Arrhythmogenesis in Horses

By

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It is also important for me to mention that this work is a result of my lifelong love of horses. It would not have been possible without the horses who have inspired me to walk this path. I dedicate this work to two of the greatest loves of my life, Dante and Gallon. The horse who brought me to Davis, and the horse who kept me here.

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Overarching Abstract

Electrical disturbances in the heart, known as cardiac arrhythmias, are common in the horse but their clinical signficance remains a topic of debate. Due to their electric nature, arrhythmias have a tendency to go unnoticed, as there are often a lack of clincal signs associated with them. While arrhythmias have been associated with sudden cardiac death, in other instances the presence of a cardiac arrhythmia is not indicative of disease. This discrepency inspires the need to determine when cardiac arrhythmias are clinically relevant and what factors may predispose a horse to develop them. Perhaps more iminently, there is a need to scratch the surface on what a veterinarian may consider "normal".

I begin this thesis by exploring, in Chapter 1, the prevalence and risk factors for cardiac arrhythmias in a sedentary mixed breed equine population over the course of 24-hour electrocardiogram recordings. This chapter served as an exploration of what is to be expected in a sedentary herd of horses and aims to provide baseline data on prevalence in an average herd of North American horses. Our data shows that arrhythmias occur at a high prevalence despite a lack of clinical signs, and that increased body condition may put a horse at risk for the development of arrhythmias.

Cardiac remodeling, due to stress from disease or exercise, alters the structure of the heart, which in turn effects electrical conduction in the heart. The relationship between arrhythmogenesis and cardiac remodeling remains unclear. In Chapter 2, the investigation of arrhythmogenesis continues by introducing and investigating the relationship between structure and electric functionality of the heart. Here, we demonstrate that myocardial staining for fibrosis and the matricellular protein tenascin C may serve as valuable indicators of myocardial remodeling following damage from exercise or disease.

The continued investigation of equine arrhythmogenesis may provide the basis for the development of screening protocols to reduce the incidence of potential life-threatening cardiac changes. Collectively this work demonstrates the complexity involved in both diagnosing and categorizing cardiac disease status in the horse, as well as providing inspiration for the work that remains to be done.

Chapter 1: Prevalence and risk factors for cardiac arrhythmias in electrocardiograms of sedentary horses

Abstract

Cardiac arrhythmias are common in horses but their significance remains controversial. The objectives of the study were to describe the prevalence of cardiac arrhythmias in a sedentary herd and identify risk factors for arrhythmias that may warrant additional screening. It was hypothesized that age, weight, cardiac troponin I levels, and the presence of valvular regurgitation would be associated with the detection of cardiac arrhythmias at rest. Ninety-six clinically healthy sedentary horses from a university-owned herd underwent 24-hour electrocardiograms (ECGs) and echocardiograms in a prospective cross-sectional study. Potential risk factors were recorded for all horses. Affected individuals were defined as those with >1 supraventricular premature complex/hour or with a ventricular complex detected. Risk factors were compared between affected and unaffected horses using parametric and non-parametric testing as appropriate. A p-value of < 0.05 was considered significant. During recording, 92.7% (89/96) of horses experienced an arrhythmia. Supraventricular premature complexes were present in 86.5% (83/96) of the population and ventricular complexes were present in 26.0% (25/96) of the population. Increased body condition score was associated with detection of arrhythmias (p=0.039). Arrhythmias occurred at a high frequency in this population despite lack of clinical signs. Increased body condition score was identified as a potential risk factor for arrhythmias.

Introduction

Cardiac arrhythmias in the horse have been associated with poor performance and sudden death[1,2]. This poses welfare, safety, and public relations concerns for horses, riders, drivers, and handlers[3,4]. Although arrhythmias are common, the practical implications on performance and safety as well as the expected frequency of arrhythmias in clinically normal horses remains poorly understood[5,6]. Because arrhythmias can be both intermittent and situational, they pose a diagnostic challenge. The number of

arrhythmias detected is dependent upon the duration of observation and the activity performed during the observation period. A continuous 24-hour ambulatory electrocardiogram (aECG) is recommended for characterization of intermittent arrhythmias[1]. Previous reports on arrhythmia prevalence have reported supraventricular premature complexes at rates of 4-85% and ventricular arrhythmias in up to 60% of horses, with variation attributable to differences in recording duration, the population evaluated, and definition of arrhythmias[5,7–11]. In humans, arrhythmia prevalence at rest has been reported as high as 59.4% during a 24-hour aECG, and in dogs prevalence of up to 39.5% has been reported in a hospital-based population[12–14]. This variability invokes the need to determine what is to be expected in a larger diverse equine population at rest over a 24-hour recording period. Understanding the prevalence of arrhythmias in healthy horses at rest over 24 hours is essential to understanding arrhythmias that warrant further clinical investigation.

In order to identify horses for arrhythmia screening, a better understanding of which horses are most likely to experience arrhythmias is needed**.** In humans, increased age, weight, and presence of pre-existing cardiac disease have been identified as risk factors for arrhythmias[15,16]. Sex and increased age have been identified as risk factors for arrhythmias and sudden death in racehorses[17–19]. Valvular regurgitation has been described as a risk factor for the development of arrhythmias in humans[20,21]. In horses and humans, mitral regurgitation has been described as a risk factor for the development of atrial arrhythmias, specifically atrial fibrillation[21–24]. The relationship between aortic, pulmonary, and tricuspid regurgitation and the development of arrhythmias in horses has been invesgtigated to a lesser extent. To the authors knowledge, arrhythmia prevalence and risk factors for arrhythmias have not been described in a larger population of sedentary horses. Thus, the authors set out to identify risk factors for arrhythmias in a general population of horses wth the goal of defining horses that would benefit from arrhythmia screening.

The aims of this study were to 1) describe the prevalence of cardiac arrhythmias at rest in a population of sedentary horses and 2) identify risk factors for arrhythmias that may warrant additional diagnostic testing. It was hypothesized that age, weight, cardiac troponin I levels, and the presence of valvular regurgitation would be associated with the detection of cardiac arrhythmias in a 24-hour aECG performed at rest.

Materials and Methods

Study Population

Twenty four-hour aECG recordings were collected from a sedentary mixed breed population of 98 university-owned research horses housed at the UC Davis Center for Equine Health over the course of 8 months (September 2021 to April 2022). The population selected was part of a larger equine precision health initiative and also underwent whole genome sequencing and detailed phenotyping[25]. Primary selection criteria for this cohort of horses was age less than or equal to 15 years old or less than or equal to 18 years old with a distinct phenotype. Secondary selection was based off of sex and breed. Two horses with a history of cardiovascular disease were excluded from this subset, resulting in 96 horses available for inclusion.

Horses were housed individually for an overnight acclimation period prior to the beginning of their aECG recording. Horses remained individually housed for the duration of their recording and were maintained on their routine diet of grass and/or alfalfa hay morning and evening and had water available ad libitum. Immediately prior to each aECG, horses underwent a physical examination and jugular vein venipuncture. Heart girth, height, weight, age, and body condition score were recorded for each horse at the time of the ECG. Heart girth was measured in centimeters, placing a measuring tape across the highest part of the withers and around the chest of the horse as close behind the elbows as possible. Height was measured via a height measuring stick (Schneiders, Chagrin Falls, Ohio, USA) and weight was measured via a platform scale (Transcell). Age was determined based off of university records. A body condition score (BCS) was determined on a scale from 1 to 9, utilizing the Henneke Body Condition Scoring System[26]. All procedures were approved by an institutional animal care and use committee of the University of California, Davis (Protocol 23250).

aECG Recordings

A 5-lead aECG was recorded utilizing a Burdick 48 Hr H3+ Recorder with 3 separate channels (Hill-Rom Holdings, Inc.). Hair was clipped in approximately 2”x2” squares and cleaned with 70%

isopropyl alcohol at each electrode site. Skintact W-601 Solid Gel Cloth Electrodes (Leonhard Lang USA, Inc.) were placed with three on the left side of the horse and two on the right. On the left, one electrode was placed caudal to the xiphoid process on the ventral midline, one 50cm down from the dorsal midline caudal to the long head of the triceps, and one 20cm down from the dorsal midline, caudal to the subscapular cartilage. On the right side of the horse, one electrode was placed 20cm down from the dorsal midline, caudal to the subscapular cartilage, and the second electrode on the right was placed 40cm down from the dorsal midline (Figure 1). All electrodes were secured to the horse's skin with superglue. Elastikon Elastic Tape (Johnson & Johnson, New Brunswick, New Jersey, USA) was then placed around heart girth, followed by an adjustable elastic surcingle (Equi-Essentials) and a training surcingle (Dover Saddlery) to hold the aECG monitor, leads, and electrodes in place. Horses were checked at approximately six and twelve hours after beginning of recording, with leads repositioned as needed. Vision 5 Holter Analysis Software (Hill-Rom Holdings, Inc.) was utilized for ECG review and analysis. ECGs were reviewed by a single author (LM) and erroneous complex identification was corrected prior to secondary review of the ECGs by a second author (JM). Both the author performing initial review and secondary review were masked to horse identification and demographics. The software parameters were set to identify R-R intervals with deviation >20% from previous R-R intervals, including atrial fibrillation, supraventricular premature complexes, ventricular complexes, and pauses. Supraventricular premature complexes (SVPCs) were characterized as premature atrial depolarizations with >20% shorter deviation from the previous beat (Figure 2a). Overall deviation was evaluated subjectively during ECG review and periods of sinus arrhythmia or greater than 20% overall devation in R-R interval were removed from the SVPC catagory. Ventricular complexes (VCs) were characterized as ventricular depolarization characterized by the absence of detectable P-waves and change in QRS morphology (Figure 2b). Total recording duration, total analyzed recording duration, minimum and maximum heart rate (HR) in beats per minute (BPM), average HR, number of SVPCs, VCs, and pauses (second degree atrioventricular block (2DAVB) or sinus block) were recorded, as well as frequency of singlets, couplets, or runs of 3+ SVPCs and/or VCs in each recording.

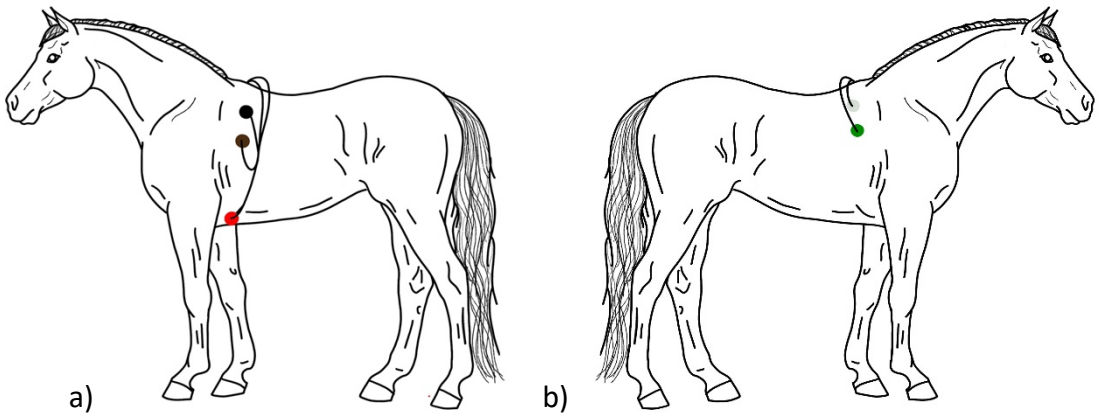

Figure 1. Electrode placement. Leads were connected to Skintact W-601 Solid Gel Cloth ECG Electrodes (Leonhard Lang USA, Inverness, Florida, USA) and then placed on each horse. (a) On the left side of the horse, one electrode was placed caudal to the xiphoid process on the ventral midline. One electrode was placed 50cm down from the dorsal midline, caudal to the long head of the triceps. One electrode was placed 20cm down from the dorsal midline, caudal to the subscapular cartilage. (b) On the right side of the horse, one electrode was placed 20cm down from the dorsal midline, caudal to the subscapular cartilage. The final electrode was placed 40cm down from the dorsal midline.

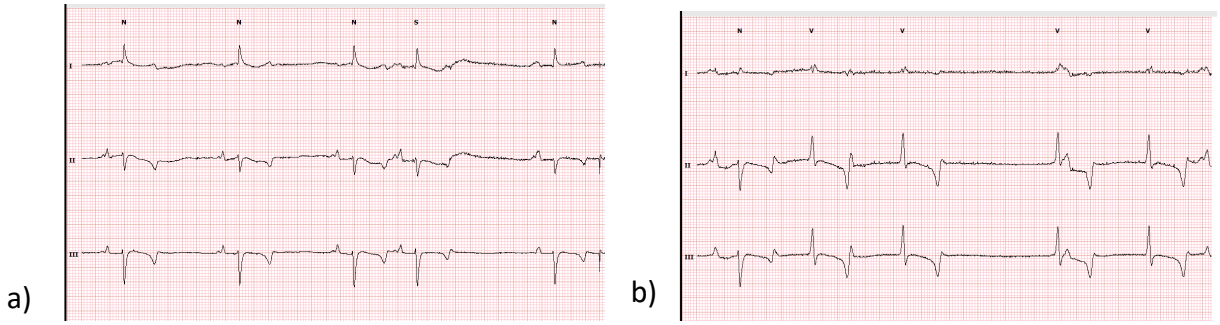

Figure 2. Representative tracing of a Supraventricular Premature Complex (SVPC) on electrocardiogram (a) and representative tracing of a Ventricular Complex (VC) on electrocardiogram (b). Tracings are displayed at 25mm/sec paper speed and 10mm/mV.

Blood Sample Handling and Analysis

Blood was collected in heparinized tubes (Becton, Dickinson and Company) and centrifuged at 3000rpm and 24°C for 15 minutes. Heparinized plasma samples were aliquoted into cryovials and stored in a -80°C freezer until analysis. Plasma samples were subsequently shipped on dry ice to the University of Pennsylvania for analysis of Cardiac Troponin I (cTnI) concentration utilizing the Straus CS immunoassay (Stratus CS, Siemens Diagnostic Healthcare, Newark, DE, USA) which was previously validated for use with equine cTnI[27].

Echocardiography

Unsedated transthoracic echocardiograms were performed on all horses enrolled in the study between May 16th, 2020 and May 13th, 2022 by two experienced clinicians (JM and EWL). The hair was clipped bilaterally and prepared with alcohol and ultrasound gel. Cine-loops and images were obtained with an ultrasound machine (LOGIQ e, Sound, Carlsbad, CA, USA) equipped with a 3.5MHz cardiac probe and simultaneous ECG recording. Standard echocardiographic examinations involving 2-dimensional, M-mode, and color doppler with imaging of both the left and right sided parasternal long and short axis views were performed, as previously described[28–30]. Mitral regurgitation (MR), aortic regurgitation (AR), pulmonary regurgitation (PR), and tricuspid regurgitation (TR) were scored from 0-4 by two observers (LM and JM) utilizing color doppler cine-loops and a previously described scoring system[31]. A score of 0 signified no regurgitation present, while a score of 4 signified severe regurgitation.

Data Analysis

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Evaluated risk factors for this study were breed, age, height, weight, heart girth, body condition score, sex, cTnI level, and presence of valvular regurgitation. Descriptive statistics were performed on arrhythmia prevalence and each risk factor. Normality was assessed on each risk factor by a Shapiro-Wilk test. Normally distributed data is reported as mean +/- standard deviation and nonparametric data as median and

interquartile range (IQR). The single draft horse in the population, a Percheron gelding, was excluded from risk factor analysis for heart girth, weight, and height due to being an outlier. Three definitions of arrhythmias were initially considered for risk factor analysis: 1. The presence of >1 SVPC/hour or the presence of a VC; 2. The presence of >1 SVPC/hour; 3. The presence of a VC. Due to the low numbers of horses with VC the first definition was selected to maximize the power to detect arrhythmia risk factors. Affected individuals were thus defined as horses with >1 SVPC/hour or the presence of a VC. Any horse with a frequency of >1 SPVC/hour or at least one VC present on recording were considered affected. Unaffected individuals were defined as horses with <1 SVPC/hour and no VCs present in their recording. Risk factors in the affected versus unaffected horses were compared with a student's t-test, Mann-Whitney, Chi-Squared or Fisher's exact test as appropriate. For breed analysis, the three most represented breeds, Quarter Horses (n = 33), Thoroughbreds (n = 29), and Warmbloods (n = 21) were considered individual groups, and the fourth group was titled "Other" and consisted of all other breeds (n = 14). Significance was defined as $P < 0.05$.

Results

Study Population

The 96 horses included consisted of 51 mares (5 of which were ovariectomized), 43 geldings, and 2 stallions. The population consisted of Quarter Horses (32), Thoroughbreds (29), Warmbloods (20), Standardbreds (6), Iberians (2), Lipizzaners (2), Appaloosas (2), an Arabian (1), a Percheron (1), and a horse of unknown breeding (1). Two Paint Horses and one Quarter Horse Cross were included in the Quarter Horse breed category. Age, height, weight, heart girth, and body condition score descriptive statistics are described in Table 1.

Table 1. Characteristics of the population of 96 horses included in the sedentary study population.

	Minimum	Maximum	Median (IQR)	Mean±SD
Age (years)	3	20	N/A	12±4
Weight (kg)	429	867	556 (523-600)	N/A
Height (hh)	14.1	19.0	16.2 (15.1-16.2)	N/A
Heart Girth (cm)	174	228	N/A	190±8.6
BCS	5	9	6 (6-7)	N/A

Arrhythmia Detection

A total of 96 aECGs were used for analysis; four were repeated, either due to poor-quality recordings (2), behavior (1), or technological difficulties (1). The recording duration ranged from 23.53 to 28.41 hours with a median duration of 24.31 hours (IQR 24.16-24.46). The analyzed duration of each recording ranged from 5.3 to 25.3 hours, with a median duration of 24.2 hours (IQR 24.1-24.4). The average heart rate throughout the recording ranged from 28 to 44 bpm, with a median average heart rate of 35 bpm (IQR 33-38). During recording, 92.7% (89/96) of horses experienced an arrhythmia (SVPC or VC). At least one SVPC was present in 86.5% (83/96) of the population, 18.8% (18/96) had >1 SVPC/hour. SVPCs ranged from 0.0 per recording to 24.6 per hour with a median of 0.2 per hour (IQR 0.04-0.5). Ventricular complexes were detected in 26.0% (25/96) of the population (Figure 3). VCs ranged from 0.0 per recording to 19.1 per hour with a median of 0.0 per hour (IQR 0.0-0.04). Pauses, defined as either 2DAVB or a sinus block, were observed in 87.5% (84/96) of the population. Pauses ranged from 0.0 per recording to 340.4 per hour, with a median of 1.7 per hour (IQR 0.2-33.3) The longest pause per recording ranged from 2.0 to 6.6 seconds, with a median of 4.2 seconds (IQR 3.7-4.8). The prevalence of individuals with >1 SVPC/hr or a VC was 39.6% (38/96). SVPC and VCs occured as singlets, couplets, or runs of 3+ complexes in a row (Table 2, Supplementary Table 1). SVPC couplets were identified in 14.5% (12/83) of horses who experienced a SVPC in their recording, and one horse had a single run of SVPCs. Of the horses that experienced them, the minimum number of SVPC couplets per recording was 1 per recording and the maximum was 9 with a median of 2 (IQR 1-6.5). VC couplets were identified in 12.0% (3/25) of horses who experienced VCs and runs were identified in 24.0% (6/25) of horses who experienced VCs. Among

the three horses with ventricular couplets, one horse had 16 couplets, one had 8, and one had 2 per recording. Among the six horses that experienced runs of VCs, the minimum number of runs was 1 and the maximum was 30 with a median of 2.5 (IQR 1.8-9.8). The maximum number of VCs per run was 117 complexes and the minimum was 3 complexes with a median of 7 complexes (IQR 4-57). No complex arrhythmias were identified.

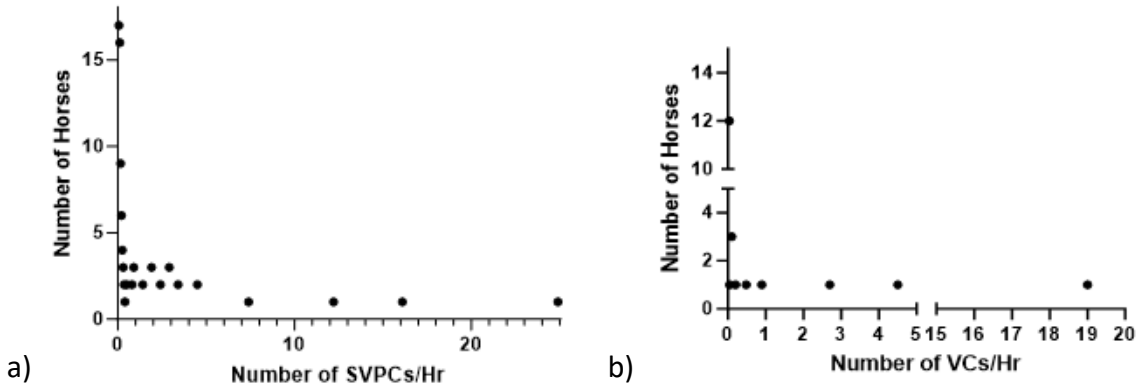

Figure 3. Number of horses with a given frequency of supraventricular and ventricular ectopy in a continuous 24-hour ECG recording (n=96).

Table 2. Ventricular complex (VC) profile for horses that experienced any VCs during their 24-hour continuous ambulatory ECG.

Horse	Sex	Age (Years)	Breed	Number of VCs	Number of VCs/Hour	Number of Singlets	Number of Couplets	Number of Runs	Max Run	Number of SVPCs	Number of SVPCs/Hour
Horse 72	M	15	Quarter Horse	459	19.046	25	16	30	117	109	4.52
Horse 66	G	18	Quarter Horse	110	4.405	61	2	3	37	0	0
Horse 6	G	7	Thoroughbred	65	2.66	43	8	2	3	3	0.123
Horse 84	G	8	Thoroughbred	21	0.862	7	0	3	0	45	1.847
Horse 7	G	6	Thoroughbred	12	0.48	2	0	2	7	9	0.36
Horse 63	M	13	Quarter Horse	5	0.205	1	0	1	4	1	0.041
Horse 69	G	20	Warmblood	3	0.124	3	0	0	0	12	0.497
Horse 24	M	14	Thoroughbred	2	0.082	2	0	0	0	2	0.082

Horse 47	O	12	Standardbred	2	0.084	2	0	0	0	1	0.042
Horse 57	M	17	TB/Appaloosa	2	0.081	2	0	0	0	71	2.88
Horse 70	G	18	Percheron	2	0.108	2	0	0	0	4	0.216
Horse 3	G	14	Thoroughbred	1	0.041	1	0	0	0	1	0.041
Horse 4	G	12	Lusitano	1	0.043	1	0	0	0	3	0.129
Horse 9	G	5	Quarter Horse	1	0.041	1	0	0	0	19	0.782
Horse 20	G	13	Warmblood	1	0.041	1	0	0	0	45	1.851
Horse 21	M	12	Thoroughbred	1	0.041	1	0	0	0	0	0
Horse 27	M	12	Warmblood	1	0.041	1	0	0	0	7	0.29
Horse 43	M	16	Standardbred	1	0.042	1	0	0	0	4	0.168
Horse 48	M	8	Warmblood	1	0.041	1	0	0	0	0	0
Horse 52	O	13	Thoroughbred	1	0.041	1	0	0	0	71	2.942
Horse 61	M	15	Appaloosa	1	0.04	1	0	0	0	2	0.081
Horse 67	M	11	Warmblood	1	0.181	1	0	0	0	0	0
Horse 88	G	15	Quarter Horse	1	0.051	1	0	0	0	1	0.051
Horse 78	M	14	Thoroughbred	1	0.041	1	0	0	0	9	0.367
Horse 98	G	6	Warmblood	1	0.04	1	0	0	0	0	0

M = Mare, G = Gelding, O = Ovariectomized Mare

Cardiac Troponin I

In our population, 5.2% (5/96) were above the reference range for Cardiac Troponin I (cTNI) in horses is 0.00 – 0.06 ng/ml[27]. One Lipizzaner mare (cTnI of 5.45 ng/ml) had a history of familial narcolepsy and had collapse episodes the day of the ECG. Four horses with elevated cTNI had no known history to explain the elevation, including one Warmblood mare (3.07 ng/ml), one Thoroughbred gelding (0.17ng/ml), one Standardbred mare (0.12 ng/ml), and one Warmblood mare (0.07 ng/ml).

Valvular Regurgitation

Valvular regurgitation was identified in 72.0% (67/93) of horses. 10.8% (10/93) of horses had mitral regurgitation, 49.5% (46/93) had aortic regurgitation, 20.9% (19/91) had tricuspid regurgitation, and 29.7% (27/91) had pulmonic regurgitation. Of the 49.5% of horses that had aortic regurgitation, 19.6% (9/46) of those horses had a score of 2 or higher. Aortic regurgitation was the only type of regurgitation that received a score of a 3 (n=1). All other types of regurgitation received a score of 2 or below. No scores

of 4 were recorded for any type of regurgitation. Three horses were excluded from this analysis due to inadequate imaging available to identify regurgitation.

Arrhythmia Risk Factors

Affected horses (n=38) were defined as having the presence of >1 SVPC/hour or the presence of a VC in their recording for the purpose of risk factor evaluation. No significant differences in age, height, weight, heart girth, breed, or sex were detected between horses with arrhythmias and those without. Arrhythmias were associated with higher body condition scores (p=0.039, Table 3, Figure 4). No significant statistical differences in arrhythmia prevalence were detected between breeds (Figure 5a). Prevalence of arrhythmias ranged from 10/20 (50.0%) of Warmbloods affected to 4/15 (26.7%) of other breeds affected (Figure 5b).

Table 3. Minimum, maximum, median, and IQR for affected horses (n=38) versus unaffected horses (n=58) for all recorded quantitative risk factors: age, weight, height, heart girth, BCS, and cTnI. *p < 0.05

Horses with >1SVPC/hr or VC Present (n=38)				Horses with <1SVPC/hr and no VC Present (n=58)		
	Minimum	Maximum	Median (IQR)	Minimum	Maximum	Median (IQR)
Age	5	20	13 (11 -15)	3	19	12 (9-15)
Weight(kg)	484	867	566 (528-610)	429	776	555 (519-588)
Height(hh)	14.1	19.0	16.0 (15.0-16.2)	14.1	18.1	15.3 (15.0-16.2)
Heart Girth(cm)	179	228	191 (185-197)	174	212	188 (183-194)
BCS	5	9	***7 (6-8)**	5	8	***6 (6-7)**
cTNI (ng/ml)	0.00	0.12	0.00 (0.00-0.01)	0.00	5.45	0.00 (0.00-0.02)

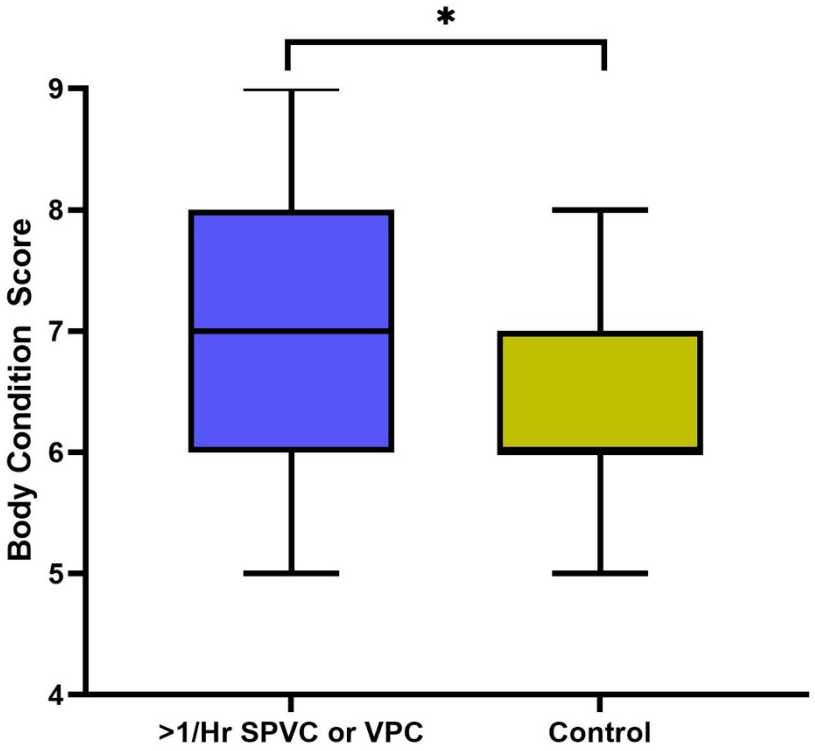

Figure 4. Box and whisker plot of body condidion score (BCS) minumum, maximum, and median in affected horses (>1 SVPC/hour or VC present on recording) compared with unaffected horses (<1 SVPC/hour and no VC present on recording). *p < 0.05.

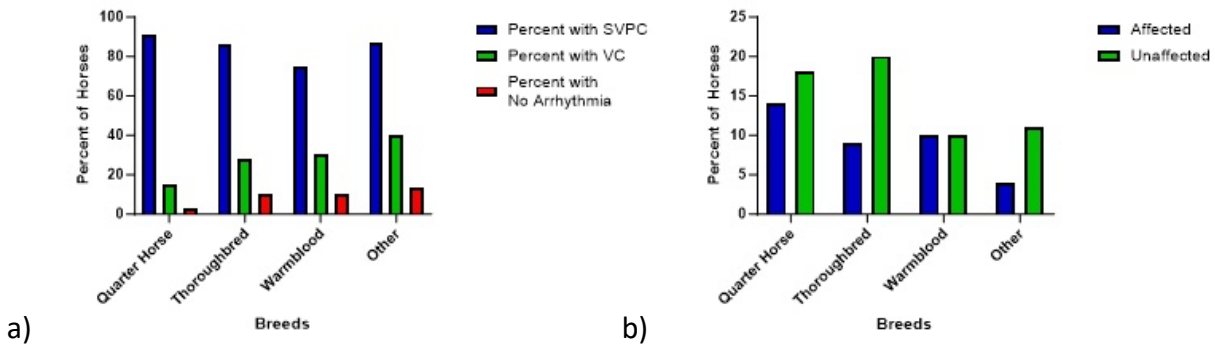

Figure 5. (a) Percent of each breed (Quarter Horses, Thoroughbreds, Warmbloods, or Other) that experienced any Superventricular Premature Complexes, Ventricular Complexes, or no arrhythmias in their

ECG recording. (b) Percent of each breed considered "Affected" (>1SVPC/hr or VC present) or "Unaffected" (<1SVPC/hr and no VC present).

Discussion

Cardiac arrhythmias occur at a high prevalence in this sedentary mixed breed equine population without a history of cardiac disease. During recording, 92.7% of horses experienced at least one arrhythmia: 86.5% of them had at least one supraventricular premature complex and 26.0% of them had at least one ventricular complex. The prevalence of individuals in the population with >1 SVPC/hour or a VC, which we considered of potential clinical significance, was 38/96 (39.6%). To the authors knowledge, this is the first study evaluating 24-aECG findings in a sedentary mixed breed population of this size. Previous reports site infrequent detection of arrhythmias at rest and have evaluated small uniform populations of horses[7,32,33]. Arrhythmia prevalence reported in sporthorses (event, dressage, jumping, and racehorses) during, and immediately post-exercise, ranges from 27.8-92.0% and is generally higher than that reported at rest[5,8,9,34–38]. Risk factor analysis indentified increased body condition score as a potential risk factor for increased arrhythmia prevalence. These data fills a literature gap by describing arrhythmia prevalence in a large, mixed breed, sedentary herd of horses over 24 hours and exploring potential risk factors in a herd of horses not trained for any specific purpose.

The prevalence of SVPCs (86.5%) in this population was higher than previously reported in sedentary Standardbreds (15.2%) or Thoroughbreds (0%), despite those breeds being represented in our population[39]. Higher SVPC prevalence comparable than observed here (70%-86.4%), has been reported in athletic populations of 10-22 horses at rest and in a hospital-based population after recovering from anesthesia (85%)[32,40]. Lower prevalence of SVPCs has also been reported in athletic populations including dressage horses (5%) and show jumpers (32%) at rest[98]. However these are based on very brief resting ECGs of 2-5 minutes in duration compared to the 24-hour recording period used in those reported above, which likely explains the decrease in arrhythmia detection. The prevalence of horses with >1 SVPC/hour in 24-hour recordings (5-18%) is similar to what we report (19%)[32,40]. A benefit of the 24-hour aECG

monitor is to allow for an increased likelihood of detecting intermittent arrhythmias due to the increased recording time and periods of increased vagal tone during overnight recordings. It is possible that our reported prevalence is high due to the subjective nature of evaluating overall beat to beat variation. Increased information on prevalence of arrhythmias in 24-hour recordings will aid in interpretation of the clinical relevance of intermittent cardiac arrhythmia findings.

Prevalence of VCs (26%) in this population is increased compared to previous reports in a sedentary population at rest (0.0-3.5%)[7,10,39]. However, the prevalence of VCs is decreased compared to reports in both athletic (50%) and a hospital-based populations (60%)[11,32].

Increased body condition score was identified as a potential risk factor for cardiac arrhythmias in this study. Horses with cardiac arrhythmias had a median BCS of 7, while the unaffected group had a median BCS of 6. Metabolic syndrome has been associated with cardiovascular disease and the development of arrhythmias in humans[41,42]. Obesity and insulin resistance has been associated with changes in cardiac structure and function in horses and dogs[43,44]. However, the single study that has arrhythmias in Equine Metabolic Syndrome (EMS) did not find an increase in arrhythmias in EMS cases compared to breed matched contorls[44]. Due to small sample size, this finding should be interpreted with caution[45,46]. No other risk factors were identified consistent with several previous reports in athletic horses at rest [8,9,34]. Some studies have identified age and sex as potential risk factors for sudden death or post-race arrhythmias in racehorses[17,18]. However, it is possible that fitness level has a relationship with these risk factors, as age has been detected as a risk factor in racehorses but not in other populations. Fitness has also been associated with increase in SVPCs with eventing and endurance horses having more SVPCs that pleasure horse controls[32]. It is likely that fitness and obesity and/or metabolic derangements represent separate physiologic stresses that increase risk of arrhytmogenesis as observed in humans[16]. Our findings combined with the existing literature suggest that increased BCS, which can be associated of metabolic derrangements, may put a horse at risk for the development of arrhythmias and warraents additional investigation.

This study had a few limitations. Whilst the relationship between the presence of arrhythmias and BCS warrants additional investigation, our population only had body condition scores ranging from 5 to 9,

not giving a true representation of BCS grades any lower than what is considered to be "ideal" (5). It is also of importance to note that BCS is inherently a subjective scoring system, although we had one observer record BCS in all horses in order to maintain consistency and reduce error due to variability. In humans, sex is associated with cardiac disease, with men being at higher risk of developing cardiac arrhythmias and experiencing life-threatening cardiac events[47–49]. In Thoroughbred racehorses, one recent study found that intact colts were at lower risk of experiencing sudden cardiac death when compared to mares and geldings[18]. However, another study found that intact males were at 39% higher risk of experiencing sudden cardiac death when compared to mares and geldings[17]. As there were only 2 stallions in our study population, our sex analysis was limited to geldings and mares, so we were unable to provide insight into this discrepancy. Investigation into the prevalence of arrhythmias in horses with lower BCSs as well as in stallions would provide additional insight into what may put horses at risk for the development of arrhythmias.

In conclusion, body condition score was found to be a potential risk factor for a higher prevalence of arrhythmias over the course of a 24-hour recording. Arrhythmias occurred at a high prevalence in this population despite a lack of clinical signs. While additional research is needed to determine what puts a horse at increased risk of developing arrhythmias and at what point these arrhythmias are clinically relevant, this data provides valuable insight to what prevalence of arrhythmias in a population may be considered normal when performing 24-hour aECG monitoring in sedentary horses.

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Supplemental Table 1. Supraventricular Premature Complex (SVPC) profile for horses that experienced any SVPCs during their recrording.

Horse	Sex	Age (Years)	Breed	Number of Supraventricular Premature Complexes	Number of Supraventricular Premature Complexes/Hour	Number of Singlets	Number of Couplets	Number of Runs	Max Run
Horse 46	M	7	Warmblood	607	24.595	607	0	0	0
Horse 13	M	15	Thoroughbred	389	16.088	373	8	0	0
Horse 87	M	12	Quarter Horse	295	12.130	295	0	0	0
Horse 10	M	18	Paint Horse	177	7.375	171	3	0	0
Horse 72	M	15	Quarter Horse	109	4.523	91	9	0	0
Horse 41	G	11	Quarter Horse	105	4.402	105	0	0	0
Horse 77	G	13	Quarter Horse	85	3.628	85	0	0	0
Horse 35	M	11	Warmblood	83	3.433	83	0	0	0
Horse 17	G	14	Quarter Horse	72	2.935	72	0	0	0
Horse 52	O	13	Thoroughbred	71	2.942	71	0	0	0
Horse 57	M	17	X	71	2.880	71	0	0	0
Horse 79	G	16	Quarter Horse	60	2.456	60	0	0	0
Horse 91	M	13	Quarter Horse	60	2.449	54	3	0	0
Horse 11	M	18	Quarter Horse	48	1.988	34	7	0	0
Horse 20	G	13	Warmblood	45	1.852	43	1	0	0
Horse 84	G	8	Thoroughbred	45	1.847	43	1	0	0
Horse 59	M	14	Warmblood	28	1.130	22	1	1	4
Horse 68	O	15	Warmblood	24	1.380	24	0	0	0
Horse 23	M	17	Thoroughbred	24	0.989	24	0	0	0
Horse 14	M	14	Thoroughbred	23	0.953	13	5	0	0
Horse 22	G	10	Paint Horse	22	0.887	22	0	0	0
Horse 12	G	11	Thoroughbred	19	0.792	19	0	0	0
Horse 9	G	5	Quarter Horse	19	0.782	17	1	0	0
Horse 83	G	11	Quarter Horse	13	0.527	13	0	0	0
Horse 69	G	20	Warmblood	12	0.497	12	0	0	0
Horse 93	G	13	Quarter Horse	10	0.414	10	0	0	0
Horse 78	M	14	Thoroughbred	9	0.367	9	0	0	0
Horse 7	G	6	Thoroughbred	9	0.360	9	0	0	0
Horse 27	M	12	Warmblood	7	0.289	7	0	0	0
Horse 64	M	11	Warmblood	7	0.280	7	0	0	0
Horse 28	G	5	Quarter Horse	7	0.278	7	0	0	0
Horse 37	G	15	Quarter Horse	6	0.254	6	0	0	0
Horse 5	M	14	Lipizzaner	6	0.250	6	0	0	0
Horse 34	G	11	Thoroughbred	6	0.245	6	0	0	0
Horse 55	M	7	Quarter Horse	6	0.240	6	0	0	0
Horse 32	G	19	Thoroughbred	5	0.207	5	0	0	0
Horse 74	M	12	Quarter Horse	5	0.206	5	0	0	0
Horse 18	M	17	Quarter Horse	5	0.205	5	0	0	0
Horse 80	M	10	Warmblood	5	0.204	5	0	0	0
Horse 19	M	16	Lipizzaner	4	0.306	4	0	0	0

Horse 70	G	18	Percheron	4	0.216	4	0	0	0
Horse 43	M	16	Standardbred	4	0.168	4	0	0	0
Horse 82	G	10	Thoroughbred	4	0.166	4	0	0	0
Horse 29	M	16	Quarter Horse	4	0.165	2	1	0	0
Horse 38	G	13	Andalusian	4	0.165	4	0	0	0
Horse 44	G	9	Warmblood	4	0.164	4	0	0	0
Horse 49	G	10	Quarter Horse	4	0.163	4	0	0	0
Horse 36	G	13	Thoroughbred	4	0.162	4	0	0	0
Horse 50	M	14	X	4	0.160	2	1	0	0
Horse 26	G	16	Quarter Horse	4	0.157	4	0	0	0
Horse 4	G	12	Lusitano	3	0.129	3	0	0	0
Horse 86	G	6	Thoroughbred	3	0.127	3	0	0	0
Horse 42	M	3	Quarter Horse	3	0.126	3	0	0	0
Horse 62	M	15	Appaloosa	3	0.123	3	0	0	0
Horse 6	G	7	Thoroughbred	3	0.123	3	0	0	0
Horse 54	G	12	Quarter Horse	3	0.122	3	0	0	0
Horse 31	G	13	Thoroughbred	3	0.118	3	0	0	0
Horse 85	G	9	Thoroughbred	2	0.084	2	0	0	0
Horse 39	M	16	Quarter Horse	2	0.083	2	0	0	0
Horse 96	S	7	Thoroughbred	2	0.083	2	0	0	0
Horse 24	M	14	Thoroughbred	2	0.082	2	0	0	0
Horse 30	G	7	Warmblood	2	0.081	2	0	0	0
Horse 51	M	14	Thoroughbred	2	0.081	2	0	0	0
Horse 94	M	16	Thoroughbred	2	0.081	2	0	0	0
Horse 61	M	15	Appaloosa	2	0.081	2	0	0	0
Horse 92	O	17	Standardbred	2	0.080	2	0	0	0
Horse 76	M	13	Thoroughbred	1	0.052	1	0	0	0
Horse 88	G	15	Quarter Horse	1	0.051	1	0	0	0
Horse 71	M	16	Warmblood	1	0.046	1	0	0	0
Horse 97	S	5	Thoroughbred	1	0.043	1	0	0	0
Horse 47	O	12	Standardbred	1	0.042	1	0	0	0
Horse 3	G	14	Thoroughbred	1	0.041	1	0	0	0
Horse 15	M	7	Warmblood	1	0.041	1	0	0	0
Horse 58	G	10	Warmblood	1	0.041	1	0	0	0
Horse 63	M	13	Quarter Horse	1	0.041	1	0	0	0
Horse 60	M	7	Quarter Horse	1	0.046	1	0	0	0
Horse 1	M	8	Standardbred	1	0.041	1	0	0	0
Horse 81	G	11	Quarter Horse	1	0.041	1	0	0	0
Horse 16	M	17	Quarter Horse	1	0.040	1	0	0	0
Horse 90	M	12	Standardbred	1	0.040	1	0	0	0
Horse 95	M	14	Warmblood	1	0.040	1	0	0	0
Horse 45	G	11	Thoroughbred	1	0.040	1	0	0	0
Horse 25	G	7	Thoroughbred	1	0.040	1	0	0	0

M = Mare, G = Gelding, O = Ovariectomized Mare, S = Stallion

Chapter 2: Evaluation of cardiac extracellular matrix remodeling in equine cardiac disease

Abstract

Cardiac disease in horses is a cause of morbitity and mortality. Understanding the difference between normal and pathologic cardiac physiology and the relationship between electrical and structural abnormalities is essential for the early detection and successful treatment of disease. Fibrosis of the myocardium as well as the expression of matriceullar protein Tenascin C (TNC) are of particular interest for their known role in remodeling of the human myocardium. The objectives of the study were to quantify the degree of fibrosis present in the cardiac extracellular matrix and evaluate the expression of matricellular protein tenascin c in the cardiac extracellular matrix of horses that have experienced cardiac events compared to controls. Myocardial samples from five heart failure cases and controls, as well as racehorse myocardial samples from two cases who experienced idiopathic sudden cardiac death and two controls who experienced death due to catastrophic musculoskeletal breakdown, were stained for fibrosis and TNC. Slides were imaged by a Nikon DS-Fi2 microscope with NIS imaging software utilizing automated tiling. Fibrosis was quantified in heart failure cases. TNC expression was grossly evaluated for both heart failure and sudden death cases. There was a non-significant trend indicating a higher percent fibrosis and greater mean intensity in heart failure cases when compared with controls ($p=0.13$). However, upon gross observation, racehorse cases do not appear to have an increased amount of fibrosis when compared with controls. Additionally, TNC expression does not appear increased in either heart failure cases or sudden death cases when compared with respective controls. Staining and imaging protocols yielded results which could be useful moving forward. Future directions for this work involve the continued investigation of cardiac remodeling in a larger population of horses that have experienced fatal cardiac events.

Introduction

Equine cardiac disease can present itself in various ways in a multitude of populations. An appreciation of normal and pathologic cardiac physiology, both electrical and structural, is essential for the diagnosis

and treatment of disease. Electrical disturbances, known as cardiac arrhythmias, pose a diagnostic challenge due to their ability to go undetected, as they are commonly intermittent and asymptomatic. The clinical signficance of specific arrhythmias remains controversial. Cardiac remodeling occurs due to molecular, cellular, and interstitial changes that manifest themselves as changes in size, mass, geometry, and/or function of the heart following injury or exercise[1]. In humans, there is evidence documenting that persistent arrhythmias lead to remodeling of the heart[2]. Cardiac enlargement due to remodeling has been seen in racehorses[3–6]. While it has been suggested that ventricular hypertrophy can cause arrhythmias in these athletic populations, remodeling of the myocardium and its role in arrhythmogenesis in horses is poorly understood[7,8]. Chronic electrical and structural cardiac changes due to intense physical activity can mimic the changes in diseased hearts; however, the disease process in equine cardiac events is not well studied[9]. Investigating remodeling of the myocardium in horses that are affected with cardiac disease (heart failure and sudden cardiac death) will allow for increased insight into the tissue level disease process that occurs on a cellular level. Understanding the underlying disease process in cardiac disease is essential for the development of effective diagnostic tools and interventions prior to death.

Cardiac fibrosis is a common finding in various forms of human cardiac disease and is known to promote ventricular tacharrhythmias[10]. Thus, it is well recognized in human medicine as a cause of morbitiy and mortality. Cardiac fibrosis is characterized by the net accumulation of extracellular matrix proteins in the cardiac interstitium either due to reparative processes or in response to disease. Initially, remodeling of the extracellular matrix can be beneficial as a protective mechanism; however, excessive remodeling leads to impaired function. Fibrotic tissue causes stiffening of the myocardial matrix, which can impair the physical output of the heart as well as electric conductance[11]. While cardiac fibrosis is associated with myocardial cell death and is known to promote the development of arrhythmias in humans, the role that fibrosis plays in cardiac disease of the horse is less well defined[12,13]. While existing literature in equids supports what is available in humans, most reports of cardiac fibrosis in the horse have been in the form of individual case studies with varying pathologies[14–16]. In a 2019 study investigating fibrosis present in Thoroughbred racehorses who died of sudden cardiac death, mean fibrosis was found to be higher in the

sudden cardiac death group compared against matched controls who died of non-cardiac causes[17]. This suggests that increased fibrosis could be a precursor for the development of arrhythmias and potentially sudden cardiac death.

TNC is a matricellular protein of particular interest due to it's role in pressure overload and ability to serve as a precursor to fibrosis. In humans, TNC is not commonly detected in normal adults, but is strongly up-regulated with inflammation and is expressed in diseased hearts[18]. Previous clinical studies in humans suggest that TNC facilitattes cardiac fibrosis by promoting inflammation with a positive feedback loop[19]. In horses, TNC has previously been found to be an important factor in tendon wound healing, in addition to playing a crucial role in the loading of tendon-like tissues during exercise in other species[20–22]. In swine and sheep, TNC was previously found to promote valvular remodeling[23]. To the authors knowledge, TNC expression in equine cardiac tissue has not been previously investigated.

In this study we aimed to 1) Quantify the degree of fibrosis present in the cardiac extracellular matrix and 2) Evaluate the expression of matricellular TNC in the cardiac extracellular matrix of horses that have experienced cardiac events compared to age-matched controls. We hypothesized that horses that expereince a cardiac event would have increased myocardial fibrosis as well as increased expression of matricellular TNC when compared to age-matched controls. This work is important for the future development of a protocol for the early detection of cardiac disease in the horse.

Materials & Methods

Animals and Tissue Collection: Heart Failure Cases

A total of ten horses were utilized to evaluate cardiac remodeling in horses with cardiac disease. Myocardial samples from five cases and five breed and age-matched controls were acquired retrospectively from banked tissue blocks made available by the UC Davis Anatomic Pathology Service. The horses ranged in age from 7 months to 14 years of age with cases defined as horses who died of various forms of heart failure and controls having varying non-cardiovascular causes of death (Table 1). The samples were

formalin-fixed and embedded in paraffin and then cryosectioned by a microtome and mounted onto glass slides.

Table 1. Heart Failure case and control signalment. TB = Thoroughbred, QH = Quarter Horse. G = Gelding, S = Stallion, M = Mare.

Cases (n=5)					Controls (n=5)				
Horse	**Breed**	**Sex**	**Age**	**Cause of Death**	**Horse**	**Breed**	**Sex**	**Age**	**Cause of Death**
1	TB	G	3	Ruptured mitral chordae tendineae	1	TB	G	2	Enterocolitis, typhlitis
2	TB	S	14	Myocardial degeneration and necrosis	2	TB	G	13	Cachexia
3	Paint	G	5	VSD and Cardiomegaly	3	Paint	M	5	Splenic foreign body
4	QH	G	11	Ruptured mitral chordae tendineae	4	QH	M	12	Displaced left ischial fracture
5	QH	M	1	VSD and Cardiomegaly	5	QH	M	0.7	C5/C6 Osteoarthritis

Racehorse Sudden Death Cases

A total of four racehorses (3 Thoroughbreds, 1 Quarter Horse) were available for this study. Myocardial samples from two cases (1 mare, 1 colt) who experienced idiopathic sudden cardiac death and two age-matched controls (1 gelding, 1 mares) who experienced death due to catastrophic musculoskeletal breakdown were previously banked. The horses ranged in age from 2 to 5 years of age (Table 2). Myocardial samples were made available by the California Animal Health and Food Safety (CAHFS) Lab San Bernadino and the California Horse Racing Board (CHRB) Post-Mortem Program. Auricle samples were obtained using a standardized post-mortem protocol[24]. The samples were formalin-fixed and embedded in paraffin and then cryosectioned by a microtome and mounted onto glass slides.

Table 2. Racehorse case and control signalment. TB = Thoroughbred, QH = Quarter Horse. G = Gelding, S = Stallion, M = Mare.

Cases (n=2)				Controls (n=2)				
Horse	**Breed**	**Sex**	**Age**	**Horse**	**Breed**	**Sex**	**Age**	**Cause of Death**
2	TB	M	5	2	TB	M	3	Fracture of right ilium
3	TB	S	2	3	QH	G	2	Spinal fracture

Immunohistochemical Analysis of Myocardium

Masson's trichrome (MTRI) staining was utilized to semi-quantitatively determine fibrosis present in the tissue samples, as previously described[25]. Immunohistochemistry (IHC) was utilized to detect and quantify expression of tenascin C (Supplemental 1). IHC for tenascin C took place over two days. Slides were deparaffinized in xylene, 100% ethanol, and 95% ethanol. A sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) was utilized for antigen retrieval at 65°C for 30 minutes. Following the sodium citrate buffer incubation, slides were incubated for 10 minutes in 3% H2O2/Methanol (1:10 dilution) to block endogenous peroxidases. An Innovex Universal Animal IHC Kit (Innovex Biosciences Inc.) was used for the remainder of the protocol. Background buster was applied for 30 minutes. After a PBS and PBST wash, Human/Mouse Tenascin C Antibody #MAB2138 (R&D Systems, Minneapolis, MN), was then added overnight at room temperature at a 1:50 dilution with 5% BSA/PBST. The following morning, slides were incubated with a multivalent linking antibody for 10 minutes, followed by a peroxidase for 10 minutes. 1 drop of DAB chromogen was added per milliliter of Innovex substrate, then tissue was incubated for 5 minutes. A counterstain was then applied with Innovex aqueous hematoxylin for 10-15 seconds. Slides were then rinsed in tap water followed by distilled water. After dehydrating the tissue via 95% ethanol, 100% ethanol, and xylene, slides were cover slipped with Vectamount medium. Both a negative and positive control were utilized for validation of TNC staining protocol. A negative control was utilized to identify non-specific staining by staining duplicate myocardial samples at the same time as cases and controls, without primary antibody. A positive control of equine lung tissue, known to express TNC, was utilized to confirm success of the staining.

Microscopic Imaging & Quantification

Slides were imaged by a Nikon DS-Fi2 microscope with NIS imaging software utilizing automated tiling (Supplemental 2). Prior to the beginning of analysis, slide samples were all arbitrarily named to reduce selection bias. For MTRI stained slides, a pathologist trained in identifying cardiac structures (SK) reviewed images and outlined areas to investigate, as well as which areas on each slide to exclude (vessels, anchoring, valves, endocardium, epicardium). Fiji (ImageJ) was used to select regions of interest (ROI) for quantification in the masked samples[26]. Slide images were stitched together via MATLAB 2020a (MathWorks, Inc., Natick, MA, USA) using phase correlation as previously described[27]. For fibrosis quantification, the images were demultiplexed[28] to split the RGB images into the respective histological stains. From the fibrosis channel, mean intensity and the percentage of the fibrosis area were calculated. Mean intensity was defined as the sum of fibrosis signal divided by the number of positive pixels in the ROI. Percentage of fibrosis area was defined as the number of threshold fibrosis pixels divided by the number of the pixels in the ROI. Only heart failure cases and controls had fibrosis quantified.

Statistical Analysis

Statistical analysis was be performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data was assessed for normality by a Shapiro-Wilk test. Normally distributed data is reported as mean +/- standard deviation and nonparametric data as median and interquartile range (IQR). Data were compared using a paired T-test or Wilcox rank-sum test as appropriate. Statistical significance was defined as $P < 0.05$.

Results

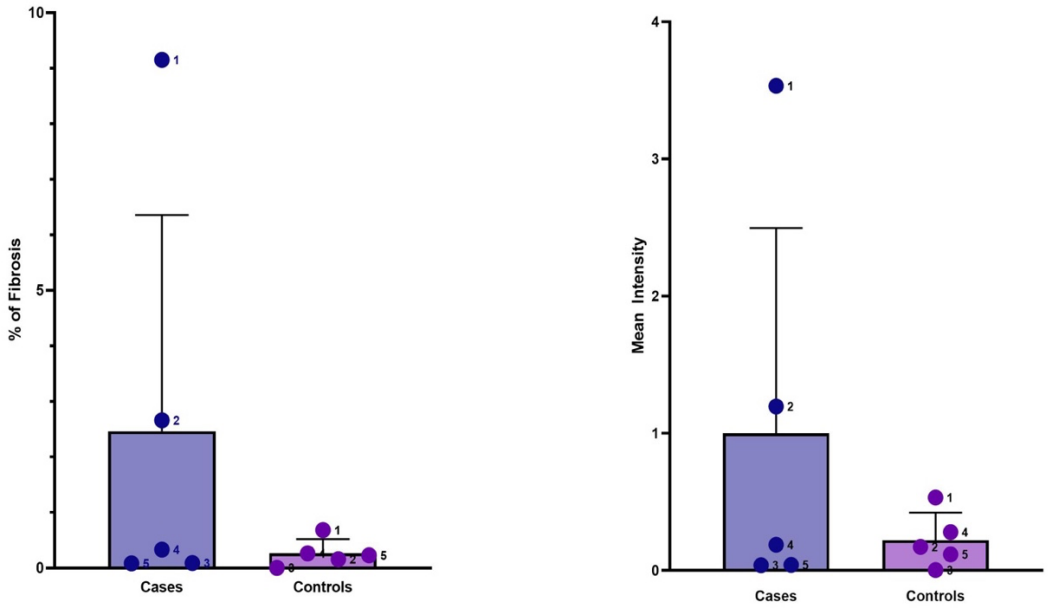
Fibrosis Quantification

Percent fibrosis in heart failure cases ranged from 0.09 to 9.15% with a median of 0.33% (IQR 0.09-5.91). Percent fibrosis in heart failure controls ranged from 0.005 to 0.68% with a mean of 0.27% (SD±0.25) (Figure 1a). Mean intensity, the mean intensity across pixels, of fibrosis in heart failure cases

ranged from 0.038 to 3.53% with a median of 0.19% (IQR 0.040-2.37). Mean intensity of fibrosis in heart failure controls ranged from 0.0028 to 0.53% with a mean of 0.22% (SD±0.20) (Figure 1b, Table 1). There was a non-significant trend for a higher percent fibrosis and higher mean intensity in heart failure cases (p=0.13) (Figure 2). On gross observation, racehorse cases do not appear to have an increased amount of fibrosis when compared with controls (Figure 3) but have not been objectively quantified.

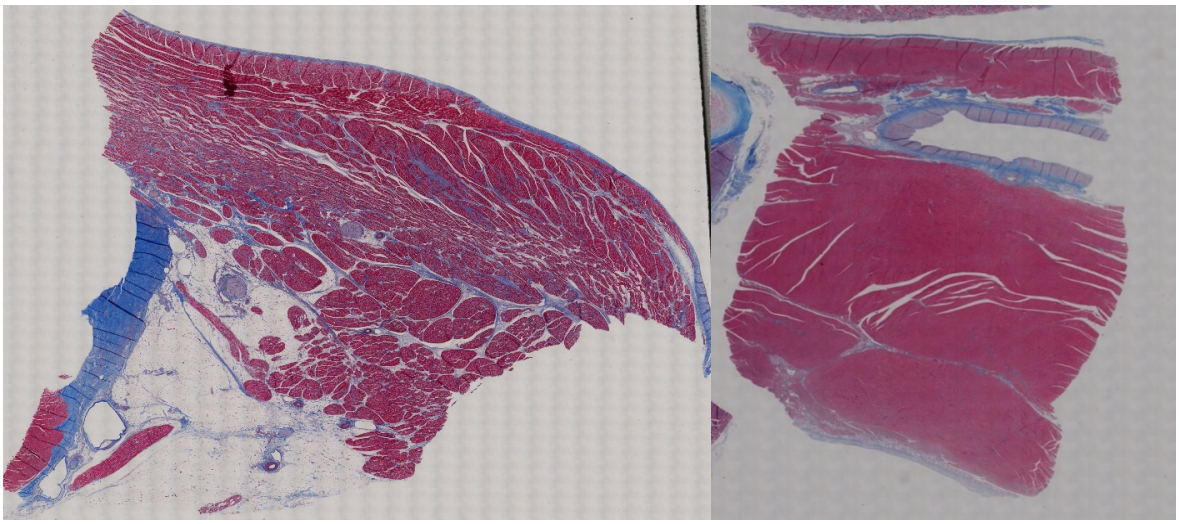
Table 3. Percent fibrosis and mean intensity (MINT) of fibrosis for heart failure cases and controls.

HF Cases (n=5)			**HF Controls (n=5)**		
Horse	**% Fibrosis**	**MINT**	**Horse**	**% Fibrosis**	**MINT**
1	9.150	3.533	1	0.684	0.532
2	2.659	1.196	2	0.157	0.173
3	0.089	0.038	3	0.006	0.003
4	0.332	0.188	4	0.265	0.280
5	0.086	0.041	5	0.227	0.118



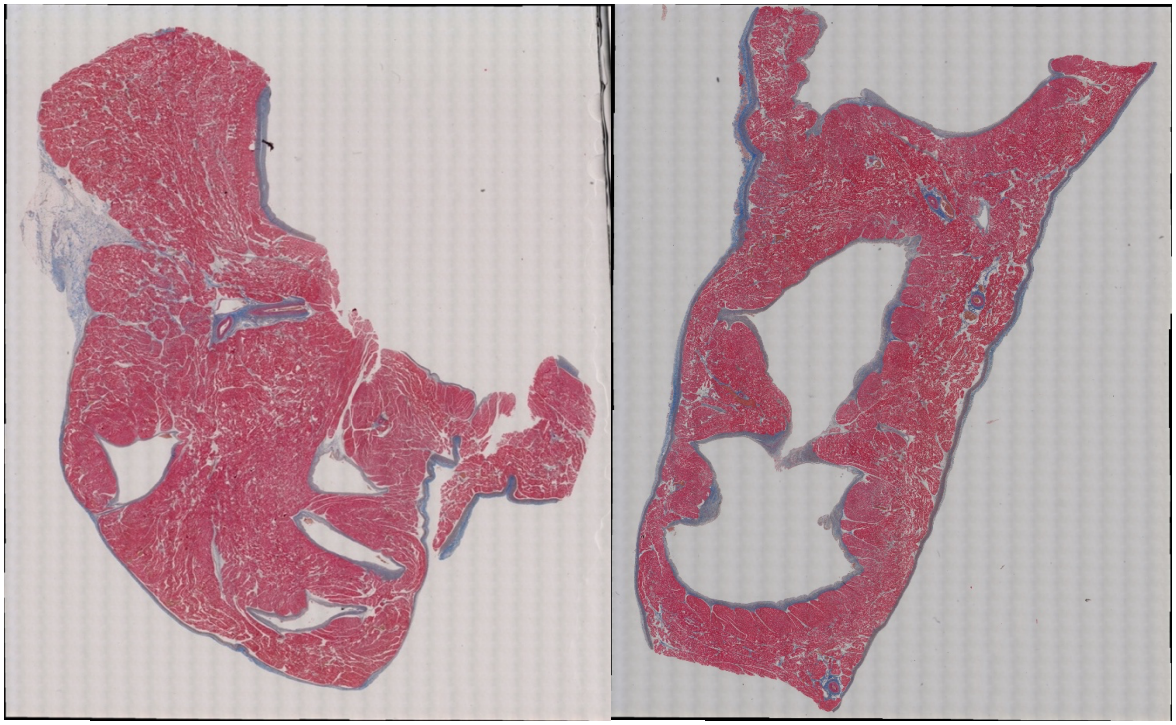
(a) (b)

Figure 1. a) Percent fibrosis in heart failure cases (n=5) and controls (n=5) b) Mean intensity in heart failure cases and controls.



(a) (b)

Figure 2. Representative images from mason trichrome stained cardiac tissue including a heart failure case (a) and control (b). Images were acquired at 10X and stitched together to generate these composites.



(a) (b)

Figure 3. Representative images from mason trichrome stained cardiac tissue taken from the auricle of a racehorse sudden death case and control. Images were acquired at 10X and stitched together to generate these composites.

TNC Expression

TNC expression and localization was comparable in both heart failure cases and sudden death cases when compared with respective controls. Expression was present in the interstitial space, with uptake apparent surrounding vessels and purkinjie fibers as well as on the peripheral edges of tissue sections. Expression in cardiac tissue was decreased compared to expression in the lung tissue used as a positive control, as expected.

Discussion

This study investigated the potential utility of the evaluation of fibrosis and tenascin c expression in horses that have experienced fatal cardiac events. There was a non-significant trend for a higher percent fibrosis and higher mean intensity in heart failure cases when compared with controls; consistent with previous reports in the literature[17]. Upon histological examination, there did not appear to be a marked increase of TNC expression in heart failure or sudden death cases when compared with controls. This partially supports our hypothesis that cardiac disease will be associated with increased myocardial fibrosis. However, it does not support that cardiac disease will be associated with increased expression of matricellular protein tenascin C.

Following myocardial injury, due to disease or exercise-remodeling, the cardiac remodeling process can increase risk for arrhythmogenesis as well as affect the mechanical function of the heart due to changes in structure and contractility[15,29]. In one case study in horses, myocardial fibrosis has been reported to induce sudden death as well as sustained ventricular tachycardia[30]. This is consistent with the trend toward increasing fibrosis observed in our heart failure population. In a study investigating sudden cardiac death in racehorses, increased mean fibrosis was seen in the sudden cardiac death group when compared with controls that died of non-cardiac related reasons[17]. In another prior study investigating 5 racehorses who died of sudden cardiac death, myocardial fibrosis was found in the right atrium close to the sinoatrial (SA) node as well as in the upper portion of the interventricular septum, including atrioventricular (AV) conduction system[31]. In our heart failure population, there were varying types of heart failure, which could have affected our results as the etiology of each condition varies. There were no apparent trends associated with specific types of heart failure. Gross observation of racehorse cases and controls revealed no apparent increase of fibrosis in cases. These results were contradicted in the 2019 study that investigated fibrosis present in Thoroughbred racehorses who died of sudden cardiac death[17]. In this study, fibrosis was assessed from the left and right ventricular free wall and left ventricular papillary muscle rather than the auricle, which may have influenced the results. The summation of this evidence

validates the need for continued investigation on the relationship between fibrosis, arrhythmogenesis, and cardiac disease in horses.

To our knowledge, there are no previous reports of TNC expression evaluated in the myocardium of the horse. On gross observation, heart failure and sudden cardiac death cases did not appear to have an increased expression of TNC when compared with controls. Expression was apparent in the interstitial space, with expression localized to surrounding vessels and on the periphery of the tissue sections. In humans, it has been reported to be expressed in diseased hearts[18]. TNC is rarely detected in the healthy adult heart, but is re-expressed under pathological conditions associated with inflammation, such as myocardial infarction, hypertensive cardiac fibrosis, myocarditis, dilated cardiomyopathy, and Kawasaki disease[32]. A previous study in humans indicated that there was no detectable positive immunostaining for TNC in healthy cardiac tissue[33]. In diseased hearts in humans and rats, intense positive staining for TNC has been observed in areas of inflammation[32,33]. This varied from our results, where mild TNC expression appeared in both cases and controls. Although our results were inconclusive, the continued investigation of expression of TNC in horses that have died of cardiac disease is warranted. Quantitative analysis of TNC expression in equine myocardium, as has been previously performed in human myocardial samples, to determine the ratio of TNC positive area to the whole myocardium would be beneficial to reduce subjectivity and allow for statical analysis[33].

This study had several limitations. The largest limitation for both horses in heart failure and racehorse populations was a limited sample size and the lack of consistent sampling technique in heart failure population due to the retrospective nature of its collection. A larger more uniform population is needed to determine if these preliminary findings are valid.

In conclusion, this demonstrates successful staining and imagining protocols for equine tissue and provides preliminary data for the investigation of cardiac remodeling in the horse. Understanding the pathogenesis of cardiovascular disease is the first step in developing preventative strategies. Future directions for this work involve the continued investigation of cardiac remodeling in a larger population of horses that have experienced fatal cardiac disease. A deeper understanding may eventually lead to a

decrease in the incidence of cardiovascular disease in horses. This could improve the welfare of these horses by allowing for the development of targeted screening protocols and potentially reducing the incidence of death, while also reducing the risk of injury for jockeys or handlers.

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Supplemental Materials

Supplemental 1. Immunohistochemistry (IHC) Tenascin-C Protocol

Day 1

1. Heat slides at 65C, 5-10 minutes
2. Deparaffinize sections in xylene, 2x, 5 minutes each
3. Hydrate with 100% ethanol, 2x, 3 minutes each
4. Hydrate with 95% ethanol, 1 minute
5. Rinse quickly in distilled water 3x using cassettes, leaving in final cassette for 5 minutes
6. Place in sodium citrate buffer at 65C for antigen retrieval, 30 minutes
7. Rinse quickly in distilled water 3x using cassettes, leaving in final cassette for 5 minutes
8. Place in 3% H2O2/Methanol (1:10 dilution, 2mL H2O2: 28mL methanol) for endogenous peroxidase blocking, 10 minutes
9. Rinse quickly in distilled water 3x using cassettes, leaving in final cassette for 5 minutes
10. Use pap pen around tissue sections
11. Place slides incubation tray with water
12. Block with background buster in Universal IHC Kit, 30 minutes
13. Rinse quickly in PBS 3x using cassettes, leaving in final cassette for 5 minutes
14. Rinse quickly in PBST 3x using cassettes, leaving in final cassette for 5 minutes
15. Place slides back in incubation tray with water
16. Add primary antibody overnight at room temperature (5% BSA/PBST), 1:50 antibody dilution

Day 2

17. Rinse quickly in PBS 3x using cassettes, leaving in final cassette for 5 minutes
18. Add solution #1, multivalent linking antibody, 10 minutes
19. Rinse quickly in PBS 3x using cassettes, leaving in final cassette for 1 minute
20. Add solution #2, peroxidase, 10 minutes
21. Per 1mL of Innovex substrate, add 100uL of DAB chromagen, 90 seconds

22. Rinse quickly in PBS 3x using cassettes, leaving in final cassette for 5 minutes
23. Counterstain with Innovex aqueous hematoxylin
24. Rinse quickly in tap water 1x, then in distilled water, 5 minutes
25. Dehydrate with 95% ethanol, 2 minutes
26. Dehydrate with 100% ethanol, 2x, 3 minutes each
27. Coverslip with Vectamount medium, nonaqueous

Supplemental 2. Imaging Protocol

1. Turn on camera, microscope, and computer
    a. Computer is to the right – turn on first
    b. Microscope Power switch is on the right of microscope
    c. Nikon box (camera) is to the left of the microscope,
        i. Make sure the slide above the objectives is forward to the Nikon setting
    d. Turn on ASI box (Poser switch in back)
3. Open NIS Elements on the computer
    a. Select Morgan Lab and use password JM2328
    b. Select Nikon DS-U3 in NIS Elements and click OK
4. Lower condenser and move to lowest objective so they do not get hit when the stage moves
5. Set the condenser to Phase A and open aperture about half way (usually already done)
    a. Aperture is on the front of the condenser
    b. Dial for light is on the left
6. On NIS, hit “Devices” --> “Initiate Stage” --> “Don’t Escape & Initiate” (It will start moving)
7. Place Slide on Stage
8. Perform Kohler illumination
    a. Raise condenser with black knob to the left and back of scope
    b. Close down the aperture all the way

c. Adjust the condenser until the hexagon is crisply outlined

d. Open the aperture to provide even lighting

9. Hit the “Play” button on the computer screen (green triangle)
10. Choose auto exposure
11. Focus on tissue on the objective that will be used for imaging
12. Auto white balance:
    a. Move to blank/white portion of the slide
    b. “Acquire” --> “Shading Correction” --> “Capture Correction Image”
        i. Select “Multiplicative”, take 3 images from blank parts of the slide, moving to a new blank location every time you take an image, click “Next” between 1 and 2 and 2 and 3, and then click “Finish”
13. Click the “Play” button for a live image, make sure shading correction looks correct
14. Image acquisition:
    a. “Acquire” --> “Scan Large Image”
    b. Select correct objective (Drop down in top left corner)
    c. Set boundaries of tissue with 4 arrows
    d. Select focusing preferences (Every 1 field - on right side, or z-stack to account for changes in elevation on tissue to run automatically without manual focus)
15. After image has been acquired, save to “Sudden Death” folder and then export onto external drive