ORIGINAL RESEARCH ARTICLE

Effects of different exercise modalities on cardiac dysfunction in heart failure with preserved ejection fraction

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Abstract

Aims Heart failure with preserved ejection fraction (HFpEF) is an increasingly prevalent disease. Physical exercise has been shown to alter disease progression in HFpEF. We examined cardiomyocyte Ca^{2+} homeostasis and left ventricular function in a metabolic HFpEF model in sedentary and trained rats following 8 weeks of moderate-intensity continuous training (MICT) or high-intensity interval training (HIIT).

Methods and results Left ventricular *in vivo* function (echocardiography) and cardiomyocyte Ca^{2+} transients (CaTs) (Fluo-4, confocal) were compared in ZSF-1 obese (metabolic syndrome, HFpEF) and ZSF-1 lean (control) 21- and 28-week-old rats. At 21 weeks, cardiomyocytes from HFpEF rats showed prolonged Ca^{2+} reuptake in cytosolic and nuclear CaTs and impaired Ca^{2+} release kinetics in nuclear CaTs. At 28 weeks, HFpEF cardiomyocytes had depressed CaT amplitudes, decreased sarcoplasmic reticulum (SR) Ca^{2+} content, increased SR Ca^{2+} leak, and elevated diastolic $[Ca^{2+}]$ following increased pacing rate (5 Hz). In trained HFpEF rats (HIIT or MICT), cardiomyocyte SR Ca^{2+} leak was significantly reduced. While HIIT had no effects on the CaTs (1–5 Hz), MICT accelerated early Ca^{2+} release, reduced the amplitude, and prolonged the CaT without increasing diastolic $[Ca^{2+}]$ or cytosolic Ca^{2+} load at basal or increased pacing rate (1–5 Hz). MICT lowered pro-arrhythmogenic Ca^{2+} sparks and attenuated Ca^{2+}-wave propagation in cardiomyocytes. MICT was associated with increased stroke volume in HFpEF.

Conclusions In this metabolic rat model of HFpEF at an advanced stage, Ca^{2+} release was impaired under baseline conditions. HIIT and MICT differentially affected Ca^{2+} homeostasis with positive effects of MICT on stroke volume, end-diastolic volume, and cellular arrhythmogenicity.

Introduction

Obesity-related heart failure (HF) with preserved ejection fraction (HFpEF) is a highly prevalent condition with significant morbidity and mortality, yet pharmacological therapies for this condition remain elusive.

Physical exercise has been proposed as an approach to mitigate the course of the disease. For example, in the randomized clinical Ex-DHF pilot study, investigating the impact of supervised exercise training in HFpEF, diastolic dysfunction was partly mitigated associated with improved quality of life.[1] The mechanisms by which physical exercise alters

disease progression in HFpEF are not understood. In a hypertensive rat model of HFpEF, high-intensity interval training (HIIT), initiated before the onset of HFpEF, was associated with improved skeletal muscle performance.[2] In contrast, in a rat model of obesity-related HFpEF, neither HIIT nor moderate-intensity continuous training (MICT) treadmill exercise improved impaired contractile forces in skeletal muscle.[3]

In HF with reduced ejection fraction (HFrEF), depressed Ca^{2+} transients (CaTs) in cardiomyocytes contribute to contractile dysfunction, and exercise training can improve left ventricular (LV) cardiomyocyte Ca^{2+} homeostasis.[4,5] In an animal model of cardiorenal HFpEF, cardiac remodelling and contractile dysfunction have also been linked to impaired Ca^{2+} homeostasis in LV cardiomyocytes.[6,7] Similarly, in a model of obesity-related HFpEF, overt HF was associated with altered cytosolic Ca^{2+}.[8] The role of exercise training on cardiomyocyte Ca^{2+} homeostasis has not been evaluated yet.

Vasculature and heart adaption to exercise is highly dependent on the intensity, duration, and frequency of exercise training.[9] Both MICT and HIIT have been associated with T-tubular reverse remodelling and differential contractile *in vitro* response in the setting of hypertensive heart disease.[10] Moreover, especially MICT has been shown to improve regional cardiac function and reduce cardiomyocyte cross-sectional area.[11] Here, we compared the two popular exercise programmes, MICT and HIIT, and investigated molecular Ca^{2+}-related mechanisms of *in vitro* dysfunction in LV myocytes from a well-characterized obesity-related HFpEF model.

We hypothesized that LV myocytes from HFpEF rats have impaired Ca^{2+} handling when compared with control. Furthermore, we hypothesized that both exercise programmes improve LV function, measured by echocardiography, and normalize LV cardiomyocyte Ca^{2+} handling.

Methods

Animal model

ZSF-1 rats were acquired (Charles River Laboratories; at 8 weeks of age) and kept in identical conditions of 12 h light/dark cycles and free access to food and water. The model is based on a leptin receptor mutation leading to a lean ($ZSF^{+/-}$; CT) and obese ($ZSF^{+/+}$; HFpEF) phenotype. At 20 weeks, the obese rats have repeatedly been shown to develop clinical signs of HFpEF.[12–14]

All procedures were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All procedures were approved by the Norwegian Animal Research Authority in accordance with the Use of Laboratory Animals by the European Commission Directive 86/609/EEC.

Study design

The study design is visualized in *Figure 1*. The pathological HFpEF phenotype was validated *in vivo*,[15] and Ca^{2+} cycling of LV cardiomyocytes was investigated at 20–21 weeks in CT and HFpEF ($n = 10$ per group). Subsequently, HFpEF rats were randomly assigned to undergo either MICT or HIIT or to remain sedentary (Sed.) for 8 weeks until final *in vivo* and *in vitro* evaluation at 28 weeks ($n = 4$ per group). The experimenter was blinded for all *in vivo* and *in vitro* experiments.

Training

High-intensity interval training was performed on a treadmill at an inclination of 25° three times per week (four intervals at 90% VO_{2peak} for 4 min, with 3 min of recovery at 60% VO_{2peak}) for 8 weeks. MCT was performed on a treadmill at an inclination of 25° five times per week (60% VO_{2peak} for 1 h, followed by 10 min of running at 40% to 50% VO_{2peak}) for 8 weeks.

Echocardiography

Transthoracic echocardiography (Vevo 2,100; VisualSonics, Ontario, Canada) was performed as previously described in detail.[2] In brief, lightly anaesthetized rats (1.5–2% isoflurane) and spontaneously breathing rats in supine position were imaged using a 24 MHz transducer. Diastolic and systolic volumes, as well as global longitudinal strain (speckle tracking), were calculated from images obtained in parasternal long-axis view.

Single-cell isolation

Isolated cardiomyocytes were acquired in 21- and 28-week-old rats by enzymatic digestion using a Langendorff system as previously described in detail.[16] In brief, animals were sacrificed by cervical dislocation, the heart was excised, and the aorta was cannulated. The heart was mounted to the Langendorff system and perfused with nominally Ca^{2+}-free perfusion buffer containing highly purified collagenase (Liberase) at 37°C until satisfactory digestion of the LV was reached. LV tissue was dispersed, cardiomyocytes were allowed to settle, and external $[Ca^{2+}]$ increased to 2 mmol/L in a stepwise manner. LV cardiomyocytes were placed on laminin-coated coverslips for subsequent *in vitro* experiments.

Figure 1 Schematic outline of the present study. At 20 weeks, obese rats were randomly assigned to undergo moderate-intensity continuous exercise training (MICT) or high-intensity interval training (HIIT) or to remain sedentary (Sed.). The first part of the study investigates pathological cellular mechanisms of the sedentary obese rats [heart failure with preserved ejection fraction (HFpEF)] vs. their lean litter mates (CT) in early (*Figure* 2; 21-week-old rats) and advanced disease progression (28-week-old rats). The second part evaluates how the different modalities of exercise training can alter these pathologies and consecutively change cardiac function in HFpEF.
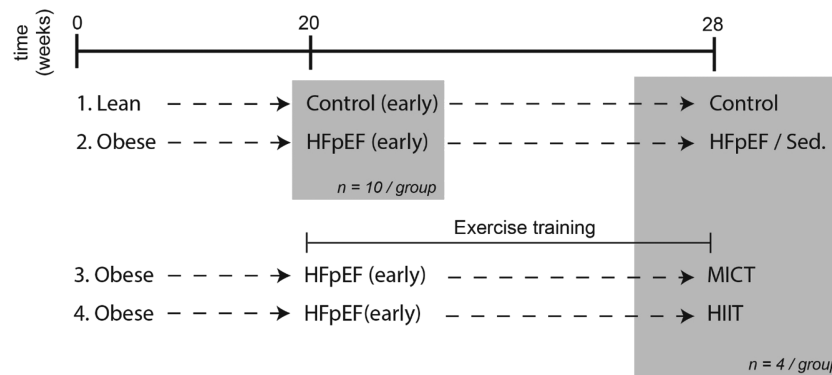
Solution and chemicals

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless noted otherwise. The fluorescent Ca^{2+} indicator Fluo-4 AM was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Tyrode's solution contained (in mmol/L): 130 NaCl, 4 KCl, 2 CaCl, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. Tyrode's solution without sodium and Ca^{2+} (0Na0Ca) contained (in mmol/L): 130 LiCl, 4 KCl 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with LiOH.

Confocal Ca^{2+} measurements

Cells were loaded with Fluo-4 AM as previously described.[13] Confocal line scan images were recorded along the longitudinal axis of the cell at either 1041 or 870 lines per second (pixel size: 0.12 μm) using a ×40 oil-immersion objective lens with a Zeiss LSM 510 system. The cells were stimulated in an electrical field using a pair of platinum electrodes (voltage: ~50% above threshold) at varying frequencies (according to the experimental protocol), and CaTs were recorded.

Experimental protocol

Left ventricular cardiomyocytes of 21-week-old rats were perfused at 37°C with Tyrode's solution containing 2 mmol/L Ca^{2+} and stimulated at 1 Hz for 3 min, allowing them to reach a steady state of Ca^{2+} cycling. Cytosolic and nuclear CaTs were recorded for the last 10 s. Pacing was increased to 3 Hz. For a graphical outline of the experimental protocol, refer to *Figure 2A*.

Left ventricular cardiomyocytes of 28-week-old rats were perfused at 37°C with Tyrode's solution containing 2 mmol/L Ca^{2+} and stimulated at 1 Hz for 3 min, allowing them to reach a steady state of Ca^{2+} cycling. CaTs were recorded for the last 10 s. Pacing was increased to 2, 3, and 5 Hz and decreased to 1 Hz. Perfusion was immediately changed to 0Na0Ca solution, and electrical pacing was paused. The cell was imaged for 10 s to record spontaneous Ca^{2+} release events (sparks and waves) and quantify the change in cytosolic resting $[Ca^{2+}]$. $\Delta F/F_0$ during 0Na0Ca was used as a measure of cytosolic Ca^{2+} leak. Perfusion was changed to 0Na0Ca solution containing 20 mmol/L caffeine, and the caffeine-induced CaTs were recorded. For a graphical outline of the experimental protocol, refer to *Figures 3A* and *4A*.

Image analysis

Changes in Ca^{2+} are expressed as the amplitude $\Delta F/F_0$, where F represents time-dependent Fluo-4 fluorescence levels under steady-state conditions during electrical stimulation, and $\Delta F = F - F_0$. Tau of a mono-exponential fit of the decay of CaTs was obtained as a parameter of Ca^{2+} removal. F_{50} was defined as 50% of the CaT amplitude, and the corresponding time to F_{50} (TF_{50}) was calculated as an indicator of early release (ER).

Transient amplitudes of caffeine-induced contractures ($\Delta F/F_0$) were determined as an indicator of sarcoplasmic reticulum (SR) Ca^{2+} content and tau of Ca^{2+} decay as in indicator of NCX forward-mode function.[17,18]

For early site analysis

Scan lines along the longitudinal axis were grouped into 1 μm intervals, indicating active couplons.[19,20] ER was defined to be smaller than the average TF_{50} of the control

Figure 2 Representative samples of the experimental protocol (A). Left ventricular cardiomyocytes of 21-week-old rats were exposed at 1 Hz electrical stimulation, and cytosolic Ca^{2+} transients were analysed for their Ca^{2+} release amplitude (B), time to half peak (TF_{50}; C), time to peak (TTP; D), and Ca^{2+} decay (tau; E). Consecutively, cells were paced at 3 Hz, and cytosolic Ca^{2+} transients were analysed for their Ca^{2+} release amplitude (F), time to half peak (TF_{50}; G), time to peak (TTP; H), and Ca^{2+} decay (tau; I). Nuclear Ca^{2+} transients were recorded at 1 Hz electric pacing, and cytosolic Ca^{2+} transients were analysed for their Ca^{2+} release amplitude (J), time to half peak (TF_{50}; K), time to peak (TTP; L), and Ca^{2+} decay (tau; M) determined. Statistical analysis: two-tailed, unpaired Student's t-test. P-values: [1]0.027, [2] < 0.0001, [3]0.035, and [4]0.027. n = cells from 10 animals per group. HFpEF, heart failure with preserved ejection fraction.
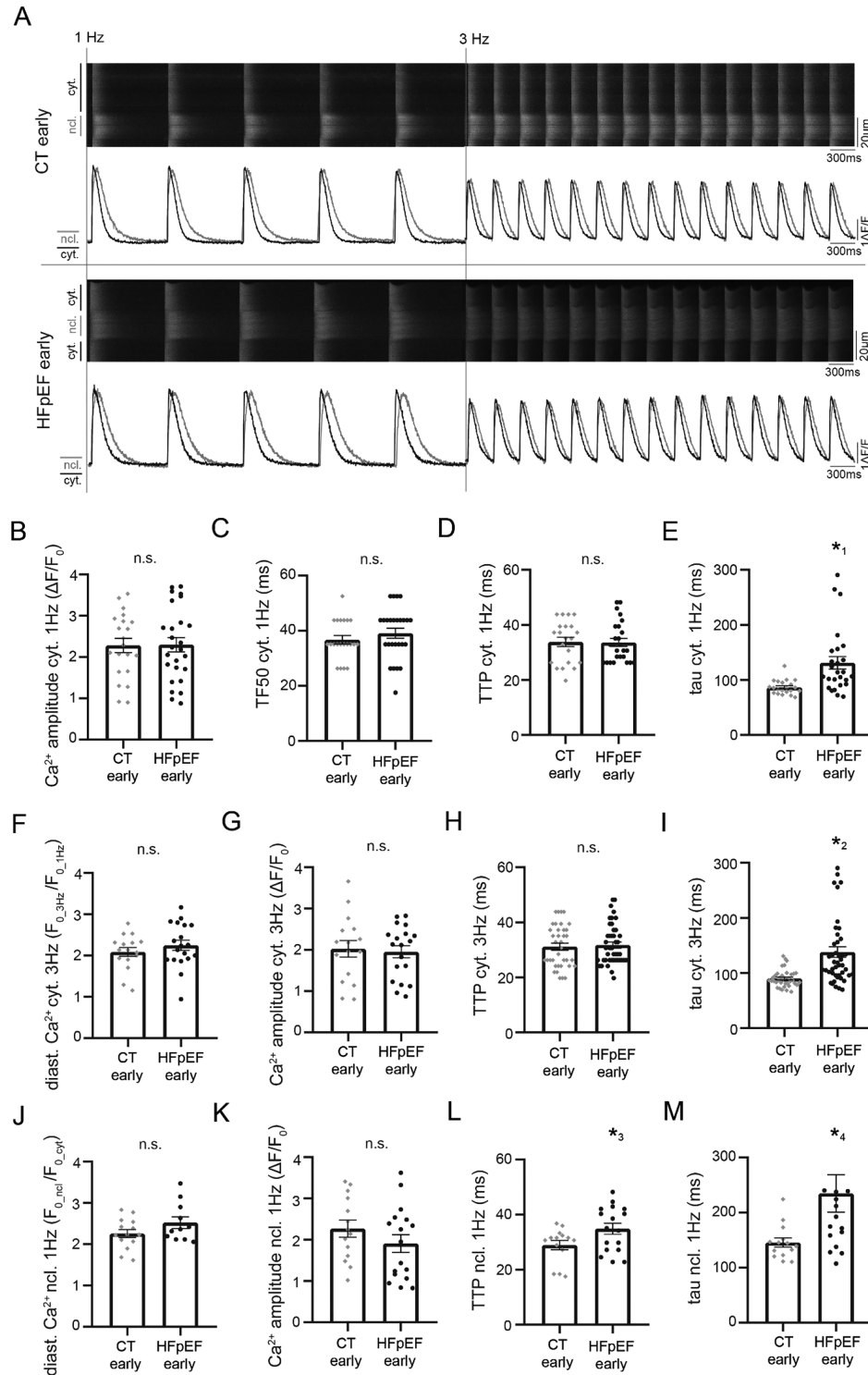
Figure 3 Representative samples of the experimental protocol (A). Left ventricular cardiomyocytes of 28-week-old rats were exposed to varying pacing frequencies by electrical stimulation, and Ca^{2+} transients were analysed for their time to peak (TTP; B), Ca^{2+} release amplitude (C), Ca^{2+} decay (tau; D), and diastolic Ca^{2+} (E). Ca^{2+} leak from the sarcoplasmic reticulum (SR; F) during sodium–Ca^{2+} exchanger inactivation. Ca^{2+} load of the SR quantified by application of caffeine (G) and subsequent Ca^{2+} decay (H). Statistical analysis: two-way ANOVA followed by *post hoc* Bonferroni (B–D: 1–5 Hz); two-tailed, unpaired Student's *t*-test (B–D: 1 Hz rec., F–H). *P*-values: [1]0.0001, [2] < 0.0001, [3]0.001, [4]0.02, [5]0.0007, [6]0.047, [7]0.02, [8]0.039, and [9]0.025. n = cells from four animals per group. (B–E) CT, $n = 23$; heart failure with preserved ejection fraction (HFpEF), $n = 19$.
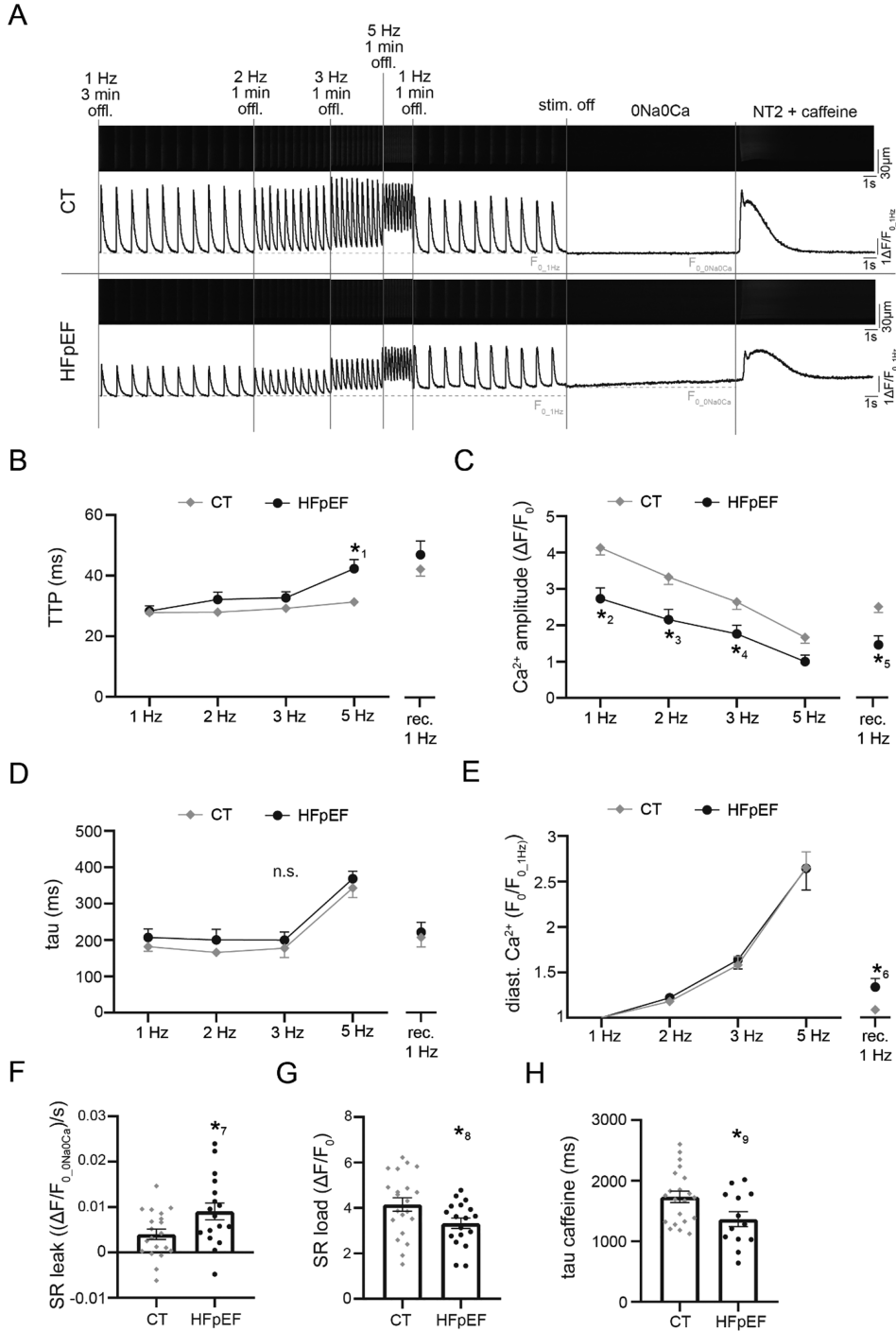
Figure 4 Representative examples of the experimental protocol (A). Ca^{2+} transients (CaTs) were analysed for their Ca^{2+} release amplitude (B), the area under the curve (AUC; C), Ca^{2+} decay during CaTs (tau CaT; D), and diastolic Ca^{2+} (E). Ca^{2+} leak from the sarcoplasmic reticulum (SR; F) during sodium–Ca^{2+} exchanger inactivation. Ca^{2+} load of the SR quantified by application of caffeine (G) and subsequent Ca^{2+} decay (H). Statistical analysis: paired, two-way (B–E: 1–5 Hz) and one-way ANOVA (B–E: 1–5 Hz rec., F–H) followed by *post hoc* Bonferroni vs. Sed. P-values: [1]0.016, [2]0.004, [3]0.006, [4]0.007, [5]0.005, [6]0.04, [7]0.049, [8]0.046, [9]0.028, and [10]0.006. n = cells from four animals per group. (B–E) Sed., n = 20; moderate-intensity continuous training (MICT), n = 22; and high-intensity interval training (HIIT), n = 27.
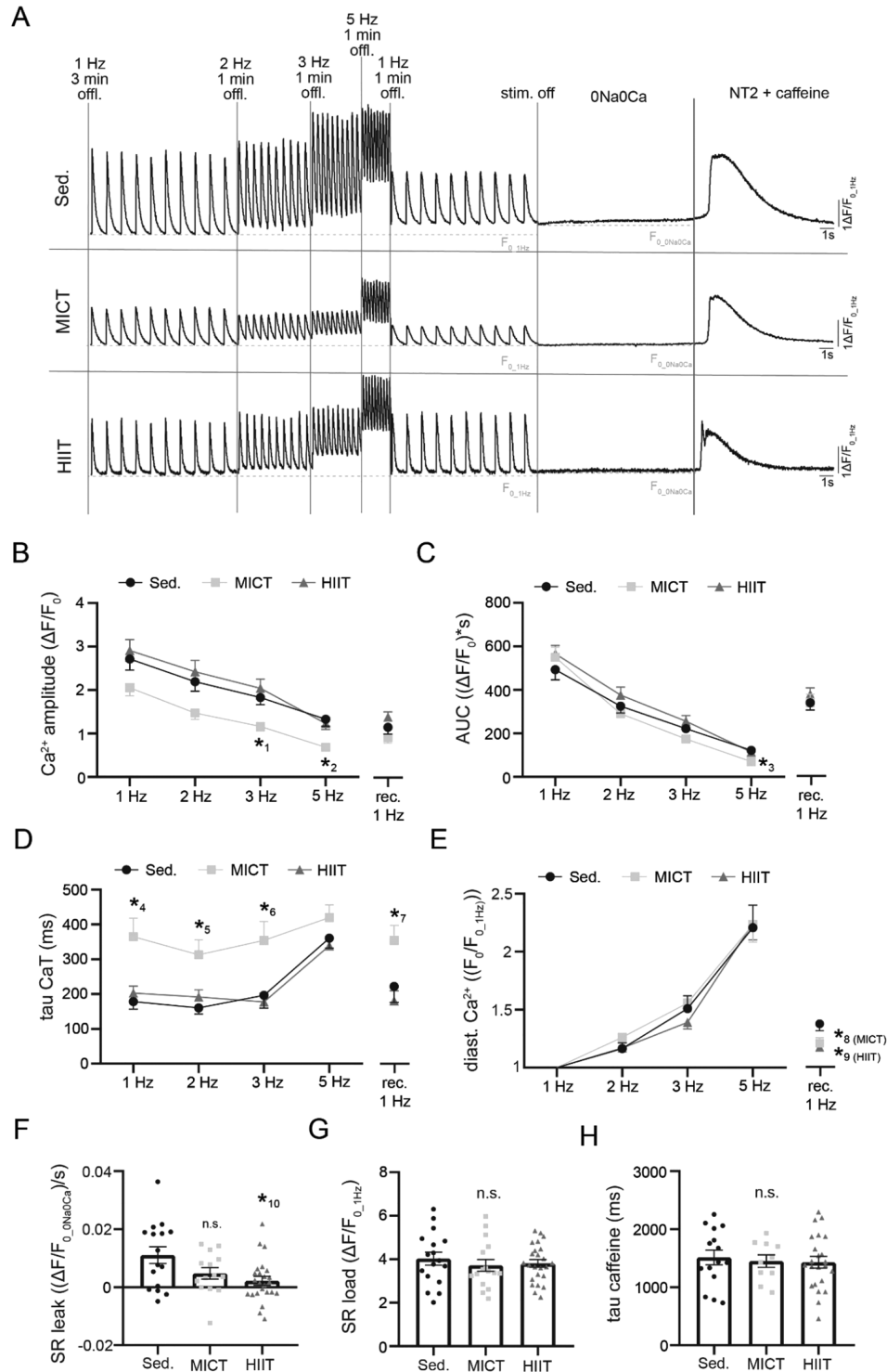
Figure 5 Echocardiographic evaluation after 8 weeks of exercise training [moderate-intensity continuous training (MICT) and high-intensity interval training (HIIT)] or the absence thereof (Sed.). End-diastolic volume (A; EDV) and stroke volume (B; SV) were calculated from diastolic and systolic volumes, global longitudinal strain (GLS) by speckle tracking (C) and heart rate in beats per minute (b.p.m.; D). Statistical analysis: one-way ANOVA followed by a *post hoc* Bonferroni vs. Sed. (A, B) or vs. all groups (C, D). *P*-values: [1]0.03, [2]0.04, and [3]0.0004. *n* = animals.
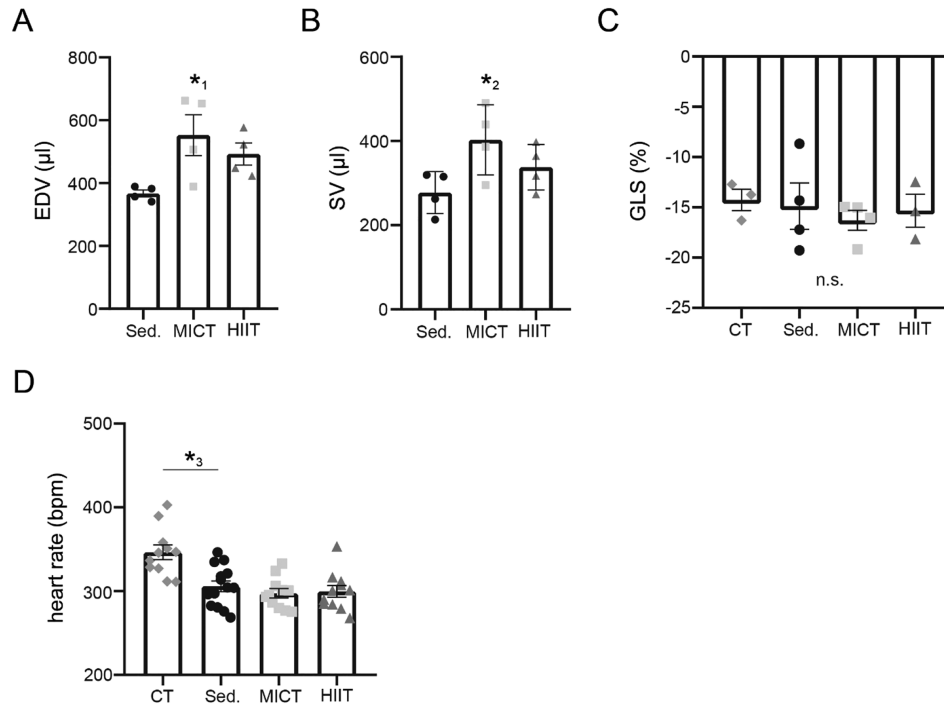
group (CT; $TF_{50} < 10.5$ ms), and an ER site was defined to be an active couplon with ER events in at least three out of 10 consecutive stimulation cycles. The fraction of ER events ER sites in 10 consecutive cycles was quantified as the probability of ER.

Code availability

Image analysis was mostly performed with the freely available software ImageJ (http://imagej.nih.giv). Calcium transient analysis was performed with custom code (Interactive Data Language), which is not publicly available.

The analyser was blinded towards group and/or treatment for all *in vivo* and *in vitro* experiments.

T-tubular network

T-tubules were visualized as previously described.[19] In brief, two-dimensional images of LV cardiomyocytes were obtained after staining with the fluorescence probe di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate, subjected to local thresholding, and the fraction of signal positive pixels in relation to the cell surface was taken as a measure of T-tubular density.

Western blotting

Left ventricular tissue samples were homogenized at 4°C in lysis buffer (in mmol/L: 20 Tris–HCl (pH 7.4), 137 NaCl, 20 NaF, 1 sodium pyrophosphate, 50 β-glycerophosphate, 10 EDTA, 1 EGTA, 1 PMSF, 10% glycerol, 1% NP 40, 4 μg/mL aprotinin, 4 μg/mL pepstatin A, and 4 μg/mL leupeptin); 30 μg of tissues homogenates was run on 4–12% Bis–Tris polyacrylamide gels and transferred to nitrocellulose membranes for 1 h. Proteins on membrane were stained with Ponceau S. Non-specific binding was blocked with 5% dried milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20. Membranes were probed with anti-phospho-Thr17 PLB, anti-phospho-Ser16 PLB, anti-SERCA2a (Badrilla, Leeds, UK), and anti-PLB (Santa Cruz, Heidelberg, Germany) overnight at 4°C. Anti-rabbit IgG linked with IRDye 680RD or anti-mouse linked with 800CW (LI-COR, Lincoln, Nebraska, USA) were used as a secondary antibody. The signal was detected with Odyssey CLx System. The band intensities and total proteins stain were determined by Image Studio software (LI-COR).

Data analysis and statistics

Results are shown as mean ± standard error. Individual data points are shown where spatially feasible. Statistical tests,

n, and P-values are supplied for each graph in the figure legend. A P-value of <0.05 was considered to be of statistical significance.

Results

Cardiomyocytes of early heart failure with preserved ejection fraction animals (21 weeks) show impaired Ca^{2+} reuptake

Freshly isolated cardiomyocytes of 21-week-old animals were stimulated at 1 and 3 Hz pacing frequencies, and CaTs of the cytosolic and nuclear compartment were examined (*Figure 2A*). Compared with controls, cytosolic CaTs of early HFpEF animals did not show alterations in Ca^{2+} release amplitude (*Figure 2B*) and release kinetics (*Figure 2C* and *2D*), but Ca^{2+} reuptake was prolonged (*Figure 2E*) at 1 Hz pacing. Similar results were observed at 3 Hz (*Figure 2F–2I*). In the nucleus, diastolic Ca^{2+} and Ca^{2+} release amplitudes remained unaltered in early HFpEF (*Figure 2J* and *2K*), but Ca^{2+} release and reuptake kinetics were significantly slower (*Figure 2J–2M*). Differences could be detected neither in cell size nor in T-tubular density (Supporting Information, *Figure S1*).

Cardiomyocytes of heart failure with preserved ejection fraction animals (28 weeks) show impaired Ca^{2+} release

At 28 weeks, CaTs of LV cardiomyocytes were analysed during increasing pacing frequencies and during recovery to the initial pacing frequency (*Figure 3A*). Compared with controls, time to peak Ca^{2+} (TTP; *Figure 3B*) was unchanged in HFpEF at lower frequencies 1–3 Hz but significantly prolonged at 5 Hz. Both groups showed a significantly increased TTP Ca^{2+} upon recovery to 1 Hz vs. initial 1 Hz, with no significant difference between the groups. The Ca^{2+} amplitude was significantly decreased in HFpEF at 1–3 Hz vs. CT and after recovery to 1 Hz (*Figure 3C*). No difference in cytosolic Ca^{2+} removal kinetics could be detected at various frequencies (*Figure 3D*).

Diastolic Ca^{2+} and sarcoplasmic reticulum leak are increased, and sarcoplasmic reticulum load decreased after pacing in heart failure with preserved ejection fraction

Diastolic Ca^{2+} of HFpEF cardiomyocytes rose to a similar extent as CT with increasing pacing frequencies from 1 to 5 Hz but showed an impaired recovery to baseline values vs. CT with return to 1 Hz stimulation (*Figure 3E*). Resting cardiomyocytes from HFpEF (following stimulation) showed a significantly increased rate of cytosolic Ca^{2+} accumulation (*Figure 3F*), suggesting SR Ca^{2+} leak. In line with this finding, a decreased SR Ca^{2+} load could be detected in HFpEF (*Figure 3G*). In addition, HFpEF showed a faster Ca^{2+} decay following caffeine-induced contractures (*Figure 3H*), indicating enhanced NCX forward-mode function.

Moderate-intensity continuous training but not high-intensity interval training is associated with a lower Ca^{2+} transient amplitude and prolonged Ca^{2+} transient decay

The same protocol (as *Figure 3A*) was employed to study changes of intracellular Ca^{2+} cycling in LV cardiomyocytes after 8 weeks of MICT or HIIT vs. sedentary animals (Sed.). In HFpEF animals that had performed MICT, CaT amplitude was lower at 2–5 Hz vs. HFpEF Sed. (*Figure 4B*), yet the area under the curve of the CaTs was only slightly decreased at 5 Hz in the MICT group (*Figure 4C*). This may in part be mediated by a slowed Ca^{2+} decay at 1–3 Hz in MICT (*Figure 4D*).

Moderate-intensity continuous training and high-intensity interval training protect from pacing-induced diastolic Ca^{2+} increase

Neither MICT nor HIIT had an influence on diastolic $[Ca^{2+}]$ with increased pacing rate (2–5 Hz, *Figure 4E*). However, restitution of diastolic cytosolic Ca^{2+} with return to 1 Hz stimulation was improved with MICT and HIIT vs. Sed. A lower diastolic SR Ca^{2+} leak from the SR in the HIIT (significant) and MICT (trend) groups vs. Sed. was identified as a possible contributor to this phenomenon (*Figure 4F*). SR Ca^{2+} load was unchanged MICT and HIIT (*Figure 4G*), as was the decay of the caffeine transient (*Figure 4H*).

Moderate-intensity continuous training associates with improved diastolic filling and stroke volume

In vivo, MICT but not HIIT resulted in significantly increased diastolic filling and stroke volume (SV; *Figure 5A* and *5B*). No difference in LV global longitudinal strain could be observed in either group (*Figure 5C*).

Moderate-intensity continuous training lowers spark incidence and decreases wave propagation velocity

Another approach to quantify SR Ca^{2+} leak is spontaneous and spatially limited (sparks) and propagated (waves)

spontaneous Ca^{2+} release events (*Figure 6A*). MICT, but not HIIT, lowered the incidence of sparks (*Figure 6B*). A statistically relevant difference in the incidence of waves was not observed (OS: 0.020 ± 0.012 vs. MICT: 0.024 ± 0.016 vs. HIIT: 0.017 ± 0.012 waves per second per 100 μm, n.s.). Assessment of Ca^{2+}-wave propagation velocity revealed a significant reduction in MICT vs. Sed. (*Figure 6C*). Wave propagation velocity of HIIT could not be assessed because of the very low wave incidence (two waves in 27 measured cells).

## Moderate-intensity continuous training improves early Ca^{2+} release

While time to peak of Ca^{2+} release remained unchanged by MICT and HIIT (*Figure 7A*), significant acceleration of ER of Ca^{2+} could be observed following MICT at 5 Hz and upon recovery to 1 Hz after pacing [time to half-maximum amplitude (TF_{50}; *Figure 7B*]. Adjacency and coupling of RyR to the sarcolemma through Ca^{2+}-induced Ca^{2+} release have previously been shown to be important drivers of early Ca^{2+} release in cardiomyocytes.[20,21] Scan lines of confocally acquired CaTs (*Figures 3A* and *4A*) were grouped into active couplons, and their corresponding TF_{50} was analysed in 10 consecutive cycles (*Figure 7C*). In cardiomyocytes from animals following MICT, the fraction of active ER sites was preserved at higher frequencies and during recovery (*Figure 7D*). In addition, the probability of Ca^{2+} release from active ER sites was significantly higher at 2 Hz in MICT vs. Sed. or HIIT (*Figure 7E*).

Discussion

In this study, we investigated the effect of two different exercise modalities on myocardial function *in vitro* and *in vivo* in a model of metabolic HFpEF.[15] Exercise training has been shown to improve diastolic dysfunction in human HFpEF.[1] While a positive effect of chronic low-intensity exercise has been previously reported in afterload-dependent HFpEF, the effect of different exercise regimes on metabolic HFpEF remained elusive.[22] At the age of 28 weeks, we found the cytosolic CaT amplitude in LV cardiomyocytes to be significantly reduced, despite a preserved ejection fraction.[15] Lower cytosolic Ca^{2+} release in HFpEF was related to a decreased SR Ca^{2+} load and an increased diastolic SR Ca^{2+} leak (Supporting Information, *Figure S3*). Also, in this study, we show that MICT and HIIT significantly reduced diastolic SR Ca^{2+} leak in HFpEF, associated with a significantly improved stroke volume in MICT. We found that MICT and HIIT affected cytosolic CaTs differently: only MICT synchronized early cytosolic Ca^{2+} release and reduced the CaT amplitude and the rate of Ca^{2+} decay.

The cellular pathomechanisms of HFpEF are not well understood. However, in a variety of animal models and in human myocardium, diastolic dysfunction in HFpEF has been linked to alterations in the cytosolic CaTs in LV cardiomyocytes.[6,7,23–25] CaT amplitudes have been reported as higher (abdominal aortic banding model,[24] or hypertrophic heart rat[26]), unchanged (ZSF-1 rat[25]), or lower (aortic banding rat[27]) as compared with control animals, suggesting that adaptation of the CaT may depend on the pathological trigger of HFpEF and probably the disease stage. In accordance, we have shown earlier in a cardiorenal model of HFpEF that an unchanged CaT amplitude in early HFpEF may deteriorate

Figure 6 Representative examples of spontaneous Ca^{2+} activity during inactivation of the sodium–Ca^{2+} exchanger (A). Occurrence of Ca^{2+} sparks (B) and wave propagation velocity [C; high-intensity interval training (HIIT): insufficient events for quantification]. One-way ANOVA followed by *post hoc* Bonferroni vs. Sed. (B) and two-tailed, unpaired Student's *t*-test (C). *P*-values: [1]0.046 and [2]0.02. *n* = cells from four animals per group. MICT, moderate-intensity continuous training.
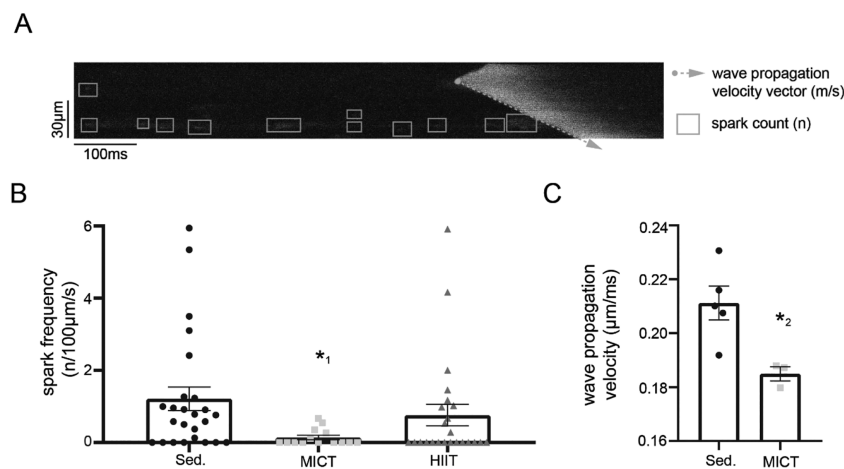
Figure 7 Ca^{2+} transients were analysed for their time to reach maximum amplitude (A) and their time to reach half-maximum amplitude (TF_{50}; B). Example of the spatial distribution of early and late Ca^{2+} release in left ventricular cardiomyocytes (shown: Sed.), as well as their consecutive beat-to-beat variation (C). The amount of early release sites was quantified (>3/10 early release events; D), and their probability of early release was determined in 10 consecutive cycles (E). Statistical analysis: either a two-way (A–E: 1–5 Hz) or one-way (A–E: 1 Hz rec.) ANOVA followed by a *post hoc* Bonferroni vs. Sed. (A–C). *P*-values: [1]0.0494, [2]0.03, [3]0.02, and [4]0.03. *n* = cells from four animals per group. (A, B) Sed., *n* = 20; moderate-intensity continuous training (MICT), *n* = 22; and high-intensity interval training (HIIT), *n* = 27. (D, E) Sed., *n* = 39; MICT, *n* = 22; and HIIT, *n* = 27.
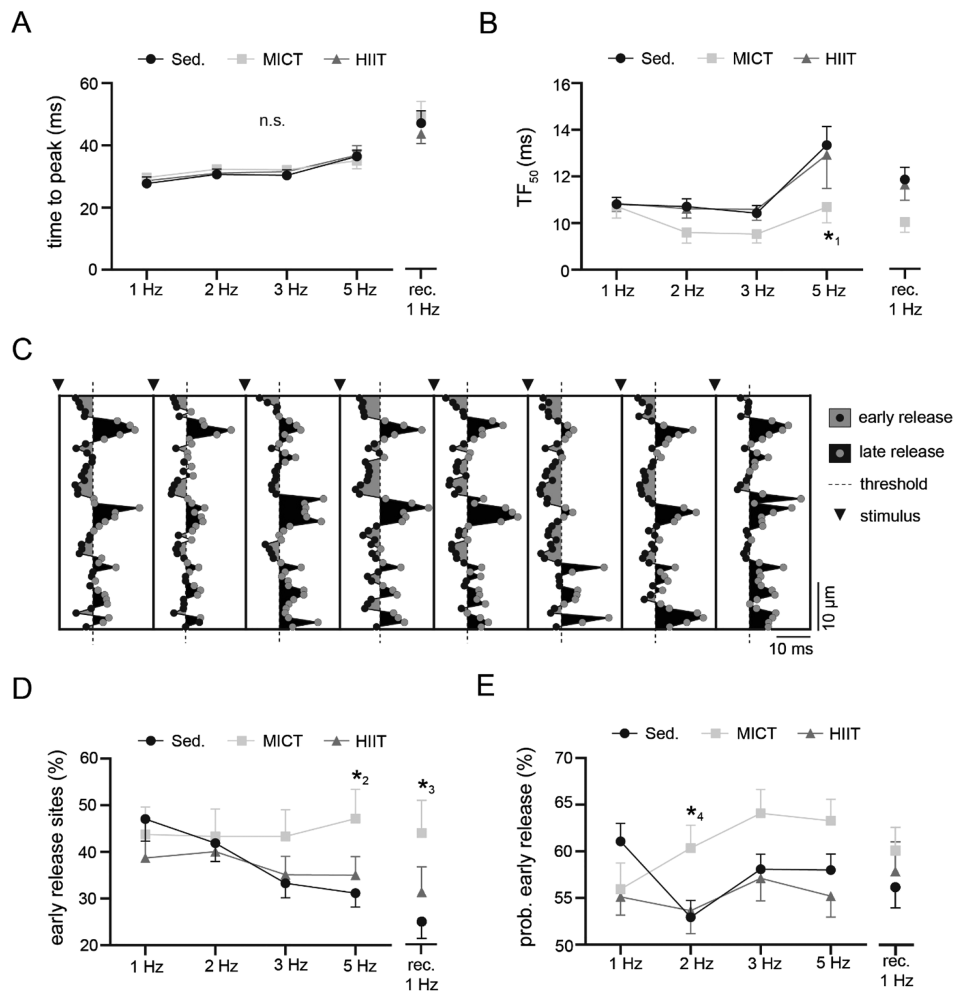
with progressive remodelling despite preserved ejection fraction.[6,7] Indeed, also in the present model, a normal systolic CaT amplitude has been reported at earlier disease stages,[25] indicating similar dynamic adaptations in Ca^{2+} homeostasis with HFpEF disease progression. Disease stage-dependent adaptations in cardiomyocyte Ca^{2+} signalling were also observed in atrial cardiomyocytes in this HFpEF model.[13,28]

In the present model of advanced metabolic HFpEF, we identified a lower SR Ca^{2+} load and increased SR Ca^{2+} leak as a contributing mechanism for reduced CaT amplitudes. Modelling of human myocardium suggested that a concentrically hypertrophied ventricular wall can maintain a preserved EF despite reduced sarcomere shortening at the cardiomyocyte level.[29]

Interestingly, also in the cardiorenal model of HFpEF deterioration of the CaT amplitude was associated with the occurrence of SR Ca^{2+} leak and a reduced SR Ca^{2+} load,[7] suggesting a common cellular pathomechanism in advanced stages of HFpEF.

In untrained conditions, diastolic Ca^{2+} was unchanged at baseline, and this was confirmed at stimulation frequencies close to the *in vivo* heart rate (i.e. 5 Hz, *Figure 3E*). Interestingly, cytosolic [Ca^{2+}] remained significantly elevated vs.

control during the recovery period after 5 Hz pacing, indicating impaired Ca^{2+} removal in HFpEF following cellular stress.

Moderate-intensity continuous training and HIIT have both been proven to be effective interventions to reduce endothelial dysfunction in the ZSF-1 metabolic HFpEF model.[30] HIIT was associated with improved clinical outcome in human HFpEF,[31] however potentially related to non-cardiac training effects.[32] In the present study, MICT and HIIT significantly reduced resting SR Ca^{2+} leak in LV cardiomyocytes. As we and others have shown earlier in other types of HF, a reduction in SR Ca^{2+} leak may attenuate cardiac remodelling and deterioration of contractile function *in vivo*.[33,34]

High-intensity interval training had no significant effect on the CaT in LV cardiomyocytes at low or elevated pacing frequencies in this metabolic HFpEF model. While this observation argues against a positive effect of HIIT on active (i.e. Ca^{2+}-dependent) cardiomyocyte contraction and relaxation, our results do not exclude beneficial effects of HIIT on diastolic function, especially because *in vivo* parameters like SV and EDV showed a trend towards improvement upon HIIT. Moreover, HIIT decreased SR Ca^{2+} leak and, as opposed to MICT, had no effect on CaT tau (i.e. cytosolic Ca^{2+} removal). Indeed, in previous studies, positive effects of HIIT on diastolic function were attributed to decreased stiffness or improved cardiac vagal tone.[32,35]

In MICT, additional parameters of *in vivo* function were improved as both SV and EDV increased upon training. Interestingly, CaT amplitudes were smaller in LV cardiomyocytes from MICT. In addition, CaT decay was prolonged. However, as opposed to HFrEF models, where impaired contractility is frequently associated with Ca^{2+} overload,[36] total cytosolic Ca^{2+} exposure (area under the curve) in our trained HFpEF model was unchanged and diastolic Ca^{2+} even decreased during the recovery period after high-frequency stimulation, which argues against cellular Ca^{2+} overload in MICT. It is of note that slowed Ca^{2+} decay in MICT occurred without a detectable increase in end-diastolic $[Ca^{2+}]$ also at higher pacing frequencies. This might lead to an increased cytosolic availability of Ca^{2+} during systole. Ca^{2+} sensitization and a prolonged exposure of myofilaments to Ca^{2+} are used in the clinic for the treatment of HFrEF, and components of systolic dysfunction are often also observed in HFpEF.[37–39] Following this concept, the Ca^{2+} sensitizer levosimendan is currently evaluated in clinical trials for the treatment of HFpEF.[40,41] As the decay of the caffeine-induced Ca^{2+} release as a measure for Na^+/Ca^{2+} exchanger-dependent Ca^{2+} extrusion was unchanged, slower cytosolic Ca^{2+} removal in MICT might be related to altered SERCA activity (see also Supporting Information, *Figure S2*).

In contrast to HIIT, MICT also significantly increased the number and open probability of functional early Ca^{2+} release sites (dyads) within the cardiomyocytes resulting in an accelerated early rise in cytosolic $[Ca^{2+}]$ and suggesting an improved gain of Ca^{2+}-induced Ca^{2+} release.[42]

Training also reduces arrhythmias in the setting of HF.[43] Here, we show that MICT significantly decreased pro-arrhythmic Ca^{2+} sparks and slowed Ca^{2+} wave propagation.

In conclusion, we show a novel pattern of Ca^{2+} dysregulation in a metabolic model of HFpEF. In addition, MICT and HIIT improved SR Ca^{2+} leak in cardiomyocytes, but only MICT was associated with profound effects on the cytosolic CaT and improved stroke volume *in vivo*.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. (A) Cell surface of isolated LV cardiomyocytes quantified from two-dimensional light microscopic images. (B) Representative example of the t-tubular network visualized by fluorescence probe di-8-ANNEPS in isolated LV cardiomyocytes and (C) quantification of t-tubular density after thresholding. Statistical analysis: Two-tailed, unpaired students t-test.

Figure S2. (A) Original images of Western Blot analysis showing LV myocardial expression of (B) sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), (C) total

phospholamban (PLB_{total}), (D) ratio of phosphorylated PLB at serine 16 (p-PLB_{Ser16}) to PLB_{total} and (E) ratio of phosphorylated PLB at threonine 17 (p-PLB_{Thr17}) to PLB_{total}. Statistical analysis: one-way ANOVA followed by a post-hoc Fishers LSD test *vs.* Sed. *p*-values: [1]0.034, [2]0.046, [3]0.045, [4]0.028, [5]0.036. n = animals.

Figure S3. Correlation of calcium transient (CaT) amplitude with (A) sarcoplasmic reticulum (SR) Ca^{2+} load and (B) SR leak. *p*-values (deviation from zero): [1]0.0002, [2]0.013. n = cells from 4 animals per group.

Table S1. List of used equipment and chemicals.

Table S2. Distribution of measured LV cardiomyocytes in 28-week-old animals.

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