CARDIAC MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL CHANGES INDUCED BY SUSTAINED, HIGH-INTENSITY ENDURANCE TRAINING IN LARGE ANIMAL EXPERIMENTAL MODELS

Ph.D. thesis

Alexandra Júlia Polyák, MD

Szeged, Hungary

2023

CARDIAC MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL CHANGES INDUCED BY SUSTAINED, HIGH-INTENSITY ENDURANCE TRAINING IN LARGE ANIMAL EXPERIMENTAL MODELS

Ph.D. thesis

Alexandra Júlia Polyák, MD

Supervisor: Attila Farkas, MD, Ph.D.

Doctoral School of Multidisciplinary Medical Sciences

Department of Medicine &

Department of Pharmacology and Pharmacotherapy

Albert Szent-Györgyi Medical School,
University of Szeged

Szeged, Hungary

2023

TABLE OF CONTENTS

LIST OF PUBLICATIONS i
ACRONYMS AND ABBREVIATIONS iv
ABSTRACT .... - 1 -
1. INTRODUCTION .... - 2 -
1.1 Balancing cardiovascular benefits and risks in sport: the U-shaped relationship .... - 2 -
1.2 Structural and electrical heart adaptations in elite athletes .... - 2 -
1.3 Sudden cardiac death in athletes: a multifactorial perspective .... - 4 -
1.4 Enhancing translational research: non-rodent animal models for investigating cardiac adaptations to high-intensity exercise .... - 8 -
2. AIMS OF THE STUDY .... - 9 -
3. MATERIALS AND METHODS .... - 10 -
3.1 General methods .... - 10 -
3.2 Experimental set-up and training protocols .... - 10 -
3.3 Echocardiography measurements .... - 12 -
3.4 Electrocardiography measurements .... - 13 -
3.5 Atropine and heart rate response in canines *in vivo* .... - 14 -
3.6 Dofetilide and QT interval prolongation challenge in canines *in vivo* .... - 14 -
3.7 Open-chest arrhythmia provocation in anaesthetised canines .... - 14 -
3.8 Morphometry and histology .... - 15 -
3.9 Conventional microelectrode techniques in canine hearts .... - 15 -
3.10 Patch-clamp measurements in canine hearts .... - 16 -
3.10.1 Measurement of L-type calcium current .... - 16 -
3.10.2 Measurement of potassium currents .... - 17 -
3.10.3 Measurement of late sodium current .... - 17 -
3.10.4 Measurement of NCX current .... - 17 -
3.10.5 Measurements of single cell action potentials .... - 17 -
3.11 Western blot analysis of KChIP2 and Kv4.3 proteins in canine hearts .... - 18 -
3.12 Immunocytochemistry of KChIP2, Kv4.3, and HCN1, HCN2, and HCN4 proteins in canine hearts .... - 18 -
3.13 Gene expression of fibrosis markers in rabbit hearts using real-time qRT-PCR .... - 19 -
4. STATISTICS .... - 20 -
5. RESULTS .... - 21 -
5.1 Sustained exercise-induced cardiac hypertrophy and fibrosis .... - 21 -

5.2 The effect of training on heart rate and its variability in conscious canines and rabbits, and on spontaneous beating rate in isolated canine right atrial tissue preparations .... - 24 -

5.3 Heart rate changes in heavily trained canines under atropine challenge .... - 26 -

5.4 ECG changes and increased proarrhythmic response following long-term sustained training .... - 26 -

5.5 Repolarization sensitivity to the proarrhythmic agent dofetilide in conscious canines- 28 -

5.6 Influence of prolonged training on cardiac APD and its short-term variability in left ventricular preparations of heavily trained sedentary and trained canines...................... - 29 -

5.7 Effects of chronic sustained training on various transmembrane ionic currents in canine left ventricular myocytes .... - 30 -

5.8 The relative density of transmembrane Kv4.3 and KChiP2 proteins in trained and sedentary canine hearts .... - 31 -

5.9 HCN4 channel upregulation in left ventricles after sustained training .... - 32 -

5.10 Gene expression of fibrosis biomarkers in rabbit hearts after 16 weeks of training - 32 -

5.11 Testosterone levels in canines .... - 34 -

6. DISCUSSION .... - 35 -

6.1 Animal models of the athlete's heart: Insights from rabbits and canines .... - 36 -

6.2 Cardiac morphological remodelling in response to chronic exercise .... - 39 -

6.3 The complex mechanism of bradycardia .... - 40 -

6.4 Mechanistic basis of arrhythmias in the athlete's heart model .... - 41 -

6.5 Impacts of exercise on ventricular repolarization in the canine and the rabbit athlete's heart models .... - 44 -

6.6 Steroid-induced cardiovascular risk among endurance athletes .... - 47 -

7. CONCLUSION .... - 49 -

8. LIMITATION .... - 50 -

9. ACKNOWLEDGEMENT vi

10. FUNDING vii

REFERENCES viii

LIST OF PUBLICATIONS

The publications related to the subject of the Ph.D. thesis:

I.) **A. Polyák,** L. Topal, N. Zombori-Tóth, N. Tóth, J. Prorok, Zs. Kohajda, Sz. Déri, V. Demeter-Haludka, P. Hegyi, V. Venglovecz, G. Ágoston, Z. Husti, P. Gazdag, J. Szlovák, T. Árpádffy-Lovas, M. Naveed, A. Sarusi, N. Jost, L. Virág, N. Nagy, I. Baczkó, A. S. Farkas, A. Varró. Cardiac electrophysiological remodelling associated with enhanced arrhythmia susceptibility in a canine model of elite exercise.
Elife, 2023, doi: 10.7554/eLife.80710. (**IF:** 8.14, **D1**)

II.) Péter Kui*, **Alexandra Polyák*,** Nikolett Morvay, László Tiszlavicz, Norbert Nagy, Balázs Ördög, Hedvig Takács, István Leprán, András Farkas, Julius Gy. Papp, Norbert Jost, András Varró, István Baczkó and Attila S. Farkas. Long-term endurance exercise training alters repolarization in a new rabbit athlete's heart model.
Frontiers in Physiology, 2022, doi: 10.3389/fphys.2021.741317. (**IF:** 4.566, **Q2**)

III.) **Alexandra Polyák,** Péter Kui, Nikolett Morvay, István Leprán, Gergely Ágoston, Albert Varga, Norbert Nagy, István Baczkó, András Farkas, Julius Gy. Papp, András Varró and Attila S. Farkas. Long-term endurance training-induced cardiac adaptation in new rabbit and dog animal models of the human athlete's heart.
Reviews in Cardiovascular Medicine, 2018, doi: 10.31083/j.rcm.2018.04.4161. (**IF:** 2.93, **Q3**)

IV.) **Polyák Alexandra,** Kui Péter, Morvay Nikolett, Leprán István, Ágoston Gergely, Varga Albert, Baczkó István, Farkas András, Papp Gyula, Varró András, Farkas Attila. Hosszú időtartamú állóképességi tréning kardiovaszkuláris hatásainak vizsgálata nyúlban és kutyában. *Cardiologia Hungarica, 2017,*
doi: http://doi.org/10.26430/CHUNGARICA.2017.47.suG.40

Impact factor of publications related to the subject of the Ph.D. thesis: 15.636

Other publications published under Ph.D. scholarship:

V.) L. Topal*, **A. Polyák***, N. Tóth, G. Ágoston, P. Bencsik, Zs. Kohajda, J. Prorok, Sz. Déri, N. Nagy, N. Jost, L. Virág, A.S. Farkas, A. Varró, and I. Baczkó. Endurance training-induced cardiac remodeling in a guinea pig athlete's heart model.
Can. J. Physiol. Pharmacol., 2022, doi: dx.doi.org/10.1139/cjpp-2022-0073. (**IF:** 2.273, **Q2**)

VI.) Arie O. Verkerk, Illés J. Doszpod, Isabella Mengarelli, Tibor Magyar, **Alexandra Polyák,** Bence Pászti, Igor R. Efimov, Ronald Wilders, and István Koncz. Acetylcholine Reduces L-Type Calcium Current without Major Changes in Repolarization of Canine and Human Purkinje and Ventricular Tissue.
Biomedicines, 2022, doi: https://doi.org/10.3390/biomedicines10112987. (**IF:** 5.612, **Q2**)

VII.) Noémi Tóth, Alexandra Soós, Alex Váradi, Péter Hegyi, Benedek Tinusz, Anna Vágvölgyi, Andrea Orosz, Margit Solymár, **Alexandra Polyák,** András Varró, Attila S. Farkas, Norbert Nagy. Effect of ivabradine in heart failure: a meta-analysis of heart failure patients with reduced versus preserved ejection fraction. *Canadian Journal of Physiology and Pharmacology 2021,* doi: 10.1139/cjpp-2020-0700. (**IF:** 2.273, **Q3**)

VIII.) Péter Gazdag, Kinga Oravecz, Károly Acsai, Vivien Demeter-Haludka, Balázs Ördög, Jozefna Szlovák, Zsófa Kohajda, **Alexandra Polyák,** Bálint András Barta, Attila Oláh, Tamás Radovits, Béla Merkely, Julius Gy. Papp, István Baczkó, András Varró, Norbert Nagy, and János Prorok. Increased Ca2+ content of the sarcoplasmic reticulum provides arrhythmogenic trigger source in swimming-induced rat athlete's heart model.
Scientific Reports, 2020. doi: https://doi.org/10.1038/s41598-020-76496-2. (IF: 4.379, **D1**)

IX.) Péter Orvos, Bence Pászti, Leila Topal , Péter Gazdag, János Prorok, **Alexandra Polyák,** Tivadar Kiss, Edit Tóth-Molnár, Boglárka Csupor-Löffler, Ákos Bajtel, András Varró, Judit Hohmann, László Virág, and Dezső Csupor. The electrophysiological efect of cannabidiol on hERG current and in guinea-pig and rabbit cardiac preparations.

Scientific Reports, 2020. doi: https://doi.org/10.1038/s41598-020-73165-2. (**IF:** 4.379, **D1**)

X.) A. Regev, H. Takacs, A.S. Farkas, F. Rarosi, **A. Polyak,** H. Papp, E. Ivany, J.G. Papp , A. Varro, A. Farkas. Application of ventricular tachyarrhythmia definitions of the updated lambeth conventions provides incompatibility with earlier results, masks antifibrillatory activity and reduces inter-observer agreement.
Journal of Physiology and Pharmacology, 2019, doi: 10.26402/jpp.2019.1.03. (**IF:** 3.011, **Q2**)

XI.) Papp Henriett, Sarusi Annamária, Farkas Attila, **Polyák Alexandra**, Papp Gyula, Varró András, Farkas András. Repolarizációs tartalékszűkítésen alapuló új proaritmia-modell izolált tengerimalac-szívben. *Cardiologia Hungarica, 2017,* doi: http://doi.org/10.26430/CHUNGARICA.2017.47.suG.15

Number of full publications: 11

Cumulative impact factor: 37.5

ACRONYMS AND ABBREVIATIONS

AAS: anabolic-androgenic steroid

ACTB: β-actin

AF: atrial fibrillation

ANS: autonomous nervous system

APD_{90}: action potential duration measured at 90 % repolarization

BSA: body surface area

BW: body weight

CAD: coronary artery disease

cDNA: complementary deoxyribonucleic acid

COL1A1 and COL3A1: pro-alpha1 chain of type I collagen and type III collagen

DMSO: dimethyl sulfoxide

DOP: doping

ECG: electrocardiogram

ECHO: echocardiogram

ECM: extracellular matrix

EF: ejection fraction

ERP: effective refractory period

ES: extrasystole

ESV and EDV: end-systolic and diastolic volumes

EX: exercised

FN-1: fibronectin-1

FS: fractional shortening

HCN: hyperpolarization-activated cyclic nucleotide-gated potassium channel

HRV: heart rate variability

I_{CaL}: L-type calcium current

I_{f}: hyperpolarization-activated "funny" current

I_{K1}: inward rectifier potassium current

I_{Kr}: rapid delayed rectifier potassium current

I_{Ks}: slow delayed rectifier potassium current

I_{NaL}: late sodium current

I_{NCX}: sodium-calcium exchanger current

I_{to}: transient outward potassium current

IVS: interventricular septal thickness

LAV and LAV_i: left atrial volume and left atrial volume index

LQTS: long QT syndrome

LVESD and LVEDD: left ventricular end-systolic and diastolic diameters

LVM and LVM_i: left ventricular mass and left ventricular mass index

LVPW: thickness of the left ventricular posterior wall

MMP-2: matrix metalloproteinase-2

mRNA: messenger ribonucleic acid

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PKA and PKC: protein kinase A and protein kinase C

PVDF: polyvinylidene difluoride

QT_c: heart rate-corrected QT interval

RMS: root mean square

rmsSD: root mean square of successive differences

RNA: ribonucleic acid

RPS5: ribosomal protein S5

SAN: sinoatrial node

SCD: sudden cardiac death

sdSD: standard deviation of the successive differences

SED: sedentary

SRP14: signal recognition-particle assembly 14

STV: short-term variability

TdP: Torsades de Pointes

TGF-β: transforming growth factor-β1

TIMP-1: tissue inhibitor of metalloproteinase-1

TRN: trained

VF: ventricular fibrillation

VPB: ventricular premature beat

VT: ventricular tachycardia

VVI: ventricular pacing and sensing

ABSTRACT

Despite the well-known benefits of regular exercise, there is a growing body of evidence suggesting that elite athletes who engage in intense training beyond a certain threshold are more susceptible to harmful ventricular cardiac arrhythmias, and in some cases, even sudden cardiac death, although the mechanisms behind this remain unclear. To gain a better understanding of cardiac remodelling in response to chronic vigorous exercise, this study explores the effects of such exercise on cardiac structure and electrophysiology in new rabbit and dog models of the athlete's heart.

In the first experiments, rabbits and dogs were divided into sedentary (SED), exercised (EX) subjected to 16 weeks of chronic treadmill exercise, and a testosterone-treated group in dogs (DOP). In the second experiments, a more intense training protocol for dogs was introduced, and changes between trained (TRN) dogs and their sedentary (SED) counterparts were studied.

Various tests, including echocardiography and electrocardiograms, were conducted, along with assessing proarrhythmic sensitivity and autonomic responses in conscious dogs. In vitro studies, such as electrophysiological measurements, immunocytochemistry, and histopathological analysis, were carried out after heart removal.

Results showed that EX animals, both rabbits and dogs, displayed left ventricular enlargement and bradycardia, indicating an increased vagal tone. EX and DOP dogs showed a lower response to the parasympatholytic agent atropine and more pronounced QT_c interval lengthening after dofetilide challenge compared to the SED group. No significant morphological or functional changes were observed in dogs treated with steroids.

In conscious trained dogs, ECG recordings indicated bradycardia, prolonged QT_c intervals, and increased QT interval variability, reflecting elevated repolarization dispersion. At the cellular level, prolonged action potential duration and reduced magnitude of the transient outward potassium current were observed in the left ventricular myocytes of trained dogs. Left ventricular fibrosis and increased HCN4 protein expression were also noted.

Our findings provide *in vivo*, cellular electrophysiological, and molecular biological evidence for the enhanced susceptibility to ventricular arrhythmias in the large animal model of chronic exercise. The sustained 4-month training regimen resulted in echocardiographic changes that are consistent with the morphology of the hearts of endurance-trained human athletes. These animal models hold promise for further investigations into the cardiovascular effects of competitive training.

1. INTRODUCTION

1.1 Balancing cardiovascular benefits and risks in sport: the U-shaped relationship

Physical activity provides substantial cardiovascular benefits, improving both quality of life and longevity, while physical inactivity significantly contributes to cardiovascular morbidity and mortality [1, 2]. Regular exercise yields a range of advantages, including improved cardiovascular function, enhanced mental well-being, and decreased susceptibility to chronic diseases, thereby promoting both physiological and psychological well-being.

Nonetheless it is essential to acknowledge the existence of an optimal exercise intensity level. Exceeding this threshold can result in diminishing or adverse health outcomes. Recent insights, emerged from an extensive meta-analysis involving eight prospective cohort studies, indicate that the most significant risk reductions for premature mortality associated with moderate to vigorous physical activity were attained with a daily commitment of 24 minutes, equivalent to a total of 168 minutes per week [3]. These findings closely align with the current global recommendations, which advocate that adults should engage in 150 to 300 minutes of physical activity per week [4]. The Guidelines Development Group has recently affirmed that participating in 150 to 300 minutes of moderate-intensity aerobic physical activity per week reduces the risk of various health issues. However, the risk reduction levels off as one exceeds the 300-minute per week threshold [4]

The U-shaped model that represents the relationship between exercise intensity and the risk of adverse cardiovascular events emphasizes that while moderate and regular exercise confers numerous health benefits, extreme exercise intensity and duration may lead to detrimental health consequences **(Figure 3)** [5]. In the context of competitive sports, additional considerations arise, especially for athletes with underlying cardiovascular conditions that compromise repolarization reserve. These conditions encompass hypertrophic cardiomyopathy, long QT syndrome, diabetes, electrolyte imbalances, doping, or seemingly innocuous presumed medications and often remain silent, with the first clinical sign being sudden cardiac death. Therefore, a nuanced approach is essential to balance the cardiovascular advantages of exercise with potential risks, especially for competitive athletes.

1.2 Structural and electrical heart adaptations in elite athletes

Elite athletes, admired for their remarkable physical abilities, undergo cardiac morphological and functional adaptations known as the “athlete's heart” phenomenon due to intense physical training [6]. These adaptations include increased left ventricular chamber size,

wall thickness, cardiac mass, and stroke volume, optimizing cardiac performance, especially during demanding activities like intense exercise.

It is worth noting that the type and frequency of athletic activities influence these cardiac changes [7], categorized athletic activities into “isometric” and “isotonic” types based on the presumed hemodynamic stresses associated with each type of activity. In “isotonic” or “high dynamic exercise” activities like long-distance running, cycling, and swimming, the alterations in left ventricular (LV) architecture could be considered a form of eccentric remodelling, characterized by increases in both chamber size and LV mass. This is similar to those observed in chronic volume overloads [8]. D’Andrea *et al.* also reported significantly greater LV end-diastolic diameter in endurance-trained athletes compared to strength-trained athletes and control participants [9].

However, recent researches [10, 11] have revealed a more complex scenario on the geometric patterns observed in the athlete's heart. Endurance-trained runners, traditionally thought to develop purely eccentric left ventricular hypertrophy, exhibited a more significant increase in wall thickness than expected. In contrast, strength-trained athletes, including weightlifters, throwers, and bodybuilders, typically associated with developing purely concentric left ventricular hypertrophy, exhibited augmented absolute and relative wall thickness alongside a significant increase in left ventricular diameter. These findings align with Spirito *et al.'s* extensive study involving 947 elite athletes across 27 sports, focusing on athlete's heart morphology [12]. This expanding body of research highlights the complex myocardial adaptations in athletes and underscores the need for further investigation to achieve a comprehensive understanding of cardiac morphology in various sports and its differentiation from pathological conditions.

Elite athletes often exhibit distinctive cardiac electrical adaptations, characterized by a reduced resting heart rate and increased heart rate variability **(Figure 1)**. These adaptations are primarily attributed to enhanced autonomic nervous system control over the heart and are considered indicators of cardiovascular fitness and training-related myocardial remodelling [13]. Heart rate variability (HRV) reflects the balance between sympathetic and parasympathetic influences on the heart. In well-trained athletes, HRV tends to be higher, indicating superior autonomic nervous system control over the heart [14]. This increased HRV enhances cardiovascular performance during both rest and exercise by promoting efficient energy utilization and overall cardiovascular health. Investigating HRV is an important indicator of workout efficiency and post-workout recovery.

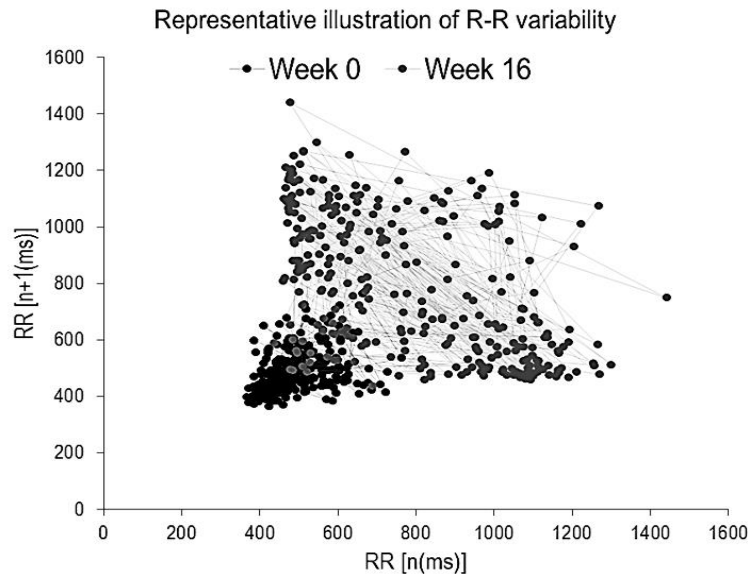

Figure 1. A Poincaré plot, provided to facilitate a deeper understanding of the marked increase in RR interval variability. This dataset was collected from the same trained dog both before and after a 16-week training regimen.

However, a recent study by Boyett et al. suggested that training-induced bradycardia in rats may be due to reduced expression of the "hyperpolarization-activated cyclic nucleotide-gated potassium channel 4" (HCN4) gene, specifically the downregulation of channels conducting the cardiac pacemaker or "funny" (I_f) current, rather than changes in cardiac autonomic regulation [15]. This finding challenges the conventional understanding of bradycardia in elite athletes. Nevertheless, translating these findings from rats to humans requires further research to confirm their relevance to human athletes.

1.3 Sudden cardiac death in athletes: a multifactorial perspective

Sudden cardiac death (SCD) among athletes is defined as an abrupt, unexpected, non-traumatic event occurring during or shortly after sports activities. The leading causes of SCD are predominantly underlying cardiac issues, such as arrhythmias or structural heart abnormalities [16].

The timeframe for classifying a death as SCD among athletes can vary, with some definitions focusing on events during or shortly after exertion (within an hour), while others encompass any SCD event involving an athlete, whether during physical activity or at rest. Additionally, some definitions include cases of successfully resuscitated sudden cardiac arrest in athletes [16]. These variations in definitions contribute to a wide range of reported incidence rates, ranging from 1 in 3,000 to as low as 1 in 1 million [17]. Although SCD in athletes is relatively rare, its incidence is two to four times higher than in their non-athletic counterparts

[18]. For instance, data from Italy revealed a 2.8-fold greater risk of SCD among elite athletes compared to age-matched non-athletes [19].

Research on National Collegiate Athletic Association athletes has identified specific risk factors for SCD, including male gender and black race. Notably, male Division 1 basketball players face a risk estimated at over ten times that of the overall athlete population. While Marfan syndrome cases leading to aortic dissection are more common in male basketball players, they constitute only a small portion of SCD cases in this athlete subgroup [20], and the underlying reasons for this heightened risk are not fully understood. SCD seems to be more frequent among elite football and basketball players [20, 21] suggesting that individuals engaged in high dynamic and low isometric intensity sports face a higher risk of SCD.

Emerging research indicates that chronic, high-level exercise in elite athletes can trigger cardiac arrhythmias, including atrial fibrillation [22, 23], ventricular fibrillation (VF), or sustained ventricular tachycardia (VT). These arrhythmias disrupt normal cardiac electrical activity, with VF and VT potentially leading to an abrupt cessation of effective cardiac output [24, 25], consequently resulting in SCD [26].

Various structural, electrical, and acquired cardiovascular abnormalities, such as hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy (genetic heart muscle diseases), and atherosclerotic coronary artery disease (CAD), are recognized as contributing factors to SCD in different scenarios. For example, CAD can lead to scar formation in the heart muscle, creating a substrate for the initiation and propagation of malignant arrhythmias in athletes, particularly those over 35 years old [27, 28].

Previously, SCD was attributed to ventricular fibrillation of ischemic origin, even in younger athletes. However, this explanation is questionable because SCD often occurs during warm-up, cool-down, or rest periods, challenging the notion that it primarily results from increased myocardial oxygen demand during peak performance. Therefore, the cause and underlying mechanism of sports-related SCD among young athletes require further exploration. In an astounding 3–6 % of cases, the exact cause of SCD remains elusive [28, 29]. Equally noteworthy, autopsy findings yield negative results [30], suggesting uninvestigated and still unknown factors contributing to these tragic events.

A complex interplay of factors, classically referred to as the "arrhythmic triangle," may render athletes susceptible to life-threatening arrhythmias, such as Torsades de Pointes (TdP), even in individuals with ostensibly healthy hearts following prolonged and intensive training [31, 32]. According to this classical concept, arrhythmias occur when specific combinations of substrates, triggers, and arrhythmia-promoting modulators are present **(Figure 2)**. It important

to note that, regardless of the underlying cause of bradycardia, a reduced heart rate in itself can result in a prolonged action potential duration and increased dispersion of cardiac repolarization. This phenomenon may contribute to an elevated arrhythmic substrate. Additionally, bradycardia, which leads to longer diastolic intervals, may enhance the likelihood of spontaneous diastolic depolarization reaching the firing threshold, potentially acting as an arrhythmia trigger [31]. Athletes have also been reported to exhibit a prolongation of the ECG QT_c interval, bradycardia [33], and increased spatial and temporal dispersion of repolarization. This is evident through the extension of the T_pT_e interval and the short-term variability of the QT interval observed on the ECG [34]. For example, early research findings indicated elevated repolarization parameters in elite soccer players [34]. Furthermore, an increasing number of histopathological studies have explored the presence of fibrosis, a known arrhythmogenic substrate, in the left ventricular muscle of endurance athletes [35-38]. The elevated level of myocardial fibrosis can serve as a potential substrate for arrhythmias by reducing impulse conduction and disrupting impulse propagation. Triggers may originate from the pulmonary veins in atrial fibrillation [39] or Purkinje fibres in ventricular fibrillation [40].

Postulated contributory mechanisms include inherited arrhythmia disorders, such as long QT syndrome (LQTS) and Brugada syndrome [41, 42], electrolyte imbalances, autonomous nervous system (ANS) dysregulation, certain drugs prolonging the QT interval of the ECG [43], and performance-enhancing drugs associated with exercise.

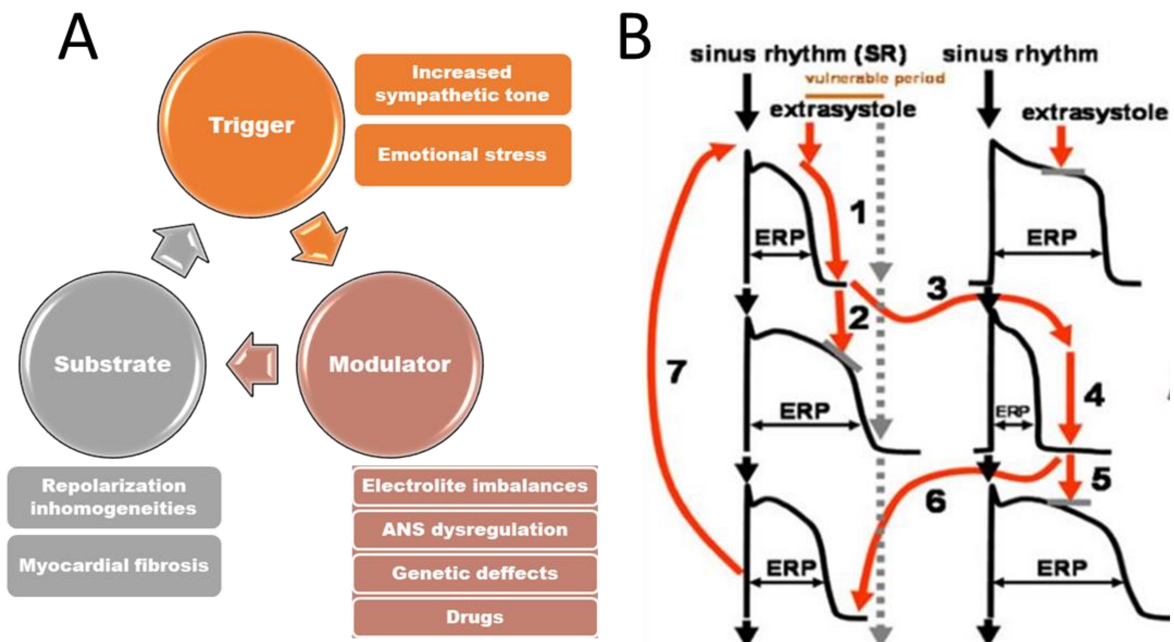

Figure 2. A. **The "triangle of arrhythmogenesis"**, the interaction of trigger-substrate-modulator leading to arrhythmias [31, 44]. **B**. **schematic representation of re-entry in tissue,** showing variations in early and late repolarization due to short or long action potential durations ("substrate"). A premature beat or extrasystole (ES) ("trigger", marked with red arrows) can only propagate through pathways

where cells are not in a refractory state, and their action potentials are in their vulnerable periods (1, 3, 4, and 6). It gets blocked in directions where cell action potentials are in the refractory period (2 and 5). Abnormal impulses can follow re-entry paths created by inhomogeneous repolarization (7), restarting this circuit. The interactions between triggers and substrates are strongly influenced by modulators. ERP, effective refractory period. This figure has been modified from *Varró and Baczkó (2010)* [31] with permission.

These factors may collectively contribute to cardiac repolarization inhomogeneity, potentially leading to life-threatening arrhythmias [31, 45]. While these adaptations may appear insignificant individually, their cumulative effects, along with other factors, may significantly predispose athletes to arrhythmias. This multifactorial understanding **(Figure 3)** of SCD among elite athletes challenges the conventional perception of top athletic hearts as models of health and vitality, emphasizing the need for ongoing research in this field, both at the level of *in vivo* individuals and cellular electrophysiological levels.

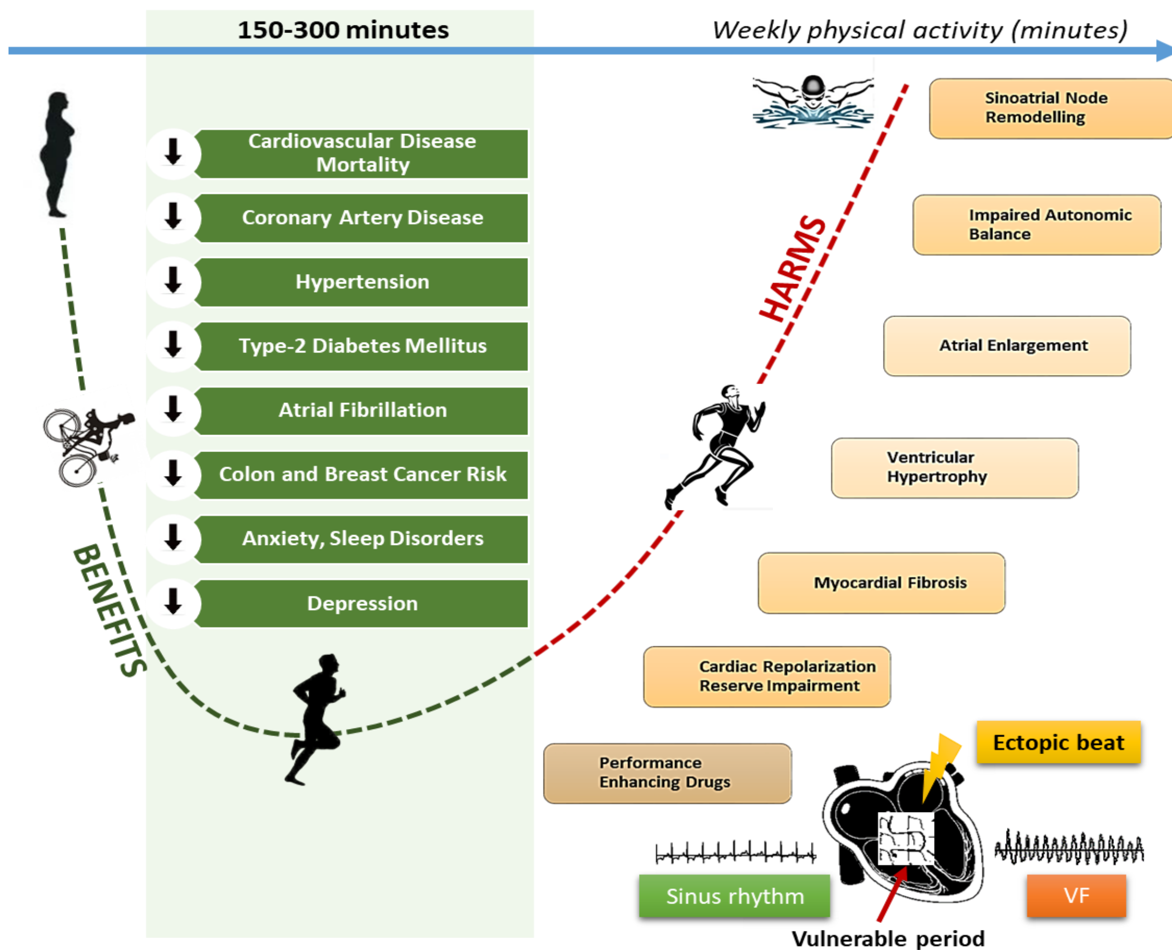

Figure 3. Dose–response relationship between exercise and cardiovascular morbidity. It emphasizes that higher levels of physical activity reduce the risk for multiple health outcomes but start to plateau beyond 300 minutes per week. Vigorous exercise at a competitive level may even be harmful to the cardiovascular system [4, 5]. For example, inhomogeneous repolarization can lead to re-entry pathways, and during a vulnerable period, an extrasystole can trigger ventricular arrhythmias, such as ventricular fibrillation (VF), potentially resulting in sudden cardiac death.

1.4 Enhancing translational research: non-rodent animal models for investigating cardiac adaptations to high-intensity exercise

While there are numerous screening strategies for athletes, early detection of an increased susceptibility to sudden cardiac events in seemingly healthy individuals remains a challenge. Evaluating athletes presents diagnostic complexities, particularly in distinguishing physiological adaptations with associated electrocardiographic and echocardiographic changes characteristic of the "athlete's heart" from cardiac pathologies that could lead to sudden cardiac death. The study of the intricate mechanisms underlying sports-related SCD in humans is a formidable task, often constrained or even impossible. Therefore, there is a compelling need for more fundamental research to assess the physiological adaptations of the cardiovascular system and the regulatory mechanisms exerted by the autonomic nervous system on cardiovascular functions in athletes.

Recent studies have highlighted the electrophysiological remodelling effects of high-intensity exercise in animal models, including atrial fibrillation [22, 46], sinus bradycardia [47], and atrioventricular node dysfunction [48]. Additionally, investigations have revealed fibrotic changes in the hearts of rodents following chronic, high-intensity exercise, accompanied by diastolic dysfunction in both left and right ventricles. These findings were associated with an increased vulnerability to supraventricular arrhythmias [49].

It is essential to note that a significant portion of animal experiments focused on chronic endurance training-induced atrial fibrillation have been carried out in mice or rats. However, these rodent models differ significantly from humans in terms of cardiac function, including heart rate and repolarization properties [22, 46, 47, 49]. To address these differences and improve the translational relevance of our research, we have carefully selected animal non-rodent animal models that closely mimic human cardiac morphological, electrophysiological, and autonomic neural properties. Our rabbit and dog models offer valuable insights into the electrophysiological characteristics of the athlete's heart and the heightened risk of ventricular arrhythmias associated with strenuous exercise. To the best of our knowledge, apart from a recent report on atrioventricular dysfunction in racehorses [48], no experimental studies in larger animals with higher translational relevance have been conducted. Rabbits provide a more cost-effective and experimentally suitable option for investigating cardiovascular adaptations related to exercise, while canines, due to their stronger resemblance to human cardiac electrophysiology, emerge as ideal candidates for exploring the effects of endurance training at levels similar to those experienced by elite athletes.

2. AIMS OF THE STUDY

The present study was designed to develop and characterize animal models that closely mimic human cardiac physiology. Its primary aim is to enhance our understanding of the physiological effects of chronic vigorous exercise on cardiac structure and function within the athlete's heart. Furthermore, special attention was given to identifying potential adverse changes in myocardial structure and function and increased arrhythmia sensitivity in both *in vivo* and *ex vivo* settings.

The primary aims of this study are as follows:

I. To gain a comprehensive understanding of the complex electrophysiological changes resulting from prolonged intense exercise, particularly in non-rodent species that closely resemble the human heart in terms of electrophysiological properties.

II. To investigate potential adverse myocardial morphological and functional alterations induced by sustained intensive exercise training.

III. To assess whether prolonged intense exercise leads to increased susceptibility to arrhythmias in both *in vivo* and *ex vivo* settings.

IV. To evaluate the influence of chronic anabolic steroid treatment on cardiac structure and function in animals subjected to long-term exercise regimens.

3. MATERIALS AND METHODS

3.1 General methods

Animal maintenance and research were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures involving animals were approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (approval numbers: I-74-15-2017 and I-74-24-2017) and by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (authority approval numbers: XIII/3330/2017 and XIII/3331/2017) and conformed to the rules and principles of the 2010/63/EU Directive. The animals were purchased from an experimental animal breeder, Ásotthalom, Hungary (breeder's authority approval number: XXXV/2018) certified by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development, Hungary.

3.2 Experimental set-up and training protocols

Our experiments consisted of two main sets. In the first set, for model setup, we used New Zealand white rabbits aged 11 months, weighing 3.5-4.0 kg, and mongrel dogs aged 12-18 months, weighing 7.0-7.5 kg, of both sexes. The animals were randomly assigned to sedentary groups (SED, rabbits n = 7; dogs n = 2) and exercised (EX rabbits n = 7; dogs n = 2) groups. In the second set of experiments involving dogs, a doping group (DOP, dogs n = 2) was introduced, receiving long-acting anabolic-androgenic steroid (AAS) medication, testosterone undecanoate (Nebido, Bayer AG, Germany), via intramuscular injection at a dosage of 14.3 mg per kg of body weight at four-week intervals. See the protocol in **Figure 4A** and **Figure 4B**.

Running sessions were conducted using a custom treadmill system for rabbits with two separate corridors and a specialized canine treadmill system (Dogrunner K9 Racer Treadmill, Dendermonde, Belgium) with adjustable gradient and speed intensity for dogs. EX and DOP animals underwent a 16-week training session, while the SED group did not participate in training. Before starting the experiments, animals were conditioned to the training protocol, becoming familiar with research personnel and undergoing a few minutes of continuous walking on the treadmill to minimize distress.

The protocol began with a 2-week warm-up period, followed by training sessions conducted 5 days a week. Rabbits underwent daily 20-minute running sessions at speeds between 2.5 and 3 $km \cdot h{-1}$, while dogs engaged in two 90-minute sessions per day, with speeds ranging from 6 to 10 $km \cdot h{-1}$, all sustained over a 16-week period. Training intensity was maintained by adjusting the inclination, which ranged between 5 % and 12 %. Training sessions

for DOP dogs were identical to those for TRN dogs, ensuring that any observed effects of testosterone could not be attributed to varying training conditions. Blood samples were collected from dogs via the cephalic or saphenous vein and serum testosterone levels were measured.

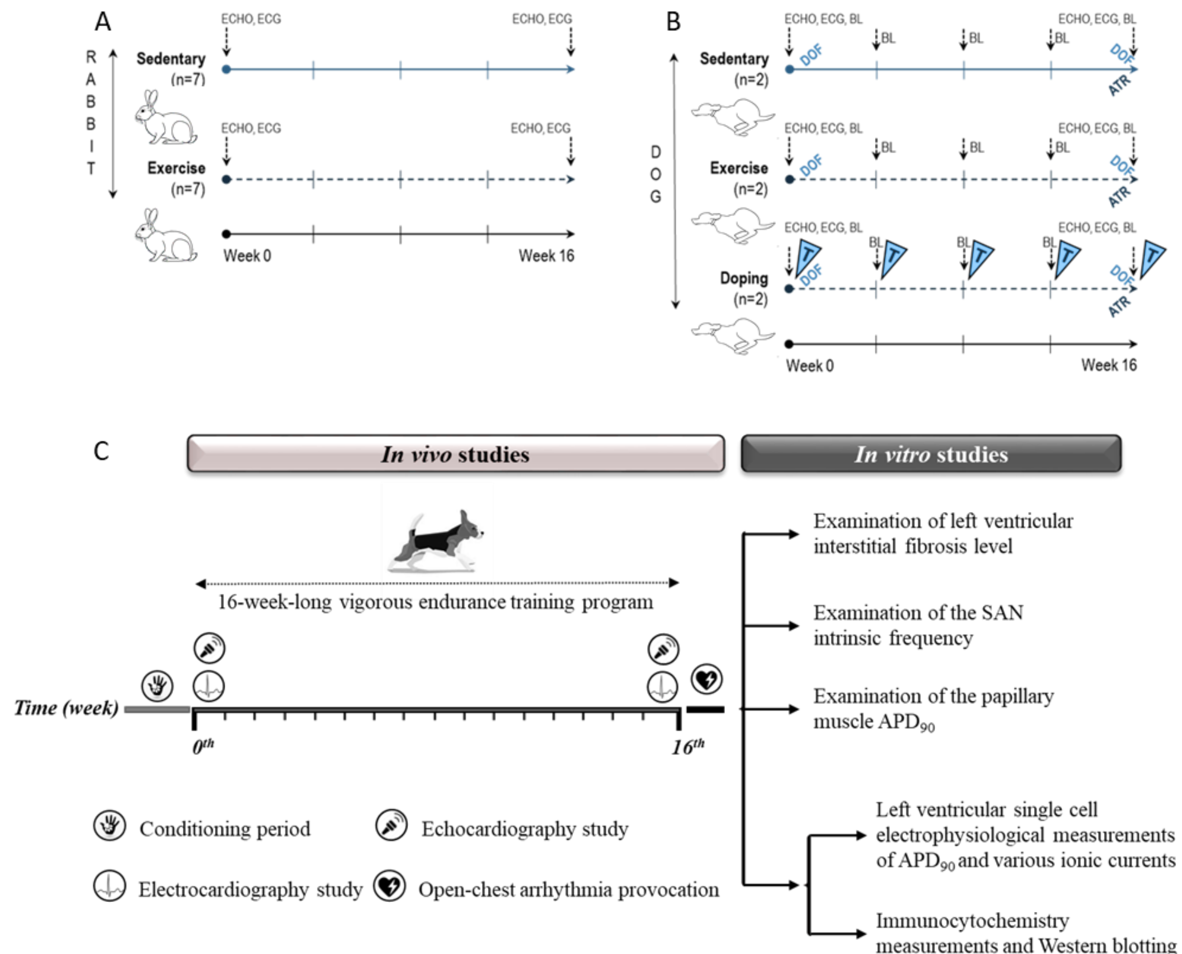

Figure 4. Experimental protocol for both rabbits (A) and dogs (B). Continuous lines correspond to the groups that did not participate in training sessions (SED), while dotted lines represent groups that engaged in training sessions (EX and DOP). The timeline of the experiments is illustrated, including various procedures and challenges: ECHO, Echocardiography; ECG, Electrocardiography; BL, Blood collection; DOF, intravenous dofetilide challenge (0.035 mg/kg); ATR, intravenous atropine sulphate challenge (0.04 mg/kg); T, intramuscular testosterone-undecanoate treatment, (14.3 mg/kg).
C. information about the second set of experiments conducted on dogs, outlining the conditioning and training period, as well as summarizing the *in vivo* and *in vitro* experiments performed. The symbols in the figure represent the *in vivo* studies conducted during the 16-week-long vigorous training. On the right side, the *in vitro* experiments are listed. SAN, sino-atrial node; APD_{90}, action potential duration measured at 90 % repolarization.

Following promising initial results with rabbit and canine models, we proceeded to the next phase of experiments using beagle dogs, who underwent a more intense treadmill exercise program compared to the previous canine experiments. After a three-week conditioning period, beagle dogs of both sexes, weighing 9–15 kg, were randomly divided into sedentary (SED, n = 12) and trained (TRN, n = 12) groups. TRN animals underwent a 16-week training period, while

the SED group remained untrained. During this 16-week period, TRN animals trained for 5 days a week. These training sessions included 2x90-minute sessions per day with speeds ranging from 12 to 18 km·h−1 (gradually increasing protocol) and 2×50-minute interval running sessions at fixed speeds of 4 and 22 km·h−1. Regular rest periods were included to maintain proper hydration, with daily training interruptions not exceeding 1.5 hours. Training intensity was controlled by adjusting the inclination between 5 % and 14 %. **Figure 4C** summarizes the *in vivo* and *in vitro* experiments performed.

3.3 Echocardiography measurements

At 0 and 16 weeks of the training protocol, two-dimensional M-mode and Doppler echocardiographic examinations were conducted on orally acepromazine-sedated rabbits and dogs, following the American Society of Echocardiography criteria. An 11.5 MHz transducer (GE 10S-RS, GE Healthcare, Chicago, IL, USA) connected to an echocardiographic imaging unit (Vivid S5, GE Healthcare, Chicago, IL, USA) was used. All parameters were in a randomised and blinded manner, calculating mean values from three measurements for statistical evaluation.

Various cardiac parameters were evaluated. Left atrial volume (LAV) was measured from standard apical 4-chamber views at end-systole and it was corrected for body surface area (LAV_i, left atrial volume index). Left ventricular end-systolic and diastolic diameters (LVESD and LVEDD) were measured by means of M-mode echocardiographic images from long-axis and short-axis views between the endocardial borders. Wall thickness parameters (LVPW, thickness of the left ventricular posterior wall and IVS, interventricular septal thickness) were obtained from parasternal short-axis view and long-axis view. Left ventricular mass (LVM) was calculated using the following formula: $LVM (g) = 0.0008 \times \{[1.04 \times (LVEDD+LVPW+IVS)^3 – LVEDD^3] + 0.6\}$. Left ventricular mass index (LVM_i) was then calculated by dividing the LVM by the body surface area (BSA).

The IVS and LVPW parameters were normalized to body weight (BW), while LVESD, LVEDD parameters, end-systolic and diastolic volumes (ESV and EDV) were normalized to body surface area (BSA). Animal body weight was measured immediately before echocardiography. The BSA calculation method for dogs was based on Cornell *et al.* [19].

To determine ejection fraction (EF), the echocardiography device, which featured built-in software, employed the Teicholz formula: End-diastolic volume (EDV) = $[7 / (2.4 + LVEDD)] \times LVEDD^3$; End-systolic volume (ESV) = $[7 / (2.4 + LVESD)] \times LVESD^3$; Left ventricular Ejection fraction (EF) = (EDV - ESV) / EDV.

3.4 Electrocardiography measurements

In anaesthetized rabbits, ECG recordings were conducted using subcutaneously placed needle electrodes in all four limbs following the method of Farkas *et al.* [50]. For conscious dogs, precordial leads were utilized for ECG recordings at both 0 and 16 weeks, as described by Polyák *et al.* [51]. ECG data were recorded simultaneously using National Instruments data acquisition hardware (PC card, National Instruments, Austin, TX., U.S.A.) and SPEL Advanced Haemosys software (version 3.26, Experimetria Ltd. and Logirex Software Laboratory, Budapest, Hungary).

Various ECG intervals were measured, including RR, PQ, QRS, QT, and T_{peak}-T_{end} (T_pT_e) intervals by manually positioning on-screen markers on 40 consecutive sinus beats recorded at the 10th minute after initiation of the recording. Mean values were then calculated. Heart rate was derived from the RR interval.

Since the QT interval is influenced by heart rate, baseline data for ventricular heart rates and QT intervals were used to establish the relationship between the RR interval and QT interval in sinus rhythm, following the method of Kui *et al.* [52]. Simple linear regression demonstrated a positive correlation between QT and RR intervals in rabbits ($QT_{rabbit} = 0.354 \cdot RR + 51.7$) and dogs ($QT_{dog} = 0.04 \cdot RR + 188.5$). The equations were rearranged to calculate the rate-corrected QT interval in rabbits at an RR interval of 295 ms (corresponding to a ventricular rate of 203 beats·min−1) using the formula $QT_{cx} = QT_x - 0.354 \cdot (RR_{x\text{-}1} - 295)$, and in dogs at an RR interval of 613 ms (corresponding to a ventricular rate of 98 beats·min−1) using the formula $QT_{cx} = QT_x - 0.04 \cdot (RR_{x\text{-}1} - 613)$. These equations enable the calculation of $QT_{c\text{-}rabbit}$ and $QT_{c\text{-}dog}$, effectively eliminating the influence of heart rate when plotted against the corresponding RR interval, resulting in a regression line with a slope of zero.

Parameters related to beat-to-beat variability and instability of RR and QT intervals, such as the “root mean square of successive differences” (rmsSD-RR) and the “short-term variability” of the QT intervals (STV-QT), were derived from 40 consecutive sinus beats, using methods described earlier [51, 53].

Arrhythmia incidence was assessed by reviewing offline ECG recordings collected over three 20-minute sessions on three consecutive days during the 16th week. Definitions for ventricular tachyarrhythmias from Lambeth Conventions [54] were applied, along with all other ventricular arrhythmia definitions from Lambeth Conventions (II) [55]. The total number of arrhythmic beats was calculated as the sum of all ventricular arrhythmic beats in any type of arrhythmia.

3.5 Atropine and heart rate response in canines *in vivo*

In conscious dogs at week 16, we evaluated the heart rate response by intravenously administering the parasympatholytic agent atropine sulphate (Egis Pharmaceuticals PLC, Budapest, Hungary). After recording resting ECGs for 20 minutes (Baseline), we administered 0.04 mg per kg atropine, dissolved in saline. The mean values of 40 consecutive RR intervals were measured during the drug-free control period (T1) and at 2-minute intervals for a total duration of 50 minutes after atropine treatment (T2).

3.6 Dofetilide and QT interval prolongation challenge in canines *in vivo*

In heavily trained conscious dogs, dofetilide, a class III antiarrhythmic agent, was administered at 0 and 16 weeks to evaluate its potential to prolong the QT interval and test proarrhythmic sensitivity before and after a long-term training protocol. Control ECG data were recorded for 20 minutes, and resting ECG values were determined at the 19th minute after the start of recording (Baseline; 1 minute before dofetilide treatment). Then, intravenous dofetilide was administered as a bolus at a dosage of 0.035 mg per kg. ECG intervals were measured 15 minutes after dofetilide administration, in the 40th experimental minute (Dof). The SED and TRN experimental groups were compared. Dofetilide was dissolved in dimethyl sulfoxide (DMSO) and obtained from Sigma-Aldrich, Inc. (Vienna, Austria).

3.7 Open-chest arrhythmia provocation in anaesthetised canines

Following premedication with 0.5 mcg/kg intravenous sufentanyl (Sufentanyl Torrex 5 µg/ml; Chiesi Pharmaceuticals GmbH, Vienna, Austria) and anaesthesia induction with 150 mg/kg intravenous pentobarbital (Release 300 mg/ml; WDT, Garbsen, Germany), left thoracotomy was performed on all animals (SED, n = 10; TRN, n = 10). The dogs were endotracheally intubated and mechanically ventilated (UGO Basile S.R.L. respirator; Biological Research Apparatus VA Italy). Physiological parameters (non-invasive blood pressure, oxygen saturation, and electrocardiography) were continuously monitored during surgery and experiments (InnoCare-VET Patient Care Monitor; Innomed Medical Inc., Budapest, Hungary). The ECG was recorded using precordial leads and digitized as described.

Under pentobarbital anaesthesia, an epicardial pacemaker electrode (Biotronik Solia S 60; Biotronik Hungary Ltd., Hungary) was positioned into the left ventricular apex and connected to a pacemaker (Effecta D; Biotronik Hungary Ltd., Hungary). Pacemakers were programmed in VVI (V – pacing in the ventricle; V – sensing in the ventricle; I – inhibit) mode using Biotronik IC: 4808A-Renamic programmer to prevent the potential bradycardic effects of general anaesthesia, which could lead to hemodynamic instability. In VVI pacing mode, the

pacemaker is designed to sense the electrical activity, and initiate pacing at a pre-programmed rate when no electrical impulse is detected, but pacing is inhibited if an electrical impulse is detected. However, the pacemakers were not activated in our experiments due to the absence of bradycardia. Ventricular threshold was measured before arrhythmia induction in all animals. Ventricular pacing was set to three times the measured threshold in unipolar electrode configuration.

Ventricular arrhythmia inducibility and incidence were tested in both groups using consecutive ventricular burst pacing for varying durations (1, 3, 6, and 9 seconds), using Effecta D pacemaker in unipolar electrode configuration, with a pacing frequency of 800 beats per minute. Ventricular stimulation was delivered epicardially into the apex of the left ventricle with a three-fold threshold level. The incidence of induced arrhythmias was compared between control and trained animals during the experiments.

3.8 Morphometry and histology

At the end of the training protocol, rabbits were anticoagulated with sodium heparin and anesthetized with 80 mg/kg of sodium pentobarbital injected into the marginal ear vein. Hearts were rapidly removed via thoracotomy. For dogs, at the end of open-chest arrhythmia provocation, an intravenous injection of 400 IU/kg of sodium heparin and a sedative (1 mg/kg of xylazine, intravenously) were administered, followed by euthanasia with 150 mg/kg of intravenous pentobarbital sodium. After confirming the disappearance of the corneal reflex, the hearts were excised.

The atria were removed from the hearts, and ventricles were weighed separately. Left ventricular mass index (LVM_i) was calculated by dividing the measured LVM by the BSA. Ventricular (LVPW) and septal (IVS) wall thicknesses were also measured using a digital calliper, and were normalized to body weight (BW).

Samples were taken from the ventricular free wall for histology. Paraffin sections were stained with Crossman's trichrome staining to identify collagen deposition. An independent pathologist performed semi-quantitative analysis, scoring the degree of interstitial fibrosis as follows: 0 = negative; 1 = mild; 2 = moderate.

3.9 Conventional microelectrode techniques in canine hearts

For the investigation of heavily trained canine hearts, action potentials were recorded in left ventricular papillary muscle preparations obtained from the hearts of the trained and sedentary dogs using the conventional microelectrode techniques previously described in detail [56]. Briefly, the preparations were mounted in a tissue chamber of 50 ml volume individually.

The experiments were performed using a modified Locke's solution with the following composition (in mM): (in mM): NaCl 128.3, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 0.42, $NaHCO_3$ 21.4, and glucose 10. The pH of this solution was set between 7.35 and 7.4 when gassed with 95 % O_2 and 5 % CO_2 at 37 °C. Each preparation was stimulated through a pair of platinum electrodes, maintaining a constant basic cycle length of 1000 ms. Transmembrane potentials were recorded after an equilibration period of 60 minutes following mounting, using conventional glass microelectrodes filled with 3 M KCl. Measurements were excluded from analysis if the resting membrane potential of the recorded action potential was more positive than -70 mV and/or if the action potential amplitude was less than 90 mV.

3.10 Patch-clamp measurements in canine hearts

Ventricular myocytes were enzymatically dissociated as previously described [57]. A single droplet of cell suspension was placed in a transparent recording chamber mounted on the stage of an inverted microscope (Olympus IX51, Olympus, Tokyo, Japan). Individual myocytes were allowed to settle and adhere to the chamber bottom for at least 5–10 min before superfusion was initiated and maintained by gravity. Only rod-shaped cells with clear striations were used. The normal superfusate was HEPES-buffered Tyrode's solution (composition in mM: NaCl 144, NaH_2PO_4 0.4, KCl 4.0, CaCl2 1.8, $MgSO_4$ 0.53, glucose 5.5, and HEPES 5.0, pH 7.4).

Micropipettes were made from borosilicate glass capillaries (Science Products GmbH, Hofheim, Germany), using a P-97 Flaming/Brown micropipette puller (Sutter Co, Novato, CA, USA), and had a resistance of 1.5–2.5 MOhm when filled with pipette solution. The membrane currents were recorded with Axopatch-200B amplifiers (Molecular Devices, Sunnyvale, CA, USA) by means of the whole-cell configuration of the patch-clamp technique. The membrane currents were digitized with 250 kHz analogue-to-digital converters (Digidata 1440A, Molecular Devices, Sunnyvale, CA, USA) under software control (pClamp 10, Molecular Devices, Sunnyvale, CA, USA). All patch-clamp experiments were conducted at 37°C.

3.10.1 Measurement of L-type calcium current

The L-type calcium current (I_{CaL}) was recorded in HEPES-buffered Tyrode's solution supplemented with 3 mM 4-aminopyridine. The micropipettes were filled with a special solution (composition in mM: CsCl 125, TEACl 20, MgATP 5, EGTA 10, HEPES 10, pH adjusted to 7.2 with CsOH).

3.10.2 Measurement of potassium currents

The inward rectifier (I_{K1}), the transient outward (I_{to}), the rapid (I_{Kr}) and the slow (I_{Ks}) delayed rectifier potassium currents were recorded in HEPES-buffered Tyrode's solution. The pipette solution composition (in mM) was: KOH 110, KCl 40, K_2ATP 5, $MgCl_2$ 5, EGTA 5, and HEPES 10 (pH was adjusted to 7.2 by aspartic acid). 1 μM nisoldipine was added to the bath solution to block I_{CaL}. When recording I_{Kr}, selective I_{Ks} blocker HMR-1556 (0.5 μM) was used to inhibit I_{Ks}. For I_{Ks} measurements, I_{Kr} was blocked with 0.1 μM dofetilide, and the bath solution contained 0.1 μM forskolin.

3.10.3 Measurement of late sodium current

The late sodium current (I_{NaL}) was activated by depolarizing voltage pulses of 2 s at -20 mV from holding potential of -120 mV with pulsing cycle lengths of 5 seconds. After 5–7 minutes of drug incubation, the external solution was replaced with a solution containing 20 μM TTX, which at this concentration completely blocked the I_{NaL}. The external solution was HEPES-buffered Tyrode's solution supplemented with 1 μM nisoldipine, 0.5 μM HMR-1556 and 0.1 μM dofetilide to block I_{CaL}, I_{Ks} and I_{Kr} currents. The pipette solution composition (in mM) was: CsCl 125, TEACl 20, MgATP 5, EGTA 10, HEPES 10, pH was adjusted to 7.2 by CsOH).

3.10.4 Measurement of NCX current

To measure the Na^+/Ca^{2+} exchanger current (I_{NCX}), the method of Hobai *et al.* [58] was applied. The NCX current is defined as a Ni^{2+}-sensitive current and measured in a special K^+-free solution (composition in mM: NaCl 135, CsCl 10, $CaCl_2$ 1, $MgCl_2$ 1, $BaCl_2$ 0.2, NaH_2PO_4 0.33, TEACl 10, HEPES 10, glucose 10 and ouabain 20 μM, nisoldipine 1 μM, and lidocaine 50 μM, at pH 7.4) as described earlier in detail [57]. The pipette solution used for recording I_{NCX} contained (in mM) CsOH 140, aspartic acid 75, TEACl 20, MgATP 5, HEPES 10, NaCl 20, EGTA 20 and $CaCl_2$ 10, pH was adjusted to 7.2 by CsOH.

3.10.5 Measurements of single cell action potentials

Measurements of single cell action potentials were conducted using the perforated patch-clamp technique on isolated left ventricular myocytes obtained from both trained and sedentary canines. The membrane potential was recorded in current clamp configuration. The myocytes were paced with a rapid rectangular pulse (ranging from 0 to 180 mV, 5 ms) at a frequency of 1 Hz to elicit the action potential. A normal Tyrode solution was used as the extracellular solution containing (in mM): 144 NaCl, 0.4 NaH_2PO_4, 4 KCl, 0.53 $MgSO_4$, 1.8

$CaCl_2$, 5.5 glucose and 5 HEPES, titrated to pH = 7.4. The patch pipette solution contained (in mM): 120 K-gluconate, 2.5 NaCl, 2.5 MgATP, 2.5 Na_2ATP, 5 HEPES, 20 KCl, titrated to pH 7.2 with KOH. Additionally, 50 µM β-escin was added to the pipette solution to achieve the membrane patch perforation. Membrane voltage recordings were obtained by using an Axoclamp 1-D amplifier (Molecular Devices, Sunnyvale, CA, USA) connected to a Digidata 1440A (Molecular Devices, Sunnyvale, CA, USA) analogue-digital converter. The membrane voltage was recorded by Clampex 10.0 (Molecular Devices, Sunnyvale, CA, USA), with a minimum of 60 beats recorded for each measurement. The action potential duration was measured at 90 % repolarization (APD_{90}). Short-term APD variability (STV-APD) was calculated based on the analysis of 30 consecutive action potentials.

3.11 Western blot analysis of KChIP2 and Kv4.3 proteins in canine hearts

Membrane fractions were isolated from myocardial samples of TRN (n = 12) and SED (n = 12) dogs, obtained from the left ventricular free wall, using a previously described method (Papp et al., 2007). Protein concentrations were determined using the Lowry method, and 20 µg of each sample was separated on 8 % polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride (PVDF) Western blotting membrane. The membrane was blocked with 2.5 % non-fat milk for 1 hour at room temperature and then immunolabelled overnight at 4 °C with anti-KChIP2 (Alomone, #APC-142, RRID:AB_2756744) and anti-Kv4.3 (Alomone, #APC-017, RRID:AB_2040178) primary antibodies, both diluted at 1:1000. This was followed by incubation for 1 hour with Goat anti-Rabbit IgG-HRP (SouthernBiotech, 4030-05, RRID:AB_2687483) secondary antibody at a dilution of 1:8000. Band densities were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare) and a ChemiDoc Imaging System (Bio-Rad). Equal loading was confirmed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) labelling (Thermo Fisher, PA1-988, RRID: AB_2107310). The pixel intensity of each band was measured using ImageJ software. Three parallel Western blots were performed for statistical analysis.

3.12 Immunocytochemistry of KChIP2, Kv4.3, and HCN1, HCN2, and HCN4 proteins in canine hearts

Cardiomyocytes were isolated from left ventricular tissue of TRN (n = 6) and SED (n = 6) dogs and then fixed on glass coverslips using acetone [59]. Prior to immunolabelling, samples were rehydrated with calcium-free phosphate-buffered saline (PBS) and blocked for 1 hour with PBST (PBS with 0.01 % Tween) containing 2.5 % bovine serum albumin (BSA) at room temperature. Following the incubation period, cells were labelled overnight at 4 °C with

anti-KChIP2 (Alomone, #APC-142, RRID:AB_2756744), anti-Kv4.3 (Alomone, #APC-017, RRID:AB_2040178), anti-HCN1 (Alomone, #APC-056, RRID:AB_2039900), anti-HCN2 (Alomone, #APC-030, RRID:AB_2313726) and anti-HCN4 (Alomone, #APC-052, RRID:AB_2039906) primary antibodies diluted 1:50. Subsequently, the cells were incubated with goat anti-rabbit IgG Alexa Fluor 488 secondary antibody (ThermoFisher, A-11034, RRID:AB_2576217) at a dilution of 1:500 the following day. Fluorescent images were acquired using an LSM 880 (Zeiss) laser scanning confocal microscope. Images were quantitatively analysed by the ImageJ software. Control samples were incubated only with secondary antibodies.

3.13 Gene expression of fibrosis markers in rabbit hearts using real-time qRT-PCR

Real-time quantitative polymerase chain reaction (PCR) was performed on cardiac samples from exercised rabbits and their sedentary controls. Ribonucleic acid (RNA) was isolated from left ventricular free wall samples using the Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA, United States, Cat. No. R2051). Complementary deoxyribonucleic acid (cDNA) molecules were synthesized from messenger ribonucleic acid (mRNA) templates by reverse transcription, using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, United States, Cat. No. 4368814). Real-time PCR was conducted with gene-specific primers and SYBR green (Thermo Fisher Scientific, Waltham, MA, United States, Cat. No. K0222) on the ABI PRISM R 7000 Sequence Detection System (Thermo Fisher Scientific, Waltham, MA, United States, Cat. No. K0222). After each run, a melting point analysis was performed by measuring fluorescence intensity. The expression levels of genes encoding fibrotic biomarkers were examined, including transforming growth factor-β1 (TGF-β), fibronectin-1 (FN-1), pro-alpha1 chain of type I collagen (COL1A1), pro-alpha1 chain of type III collagen (COL3A1), matrix metalloproteinase-2 (MMP-2), and tissue inhibitor of metalloproteinase-1 (TIMP-1). The relative copy numbers of mRNAs were calculated by normalizing each cDNA to the geometric average of β-actin (ACTB), signal recognition-particle assembly 14 (SRP14), and ribosomal protein S5 (RPS5) expression.

4. STATISTICS

IBM SPSS Statistics V25, Microsoft Excel (Microsoft Office Professional Plus 2016) and Origin software (2021b, OriginLab) packages were used for statistical analysis. Continuous data were expressed as mean ± standard error of the mean (S.E.M.). Each figure indicates the number of observations made ('n'), representing the biological replicates of the experiment. The 'n' refers to the number of animals, except for action potential, patch clamp, Western blotting, and immunocytochemistry measurements, where it refers to the number of preparations/cells, followed by the number of animals from which preparations/cells were obtained.

After assessing the normality of our data using Kolmogorov-Smirnov test, paired and unpaired Student's t-test were applied to estimate whether there was a statistically significant difference between the means of the self-control or independent group arrangements, respectively. When data did not follow normal distribution, Mann–Whitney U test was applied instead of Student's t-test. When data could be described by a discrete variable, the chi-square (χ^2) test was applied. Data were considered statistically significant when $p \leq 0.05$.

5. RESULTS

5.1 Sustained exercise-induced cardiac hypertrophy and fibrosis

This chapter outlines the impact of 16 weeks of endurance training on various animal groups, including rabbits (SED and EX groups), dogs (SED, EX, and DOP groups), and dogs subjected to more intense endurance training (SED and TRN groups). The primary focus was to assess changes in their left ventricular cardiac parameters using echocardiographic measurements and histological findings. The 16-week training program resulted in significant cardiac adaptations, with more pronounced effects seen in vigorously trained dogs.

Specifically, the 16-week training regimen led to a significant increase in the left ventricular end-diastolic diameter (LVEDD) in EX rabbits, with a similar trend in EX dogs. Both EX rabbits and dogs showed a moderate increase in the internal end-systolic diameter of the left ventricle (LVESD). Notably, there were no significant differences in the thickness of the left ventricular posterior wall (LVPW) or the interventricular septum (IVS) between the groups in either the rabbit or dog models. This suggests that endurance exercise training led to left ventricular dilation and enlargement without a concurrent increase in ventricular wall thickness. Ejection fraction (EF) and fractional shortening (FS) did not differ significantly among the groups. The influence of steroid on cardiac morphology was not prominent.

After 16 weeks of more vigorous endurance training, the TRN dog group showed an increase in left atrial volume (LAV), indicating left atrial enlargement. Trained animals also exhibited thickening of the interventricular septum (IVS) and left ventricular posterior walls (LVPW), along with greater end-diastolic diameter (LVEDD) in the left ventricle and increased left ventricular mass (LVM and LVM_i), indicating left ventricular hypertrophy in response to the increased workload. Trained dogs had a greater end-diastolic left ventricular volume, indicating that the left ventricle was holding more blood during the filling phase. Even when adjusting the echocardiographic parameters for body weight (IVS/BW; LVPW/BW) or body surface area (LAV_i; LVESD/BSA; LVEDD/BSA; EDV/BSA), the differences between the trained and sedentary dogs persisted. This means that the observed cardiac changes were not solely due to differences in body size or weight. No significant differences were observed in end-systolic volume (ESV), ESV adjusted for body surface area (ESV/BSA), and ejection fraction (EF) between the trained and sedentary groups. Detailed echocardiographic parameters for the initial and subsequent sets of experiments are provided in **Table 1** and **Table 2**, respectively.

At 16th week	Group	LVEDD (mm)	LVESD (mm)	IVS (mm)	LVPW (mm)	EF (%)	FS (%)
RABBIT (n=7)	SED	13.6±0.7	9.2±0.7	3.3±0.2	3.2±0.2	64.7±4.1	33.0±3.1
	EX	**17.5±0.6***	10.6±0.6	3.2±0.2	2.7±0.1	70.7±2.8	37.7±2.4
DOG (n=2)	SED	24.0±3.0	12.5±0.5	7.4±0.3	7.0±0.1	80.4±3.7	47.0±4.2
	EX	27.2±0.2	14.9±0.1	7.3±0.6	6.5±0.5	78.8±0.5	45.6±0.5
	DOP	22.5±1.5	13.5±0.5	6.6±0.3	6.5±0.4	72.4±4.5	39.8±4.0

Table 1. Echocardiographic measurements in rabbits (above) and dogs (below) after 16 weeks of endurance exercise. Refer to the text above for the complete descriptions of the abbreviations. All values are presented as means ± SEM. *p<0.05.

ECHOCARDIOGRAPHIC PARAMETERS BEFORE AND AFTER LONG-TERM VIGOROUS TRAINING				
	Before the training protocol (at 0th week)		After the training protocol (at 16th week)	
	'SED' group	'TRN' group	'SED' group	'TRN' group
IVS, mm	7.1 ± 0.3	6.8 ± 0.2	7.4 ± 0.2	**8.13 ± 0.2**$^{*\#}$
IVS/BW, mm/kg	0.6 ± 0.03	0.5 ± 0.03	0.6 ± 0.03	**0.74 ± 0.03**$^{*\#}$
LVPW, mm	7.1 ± 0.2	6.95 ± 0.2	7.4 ± 0.3	7.64 ± 0.3
LVPW/BW, mm/kg	0.6 ± 0.03	0.6 ± 0.03	0.6 ± 0.03	**0.70 ± 0.04**$^{*\#}$
LVESD, mm	14.2 ± 0.3	**17.6 ± 0.7***	18.7 ± 0.5	18.4 ± 0.1
LVESD/BSA, mm/m²	25.8 ±1.1	**32 ± 1.03***	34.2 ± 1.5	**36.5 ± 2.1**$^{\#}$
LVEDD, mm	28.7 ± 0.7	29 ± 0.96	30.4 ± 0.7	**32.0 ± 0.7**$^{\#}$
LVEDD/BSA, mm/m²	51.7 ± 1.4	52.7 ± 1	55.3 ± 1.9	**63.5 ± 1.3**$^{*\#}$
LVM, g	46.7 ± 3.4	45.1 ± 2.1	54.1 ± 3.9	**63.6 ± 2.8**$^{\#}$
LVMi, g/m²	83.6 ± 5.6	81.2 ± 2.8	97.7 ± 6.4	**125.8 ±4.3**$^{*\#}$
EDV, ml	32.3 ± 2	32.6 ± 2.5	37.9 ± 2.2	**40.6 ± 1.7**$^{\#}$
EDV/BSA, ml/m²	57.5 ± 2.5	58.4 ± 2.9	68.4 ± 3.4	**80.3 ± 2.3**$^{*\#}$
ESV, ml	6.1 ± 0.6	**9.5 ± 1.03***	11.4 ± 0.8	10.2 ± 1.3
ESV/BSA, ml/m²	10.9 ± 0.9	**17± 1.5***	20.7 ± 1.3	20.1 ± 2.5
EF, %	80.4 ± 1.7	**70.9 ± 2.3***	69.7 ± 1.3	75.1 ± 2.7
LAV, ml	9 ± 0.9	8.9 ± 0.6	10.4 ± 0.9	**11.4 ± 1.4**$^{\#}$
LAVi, ml/m²	16 ± 1.4	16 ± 0.8	18.7 ± 1.3	**22.4 ± 2.3**$^{\#}$

AUTOPSY FINDINGS AFTER THE LONG-TERM VIGOROUS TRAINING						
	IVS, mm	IVS/BW, mm/kg	LVPW, mm	LVPW/BW, mm/kg	LVM, g	LVMi, g/m²
'SED' group	3.62 ± 0.6	0.28 ± 0.04	2.54 ± 0.4	0.2 ± 0.03	79.3±4.4	144.1 ± 4.1
'TRN' group	4.25 ± 0.3	**0.039 ± 0.03***	**3.42 ± 0.3***	**0.031 ± 0.02***	83.3±4.8	**167.2 ± 5.7***

Table 2. Echocardiographic measurements and autopsy findings in heavily trained dogs before (at 0th week, control measurements) and after (at 16th week) endurance training. Refer to the text above for the complete descriptions of the abbreviations. All values are presented as means ± SEM. *p

< 0.05 Trained vs. Sedentary group at 16th week. $^{\#}p < 0.05$ Trained group at 16th week vs. Trained group at 0th week.

Autopsy results confirmed cardiac hypertrophy in trained canine hearts, as indicated by increased left ventricular mass index (LVM_i), thickening of the interventricular septum (IVS/BW), and left ventricular posterior wall (LVPW and LVPW/BW) compared to sedentary hearts **(Table 2)**. Additionally, semiquantitative fibrosis score analysis showed higher fibrosis levels in the left ventricles of trained canine hearts when compared to sedentary hearts **(Figure 5).** These findings also align with the relative gene expression results observed in our exercised rabbit hearts.

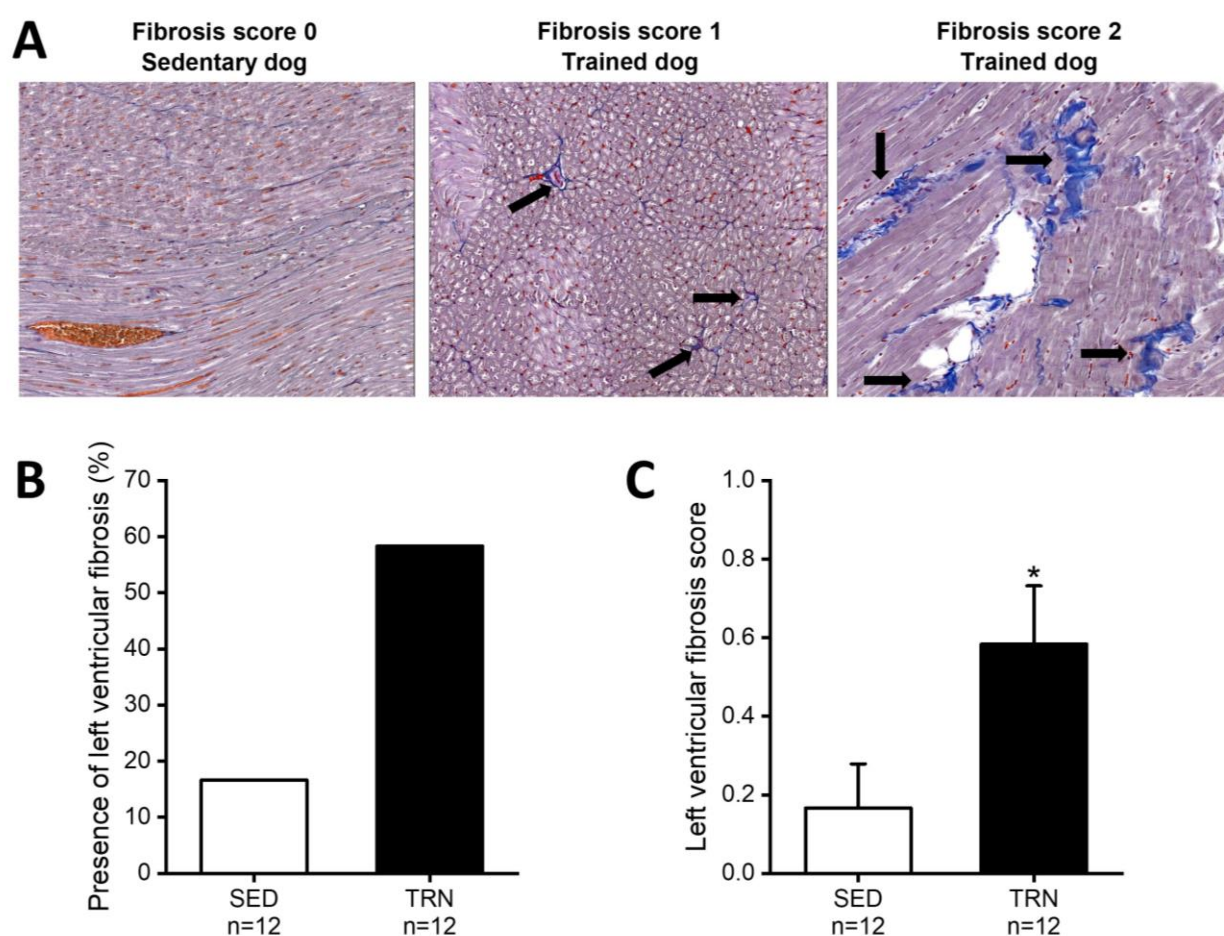

Figure 5. Increased level of myocardial fibrosis in the canine left ventricle. A. Representative histological images of left ventricular free-wall connective tissue visualized by Crossman's trichrome staining taken from SED dogs (fibrosis score 0 = negative outcome), and the TRN dogs (fibrosis score 1 = mild and fibrosis score 2 = moderate level of fibrosis). Black arrows indicate the presence of fibrosis. **B.** Bar chart showing the incidence of fibrosis, expressed as the percentage of the total number of animals, irrespective of the degree of fibrosis. **C.** Bar chart estimating the amount of scarring via fibrosis scoring in SED and TRN dogs. The n numbers refer to the number of dogs included. Data are expressed as mean ± SEM.*$p < 0.05$ TRN vs. SED group at 16th week.

5.2 The effect of training on heart rate and its variability in conscious canines and rabbits, and on spontaneous beating rate in isolated canine right atrial tissue preparations

In our initial experiments, the ECG revealed prolonged mean RR intervals in all exercise groups of both dogs and rabbits, indicating the presence of training-induced bradycardia. In canines exposed to the less intense training regimen, the RR interval changes were more subtle, with a slight prolongation of the RR interval observed in the doping group, but the differences did not reach statistical significance (**Figure 6A** and **Figure 6B**).

Beat-to-beat variability parameters, such as root mean square (Rabbit RMS-RR EX vs. SED: 335 ± 15 vs. 288 ± 19 ms; Dog RMS-RR EX vs. DOP vs. SED: 660 ± 26 vs. 736 ± 7 vs. 611 ± 57 ms), root mean square of successive differences (Rabbit rmsSD-RR EX vs. SED: 2.1 ± 0.5 vs. 1.3 ± 0.1 ms; Dog rmsSD-RR EX vs. DOP vs. SED: 236 ± 55 vs. 184 ± 48 vs. 159 ± 73 ms), and standard deviation of the successive differences (Rabbit sdSD-RR EX vs. SED: 2.1 ± 0.5 vs. 1.4 ± 0.1 ms; Dog sdSD-RR EX vs. DOP vs. SED: 238 ± 56 vs. 218 ± 82 vs. 161 ± 74 ms), tend to increase in both trained models, indicating vagal enhancement.

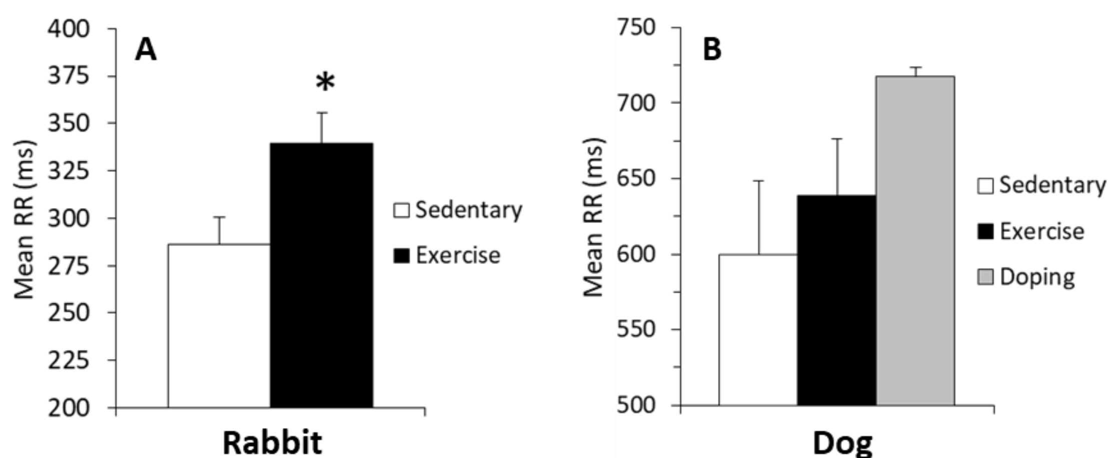

Figure 6. The mean *in vivo* RR intervals in rabbits (A) and dogs (B) at 16th week. Data are expressed as mean ± SEM. ∗ $p < 0.05$ vs. Sedentary.

In the second set of dog experiments, sustained training resulted in significant bradycardia **(Figure 7A)** and an increase in numerous heart rate variability parameters (e.g., rmsSD-RR, root mean square of successive differences of RR interval) in the TRN dogs **(Figure 7B)** compared to the sedentary controls and their baseline values at week 0.

To investigate changes independent of the autonomic nervous system, we examined the intrinsic beating rate in spontaneously beating isolated right atrial tissue preparations from SED and TRN dogs. The spontaneous rate was significantly slower in the trained canine tissue

preparations than in the sedentary subjects **(Figure 7C)**, further indicating that the observed bradycardia in trained dogs is not entirely due to an enhancement of vagal tone.

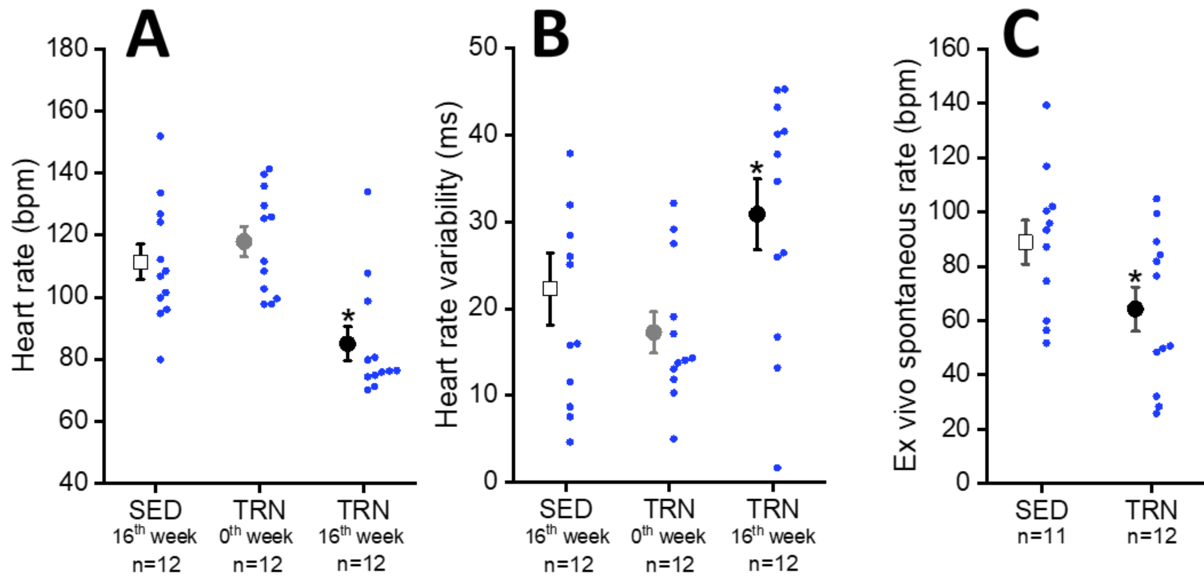

Figure 7. A. Heart rate**, B.** heart rate variability values in conscious dogs from the SED group at the end of the training protocol (16th week; n = 12 dogs), the TRN group before chronic endurance training (0th week; n = 12), and the TRN group after chronic endurance training (16th week; n = 12 dogs). RmsSD-RR represents the root mean square of successive differences of RR interval. **C.** Spontaneous beating rate in isolated right atrial tissue preparations from SED (n = 11) dogs and TRN (n = 12) dogs. The 'n' numbers indicate the number of dogs included. Data are expressed as mean ± SEM. Blue dots represent individual data points. *P < 0.05 TRN vs. SED group at 16th week. # P < 0.05 TRN group at 16th week vs. TRN group at 0th week.

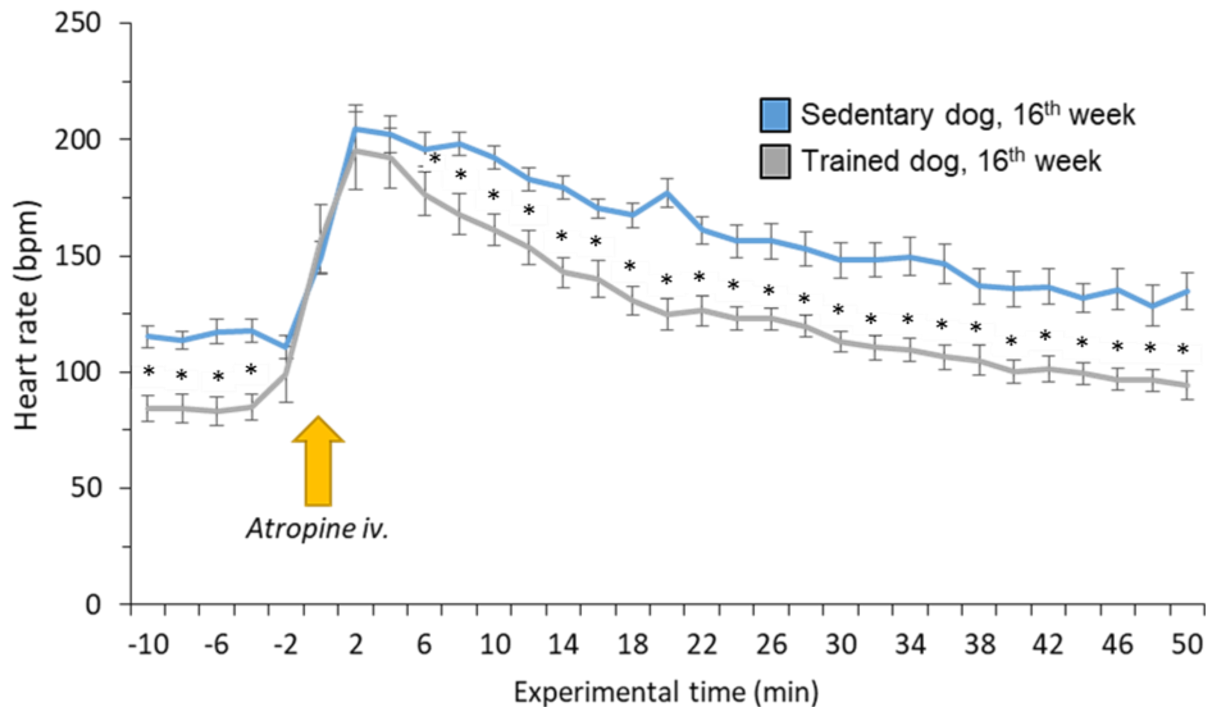

Figure 8. Mean RR intervals in dogs after parasympatholytic atropine challenge at the 16th week. All values are presented as means ± SEM. ∗ p < 0.05 vs. 'Sedentary'.

5.3 Heart rate changes in heavily trained canines under atropine challenge

Repetitive heart rate measurements during the drug-free period (prior to atropine challenge) confirmed the bradycardia induced by training in TRN dogs. Then, the continuous infusion of atropine failed to elevate the heart rate of the TRN group to the level of the SED group. Instead, a moderate increase in heart rate was observed in the trained animals **(Figure 8)**, consistent with the findings of spontaneous heart rate measurements **(Figure 7C)**. This indicates that the observed bradycardia in trained dogs may not be solely attributed to an enhancement of vagal tone.

5.4 ECG changes and increased proarrhythmic response following long-term sustained training

In our initial experiments with rabbits and dogs, there were no differences in depolarizing PQ and QRS intervals following the completion of the training protocol (Rabbit mean-PQ EX vs. SED: 74.0 ± 6.5 vs. 67.9 ± 2.0 ms; Dog mean-PQ EX vs. DOP vs. SED: 90.8 ± 8.0 vs. 95.2 ± 3.2 vs. 91.2 ± 3.5 ms; Rabbit mean-QRS EX vs. SED: 49.8 ± 4.2 vs. 56.5 ± 5.2 ms; Dog mean-QRS EX vs. DOP vs. SED: 60.3 ± 3.6 vs. 56.5 ± 0.8 vs. 50.8 ± 9.3 ms).

Heart rate-corrected QT intervals (QT_c) also did not vary between the groups after the long-term physical exercise (Rabbit $QT_{c:}$ EX vs. SED: 169 ± 7.6 vs. 178 ± 5 ms; Dog QT_c EX vs. DOP vs. SED: 218 ± 7.4 vs. 213 ± 16 vs. 204 ± 4.3 ms). Some beat-to-beat QT variability values showed minor increases in EX rabbits (e.g., short-term variability (STV): EX vs. SED: 4.4 ± 0.4 vs. 4.0 ± 0.5 ms), while no differences were observed in dogs (STV: EX vs. DOP vs. SED: 3.6 ± 0.2 vs. 2.8 ± 0.4 vs. 4.5 ± 0.7 ms).

However, in heavily trained dogs, the 16-week sustained training protocol led to significant prolongation of RR, PQ, QT, heart-rate corrected QT (QT_c), and T_pT_e intervals. It also widened the QRS complex on the electrocardiogram **(Table 3)**. The prolonged QT interval was also associated with significantly increased QT interval variability (e.g., short-term variability of the QT intervals; STV-QT), indicating elevated dispersion of repolarization compared to sedentary controls after the training protocol **(Table 3)**.

As some arrhythmias occurred, we conducted an arrhythmia analysis in our heavily trained dogs. In conscious, sedentary (SED) animals, only a few ventricular beats were observed during the 3 x 20-minute ECG recordings at rest. However, trained (TRN) animals exhibited a significantly higher incidence of ventricular beats, as illustrated in **Figure 9B**. The majority of these ventricular beats were identified as ventricular escape beats, as shown in **Figure 9A** and **Figure 9C**. Although the incidence of premature beats was low in both experimental groups,

they were more prevalent in the TRN group compared to the sedentary subjects **(Figure 9C)**. More complex arrhythmias were not observed at rest in either group.

During electrical burst stimulation in open-chest, anaesthetized dogs, ventricular fibrillation was induced in 6 out of 10 TRN dogs, whereas only 3 out of 10 of the sedentary control dogs experienced ventricular fibrillation, as indicated in **Figure 9D** and **Figure 9E**.

ELECTROCARDIOGRAPHIC PARAMETERS BEFORE AND AFTER LONG-TERM VIGOROUS TRAINING				
	Before the training protocol (at 0th week)		After the training protocol (at 16th week)	
	'SED' group (n = 12)	'TRN' group (n = 12)	'SED' group (n = 12)	'TRN' group (n = 12)
RR, ms	588.4 ± 32.1	579.3 ± 33.2	644.2 ± 58.6	**841.8 ± 62.8*#**
PQ, ms	103.2 ± 2.0	98.3 ± 2.9	102.8 ± 3.2	**110.7 ± 3.6#**
QRS, ms	59.6± 1.7	60.5 ± 2.4	56.3 ± 2.6	**70.8 ± 1.6*#**
QT, ms	218.7 ± 5.7	215.9 ± 2.9	223.0 ± 6.4	**251.3 ± 3.2*#**
QTc, ms	216 ± 4.7	213.6 ± 2.8	217.7 ± 4.5	**237.1 ± 3.4*#**
STV-QT, ms	2.6 ± 0.2	2.5 ± 0.2	2.6 ± 0.2	**3.6 ± 0.4*#**
TpTe, ms	27.3 ± 2.3	27.9 ± 2.5	30.9 ± 2.4	**36.5 ± 1.7#**

Table 3. The effect of 16-week-long vigorous training on electrocardiographic intervals in conscious dogs. Electrocardiographic parameters values were measured before (at 0th week, control measurements) and after (at 16th week) the training protocol. QT interval variability ("short-term variability" of the QT intervals; STV-QT). All values are mean ± SEM. *$p < 0.05$ TRN vs. SED group at 16th week. #$p < 0.05$ TRN group at 16th week vs. TRN group at 0th week.

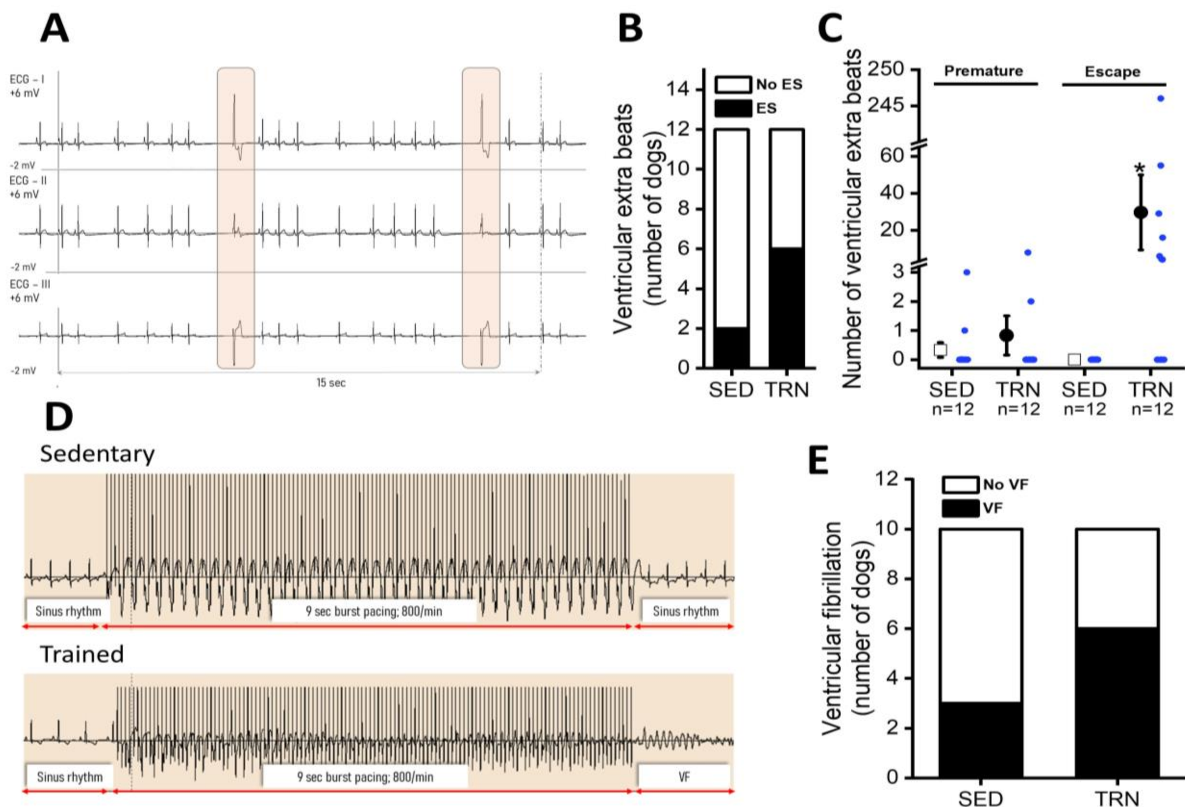

Figure 9. A. representative ECG recordings of escape beats and increased R-R interval variability at rest in a conscious TRN dog at 16th week. **B**. Bar chart plotting the increased incidence of different

ventricular extra beats (premature and escape) at rest in the TRN group vs. SED group at 16th week. **C.** average of premature and ventricular escape beats across animals at rest in conscious SED and TRN dogs at 16th week. **D**. Representative burst pacing ECG image in anaesthetized SED and TRN dogs at 16th week. **E.** Bar chart plotting the increased incidence of ventricular fibrillation (VF) in the TRN group vs. SED group following burst arrhythmia provocation. The 'n' numbers refer to the number of dogs included. Data are expressed as mean ± SEM. Blue dots represent individual data. *P < 0.05 'TRN' vs. 'SED' group at 16th week.

5.5 Repolarization sensitivity to the proarrhythmic agent dofetilide in conscious canines

To assess the repolarization sensitivity of athletes' hearts, an I_{Kr} inhibitor, dofetilide, was administered to dogs from both sets of experiments, as described in the exercise training (EX, DOP, and SED groups) and heavily intense training (TRN and SED groups) protocols for dogs in the methods, following the completion of the 16-week training program. As expected, dofetilide markedly increased the heart rate-corrected QT interval (QT_c) in each group, with more pronounced QT_c lengthening effect observed in animals that had undergone any kind of training protocol (**Figure 10A** and **Figure 10B**). There were no meaningful differences in QT_c intervals between the exercised (EX) and doping (DOP) groups (**Figure 10A**). Nonetheless, the percentage of QT_c prolongation calculated from baseline and post-dofetilide values did not differ significantly among the groups. This may indicate that the QT_c lengthening effect induced by training and the QT_c lengthening effect induced by dofetilide were additive but not superadditive in this experiment (QT_c lengthening in percentage: EX vs. DOP vs. SED: 15 ± 2 vs. 17 ± 2 vs. 8 ± 3 %; TRN vs. SED: 13 ± 2 vs. 16 ± 2 %).

Dofetilide treatment increased the beat-to-beat variability and instability parameters in each examined groups, without significant difference between the groups (e.g., STV-DOF EX vs. DOP vs. SED: 4.7 ± 0.6 vs. 4.4 ± 1.0 vs. 4.6 ± 1.0 ms; STV-DOF TRN vs. SED: 4.5 ± 1.7 vs. 3.6 ± 0.2 ms). Notably, between the heavily trained animals and their controls, an almost significant difference was found, suggesting some degree of more pronounced repolarisation inhomogeneity in those trained hearts.

In canines subjected to the less intensive training program, there was a modest occurrence of ventricular premature beats during dofetilide perfusion, but no significant intergroup difference was observed. Complex arrhythmias, such as Torsades de Pointes, did not manifest during dofetilide perfusion across all groups (data not presented). In contrast, dogs subjected to the heavy training protocol exhibited an elevated frequency of ventricular beats, accompanied by the emergence of more complex arrhythmias, thereby heightening the risk of life-threatening arrhythmia development (**Figure 10C**).

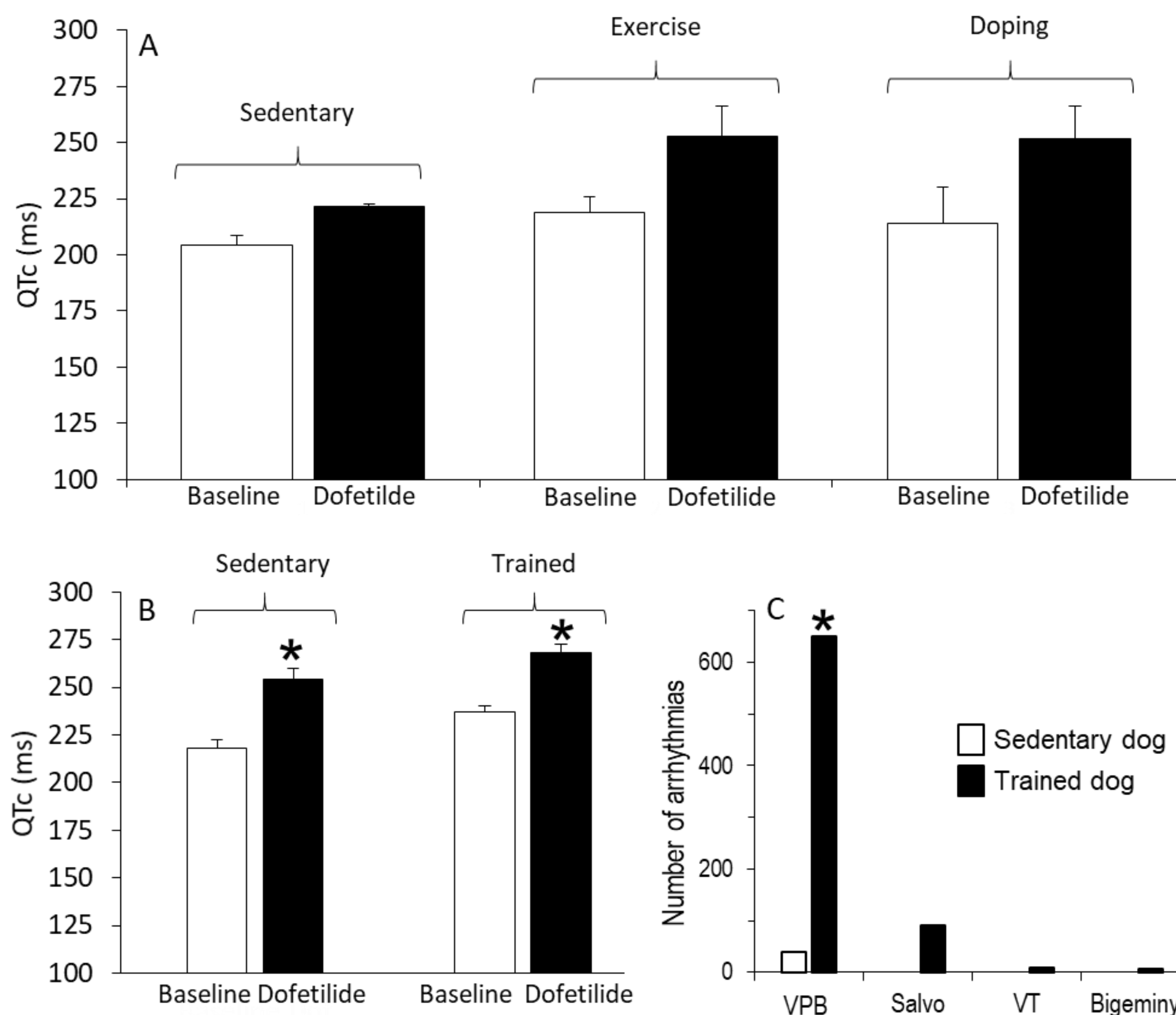

Figure 10. The effect of the selective I_{Kr} channel inhibitor dofetilide on QT_c interval lengthening in exercised, doping (A) and heavily trained dogs (B) as well as in their corresponding sedentary controls. **C**. Number of arrhythmias after dofetilide treatment in the heavily trained dog group. 'Baseline' represents the measurement taken 1 minute before dofetilide treatment, while 'Dofetilide' represents the measurement taken 15 minutes after intravenous bolus dofetilide administration at a dosage of 0.035 mg per kg. VPB, ventricular premature beat; Salvo, a run of 2 or 3 consecutive VPBs; Bigeminy, the minimum sequence VPB-normal sinus beat-VPB, in which the VPBs have the same shape and timing; VT, ventricular tachycardia, a run of four or more consecutive VPBs. Data are expressed as mean ± SEM. *$p < 0.05$ 'Dofetilide' vs. 'Baseline' time points at 16th week.

5.6 Influence of prolonged training on cardiac APD and its short-term variability in left ventricular preparations of heavily trained sedentary and trained canines

Cardiac ventricular action potentials were assessed in two distinct types of preparations: isolated left ventricular papillary muscles (subendocardial origin) and enzymatically isolated left ventricular single myocytes (midmyocardial origin). **Figure 11B** shows that the cardiac action potential duration at 90 % repolarization (APD_{90}) of left ventricular papillary muscle preparations did not differ significantly between the groups. However, enzymatically isolated left ventricular myocytes from TRN dogs showed a significant lengthening of APD_{90}

(**Figure 11A** and **Figure 11C**) and an increase in STV-APD (**Figure 11D** and **Figure 11E**) compared to SED animals.

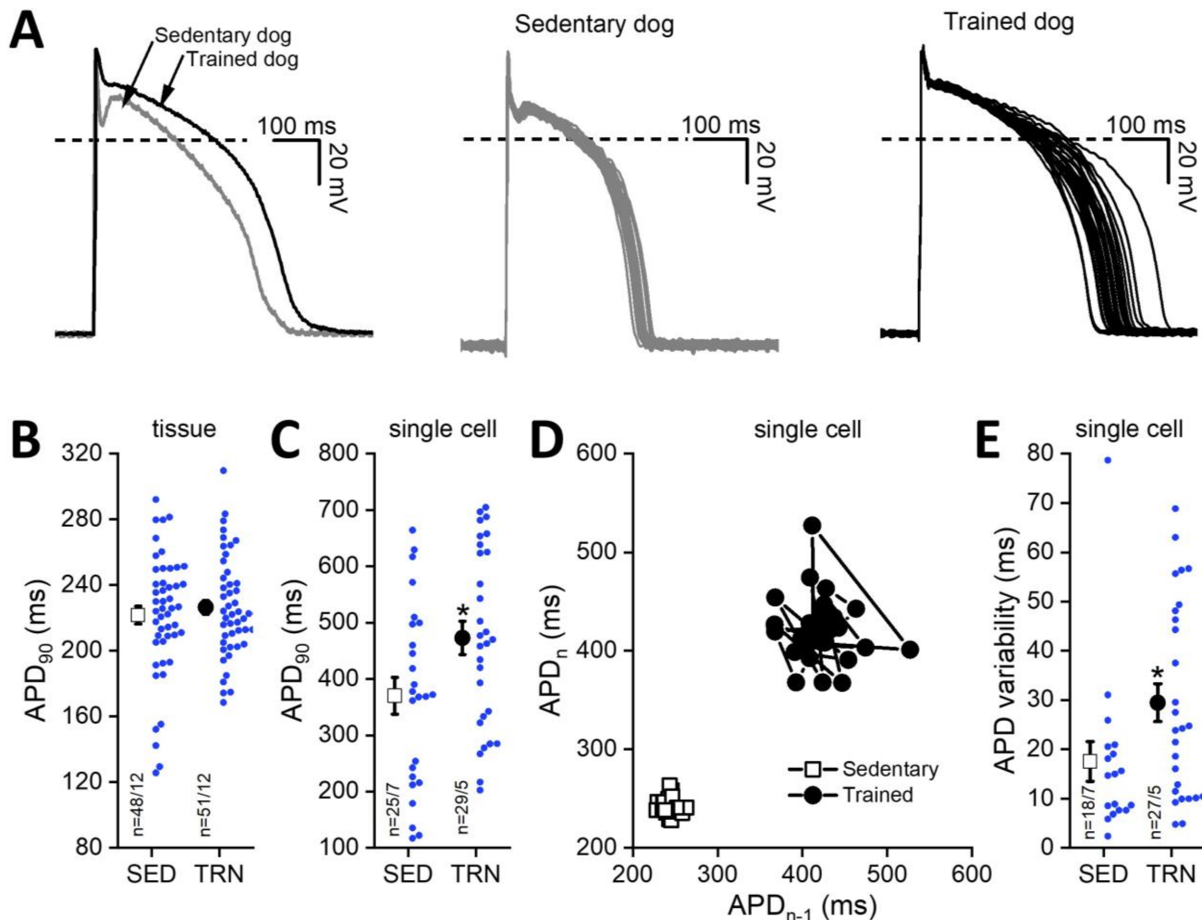

Figure 11. Various aspects of our study on the effects of long-term sustained training on cardiac action potential duration in dogs. A Left panel**.** Representative action potential curves recorded from isolated left ventricular myocytes of SED and TRN dogs, showing the prolonged action potential duration in TRN dog. Central (SED) and right (TRN) panels, 30–30 representative action potential curves, highlighting increased variability in action potential duration in TRN dogs, recorded from ventricular myocytes, respectively. **B.** presents a graph that illustrates the action potential duration at 90 % repolarization (APD_{90}) in SED and TRN dogs, recorded from papillary muscle (multicellular tissue preparations; n = 48 preparations/12 dogs in SED and n = 51 preparations/12 dogs in TRN groups). **C.** Graph showing the prolonged APD_{90} of enzymatically isolated left ventricular single myocytes from TRN dogs (n = 25 cells/7 dogs in SED and n = 29 cells/5 dogs in TRN groups). **D.** Representative Poincare plot demonstrating increased APD_{90} variability. **E.** Effect of long-term sustained training on APD_{90} variability of a single left ventricular myocyte (n = 18 cells/7 dogs in SED and n = 27 cells/5 dogs in TRN groups). The 'n' numbers refer to the number of preparations or cells followed by the number of dogs from which preparations or cells were obtained. Data are expressed as mean ± SEM, with blue dots representing individual data points. $^*p < 0.05$ TRN vs. SED group at 16th week.

5.7 Effects of chronic sustained training on various transmembrane ionic currents in canine left ventricular myocytes

As **Figure 12A** shows, the magnitude of the I_{to} current was significantly smaller in myocytes obtained from the trained dogs compared to the sedentary animals. However, there

were no significant differences in the magnitudes of the I_{NaL} (**Figure 12B**), I_{NCX} (**Figure 12C**), L-type I_{Ca} (**Figure 12D**), I_{K1} (**Figure 12E**), I_{Kr} (**Figure 12F**), and I_{Ks} currents (**Figure 12G**).

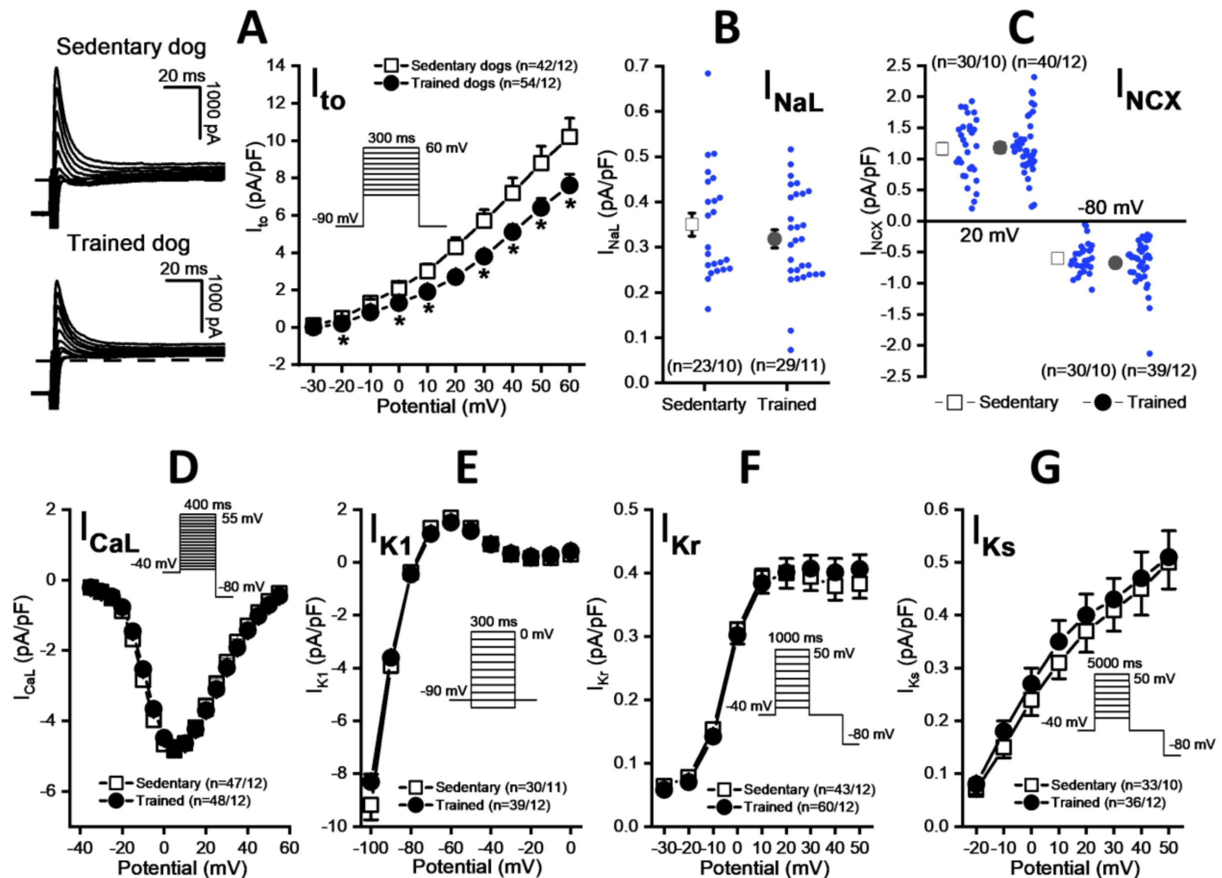

Figure 12. A. Effect of sustained training on transient outward potassium current (I_{to}); representative current recordings (left) and current-voltage relationships (right) in SED and TRN subjects. **B and C.** Graphs indicating that sustained training has no effect on either the late Na^+ current (I_{NaL}) or the Na^+/Ca^{2+} exchange current (I_{NCX}). **D–12G,** current-voltage relationships of the L-type Ca^{2+} current (I_{CaL}), the inward rectifier K^+ current (I_{K1}), the rapid (I_{Kr}) and the slow (I_{Ks}) delayed rectifier K^+ currents are similar for SED and TRN subject. Insets show the voltage protocols. The 'n' numbers refer to the number of cells followed by the number of dogs from which cells were obtained. Data are expressed as mean ± SEM. Blue dots represent individual data. *$p < 0.05$ TRN vs. SED group at 16th week.

5.8 The relative density of transmembrane Kv4.3 and KChiP2 proteins in trained and sedentary canine hearts

Further investigations were conducted to explore the molecular basis of the I_{to} current in the TRN dog heart. Specifically, the expression levels of the Kv4.3 alpha and KChiP2 beta channel subunits, which are considered the primary channel proteins underlying I_{to}, were assessed through Western blotting and immunocytochemistry measurements. As indicated in **Figure 13A-F**, no significant differences in the expression of Kv4.3 and KChiP2 proteins were observed between the left ventricular samples of the SED and TRN dog hearts. This suggests that the decrease in the magnitude of I_{to} current may be attributed to the changes in other less

well-characterized accessory proteins or alternatively to post-translational changes in ion channel proteins.

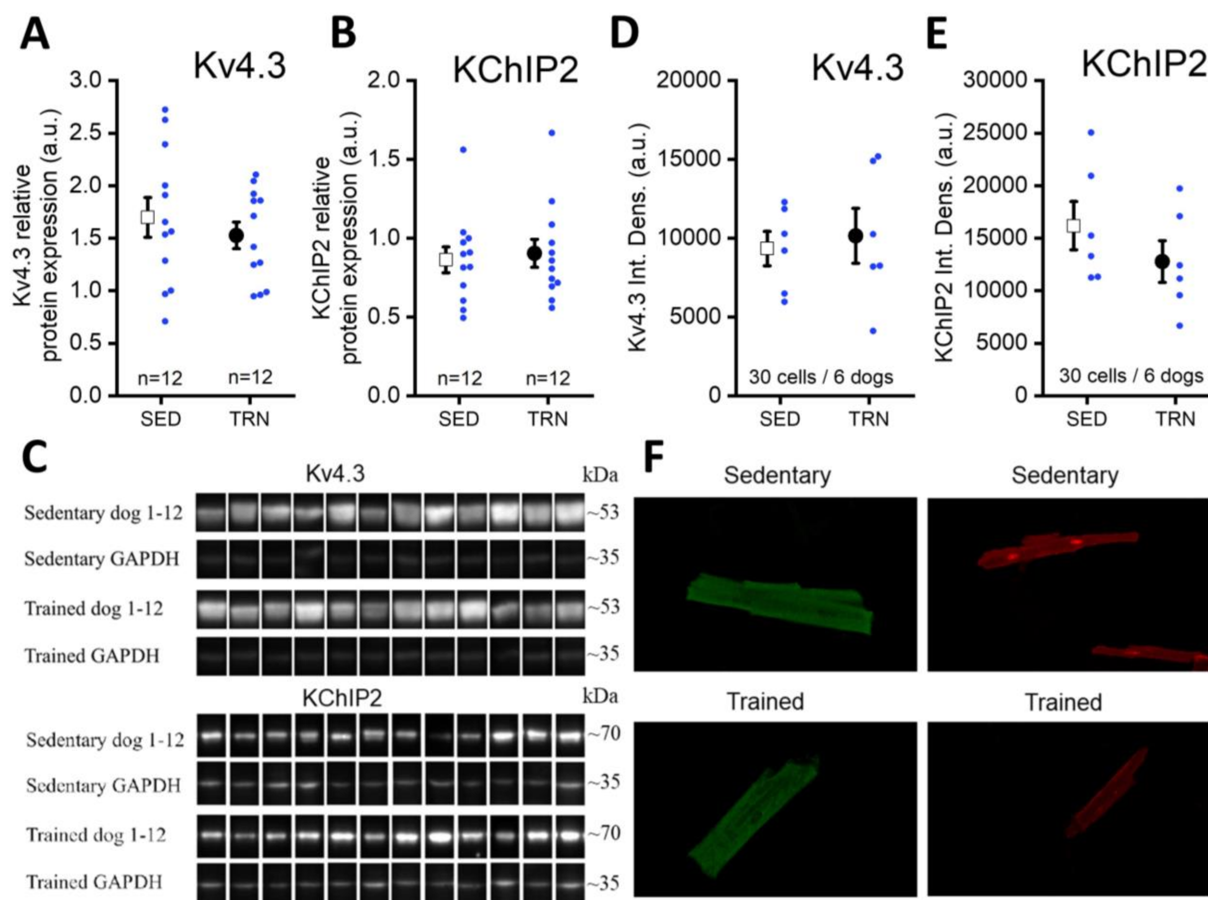

Figure 13. **A and B.** Relative protein expression of Kv4.3 and KChiP2 subunits determined by Western blotting in left ventricular samples of SED (n = 12) and TRN (n = 12) dogs, respectively. **C.** Representative image of Kv4.3 and KChIP2 bands and their corresponding loading controls (GAPDH). **D and E.** Relative densities of Kv4.3 and KChiP2 protein immunolabelling obtained from SED (n = 30 cells/6 dogs) and TRN (n = 30 cells/6 dogs) cardiomyocytes. **F.** Representative immunofluorescence images of canine cardiomyocytes with Kv4.3 and KChiP2 immunolabelling. The 'n' numbers refer to the number of dogs (12A and 12B) or the number of cells followed by the number of dogs from which the cells were obtained (12D and 12E). Data are expressed as mean ± SEM. Blue dots represent individual data.

5.9 HCN4 channel upregulation in left ventricles after sustained training

Immunocytochemistry of left ventricular myocytes in TRN dog hearts revealed significantly enhanced HCN4 protein expression compared to SED dogs (**Figure 14A**). No differences were observed in the expression of HCN1 and HCN2 proteins between the two groups (**Figure 14B** and **Figure 14C**).

5.10 Gene expression of fibrosis biomarkers in rabbit hearts after 16 weeks of training

The relative gene expression of fibrosis-related markers in rabbit left ventricular free wall was assessed after 16 weeks of endurance training. In the EX group, COL3A1, MMP-2, and TIMP-1 expression significantly increased compared to the SED group (**Figure 15**).

COL1A1 and FN-1 expression showed a modest, nonsignificant increase in the exercise group, while TGF-ß expression remained similar in both groups (**Figure 15**).

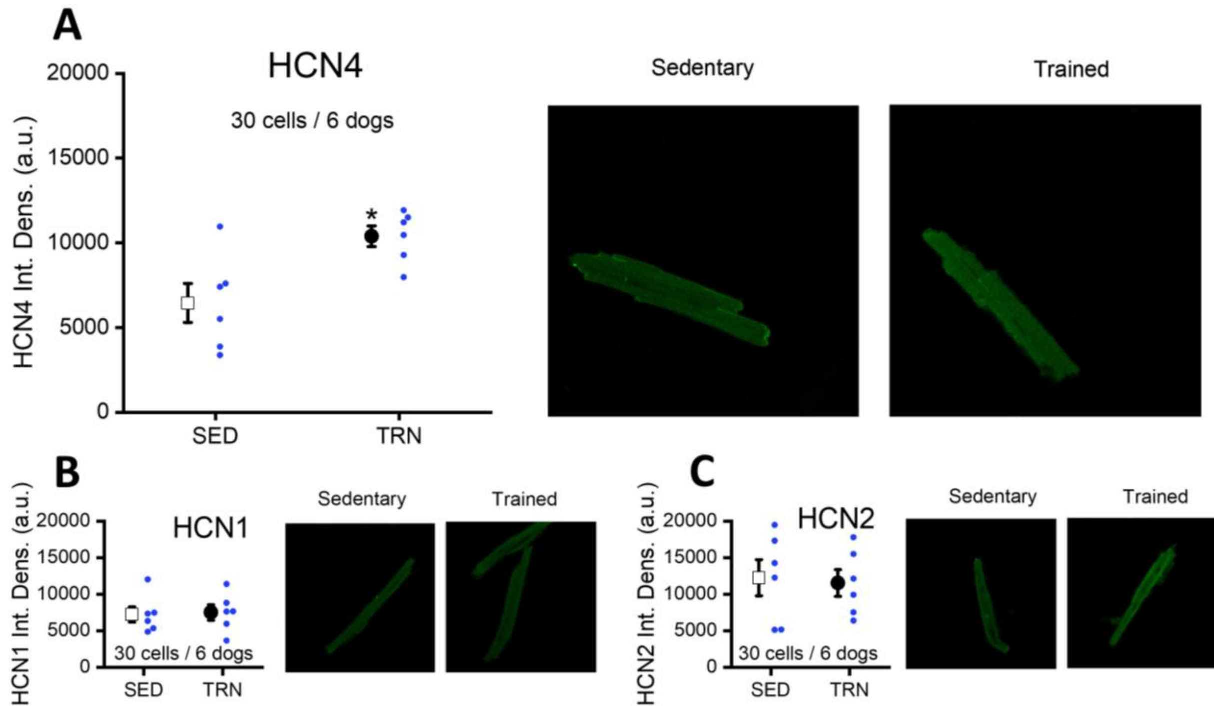

Figure 14. A. Relative density of HCN4 protein immunolabelling obtained from enzymatically isolated left ventricular myocytes of SED (n = 30 cells/6 dogs) and TRN (n = 30 cells/6 dogs) groups. **B and C,** No significant changes in the relative density of HCN1 and HCN2 proteins immunolabelling in left ventricular myocytes from SED (n = 30 cells/6 dogs) and TRN (n = 30 cells/6 dogs) groups. Representative original immunofluorescence images are shown on the right. The 'n' numbers refer to the number of cells followed by the number of dogs from which the cells were obtained. Data are expressed as mean ± SEM. Blue dots represent individual data. *p < 0.05 TRN vs. SED group at 16th week.

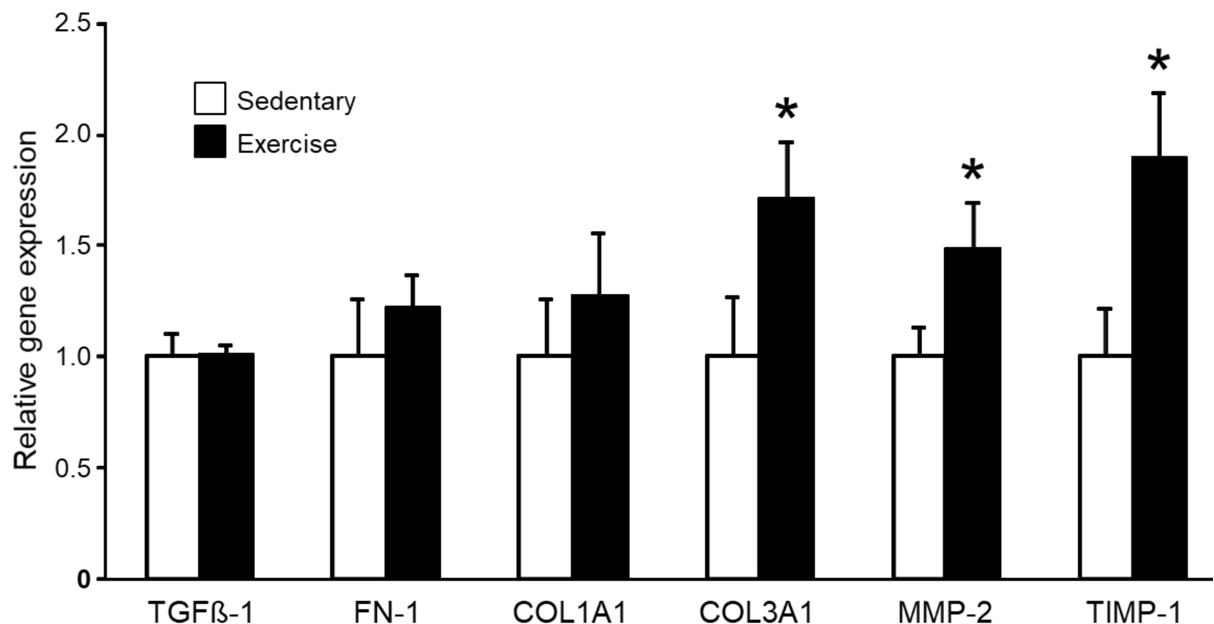

Figure 15. The relative expression levels of different fibrosis synthesis and degradation biomarker genes determined by real-time quantitative PCR (qRT-PCR). The mRNA levels were quantified in

tissue samples collected from the left ventricle (LV). The data are presented as mean ± SEM. *$p < 0.05$ vs. "Sedentary".

5.11 Testosterone levels in canines

Testosterone levels in DOP dogs' blood samples were higher over the 16-week training period than in the testosterone-free dogs (DOP vs. EX vs. SED in male: 19.4 ± 3.2 vs. 10.2 ± 1.4 vs. 11.2 ± 1.4 nmol/l, $p < 0.05$; in female: 18 ± 4.1 vs. < 0.43 vs. < 0.43 nmol/l, $p < 0.05$). No differences were observed between the groups in any other examined laboratory parameters, including electrolytes, renal, and hepatic functions (data not shown).

6. DISCUSSION

In this study, long-term endurance exercise was conducted on two non-rodent species with cardiac morphological, electrophysiological, and autonomic neural properties similar to humans. These animal models provided important insights into the electrophysiological characteristics of the athlete's heart and its associated elevated risk of ventricular arrhythmias.

The main findings of the study include:

1) Successful establishment of training protocols that address both morphology and electrophysiology, enabling the replication of the athlete's heart phenotype in two distinct, human-relevant animal models.
2) Sustained endurance training in dogs resulted in athlete's heart characteristics, including left ventricular hypertrophy and increased atrial and ventricular volumes, while less intense training protocols in rabbits and dogs did not affect ventricular wall thickness. This underscores the impact of training intensity on cardiac remodelling.
3) Sustained endurance training increased heart rate variability, indicating an increased parasympathetic tone, and decreased resting heart rate in both whole animal and *in vitro* experiments. These outcomes propose a parasympathetic impact and other factors besides vagal tone contributing to training-induced bradycardia. Parasympatholytic agent experiments in dogs further validated this hypothesis.
4) Sustained endurance training consistently prolonged cardiac repolarization, resulting in *in vivo* ECG QT_c lengthening and APD prolongation with reduced I_{to} current density in cellular measurements. These findings were associated with increased variability of cardiac repolarization, indicating impaired repolarization following intensive training.
5) No significant differences were observed in the expression of Kv4.3 alpha or KChIP2 beta accessory channel proteins between SED and TRN dog hearts, implying that reduced I_{to} current density may be influenced by other factors.
6) Trained dogs from both experiments exhibited higher sensitivity to the QT_c prolongation effect of the class III antiarrhythmic agent dofetilide during an *in vivo* proarrhythmic challenge, with an increase in the number and complexity of arrhythmias. However, the QT_c lengthening effect induced by training and dofetilide was additive rather than superadditive in this experiment.
7) Sustained endurance training increased the risk of ventricular fibrillation during electrical stimulation, indicating higher susceptibility to arrhythmias in trained dog hearts.

8) Sustained endurance training upregulated HCN4 channels in dog ventricular myocardium, potentially promoting arrhythmogenesis by enhancing ectopic electrical activity.
9) Sustained endurance training increased fibrosis in dog ventricular myocardium and elevated the expression of fibrotic genes in rabbit hearts, potentially contributing to arrhythmia sensitivity by creating arrhythmogenic substrates.
10) Administration of testosterone at a specific dose, along with a standardized endurance exercise program, did not induce an increase in cardiac muscle mass and had no significant impact on other structural and *in vivo* repolarization parameters in dogs.

6.1 Animal models of the athlete's heart: Insights from rabbits and canines

This study aimed to address the issue of increased unexpected death among elite athletes [19], which remains poorly defined, with underlying causes that are still open to investigation [29]. Our working hypothesis centred on exploring whether these tragic events are linked to changes in ventricular repolarization and fibrosis resulting from high-intensity exercise in competitive athletes [36, 60]. Due to various limitations, it is challenging to unravel the relevant mechanisms in humans; therefore, the use of suitable animal models with human relevance is essential. However, the model of choice needs to be considered carefully, since it vitally affects experimental outcomes. Larger animals, such as rabbit and dog characterize the human heart more accurately than those of smaller mammals in terms of oxygen uptake kinetics, cardiac mechanics, repolarization, excitation-contraction coupling, collateral coronary circulation, and cellular-subcellular architecture. Taking into account the kinetic properties of ion channels, such as the I_{Ks} current, measurements obtained from dog ventricles [61] and rabbit ventricles [62] closely resemble those observed in human hearts. In this regard, the current study aimed to examine the impact of rigorous endurance exercise training on cardiac remodelling in two distinct animal models: rabbits and canines. We implemented our self-developed, species-specific training protocols to ensure a targeted approach for each animal group. Based on recent research, the treadmill running model appears to be the preferred option for investigating the impact of chronic physical exercise due to its ability to provide uniform and well-controlled exercise workloads [63].

Previous research studies in this field mostly utilized small animal models, such as rodents, which provided valuable insights into electrophysiological remodelling, cardiac fibrosis, bradycardia development, and increased atrial fibrillation risk following exercise training [22, 46, 47, 49, 64, 65]. However, it must be emphasized that rodents have distinctly

different cardiac remodelling signs and ion channel expression patterns than other mammals, including humans. For example, rodent hearts must contract and relax more rapidly to maintain cardiac output at very high heart rates [66]. Furthermore, rodent action potentials have a rapid repolarization and lack a prominent plateau phase compared to human cardiomyocytes [67]. Rat and mouse myocytes also lack functional sarcolemmal I_{Kr} and I_{Ks} potassium currents [68], thus repolarization-related arrhythmias cannot be examined accurately in these species, and results cannot be directly extrapolated to humans. On the other hand, rabbits have a more comparable expression pattern of ion channels to human hearts, making them a suitable species for studying cardiovascular adaptations to exercise.

Rabbits offer advantages over small animal models like rodents, as they exhibit a more comparable expression pattern of ion channels and provide a closer resemblance to human cardiac electrophysiology. Additionally, rabbits are relatively cost-effective compared to larger animals such as canines, reducing expenses associated with the purchase, housing, and maintenance of animals. Their smaller size and ease of handling simplify experimental procedures, reducing the need for specialized facilities and equipment. Moreover, their higher reproduction rate enables researchers to generate larger sample sizes in a shorter time.

While some studies have explored acute and chronic rabbit exercise models, limited attention has been given to investigating the cardiovascular effects of exercise, especially with a specific emphasis on electrophysiological parameters [69]. Previous studies demonstrated that rabbits respond to cardiovascular training by adjusting intensity, duration, and frequency of exercise [70, 71]. Gaustad *et al.* [72] tested a maximal oxygen uptake protocol in rabbits and found them to be suitable for studying responses to training and revealing novel cellular cardiac adaptations. Hexeberg *et al.* [73] observed structural myocardial remodelling and increased contractile reserve in rabbits after a 10-week exercise training program, supporting their potential as a model for studying the effects of endurance training. Carroll and Kyser [74] employed a rabbit model to investigate the impact of exercise training during obesity development. Their study demonstrated that exercise-trained rabbits, subjected to a 12-week treadmill protocol at a maximum speed of 18–20 meters/minute (1.2 km/h) with daily running sessions lasting 50–60 minutes, exhibited slower resting heart rates compared to non-exercised rabbits. Such *et al.* [75] also found lower resting heart rates and longer ventricular effective refractory periods in rabbits during similar workload conditions.

In this study, we present a rabbit model that closely mimics a human athlete's heart, particularly at cardiac electrophysiological level. Using the New Zealand white rabbit, which is a relatively physically inactive species, we employed a treadmill running protocol that

induced physical tiredness and exhaustion, effectively simulating regular, high-intensity exercise training in humans.

Although some chronic exercise models have been reported in rabbits, there remains a lack of extensive data on cardiac electrophysiological remodelling following sustained exercise in larger animals like dogs, which have high relevance to human cardiac electrophysiology. Canine heart rate, heart weight, excitation-contraction coupling, action potential duration, and expression patterns of various ion channels are more comparable to humans [76]. Moreover, canines can increase their heart rate by approximately 96–136 % during maximal exercise, which is close to the 140–170 % increase observed in humans [77-79]. Existing data in canine models suggest that both acute [80] and adequate levels of chronic exercise [81, 82] can be beneficial in preventing acute ischaemia-induced ventricular fibrillation through delayed preconditioning [80] or restoration of the distorted parasympathetic–sympathetic balance observed after myocardial infarction [82, 83]. However, responses to training at above-optimal levels by elite athletes are still unclear. Additionally, limited data are available on cardiac electrophysiological remodelling after sustained exercise in sled dogs, with most investigations focused on *in vivo* ECG recordings. Studies documenting widened QRS complexes and prolonged QT intervals in these animals are scarce [84, 85], and investigations on arrhythmogenesis and cellular electrophysiological changes in association with these alterations are warranted.

Our study fills this research gap by providing comprehensive experimental data on arrhythmic electrophysiological remodelling and the associated increased arrhythmic risk at ventricular level in animal models of sustained chronic training. To the best of our knowledge, this is the first study to provide comprehensive experimental data on arrhythmic electrophysiological remodelling and the associated increased arrhythmic risk at the ventricular level in a canine model of sustained chronic training. Our findings suggest a novel hypothesis linking sudden cardiac death with vigorous endurance exercise. According to our hypothesis, severe ventricular arrhythmias may be associated with changes in cardiac repolarization observed after endurance exercise.

Consequently, both rabbit and canine models provide essential cardiac electrophysiological information on the athlete's heart. While the rabbit model offers advantages in terms of cost, time, and resource-effectiveness compared to larger animal models, the canine model represents the most human-relevant data. The selection of an animal model should always be guided by meticulous consideration of research questions, biological relevance, and overall study objectives.

6.2 Cardiac morphological remodelling in response to chronic exercise

The exercise-induced physiological cardiac response and adaptation are profoundly influenced by the frequency and specific nature of each sporting activity [7]. Therefore, it was crucial to verify the cardiac morphological changes resulting from the applied exercise modality in our experimental models.

Our endurance-trained rabbits exhibited increased cardiac left ventricular end-diastolic diameters and relatively greater aortic root diameters compared to the control group, indicating the effect of cardiac volume overload. These findings are consistent with a large cohort study of competitive athletes, where male endurance athletes showed greater aortic and left ventricular cavity dimensions than sex-matched strength athletes [11]. However, there were no significant changes in interventricular septum or posterior wall thickness, and left ventricular contractile function (e.g., ejection fraction and fractional shortening) remained normal in this rabbit model. Similarly, Such *et al.* [75] did not observe differences between sedentary and exercised rabbit hearts in terms of hypertrophy.

Endurance-trained athletes, including those engaged in activities like long-distance running or cross-country skiing, frequently demonstrate signs of cardiac enlargement, which may or may not be accompanied by noticeable increases in the thickness of the left ventricular wall [9, 86-88]. The discrepancies in echocardiography results concerning hypertrophy may arise from different hemodynamic and loading conditions of the heart, depending on various training activities [89]. Furthermore, the duration of long-term exercise can also impact the physiological remodelling of myocardial structure and function in athletes [90]. It is possible that after several months of continuous training, the morphological signs of wall thickness and ventricular hypertrophy may become evident in this rabbit model.

In our canine model, the structural changes were left ventricular chamber enlargement with increased wall thickness occurring in the trained group, as confirmed by both echocardiography and autopsy measurements. However, similar to human data, the ejection fraction remained unchanged [87, 91]. Our structural and hemodynamic outcomes typically correlate with the previously described exercise-induced cardiac remodelling in elite endurance athletes [10, 92]. Although the limitation of the model is that it does not correlate perfectly with any human physical activity, the change in left ventricular architecture suggests that it is close to that of "isotonic exercise" activities (e.g., long-distance running, cycling, and swimming), as both ventricular size and left ventricular mass increased, similar to chronic volume overload [93]. The differences in wall thickness changes between the two models may be attributed to interspecies factors; however, it is more plausible that they are influenced by the nature and

duration of the physical activity performed by the animals. Given the anatomical features of dogs, they are naturally suited for sustained dynamic running over long distances, unlike rabbits. This inherent difference may potentially explain the observed variations in wall thickness changes between the two animal models.

Our findings in the atria of trained dogs correspond to highly trained athletes, who also develop moderately enlarged left atrial volume and left atrial volume index, as a potential physiologic adaptation to exercise conditioning [9]. Although our work has focused primarily on changes in the left ventricle, it is important to highlight that morphological changes involving the atria may also be the origin of arrhythmias such as atrial fibrillation that is commonly observed in athletes [94].

6.3 The complex mechanism of bradycardia

In the present study, prolonged PQ intervals (representing the atrioventricular conduction time) and significant reductions in baseline heart rate accompanied by significant increases in almost all examined heart rate variability parameters were found in ECG recordings in both rabbits and dogs after chronic exercise. Furthermore, only a moderate heart rate increment was found after the administration of a parasympatholytic agent, and the bradycardia also persisted under *in vitro* conditions, examined in dogs.

Although bradycardia is a general and well established finding in elite athletes [95] and in animal models of endurance exercise [96, 97], its exact mechanism is still a matter of debate [47, 96-100]. The most common interpretation attributes bradycardia to increased vagal tone both in athletes [97] and in animal exercise studies [96, 100] as well. However, this theory has generated some disagreement [47, 101, 102], and a more satisfactory approach remains to be explored. A very recent publication by Mersica *et al.* [48] revealed that the slowing of atrioventricular conduction persisted after vegetative blockade in racehorses and in mice after swimming-induced exercise. This study further argues for the electrical remodelling of the sinoatrial node (SAN), more specifically the reduction in hyperpolarization-activated "funny" current (I_f) density and the remodelling of the underlying HCN4 ionic channel, as previously published in SAN preparations of mice [47].

The present data can only partially support the observations of Souza *et al.* [47] and Mersica *et al.* [48], as ionic mechanisms underlying SAN cell automaticity including the I_f and the SAN hyperpolarization-activated cyclic nucleotide-gated channel (HCN) isoforms have not been investigated in this work. Although the data available in our study were limited, our model appeared to be consistent with the results of the previously mentioned studies. Specifically, it

demonstrated a notable level of sinus bradycardia in isolated right atrial preparations from trained dog hearts following the termination of the autonomic system. Moreover, the observed moderate heart rate increment after administering a parasympatholytic atropine infusion in the exercised dog group suggests that long-term endurance training may lead to intrinsic adaptations in the sinoatrial node in addition to the previously identified increased vagal tone.

On the other hand, the increased beat-to-beat variability of cycle length and first-degree atrioventricular block argue for an important contribution of enhanced vagal tone as well, as these values are considered parameters of parasympathetic activity [13] and are especially common among athletes with high aerobic resistance.

It should also be emphasized that the pacemaker function of the SAN is complex and cannot be satisfactorily explained by the I_f current alone. Since activation of I_f occurs largely at voltages more negative than the maximal diastolic potential of SAN cells, the special importance of I_f as the main pacemaker current of the SAN has even been questioned [103, 104]. In addition, other mechanisms based on calcium handling [105-107] or on the contribution of other ion channels have been proposed [108-110] to explain the cardiac pacemaker effect of SAN. In summary, our present data suggest that both changes in the cardiac autonomic regulation and intrinsic SAN changes are parallel responses to vigorous exercise, regardless of the still unexplored mechanisms.

6.4 Mechanistic basis of arrhythmias in the athlete's heart model

Irrespective of the underlying cause of bradycardia, a reduced heart rate can result in a longer action potential duration and increased dispersion of cardiac repolarization. This phenomenon may contribute to an increase in the arrhythmic substrate factor in the classical concept of the “arrhythmic triangle”, which suggests that arrhythmias occur when certain combinations of substrates, triggers, and arrhythmia-promoting modulators are present [31, 32].

Moreover, bradycardia resulting in longer diastolic intervals may enhance the likelihood of spontaneous diastolic depolarization reaching the firing threshold, thereby serving as a potential arrhythmia trigger [31].

Notably, bradycardia, hypokalaemia, and female gender are well-known risk factors for drug-induced arrhythmias, including Torsades de Pointes [111, 112]. Specifically, bradycardia increases the torsadogenicity of drugs that block I_{Kr} channels, as these medications block K^+ channels in a reverse-use-dependent manner [113, 114]. Therefore, it is crucial not to underestimate the substantial reduction in heart rate observed in athletes, particularly when hypokalaemia accompanies extensive sweating. Under such circumstances, seemingly harmless

drugs with minimal effects on cardiac repolarization could potentially precipitate life-threatening arrhythmias.

Furthermore, it is important to highlight that significant bradycardia in the present study was associated with an increased number of escape beats. Well-trained athletes often exhibit slow heart rates, occasionally experiencing sinus pauses and frequently manifesting multiple escape beats. Escape arrhythmia is considered a compensatory mechanism induced by increased vagal tone and/or disturbances in the SAN or other components of the cardiac conduction system. It may also be interpreted as a form of ectopic pacemaker activity unveiled when other pacemakers fail to stimulate the ventricles. While escape arrhythmia is generally regarded as a benign ECG pattern in athletes, which vanishes during exercise as vagal tone decreases, an earlier study demonstrated that vagally mediated heart rate reductions were necessary for the induction of TdP in $\alpha 1$-adrenoceptor-stimulated anaesthetized rabbits. In this context, vagotomy prevented TdP in rabbits given phenylephrine [115]. Consequently, alterations in vagal activity may also precipitate episodes of drug-induced TdP in human athletes, necessitating careful consideration when prescribing drugs in this population.

The coexistence of the widened QRS complex, which is associated with the P wave on the ECG in our experiments also suggests electrical conduction abnormalities [116], further underscoring the significant impact of intense exercise on the cardiac conduction system, which is still not fully elucidated.

Pacemaking is a fundamental physiological process, and the cellular mechanisms involved in this function have always attracted the keen attention of investigators. The hyperpolarization-activated cyclic nucleotide–gated 4 (HCN4) channel serves as the major constitutive subunit of f-channels in pacemaker cells of the sinoatrial node [117-119]. HCN channels are also found in adult atrial and ventricular cardiomyocytes, where their physiological roles are currently under investigation. Dysfunctional HCN channels have been implicated in various cardiac pathologies as direct contributors to rhythm disorders. Loss-of-function mutations of HCN channels are associated with sinus bradycardia, while HCN channel gain-of-function may enhance ectopic electrical activity and promote arrhythmogenesis in conditions like atrial fibrillation, ventricular hypertrophy, and heart failure. Emerging evidence indicates high expression of HCN channels in the left ventricle of hypertrophic heart and heart failure, indicating their involvement in augmented arrhythmogenic activity [120, 121].

Notably, despite the observed bradycardia in both *in vivo* and *in vitro* settings, which would typically indicate lower expression of HCN channels, a significant increase in HCN4 protein expression was observed in left ventricular myocytes after chronic exercise in this

model. This apparent contradiction might be attributed to tissue-specific regulation of HCN4 channels in the heart. Furthermore, one limitation of the study is that ionic mechanisms underlying SAN cell automaticity, including the I_f current and the SAN hyperpolarization-activated cyclic nucleotide-gated channel isoforms, have not been investigated, as previously mentioned.

In addition to the increased HCN4 expression in left ventricular myocytes and other potential arrhythmia-promoting factors discussed earlier, the incidence of ventricular electrical stimulation-induced ventricular fibrillation also increased in trained animals. This observation supports the hypothesis that HCN4 protein overexpression in the ventricle may facilitate enhanced ectopic electrical activity and promote arrhythmogenesis. Notably, to the best of our knowledge, this is the first time such findings have been described, not only in pathological processes, but also in the context of well-trained athletes with a considered healthy heart.

Examining the myocardial extracellular matrix (ECM) is essential in elucidating the remodelling process within the human athlete's heart, as it constitutes a complex microenvironment with various matrix proteins, signalling molecules, proteases, and cells that play a crucial role in remodelling [122].

Focused on this particular aspect, a consistent histopathological finding in our study revealed a higher level of fibrosis in the left ventricular muscle of trained rabbits and dogs. These results align with previous studies on chronic endurance exercise in humans and animal experiments [35, 123, 124]. For example, increased fibrosis and inflammatory infiltrates have been identified in well-trained athletes [91], in forced swimming rats [125], and in treadmill running guinea pigs [124]. Studies on “marathon rats” after treadmill exercise demonstrated increased atrial and ventricular inflammation, fibrosis, and a higher risk of ventricular arrhythmias [49].

In correlation with the histopathological findings, our results showed significantly increased gene expressions of MMP-2 and TIMP-1, pivotal regulators of cardiac ECM degradation [126], indicating higher fibrotic activity in exercised hearts. Similar findings were reported in rats after acute exhaustive swimming [127] and explanted human hearts with heart failure due to various cardiomyopathies [128].

Furthermore, we observed a significant increase in the relative gene expression of collagen type III. Since collagen type III is a major component of the cardiac ECM, its qualitative-quantitative changes may have important implications for cardiac pathophysiology. The higher collagen synthesis promotes increased expression of TIMP and MMP, contributing to fibrosis.

Similar findings were reported in studies investigating cardiac remodelling in heart failure [129] and in spontaneous hypertensive rats, in which the ratio of collagen type I to type III was decreased after 10 weeks compared to normotensive control rats, while the total collagen concentration did not differ between the groups [130]. Additionally, increased levels of type III collagen have been observed in various other fibrotic conditions, such as liver and kidney fibrosis [131-133]. These observations further support the role of collagen type III in the fibrotic processes in different tissues and pathological conditions.

Histological analysis of the rabbit and dog hearts in our study supported the presence of long-term exercise-induced fibrosis in the myocardium, consistent with our rabbit heart gene expression data. The accumulation of collagen and subsequent myocardial fibrosis may result from an imbalance between synthetic and degradative processes, necessitating further investigations in this area. Although this structural abnormality may not be evident in non-invasive methods like electrocardiography and echocardiography, it could greatly impact the onset and the modulation of re-entry ventricular arrhythmias, representing a potentially dangerous myocardial arrhythmia substrate.

6.5 Impacts of exercise on ventricular repolarization in the canine and the rabbit athlete's heart models

Prolongation of the ECG QT_c interval, bradycardia [33], and increased spatial and temporal dispersion of repolarization have been reported in athletes, as evidenced by the prolongation of the T_pT_e interval and short-term variability of the QT interval observable on ECG [34]. For instance, early research findings indicated that repolarization parameters are elevated in elite soccer players [34]. However, it remains uncertain whether these observed changes in athletes, which are generally thought to be potentially harmful, are directly associated with an increased risk of mortality. Additionally, there is limited data available regarding cardiac repolarization in exercise-induced physiological cardiac remodelling, particularly in animal models, thus we conducted experiments in both dog and rabbit hearts to further investigate cardiac repolarization impairment.

In our experiments, we consistently observed cardiac repolarization impairment in both dog and rabbit hearts. This was evident as a prolonged QT_c interval in conscious dogs *in vivo* and as a prolonged action potential duration at the cellular level in isolated left ventricular myocytes in the trained dog hearts. These findings were associated with increased variability parameters, specifically the short-term variability (STV) of the QT interval on the ECG, and also the STV of APD in cellular canine measurements. This suggests an increased spatial and

temporal dispersion of repolarization in our dog model. In our rabbit study, some of the QT interval variability parameters also showed a tendency to increase in the exercised rabbits. It is worth noting that higher instability in repolarization variability parameters increases proarrhythmic activity, even in the presence of lengthened or shortened ADP, as reported by Hondeghem *et al.* [134]. In addition, STV QT is known as a reliable predictor of proarrhythmia in reduced repolarization reserve [135]; therefore, its increment may represent a greater risk for arrhythmia development.

It has been previously hypothesized that endurance-trained animals may exhibit a certain degree of repolarization impairment, even in the absence of clinical symptoms or measurable QT interval prolongation during baseline assessment, and this effect might be unmasked through potassium channel inhibition [136, 137]. To test this hypothesis, our current study examined the impact of dofetilide, a selective I_{Kr} channel blocker, on QT_c interval prolongation in exercised dogs *in vivo*, comparing it to the sedentary group. Dofetilide exhibited a propensity to equally extend QT_c intervals in exercised and sedentary dogs *in vivo*. In another unpublished study conducted by our research group, however, dogs subjected to a more intensive training protocol and exposed to the same concentration of dofetilide exhibited greater QT_c interval prolongation, an elevated frequency of ventricular beats, and the emergence of more complex arrhythmias. According to Farkas *et al.*, the complexity of the arrhythmic beats is an important factor in TdP genesis [138]. These findings underscore the role of intense training in inducing repolarization impairment and heightening the risk of life-threatening arrhythmia development.

In trained, isolated rabbit hearts exposed to the I_{Kr} blocker dofetilide, we also observed a nearly significant QT_c prolongation. The papillary muscles from trained rabbit myocardium exhibited increased sensitivity to the effects of dofetilide, including APD prolongation and increased triangulation, particularly under low potassium concentrations. This phenomenon may play a role in the development of arrhythmias. It is worth noting that hypokalaemia is known to enhance I_{Ks} and decrease I_{Kr} current [139]. Consequently, the use of dofetilide, which inhibits I_{Kr} current, could have a more pronounced impact on repolarization when I_{Ks} is downregulated [52], and hypokalaemia cannot effectively compensate for the reduction in I_{Kr} density. These specific findings were not presented in this dissertation [123], but they contribute to a deeper understanding of repolarization impairment, a phenomenon that can be detected in both models following intense, chronic exercise.

In our long term trained animal models, we investigated the possible cellular mechanism underlying repolarization impairment. In the canine model, we found that a reduction in the

magnitude of the I_{to} current in midmyocardial myocytes could be responsible for this effect. The I_{to} current plays a crucial role in phase 1 repolarization of the action potential in left ventricular midmyocardial myocytes, as established by previous research [140]. These findings align with consistent results published in failing canine [141] and human [142] hearts, where APD prolongation correlated with reduced I_{to} current. In alignment with the transmembrane current data, notable prolongation of the APD was exclusively observed in myocytes derived from the midmyocardial region of the left ventricle, where a robust I_{to} current is anticipated [143, 144]. Conversely, in subendocardial left ventricular papillary muscle preparations, where a relatively weak magnitude of I_{to} current has been reported [144, 145], no significant APD prolongation was detected.

Interestingly, in our current experimental patch-clamp measurements, we did not observe significant differences in the magnitudes of various transmembrane ionic currents, which are crucial for the initiation and maintenance of repolarization as well as the plateau phase of the action potential (such as I_{Kr}, I_{Ks}, I_{K1}, I_{CaL}, I_{NaL}, and I_{NCX}), between the examined groups in native canine left ventricular myocytes. However, it is worth noting that, under different conditions, possible intracellular signalling pathways might influence their function.

In our current study, the observed lower I_{to} current density in trained dogs did not seem to be the result of reduced expression of Kv4.3 alpha or KChIP2 beta accessory channel proteins, as neither Western blotting nor immunohistochemistry revealed any difference in protein expression between sedentary and trained heart. Similar findings, specifically a decrease in I_{to} current density without alterations in the expression of Kv4.2 and KChIP2 proteins, were previously reported in chronically exercised rats [146].

While Kv4.3 and KChIP2 are regarded as the primary proteins determining I_{to} in the canine heart [147], it is essential to emphasize that other I_{to} accessory proteins, which were not examined in the present study, such as Kvbeta1, Kvbeta2, [148, 149], I_{Na} beta1 [150], DPP6 [151, 152], DPP10 [153], could also influence the function of the I_{to} channel [148]. Additionally, I_{to} may be modulated by the activation of protein kinase A (PKA), protein kinase C (PKC), or both [154], and its characteristics may change following exercise. Since we did not investigate this aspect in the present study, the potential effects of PKA and PKC modulation cannot be ruled out.

We believe that the repolarization changes induced by training are mild in both of our models, similar to that is observed in the human athlete's heart. Even if these effects are found to be minimal, they may accumulate with other potentially harmful factors (such as non-steroidal agents, histamine H1 receptor antagonist, dietary components, doping, or mild fibrosis

in the ventricles after strenuous exercise), leading to a dangerous increase in repolarization inhomogeneity, thereby creating a significant substrate for arrhythmias in the athlete's heart. Moreover, the observed changes may expand the vulnerable window for extrasystoles to trigger ventricular arrhythmias [31]. It is worth noting that repolarization variability changes were detected both *in vivo* and *in vitro*, suggesting that this variability parameter may serve as a valuable and easily measurable biomarker, even in future human studies. It can serve as an indicator of repolarization abnormalities on the electrocardiogram, which may potentially exist at the cellular level.

6.6 Steroid-induced cardiovascular risk among endurance athletes

Anabolic-androgenic steroids (AAS) are synthetic compounds that mimic testosterone, gaining popularity in sports due to their muscle-building and performance-enhancing effects. While AAS are commonly associated with muscle growth and enhanced strength, there has been limited research on the misuse of steroids among endurance athletes, such as long-distance runners and cyclists, who prioritize fitness and stamina over muscle mass and explosive power. Nevertheless, documented cases of steroid misuse exist among endurance athletes, driven by various motivations, including faster recovery between training sessions or competitions, preservation of lean muscle mass during intense training or calorie restriction, and the psychological benefits that include increased confidence, focus, energy levels, and aggressiveness, all perceived as advantageous in competitive settings [155].

It is essential to recognize that steroid misuse poses significant cardiovascular risks. AAS can elevate blood pressure through mechanisms such as sodium retention and activation of the sympathetic nervous system, thereby increasing the risk of atherosclerosis. They may have a negative impact on lipid profiles, further contributing to the development of atherosclerosis and coronary artery diseases. Additionally, steroid abuse may predispose athletes to thrombotic events, potentially leading to stroke or myocardial infarction [156]. Steroids may induce cardiac hypertrophy and impair ventricular diastolic function, thereby increasing the risk of heart failure. This is especially concerning when combined with exercise, as it can shift the physiological cardiac remodelling process, including training-induced cardiac hypertrophy and some fibrosis - elements that, as previously discussed, might already predispose to arrhythmias - towards pathological hypertrophy, thus raising the risk of life-threatening arrhythmias [157, 158]. AAS users often exhibit abnormal cardiac repolarization, including prolonged QT_c intervals and increased QT dispersion, even at rest and after exercise, along with diminished heart rate recovery compared to non-AAS users. Moreover, unusual

findings in signal-averaging electrocardiography after extended use of AAS indicate the possible existence of a re-entry mechanism that contributes to arrhythmias. These abnormalities, combined with acute exercise-induced sympathetic activation that lowers the ventricular fibrillation threshold, increase the risk of tachyarrhythmias and potential sudden cardiac death [159-161]. While a number of studies link AAS abuse to cardiac arrhythmias and SCD [162], the exact connection between AAS abuse and cardiac arrhythmias and sudden cardiac death may be underestimated due to limited access to autopsy data and athletes' reluctance to report steroid use, given the potential penalties, including disqualification and suspension from competitions [163].

Our research focused on the relatively understudied area of investigating the cardiovascular impacts of testosterone in the context of endurance sports. Specifically, we examined the potential of testosterone undecanoate [164], an injectable, long-acting form of testosterone commonly used in hormone replacement therapy, to induce direct cardiac electrophysiological changes and enhance the risk of proarrhythmia in hearts adapted to endurance exercise. We selected testosterone undecanoate due to its legal accessibility and popularity among athletes, as indicated by our preliminary surveys among this population, making it a practical choice for experimentation. Additionally, its extended duration of action allowed us to administer it at a supra-therapeutic dosing frequency, thereby achieving elevated testosterone levels in our animal subjects while minimizing discomfort and the risk of infections. To simulate real-world scenarios, we combined testosterone administration with our standardized endurance exercise program.

The results showed minimal changes in mood and libido, slight enhancements in body muscle mass, and no significant changes in cardiac muscle mass or other structural and repolarization parameters, with unaffected serum electrolyte and haematocrit levels, compared to those endurance-trained animals who did not receive any testosterone treatment.

The relatively mild changes observed in our study could be attributed to several factors. Firstly, some endurance athletes and bodybuilders may use higher doses of anabolic steroids than those employed in this study. Determining the precise steroid dosages employed by athletes in real-life scenarios is challenging due to their unpredictable and fluctuating patterns of use. Additionally, it is well-established that AASs induce cardiac hypertrophy by directly targeting cardiac androgenic receptors, with the extent of these effects directly correlated with the dose, duration, and frequency of administration [165]. Our study primarily focused on developing a doping animal model with mildly and consistently elevated serum testosterone levels. It is

conceivable that more pronounced effects could be observed with larger doses and more frequent administration of testosterone.

It is also essential to consider that AAS users frequently combine these substances with other drugs, such as cocaine, methamphetamine, and smart drugs. This co-administration of multiple substances amplifies the risk of adverse drug interactions, exacerbating potential adverse effects, including the heightened risk of sudden cardiac death [166]. Furthermore, it is plausible that the impact of steroid doping may vary across different sports disciplines.

In summary, our research contributes to the expansion of scientific knowledge regarding the cardiovascular impacts of testosterone in the context of endurance sports. While we observed relatively minor steroid-related effects in our animals, using our specific training protocol, this has improved our understanding of the complex relationship between hormone administration and cardiovascular factors in this athletic context. Nonetheless, further research is essential to gain a deeper understanding of the mechanisms underlying these cardiovascular implications. This will empower healthcare professionals, athletes, and coaches to better recognize these risks and emphasize the importance of actively promoting education, prevention, and harm reduction strategies to mitigate the potentially life-threatening consequences associated with the misuse of steroids in sports.

7. CONCLUSION

The present study suggests that running-specific vigorous endurance training leads to the prolongation of cardiac repolarization and an increase in repolarization instability. These changes are associated with mild ventricular fibrosis in animal models mimicking the human athlete's heart. While exercise-induced adaptive mechanisms in the athlete's heart are typically seen as distinct from pathological conditions, our findings indicate that intense exercise may not always be entirely beneficial for the cardiovascular system. In fact, increased arrhythmia susceptibility may also develop after vigorous exercise. It is important to note that this does not necessarily mean that exercise at a competitive level is harmful, as it tends to induce ventricular arrhythmias in a normal, healthy heart. However, in certain individuals or in situations where the repolarization reserve is impaired due to underlying conditions like hypertrophic cardiomyopathy, long QT syndromes, diabetes, electrolyte imbalances, or the use of doping substances or seemingly benign medications, endurance training could pose an additional risk factor that needs to be considered to prevent potential adverse events in competitive sports.

In summary, the mechanisms contributing to arrhythmias in the athlete's heart model involve a complex interplay of various factors. This study provides valuable insights into

understanding the multifaceted nature of arrhythmogenesis in the athlete's heart model. These insights may have implications for ensuring the safe prescription of medications and exercise protocols within this specific population.

8. LIMITATION

Our study has some limitations. First, rabbits do not tolerate heavy exercise well, resulting in relatively modest exercise-induced changes. In relation to the enhanced myocardial fibrosis mechanism in our rabbit hearts, it is worth noting that mRNA levels not always correspond to protein levels, allowing for the possibility of different protein levels compared to mRNA levels. Taking into consideration that the maximum number of animals used in each training session is usually less than in rodent studies, the advantage of the rabbit and canine experiments itself is also their limitation. In the initial dog experiments, a low ‘n’ number was utilized, hindering proper group comparisons. This protocol was less rigorous than the one used in the second set of experiments, and these differences in training intensity are also reflected in our results.

Canine experiments, while having high translational value [167], still exhibit significant electrophysiological differences in repolarization reserve compared to the human heart [56]. Additionally, QT_c measurements in rabbits are somewhat uncertain due to the distinct frequency-dependent repolarization relationships compared to humans [168]. Rabbit myocardium also differs from the human heart in terms of ionic channel profiles (e.g., lower expression of I_{Ks} in rabbits) [169, 170]. In our experiments, we used open chest procedures and epicardial stimulation, rather than using a transvenous catheter approach, to induce ventricular arrhythmias due to experimental setup and time constraints. Furthermore, we lacked dedicated software for fully quantitative fibrotic area measurements, so the otherwise widely accepted semi-quantitative method was used. In *in vitro* studies, due to limitations in our research facility, our studies mainly focused on left ventricular transmembrane ionic currents, without delving into other cellular mechanisms or assessing the right ventricular myocardium.

Elite athletes undergo several years of training to achieve peak performance, while our study was limited to a 4-month intense training period, making cross-species exercise duration translation challenging. Ventricular arrhythmias related to athlete's heart are rare, and the associated cardiac electrophysiological changes are relatively small. Additionally, it is essential to note that cardiac arrhythmias and sudden cardiac death in athletes often result from a combination of factors, such as hypertrophic cardiomyopathy, drug use, and hypokalaemia. However, these factors were not investigated in our study.

9. ACKNOWLEDGEMENT

This work was carried out at the Department of Pharmacology and Pharmacotherapy, Szent-Györgyi Albert Medical School, University of Szeged. I extend my sincere appreciation to **Professor István Baczkó, MD, Ph.D.**, the Head of the Department, for providing the opportunity to engage in scientific research as a Ph.D. student. I am also grateful to **Professor László Dux, MD, Ph.D., DSc.**, the Head of the Doctoral School of Multidisciplinary Medical Sciences, for allowing me to conduct doctoral studies at the University. I would also like to thank **Professor Tamás Forster, MD, Ph.D., DSc.,** the former Head of the Department of Internal Medicine and Cardiology Centre, for his support during my Ph.D. studies.

I would like to express my deepest gratitude to **Professor András Varró, MD, Ph.D., DSc**, the former Head of the Department, for his invaluable guidance and mentorship throughout my Ph.D. studies. His expertise and support have played a pivotal role in shaping the direction and quality of this work.

Most importantly, I would like to express my sincere gratitude to my all-time Ph.D. supervisor since undergraduate years, **Attila Farkas, MD, Ph.D.,** for introducing me to the field of cardiology and cardiac electrophysiology and for teaching me the professional skills and critical thinking I need at scientific work. His stimulating enthusiasm, kindness, and continuous support guided me through my doctoral studies.

I also extend my thanks to **András Farkas, MD, Ph.D.,** for his invaluable assistance during my Ph.D. studies. His teaching and encouragement were also essential to me through the first steps of my scientific career. I am grateful to **Professor Gyula Papp, MD, Ph.D., Dsc, Professor Norbert Jost Ph.D., DSc,** and **László Virág Ph.D.,** for their professional help, suggestions and support, as well as inspiring discussions and excellent advice.

I am also grateful for my closest colleagues and friends at the same time, **Noémi Zombori-Tóth, MD, Leila Topal, Pharm.D, Noémi Tóth, MD, Ph.D., Jenő Pintér, MD, Zoltán Husti, MD, Ph.D., Tibor Hornyik, MD, Ph.D., Gergely Ágoston MD, Ph.D., Norbert Nagy, Ph.D., Zsófia Nagy, Ph.D., Nikolett Morvay, Ph.D., Szilvia Déri, Msc, Vivien Demeter-Haludka, Ph.D., Péter Kui, MD, Péter Gazdag, Ph.D., Balázs Ördög, Ph.D.,** and **János Prorok, Ph.D.** Their support, assistance, and collaboration over the years have been indispensable. Without their expertise, perennial curiosity, and perseverance, the scientific work presented above would have been poorer.

I also express my gratitude to the entire staff of the Department of Pharmacology and Pharmacotherapy in Szeged for their continuous support. Special thanks to **Mária Kosztka,**

Melinda Molnár, Anikó Deákné Tóth, Gábor Girst, Gábor Dobai, and **Róbert Motzwickler** for their excellent administrative and technical support. Above all, I would like to acknowledge the unwavering support of my family and friends, whose encouragement and understanding sustained me throughout this endeavor.

10. FUNDING

The work based on the "Cardiac electrophysiological remodelling associated with enhanced arrhythmia susceptibility in a canine model of elite exercise" article the National Research Development and Innovation Office (NKFIH K 135464 to AV, NKFIH PD-125402 and FK-129117 to NN, NKFIH K 128851 to IB, SNN-134497 to VV and GINOP-2.3.2.-15-2016-00047 and TKP2021-EGA-32), the Ministry of Human Capacities Hungary (20391 3/2018/FEKUSTRAT and EFOP-3.6.2-16-2017-00006), the UNKP-20-5-SZTE-165, János Bolyai Research Scholarship of the Hungarian Academy of Sciences (to NN), the Eötvös Loránd Research Network and by the Albert Szent-Györgyi Medical School institutional grant (SZTE ÁOK KKA 2021 to LV).

The work based on the "Long-term endurance training-induced cardiac adaptation in new rabbit and dog animal models of the human athlete's heart" article was supported by Hungarian Scientific Research Fund [OTKA K 119992], the UNKP-17- 4 and UNKP-18-4-SZTE95 new National Excellence Program of The Ministry of Human Capacities, GINOP-2.3.2-15-2016-00047, the EFOP-3.6.2- 16-2017-00006 and János Bolyai Research Scholarship of the Hungarian Academy of Sciences (N.N), the NKFIH PD-125402 (N.N) and by FK-129117 (N.N) projects.

The work based on the "Long-term endurance exercise training alters repolarization in a new rabbit athlete's heart model" article was supported by the National Research Development and Innovation Office (NKFIH K-135464, NKFIH K-128851, FK-129117, and GINOP-2.3.2-15-2016-00047), the Ministry of Human Capacities Hungary (20391-3/2018/FEKUSTRAT and EFOP3.6.2-16-2017-00006), and from the Eötvös Loránd Research Network.

REFERENCES

1. Lawler, P.R., K.B. Filion, and M.J. Eisenberg, *Efficacy of exercise-based cardiac rehabilitation post-myocardial infarction: a systematic review and meta-analysis of randomized controlled trials.* Am Heart J, 2011. **162**(4): p. 571-584 e2 DOI: 10.1016/j.ahj.2011.07.017.
2. Quindry, J.C. and B.A. Franklin, *Exercise Preconditioning as a Cardioprotective Phenotype.* Am J Cardiol, 2021. **148**: p. 8-15 DOI: 10.1016/j.amjcard.2021.02.030.
3. Ekelund, U., et al., *Dose-response associations between accelerometry measured physical activity and sedentary time and all cause mortality: systematic review and harmonised meta-analysis.* BMJ, 2019. **366**: p. l4570 DOI: 10.1136/bmj.l4570.
4. Chaput, J.P., et al., *2020 WHO guidelines on physical activity and sedentary behaviour for children and adolescents aged 5-17 years: summary of the evidence.* Int J Behav Nutr Phys Act, 2020. **17**(1): p. 141 DOI: 10.1186/s12966-020-01037-z.
5. Merghani, A., A. Malhotra, and S. Sharma, *The U-shaped relationship between exercise and cardiac morbidity.* Trends Cardiovasc Med, 2016. **26**(3): p. 232-40 DOI: 10.1016/j.tcm.2015.06.005.
6. O'Keefe, J.H., et al., *Potential adverse cardiovascular effects from excessive endurance exercise.* Mayo Clin Proc, 2012. **87**(6): p. 587-95 DOI: 10.1016/j.mayocp.2012.04.005.
7. Morganroth, J., et al., *Comparative left ventricular dimensions in trained athletes.* Ann Intern Med, 1975. **82**(4): p. 521-4 DOI: 10.7326/0003-4819-82-4-521.
8. Kim, J.H. and A.L. Baggish, *Physical Activity, Endurance Exercise, and Excess-Can One Overdose?* Curr Treat Options Cardiovasc Med, 2016. **18**(11): p. 68 DOI: 10.1007/s11936-016-0490-6.
9. D'Andrea, A., et al., *Left ventricular myocardial velocities and deformation indexes in top-level athletes.* J Am Soc Echocardiogr, 2010. **23**(12): p. 1281-8 DOI: 10.1016/j.echo.2010.09.020.
10. Pluim, B.M., et al., *The athlete's heart. A meta-analysis of cardiac structure and function.* Circulation, 2000. **101**(3): p. 336-44 DOI: 10.1161/01.cir.101.3.336.
11. Pelliccia, A., et al., *Physiologic left ventricular cavity dilatation in elite athletes.* Ann Intern Med, 1999. **130**(1): p. 23-31 DOI: 10.7326/0003-4819-130-1-199901050-00005.
12. Spirito, P., et al., *Morphology of the "athlete's heart" assessed by echocardiography in 947 elite athletes representing 27 sports.* Am J Cardiol, 1994. **74**(8): p. 802-6 DOI: 10.1016/0002-9149(94)90439-1.

13. Aubert, A.E., B. Seps, and F. Beckers, *Heart rate variability in athletes.* Sports Med, 2003. **33**(12): p. 889-919 DOI: 10.2165/00007256-200333120-00003.
14. Singh, N., et al., *Heart Rate Variability: An Old Metric with New Meaning in the Era of Using mHealth technologies for Health and Exercise Training Guidance. Part Two: Prognosis and Training.* Arrhythm Electrophysiol Rev, 2018. **7**(4): p. 247-255 DOI: 10.15420/aer.2018.30.2.
15. Boyett, M.R., et al., *Viewpoint: is the resting bradycardia in athletes the result of remodeling of the sinoatrial node rather than high vagal tone?* J Appl Physiol (1985), 2013. **114**(9): p. 1351-5 DOI: 10.1152/japplphysiol.01126.2012.
16. Wasfy, M.M., A.M. Hutter, and R.B. Weiner, *Sudden Cardiac Death in Athletes.* Methodist Debakey Cardiovasc J, 2016. **12**(2): p. 76-80 DOI: 10.14797/mdcj-12-2-76.
17. Harmon, K.G., et al., *Incidence of sudden cardiac death in athletes: a state-of-the-art review.* Br J Sports Med, 2014. **48**(15): p. 1185-92 DOI: 10.1136/bjsports-2014-093872.
18. Marijon, E., et al., *Sports-related sudden death in the general population.* Circulation, 2011. **124**(6): p. 672-81 DOI: 10.1161/CIRCULATIONAHA.110.008979.
19. Corrado, D., et al., *Pre-participation screening of young competitive athletes for prevention of sudden cardiac death.* J Am Coll Cardiol, 2008. **52**(24): p. 1981-9 DOI: 10.1016/j.jacc.2008.06.053.
20. Harmon, K.G., et al., *Incidence, Cause, and Comparative Frequency of Sudden Cardiac Death in National Collegiate Athletic Association Athletes: A Decade in Review.* Circulation, 2015. **132**(1): p. 10-9 DOI: 10.1161/CIRCULATIONAHA.115.015431.
21. Maron, B.J., et al., *Sudden death in young competitive athletes. Clinical, demographic, and pathological profiles.* Jama, 1996. **276**(3): p. 199-204.
22. Aschar-Sobbi, R., et al., *Increased atrial arrhythmia susceptibility induced by intense endurance exercise in mice requires TNFalpha.* Nat Commun, 2015. **6**: p. 6018 DOI: 10.1038/ncomms7018.
23. Baldesberger, S., et al., *Sinus node disease and arrhythmias in the long-term follow-up of former professional cyclists.* Eur Heart J, 2008. **29**(1): p. 71-8 DOI: 10.1093/eurheartj/ehm555.
24. Zipes, D.P. and H.J. Wellens, *Sudden cardiac death.* Circulation, 1998. **98**(21): p. 2334-51 DOI: 10.1161/01.cir.98.21.2334.
25. Al-Khatib, S.M., et al., *2017 AHA/ACC/HRS Guideline for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death: Executive Summary: A Report of the American College of Cardiology/American Heart*

Association Task Force on Clinical Practice Guidelines and the Heart Rhythm Society. Circulation, 2018. **138**(13): p. e210-e271 DOI: 10.1161/CIR.0000000000000548.

26. Maron, B.J. and A. Pelliccia, *The heart of trained athletes: cardiac remodeling and the risks of sports, including sudden death.* Circulation, 2006. **114**(15): p. 1633-44 DOI: 10.1161/CIRCULATIONAHA.106.613562.
27. Corrado, D., et al., *Does sports activity enhance the risk of sudden death in adolescents and young adults?* J Am Coll Cardiol, 2003. **42**(11): p. 1959-63 DOI: 10.1016/j.jacc.2003.03.002.
28. Maron, B.J., et al., *Sudden deaths in young competitive athletes: analysis of 1866 deaths in the United States, 1980-2006.* Circulation, 2009. **119**(8): p. 1085-92 DOI: 10.1161/CIRCULATIONAHA.108.804617.
29. Raukar, N., et al., *Cardiovascular pre-participation screening in the young athlete: addressing concerns.* Phys Sportsmed, 2017. **45**(4): p. 365-369 DOI: 10.1080/00913847.2017.1363622.
30. Asif, I.M., A.L. Rao, and J.A. Drezner, *Sudden cardiac death in young athletes: what is the role of screening?* Curr Opin Cardiol, 2013. **28**(1): p. 55-62 DOI: 10.1097/HCO.0b013e32835b0ab9.
31. Varro, A. and I. Baczko, *Possible mechanisms of sudden cardiac death in top athletes: a basic cardiac electrophysiological point of view.* Pflugers Arch, 2010. **460**(1): p. 31-40 DOI: 10.1007/s00424-010-0798-0.
32. Coumel, P., *The management of clinical arrhythmias. An overview on invasive versus non-invasive electrophysiology.* Eur Heart J, 1987. **8**(2): p. 92-9 DOI: 10.1093/oxfordjournals.eurheartj.a062259.
33. Bjornstad, H., et al., *Electrocardiographic findings in athletic students and sedentary controls.* Cardiology, 1991. **79**(4): p. 290-305 DOI: 10.1159/000174893.
34. Lengyel, C., et al., *Increased short-term variability of the QT interval in professional soccer players: possible implications for arrhythmia prediction.* PLoS One, 2011. **6**(4): p. e18751 DOI: 10.1371/journal.pone.0018751.
35. Malek, L.A. and C. Bucciarelli-Ducci, *Myocardial fibrosis in athletes-Current perspective.* Clin Cardiol, 2020. **43**(8): p. 882-888 DOI: 10.1002/clc.23360.
36. Malek, L.A. and C. Bucciarelli-Ducci, *Myocardial fibrosis in athletes: Additional considerations.* Clin Cardiol, 2020. **43**(11): p. 1208 DOI: 10.1002/clc.23466.

37. Zhang, C.D., et al., *Prevalence of Myocardial Fibrosis in Intensive Endurance Training Athletes: A Systematic Review and Meta-Analysis.* Front Cardiovasc Med, 2020. **7**: p. 585692 DOI: 10.3389/fcvm.2020.585692.

38. Rajanayagam, J. and M. Alsabri, *Intense Endurance Exercise: A Potential Risk Factor in the Development of Heart Disease.* Cureus, 2021. **13**(1): p. e12608 DOI: 10.7759/cureus.12608.

39. Haissaguerre, M., et al., *Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins.* N Engl J Med, 1998. **339**(10): p. 659-66 DOI: 10.1056/NEJM199809033391003.

40. Haissaguerre, M., et al., *Role of Purkinje conducting system in triggering of idiopathic ventricular fibrillation.* Lancet, 2002. **359**(9307): p. 677-8 DOI: 10.1016/S0140-6736(02)07807-8.

41. Schwartz, P.J., L. Crotti, and R. Insolia, *Long-QT syndrome: from genetics to management.* Circ Arrhythm Electrophysiol, 2012. **5**(4): p. 868-77 DOI: 10.1161/CIRCEP.111.962019.

42. Brugada, P. and J. Brugada, *Right bundle branch block, persistent ST segment elevation and sudden cardiac death: a distinct clinical and electrocardiographic syndrome. A multicenter report.* J Am Coll Cardiol, 1992. **20**(6): p. 1391-6 DOI: 10.1016/0735-1097(92)90253-j.

43. Roden, D.M., *Drug-induced prolongation of the QT interval.* N Engl J Med, 2004. **350**(10): p. 1013-22 DOI: 10.1056/NEJMra032426.

44. Coumel, P., J.F. Leclercq, and A. Leenhardt, *Arrhythmias as predictors of sudden death.* Am Heart J, 1987. **114**(4 Pt 2): p. 929-37 DOI: 10.1016/0002-8703(87)90590-4.

45. Farkas, A.S. and S. Nattel, *Minimizing repolarization-related proarrhythmic risk in drug development and clinical practice.* Drugs, 2010. **70**(5): p. 573-603 DOI: 10.2165/11535230-000000000-00000.

46. Guasch, E., et al., *Atrial fibrillation promotion by endurance exercise: demonstration and mechanistic exploration in an animal model.* J Am Coll Cardiol, 2013. **62**(1): p. 68-77 DOI: 10.1016/j.jacc.2013.01.091.

47. D'Souza, A., et al., *Exercise training reduces resting heart rate via downregulation of the funny channel HCN4.* Nat Commun, 2014. **5**: p. 3775 DOI: 10.1038/ncomms4775.

48. Mesirca, P., et al., *Intrinsic Electrical Remodeling Underlies Atrioventricular Block in Athletes.* Circ Res, 2021. **129**(1): p. e1-e20 DOI: 10.1161/CIRCRESAHA.119.316386.

49. Benito, B., et al., *Cardiac arrhythmogenic remodeling in a rat model of long-term intensive exercise training.* Circulation, 2011. **123**(1): p. 13-22 DOI: 10.1161/CIRCULATIONAHA.110.938282.

50. Farkas, A., A.J. Batey, and S.J. Coker, *How to measure electrocardiographic QT interval in the anaesthetized rabbit.* J Pharmacol Toxicol Methods, 2004. **50**(3): p. 175-85 DOI: 10.1016/j.vascn.2004.05.002.

51. Polyak, A., et al., *Long-term endurance training-induced cardiac adaptation in new rabbit and dog animal models of the human athlete's heart.* Rev Cardiovasc Med, 2018. **19**(4): p. 135-142 DOI: 10.31083/j.rcm.2018.04.4161.

52. Kui, P., et al., *New in vitro model for proarrhythmia safety screening: IKs inhibition potentiates the QTc prolonging effect of IKr inhibitors in isolated guinea pig hearts.* J Pharmacol Toxicol Methods, 2016. **80**: p. 26-34 DOI: 10.1016/j.vascn.2016.04.005.

53. Sarusi, A., et al., *Absolute beat-to-beat variability and instability parameters of ECG intervals: biomarkers for predicting ischaemia-induced ventricular fibrillation.* Br J Pharmacol, 2014. **171**(7): p. 1772-82 DOI: 10.1111/bph.12579.

54. Walker, M.J., et al., *The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion.* Cardiovasc Res, 1988. **22**(7): p. 447-55 DOI: 10.1093/cvr/22.7.447.

55. Curtis, M.J., et al., *The Lambeth Conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias.* Pharmacol Ther, 2013. **139**(2): p. 213-48 DOI: 10.1016/j.pharmthera.2013.04.008.

56. Jost, N., et al., *Ionic mechanisms limiting cardiac repolarization reserve in humans compared to dogs.* J Physiol, 2013. **591**(17): p. 4189-206 DOI: 10.1113/jphysiol.2013.261198.

57. Jost, N., et al., *ORM-10103, a novel specific inhibitor of the Na+/Ca2+ exchanger, decreases early and delayed afterdepolarizations in the canine heart.* Br J Pharmacol, 2013. **170**(4): p. 768-78 DOI: 10.1111/bph.12228.

58. Hobai, I.A., D. Khananshvili, and A.J. Levi, *The peptide "FRCRCFa", dialysed intracellularly, inhibits the Na/Ca exchange in rabbit ventricular myocytes with high affinity.* Pflugers Arch, 1997. **433**(4): p. 455-63 DOI: 10.1007/s004240050300.

59. Nagy, N., et al., *Does small-conductance calcium-activated potassium channel contribute to cardiac repolarization?* J Mol Cell Cardiol, 2009. **47**(5): p. 656-63 DOI: 10.1016/j.yjmcc.2009.07.019.

60. Domenech-Ximenos, B., et al., *Prevalence and pattern of cardiovascular magnetic resonance late gadolinium enhancement in highly trained endurance athletes.* J Cardiovasc Magn Reson, 2020. **22**(1): p. 62 DOI: 10.1186/s12968-020-00660-w.
61. Liu, D.W. and C. Antzelevitch, *Characteristics of the delayed rectifier current (IKr and IKs) in canine ventricular epicardial, midmyocardial, and endocardial myocytes. A weaker IKs contributes to the longer action potential of the M cell.* Circ Res, 1995. **76**(3): p. 351-65 DOI: 10.1161/01.res.76.3.351.
62. Lengyel, C., et al., *Pharmacological block of the slow component of the outward delayed rectifier current (I(Ks)) fails to lengthen rabbit ventricular muscle QT(c) and action potential duration.* Br J Pharmacol, 2001. **132**(1): p. 101-10 DOI: 10.1038/sj.bjp.0703777.
63. Hoydal, M.A., et al., *Running speed and maximal oxygen uptake in rats and mice: practical implications for exercise training.* Eur J Cardiovasc Prev Rehabil, 2007. **14**(6): p. 753-60 DOI: 10.1097/HJR.0b013e3281eacef1.
64. Gazdag, P., et al., *Increased Ca(2+) content of the sarcoplasmic reticulum provides arrhythmogenic trigger source in swimming-induced rat athlete's heart model.* Sci Rep, 2020. **10**(1): p. 19596 DOI: 10.1038/s41598-020-76496-2.
65. Oh, Y., et al., *Transcriptomic Bioinformatic Analyses of Atria Uncover Involvement of Pathways Related to Strain and Post-translational Modification of Collagen in Increased Atrial Fibrillation Vulnerability in Intensely Exercised Mice.* Front Physiol, 2020. **11**: p. 605671 DOI: 10.3389/fphys.2020.605671.
66. Janssen, P.M. and M. Periasamy, *Determinants of frequency-dependent contraction and relaxation of mammalian myocardium.* J Mol Cell Cardiol, 2007. **43**(5): p. 523-31 DOI: 10.1016/j.yjmcc.2007.08.012.
67. Nerbonne, J.M., *Studying cardiac arrhythmias in the mouse--a reasonable model for probing mechanisms?* Trends Cardiovasc Med, 2004. **14**(3): p. 83-93 DOI: 10.1016/j.tcm.2003.12.006.
68. Tamargo, J., et al., *Pharmacology of cardiac potassium channels.* Cardiovasc Res, 2004. **62**(1): p. 9-33 DOI: 10.1016/j.cardiores.2003.12.026.
69. Lozano, W.M., et al., *Exercise Training Protocols in Rabbits Applied in Cardiovascular Research.* Animals (Basel), 2020. **10**(8) DOI: 10.3390/ani10081263.
70. DiCarlo, S.E. and V.S. Bishop, *Regional vascular resistance during exercise: role of cardiac afferents and exercise training.* Am J Physiol, 1990. **258**(3 Pt 2): p. H842-7 DOI: 10.1152/ajpheart.1990.258.3.H842.

71. Liu, J.L., et al., *Chronic exercise reduces sympathetic nerve activity in rabbits with pacing-induced heart failure: A role for angiotensin II.* Circulation, 2000. **102**(15): p. 1854-62 DOI: 10.1161/01.cir.102.15.1854.
72. Gaustad, S.E., N. Rolim, and U. Wisloff, *A valid and reproducible protocol for testing maximal oxygen uptake in rabbits.* Eur J Cardiovasc Prev Rehabil, 2010. **17**(1): p. 83-8 DOI: 10.1097/HJR.0b013e32833090c4.
73. Hexeberg, E., et al., *Effects of endurance training on left ventricular performance: a study in anaesthetized rabbits.* Acta Physiol Scand, 1995. **154**(4): p. 479-88 DOI: 10.1111/j.1748-1716.1995.tb09933.x.
74. Carroll, J.F. and C.K. Kyser, *Exercise training in obesity lowers blood pressure independent of weight change.* Med Sci Sports Exerc, 2002. **34**(4): p. 596-601 DOI: 10.1097/00005768-200204000-00006.
75. Such, L., et al., *Effects of chronic exercise on myocardial refractoriness: a study on isolated rabbit heart.* Acta Physiol (Oxf), 2008. **193**(4): p. 331-9 DOI: 10.1111/j.1748-1716.2008.01851.x.
76. Szel, T., et al., *Class I/B antiarrhythmic property of ranolazine, a novel antianginal agent, in dog and human cardiac preparations.* Eur J Pharmacol, 2011. **662**(1-3): p. 31-9 DOI: 10.1016/j.ejphar.2011.04.042.
77. Haidet, G.C., et al., *Cardiovascular effects of dobutamine during exercise in dogs.* Am J Physiol, 1989. **257**(3 Pt 2): p. H954-60 DOI: 10.1152/ajpheart.1989.257.3.H954.
78. Musch, T.I., et al., *Regional distribution of blood flow of dogs during graded dynamic exercise.* J Appl Physiol (1985), 1987. **63**(6): p. 2269-77 DOI: 10.1152/jappl.1987.63.6.2269.
79. Stratton, J.R., et al., *Cardiovascular responses to exercise. Effects of aging and exercise training in healthy men.* Circulation, 1994. **89**(4): p. 1648-55 DOI: 10.1161/01.cir.89.4.1648.
80. Babai, L., et al., *Delayed cardioprotective effects of exercise in dogs are aminoguanidine sensitive: possible involvement of nitric oxide.* Clin Sci (Lond), 2002. **102**(4): p. 435-45.
81. Bonilla, I.M., et al., *Endurance exercise training normalizes repolarization and calcium-handling abnormalities, preventing ventricular fibrillation in a model of sudden cardiac death.* J Appl Physiol (1985), 2012. **113**(11): p. 1772-83 DOI: 10.1152/japplphysiol.00175.2012.

82. Holycross, B.J., et al., *Exercise training normalizes beta-adrenoceptor expression in dogs susceptible to ventricular fibrillation.* Am J Physiol Heart Circ Physiol, 2007. **293**(5): p. H2702-9 DOI: 10.1152/ajpheart.00763.2007.
83. Billman, G.E., *Cardiac autonomic neural remodeling and susceptibility to sudden cardiac death: effect of endurance exercise training.* Am J Physiol Heart Circ Physiol, 2009. **297**(4): p. H1171-93 DOI: 10.1152/ajpheart.00534.2009.
84. Constable, P.D., et al., *Athletic heart syndrome in dogs competing in a long-distance sled race.* J Appl Physiol (1985), 1994. **76**(1): p. 433-8 DOI: 10.1152/jappl.1994.76.1.433.
85. Constable, P.D., et al., *Effects of endurance training on standard and signal-averaged electrocardiograms of sled dogs.* Am J Vet Res, 2000. **61**(5): p. 582-8 DOI: 10.2460/ajvr.2000.61.582.
86. Mitchell, J.H., et al., *Task Force 8: classification of sports.* J Am Coll Cardiol, 2005. **45**(8): p. 1364-7 DOI: 10.1016/j.jacc.2005.02.015.
87. Pelliccia, A., et al., *Long-term clinical consequences of intense, uninterrupted endurance training in olympic athletes.* J Am Coll Cardiol, 2010. **55**(15): p. 1619-25 DOI: 10.1016/j.jacc.2009.10.068.
88. Toufan, M., et al., *Assessment of electrocardiography, echocardiography, and heart rate variability in dynamic and static type athletes.* Int J Gen Med, 2012. **5**: p. 655-60 DOI: 10.2147/IJGM.S33247.
89. Hoogsteen, J., et al., *Myocardial adaptation in different endurance sports: an echocardiographic study.* Int J Cardiovasc Imaging, 2004. **20**(1): p. 19-26 DOI: 10.1023/b:caim.0000013160.79903.19.
90. Weiner, R.B., et al., *Exercise-Induced Left Ventricular Remodeling Among Competitive Athletes: A Phasic Phenomenon.* Circ Cardiovasc Imaging, 2015. **8**(12) DOI: 10.1161/CIRCIMAGING.115.003651.
91. Tahir, E., et al., *Impact of Myocardial Fibrosis on Left Ventricular Function Evaluated by Feature-Tracking Myocardial Strain Cardiac Magnetic Resonance in Competitive Male Triathletes With Normal Ejection Fraction.* Circ J, 2019. **83**(7): p. 1553-1562 DOI: 10.1253/circj.CJ-18-1388.
92. Grazioli, G., et al., *Echocardiography in the evaluation of athletes.* F1000Res, 2015. **4**: p. 151 DOI: 10.12688/f1000research.6595.1.

93. Kim, J.H. and A.L. Baggish, *Differentiating Exercise-Induced Cardiac Adaptations From Cardiac Pathology: The "Grey Zone" of Clinical Uncertainty.* Can J Cardiol, 2016. **32**(4): p. 429-37 DOI: 10.1016/j.cjca.2015.11.025.

94. Sorokin, A.V., et al., *Atrial fibrillation in endurance-trained athletes.* Br J Sports Med, 2011. **45**(3): p. 185-8 DOI: 10.1136/bjsm.2009.057885.

95. Langdeau, J.B., et al., *Electrocardiographic findings in athletes: the prevalence of left ventricular hypertrophy and conduction defects.* Can J Cardiol, 2001. **17**(6): p. 655-9.

96. Billman, G.E., et al., *Exercise training-induced bradycardia: evidence for enhanced parasympathetic regulation without changes in intrinsic sinoatrial node function.* J Appl Physiol (1985), 2015. **118**(11): p. 1344-55 DOI: 10.1152/japplphysiol.01111.2014.

97. Gourine, A.V. and G.L. Ackland, *Cardiac Vagus and Exercise.* Physiology (Bethesda), 2019. **34**(1): p. 71-80 DOI: 10.1152/physiol.00041.2018.

98. Billman, G.E., *Rebuttal from Billman on Point:Counterpoint: Exercise training-induced bradycardia.* J Appl Physiol (1985), 2017. **123**(3): p. 690-691 DOI: 10.1152/japplphysiol.00607.2017.

99. Boyett, M.R., et al., *Point: Exercise training-induced bradycardia is caused by changes in intrinsic sinus node function.* J Appl Physiol (1985), 2017. **123**(3): p. 684-685 DOI: 10.1152/japplphysiol.00604.2017.

100. Flannery, D., et al., *Point:Counterpoint.* J Appl Physiol (1985), 2017. **123**(3): p. 692-693 DOI: 10.1152/japplphysiol.00546.2017.

101. D'Souza, A., S. Sharma, and M.R. Boyett, *Rebuttal from Alicia D'Souza, Sanjay Sharma and Mark R. Boyett.* J Physiol, 2015. **593**(8): p. 1755 DOI: 10.1113/JP270256.

102. Coote, J.H. and M.J. White, *CrossTalk proposal: bradycardia in the trained athlete is attributable to high vagal tone.* J Physiol, 2015. **593**(8): p. 1745-7 DOI: 10.1113/jphysiol.2014.284364.

103. Morad, M. and X.H. Zhang, *Mechanisms of spontaneous pacing: sinoatrial nodal cells, neonatal cardiomyocytes, and human stem cell derived cardiomyocytes.* Can J Physiol Pharmacol, 2017. **95**(10): p. 1100-1107 DOI: 10.1139/cjpp-2016-0743.

104. Noma, A., M. Morad, and H. Irisawa, *Does the "pacemaker current" generate the diastolic depolarization in the rabbit SA node cells?* Pflugers Arch, 1983. **397**(3): p. 190-4 DOI: 10.1007/BF00584356.

105. Maltsev, V.A. and E.G. Lakatta, *Funny current provides a relatively modest contribution to spontaneous beating rate regulation of human and rabbit sinoatrial*

node cells. J Mol Cell Cardiol, 2010. **48**(4): p. 804-6 DOI: 10.1016/j.yjmcc.2009.12.009.

106. Verkerk, A.O., M.M. van Borren, and R. Wilders, *Calcium transient and sodium-calcium exchange current in human versus rabbit sinoatrial node pacemaker cells.* ScientificWorldJournal, 2013. **2013**: p. 507872 DOI: 10.1155/2013/507872.

107. Vinogradova, T.M., et al., *Rhythmic Ca2+ oscillations drive sinoatrial nodal cell pacemaker function to make the heart tick.* Ann N Y Acad Sci, 2005. **1047**: p. 138-56 DOI: 10.1196/annals.1341.013.

108. Hu, W., et al., *Physiological Roles of the Rapidly Activated Delayed Rectifier K(+) Current in Adult Mouse Heart Primary Pacemaker Activity.* Int J Mol Sci, 2021. **22**(9) DOI: 10.3390/ijms22094761.

109. Kohajda, Z., et al., *Novel Na(+)/Ca(2+) Exchanger Inhibitor ORM-10962 Supports Coupled Function of Funny-Current and Na(+)/Ca(2+) Exchanger in Pacemaking of Rabbit Sinus Node Tissue.* Front Pharmacol, 2019. **10**: p. 1632 DOI: 10.3389/fphar.2019.01632.

110. Ono, K., S. Shibata, and T. Iijima, *Pacemaker mechanism of porcine sino-atrial node cells.* J Smooth Muscle Res, 2003. **39**(5): p. 195-204 DOI: 10.1540/jsmr.39.195.

111. Haverkamp, W., et al., *The potential for QT prolongation and pro-arrhythmia by non-anti-arrhythmic drugs: clinical and regulatory implications. Report on a Policy Conference of the European Society of Cardiology.* Cardiovasc Res, 2000. **47**(2): p. 219-33 DOI: 10.1016/s0008-6363(00)00119-x.

112. Gupta, A., et al., *Current concepts in the mechanisms and management of drug-induced QT prolongation and torsade de pointes.* Am Heart J, 2007. **153**(6): p. 891-9 DOI: 10.1016/j.ahj.2007.01.040.

113. Hondeghem, L.M. and D.J. Snyders, *Class III antiarrhythmic agents have a lot of potential but a long way to go. Reduced effectiveness and dangers of reverse use dependence.* Circulation, 1990. **81**(2): p. 686-90 DOI: 10.1161/01.cir.81.2.686.

114. Tande, P.M., et al., *Rate-dependent class III antiarrhythmic action, negative chronotropy, and positive inotropy of a novel Ik blocking drug, UK-68,798: potent in guinea pig but no effect in rat myocardium.* J Cardiovasc Pharmacol, 1990. **16**(3): p. 401-10 DOI: 10.1097/00005344-199009000-00008.

115. Farkas, A.S., et al., *Importance of extracardiac alpha1-adrenoceptor stimulation in assisting dofetilide to induce torsade de pointes in rabbit hearts.* Eur J Pharmacol, 2006. **537**(1-3): p. 118-25 DOI: 10.1016/j.ejphar.2006.03.014.

116. Crescenzi, C., et al., *Ventricular arrhythmias and risk stratification of cardiac sudden death in athletes.* Minerva Cardioangiol, 2020. **68**(2): p. 110-122 DOI: 10.23736/S0026-4725.20.05178-6.

117. Brown, H. and D. Difrancesco, *Voltage-clamp investigations of membrane currents underlying pace-maker activity in rabbit sino-atrial node.* J Physiol, 1980. **308**: p. 331-51 DOI: 10.1113/jphysiol.1980.sp013474.

118. Yanagihara, K. and H. Irisawa, *Inward current activated during hyperpolarization in the rabbit sinoatrial node cell.* Pflugers Arch, 1980. **385**(1): p. 11-9 DOI: 10.1007/BF00583909.

119. Baruscotti, M., A. Bucchi, and D. Difrancesco, *Physiology and pharmacology of the cardiac pacemaker ("funny") current.* Pharmacol Ther, 2005. **107**(1): p. 59-79 DOI: 10.1016/j.pharmthera.2005.01.005.

120. Cerbai, E., et al., *Characterization of the hyperpolarization-activated current, I(f), in ventricular myocytes from human failing heart.* Circulation, 1997. **95**(3): p. 568-71 DOI: 10.1161/01.cir.95.3.568.

121. Cerbai, E., et al., *The properties of the pacemaker current I(F)in human ventricular myocytes are modulated by cardiac disease.* J Mol Cell Cardiol, 2001. **33**(3): p. 441-8 DOI: 10.1006/jmcc.2000.1316.

122. Spinale, F.G., *Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function.* Physiol Rev, 2007. **87**(4): p. 1285-342 DOI: 10.1152/physrev.00012.2007.

123. Kui, P., et al., *Long-Term Endurance Exercise Training Alters Repolarization in a New Rabbit Athlete's Heart Model.* Front Physiol, 2021. **12**: p. 741317 DOI: 10.3389/fphys.2021.741317.

124. Topal, L., et al., *Endurance training-induced cardiac remodeling in a guinea pig athlete's heart model.* Can J Physiol Pharmacol, 2022. **100**(10): p. 993-1004 DOI: 10.1139/cjpp-2022-0073.

125. Chen, Y., et al., *Cardiac troponin T alterations in myocardium and serum of rats after stressful, prolonged intense exercise.* J Appl Physiol (1985), 2000. **88**(5): p. 1749-55 DOI: 10.1152/jappl.2000.88.5.1749.

126. Brown, R.D., et al., *The cardiac fibroblast: therapeutic target in myocardial remodeling and failure.* Annu Rev Pharmacol Toxicol, 2005. **45**: p. 657-87 DOI: 10.1146/annurev.pharmtox.45.120403.095802.

127. Olah, A., et al., *Cardiac effects of acute exhaustive exercise in a rat model.* Int J Cardiol, 2015. **182**: p. 258-66 DOI: 10.1016/j.ijcard.2014.12.045.

128. Polyakova, V., et al., *Fibrosis in endstage human heart failure: severe changes in collagen metabolism and MMP/TIMP profiles.* Int J Cardiol, 2011. **151**(1): p. 18-33 DOI: 10.1016/j.ijcard.2010.04.053.

129. Zannad, F., P. Rossignol, and W. Iraqi, *Extracellular matrix fibrotic markers in heart failure.* Heart Fail Rev, 2010. **15**(4): p. 319-29 DOI: 10.1007/s10741-009-9143-0.

130. Mukherjee, D. and S. Sen, *Collagen phenotypes during development and regression of myocardial hypertrophy in spontaneously hypertensive rats.* Circ Res, 1990. **67**(6): p. 1474-80 DOI: 10.1161/01.res.67.6.1474.

131. Fogo, A.B., et al., *AJKD Atlas of Renal Pathology: Type III Collagen Glomerulopathy.* Am J Kidney Dis, 2017. **69**(6): p. e25-e26 DOI: 10.1053/j.ajkd.2017.04.004.

132. Karsdal, M.A., et al., *The good and the bad collagens of fibrosis - Their role in signaling and organ function.* Adv Drug Deliv Rev, 2017. **121**: p. 43-56 DOI: 10.1016/j.addr.2017.07.014.

133. Ricard-Blum, S., G. Baffet, and N. Theret, *Molecular and tissue alterations of collagens in fibrosis.* Matrix Biol, 2018. **68-69**: p. 122-149 DOI: 10.1016/j.matbio.2018.02.004.

134. Hondeghem, L.M., L. Carlsson, and G. Duker, *Instability and triangulation of the action potential predict serious proarrhythmia, but action potential duration prolongation is antiarrhythmic.* Circulation, 2001. **103**(15): p. 2004-13 DOI: 10.1161/01.cir.103.15.2004.

135. Orosz, S., et al., *Assessment of efficacy of proarrhythmia biomarkers in isolated rabbit hearts with attenuated repolarization reserve.* J Cardiovasc Pharmacol, 2014. **64**(3): p. 266-76 DOI: 10.1097/FJC.0000000000000116.

136. Lengyel, C., et al., *Role of slow delayed rectifier K+-current in QT prolongation in the alloxan-induced diabetic rabbit heart.* Acta Physiol (Oxf), 2008. **192**(3): p. 359-68 DOI: 10.1111/j.1748-1716.2007.01753.x.

137. Farkas, A.S., et al., *The role of the Na+/Ca2+ exchanger, I(Na) and I(CaL) in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts.* Br J Pharmacol, 2009. **156**(6): p. 920-32 DOI: 10.1111/j.1476-5381.2008.00096.x.

138. Farkas, A.S., et al., *Biomarkers and endogenous determinants of dofetilide-induced torsades de pointes in alpha(1) -adrenoceptor-stimulated, anaesthetized rabbits.* Br J Pharmacol, 2010. **161**(7): p. 1477-95 DOI: 10.1111/j.1476-5381.2010.00965.x.

139. Varro, A., et al., *Cardiac transmembrane ion channels and action potentials: cellular physiology and arrhythmogenic behavior.* Physiol Rev, 2021. **101**(3): p. 1083-1176 DOI: 10.1152/physrev.00024.2019.

140. Virag, L., et al., *Analysis of the contribution of I(to) to repolarization in canine ventricular myocardium.* Br J Pharmacol, 2011. **164**(1): p. 93-105 DOI: 10.1111/j.1476-5381.2011.01331.x.

141. Kaab, S., et al., *Ionic mechanism of action potential prolongation in ventricular myocytes from dogs with pacing-induced heart failure.* Circ Res, 1996. **78**(2): p. 262-73 DOI: 10.1161/01.res.78.2.262.

142. Kaab, S., et al., *Molecular basis of transient outward potassium current downregulation in human heart failure: a decrease in Kv4.3 mRNA correlates with a reduction in current density.* Circulation, 1998. **98**(14): p. 1383-93 DOI: 10.1161/01.cir.98.14.1383.

143. Antzelevitch, C., *Molecular basis for the transmural distribution of the transient outward current.* J Physiol, 2001. **533**(Pt 1): p. 1 DOI: 10.1111/j.1469-7793.2001.0001b.x.

144. Zicha, S., et al., *Molecular basis of species-specific expression of repolarizing K+ currents in the heart.* Am J Physiol Heart Circ Physiol, 2003. **285**(4): p. H1641-9 DOI: 10.1152/ajpheart.00346.2003.

145. Zicha, S., et al., *Transmural expression of transient outward potassium current subunits in normal and failing canine and human hearts.* J Physiol, 2004. **561**(Pt 3): p. 735-48 DOI: 10.1113/jphysiol.2004.075861.

146. Stones, R., et al., *The role of transient outward K+ current in electrical remodelling induced by voluntary exercise in female rat hearts.* Basic Res Cardiol, 2009. **104**(6): p. 643-52 DOI: 10.1007/s00395-009-0030-6.

147. Akar, F.G., et al., *Molecular mechanisms underlying K+ current downregulation in canine tachycardia-induced heart failure.* Am J Physiol Heart Circ Physiol, 2005. **288**(6): p. H2887-96 DOI: 10.1152/ajpheart.00320.2004.

148. Patel, S.P. and D.L. Campbell, *Transient outward potassium current, 'Ito', phenotypes in the mammalian left ventricle: underlying molecular, cellular and biophysical mechanisms.* J Physiol, 2005. **569**(Pt 1): p. 7-39 DOI: 10.1113/jphysiol.2005.086223.

149. Perez-Garcia, M.T., J.R. Lopez-Lopez, and C. Gonzalez, *Kvbeta1.2 subunit coexpression in HEK293 cells confers O2 sensitivity to kv4.2 but not to Shaker channels.* J Gen Physiol, 1999. **113**(6): p. 897-907 DOI: 10.1085/jgp.113.6.897.

150. Deschenes, I. and G.F. Tomaselli, *Modulation of Kv4.3 current by accessory subunits.* FEBS Lett, 2002. **528**(1-3): p. 183-8 DOI: 10.1016/s0014-5793(02)03296-9.

151. Nadal, M.S., et al., *The CD26-related dipeptidyl aminopeptidase-like protein DPPX is a critical component of neuronal A-type K+ channels.* Neuron, 2003. **37**(3): p. 449-61 DOI: 10.1016/s0896-6273(02)01185-6.

152. Radicke, S., et al., *Expression and function of dipeptidyl-aminopeptidase-like protein 6 as a putative beta-subunit of human cardiac transient outward current encoded by Kv4.3.* J Physiol, 2005. **565**(Pt 3): p. 751-6 DOI: 10.1113/jphysiol.2005.087312.

153. Jerng, H.H., Y. Qian, and P.J. Pfaffinger, *Modulation of Kv4.2 channel expression and gating by dipeptidyl peptidase 10 (DPP10).* Biophys J, 2004. **87**(4): p. 2380-96 DOI: 10.1529/biophysj.104.042358.

154. Fedida, D., A.P. Braun, and W.R. Giles, *Alpha 1-adrenoceptors in myocardium: functional aspects and transmembrane signaling mechanisms.* Physiol Rev, 1993. **73**(2): p. 469-87 DOI: 10.1152/physrev.1993.73.2.469.

155. Smit, D.L., et al., *Baseline characteristics of the HAARLEM study: 100 male amateur athletes using anabolic androgenic steroids.* Scand J Med Sci Sports, 2020. **30**(3): p. 531-539 DOI: 10.1111/sms.13592.

156. Dhar, R., et al., *Cardiovascular toxicities of performance-enhancing substances in sports.* Mayo Clin Proc, 2005. **80**(10): p. 1307-15 DOI: 10.4065/80.10.1307.

157. Hernandez-Guerra, A.I., et al., *Sudden cardiac death in anabolic androgenic steroids abuse: case report and literature review.* Forensic Sci Res, 2019. **4**(3): p. 267-273 DOI: 10.1080/20961790.2019.1595350.

158. Fineschi, V., et al., *Anabolic steroid- and exercise-induced cardio-depressant cytokines and myocardial beta1 receptor expression in CD1 mice.* Curr Pharm Biotechnol, 2011. **12**(2): p. 275-84 DOI: 10.2174/138920111794295792.

159. Sculthorpe, N., et al., *Evidence of altered cardiac electrophysiology following prolonged androgenic anabolic steroid use.* Cardiovasc Toxicol, 2010. **10**(4): p. 239-43 DOI: 10.1007/s12012-010-9090-y.

160. Gatzoulis, K.A., et al., *Signal-averaged electrocardiography: Past, present, and future.* J Arrhythm, 2018. **34**(3): p. 222-229 DOI: 10.1002/joa3.12062.

161. Maior, A.S., et al., *Abnormal cardiac repolarization in anabolic androgenic steroid users carrying out submaximal exercise testing.* Clin Exp Pharmacol Physiol, 2010. **37**(12): p. 1129-33 DOI: 10.1111/j.1440-1681.2010.05452.x.

162. Melchert, R.B. and A.A. Welder, *Cardiovascular effects of androgenic-anabolic steroids.* Med Sci Sports Exerc, 1995. **27**(9): p. 1252-62.

163. Torrisi, M., et al., *Sudden Cardiac Death in Anabolic-Androgenic Steroid Users: A Literature Review.* Medicina (Kaunas), 2020. **56**(11) DOI: 10.3390/medicina56110587.

164. Saad, F., et al., *More than eight years' hands-on experience with the novel long-acting parenteral testosterone undecanoate.* Asian J Androl, 2007. **9**(3): p. 291-7 DOI: 10.1111/j.1745-7262.2007.00275.x.

165. Montisci, M., et al., *Anabolic androgenic steroids abuse and cardiac death in athletes: morphological and toxicological findings in four fatal cases.* Forensic Sci Int, 2012. **217**(1-3): p. e13-8 DOI: 10.1016/j.forsciint.2011.10.032.

166. Sessa, F., et al., *Anabolic-androgenic steroids and brain injury: miRNA evaluation in users compared to cocaine abusers and elderly people.* Aging (Albany NY), 2020. **12**(15): p. 15314-15327 DOI: 10.18632/aging.103512.

167. Nanasi, P.P., et al., *Canine Myocytes Represent a Good Model for Human Ventricular Cells Regarding Their Electrophysiological Properties.* Pharmaceuticals (Basel), 2021. **14**(8) DOI: 10.3390/ph14080748.

168. Arpadffy-Lovas, T., et al., *Electrical Restitution and Its Modifications by Antiarrhythmic Drugs in Undiseased Human Ventricular Muscle.* Front Pharmacol, 2020. **11**: p. 479 DOI: 10.3389/fphar.2020.00479.

169. Lu, H.R., et al., *Drug-induced long QT in isolated rabbit Purkinje fibers: importance of action potential duration, triangulation and early afterdepolarizations.* Eur J Pharmacol, 2002. **452**(2): p. 183-92 DOI: 10.1016/s0014-2999(02)02246-x.

170. Dumaine, R. and J.M. Cordeiro, *Comparison of K+ currents in cardiac Purkinje cells isolated from rabbit and dog.* J Mol Cell Cardiol, 2007. **42**(2): p. 378-89 DOI: 10.1016/j.yjmcc.2006.10.019.