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ORIGINAL ARTICLE

Modifications of short-term intrinsic pacemaker variability in diet-induced metabolic syndrome: a study on isolated rabbit heart

Conrado J. Calvo[1,2] · Wilson M. Lozano[1] · Óscar J. Arias-Mutis[1,2,3] · Luis Such-Miquel[4] · Luis Such[1] · Patricia Genovés[1,2,3] · Antonio Guill[5] · José Millet[5] · Francisco J. Chorro[2,3] · Antonio Alberola[1] · Sandeep V. Pandit[6] · Manuel Zarzoso[4]

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Abstract

Metabolic syndrome (MetS) describes a condition associated with multiple diseases concomitantly such as diabetes, hypertension, obesity, and dyslipidemia. It has been linked with higher prevalence of cardiovascular disease, atrial fibrillation, and sudden cardiac death. One of the underlying mechanisms could be altered automaticity, which would reflect modifications of sinus node activity. These phenomena can be evaluated analyzing the components of heart rate variability (HRV). Our aim was to examine the modifications of sinus node variability in an isolated heart model of diet-induced obesity and MetS. Male NZW rabbits were randomly assigned to high-fat (HF, $n = 8$), control (HF-C, $n = 7$), high-fat, high-sucrose (HFHS, $n = 9$), and control (HFHS-C, $n = 9$) groups, fed with their respective diets during 18/28 weeks. After euthanasia, their hearts were isolated in a Langendorff system. We recorded 10–15 min of spontaneous activity. Short RR time series were analyzed, and standard HRV parameters were determined. One-way ANOVA, Kruskal-Wallis test, and bivariate correlation were used for statistical analysis ($p < 0.05$). We did find an increase in the complexity and irregularity of intrinsic pacemaker activity as shown by modifications of approximate entropy, sample entropy, minimum multiscale entropy, and complexity index in HFHS animals. Even though no differences were found in standard time and frequency-domain analyses, spectral heterogeneity increased in HFHS group. Animal weight and glucose intolerance were highly correlated with the modifications of intrinsic pacemaker variability. Finally, modifications of intrinsic HRV seemed to be reliant on the number of components of MetS present, given that only HFHS group showed significant changes towards an increased complexity and irregularity of intrinsic pacemaker variability.

Keywords Metabolic syndrome · HRV · Atrial fibrillation · Sudden cardiac death

Conrado J. Calvo, Wilson M. Lozano and Óscar J. Arias-Mutis contributed equally to this work.

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✉ Manuel Zarzoso
manuel.zarzoso@uv.es

[1] Department of Physiology, Universitat de València, Valencia, Spain

[2] CIBERCV, Instituto de Salud Carlos III, Madrid, Spain

[3] INCLIVA, Valencia, Spain

[4] Department of Physiotherapy, Universitat de València, Valencia, Spain

[5] ITACA Institute, Universidad Politécnica de Valencia, Valencia, Spain

[6] Center for Arrhythmia Research, University of Michigan, Ann Abor, MI, USA

Introduction

It is well known that an excessive intake of calories from saturated fat and refined sugar can produce deleterious effects on health, as it has been shown by epidemiological, clinical, and experimental studies [11]. In addition, when this increased intake is coupled with other factors such as genetic predisposition to obesity or lack of physical activity, an imbalance between caloric intake and energy expenditure occurs, leading to overweight, obesity, and metabolic syndrome (MetS). Obesity and MetS are becoming a global epidemic, and their prevalence during the last 15 years has dramatically increased in both children and adults [2]. MetS describes a cluster of cardiovascular and metabolic alterations such as abdominal obesity, reduced HDL and elevated LDL cholesterol, elevated triglycerides, glucose intolerance, and

hypertension. Diagnosis requires that any three out of these five criteria are present [2].

Obesity and MetS increase the risk of developing metabolic and cardiovascular pathologies such as type 2 diabetes and cardiovascular and cerebrovascular disease [15]. Furthermore, these pathological conditions have been linked with a higher prevalence of supraventricular arrhythmias, atrial fibrillation (AF), and sudden cardiac death (SCD) [26]. Undeniably, AF is the most common tachyarrhythmia found in the clinical practice, but the underlying mechanisms through which MetS produces AF are not well understood [17].

Heart rate responds dynamically to physiologic and pathologic perturbations, and this cyclic fluctuation of RR intervals can be analyzed using heart rate variability (HRV). One of the proposed mechanisms of cardiac arrhythmia is abnormal impulse formation due to altered automaticity [5, 27]. Indeed, it has been shown that aged obese rats had a lower intrinsic heart rate than its young counterparts and a shift of the leading pacemaker site towards the inferior vena cava was observed [35], suggesting a decline in the function of sinoatrial node (SAN) that has been correlated with arrhythmias such as sick sinus syndrome. On the other hand, Albarado-Ibañez et al. [1] showed that rats fed with 20% sucrose in the drinking solution during 8 weeks presented a higher HRV both in vivo and in SAN preparations that could be related to the appearance of atrial arrhythmias.

Even though there are studies that have analyzed, individually, the effect of some components of MetS on the abnormal remodeling of the atria [1, 34], the specific alterations of cardiac electrophysiological properties such as SAN automaticity, induced by its combination, have not been explored in detail. This is of crucial importance given that the different components of MetS do not manifest individually and the pathological remodeling is aggravated when several of them appear concomitantly [19]. Thus, our aim is twofold: (1) determine the changes produced by an experimental model of diet-induced obesity and MetS on short-term intrinsic pacemaker variability, analyzed using HRV (time-domain, frequency-domain, and non-linear analyses), and (2) investigate if the severity of the changes depends on the number of components of MetS. Our working hypothesis is that obesity and MetS produce a pathological structural and electrophysiological remodeling in the heart that, in turn, modifies the different components of intrinsic pacemaker variability, resulting in an increased propensity for atrial arrhythmias.

Materials and methods

Animals and diets Adult male New Zealand white rabbits (n = 33) were used in the present study. The animals were randomly assigned into two different dietary regimes using either high-fat chow (n = 15) or high-fat, high-sucrose diet (n = 18). Animal care and the experimental protocols used in this study complied with EU directive 2010/63 on the protection of animals used for scientific purposes and with the United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised 1996). All procedures were approved by the Institutional Animal Care and Use Committee of the Universitat de València (2015/VSC/PEA/00049) and the University Committee on Use and Care of Animals (UCUCA) of the University of Michigan, Ann Arbor.

A. High-fat diet group (HF)

The animals were housed under controlled conditions of temperature (20 ± 1 .5 °C) and humidity (50 ± 5%), with a 12-h light cycle, and fed standard rabbit chow (laboratory rabbit diet Purina 5326, LabDiet, MO, USA). After a period of 2–3 weeks of acclimation, the rabbits were randomly assigned to the control group (HF-C; n = 7), which was fed with the normal chow (120 g day^{-1}), or to the experimental group (HF; n = 8), which was fed ad libitum with a high-fat diet. The standard rabbit chow used was Laboratory Rabbit Diet High Fiber #5326 (LabDiet, St. Louis, MO, USA) with a 23.1% of calories provided by proteins, 7.9% by fat, and 69.0% by carbohydrates (2.03 kcal g^{-1}). The high-fat group was fed ad libitum with a high-fat diet, which consisted of the standard rabbit chow with an additional 15% fat (Research Diets Inc., NJ, USA, yielding 3.1 kcal g^{-1}), as described previously [36]. Both groups were kept on their respective diets for 18 weeks. As previously reported, animals with this dietary regime developed central obesity and glucose intolerance, but no changes in total cholesterol, triglycerides, and HDL were reported [36].

B. High-fat, high-sucrose group (HFHS)

Rabbits were housed in the same conditions as HF group. Briefly, after an acclimation of 2–3 weeks in which rabbits were fed 120 g of standard rabbit chow (V2333–000, Ssniff, Soest, Germany), animals were randomly assigned to a control (HFHS-C, n = 9) or high-fat, high-sucrose group (HFHS, n = 9). Control animals followed the same dietary regime as HF-C, which has been shown to be appropriate for the maintenance of the adult rabbit [13]. Animals in the HFHS group were fed ad libitum during 28 weeks with added high-fat (diet #S9052-E020, Ssniff, Soest, Germany) and high-sucrose (15% dissolved in water) diet. Control diet contained 23.4% protein, 11.1% fat, and 65.5% carbohydrates (2.7 kcal g^{-1}). High-fat chow was composed mainly by 15.7% protein, 43.1% fat, and 41.2% carbohydrates (3.7 kcal g^{-1}), and the animals consumed 0.6 kcal mL^{-1} of the drinking solution [6].

With this dietary regime, animals developed central obesity, a state of prediabetes characterized by impaired fasting glucose and glucose intolerance, mild hypertension, and alterations in the lipid profile revealed by an increase in triglycerides and LDL, a decrease of HDL, and no changes in total cholesterol [6].

Isolated heart preparation Following heparinization (2500 IU), animals were euthanized with an overdose of sodium pentobarbitone (100 mg kg^{-1}) administered via injection through the left ear marginal vein. After thoracotomy, the heart was quickly removed and immersed in cold Tyrode solution (4 °C) for further preparation. After dissection, the aorta was cannulated and connected to a Langendorff system to provide the heart with warmed, oxygenated Tyrode solution containing (in mM) 130 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 0.6 $MgCl_2$, 1.4 NaH_2PO_4, 25 $NaHCO_3$, and 12.2 glucose. Oxygenation was carried out with a mixture of 95% O_2 and 5% CO_2. Tyrode temperature was constant throughout the experiment (37 ± 0 .5 °C), and perfusion pressure was maintained at 60 mmHg.

Two bipolar stainless surface electrodes were positioned on the heart, one in the right atrial appendage and another in the ventricle, right below the apex, for atrial and ventricular electrogram recording. The indifferent electrode was a 6-mm stainless steel plaque located over the cannulated aorta. After stabilization, 10–15 min recordings of spontaneous cardiac activity (2969 ± 750 complexes) were obtained with an electrophysiology data acquisition system (Axoscope, Molecular Devices, Sunnyvale, CA, USA). The sampling rate was 1 kHz.

Physiological parameters and calculations Standard HRV parameters were quantified using a custom-made software. Ventricular electrogram recordings were analyzed offline using custom scripts for pre-processing, visualization, and quantification of heart rate variability markers. After band-pass filtering between 0.5–250 Hz, baseline wander was removed using a bidirectional filtering strategy. Finite differential methods and wavelet transform were used for fiducial point estimation. *R*-peak detection was robustly estimated by parabolic fitting of the coiflet wavelet transform and detection of the maximum magnitude point. All *R* detections, defined as the peak of the ventricular activation wave, were supervised. RR series underwent high-pass filtering to ensure stationary results [28]. All recordings that were included for analysis were in sinus rhythm, and ectopic beats were excluded of the analyses by means of interpolation (4 Hz). The following parameters were quantified: (1) Time-domain: NN, SDNN, HR, SDHR, RMSSD, NN50, triangular index (Ti), and TINN; (2) Frequency-domain: very low frequency (VLF), low frequency (LF), high frequency (HF) peak, power and normalized, and LF-HF ratio, using FFT (Welch periodogram, window width 256 s and 50% overlap, interpolation rate 4 Hz); (3) Time-frequency spectral concentration analysis in order to calculate the spectral concentration ratio (SpCR) as a measure of spectral heterogeneity. SpCR was defined as the mean integration of the instantaneous power ratio of the power contained within 0.05 Hz around the maximum peak (± 0.05 Hz) divided to the total power within each band (VLF+ LF, HF); and (4) Non-linear analysis: Poincaré plot (SD1, SD2), detrended fluctuations (DFA α-1 and α-2), approximate entropy (ApEn), sample entropy (SampEn), and multiscale entropy (MSE) analyses. Additionally, we calculated the complexity index (CI_{1-20}) as the mean of entropies on all 20 scales of MSE [22]. All the measurements were performed according to the standards determined by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology [38]. A detailed description of the parameters can be found in the Supplementary Tables (Tables S1-S3).

Statistical analysis Values are reported as mean (SD) unless stated otherwise. Normality of data distribution was assessed using Shapiro-Wilk test. One-way analysis of variance with Bonferroni test for multiple comparisons was performed to analyze the differences between groups. When data was not normally distributed, Kruskal-Wallis test with Mann-Whitney's *U* test for pairwise comparisons were used. Bivariate correlation (Pearson's *r*) was used to study the association among parameters (SPSS, version 24.0 for Windows). Differences were considered significant at a two-tailed $p < 0.05$.

Results

Time-domain HRV analysis in isolated heart

The time-domain analysis of short-term (10–15 min) HRV showed no differences when comparisons were made between the four experimental groups in all the parameters studied: RR, SDRR, HR, SDHR, RMSSD, NN50, triangular index, and TINN (Table 1). However, when examining the time-course evolution of RR within groups, we observed a differential predominance of animals in the HFHS group to present slower pacemaker rhythms as compared with the other experimental groups (Fig. 1a). This finding was further supported by observing an increase in the percentage of beats in the higher RR bands ($H(3) = 8.32$; $p < 0.05$) and a decrease in the lower bands ($H(3) = 11.26$; $p < 0.05$) in the HFHS group. Indeed, pairwise comparisons showed significant differences in 225–250 and 300–325 ms bands when comparing HFHS group to HF (225–250 band $p = 0.047$; 300–325 band $p = 0.037$), HF-C (225–250 band $p = 0.043$; 300–325 band $p = 0.044$), and HFHS-C (225–250 band $p = 0.030$; 300–325 band

Table 1 Time-domain parameters of short-term HRV analysis in isolated heart

Parameter	HF-C	HF	HFHS-C	HFHS
NN (ms)	269 (23)	251 (27)	281 (46)	286 (31)
SDNN (ms)	7 (2)	6 (3)	8 (5)	9 (5)
HR (beats·min^{-1})	224 (18)	242 (28)	218 (36)	210 (25)
SDHR (beats·min^{-1})	7 (3)	6 (2)	6 (3)	6 (4)
RMSSD (ms)	5 (3)	4 (5)	8 (7)	11 (9)
NN50 (count)	4 (5)	10 (25)	10 (13)	40 (77)
Triangular index (ms)	2.1 (0.4)	2.3 (0.8)	2.0 (0.9)	2.3 (0.8)
TINN (ms)	76 (71)	91 (54)	190 (156)	81 (60)

Values are expressed as mean (SD). HF-C $n = 7$, HF $n = 8$, HFHS-C $n = 9$, HFHS $n = 9$

$p = 0.031$), and as suggested by the normal distribution fits for all groups (Fig. 1b, c).

Non-linear analysis of intrinsic pacemaker variability

When analyzing the complexity and irregularity of the RR time series, we found an increase in ApEn ($F(3,29) = 4.517$; $p = 0.01$; $\eta^2 = 0.32$; Fig. 2a) and SampEn ($F(3,29) = 4.90$; $p < 0.01$; $\eta^2 = 0.34$; Fig. 2b Table 2). Regarding ApEn, pairwise comparisons showed significant differences when comparing HFHS with HF ($p = 0.011$), HF-C ($p = 0.002$), and HFHS-C ($p = 0.019$). With respect to SampEn, the same trend was found comparing HFHS with HF ($p = 0.009$), HF-C ($p = 0.001$), and HFHS-C ($p = 0.015$) (Table S4). Regarding MSE, the results of the different scales are depicted in Fig. 3 c for each one of the experimental groups. We observed an increase of MSE_{min} ($F(3,29) = 3.34$; $p < 0.05$; $\eta^2 = 0.26$; Fig. 3a, Table 2) and CI_{1-20} ($F(3,29) = 3.43$; $p < 0.05$; $\eta^2 = 0.26$; Fig. 3d, Table 2) in the HFHS group, as shown by post hoc analysis when comparing HFHS to HF (MSE_{min} $p = 0.010$; CI_{1-20} $p = 0.006$), HF-C (MSE_{min} $p = 0.033$; CI_{1-20} $p =$

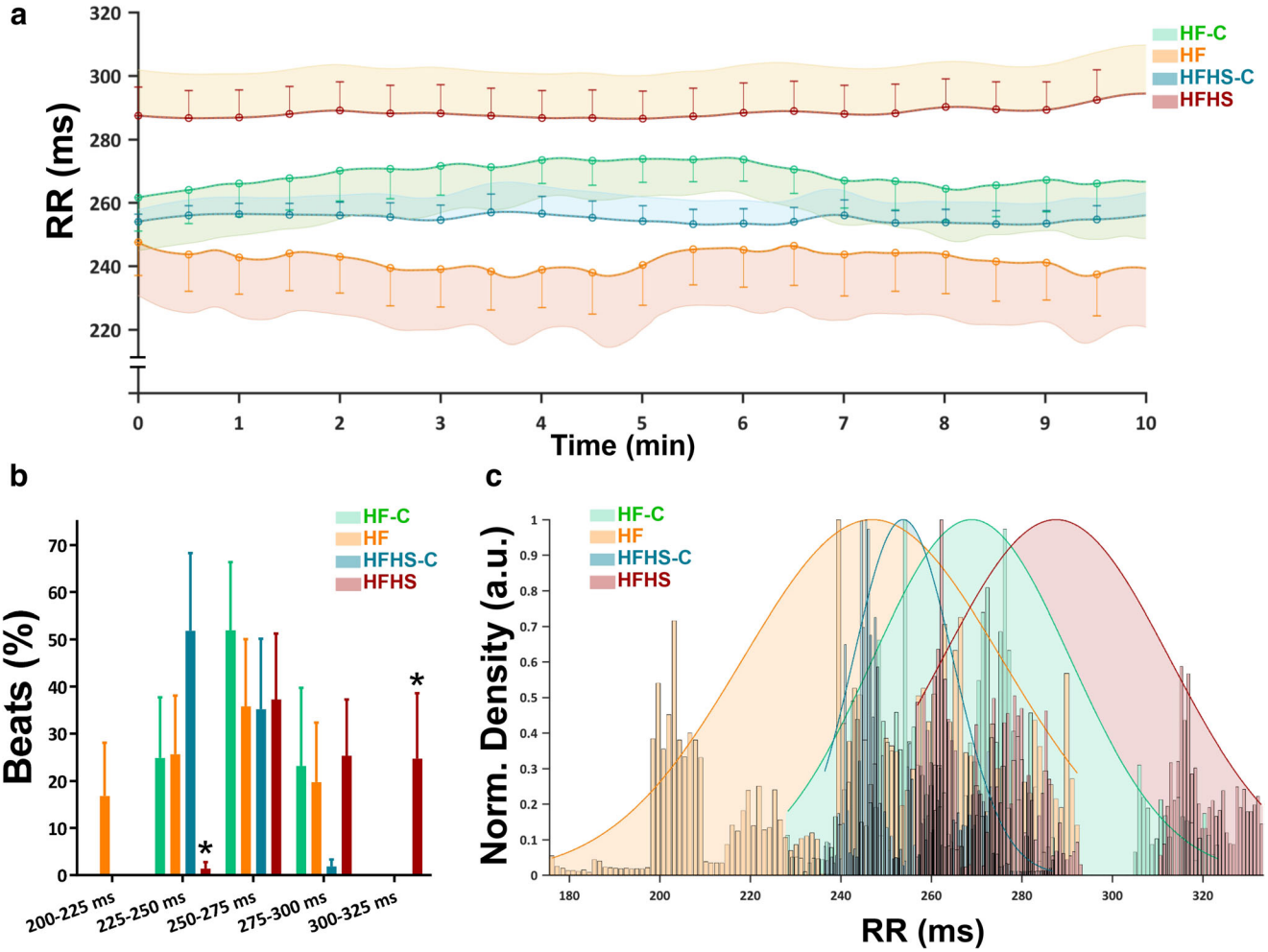

Fig. 1 Time-domain analysis of short-term intrinsic pacemaker variability. Averaged RR time course series are shown in panel **a**. Upper and lower shading represents standard deviation and error bars of the SEM. Panel **b** shows a comparison of band-segmented quantification of percentages of beats within each experimental group. Histogram representation of RRs for all animals within each group together with a normal distribution fit for each group is depicted in panel **c**. HF-C $n = 7$, HF $n = 8$, HFHS-C $n = 9$, HFHS $n = 9$; *$p < 0.05$ vs. HF-C, HF, and HFHS-C

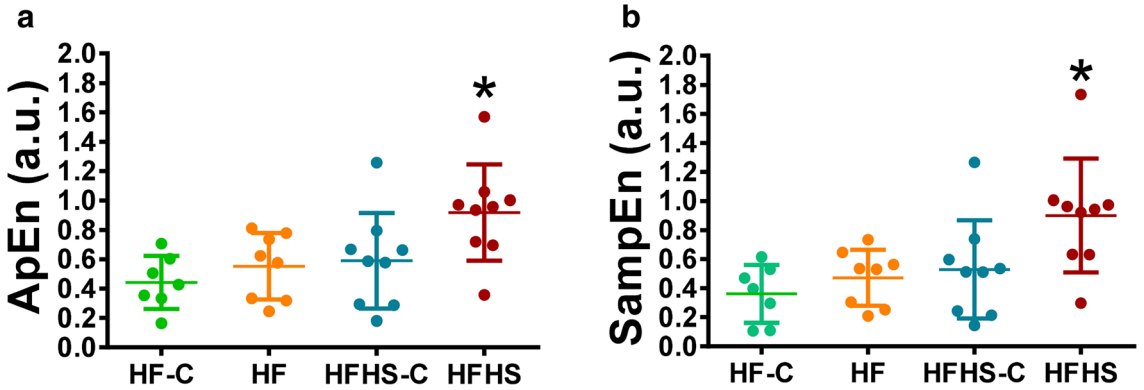

Fig. 2 Non-linear analysis of the regularity in short-term intrinsic pacemaker variability. Panel **a** shows changes in approximate entropy (ApEn). Sample entropy (SampEn) is displayed in panel **b**. HF-C $n = 7$, HF $n = 8$, HFHS-C $n = 9$, HFHS $n = 9$; *$p < 0.05$ vs. HF-C, HF, HFHS-C

0.047), and HFHS-C (MSE_{min} $p = 0.016$; $CI_{1–20}$ $p = 0.021$), indicative of a higher complexity in the RR dynamics of the isolated heart (Tables 2, S4). In the case of MSE_{max}, we found a tendency towards the increase in HFHS group, but differences were not statistically significant ($F(3,29) = 2.34$; $p = 0.09$; Fig. 3b, Table 2).

Finally, we performed Poincaré plot and detrended fluctuation analyses in order to identify dynamic changes on intrinsic heart rate. In the case of SD1 and SD2, observation of the Poincaré plots suggested a higher variability in HFHS group (Fig. 4a). However, the statistical analysis did not detect a main effect of the type of diet given the dispersion of the data, and therefore no differences were observed between groups in SD1 and SD2 of Poincaré plot (Fig. 4b). In addition, we did find higher variability in HFHS group as shown by quantification of RR differences distributions (Fig. 4c), with higher deviation and non-symmetrical distributions. No change was observed either in the detrended fluctuation analysis with DFA-α1 and DFA-α2 (Table 2).

Frequency-domain HRV analysis in isolated heart

In the frequency-domain analysis of short-term HRV using FFT, we did not find any difference between groups when

Table 2 Frequency-domain and non-linear parameters of short-term HRV in isolated heart

Parameter	HF-C	HF	HFHS-C	HFHS
VLF peak (Hz)	0.0027 (0.0019)	0.0063 (0.0130)	0.0026 (0.0020)	0.0009 (0.0017)
VLF power (%)	83.7 (18.8)	90.6 (11.1)	83.3 (18.7)	82.5 (20.5)
LF peak (Hz)	0.062 (0.025)	0.117 (0.161)	0.059 (0.033)	0.131 (0.258)
LF power (%)	5.7 (6.5)	3.7 (4.1)	5.8 (6.9)	8.1 (9.9)
LF norm. (n.u.)	0.44 (0.20)	0.53 (0.24)	0.52 (0.27)	0.48 (0.17)
HF peak (Hz)	0.20 (0.05)	0.25 (0.08)	0.28 (0.10)	0.27 (0.11)
HF power (%)	10.6 (12.6)	5.6 (7.5)	11.8 (15.5)	9.4 (10.7)
HF norm. (n.u.)	0.55 (0.20)	0.47 (0.24)	0.46 (0.27)	0.51 (0.17)
LF/HF (n.u.)	1.3 (1.2)	2.1 (2.2)	2.8 (3.8)	1.3 (1.1)
ApEn (a.u.)	0.44 (0.18)	0.55 (0.23)	0.59 (0.33)	0.92 (0.33)*
SampEn (a.u.)	0.36 (0.19)	0.48 (0.20)	0.53 (0.34)	0.90 (0.39)*
MSE_{min} (a.u.)	0.31 (0.19)	0.26 (0.17)	0.29 (0.16)	0.56 (0.33)*
MSE_{max} (a.u.)	0.75 (0.46)	0.65 (0.24)	0.72 (0.33)	1.11 (0.48)
$CI_{1–20}$ (a.u.)	0.54 (0.32)	0.41 (0.21)	0.50 (0.19)	0.83 (0.38)*
SD1 (ms)	3.2 (2.2)	2.9 (3.6)	11.7 (19.1)	20.4 (38.1)
SD2 (ms)	9.2 (3.3)	9.5 (4.9)	18.9 (25.9)	25.1 (37.8)
$SpCR_{HF}$ (n.u.)	0.47 (0.03)	0.53 (0.17)	0.38 (0.03)	0.33 (0.03)*
$SpCR_{VLF-LF}$ (n.u.)	0.62 (0.08)	0.61 (0.06)	0.63 (0.07)	0.53 (0.06)*
DFA-α1 (a.u.)	0.55 (0.49)	0.66 (0.16)	0.53 (0.18)	0.63 (0.22)
DFA-α2 (a.u.)	1.01 (0.34)	1.15 (0.46)	1.11 (0.35)	1.08 (0.35)

Values are expressed as mean (SD). HF-C $n = 7$, HF $n = 8$, HFHS-C $n = 9$, HFHS $n = 9$. *$p < 0.05$ vs. HF-C, HF, and HFHS-C

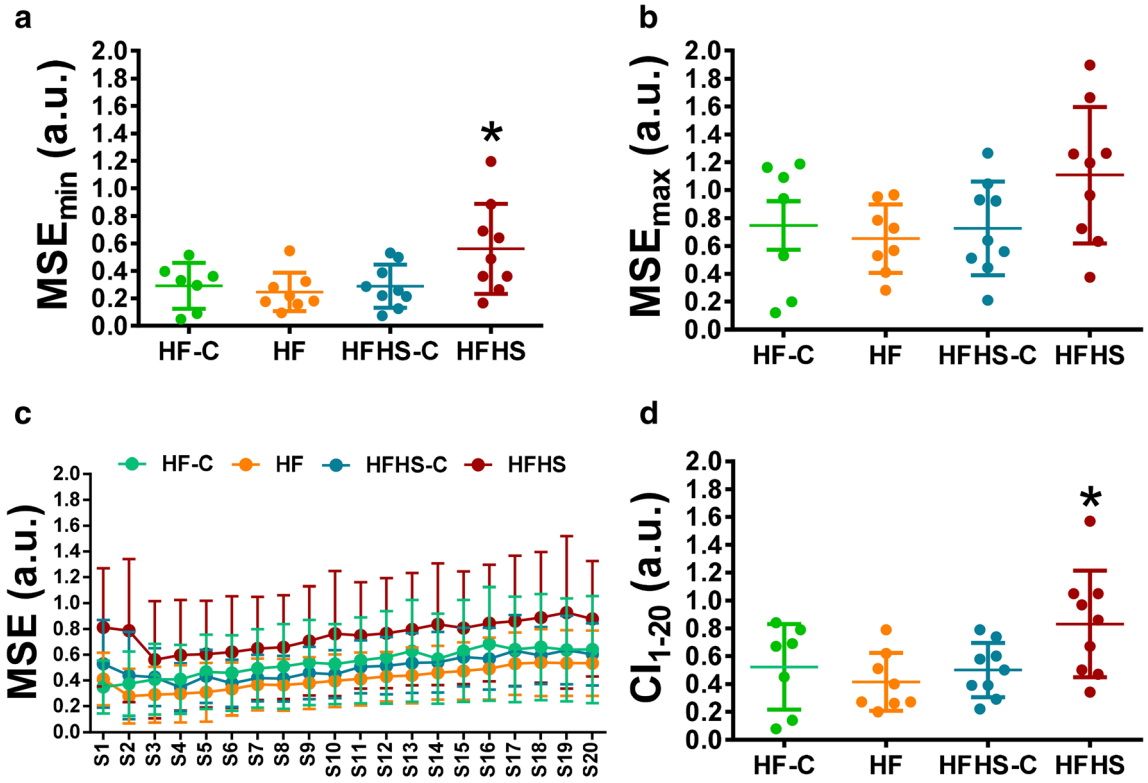

Fig. 3 Non-linear analysis of the complexity in short-term intrinsic pacemaker variability. Quantification of the minimum and maximum multiscale entropy (MSE_{min} and MSE_{max}, respectively) is shown in panels **a** and **b**. The results obtained in each one of the scales within each experimental group is depicted in panel **c**. Panel **d** shows the quantification of the complexity index ($CI_{1–20}$). HF-C $n = 7$, HF $n = 8$, HFHS-C $n = 9$, HFHS n = 9; *$p < 0.05$ vs. HF-C, HF, and HFHS-C

analyzing peak frequency, power, and normalized VLF, LF, HF, and LF/HF ratio in isolated heart (Table 2). Most of the power spectrum density was found in the range of VLF (0.0033–0.04 Hz), showing the lack of important fluctuations in frequency produced by autonomic nervous system, as we would expect in the isolated heart.

Time-frequency analysis, on the other hand, allowed us to find differences in $SpCR_{VLF+LF}$ ($F(3,29) = 3.95$; $p < 0.05$; $\eta^2 = 0.29$; Fig. 5b) and $SpCR_{HF}$ ($F(3,14.7) = 8.61$; $p < 0.05$; $\eta^2 = 0.47$; Fig. 5c) in the HFHS group. Indeed, pairwise comparisons showed a significant decrease of $SpCR_{VLF+LF}$ in HFHS animals when compared to HF ($p = 0.018$), HFHS-C ($p = 0.004$), and HF-C ($p = 0.023$) (Table 2, Table S4). With respect to $SpCR_{HF}$, we found a similar result, with a significant decrease in HFHS animals when compared to HF ($p = 0.002$), HFHS-C ($p = 0.027$), and HF-C ($p = 0.050$) (Tables 2, S4). Collectively, these results could point out towards a higher heterogeneity in both frequency bands in HFHS animals, showing a possible implication of dispersion in beat-to-beat pacemaker frequency through low- and high-frequency oscillations.

Correlation with the different components of MetS

Finally, we tried to correlate the parameters that were modified in the HFHS group with several biomarkers that characterize the different components of MetS, namely central obesity (weight, abdominal perimeter, BMI), glucose intolerance (fasting glucose, area under the curve of the in vivo glucose tolerance test), dyslipidemia (total cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides), and hypertension (systolic, diastolic, and mean blood pressure), measures taken from a previous study in the same animals [6]. The results suggested that weight was significantly correlated with ApEn, SampEn, MSE_{min}, and $CI_{1–20}$. Pearson's correlation coefficient indicated that this relation was positive and, therefore, direct (Table 3). Interestingly, modifications in spectral complexity ($SpGPF_{VLF+LF}$ and $SpGPF_{HF}$) were correlated with the parameters used to assess glucose intolerance, that is, fasting glucose and the area under the curve of the glucose tolerance test (Table 3).

Discussion

The aim of this study was to study the changes produced by an experimental model of diet-induced MetS on short-term intrinsic pacemaker variability, analyzed by exploring RR oscillations, and to investigate if the severity of the changes was dependent on the number of components of MetS. Our results showed that (1) HFHS diet administration increased the

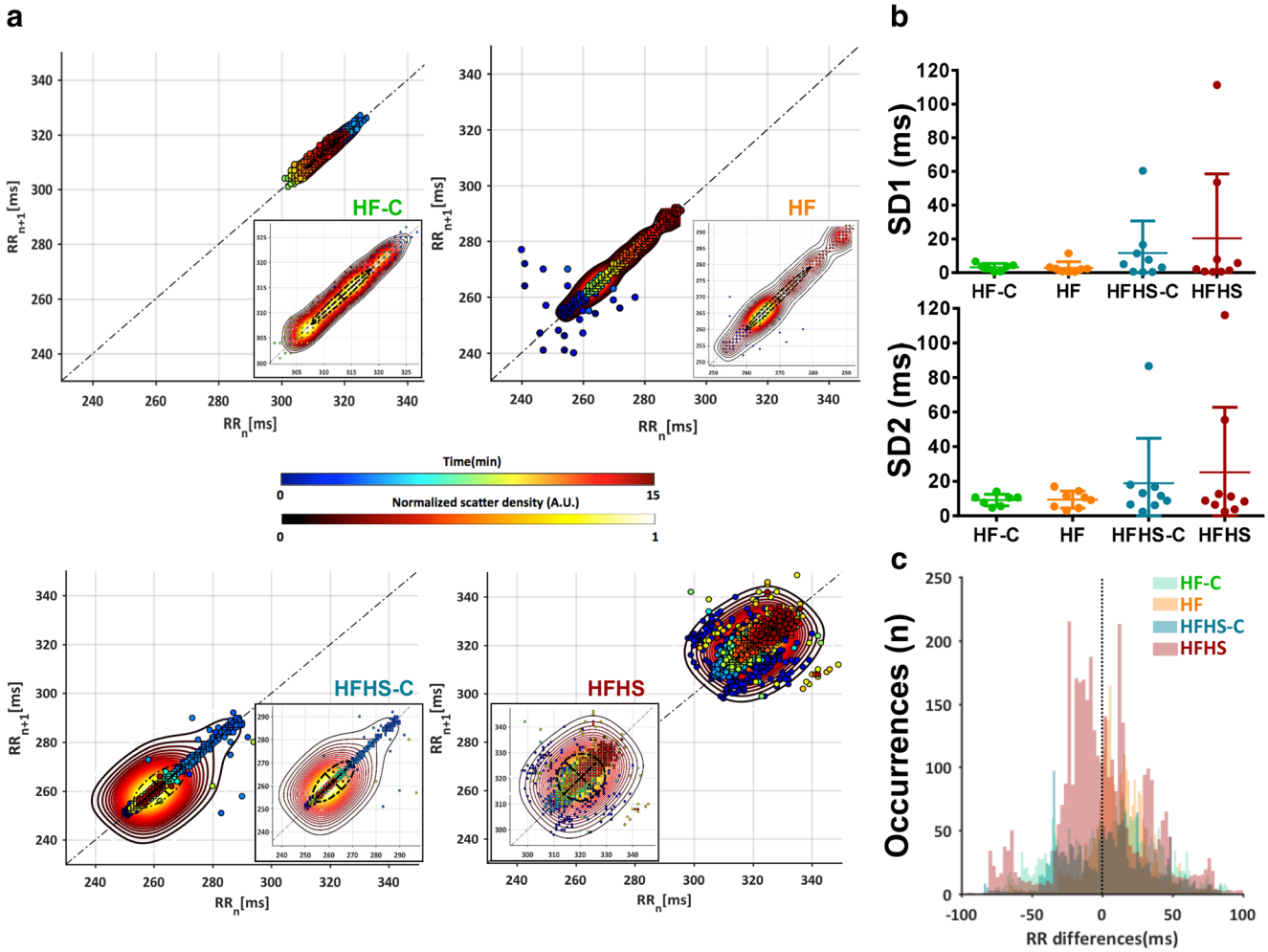

Fig. 4 Poincaré beat-to-beat relationships of pacemaker activation. Panel **a**, color-coded Poincaré patterns for each group and their distributions. Color represents transitions during 12–15 min and point-cloud density on the Poincaré beat-to-beat distribution. Panel **b**, quantified SD1, SD2 amongst groups. Panel **c**, superimposed distributions of RR differences showing greater dispersion in treated groups. HF-C $n = 7$, HF $n = 8$, HFHS-C $n = 9$, HFHS $n = 9$

complexity and irregularity of the intrinsic pacemaker activity, as shown by the higher values obtained in non-linear parameters of HRV such as ApEn, SampEn, MSEmin, and CI_{1-20}; (2) spectral complexity was modified in HFHS group, increasing in both the low and high frequency bands; (3) the modification of intrinsic HRV could be reliant on the number of components of MetS present, given that obese animals with only glucose intolerance (HF group) did not show any modification of intrinsic HRV; (4) animal weight was correlated with the modifications of intrinsic pacemaker variability showing that, when the weight of the animals increased, the same happened to the complexity and irregularity of heart rate; on the other hand fasting glucose and the area under the curve of the glucose tolerance test were correlated with spectral complexity markers, showing a direct association between glucose intolerance and spectral complexity; and (5) in short-term recordings, even though there were no modifications on the time-domain or frequency-domain analysis of HRV, when analyzing HR, we did find a decrease of the percentage of beats in the lower cycle length bands, and an increase in the higher bands, pointing towards a decrease in intrinsic heart rate.

Many studies performed over the last two decades have shown that alterations of pacemaker automaticity, both reduced and increased, can cause arrhythmic activity and reflect SAN dysfunction [5, 27]. HRV analysis is a very useful and inexpensive tool that enables to analyze heart rate dynamics. In our study, we used short-term (up to 15 min) recordings to analyze intrinsic pacemaker activity. The advantages of short-term HRV analysis include easier usability and analysis, the ability to collect data in a very controlled environment, and better suitability for spectral analysis that require stationary conditions [18, 38].

It has been reported that MetS modifies in vivo some of the time-domain parameters of short-term HRV analysis, decreasing SDNN and RMSSD, and increasing HR [12, 24, 25],

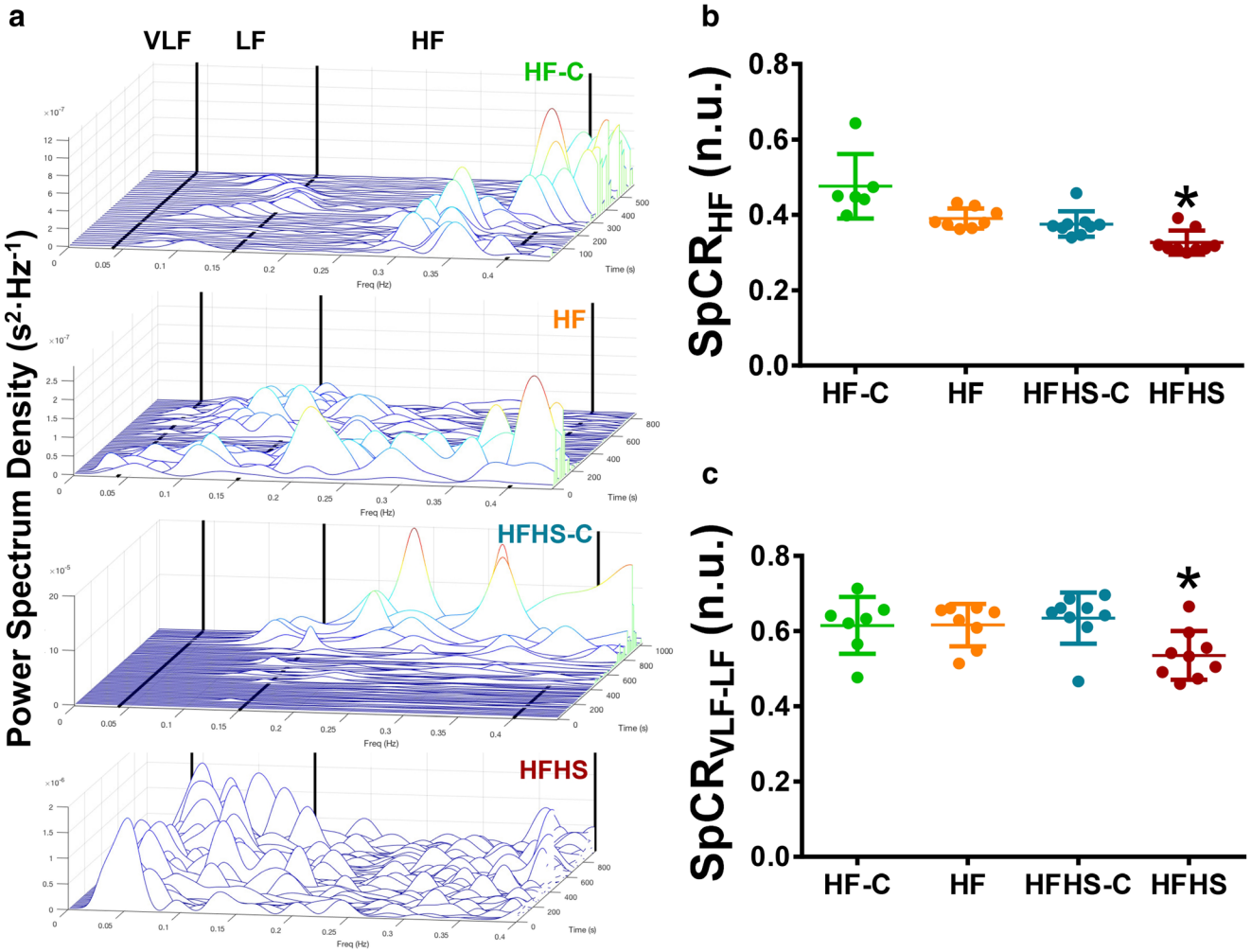

Fig. 5 Frequency-domain analysis of short-term intrinsic pacemaker variability displaying increased spectral complexity in HFHS group as well as decreased low- and high-frequency components. Panel **a**, segmented wavelet-derived time-frequency representations of RR oscillations highlighting spectral contribution in the main bands of interest (VLF+LF, HF). Panel **b**, quantification of spectral complexity by global power-frequency fraction estimation amongst groups. HF-C $n = 7$, HF $n = 8$, HFHS-C $n = 9$, HFHS $n = 9$; $^*p < 0.05$ vs. HF-C, HF, and HFHS-C

Table 3 Correlation (Pearson's *r*) between non-linear, frequency-domain parameters, and biomarkers of the different components of metabolic syndrome

	ApEn	SampEn	MSE_{min}	$CI_{1–20}$	$SpCR_{VLF+LF}$	$SpCR_{HF}$
Fasting glucose	−0.374	−0.395	−0.208	−0.316	−0.695*	−0.791*
AUC	−0.424	−0.438	−0.223	−0.364	−0.782*	−0.714*
Weight	0.669*	0.730*	0.764*	0.746*	−0.048	−0.329
BMI	−0.333	−0.305	−0.012	−0.183	−0.202	−0.637
Abdominal perimeter	0.329	0.348	0.115	0.172	−0.022	−0.106
TC	−0.261	−0.255	−0.259	−0.409	−0.045	−0.011
HDL	−0.073	−0.117	−0.372	−0.312	0.142	0.652
LDL	−0.050	−0.091	−0.397	−0.327	−0.571	−0.300
TG	0.301	0.463	0.638	0.544	0.232	0.529
Systolic BP	−0.130	−0.142	−0.089	0.032	0.447	0.267
Diastolic BP	0.300	0.372	0.478	0.411	0.291	−0.251
Mean BP	−0.021	0.022	0.188	0.163	0.448	−0.103

AUC, area under the curve; *BMI*, body mass index; *TC*, total cholesterol; *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein; *TG*, triglycerides; *BP*, blood pressure; HFHS $n = 9$; $^*p < 0.05$

whereas others have found no changes in time-domain HRV analysis [34]. However, information regarding intrinsic changes in HRV produced by MetS is scarce. Our results showed that the different dietary modifications did not alter any of the time-domain parameters studied analyzing the mean values during short-term (up to 12 min) recordings. These results differ from Yanni et al. [35] and Albarado-Ibañez et al. [1] who used a model of experimental obesity in old mice and rats, respectively, and both found a decrease on the intrinsic rate of discharge of the SAN. The different experimental preparations used (isolated mouse and rat right atria) and the duration of their recordings (≤ 60 s) could underlie in these differences. Interestingly, when we analyzed the incidence of beats by intervals of different cycle length, we did find a decrease in the percentage of beats in the shortest bands and an increase in the longer band. In our experimental model of isolated rabbit heart, the organ is not submitted to extrinsic neural or humoral mechanisms, but the intrinsic cardiac nervous system (ICNS) is intact. The ICNS represents the final relay station for the coordination of cardiac activity. It is composed of sensory (afferent), interconnecting (local circuit), and motor (adrenergic and cholinergic efferent) neurons that communicate with others in intrathoracic extracardiac ganglia, comprising a local distributive network that processes both centripetal and centrifugal information in cardiac control, all under the influence of central neuronal command and circulating catecholamines [7]. We could hypothesize that alterations within the ICNS that would alter its activity in a more stochastic manner could explain this observation. Indeed, it has been shown that electrical or nicotinic stimulation of intracardiac ganglia can produce either bradycardia, tachycardia, or a biphasic brady-tachycardia [3, 37]. It has also been reported that metabolic disorders such as type II diabetes produced a structural remodeling of intracardiac ganglia [10]. Unfortunately, we did not perform a negative control with complete autonomic or ganglionic blockade in both groups to test this hypothesis.

Regarding non-linear dynamics of the pacemaker time series, first we analyzed entropy-derived physiological markers. Our results showed an increase of ApEn, SampEn, MSE_{min}, and $CI_{1–20}$ only in the HFHS group. ApEn and SampEn quantify the irregularity and randomness of a time series, being the later less biased and more reliable when obtained with short-length time series [20, 38]. MSE, on the other hand, is a measure of the complexity of physiological time series taking into account the complex temporal fluctuations conveyed in different scales. Derived from MSE, the $CI_{1–20}$—which includes the global entropy of all 20 scales—can be computed. These measures, known to be useful for the assessment of cardiovascular risk and sudden cardiac death [38], are a well-recognized way to distinguish between physiological and pathological conditions [22]. In a healthy isolated heart, a deterministic behavior and thus lower values of entropy would be expected, since the organ is perfused in a stable and controlled environment, and the heart has no mechanical load. Collectively, the increase in the different measures of entropy indicates low predictability of cardiac fluctuations and higher randomness and complexity in SAN pacemaker activity, which has been related by others to cardiac pathological states [23].

The frequency-domain analysis of in vivo short-term recordings has shown important modifications of HRV, with most of the studies reporting a decrease of VLF, LF, or both components [8, 12, 14, 18, 24, 25, 34]. VLF and LF have been related to neurogenic sympathetic activity, intrinsic myogenic activity modulated both by sympathetic and parasympathetic nervous system, and intrinsic mechanisms occurring at the myocardial tissue level [30, 33]. Regarding HF, which reflects respiratory activity and thus is considered a marker of parasympathetic activity, results are more heterogeneous. Some studies have shown a decrease of the HF component [12, 14, 18, 24, 25], whereas others reported no change on this parameter [8, 35] in MetS. In isolated heart, and thus not submitted to extrinsic nervous or humoral influences, HRV is still present but markedly reduced, as it happens in heart transplant recipients [32]. Janousek et al. [16] compared short-term HRV in vivo with isolated heart and showed that parameters of HRV in the frequency domain significantly differ from those in intact hearts. Indeed, the power (%) of the VLF component increased greatly in isolated heart whereas the relative power in LF and HF bands diminished or remained unchanged, respectively. In our experiments, we found similar results in the isolated heart, and most of the relative power was found in the VLF band in all groups. In this line, it has been suggested that VLF rhythm has a cardiac origin and it is generated by the stimulation of afferent sensory neurons in the ICNS [21, 34]. When we studied the effects of the different dietary regimes and comparisons were made between groups, Fourier FFT spectral analysis showed no changes in any of the standard frequency-domain parameters studied.

While traditional methods allow for a limited global interpretation of the different frequency components, time-frequency techniques using wavelet transformation, on the other hand, provide an optimal time localization and resolution of the high-frequency and low-frequency components of HRV [21]. Using this method, we computed the band-normalized spectral concentration ratios (SpCR) as a measure to determine spectral heterogeneity in the low and high frequency bands, in such a way that higher values of SpCR would reflect a homogeneous distribution of the frequencies around the maximum peak (i.e., Fig. 5a, top panel), whereas lower values would show a higher heterogeneity of frequencies around the maximum peak (i.e., Fig. 5a, bottom panel). Interestingly, when comparisons were made between groups, only animals in HFHS groups showed lower values of $SpCR_{HF}$ and $SpCR_{VLF+LF}$ when compared with the rest of the groups, therefore showing higher spectral heterogeneity caused by a more erratic and irregular pacemaker activity. These results highlight the presence of more heterogeneous perturbations contributing to RR oscillations in MetS.

Regarding the associations between short-term HRV and individual metabolic syndrome risk factors, we found a direct correlation between animal weight and non-linear HRV analysis, specifically in ApEn, SampEn, MSE_{min}, and $CI_{1–20}$, showing that the complexity and irregularity of the intrinsic pacemaker activity increased with weight. Furthermore, we also found an inverse correlation between glucose tolerance, determined using fasting glucose and the AUC of the in vivo glucose tolerance test, and the spectral heterogeneity ($SpCR_{VLF+LF}$, $SpCR_{HF}$). This finding suggests that glucose intolerance aggravates the reduction in the SpCR coefficients which in turn reflects an increase in spectral heterogeneity.

In our isolated heart experiments and thus not submitted to extrinsic neural or humoral influences, intrinsic modifications in non-linear parameters and spectral heterogeneity were present in HFHS animals, but absent in the HF group. Therefore, despite our limitations, it would seem that the number of cardiovascular risk factors impact the observed modifications given that HF animals presented only central obesity and glucose intolerance [36], whereas HFHS group developed obesity, a state of prediabetes characterized by impaired fasting glucose and glucose intolerance, mild hypertension, and alterations in the lipid profile revealed by an increase in triglycerides and LDL, a decrease of HDL, and no changes in total cholesterol [6]. Indeed, several in vivo studies have shown modifications of HRV parameters with increasing number of MetS components [8, 14, 18, 25], and it has been shown that time-domain parameters are modified when one or more risk factors were present [14], while frequency-domain measures of HRV were not modified until three or more components were present [8, 14, 34].

Noteworthy, the mechanisms underlying the observed modifications of HRV and the electrical activity of the SAN were not explored in detail. Future studies should investigate possible alterations of the "calcium clock" and intracellular calcium handling in nodal cardiomyocytes or changes in the functional properties of the ionic currents that shape SAN cells action potential (especially I_f). Furthermore, the link between cardiac adipose tissue and arrhythmogenesis seems to be well established nowadays, which might be important during metabolic disorders and diabetes through the local release of cytokines, enhancing oxidative stress, modulating the effect of the intrinsic nervous system, or increasing interstitial fibrosis [4, 9, 29]. In addition, an increase in the complexity and fragmentation of atrial electrograms during atrial fibrillation has been shown during chronic atrial dilation in patients, likely due to structural remodeling of the atria, which creates a suitable environment for the maintenance of the arrhythmia [31].

The use of an isolated, non-working heart has enabled us to discard the possible influence of pressure modifications, acute stretch, extrinsic nervous activity, or humoral influences in the observed modifications and study the intrinsic SAN activity in the whole organ. However, the following limitations of our study should be considered: (1) we did not study the basic mechanisms underlying the observed modifications of HRV so our explanation is mainly speculative; (2) since the period of diet administration was different between HF and HFHS groups (18 vs. 28 weeks), the age of the animals was different at the end of the protocol, but this circumstance was mitigated by the existence of its corresponding control group; (3) the lack of in vivo measurements do not enable to analyze the effects of the different dietary regimes on HRV and make comparisons with the isolated heart experiments; (4) we cannot exclude the role of the ICNS in the observed modifications of HRV since we did not use a negative control with autonomic or ganglionic blockade. Despite these limitations, our study provides new insights in the intrinsic modifications of pacemaker activity and variability produced by MetS, which may have important implications for cardiac pathophysiology and arrhythmogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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