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Evaluation of anti-desmoglein-2 antibodies and cardiac biomarkers in Boxer dogs with arrhythmogenic right ventricular cardiomyopathy

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KEYWORDS
NT-proBNP;
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Abstract *Introduction/Objective:* The aim of this study was to evaluate whether circulating anti-desmoglein-2 antibodies distinguish Boxer dogs with arrhythmogenic right ventricular cardiomyopathy from screened controls and assess the diagnostic utility of anti-desmoglein-2, cardiac troponin I (cTnI), high-sensitivity cTnI (HS-cTnI), and N-terminal pro-B-type natriuretic peptide (NT-proBNP) relative to arrhythmia burden.
Animals, Materials and Methods: This prospective cross-sectional study included 52 Boxer dogs (28 arrhythmogenic right ventricular cardiomyopathy—affected and 24 screened controls). Diagnosis was based on Holter monitoring (>300 ventricular premature complexes [VPCs]/24 h or documented ventricular tachycardia). Anti-desmoglein-2 antibodies, cTnI, HS-cTnI, and NT-proBNP were measured. Receiver operating characteristic analysis and correlations with VPC burden were performed.
Results: Anti-desmoglein-2 antibody concentrations did not differ between groups by either method (enzyme-linked immunosorbent assay: P=0.687; Western blot: P=0.293). Receiver-operating characteristic analysis showed poor discriminatory ability for anti-desmoglein-2 (enzyme-linked immunosorbent assay: area under the receiver operating characteristic curve [AUC] = 0.534, P=0.693; Western blot: AUC = 0.600, P=0.295). In contrast, cutoffs for NT-proBNP (1530 pmol/L, AUC = 0.856), HS-cTnI (0.21

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ng/mL, AUC = 0.805), and cTnI (0.08 ng/mL, AUC = 0.795) all demonstrated good performance in differentiating groups ($P<0.001$) and showed positive correlations with VPCs ($P\leq0.001$) for NT-proBNP ($r = 0.60$), HS-cTnI ($r = 0.549$), and cTnI ($r = 0.471$).
Limitations: The cross-sectional design and lack of pedigree data may have limited detection of subclinical disease. Disease stage and therapy may also have influenced biomarker concentrations.
Conclusions: Anti-desmoglein-2 antibody lacked diagnostic value for arrhythmogenic right ventricular cardiomyopathy in Boxer dogs, whereas NT-proBNP, cTnI, and HS-cTnI showed promise as reliable clinical biomarkers.

Abbreviations

ARVC	arrhythmogenic right ventricular cardiomyopathy
AUC	area under the receiver operating characteristic curve
CNP	C-type natriuretic peptide
cTnI	cardiac troponin I
DCM	dilated cardiomyopathy
DSG2	desmosomal protein desmoglein-2
HS-cTnI	high-sensitivity cardiac troponin I
LV	left ventricle
NT-proBNP	N-terminal pro-B-type natriuretic peptide
ROC	receiver operating characteristic
STRN	striatin
VPC	ventricular premature complex
WB	Western blot

Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a familial cardiomyopathy associated with substantial cardiovascular morbidity and risk of sudden cardiac death in people and Boxer dogs [1,2]. In the Boxer breed, ARVC is a common progressive and predominantly right ventricular entity [3,4] manifested by fibrofatty infiltrates in the myocardium that result in potentially malignant ventricular arrhythmias, with or without cardiac dilation and depressed systolic function [5,6].

The clinical course of the disease is often unpredictable, and as Holter and echocardiographic abnormalities frequently do not develop until after 5-6 years of age, most affected animals have already produced progeny by that time [7]. Thus, novel biomarkers that could lead to earlier screening and identification of either affected individuals, or at risk for development of ARVC, are needed. Though a genetic deletion in the gene encoding for striatin (STRN), a protein that co-localizes with desmosomal proteins, has been found to be associated with development of ARVC in many U.S. Boxers [8], this mutation is not always associated with disease phenotype, and other genetic and/or non-genetic causes of ARVC are likely [9]. A predisposition to autoimmune cardiomyopathy has been proposed as a cause of idiopathic dilated cardiomyopathy (DCM) in people [10,11] and Chaterjee et al. [12] described circulating autoantibodies to the cardiac desmosomal protein desmoglein-2 (DSG2) in human patients with ARVC. In this study, the level of anti-desmoglein-2 (anti-DSG2) antibodies correlated with disease burden as measured by ventricular premature complex (VPC) count, and purified antibodies resulted in reduced gap junction function, an identified common pathway in ARVC pathophysiology [12]. Additionally, anti-DSG2 antibodies were detected in a small preliminary cohort of Boxer dogs with clinical evidence of ARVC, but not in healthy dogs with no family history of the disease. In outwardly healthy dogs with a family history of disease, five of ten dogs had circulating anti-DSG2 antibodies, raising the possibility that this could serve as an early marker of disease in animals in whom the disease has yet to manifest.

The presence of circulating desmosomal autoantibodies in both canine and human ARVC patients could suggest an autoimmune component to the underlying etiology of this disease. The theory of anti-desmosomal antibodies playing a contributory role in the pathogenesis of these structural derangements may explain the frequent finding of inflammatory infiltrates accompanying the fibrofatty infiltration of the myocardium in ARVC [1]. Thus, the identification of an autoantibody associated with ARVC may pave the way for new screening, diagnostic, and therapeutic possibilities, similar to those proposed for other autoimmune cardiomyopathies [10,13]. The goal of this study was to evaluate the prevalence of serum anti-DSG2 antibodies in Boxers

with ARVC compared to the age-matched screened comparison Boxer group.

Materials and Methods

Study population

Boxer dogs were considered to be afflicted with ARVC if they had ventricular ectopies with greater than 300 VPCs over 24 h on Holter monitoring or presented for collapse or syncope with documented ventricular tachycardia at admission, with or without echocardiographic evidence of ventricular dilation or systolic dysfunction. Dogs were assigned to the affected group if they showed no cavitary effusions or clear masses that might suggest a non-cardiac cause, including neoplastic diseases. Dogs afflicted with ARVC could be on cardiac medications as this was not an exclusion criterion for the study. Dogs were included in the screened comparison group if they were aged at least five years with no clinical signs of cardiac disease or other systemic illness, had normal blood work (complete blood count and full chemistry panel), had a normal echocardiogram with continuous electrocardiogram signal monitoring throughout the examination, were not on cardiac medications, and had $<$50 VPCs over 24 h on Holter monitoring. Exclusion criteria included aspects for equivocal disease (50—100 VPCs on Holter), a peak aortic ejection velocity of $>$2.3 m/s measured by continuous-wave Doppler echocardiography (subcostal view), dogs that were pregnant or lactating, those with congenital heart disease (*e.g.* subaortic or pulmonic stenosis or overt septal defect), and those that had moderate to marked azotemia, known infection, an inflammatory leukogram, or evidence of any other systemic disease that may impact biomarker levels and cardiovascular function.

Holter monitoring

Boxer dogs with $\geq$20 h of valid Holter data were included. Recordings were obtained at the study institution or by breeders, but all were blindly analyzed by a single tertiary center,[d] and arrhythmia number and severity were documented.

Echocardiogram

All dogs had echocardiographic data obtained while unsedated and in right lateral recumbency. Echocardiographic examinations consisting of two-dimensional, M-mode, color, and spectral Doppler analysis[e] were performed by two observers (S. M. C. and L. D. S.) with 1.5- to 4-MHz frequency range transducers. Echocardiographic data included left ventricular (LV) internal dimensions at end-diastole and end-systole from M-mode measurements normalized to body weight. Left ventricular enlargement was diagnosed when LV dimensions were outside of the 95% confidence interval (CI) (normalized LV internal diameter in diastole $>$1.85 or normalized LV internal diameter in systole $>$1.26) [3]. Aortic velocities were obtained from the subcostal view and the transthoracic long-axis four-chamber view, and the peak velocity was obtained via continuous-wave Doppler at either location and recorded. Pulmonic velocities were recorded using pulsed-wave Doppler from the right parasternal short-axis view at the level of the pulmonic valve.

Blood analysis

Each enrolled dog had blood collected at a single time point. Blood was analyzed for the following variables: complete blood count, serum biochemistry profile, high-sensitivity cardiac troponin-I[f] (HS-cTnI), point-of-care cardiac troponin-I[g] (cTnI), concentration of N-terminal pro-B-type natriuretic peptide[h](NT-proBNP), genetic analysis for STRN deletion[i] and anti-DSG2 analysis.[j]

Western blotting and enzyme-linked immunosorbent assay

Serum samples collected for anti-DSG2 antibody analysis were centrifuged and stored at -80 °C until batch analysis. Anti-DSG2 antibodies were assessed using two complementary methods, enzyme-linked immunosorbent assay (ELISA) and Western blot (WB). Western blot conditions were optimized to minimize weak or non-specific (false-positive) signal by systematically evaluating antigen loading, primary and secondary antibody dilutions, and

[d] ALBA Medical Systems, Inc., New Hyde Park, New York, USA).

[e] GE Vivid E95 Ultrasound System (GE HealthCare, Chicago, Illinois, USA).

[f] High-Sensitivity Cardiac Troponin I Assay (Texas A&M University College of Veterinary Medicine & Biomedical Sciences, Gastrointestinal Laboratory, College Station, Texas, USA).

[g] i-STAT® Cardiac Troponin I Cartridge (Abbott Point of Care Inc., Abbott Park, Illinois, USA).

[h] CardioPet® proBNP Test (IDEXX Laboratories, Inc., Westbrook, Maine, USA).

[i] Meurs Lab, North Carolina State University College of Veterinary Medicine, Raleigh, North Carolina, USA.

[j] Hamilton Lab, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada.

chemiluminescent exposure time. In parallel, an ELISA was developed and characterized in the collaborating laboratory, and the assay readout demonstrated an acceptable relationship between the assay calibration/characterized antibody concentration and signal obtained in canine serum samples. All ELISA and WB analyses were performed by the same laboratory that previously developed and validated the anti-DSG2 assays, using the same established protocols and reagents as described by Chatterjee et al. [12].

Statistical analysis

Continuous variables were expressed as mean and standard deviation for normally distributed data and as median and interquartile range for non-normally distributed data. Categorical variables were presented as absolute numbers and relative frequencies (percentages).

All data were assessed for normality using the Shapiro-Wilk test and histogram visualization. Continuous variables with a normal distribution were compared between groups using the unpaired Student's *t*-test, while non-normally distributed data were analyzed using the Mann-Whitney U test. Categorical variables, such as sex and STRN genotype, were evaluated using the chi-square test or Fisher's exact test. Correlations between the number of VPCs and circulating biomarker levels were analyzed using Spearman's or Pearson's correlation coefficients, depending on data distribution.

Receiver-operating characteristic (ROC) curve analysis was used to evaluate the diagnostic performance of serum biomarkers in identifying Boxer dogs affected by ARVC. The area under the ROC curve (AUC) was compared, with an AUC of 0.5 indicating no discriminatory ability. The bootstrap method (1,000 samples) was used to construct ROC curves, and the Delong method [14] was used to calculate and compare AUCs. The cutoff values were selected based on the highest Youden index, calculated as sensitivity + (specificity-1). A significance level of $P<0.05$ was adopted for all statistical comparisons. Statistical analysis was performed using a commercial software program.[k]

Ethics statement

This was a prospective, cross-sectional study of Boxer dogs, with data collected between January 1st, 2019, and June 1st, 2021. All procedures performed in the study were approved by the Institutional Animal Care and Use Committee. All dog owners signed a consent form at the time of enrollment.

Results

Of the 60 client-owned Boxer dogs initially enrolled in the study, two dogs from the screened comparison group were excluded. One dog developed ventricular arrhythmias at nine years of age, which subsequently led to sudden cardiac death before completion of the manuscript. The second dog exhibited 98 VPCs on Holter monitoring and was therefore classified as equivocal. After these exclusions, a total of 52 dogs met the inclusion criteria and were included in the final analysis, comprising 24 dogs in the screened comparison group and 28 dogs in the ARVC-afflicted group. No differences were observed between groups with respect to age ($P=0.831$), sex ($P=0.392$), or body weight ($P=0.783$) (Table 1). The number of VPCs in the screened comparison group (range: 0—46) was lower than in affected groups (range: 124—69,764) ($P<0.001$). The affected animal with 124 VPCs was on antiarrhythmic therapy during Holter recording.

None of the dogs in the screened comparison group were receiving antiarrhythmic or cardiac medication at the time of enrollment. In contrast, several dogs in the ARVC-afflicted group were receiving medical therapy, most commonly sotalol ($n = 12$), followed by amiodarone ($n = 4$), atenolol ($n = 1$), and pimobendan ($n = 2$). Dogs in the ARVC-afflicted group showed evidence of greater LV remodeling and systolic impairment than the screened comparison group (Table 1). Normalized LV internal diameter in diastole was higher in the ARVC-afflicted group, with a mean between-group difference of 0.170 cm (95% CI: 0.053—0.287; $P=0.005$). Similarly, normalized LV internal diameter in systole was also increased, with a mean difference of 0.176 cm (95% CI: 0.07—0.28; $P=0.001$). In contrast, fractional shortening was lower in the ARVC-afflicted group, with a mean difference of −5.13% (95% CI: −8.27 to −1.99; $P=0.002$).

Cardiac biomarkers

Anti-desmoglein-2 antibodies quantified by ELISA and WB did not differ between groups ($P=0.687$; $P=0.293$, respectively), while dogs with ARVC had higher in-house cTnI ($P<0.001$), HS-cTnI ($P<0.001$), and NT-proBNP ($P<0.001$). The difference

[k] IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, New York, USA).

Table 1 Comparison of signalment, clinical, laboratory, and left ventricular echocardiographic variables for screened comparison and ARVC-affected Boxer dogs.

Variable	Group		P-value
	Screened comparison (n = 24)	ARVC-afflicted (n = 28)	
Age (years)	6.5 [5.75–7]	6.5 [5–9.18]	0.831
Weight (kg)	28.1 [25.6–31.4]	28.1 [25.8–32.6]	0.783
VPCs/24 h	11 [5–15]	1400 [404–3677]	**<0.001**
Sex			
Female	10 (40%)	15 (60%)	0.392
Male	14 (51.9%)	13 (48.1%)	
Biomarker			
cTnI (ng/mL)	0.05 [0.02–0.10]	0.12 [0.09–0.18]	**<0.001**
HS-cTnI (ng/mL)	0.17 [0.13–0.24]	0.34 [0.24–0.49]	**<0.001**
NT-proBNP (pmol/L)	603 [421–965]	1812 [934–3049]	**<0.001**
Anti-DSG2 (WB) ($x10^3$)	202 [127–299]	209 [145–417]	0.303
Anti-DSG2 (ELISA)	0.313 [0.134–0.479]	0.321 [0.102–0.552]	0.709
Striatin genotype			
Negative	14 (60.9%)	12 (60%)	0.821
Heterozygous	7 (30.4%)	5 (25%)	
Homozygous	2 (8.7%)	3 (15%)	
Echocardiography LV			
FS (%)	35.6 (5.25)	30.5 (6.01)	**0.002**
LVIDdN	1.44 (0.17)	1.63 (0.22)	**0.005**
LVIDsN	0.87 (0.13)	1.05 (0.21)	**0.001**

Data are presented as mean (SD), median [interquartile range, IQR], or number (percentage). P-values are ARVC-afflicted group versus screened comparison group, with significant P-values in bold.
Anti-DSG2: anti-desmoglein-2; ARVC: arrhythmogenic right ventricular cardiomyopathy; cTnI: cardiac troponin I; ELISA: enzyme-linked immunosorbent assay; FS: fractional shortening; Hs-cTnI: high-sensitivity cardiac troponin I; LVIDdN: normalized left ventricular internal diameter in diastole; LVIDsN: normalized left ventricular internal diameter in systole; NT-proBNP: N-terminal pro-B-type natriuretic peptide; SD: standard deviation; VPC: ventricular premature complex; WB: Western blot.

determined by the Hodges-Lehmann estimator for NT-proBNP was 1,204.43 pmol/L (95% CI: 556–1714). Similarly, HS-cTnI demonstrated a between-group difference of 0.16 ng/mL (95% CI: 0.07–0.26), while cTnI showed a Hodges-Lehmann difference of 0.06 ng/mL (95% CI: 0.03–0.10).

The ROC analysis revealed that both ELISA and WB methods had AUC values of 0.534 (P=0.693) and 0.600 (P=0.295) in distinguishing screened Boxers from afflicted ones, respectively. On the other hand, the AUC of in-house cTnI and HS-cTnI were 0.795 (P<0.001) and 0.805 (P<0.001), respectively, and the AUC of NT-proBNP was 0.856 (P<0.001) in differentiating the groups (Table 2).

The biomarkers that showed a positive correlation with the number of VPCs/24-h Holter monitoring were cTnI, HS-cTnI, and NT-proBNP (Table 3).

Striatin deletion phenotype

There was no difference in STRN deletion genotype between groups (P=0.821). In fact, the screened comparison group included 60.9% negative and 30.4% heterozygous genotypes, while the ARVC group had 60% negative, 25% heterozygous, and 15% homozygous dogs. No correlation was found between anti-DSG2 antibody values and homozygous genotype.

Discussion

Our results showed that circulating anti-DSG2 antibodies, as measured by two methods (ELISA and WB), were present in both groups and did not discriminate groups in this study sample. The AUC values for anti-DSG2 were 0.59 (ELISA) and 0.62 (WB) in our study, indicating poor discrimination between groups. These values were substantially lower than those reported previously [12], where anti-DSG2 showed strong discriminatory performance (AUC = 0.99) for distinguishing ARVC-affected from screened Boxers. This difference may be attributed to several factors. A major factor may be that there is no reference standard diagnostic test or scoring system to accurately establish a diagnosis for ARVC in Boxer dogs prior to the development of arrhythmia. Thus, the

Table 2 Areas under the receiver-operating characteristic curve (AUCs) and diagnostic cutoffs with associated sensitivity (Se) and specificity (Sp) for the detection of arrhythmogenic right ventricular cardiomyopathy in Boxer dogs.

Variable	AUC	Cutoff	Se (%)	Sp (%)	P-value
NT-proBNP (pmol/L)	0.856	>1530	71	92	<0.001
HS-cTnI (ng/mL)	0.805	>0.21	83	65	<0.001
cTnI (ng/mL)	0.795	>0.08	77	66	<0.001
Anti-DSG2 (WB) ($x10^3$)	0.600	>177.6	72	50	0.295
Anti-DSG2 (ELISA)	0.534	>0.241	64	43	0.693

Anti-DSG2: anti-desmoglein 2; cTnI: cardiac troponin I; ELISA: enzyme-linked immunosorbent assay; Hs-cTnI: high-sensitivity cardiac troponin I; NT-proBNP: N-terminal pro-B-type natriuretic peptide; WB: Western blot.

Table 3 Correlation between cardiac biomarkers with arrhythmia burden (VPC/24-h Holter monitoring) for screened comparison and ARVC-affected Boxer dogs.

Biomarkers	r	P-value
cTnI (ng/mL)	0.471	**0.001**
HS-cTnI (ng/mL)	0.549	**< 0.001**
NT-proBNP (pmol/L)	0.6	**< 0.001**
Anti-DSG2 (WB)	0.136	0.436
Anti-DSG2 (ELISA)	0.115	0.470

Data are presented as Pearson's correlation coefficient (r) with 95% confidence interval (CI) and corresponding P-value. P-values refer to correlations assessed within the affected group. Bold values indicate statistically significant associations ($P < 0.05$).
Anti-DSG2: anti-desmoglein 2; ARVC: arrhythmogenic right ventricular cardiomyopathy; cTnI: cardiac troponin I; ELISA: enzyme-linked immunosorbent assay; Hs-cTnI: high-sensitivity cardiac troponin I; NT-proBNP: N-terminal pro-B-type natriuretic peptide; VPC: ventricular premature complex; WB: Western blot.

determination of which dogs are affected and which are 'normal' at a single time point is obviously critical to every study. As a result, accurate case classification at a single time point is challenging and can vary between studies. In the present study, dogs were classified as ARVC-afflicted if they had right ventricular—origin ventricular ectopy with >300 VPCs/24 h on Holter monitoring, while the prior study used an abnormal Holter threshold of >100 VPCs/24 h to define afflicted dogs. To classify dogs as screened comparison control, we used clinical history, normal echocardiographic parameters, Holter monitoring (<50 VPCs in 24 h), and a negative or heterozygous STRN genotype, whereas the previous study used Boxers with no pedigree history of ARVC as the control group [12]. That study's decision to exclude dogs clinically unaffected and without a pedigree history may have maximized the contrast between the two groups, resulting in the high discriminatory value of anti-DSG2 antibodies seen in their results. This is consistent with the human medical literature, where ARVC patients with higher anti-DSG2 antibodies usually had familial or genetic disease [12]. In a recent study, dogs from different breeds and with varying cardiac conditions were examined for the presence of anti-DSG2 antibodies [15]. The results revealed the presence of circulating anti-DSG2 antibodies in all the dogs that were analyzed, including Boxer dogs with ARVC, Doberman Pinschers with DCM, small-breed dogs with degenerative mitral valve disease, as well as healthy Boxers and non-Boxer dogs.

Clear-cut Holter criteria for classifying healthy and affected Boxers have not yet been established. Considering the substantial amount of day-to-day variability (up to 85%) of ventricular arrhythmias in Boxer dogs, it can become somewhat arbitrary to determine the diagnosis based solely on the number of VPCs [16]. Despite the inclusion criteria for the screened comparison control group having <100 VPCs on 24-h Holter monitoring, the dogs enrolled in this group had a low burden of VPCs, with a median of 11 VPCs (range: 0—46). As in humans, the level of anti-DSG2 antibodies did not correlate with VPC burden in ARVC-affected dogs in our study [12]. It is possible that different stages of the disease could account for some of the variations in the concentration of circulating anti-DSG2 antibodies; some of the affected animals had blood samples collected at the time of their presentation to the hospital (usually following collapsing or syncopal episodes), while other affected dogs had been chronically managed and free of recent collapsing events attributed to ventricular tachycardia. As this was a cross-sectional study, we cannot say for certain if or how the disease stage can alter or be altered by the concentration of circulating autoantibodies or their serum half-lives. It is also unclear whether the malignancy of ventricular

arrhythmias (R-on-T, triplet, and non-sustained ventricular tachycardia vs. isolated VPCs) could correlate with anti-DSG2 values in affected dogs prior to the manifestation of clinical signs (collapse, syncope, and sudden death), and this warrants longitudinal investigation.

Multiple biomarkers for the prediction of cardiovascular events and arrhythmias have been described as emerging diagnostic modalities in human cardiology [17]. Activating autoantibodies against β1-adrenergic receptors have been associated with dilated cardiomyopathy, chronic heart failure, and arrhythmias in approximately 40% of human patients with dilated cardiomyopathy [18]. Interestingly, a similar phenomenon of autoimmunity against the heart has also been described in Doberman Pinschers [19] however, it is unclear if Boxer dogs may manifest a similar autoimmune component.

For a circulating biomarker to be considered a valuable substitute for endomyocardial biopsy, it should ideally exhibit a good correlation with the concentration of tissue-based biomarkers. The early studies of human patients with cardiomyopathy failed to indicate increased circulating heart-specific antibodies compared with controls [20]. Additionally, for the anti-body-positive patients, no correlation existed between antibody positivity, the clinical course, and disease severity [21]. Circulating heart-specific autoantibodies directed against the myocardium (α-myosin and β-myosin heavy chains) were first documented in patients with idiopathic DCM using indirect immunofluorescence; however, the autoantibodies become undetectable with disease progression, illustrating the importance of tissue-based approaches for identifying disease-relevant antibody binding [22]. Even though endomyocardial biopsy is part of the task-force criteria for the diagnosis of ARVC in people [23], this intervention is not routinely performed in Boxers with suspected cardiomyopathy [7]. Thus, even though our results do not support circulating anti-DSG2 as specific to ARVC Boxer dogs, further diagnostic investigation could be considered using myocardial samples in different stages of the disease.

The more advanced manifestations of ARVC can also involve LV systolic dysfunction (DCM-phenotype) and are also seen in human patients with ARVC. This stage is typically associated with a worse prognosis [24]. In this study, the affected group had a larger LV cavity with lower systolic function. Considering that most affected dogs were on sotalol at the time of the echocardiogram (60%), its β-blocker effect could have negatively impacted the analysis of the LV function. The DCM phenotype is thought to be more prevalent in Boxers in the United Kingdom [25], but our data suggest that LV involvement was also present in our studied population. Given the high burden of ventricular arrhythmias in the affected group, tachycardia-induced systolic dysfunction may also be considered.

Authors have reported the diagnostic value of plasma C-type natriuretic peptide (CNP) in people and Boxers with ARVC [5,26]. Although elevated plasma (CNP) levels have been found in human individuals with ARVC, Baumwart and Meurs [5] noted no significant difference in plasma CNP levels between the groups of Boxers affected by ARVC and those that were clinically normal. The levels of NT-proBNP and BNP rely on the use of measurement kits available from various companies, and the reported coefficients of variation for several measurement kits are approximately 30% [27]. Additionally, degradation of plasma CNP has also been reported, which might have impacted the previous results in Boxers [5,28]. Interestingly, the use of plasma CNP has been progressively replaced by the more stable propeptide (N-terminal-proBNP) as a biomarker in people and dogs [27—29]. Our study evaluated NT-proBNP, and our results showed that this biomarker may hold promise for stratifying ARVC Boxers; a longitudinal study may be warranted to evaluate the potential role of NT-proBNP in screening for the disease in preclinical animals.

Cardiac troponin I is intrinsically tethered within the myocyte and becomes liberated into the bloodstream following cellular disruption and necrosis [30]. In our study, ARVC-affected Boxers had significantly greater serum concentrations of both cTnI and HS-cTnI than the control groups. These findings are consistent with data previously reported in the literature [31]. Recently, another study highlighted the increased HS-cTnI in Boxer dogs, suggesting that the myocardium of dogs with ARVC is more prone to releasing cTnI than in healthy canine myocardium [32].

While mutations in genes encoding desmosomal proteins are the major causes of ARVC in people [23], to date, the only genetic marker associated with the development of ARVC in Boxers is a deletion in the 3' untranslated region of the gene encoding STRN [8]. Pedigree analysis found that the STRN mutation is characterized by an autosomal dominant mode of inheritance with incomplete penetrance, which promotes variable disease expressivity. In people, the penetrance of desmosome gene variants is only approximately 30%, suggesting that disease expression in humans is also influenced by other genetic, epigenetic, or environmental factors [33]. Even with incomplete penetrance, however, Boxers that carry

the homozygous genotype for the STRN mutation are more likely to develop more severe phenotype abnormalities of the disease and severe ventricular arrhythmia at younger ages [7]. In clinical ARVC dogs, it has been previously reported that 9%—16% do not carry the STRN mutation [7]. Our results showed that 65% of the affected dogs were STRN genotype negative, and no significant difference in STRN genotype status between groups was documented (P=0.27). Possible explanations for this pronounced difference might be reflected in potential demographic confounders and the diverse origins of recruited dogs. Our results reinforce that other genetic and/or non-genetic factors of the disease are likely to play a role in the ARVC of Boxers. In our study, the affected group included only three dogs that were homozygous positive for STRN, but no significant differences were observed in terms of arrhythmia burden or echocardiographic changes at the time of enrollment. Interestingly, one of the homozygous dogs remained free of arrhythmias until reaching the age of nine years and then died suddenly one year after being diagnosed with the disease. No correlation was found between anti-DSG2 antibody values and homozygous genotype, but the low number of homozygous (3) animals should be taken into consideration.

Limitations

Given the high prevalence of ARVC in the Boxer breed, the primary limitation of this study was the lack of a gold standard for discriminating between affected and non-affected dogs. In addition to that, a lack of familial history data could have resulted in subclinically affected animals being erroneously included as controls at the time of enrollment. Even though it is unlikely that the screened dogs enrolled in the study would have more than 100 VPCs/24 h, considering the significant daily variation in arrhythmias, it is possible that some very early ARVC-affected dogs were included as the comparison group individuals. Thus, it is possible that the presence of circulating antibodies in the control dogs may represent dogs at risk for the disease but not yet displaying clinical signs. Another potential limitation of our study is the heterogeneity of the course of ARVC in Boxers, and having animals chronically managed and acutely diagnosed in the same group, we cannot ignore the possibility that the stage of the disease might affect the concentration of circulating anti-DSG2. Another potential limitation of our study was the specificity of the autoantibodies to human sequences and a low titer of circulating autoantibodies. Since these limitations could also partially explain our results, purified methods should be considered for future investigation. Detailed reagent identifiers (*e.g.* antibody clone, manufacturer, catalog, and lot numbers) were not available to the authors at the time of submission. It has recently been shown that although the incidence of anti-heart antibodies was higher in patients with ARVC and affected relatives, healthy subjects also carried autoantibodies at very low levels [12]. The authors are skeptical about the practical usefulness of anti-DSG2 antibodies as a screening tool for ARVC in real-world clinical practice, based on the data, due to the genetic heterogeneity of the Boxer population and the lack of a known pedigree history for most dogs. Alternative approaches — such as HS-cTnI— and NT-proBNP—based exclusion thresholds — could be evaluated in future, larger cohorts with longitudinal follow-up.

Conclusion

Our results showed that anti-DSG2 antibody assays did not effectively distinguish between ARVC-affected and screened Boxers, limiting their usefulness in screening and early diagnosis of ARVC in Boxer dogs. These findings will need validation in other Boxer groups in the future. Additionally, the study highlights the potential of NT-proBNP, cTnI, and HS-cTnI as biomarkers for detecting clinical ARVC in some Boxer dogs.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the author(s) used Grammarly to improve grammar, clarity, and language. After using this tool, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

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