history was first collected from the owners via phone call and by reviewing past medical records provided by primary care veterinarians. The dogs that did not meet any exclusion criteria were invited to undergo a complete cardiovascular assessment, which consisted of the following tests (baseline visit): a complete physical examination, Doppler blood pressure (BP), complete blood count (CBC) and plasma biochemical analysis, urinalysis, three-view thoracic radiography, 12-lead ECG of at least 3 minutes' duration, echocardiogram, and a 24 h Holter study using a 3-channel transthoracic system. The Holter results from the baseline visit are referred as 'baseline Holter' in this document. Dogs were excluded if their results met any of the pre-defined exclusion criteria. Remaining dogs underwent the study protocol. In the same baseline visit, blood samples were collected for genetic testing for striatin mutation and two cTnI assays - conventional point-of-care (POC) cTnI and hs-cTnI. Owners were asked to return with their dogs within 30 days for a second evaluation (ExT visit) to include a history and physical examination to screen for new-onset exclusionary findings, ExT, and repeat cTnI assays. At this visit, prior to the ExT, a blood sample was collected for measurement of pre-exercise [hs-cTnI], and each dog again was instrumented with a 24 h Holter monitor. The Holter results from the ExT visit are referred as 'ExT Holter' in this document. ExT was performed as detailed below. A resting standard 12-lead ECG was performed within ~2 minutes after the ExT protocol to document any sustained exercise-induced arrhythmia and to provide a reference ECG if excessive movement artifact affected the Holter recording. A blood sample was collected 3 hours after ExT to measure post-exercise [hs-

cTnI]. This time interval was chosen because [cTnI] has been reported to reach peak serum levels 3 hours after a cardiac event in humans.[86] Dogs were discharged to their owners wearing their Holter monitors. The owners were encouraged to maintain the dog's normal activity level during the Holter study. The monitor was removed after 24 h.

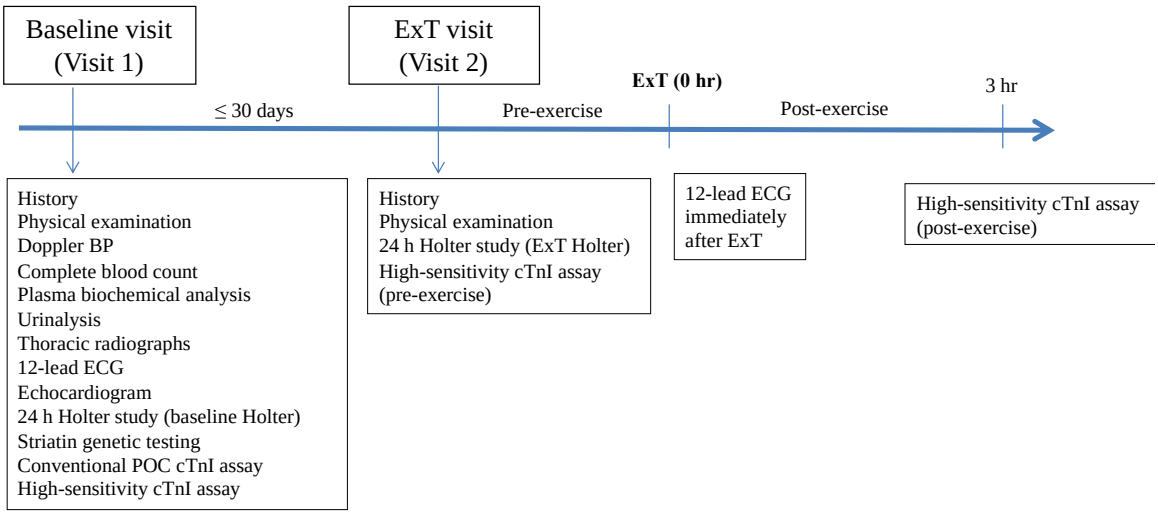

Figure 1. Study design. BP, blood pressure; cTnI, cardiac troponin I; ECG, electrocardiogram; ExT, exercise testing; h, hour; POC, point-of-care.

2.2.1. Electrocardiographic assessment

Each dog underwent a 12-lead ECG (Philips PageWriter 300pi Electrocardiograph, Philips Healthcare, Andover, MA, USA) of 3 minutes' duration while unsedated and positioned in right lateral recumbency. Dogs were instrumented with standard bipolar limb leads (I, II, and III) and unipolar augmented limb leads (aVR, aVL, and aVF).[129] For precordial lead placement, the left-sided precordial leads V2 through V6 were placed in standard fashion.[49,129] The right-sided precordial lead V1 was positioned at the costochondral junction (CCJ) of the right first intercostal space (ICS) to improve detection of right atrial and ventricular depolarizations (Figure 2).[129]

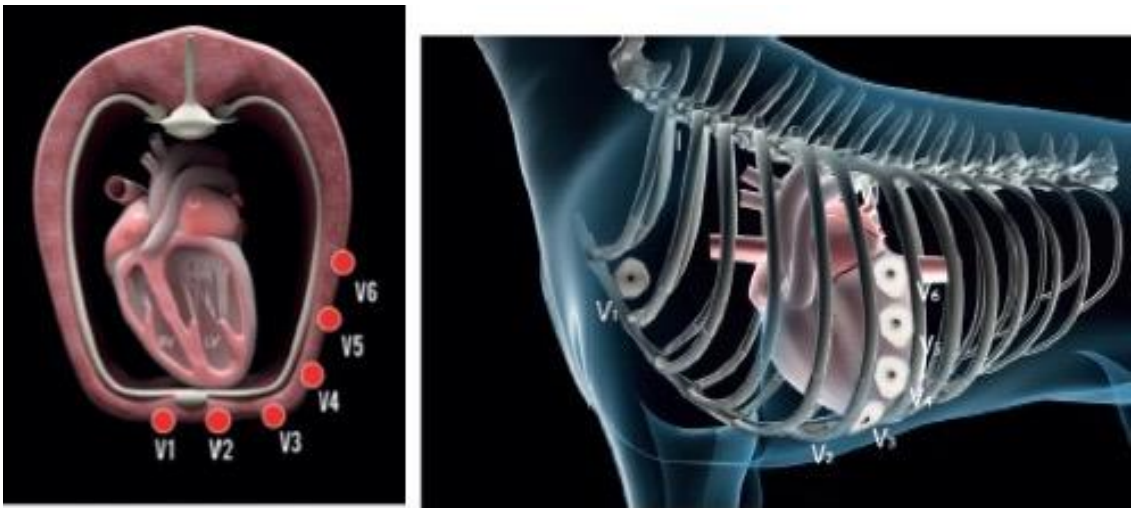

Figure 2. Schematic images of a) a transverse section of a dog's thorax depicting the precordial leads for a standard 12-lead ECG. The sixth intercostal space (ICS) was used for the electrodes for leads V2-V6. The location shown for the V1 electrode was not used. **b) the left lateral thorax depicting where the electrode for lead V1 is placed at the costochondral junction (CCJ) of the right first ICS**. With permission of the American Veterinary Medical Association.

For each 24 h Holter study, a 3-channel monitor was used. The monitor was placed on the dog in routine fashion,[130] and the dog was discharged to its owner's care to allow monitoring of the electrical activity of the dog's heart in its normal environment. Each Holter study was analyzed using the Forest Medical Trillium 5500 monitor interface (Forest Medical, LLC, East Syracuse, NY, USA). Any recording that did not have ≥ 20 hours of readable data was excluded. The total number of PVCs/24 h and the total number of PVCs occurring in the peri-exercise period, defined as from the start of exercise to 3 h after exercise was completed, was tabulated. The complexity of the ventricular arrhythmia was graded numerically from 0-3 according to the following scheme[52]: 0 = none; 1 = single, monomorphic PVCs; 2 = couplets, bigeminy, trigeminy, and multiform PVCs; and 3 = triplets, R-on-T, or ventricular tachycardia. When a Holter

showed ventricular arrhythmias of more than one grade, the grade that was recorded was the highest one on that recording.

2.2.2. Echocardiographic assessment

Image acquisition: All echocardiographic studies (Philips EPIQ 7C, Philips Healthcare, Andover, MA, USA) were performed by the same cardiology resident under the direct supervision of a board-certified veterinary cardiologist. Echocardiograms were performed using 5-8 MHz transducers. All echocardiographic recordings were made with simultaneous ECG monitoring. Each animal was gently restrained in right and then left lateral recumbency without the use of sedation. Two-dimensional (2D) and M-mode images were obtained from the right and left parasternal positions. Image planes were identified and measurements were taken according to the recommendations of the American Society of Echocardiography.[131]

Echocardiographic measurements: Echocardiographic measurements and calculations were performed by the cardiology resident and verified by a board-certified veterinary cardiologist. Values for each echocardiographic variable consisted of the average of a minimum of 3 measurements obtained during sinus rhythm or respiratory sinus arrhythmia. To avoid post-extrasystolic potentiation and its effects on ventricular function, measurements were not obtained from cardiac cycles immediately after a PVC. For 2D images, end-diastole was defined as the first frame after atrial contraction in which the mitral or tricuspid valve was closed. For M-mode images, end-diastole was determined electrocardiographically and was defined as the onset of the QRS complex.

End-systole was the frame that immediately preceded tricuspid/mitral valve opening. A leading-edge-to-leading-edge technique was used for the measurement of M-mode images. For 2D images, a trailing-edge-to-leading-edge technique was used for luminal dimensions. For measurement of wall thickness, the endocardial border furthest from the transducer was included but the more proximal was not. Left ventricular internal dimensions from the same cardiac cycle were measured at end-diastole (LVIDd) and end-systole (LVIDs) at the level of the papillary muscles from the right parasternal short-axis view using M-mode echocardiography. The ratio of left atrial to aortic root dimensions (LA/Ao) was measured on the first frame after aortic valve closure from a right parasternal short-axis view using 2D echocardiography.[132] Left ventricular internal dimensions at end-systole were normalized to body weight (LVIDsN) using the following formula: LVIDsN = LVIDs (cm) / (body weight$^{0.315}$) and LV internal dimensions at end-diastole were normalized to body weight (LVIDdN) using the following formula: LVIDdN = LVIDd (cm) / (body weight$^{0.294}$).[133] Left ventricular fractional shortening (LV FS) was calculated as ([LVIDd - LVIDs]/LVIDd) x 100. Left ventricular volumes were estimated at end-diastole (LVEDV) and end-systole (LVESV) using Simpson's method of discs from the right parasternal long-axis 4-chamber view optimized for the LV.[134] Left ventricular ejection fraction (LV EF) was calculated as ([LVEDV – LVESV]/LVEDV) x 100). Breed-specific reference values for ESV indexed to body surface area (LVESVI) were utilized to identify LV chamber enlargement in Boxers (>50 mL/m^2).[135] Dogs with trace physiologic tricuspid or pulmonary valve regurgitation were not excluded due to the high prevalence of this finding in healthy dogs.[136]

Images for estimating RV systolic function were acquired from the left apical 4-chamber view optimized for the right heart.[137] Tricuspid annular plane systolic excursion was measured in all dogs as maximal longitudinal excursion of the lateral tricuspid annulus toward the RV apex during systole.[137] Tricuspid annular plane systolic excursion was generated using standard M-mode recordings and anatomic M-mode was not used to avoid underestimation. Measurements of TAPSE were obtained at sweep speeds of 100 mm/sec. Pulsed-wave TDI velocities of longitudinal myocardial motion at the lateral tricuspid annulus were obtained to measure peak systolic annular velocity (S'). Pulsed wave sample volume was maintained at 4-5 mm. Measurements of S' were made at sweep speeds of at least 66 mm/sec. All efforts were made to ensure optimal alignment with the RV free wall during the acquisition of left apical views for the measurements of TAPSE and S'. Measurement of RV area for fractional area change (FAC) was obtained by tracing the RV endocardial border at end-diastole (RVAd) and end-systole (RVAs).[137] Right ventricular percent FAC was calculated using the formula: FAC = ([RVAd-RVAs]/RVAd) x 100.

2.2.3. Thoracic radiographs

Three-view thoracic radiographs were obtained from ventrodorsal, right, and left lateral projections without sedation. Care was taken to acquire all radiographs at maximal inspiration. A board-certified radiologist blinded to the echocardiographic results reviewed the radiographs. Radiographs were evaluated for the presence of pulmonary metastases, lymphadenopathy, cardiac enlargement, presence of pleural effusion, or any

other intra-thoracic abnormalities that, in the opinion of the investigator, could confound arrhythmia interpretation.

2.2.4. Blood pressure

Noninvasive BP was measured in all dogs using an ultrasonic Doppler flow monitor. The dogs were positioned in lateral recumbency for all measurements. The average from five consecutive measurements were recorded and considered to be normal if systolic pressure was ≤160 mmHg.[138,139] The average systolic BP >160 mmHg that occurred as a consequence of excitement or anxiety associated with the hospital environment and in the absence of a disease or condition known to cause hypertension was considered situational hypertension (white coat hypertension).[138]

2.2.5. Complete blood count and plasma biochemistry profile

Venous blood samples were drawn via jugular venipuncture and collected into blood collection tubes containing potassium ethylenediaminetetraacetic acid (K_3-EDTA) for CBC and lithium heparin for plasma biochemistry profile. The samples were processed routinely, and CBC and plasma biochemistry profiles were performed the same day samples were submitted to the Atlantic Veterinary College Diagnostic Laboratory (AVCDL).

2.2.6. Urinalysis

Alysis of urine samples collected by free catch consisted of measurement of urine specific gravity (USG) with a manual refractometer and urinary dipstick test.

2.2.7. Genotyping for striatin deletion mutation

Genotyping for the Boxer ARVC striatin deletion mutation was performed with DNA samples obtained from blood collected in K_3-EDTA tubes. The blood samples were stored at 4^0C immediately after collection until assayed. Batch analysis was performed within 30 days of sample collection by the Veterinary Cardiac Genetics Laboratory at North Carolina State University's College of Veterinary Medicine, Raleigh, NC, USA. The laboratory team members who measured biomarkers were blinded to patient, clinical and diagnostic assessment, and cTnI test results.

2.2.8. Cardiac troponin I concentration

Cardiac troponin I concentrations were measured using two different assays. They were: 1) point-of-care (POC, cage-side) cTnI assay (i-STAT; Abbott Point of Care Inc., IL, USA) with a lower detection limit of 20 ng/L or 0.02 ng/mL and 2) Advia Centaur CP® Ultra-TnI assay (hs-cTnI, Siemens Healthcare Diagnostics Inc. NY, USA) with a lower detection limit of 6 ng/L or 0.006 ng/mL. The [cTnI] was measured at baseline visit by the POC and hs-cTnI assays both. At ExT visit, pre-exercise and post-exercise [cTnI] was measured using only hs-cTnI.

Venous blood samples were drawn via jugular venipuncture and collected into blood collection tubes containing: 1) K_3-EDTA for POC cTnI and 2) no anticoagulant and without serum separator for hs-cTnI, according to the manufacturers' recommendations and diagnostic laboratory standards. The dogs were not fed for at least 6 hours before blood collection. The blood samples were stored at 4^0C immediately after collection. In accordance with manufacturer guidelines, the samples for POC cTnI were analyzed within 30 minutes of collection. For hs-cTnI, samples were centrifuged 20-25 minutes after collection, at 3000 rpm for 10 minutes, the sera extracted, transferred to microcentrifuge tubes, and stored at -80˚C for batched analysis. The batch analysis was performed within 30 days of sample collection by the Gastrointestinal Laboratory at the Texas A&M College of Veterinary Medicine and Biomedical Sciences, College Station, TX, USA. The samples were thawed once, at the time of analysis. The laboratory team members who measured biomarkers were blinded to patient, clinical and diagnostic assessment, and striatin test results.

2.2.9. Exercise testing protocol

The purpose, possible risks, and execution of ExT were explained to the dogs' owners in advance. The dogs were not fed for at least 6 hours before the test. Prior to the study, four individuals (2 veterinarians, 1 veterinary technician, and I) were designated as handlers. For each dog, two handlers (based on availability, always to include 1 veterinarian) were responsible for handling the dog. The two handlers relayed each other, switching immediately between laps (i.e., handler #1 did laps 1 and 3 and handler #2 did lap 2 with the dog) in a way that did not interrupt the dog's exercise. One additional

individual, who was a staff member or student, was responsible for operating a timer and recording time during the ExT. No one other than these 3 individuals were permitted to enter the stairwell for the duration of ExT, and signs were posted on doors on each floor to prevent people from entering the stairwell during an ExT. The ExT began with a 1-minute acclimation period at the foot of the stairwell, during which each dog was allowed to circulate on leash but was prevented from climbing the stairs. Exercise tests were performed in a 6-flight stairwell (68 steps; 42' 1" [12.83 m] vertical),[140] which each dog climbed and descended three times consecutively (i.e. 3 laps without resting in between laps) briskly with a handler. The time taken for each lap was recorded, as well as the total duration of the ExT excluding the acclimation period. During the recovery period, dogs were monitored until a full recovery was achieved as assessed when a veterinarian handler identified that the dog had a normal mentation, willingness and ability to ambulate, and respiratory effort and rate. The following ExT termination criteria were used: signs of fatigue, dyspnea, collapse, pronounced lethargy, central nervous system dysfunction (e.g., ataxia), lameness, any other clinical signs consistent with tachyarrhythmia or of concern to any investigator, and/or unwillingness to climb the stairs despite gentle coaxing.

2.3. Statistical analysis

Statistical analyses were performed using a commercial software package (Prism 7 for Mac OS X, Version 7.0c, GraphPad Software, Inc, La Jolla, CA, USA). The minimum number of animals (24 animals) to be used was calculated by a power analysis based on the following assumptions: the likelihood of type I error: 0.05, previously

reported significant difference in [cTnI] between control and Boxer dogs with ARVC [42]: 0.06 ng/mL, variance of effect size 0.05, power 0.8. A sample size of 30 was used in the study in anticipation of attrition in animals and samples. Descriptive statistics were generated. To assess normality, QQ plots were graphically inspected, and the Kolmogorov-Smirnov test was performed for all continuous data. Because results for several variables were not normally distributed, data for all variables were analyzed as non-normally distributed and are presented as median (interquartile range, IQR), unless stated otherwise.

Bland-Altman plots and Mann-Whitney U tests were used for a comparison between POC [cTnI] and [hs-cTnI]. Differences in paired continuous data (pre-exercise versus post-exercise [hs-cTnI]) were evaluated using a Wilcoxon matched-pairs signed-rank test (non-normally distributed data). The relationships between a continuous variable (number of PVCs/24 h or [cTnI]) and selected parameters (age, weight, plasma potassium concentrations [K^+], and [cTnI]) were tested using Spearman correlation analysis. Mann-Whitney U tests were used to test the difference in number of PVCs/24 h or [cTnI] for sex and striatin mutation (dichotomous categorical variables). A Kruskal-Wallis test was used to test the difference in number of PVCs/24 h or [cTnI] for grade of ventricular arrhythmias (ordinal categorical variable). Receiver operating characteristic (ROC) curve analysis was used to investigate the ability of POC [cTnI] and [hs-cTnI] to distinguish between dogs with >100 PVCs/24 h and dogs with ≤100 PVCs/24 h.

A value of $p<0.05$ was considered significant for all tests.

3. RESULTS

3.1. Study population

One hundred and fifty-eight Boxer dogs were screened individually for enrollment (Figure 3). Of these, 81 dogs were excluded because they had one or more pre-defined exclusion criteria based on history and medical records; 8 dogs did not fulfill the inclusion criteria because they were <12 months old; and for 33 dogs, the owners declined participation in the study for various reasons, leaving 36 dogs for enrollment in the study. Of these, 3 dogs then were excluded because they met one or more exclusion criteria based on physical examination and initial diagnostic test results, and 3 other dogs were excluded for other reasons listed in Figure 3. A total of 30 Boxer dogs completed the study. The results obtained from these 30 dogs were used for statistical analyses.

3.2. Demographics and clinical findings

The median age at initial assessment (baseline visit) was 4.2 years (IQR, 3.0-5.3 years). Males and females were equally distributed. Among females, 5 were intact and 10 were spayed. Among males, 5 were intact and 10 were neutered. The median body weight was 29.2 kg (IQR, 25.2–33.9 kg). Ten (33.3 %) dogs were heterozygous for the striatin deletion mutation, and the remaining 20 dogs (66.7%) were negative. None of the dogs was homozygous for the mutation. None of the dogs had received any cardiac medications before evaluation nor received any during the study period.

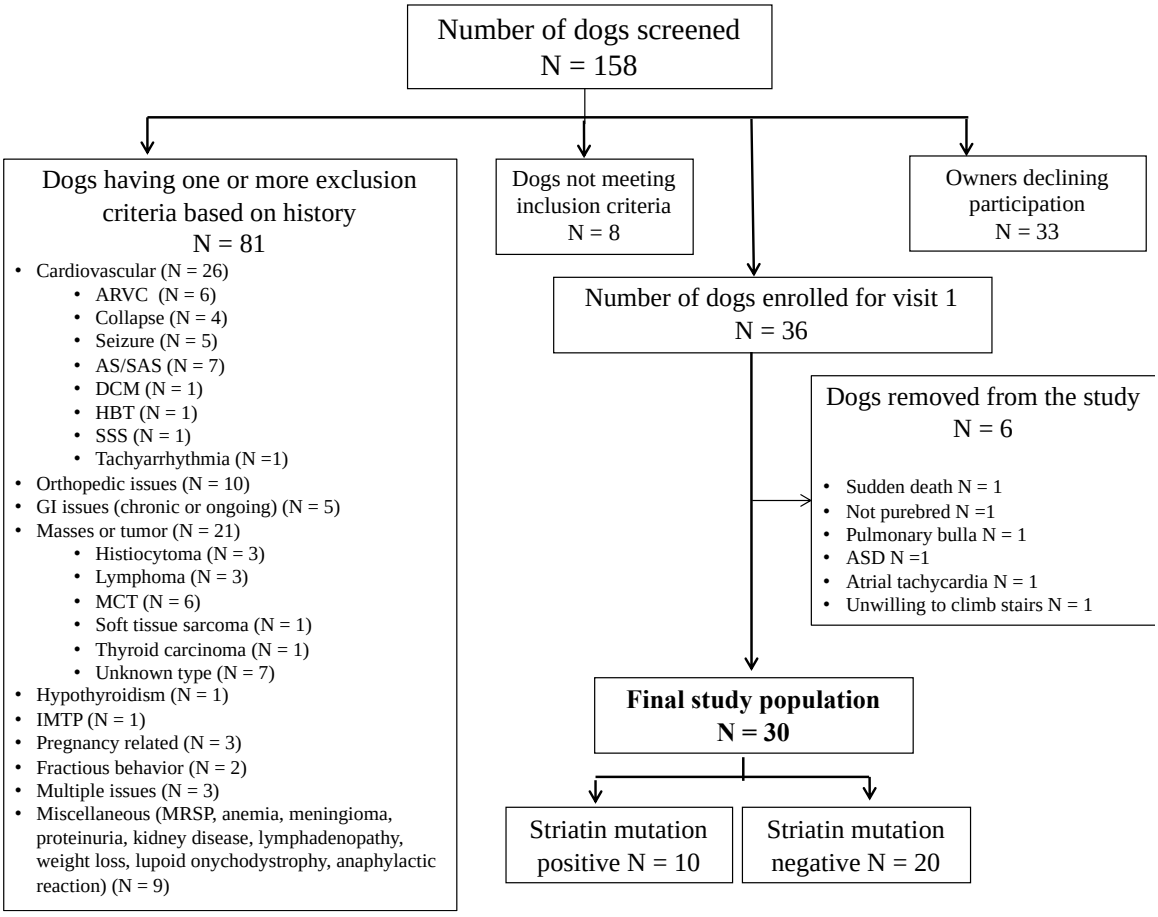

Figure 3. A flowchart illustrating the number of dogs screened for the study, the number of dogs that met pre-defined exclusion criteria based on history, the number of dogs that met exclusion criteria based on initial diagnostic assessment, the number of dogs remaining in the final population, and the striatin mutation test result for the 30 dogs that completed the study. ARVC, arrhythmogenic right ventricular cardiomyopathy; AS/SAS, aortic stenosis/subaortic stenosis; ASD, atrial septal defect; DCM, dilated cardiomyopathy; GI, gastrointestinal; HBT, heartbase tumor; IMTP, immune-mediated thrombocytopenia; MCT, mast cell tumor; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; N, number of dogs; SSS, sick sinus syndrome.

Echocardiographic data are presented in Table 1. Bloodwork (CBC and plasma biochemistry profile) did not show any significant abnormalities which could confound biomarker or arrhythmia interpretation except plasma [K^+]. The median plasma [K^+] was 3.8 mmol/L (IQR, 3.6–4.0 mmol/L) with the lowest plasma [K^+] being 2.6 mmol/L. The

median systolic BP was 132 mmHg (IQR, 113–142 mmHg). Systolic BP was normal (≤160 mmHg) in 28 dogs and the remaining 2 dogs had situational hypertension (167.5 and 169 mmHg). Thoracic radiographs and urinalysis did not show any clinically significant abnormalities.

Table 1. Echocardiographic data in apparently healthy Boxer dogs obtained at the baseline visit and breed-specific reference intervals.

Variable	Median (IQR)	Reference interval, Median (range) or mean ± SD or mean ± SD (range)
LVIDd (mm)	38.5 (35.5-41.3)	39.0 (29.0-48.0)[141]
LVIDs (mm)	26.0 (23.0-30.1)	24.5 (16.7–33.0)[141]
LVIDdN	1.44 (1.26-1.52)	-
LVIDsN	0.91 (0.79-1.04)	-
LVFS (%)	32.7 (23.9-37.2)	37.0 (21.0–51.0)[135]
LA/Ao ratio	1.5 (1.4-1.6)	1.7 (1.4-2.0)[60]
LVEDV (ml)	46.0 (29.5-59.7)	-
LVESV (ml)	18.6 (15.1-25.3)	-
LVESVI (ml/m^2)	18.7 (15.8-25.4)	36 ± 7 (22-50)[135]
LVEF (%)	60.4 (53.7-66.3)	49 ± 7[135]
RVFAC (%)	47.3 (42.3-54.4)	-
TAPSE (mm)	13.0 (12.5-15.0)	14.2 (10.2-18.2)[60]
S’ (cm/sec)	18.2 (15.9-21.3)	17.0 (10.0-28.0)[60]

IQR, interquartile range; LA/Ao, left atrial to aortic root diameter ratio; LVEDV, left ventricular (LV) end-diastolic volume; LVEF, LV ejection fraction; LVESV, LV end-systolic volume; LVESVI, LVESV indexed to body surface area; LVFS, LV fractional shortening; LVIDd, LV internal dimension at end-diastole; LVIDs, LV internal dimension at end-systole; LVIDdN, LV internal dimension at end-diastole normalized to body weight; LVIDsN, LV internal dimension at end-systole normalized to body weight; RVFAC, right ventricular fractional area change; S’, peak systolic annular velocity at the lateral tricuspid annulus; SD, standard deviation; TAPSE, tricuspid annular plane systolic excursion;

3.3. Ventricular arrhythmias at baseline visit

On baseline Holter recordings, 24 dogs had PVCs, with a median of 10 PVCs/24 h (range, 1–767 PVCs/24 h) and grades of arrhythmia ranging from 1-3 (grade 1 in 14, grade 2 in 6, and grade 3 in 4 dogs). Female (median, 28 PVCs/24 h; range, 1-767 PVCs/24 h) dogs had significantly more PVCs than did males (median, 2 PVCs/24 h; range, 1-283 PVCs/24 h) ($p=0.016$). There was a significant increase in the number of PVCs with the increase in grade of arrhythmia ($p=0.0002$). There was no correlation between the number of PVCs and age, body weight, or plasma [K^+] (Table 2). The number of PVCs did not differ between dogs with or without the striatin mutation ($p=0.191$). Based on a cutoff of 100 PVCs/24 h at baseline, 8/30 dogs (27%) had ARVC.

Table 2. Correlation (Spearman) of the number of premature ventricular complexes with selected clinical parameters in 30 Boxer dogs at baseline visit.

Clinical parameter	r	*p*
Age	0.27	0.154
Weight	-0.06	0.735
Plasma [K^+]	-0.25	0.180

3.4. Effects of ExT on number and severity of ventricular arrhythmias

The repeat Holter recording performed at the ExT visit was used to evaluate the effects of ExT on number and severity of ventricular arrhythmias. The results are shown in Table 3 and Figure 4 and were compared to the baseline Holter results. The median total exercise duration was 176.5 seconds (IQR, 163.3-221.5 seconds).

Table 3. Number of premature ventricular complexes per 24 hours and grade of ventricular arrhythmias recorded at baseline visit and at exercise testing.

	Number of PVCs/24 h				Grade of VA		
Dog #	Baseline Holter	ExT Holter	Difference between ExT and baseline Holter	% Change *	Baseline Holter	ExT Holter	Difference between ExT and baseline Holter
1	2	3	1	33	1	1	0
2	3	1	-2	-67	1	1	0
3	3	11	8	73	1	3	2
4	0	4	4	100	0	1	1
5	0	1	1	100	0	1	1
6	214	6	-208	-97	1	1	0
7	0	3	3	100	0	1	1
8	0	0	0	0	0	0	0
9	318	169	-149	-47	2	1	-1
10	2	1725	1723	100	1	2	1
11	28	0	-28	-100	3	0	-3
12	336	545	209	38	2	2	0
13	2	0	-2	-100	1	0	-1
14	570	1390	820	59	2	3	1
15	13	11	-2	-15	3	2	-1
16	11	12	1	8	3	2	-1
17	144	924	780	84	2	1	-1
18	0	0	0	0	0	0	0
19	18	36	18	50	2	2	0
20	10	20	10	50	1	3	2
21	27	55	28	51	2	1	-1

22	0	3	3	100	0	1	1
23	17	102	85	83	1	2	1
24	147	67	-80	-54	1	1	0
25	767	202	-565	-74	3	1	-2
26	10	7	-3	-30	1	1	0
27	3	171	168	98	1	1	0
28	283	64	-219	-77	1	1	0
29	1	0	-1	-100	1	0	-1
30	2	3	1	33	1	1	0

*% Change = [(Number of PVCs/24 h on ExT Holter – number of PVCs on baseline Holter)/ Number of PVCs/24 h on ExT Holter] x 100

ExT, exercise testing visit ; PVCs, premature ventricular complexes; VAs, ventricular arrhythmias. Grey shading of cells highlights dogs having ≥100 PVCs/24 h on one or both Holter recordings.

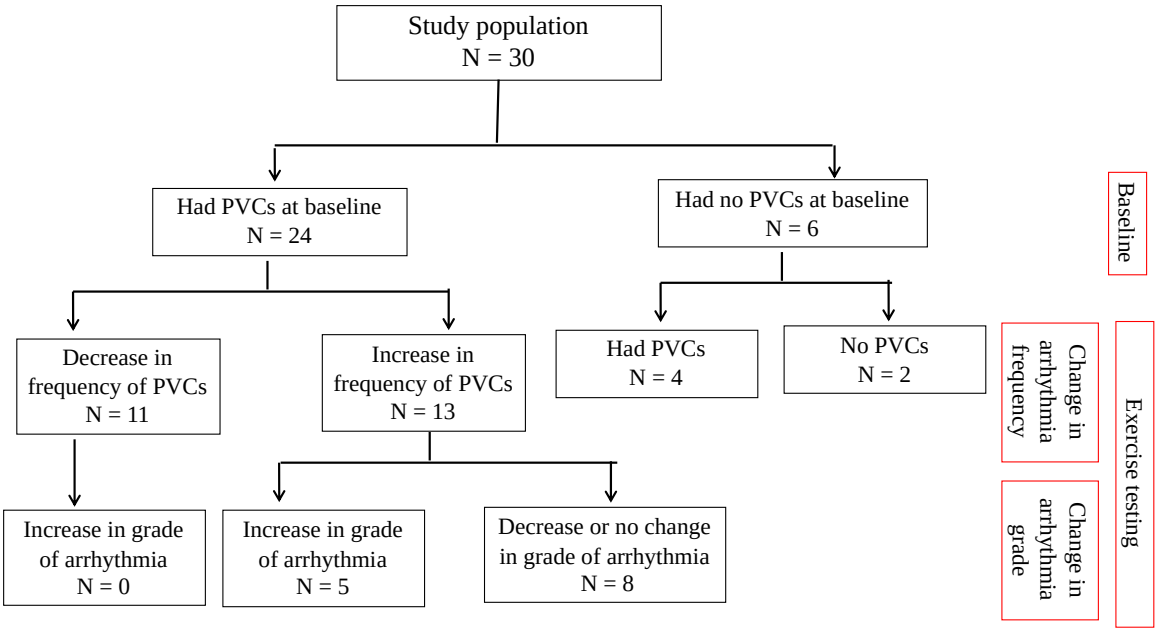

Figure 4. A flowchart illustrating the number of dogs that had or did not have PVCs on baseline Holter recording and the number of dogs exhibiting change in arrhythmia number and/or arrhythmia grade on exercise testing Holter recording. PVCs, premature ventricular complexes.

At the ExT visit, 25 dogs had PVCs, with a median of 10 PVCs/24 h (range, 1–1725 PVCs/24 h). In 17/25 dogs that had PVCs, PVCs occurred during the peri-exercise period (i.e., from onset of exercise to 3 h thereafter). The proportion of PVCs occurring in the peri-exercise period in these dogs ranged from 1.2 to 66.7% of total daily PVCs (median, 33.3%). In the remaining 8 dogs with PVCs, no PVCs occurred in the peri-exercise period.

Of 24 dogs that had PVCs at the baseline visit, 54% of dogs (13/24) had an absolute increase in the number of PVCs at the ExT visit (range, 1-1723 PVCs/24 h). In the remaining 46% of dogs (11/24), the number of PVCs at the ExT visit decreased (range, 2-565 PVCs/24 h). Additionally, of the 6 dogs that did not have any PVCs at the baseline visit, 4 dogs had PVCs at the ExT visit (range, 1-4 PVCs/24 h). The remaining 2 dogs did not exhibit any PVCs on either visit. The increase in the number of PVCs did not correlate with total exercise duration ($p=0.180$).

Among 13 dogs that had an increase in the number of PVCs/24 h on ExT Holter, 5 (38.4%) had a concurrent increase in grade of arrhythmia (range, 1-2). For the remaining 8 (61.6%), the number of PVCs increased without a concurrent increase in grade. Taken together, of 24 dogs that had PVCs on baseline visit, 9 (37.5%) had an increase in grade of arrhythmia at the ExT visit. In 11 dogs that had a decrease in the number of PVCs, there was a decrease or no change in grade of arrhythmia (none of these 11 dogs had an increase in grade of arrhythmia).

In summary, 13/30 dogs had an increase in the number of PVCs/24 h at the ExT visit, and in 4 additional dogs, ventricular arrhythmias were revealed at the ExT visit only, albeit a low number. The differences between maximum and minimum arrhythmia grade scores in these dogs were ≤3 categories.

Based on a cutoff of 100 PVCs/24 h, 8 dogs would have met the criteria for having ARVC at the baseline visit. Three additional dogs (#10, 23, 27) that would have been classified as not having ARVC at the baseline visit had >100 PVCs/24 h at the ExT visit and as such, would have been diagnosed with ARVC based on the ExT visit results. In these 3 dogs, proportions of PVCs occurring in the peri-exercise period were 14.5%, 52%, and 1.2% of total daily PVCs, respectively. One of these dogs had PVCs during the exercise test (#23).

3.5. Cardiac troponin I concentration

3.5.1. Comparison of results obtained with the POC cTnI and hs-cTnI assays

The median circulating [cTnI] detected by POC cTnI assay and hs-cTnI assay at baseline visit were 60 ng/L (IQR, 50-80 ng/L) and 140.5 ng/L (IQR, 120.3-227.5 ng/L), respectively (Figure 5). The [hs-cTnI] was significantly higher than POC [cTnI] ($p<0.0001$). There was a mean % difference of 81.0, SD of the % difference ±25.0%, 95% CI of the % difference = 71.7-90.3%, lower limit of agreement: 32.0%, and upper limit of agreement: 130.0% (Figure 6).

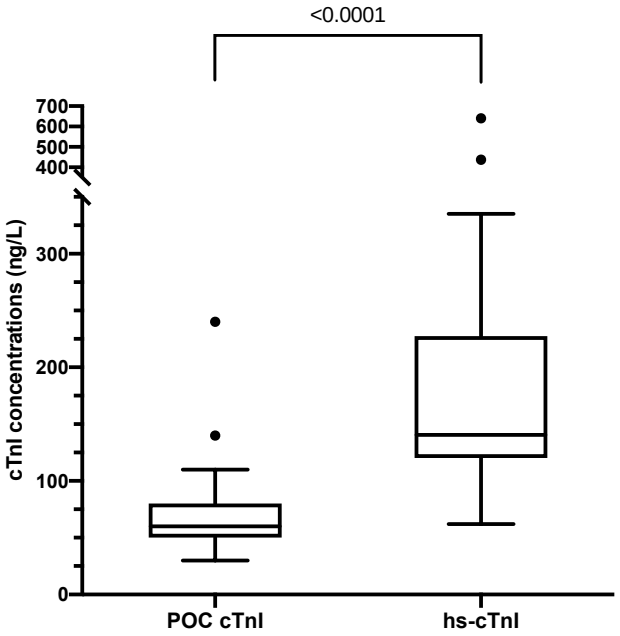

Figure 5. Box and Whisker plots comparing POC cTnI and hs-cTnI concentrations at baseline. N=30 in each group. Each box represents the IQR. Solid horizontal lines within boxes represent median values. Tukey-style whiskers extend to a maximum of 1.5 × IQR beyond the box. The dots denote outliers. cTnI, cardiac troponin I; hs-cTnI, high-sensitivity cTnI assay; IQR, interquartile range; POC cTnI, point-of-care cTnI assay.

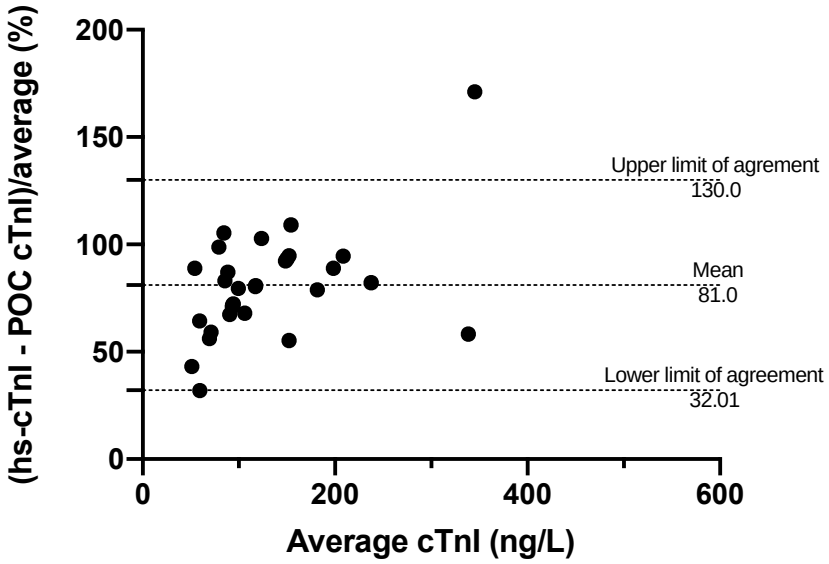

Figure 6. Bland-Altman plot displaying the agreement between cTnI concentrations obtained from POC and hs-cTnI assays. cTnI, cardiac troponin I; hs-cTnI, high-sensitivity cTnI; POC cTnI, point-of-care cTnI.

3.5.2. Clinical evaluation and comparison of results obtained with the POC cTnI and hs-cTnI assays

3.5.2.1. Baseline POC [cTnI]

The median POC [cTnI] was numerically higher in dogs with >100 PVCs/24 h (median, 70 ng/L; IQR, 52.5-77.5 ng/L) than in dogs with ≤100 PVCs/24 h (median, 60 ng/L; IQR, 50-80 ng/L); but the difference was not statistically significant (p=0.204). When dogs that had ≤100 PVCs/24 h and were negative for the striatin mutation were analyzed separately (i.e., dogs that are unlikely to have ARVC; n=16), the median POC [cTnI] was 60 ng/L (IQR, 50-80 ng/L). There was no correlation between POC [cTnI] and age, weight, or number of PVCs/24 h (Table 4). The POC [cTnI] did not differ between male and female (p=0.314) or between dogs with various grades of arrhythmia (p=0.259).

Table 4. Correlation (Spearman) of baseline POC [cTnI] and [hs-cTnI] with selected clinical parameters obtained at the baseline visit.

	POC [cTnI]		[hs-cTnI]	
Clinical parameter	r	*p*	r	*p*
Age	-0.07	0.72	0.04	0.85
Weight	-0.05	0.78	0.06	0.75
PVCs/24 h	0.30	0.10	0.53	**0.002**
[hs-cTnI], cTnI concentrations measured by the high-sensitivity cTnI assay; POC [cTnI], cTnI concentrations measured by the point-of-care assay; PVCs, premature ventricular complexes.				

Several POC [cTnI] cutoff values were tested using a receiver operating characteristic (ROC) curve to attempt to distinguish between dogs with ≤100 PVCs/24 h and dogs with >100 PVCs/24 h (baseline visit) (Table 5). The ROC analysis performed on the 30 dogs yielded an area under the curve of 0.574 (Figure 7A). At an optimal cutoff value of 55 ng/L, the sensitivity was 75% and the specificity was 41% (Table 5).

Table 5. The ability of POC [cTnI] and [hs-cTnI] to distinguish between Boxer dogs that had ≤100 PVCs/24 h and dogs with >100 PVCs/24 h (baseline visit).

Assay	Cutoff value (ng/L)	AUC	p	Sensitivity %	Specificity %	LR+
POC cTnI	65.0	0.574	0.542	62.5	63.6	1.719
	55.0	0.574	0.542	75.0	40.9	1.269
hs-cTnI	128.5	0.756	0.035	100	59.1	2.444
	153.0	0.756	0.035	75	63.6	2.063
	222.0	0.756	0.035	50.0	81.8	2.750

AUC, area under the curve; cTnI, cardiac troponin I, [hs-cTnI], cTnI concentrations measured by hs-cTnI assay; LR+, positive likelihood ratio; POC [cTnI], cTnI concentrations measured by the point-of-care assay; PVCs, premature ventricular complexes.

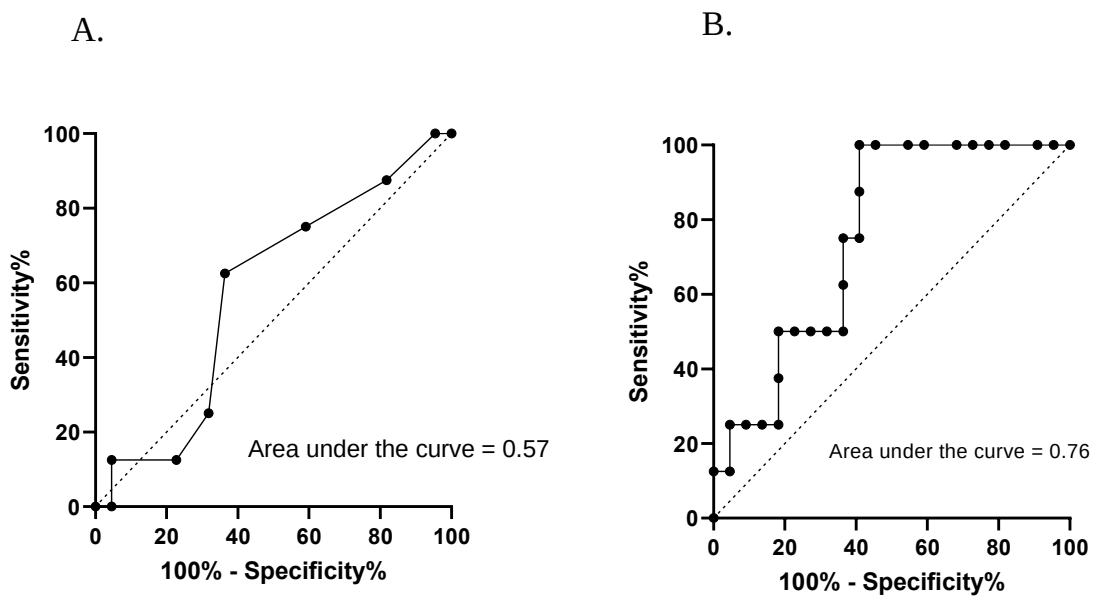

Figure 7. Receiver operating characteristic curves depicting the sensitivity and specificity of POC [cTnI] (A) and [hs-cTnI] (B) in discerning between Boxer dogs with >100 PVCs/24 h and those with ≤100 PVCs/24 h. The area under the curve for POC cTnI was 0.57 and for hs-cTnI was 0.76. cTnI, cardiac troponin I; [hs-cTnI], cTnI concentrations measured by hs-cTnI assay; POC [cTnI], cTnI concentrations measured by the point-of-care assay.

3.5.2.2. Baseline [hs-cTnI]

The baseline [hs-cTnI] was significantly higher in Boxer dogs with >100 PVCs/24 h (median, 165 ng/L; IQR 139-238 ng/L) than in Boxer dogs with ≤100 PVCs/24 h (median, 127 ng/L; IQR 90.5-205.5 ng/L; p=0.023). When dogs that had ≤100 PVCs/24 h and were also negative for the striatin mutation were analyzed separately (i.e., dogs that were unlikely to have ARVC; n = 16), the median [hs-cTnI] was 126.5 ng/L (IQR, 98.5-211.3 ng/L). A significant correlation existed between [hs-cTnI] and the number of PVCs/24 h (p=0.002; Table 4). There was no correlation between [hs-cTnI]

and age (Table 4). The [hs-cTnI] did not differ between male and female (p=0.418) or between dogs with various grades of arrhythmia (p=0.069).

Receiver operating characteristic curve analysis performed using the 30 dogs yielded an area under the curve of 0.76 to distinguish between dogs that had ≤100 PVCs/24 h and dogs that had >100 PVCs/24 h (Figure 7B). At an optimal cutoff value of 128.5 ng/L, the sensitivity was 100% and the specificity was 59% (Table 5).

3.5.2.3. Effects of exercise testing on [hs-cTnI]

The ExT resulted in a significant absolute increase in [hs-cTnI] (Figure 8). The median pre-exercise and post-exercise serum [hs-cTnI] were 121.0 ng/L (IQR, 77.3-160 ng/L) and 158.5 ng/L (IQR, 115.5-233.5 ng/L), respectively. The median difference between post- and pre-exercise [hs-cTnI] was 37.0 ng/L (CI, 26.1-71.5 ng/L; p<0.0001).

There was a weak but significant correlation between the ExT-associated increase in [hs-cTnI] and the number of PVCs/24 h at the ExT visit (r=0.45; p=0.012). The pre-exercise [hs-cTnI] was significantly higher in dogs that had PVCs during the peri-exercise period compared to those that had no PVCs during the peri-exercise period (median, 77 vs 151 ng/L; p=0.002). There was no correlation between ExT-associated increase in [hs-cTnI] and age, body weight, or exercise duration (Table 6). Sex (p=0.213) and grade of arrhythmia (p=0.281) did not have any significant effect on exercise-associated increase in [hs-cTnI].

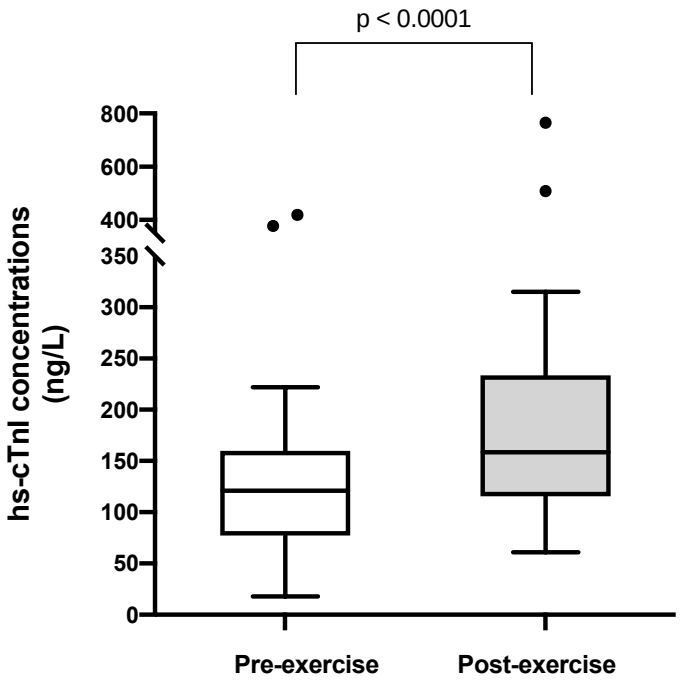

Figure 8. Box plot showing differences between pre-exercise and post-exercise [hs-cTnI] in apparently healthy Boxer dogs. N=30 in each group. Each box represents the IQR. Solid horizontal lines within boxes represent median values. Tukey-style whiskers extend to a maximum of 1.5 × IQR beyond the box. The dots denote outliers. [hs-cTnI], cardiac troponin I concentrations measured by the high-sensitivity assay; IQR, interquartile range.

The exercise-associated increases in [hs-cTnI] did not correlate with the number of PVCs/24 h at baseline (Table 6). Exercise-associated increases in [hs-cTnI] did not differ between dogs that had an increase in the number of PVCs/24 h and dogs that had no change or had a decrease in the number of PVCs/24h (46.0 vs 36.0 ng/L; p=0.173) at the ExT visit versus the baseline visit. Similarly, exercise-associated increases in [hs-cTnI] did not differ between dogs that had an increase in the grade of ventricular arrhythmia and dogs that had no change or decrease in the grade of arrhythmia (46.0 vs 36.0 ng/L; p=0.483) at the ExT visit versus the baseline visit.

Table 6. Correlation (Spearman) of change in [hs-cTnI] after exercise test (post-exercise minus pre-exercise) with selected clinical parameters in 30 Boxer dogs.

Clinical parameter	r	*p*
Age	-0.011	0.954
Weight	0.17	0.381
Exercise duration	0.28	0.134
PVCs/24 h at ExT visit	0.45	**0.012**
PVCs/24 h at baseline visit	0.24	0.202
ExT, exercise testing; [hs-cTnI], cardiac troponin I concentrations measured by the high-sensitivity assay; PVCs, premature ventricular complexes.		

Dogs with >100 PVCs/24 h had a significantly greater increase in [hs-cTnI] after exercise than did dogs with ≤100 PVCs/24 h (median, 67 vs 34.5 ng/L; p=0.006; Figure 9). Receiver operating characteristic curve analysis performed using all 30 dogs showed that the exercise-induced increase in [hs-cTnI] had an area under the curve of 0.80 (Figure 10) to distinguish Boxer dogs with >100 PVCs/24 h from Boxer dogs with ≤100 PVCs/24 h. At a cutoff value of 39.5 ng/L increase in post-exercise [hs-cTnI], the sensitivity was 87.5% and specificity was 63.6% (Figure 10, Figure 11, and Table 7).

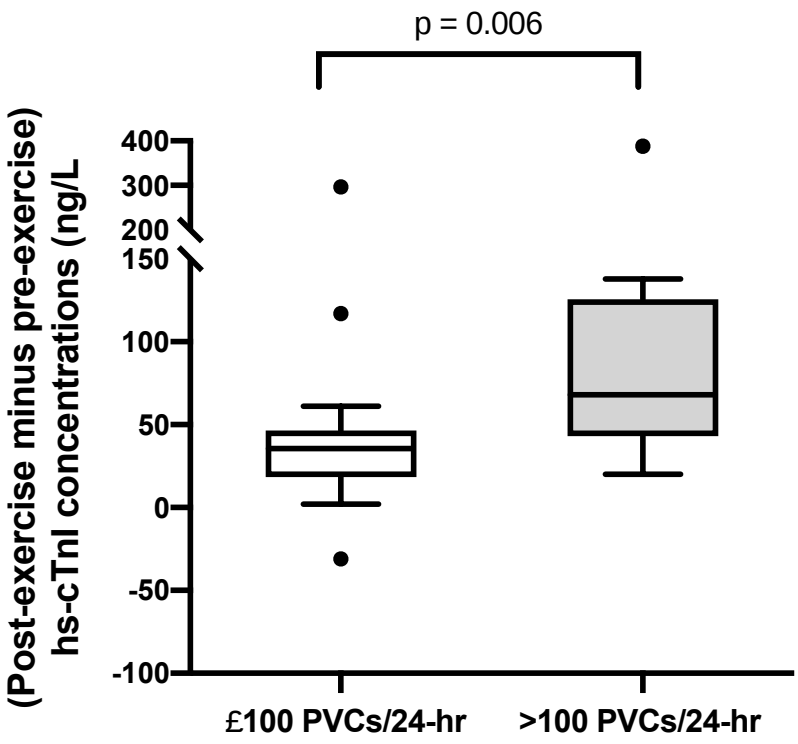

Figure 9. Box plot demonstrating exercise-associated increase in [hs-cTnI] in Boxer dogs with ≤100 PVCs/24 h and >100 PVCs/24 h. Each box represents the interquartile range. Solid horizontal lines within boxes represent median values. Tukey-style whiskers extend to a maximum of 1.5 × IQR beyond the box. The dots denote outliers. [hs-cTnI], cardiac troponin I concentrations measured by the high-sensitivity assay; PVCs, premature ventricular complexes.

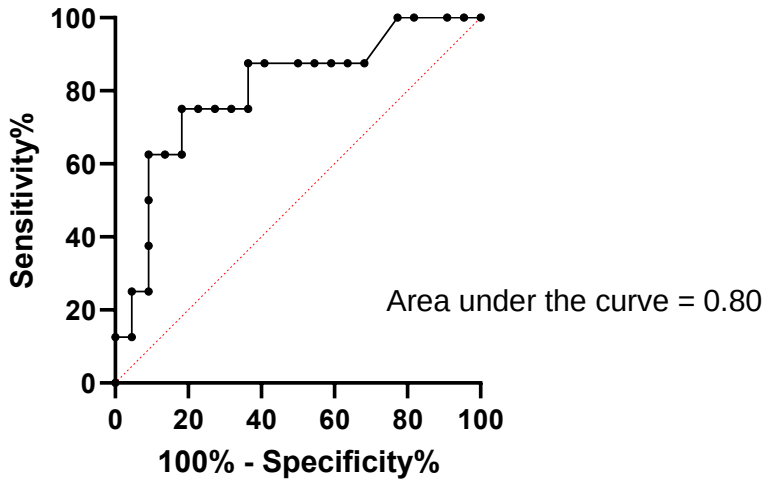

Figure 10. Receiver operating characteristic curves depicting the sensitivity and specificity of exercise-induced increase in [hs-cTnI] in discerning between dogs with ≤100 and >100 PVCs/24 h.

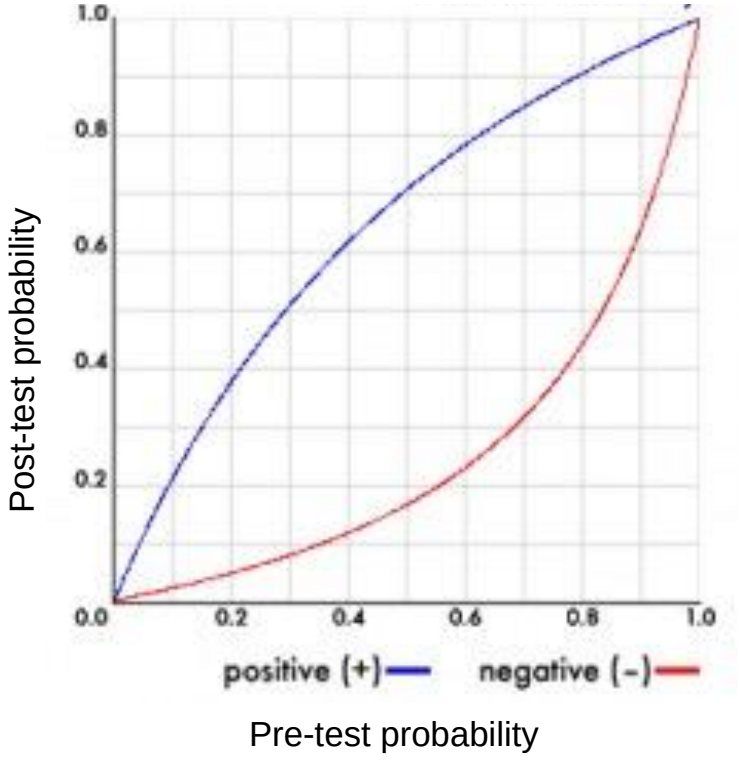

Figure 11. Relationship of pre-test probability to post-test probability of having >100 PVCs/24 h when exercise testing results in an increase in circulating [hs-cTnI], assuming sensitivity of 87.5% and specificity of 63.6% at a cutoff value of 39.5 ng/L. The blue curve represents a positive test and the red curve represents the negative test. [hs-cTnI], cardiac troponin I concentrations measured by the high-sensitivity assay.

Table 7. Test performance of exercise-associated increase in [hs-cTnI] to distinguish between dogs with ≤100 PVCs/24 h and dogs with >100 PVCs/24 h at a cutoff value of 39.5 ng/L.

	Estimate	95% CI
Sensitivity	0.875	[0.529 to 0.978]
Specificity	0.636	[0.430 to 0.803]
PPV	0.467	[0.248 to 0.699]
NPV	0.933	[0.702 to 0.988]
LR+	2.404	[1.305 to 4.436]
LR-	0.197	[0.031 to 1.262]

CI, confidence interval; [hs-cTnI], cardiac troponin I concentrations measured by the high-sensitivity assay; LR+, positive likelihood ratio; LR- negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; PVCs, premature ventricular complexes.

3.6. Relationship between [cTnI] and the striatin mutation

The baseline POC [cTnI] did not differ between dogs with or without the striatin mutation (median 60 ng/L for both; p=0.240) (Figure 12).

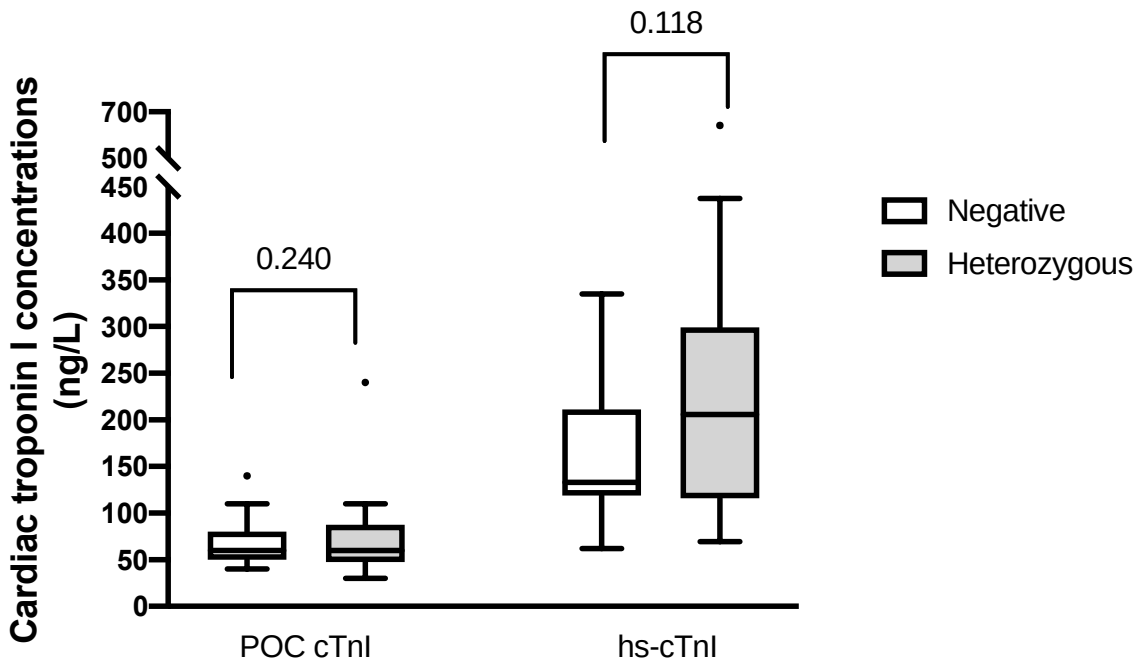

Figure 12. Box plot showing baseline [cTnI] in apparently healthy Boxer dogs with and without striatin mutation. Each box represents the interquartile range. Solid horizontal lines within boxes represent median values. Tukey-style whiskers extend to a maximum of 1.5 × IQR beyond the box. The dots denote outliers. cTnI, cardiac troponin I, [hs-cTnI], cTnI concentrations measured by hs-cTnI assay; POC [cTnI], cTnI concentrations measured by the point-of-care assay.

The baseline [hs-cTnI] was numerically higher in dogs with the striatin mutation (heterozygous) when compared to dogs without the mutation, but not significantly (p=0.118) (Figure 12). Receiver operating characteristic curve analysis performed using the 30 dogs showed that the [hs-cTnI] had an area under the curve of 0.64 to distinguish striatin-negative Boxer dogs from striatin heterozygous-positive Boxer dogs (Figure 13). At a cutoff value of 128.5 ng/L, the sensitivity was 70% and specificity was 50%.

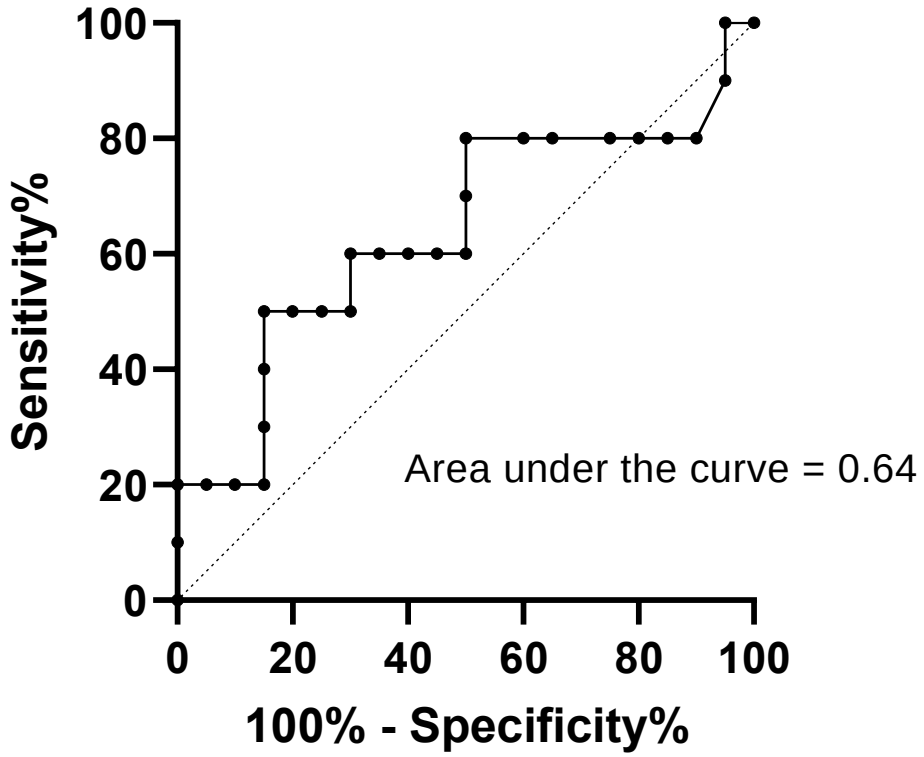

Figure 13. Receiver operating characteristic curve depicting the sensitivity and specificity of [hs-cTnI] for discerning between apparently healthy Boxer dogs with and without the striatin mutation. The area under the curve for hs-cTnI is 0.64. [hs-cTnI], cardiac troponin I concentrations measured by the hs-cTnI assay.

An exercise-associated increase in [hs-cTnI] was significantly higher in dogs with the striatin mutation compared to dogs without this mutation (Figure 14).

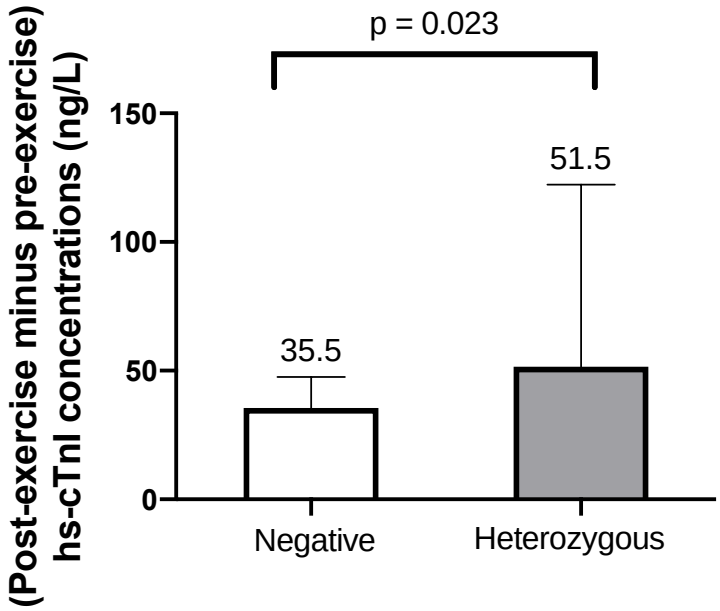

Figure 14. Bar graph displaying a difference in post-exercise and pre-exercise [hs-cTnI] (median with interquartile range) in apparently healthy Boxer dogs with and without the striatin mutation. Median values are presented above each bar. [hs-cTnI], cardiac troponin I concentrations measured by the hs-cTnI assay.

4. DISCUSSION

4.1. Effects of ExT on number and severity of ventricular arrhythmias in apparently healthy Boxer dogs

A principal finding of this study is that ExT in apparently healthy Boxer dogs may unmask ventricular arrhythmias that are not apparent on a routine cardiovascular examination and may worsen the number of PVCs in dogs that have arrhythmias on routine examination. Stair-based ExT protocol resulted in an increased number of PVCs in 13 dogs (54% of dogs that had PVCs on baseline Holter) and revealed PVCs in 4 dogs that did not have PVCs on baseline Holters, albeit a low number. Furthermore, based on the cutoff of >100 PVCs/24 h, ExT was necessary to reach a diagnosis of ARVC in 3 of these 13 dogs. In other words, in 10% (3/30) of the dogs in this study, the diagnosis of ARVC would not have been reached based on baseline Holter alone. These findings suggest that ExT can enhance the emergence of PVCs in some Boxer dogs. Since ventricular arrhythmias during ExT develop more frequently in humans with a definitive diagnosis of ARVC than healthy controls,[123,142] it is possible that these 17 Boxer dogs have occult ARVC even though many of them do not meet any of the previously proposed cutoff values[5,38,48,53–56] for the number of PVCs/24 h to differentiate between affected and nonaffected Boxer dogs. In opposition to this conclusion is our finding that 11 dogs (46% of dogs that had PVCs on baseline Holter) had a decrease in the number of PVCs/24 h on ExT Holters, suggesting that ExT decreases ventricular arrhythmias, at least in some Boxer dogs.

Three possible explanations for these findings are: 1) these dogs may not have ARVC, 2) the arrhythmogenic response to ExT could be inconsistent and, 3) there could be spontaneous temporal variability in the occurrence of PVCs. Due to the lack of an antemortem definitive diagnostic test for ARVC, it is unknown whether any individual Boxer dog in this study definitively has ARVC or not. This limits the conclusions that can be drawn on the effects of ExT on the number of PVCs/24 h. In the present study, all Boxer dogs had ExT performed at approximately the same time of the day. The percentage increase in the number of PVCs/24 h between the ExT Holter and baseline Holter ranged from 8 to 100 % and the percentage decrease ranged from -30 to -100 %. Spontaneous day-to-day variability in the number of PVCs documented on 24 h Holter from the same dog has ranged from 46% to 100%.[48] This raises the possibility that observed change (increase or decrease) in the number of PVCs/24 h on ExT visit in the present study represents spontaneous daily variability and not necessarily the effects of ExT. However, dogs' recent exercise history was not considered in the study showing 46-100% spontaneous daily variability making it difficult to exclude the confounding effects of dogs' activity level on number of PVCs/24 h in that study. Furthermore, it is important to note that this spontaneous variability does not define whether a dog is truly affected by ARVC or not but is rather a more clinically relevant concept while assessing the efficacy of therapeutic interventions.

It has been traditionally thought that PVCs that are reduced or completely suppressed during ExT in the absence of heart disease may be considered benign.[143,144] However, recent reports in humans suggest that PVCs should not be considered benign

merely because they are suppressed during ExT. In one such study, 40.5% of newly diagnosed ARVC patients exhibited a decrease or suppression of ventricular arrhythmias during ExT.[123] In another study, 24% of young ARVC patients exhibited complete suppression of PVCs at peak exercise during ExT.[122] It is not clear why some ARVC patients exhibit an increase in ventricular arrhythmias and some ARVC patients exhibit a decrease or complete suppression in ventricular arrhythmias during ExT. The physiologic response to exercise involves activation of the autonomic nervous system and both cardiac α1- and ß1-adrenergic receptors. It has been suggested that the crosstalk between α1- and ß1-adrenergic receptors may explain the inconsistent arrhythmogenic response to the ExT in these patients.[123] This explanation is supported by the finding that isoproterenol infusion (strong stimulator of ß-adrenergic receptors alone) was arrhythmogenic in all 40.5% of ARVC patients in whom PVCs were reduced or suppressed during ExT in the study mentioned above.[123]

Taken together, the results of the present study demonstrated inconsistent effects of ExT on the number of PVCs/24 h in apparently healthy Boxer dogs. Whether this is 1) a reflection of spontaneous daily variability in the number of PVCs or 2) because some Boxer dogs have arrhythmic substrate while others do not (i.e. some dogs have ARVC and some dogs do not) or 3) due to unpredictable crosstalk between α1- and ß1-adrenergic receptors cannot be definitively answered from this study. However, the results of the present study could have clinical implications both for revealing the diagnosis of ARVC in some dogs, and in accounting for the confounding effect of exercise on interpretation of Holter recordings in Boxer dogs. Past studies involving

assessment of ventricular arrhythmias in Boxer dogs have failed to take dogs' exercise history into account. Further studies would be required to establish the exact role of ExT in identification of dogs with ARVC and to make a definitive conclusion about its role in clinical decision making. However, results of the present study demonstrated that a dog's exercise history could influence the Holter results, and it should be considered when assessing Boxer dogs for PVCs via Holter.

4.2. Comparison of the results obtained with the POC cTnI and hs-cTnI assays in apparently healthy Boxer dogs

4.2.1. Analytical test comparison of the results obtained with the POC cTnI and hs-cTnI assays

Results obtained with the hs-cTnI assay reached a lower level of minimal detection (by definition for a high-sensitivity assay) and furthermore, were significantly higher compared to those obtained with the POC assay. The Bland-Altman plot (Figure 6) shows a difference between the two assays, which shows that the two assays cannot be used interchangeably. Therefore, for diagnostic and follow-up purposes, it is recommended to use the same assay and their instrument-specific reference intervals. These results were similar to those of a recent study in Doberman pinschers with and without DCM in which an hs-cTnI[a] assay measured significantly higher levels compared to a conventional[b] assay.[113 1]

[a]Advia Centaur TnI-Ultra assay; Siemens Healthcare Diagnostics
[b]Immulite 2000 troponin I test; Siemens Healthcare Diagnostics

4.2.2. Clinical evaluation and comparison of the results obtained with the POC cTnI and hs-cTnI assays

This study demonstrated that baseline [hs-cTnI] correlates with the number of PVCs/24 h recorded on a Holter monitor performed in the 24 h following the hs-cTnI measurement, whereas the POC [cTnI] does not. In this study population, the hs-cTnI assay was a more sensitive and specific test for identifying PVCs than the POC assay was.

Cardiac troponin I is an important marker of myocardial damage. An increase in [cTnI] has been reported in dogs with several naturally occurring cardiac diseases,[78,93–95] but studies evaluating [cTnI] in Boxer dogs with ARVC are scarce.[78,100,101] In the present prospective study, with a cutoff value of POC [cTnI] of >55.0 ng/L, the sensitivity was good (75%) but the specificity was low (40.9%) to identify Boxer dogs with >100 PVCs/24 h using a point-of-care instrument. With higher cutoff values, such as POC [cTnI] >65.0 ng/L, the sensitivity to detect dogs with >100 PVCs/24 h decreased to 62.5% but specificity increased to 63.6%. Based on these results and the degree of overlap seen between groups, the POC [cTnI] does not appear sufficiently sensitive or specific in distinguishing dogs with >100 PVCs and ≤100 PVCs/24 h to serve as a stand-alone screening test in a population of Boxer dogs with subclinical ventricular arrhythmia. Additionally, there was a lack of significant correlation between POC [cTnI] and the severity of ventricular arrhythmias (number of PVCs/24 h and grade of ventricular arrhythmia) in apparently healthy Boxer dogs in the present study. In the only other study that evaluated [cTnI] in Boxer dogs with ARVC, a statistically significant

positive correlation between serum [cTnI] and severity of ventricular arrhythmia was demonstrated ($r = 0.78$).[42] However, the results of this study cannot be directly compared to the present study because the cited study did not include Boxer dogs having 5-1000 PVCs/24 h (the study population comprised of Boxer dogs with ≥1000 PVCs/24 h or <5 PVCs/24 h). Equally important, the cited study had a small sample size (n=10/group), and some dogs were on antiarrhythmic medications. In the present study, subclinical Boxer dogs with less severe (less frequent and low complexity) ventricular arrhythmias were included to evaluate the utility of the POC cTnI assay in the cohort of apparently healthy Boxer dogs. Furthermore, dogs that were on any cardiac medications were excluded to prevent the potential confounding effects of the various cardiac medications.

The optimal cutoff value for the serum [cTnI], measured by conventional or high-sensitivity assay, has not yet been reported in Boxer dogs being evaluated for ARVC. Our cutoff values for the conventional POC cTnI assay to distinguish dogs with >100 PVCs from dogs with ≤100 PVCs/24 h were different from those in the study involving Doberman pinschers with ventricular arrhythmia. In that study, [cTnI] >220 ng/L using the Immulite 2000 troponin I assay had a sensitivity of 83.5% and specificity of 72.4% to detect dogs with PVCs (>300 PVCs/24 h or 2 subsequent examinations within a year showing between 50 and 300 PVCs/24 h) and a normal echocardiographic examination.[113] Such a difference can be attributed to differences in breed, assays, and pathophysiology of ventricular arrhythmia in these breeds.

In the present prospective study, unlike POC cTnI, the serum [hs-cTnI] was significantly higher in Boxer dogs with >100 PVCs/24 h than in Boxer dogs with ≤100 PVCs. Furthermore, unlike the POC cTnI assay, a significant positive correlation between serum [hs-cTnI] and number of PVCs/24 h was observed. With a cutoff value of [hs-cTnI] >128.5 ng/L, the sensitivity was excellent (100%) and specificity was modest (59.1%) to identify Boxer dogs with >100 PVCs/24 h. With higher cutoff values, such as [hs-cTnI] >222.0 ng/L, the specificity to detect dogs with >100 PVCs/24 h increased to 81.8% although the sensitivity decreased to 50%. If these results apply to the larger population of all apparently healthy Boxer dogs, then those with serum [hs-cTnI] below 128.5 ng/L are very unlikely to have >100 PVCs/24 h and those with serum [hs-cTnI] above 222.0 ng/L are very likely to have >100 PVCs/24 h. Therefore, hs-cTnI assay and these cutoff values might be investigated further for potential use in screening Boxer dogs for ARVC where previously, the diagnosis was rested only on Holter monitoring. Although there is no published study providing a cutoff value for serum [hs-cTnI] for the detection of Boxer dogs with ARVC, the cutoff presented in this study is similar to a recent study involving Doberman pinschers with PVCs.[113] Such a similarity, despite differences in breed and pathophysiology of ventricular arrhythmia in these breeds, may suggest consistency in performance of this hs-cTnI assay to detect ventricular arrhythmias in dogs.

Circulating cTnI concentrations can increase with a variety of noncardiac diseases including pulmonary hypertension.[97,98,145,146] In the present study, dogs were evaluated in depth for evidence of ongoing non-cardiac diseases, with each dog undergoing medical

history review, physical examination, CBC, serum biochemistry profile, urinalysis, arterial BP measurement, and thoracic radiographs. Furthermore, dogs were evaluated for evidence of pulmonary hypertension on echocardiography and for cardiac manifestations of systemic disorders, and significant evidence of any of these processes led to exclusion from the study. Therefore, the [cTnI] elevations noted in this study reasonably can be attributed to cardiac disease. These criteria mean that the proposed cutoff values only apply to dogs without evidence of systemic disease, to prevent extrapolation bias.

4.3. Effects of exercise on [hs-cTnI] in apparently healthy Boxer dogs

The major findings of the present study are that ExT resulted in a significant increase in [hs-cTnI] in apparently healthy Boxer dogs; that an exercise-associated increase in [hs-cTnI] correlated with the number of PVCs/24 h; and that the exercise-associated increase in [hs-cTnI] was higher in dogs with the striatin mutation.

Measurements of circulating cardiac biomarker concentrations have been used in conjunction with ExT in human cardiac patients and the general population to improve risk stratification.[128,147] In veterinary medicine, the effects of exercise on cardiac biomarkers have been studied uncommonly, particularly in cardiac patients. Wakshlag et al have investigated the effects of short-duration high-intensity exercise on plasma [cTnI] in sprint racing sled dogs.[126] They observed that strenuous exercise lasting ≤1 hour did not result in [cTnI] above the reference range for healthy dogs.[126] In a more recent study by Iwanuk et al, dogs with subclinical degenerative mitral valve disease being treated with pimobendan had a lower increase in NT-proBNP levels after exercise than those in

the placebo-group although [cTnI] did not differ between the groups.[148] In the present study, we observed that ExT resulted in a significant increase in [hs-cTnI] in apparently healthy Boxer dogs. Higher post-exercise [cTnI] may represent myocardial injury because of underlying subclinical cardiac disease. This hypothesis is supported by our finding of a significant positive correlation between the exercise-induced increase in [cTnI] and the number of PVCs/24 h. In a study involving human participants, age, body mass, baseline troponin, exercise intensity, and change in body mass during exercise were independently associated with a post-exercise troponin I increase.[128] In the present study, among several clinical parameters investigated (age, body weight, sex, total exercise duration, number of PVCs/24 h, and grade of ventricular arrhythmias), exercise-associated increase in [hs-cTnI] was correlated with the number of PVCs/24 h only. Although it does not prove a cause-and-effect relationship, the modest correlation ($r = 0.45$, $p = 0.01$) between an increase in [hs-cTnI] and electrocardiographic abnormalities suggests that exercise-associated increase in [cTnI] may be a marker of greater arrhythmic risk.

Arguably, the observed exercise-associated increase in [cTnI] may be simply a physiological and not a pathological response. Exercise-induced increases in cTnI concentrations have been considered benign because they occur frequently, are present in apparently healthy individuals, and are not accompanied by symptoms.[128,149] However, the mechanisms and clinical significance of exercise-induced increases in [cTnI] are incompletely understood.[149] A growing body of evidence suggests that exercise-induced elevations in circulating cardiac troponin concentrations are not always a benign

physiological response to exercise, but an early marker of myocardial injury that can be associated with future cardiovascular events. In humans, increased post-exercise [cTnI] was correlated with right ventricular dysfunction ($r = 0.49$, $p = 0.002$)[150] and higher exercise-induced [cTnI] independently predicted higher mortality and cardiovascular events in a cohort of older long-distance walkers.[128] In light of these reports, the results of the present study suggest that exercise-associated increase in [hs-cTnI] and a concurrent increase in the number of PVCs/24 h may be an early marker of an underlying occult myocardial disease in apparently healthy Boxer dogs.

An interesting finding in the present study was that exercise-associated increases in [hs-cTnI] were significantly higher in dogs with the striatin mutation compared to those without the mutation although baseline serum [hs-cTnI] did not differ in dogs with or without the striatin mutation. Despite reports showing that the striatin mutation is not consistently associated with all cases of ARVC, it is generally accepted that the striatin mutation co-localizes with the region in which the (yet unknown) ARVC mutation(s) exist(s).[39] Therefore, whether or not the heterozygous positive Boxer dogs in the study truly carry the gene mutation(s) causing ARVC can only be speculated. However, the results of the present study demonstrated that exercise induces a greater increase in [cTnI] in Boxer dogs with genetic susceptibility to the disease. There are at least two studies that demonstrated expression of the ARVC phenotype is accelerated by exercise training in animals (heterozygous plakoglobin-deficient mice)[151] and humans (desmosomal mutation carriers)[117] with genetic susceptibility to the disease. Therefore, it can be concluded from the present study that Boxer dogs positive for the striatin deletion

mutation may be more susceptible to myocardial injury. Whether they are also more likely to develop the ARVC phenotype may be possible to investigate by long-term follow-up of these dogs.

4.4. Cardiac troponin I concentrations in apparently healthy Boxer dogs with or without the striatin mutation

Cardiac troponin I concentrations measured by either assay did not differ between dogs with or without the striatin mutation. Furthermore, the number of PVCs/24 h did not differ between dogs with or without the mutation. These results are partly not surprising given the lack of enough evidence to support the role of the striatin mutation in the causation of ARVC in Boxer dogs.[39] A considerable proportion of normal control Boxer dogs carries the striatin mutation (~24% in one study)[35] and conversely, many affected dogs do not have this mutation (16% in another study).[38]

4.5. Strengths of the study

There are several strengths of this study. This was a prospectively designed study. The role of ExT in arrhythmia evaluation has rarely been investigated in veterinary medicine. The present study was specifically designed to examine the utility of newer, non-invasive diagnostic tools (e.g., ExT and a high-sensitivity cTnI assay) for the early identification of ventricular arrhythmia in apparently healthy Boxer dogs that did not have a large number of PVCs on routine cardiovascular assessment. This represents a Boxer dog population that has been less studied, as most of the reported studies primarily

focused on dogs with a large number of PVCs on 24 h Holter recordings. Boxer dogs in this study population were not receiving cardiac medications, including antiarrhythmics, which prevented the potential confounding effects of cardiac medications on the number of PVCs or [cTnI], as encountered in other works.[42] Ventricular arrhythmias can be the result of other cardiac diseases and systemic conditions, which should ideally be excluded before a diagnosis of ARVC is made. In the present study, a complete echocardiogram, 12-lead ECG, CBC, serum chemistry, urinalysis, thoracic radiographs, and non-invasive BP measurements, in combination with a complete history obtained from the owners and medical database and complete physical examination were performed to select a Boxer dog population where ARVC would be the leading differential diagnosis if PVCs were found. Additionally, this rigorous evaluation diminished the well-known confounding effects of comorbidities on biomarker concentrations.[84] In previous studies, such a comprehensive investigation to rule out causes other than ARVC were not performed and in most of these studies, dogs were considered to have ARVC based only on PVCs with a characteristic RV origin/LBBB pattern and lack of clinical signs of cardiac disease or other systemic illness.[42,60] This study provides an analytical and clinical evaluation and comparison of a conventional POC cTnI assay and a newer, hs-cTnI assay and also provides specificity and sensitivity values for both of these assays for detection of >100 PVCs/24 h in apparently healthy Boxer dogs.

4.6. Limitations of the study

While the sample size from the power analysis was met and exceeded, the number of dogs may not have been sufficient to allow the detection of induction of arrhythmia during exercise in some dogs. The POC cTnI and hs-cTnI assays were not benchmarked against an agreed "gold standard" test. In veterinary medicine, due to invasiveness, risk of general anesthesia, and costs, gold standards for comparison are not always available. In humans, the definitive diagnosis of ARVC is established histologically on myocardial samples, but endomyocardial or open myocardial biopsies require general anesthesia in dogs and thus are not practical. Currently, Holter recording is the diagnostic test of choice for ARVC in Boxer dogs. The lack of a gold standard in this study introduces a source of bias (i.e. errors in the reference)[152] in which the true disease status is subject to misclassification. In previous studies, various cutoff values for the number of PVCs/24 h have been selected by the investigators to define Boxer dogs as having ARVC or not (control). Based on the observations that healthy, large-breed dogs have no more than 24 PVCs/24 h[51] and normal asymptomatic adult Boxer dogs have <91 PVCs/24 h,[51,52] it has been proposed that identification of >100 PVCs per 24 h in an adult Boxer dog is strongly suggestive of ARVC.[40] However, it is not known what represents an acceptable or normal number of PVCs in Boxer dogs. For this reason, this study used the number of PVCs/24 h to investigate the association between serum [cTnI] and ventricular arrhythmia in Boxer dogs with no structural cardiac disease or evidence of systemic illnesses. This way of categorizing the study population also reduces over-diagnosis bias[152] since it is known that in many dogs with PVCs, the arrhythmia may never become a clinical problem in the absence of screening, but is detected by screening. However, it

is important to note that ventricular arrhythmias alone may not be a true indicator of disease severity. Future studies that include histologic identification of myocardial fibrofatty changes either noninvasively (via magnetic resonance imaging) or invasively (via histologic evaluation of endomyocardial biopsy specimens) could further define, or refute, the correlation between serum [cTnI] and stage of ARVC in Boxer dogs. In the present study, a rigorous diagnostic evaluation was performed to rule out comorbidities that could affect arrhythmia interpretation or biomarker concentrations. However, our comprehensive evaluation did not include advanced imaging such as abdominal ultrasound to rule out pathology in the abdomen that may be present without clinical or laboratory changes. The reproducibility of the ExT used in this study was not examined. We failed to control for physical activity leading up to the ExT and during Holter recordings. During the study, no follow-up was performed to evaluate whether serial measurements of cTnI can identify changes in the number of PVCs that may occur over time in affected animals.

5. SUMMARY AND FUTURE DIRECTIONS

Boxer dogs have a genetic predisposition for ARVC, which can be characterized by ventricular arrhythmias, syncope, exercise intolerance, or sudden death. The identification of subclinical Boxer dogs having the earliest latent stage of ARVC is challenging but important. In one study, 15 of 49 Boxer dogs (31%) in which >300 PVCs/24 h were documented anytime during a median follow-up period of 5 years (range, 3-8 years) had sudden cardiac death.[38] In the same study, none of the 23 dogs in which <50 PVCs/24 h were identified during the similar follow-up period, died due to cardiac disease.[38] Therefore, testing that can unmask ventricular arrhythmias or increase blood levels of biomarkers of myocardial injury in subclinical Boxer dogs may be beneficial in guiding monitoring and treatment recommendations. In the present study, we investigated the effects of ExT on the occurrence of ventricular arrhythmias in apparently healthy Boxer dogs, analytical and clinical comparison between POC cTnI and hs-cTnI assays, and an association between ventricular arrhythmias, [cTnI], and genetic mutation status in these Boxer dogs.

The principal findings of this study are:

1. The effects of ExT on the number and complexity of ventricular arrhythmias are inconsistent in apparently healthy Boxer dogs.
2. A high-sensitivity cTnI assay measures significantly higher values compared to a POC cTnI assay, indicating that the two assays cannot be used interchangeably

3. Baseline [hs-cTnI], but not POC [cTnI], correlates with the number of PVCs/24 h and hs-cTnI assay may be a more sensitive and specific test than the POC assay
4. Cardiac troponin I concentrations measured by either assay do not differ between dogs with or without the striatin mutation
5. Exercise testing results in a significant increase in [hs-cTnI] in some Boxer dogs.
6. Exercise-associated increases in [hs-cTnI] correlate with the number of PVCs/24 h and are higher in dogs with the striatin mutation.

The results of this study suggest that the exercise-associated increase in cTnI and number of PVCs may be a manifestation of increased propensity to the development of the ARVC phenotype in Boxer dogs. Additionally, dogs' recent exercise history should be considered when interpreting [hs-cTnI] and when quantitating PVCs via 24 h Holter monitor. Long-term follow up of these Boxer dogs may provide further evidence to address our hypotheses and conclusions, which could be a focus for future studies. It would be interesting to follow these Boxer dogs long term to evaluate whether the presence/increase in ventricular arrhythmia during or after ExT would predict the progression of the disease (i.e., whether they develop clinical signs of ARVC or have progressive changes in the number of PVCs that may occur over time in affected animals). Investigating the effects of various levels of activity on ventricular arrhythmias in Boxer dogs may also be a focus for future studies.

While exercise stress testing is a non-invasive, inexpensive, easily performed tool to assess exercise-induced abnormalities, it may not be feasible in dogs with physical (e.g., musculoskeletal disease) or behavioural (unwillingness, fear) inability to safely perform exercise testing. Pharmacological stress testing by using synthetic catecholamine (e.g., dobutamine) can be investigated as an alternative to exercise test for those patients. The effect of dobutamine on cardiac function in dogs has been evaluated and was found to have comparable effects on cardiovascular variables and indices of cardiac contractility as expected with exercise.[153] However, there are important physiological differences between the two stimuli that may lead to differences in outcomes.[154]

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