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Elevated miR-34a expression and altered transcriptional profile are associated with adverse electromechanical remodeling in the heart of male rats exposed to social stress

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Abstract

This study investigated epigenetic risk factors that may contribute to stress-related cardiac disease in a rodent model. Experiment 1 was designed to evaluate the expression of microRNA-34a (miR-34a), a known modulator of both stress responses and cardiac pathophysiology, in the heart of male adult rats exposed to a single or repeated episodes of social defeat stress. Moreover, RNA sequencing was conducted to identify transcriptomic profile changes in the heart of repeatedly stressed rats. Experiment 2 was designed to assess cardiac electromechanical changes induced by repeated social defeat stress that may predispose rats to cardiac dysfunction. Results indicated a larger cardiac miR-34a expression after repeated social defeat stress compared to a control condition. This molecular modification was associated with increased vulnerability to pharmacologically-induced arrhythmias and signs of systolic left ventricular dysfunction. Gene expression analysis identified clusters of differentially expressed genes in the heart of repeatedly stressed rats that are mainly associated with morphological and functional properties of the mitochondria and may be directly regulated by miR-34a. These results suggest the presence of an association between miR-34a overexpression and signs of adverse electromechanical remodeling in the heart of rats exposed to repeated social defeat stress, and point to compromised mitochondria efficiency as a potential mediator of this link. This rat model may provide a useful tool for investigating the causal relationship between miR-34a expression, mitochondrial (dys)function and cardiac alterations under stressful conditions, which could have important implications in the context of stress-related cardiac disease.

Keywords: miR-34a; arrhythmias; stress; cardiac disease; epigenetic

INTRODUCTION

Extensive research has shown that chronic psychosocial stress can favor the onset and progression of cardiovascular disease (CVD) in both patients with established disease and individuals with no prior history (Rozanski *et al.*, 1999; Bairey Merz *et al.*, 2002). Notably, the magnitude of psychosocial stress-related CVD risk has been reported to be comparable with that attributed to traditional CVD risk factors (Rosengren *et al.*, 2004). Potential autonomic, hormonal, and inflammatory mediators of this link have been identified (e.g., (Black & Garbutt, 2002; Girod & Brotman, 2004)). However, current scientific knowledge does not completely explain the complex pathophysiology underlying psychosocial stress-induced CVD.

It is becoming increasingly clear that epigenetic modulators might constitute an additional pathway leading to adverse cardiac remodeling under psychosocial stress conditions and therefore contribute to disease (Saban *et al.*, 2014; Muka *et al.*, 2016). Thus, a better understanding of these players may help identify novel targets for the prevention and timely treatment of psychosocial stress-related CVD. Cardiac transcriptional pathways are intimately regulated by microRNAs (miRNAs) (Small & Olson, 2011). MiRNAs are small noncoding RNAs, approximately 18-25 nucleotides in length at mature stage, which can mediate post-transcriptional gene repression by inhibiting translation and/or promoting degradation of target protein-coding mRNA by base pairing (Humphreys *et al.*, 2005). Importantly, miRNA profiling in cardiac tissue of mouse models of CVD and human biopsies has revealed signature patterns of miRNAs dysregulation in myocardial infarction (van Rooij *et al.*, 2008; Lin *et al.*, 2010; Sun *et al.*, 2017), end-stage heart failure (Ikeda *et al.*, 2007), and cardiomyopathy (Sucharov *et al.*, 2008; Quattrocelli *et al.*, 2013), opening up a new field of investigation to understand the molecular mechanisms controlling gene expression also in the context of psychosocial stress-related CVD. Indeed, studies have shown that stressful events are associated with alterations in miRNAs biogenesis, which in turn could influence gene expression in response to stress (Leung *et al.*, 2011; Malan-Muller *et al.*, 2013).

In the current study we focused on one specific family of miRNAs, the miR-34 family, and evaluated whether psychosocial stress exposure influences the cardiac expression of one of its members – the miR-34a isoform – in a rodent model. The rationale behind the choice of miR-34a as a key target of our investigation was based on the following pieces of evidence: (i) miR-34a expression is elevated in cardiac tissue of patients with heart disease or cardiac injury (Thum *et al.*, 2007; Greco *et al.*, 2012); (ii) likewise, miR-34a cardiac expression is augmented in a mouse model of myocardial infarction (Yang *et al.*, 2015); (iii) inhibition of miR-34a through anti-miR-34a therapy improves cardiac function and reverses cardiac remodeling in mouse models of myocardial infarction or pre-existing pathological hypertrophy (Bernardo *et al.*, 2014; Yang *et al.*, 2015), and in a rat model of doxorubicin cardiotoxicity (Piegari *et al.*, 2020); (iv) miR-34a expression is elevated in the medial prefrontal cortex of mice exposed to acute stress exposure (Andolina *et al.*, 2016); (v) knockout of the miR-34 family fosters behavioral resilience after acute and chronic stress exposure (Andolina *et al.*, 2016; Andolina *et al.*, 2018).

However, despite such accumulating evidence on the potential role of miR-34a in cardiac pathophysiology (Boon *et al.*, 2013) as well as in mediating neurochemical and behavioral stress responses (Andolina *et al.*, 2017), to the best of our knowledge, no studies have investigated the effects of stress exposure on miR-34a cardiac expression. Here, we adopted a rodent model of repeated social defeat stress, a highly translational, naturalistic experimental paradigm which leads to severe consequences on the electrical stability and morpho-structural properties of the heart that are indicative of enhanced CVD risk (reviewed in (Sgoifo *et al.*, 2014)). Anticipating signs of elevated miR-34a expression in the heart of rats exposed to repeated social defeat, we sought to characterize changes in putative miR34-a gene targets, in order to identify epigenetic risk factors of stress-induced CVD. Moreover, we aimed to investigate whether elevated miR-34a cardiac expression following repeated social defeat was coupled with specific alterations in (i) arrhythmia vulnerability, (ii) hemodynamic parameters, and (iii) myocardial weights.

MATERIALS AND METHODS

Animals

Experiments were conducted on 3-month-old male wild-type Groningen rats. This rat population, originally derived from the University of Groningen (The Netherlands) and currently bred in our laboratory under standard conditions, shows considerable individual differences in cardiovascular responses to environmental challenges (Sgoifo *et al.*, 2005). Experimental rats (n =17 for Experiment 1 and n = 14 for Experiment 2) were housed individually one week before the beginning of the procedures. Additional older (10-12 month-old) male wild-type Groningen rats (n= 8) were pair-housed in a different room with an oviduct-ligated female partner and used as residents in the resident-intruder paradigm (see below for details). All animals were kept in climate-controlled rooms, with a 12-h light cycle (lights on at 7 pm). Food and water were available ad libitum. Experiments were performed in accordance with the European Community Council Directive 2010/63/UE and approved by the Italian legislation on animal experimentation (D.L. 04/04/2014, n. 26, authorization n. 449/2017-PR). All efforts were made to reduce sample size and minimize animal suffering.

Social defeat stress

Social defeat stress was based on a classical "resident–intruder" paradigm (Miczek, 1979). Resident rats were screened for their level of aggression prior to inclusion in the study. Experimental rats were randomly assigned to the ''intruder" or ''control" group. Briefly, each intruder was transferred to the cage of a resident rat for 30 min after the removal of the resident's female partner. Intruders were threatened and physically assaulted by the resident rat. After exhibiting signs of subordination and social defeat (i.e., when the intruder rat assumed a supine posture that was held for at least 5s) or after 15 min, whichever came first, intruders were confined behind a Plexiglas partition within the resident's cage for the remainder of the 30-min period, allowing for psychogenic exposure to aggressive threats without physical harm. At the end of the social defeat session, which took place between 10:00 and 11:00 am, intruders were returned to their home cages. Intruders were exposed to

either one episode of social defeat stress (Experiment 1) or eight episodes on consecutive days (Experiments 1 and 2). In case of repeated episodes, intruders were exposed each day to a different resident, with a rotational design. Control rats were not present in the room during the social defeat procedure and remained in their home cages. Control manipulation consisted of handling for 60 s by the same experimenter and occurred in correspondence of each social defeat episode. Cage cleaning was done weekly for all animals by the same experimenter at the same time of the day.

Experiment 1

This experiment was designed to evaluate changes in miR-34a levels and gene expression in the heart of rats exposed to a single or repeated social defeat episodes. Rats were randomly assigned to the single social defeat (SSD, n = 5), repeated social defeat (RSD, n = 6) or control (CTR = 6) groups as described above. The CTR group served as a reference group for both the SSD and RSD groups. Twenty-four hours after the first social defeat (SSD group) or 24 h after the last social defeat/control procedure (RSD and CTR groups, respectively), rats were anesthetized using inhaled isoflurane (2% in 100% oxygen) (Zoetis, Italy). The hearts were quickly excised and perfused with a 0.9% NaCl solution to flush the residual blood from the tissue, immediately flash frozen in liquid nitrogen, and stored at -80 °C until molecular analysis.

RNA Purification

Punches of the left ventricle were dissected using stainless-steel tubes (inside diameter: 2 mm). Total RNA was extracted using total RNA purification Kit (NorgenBiotek, Thorold, Canada) according to the manufacturer’s protocols. RNA quantity was determined by absorbance at 260 nm using a NanoDrop UV-Vis spectrophotometer. The same RNA samples were used both for the quantification of miR-34a expression via quantitative real time PCR analysis and for Next Generation Quant-Seq RNA analysis. Samples showing as

outliers in a PCA (n = 1 for the RSD and n = 1 for the CTR group, Supplementary Figure 1) were excluded from these analyses.

Quantitative Real-Time RT-PCR (qPCR)

Total RNA (10 ng) from each punch sample was reverse transcribed to cDNA for miR-34a and the endogenous control sno135, using the TaqMan MicroRNA Reverse Transcription kit (Taqman Assay ID 000426 and 001230, Thermo Fisher Scientific). cDNA templates (around 0.7 ng per sample) were amplified by qPCR, using the Thermo Fisher QuantStudio 3 thermal cycler apparatus. Cycle threshold (C_T) miR-34a values were normalized to sno135 C_T values. All data were run in triplicate and expressed as fold changes according to the ΔΔC(t) method (Schmittgen & Livak, 2008).

RNA library preparation, sequencing and data analysis

mRNA sequencing was performed by NGD technology service (Pozzuoli). Total RNA from each heart sample was quantified using the Qubit 2.0 fluorimetric Assay (Thermo Fisher Scientific) and its quality was evaluated by Agilent 2100 Bioanalyzer RNA assay (Agilent technologies, Santa Clara, CA). Only RNA samples with RIN value > 7.0 were included in the study. Libraries were prepared from 100ng of total RNA using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen GmbH). Quality of libraries was assessed by using screen tape High sentisivity DNA D1000 (Agilent Technologies). Libraries were sequenced on a NovaSeq 6000 S1 system (flow cell: 100 cycles; Illumina Inc.). Illumina novaSeq base call (BCL) files were converted in fastq file through bcl2fastq (version v2.20.0.422). Sequence reads were trimmed using bbduk software (bbmap suite 37.31) to remove adapter sequences, poly-A tails, and low-quality end bases (regions with an average quality below 6). Alignment was performed with STAR 2.6.0a (Dobin *et al.*, 2013) on rn6 assembly downloaded from UCSC Genome Browser website. Gene expression levels were determined with htseq-count 0.9.1 (Anders *et al.*, 2015) by using Ensembl genes annotations (Version 6.0). Expression data were analyzed by Rosalind (https://rosalind.onramp.bio/), with

a HyperScale architecture developed by OnRamp BioInformatics, Inc. (San Diego, CA). Individual sample counts were normalized by Relative Log Expression (RLE) using DESeq2 R library (Love *et al.*, 2014). Read Distribution percentages, violin plots, identity heatmaps, and sample MDS plots were generated as part of the QC step. Gene Ontology and pathway enrichments analyses were performed on those genes whose fold changes corrected p values were <.10, using the DAVID web-based tool [http://david.abcc.ncifcrf.gov/] (Huang *et al.*, 2009).

The Quant-seq dataset is available from the GEO database under the accession number GSE169181.

Experiment 2

This experiment was designed to evaluate potential alterations in (i) vulnerability to arrhythmias, (ii) hemodynamic parameters, and (iii) myocardial weights in rats exposed to repeated episodes of social defeat. Rats were randomly assigned to RSD (n = 7) or CTR (n = 7) conditions as described above. Two weeks before the beginning of the social defeat stress/control procedure, rats were implanted, under isoflurane anesthesia (2% in 100% oxygen) (Zoetis, Italy), with radiotelemetric transmitters (TA11CTA-F40, DataSciences International, St. Paul, MN, USA) for ECG recordings (sampling frequency 1000 Hz). The transmitter body was placed in the abdominal cavity; one electrode was fixed to the dorsal surface of the xyphoid process and another electrode was placed in the anterior mediastinum close to the right atrium, according to a previously described procedure (Sgoifo *et al.*, 1996). Animals were housed individually post-surgery and were prophylactically injected for 2 days with gentamicine sulfate (Aagent, Fatro, Italy, 0.2 mL/kg, s.c.) and flunixin (Megluflen, Izo, Italy, 0.2 mL/kg, s.c.).

Vulnerability to cardiac arrhythmias

Twenty-four hours after the last social defeat/control procedure, rats were injected with the drug isoproterenol (β-adrenoceptor agonist, Sigma, St Louis, MO, USA) at a dose (0.2

mg/kg, i.p.) known to induce arrhythmias in this rat strain (Carnevali *et al.*, 2019). ECG signals were recorded during the hour that preceded and the hour that followed drug administration. The occurrence of arrhythmic events was determined offline via visual inspection of ECG traces using ChartPro 5.0 software by a trained experimenter according to (Curtis *et al.*, 2013). Specifically, arrhythmic events were assigned the following point values according to (Grippo *et al.*, 2012): a) 1 point for isolated supraventricular events (premature atrial, junctional, or atrioventricular block) or an isolated premature ventricular or ventricular escape complex; b) 2 points for supraventricular tachycardia or two or three consecutive premature ventricular complexes (salvo); c) 3 points for premature ventricular beats in a trigeminal or bigeminal pattern or non-sustained ventricular tachycardia (defined as ≥3 and ≤10 consecutive premature ventricular contractions); d) 4 points for sustained ventricular tachycardia (defined as >10 consecutive premature ventricular contractions) or asystole (absence of electrical activity during the loss of two or more consecutive cardiac cycles as defined by the length of the two preceding cardiac cycles). Using these categories, an arrhythmic burden was calculated for each rat, defined as the sum of the scored arrhythmic events during the hour that followed isoprotorenol injection. Moreover, we calculated the mean heart rate (reported in beats per minute, bpm) before and after isoproterenol injection.

Hemodynamic assessment

Three days after the isoproterenol challenge, hemodynamic data were recorded in RSD and CTR rats under ketamine chloride (Imalgene, Merial, Milan, Italy; 40 mg/kg i.p.) plus medetomidine hydrochloride (Domitor, Pfizer Italia S.r.l., Latina, Italy; 0.15 mg/kg i.p.) anesthesia. A microtip pressure transducer catheter (Millar SPC-320, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and connected to a recording system (Power Laboratory ML 845/4 channels, 2Biological Instruments, Besozzo, Italy) to collect systolic and diastolic blood pressures and heart rate. The catheter was then advanced into the left ventricle to measure left ventricular (LV) systolic pressure (LVSP), LV

end-diastolic pressure (LVEDP), the peak rate of rise ($+dP/dt_{max}$) and decline ($-dP/dt_{max}$) of LV pressure (indexes of myocardial efficiency), isovolumic contraction time (IVCT), ejection time (ET; approximated as the time interval between aortic valve opening and time of $-dP/dt_{max}$), and LV relaxation time (LVRT, computed from $-dP/dt_{max}$ to 5 mmHg above LVEDP and taken as index of isovolumic relaxation) using the software package AcqKnowledge 3.9 (Biopac Systems, Goleta, CA). Finally, we computed the myocardial performance index (MPI) as the ratio between total time spent in isovolumic activity (IVCT + isovolumic relaxation time) and ET (Salemi *et al.*, 2004). MPI is considered to reflect global cardiac function and possesses prognostic value in several pathologic conditions such as myocardial hypertrophy and infarction (Salemi *et al.*, 2004).

Myocardial weights

After hemodynamic assessment, the heart of RSD and CTR rats was rapidly excised, perfused with a 0.9% NaCl solution to flush the residual blood from the tissue and weighed. The left and right ventricles were then separated and weighed. Myocardial weights were corrected for body weight and heart weight.

Statistical analysis

All statistical analyses were performed using the IBM SPSS statistical package (International Business Machines Corporation, Armonk, NY, USA, version 25). Normal distribution of variables was checked by means of the Kolmogorov-Smirnov test. To test for the effects of stress exposure on cardiac miR-34a expression (Experiment 1), a one-way ANOVA was applied (between subject factor “stress exposure”, three levels: CTR, SSD and RSD) followed by Bonferroni's post-hoc comparisons. To test for the effects of stress exposure on cardiovascular data (Experiment 2), a series of unpaired Student’s t tests (RSD vs CTR rats) were applied. Data are reported as means ± standard error of the mean (SEM). Statistical significance was set at $p < .05$.

RESULTS

Experiment 1

Repeated social defeat stress promotes the expression of miR-34a in cardiac tissue

First, we tested whether the expression of miR-34a in the left ventricle was altered by a single episode and/or repeated episodes of social defeat stress. One factor ANOVA revealed a significant effect of stress exposure on miR-34a expression ($F_{(2,13)} = 4.167$, $p < .05$). Specifically, no significant changes were found in the cardiac expression of miR-34a between SSD and CTR rats. However, RSD rats showed a significantly larger expression of miR-34a compared to CTR rats ($p < .05$) (Figure 1).

mRNA-sequencing revealed different molecular targets potentially regulated by miR-34a in the cardiac tissue of rats exposed to repeated social defeat

We aimed at evaluating transcriptional alterations induced by repeated social defeat stress in the left ventricle and at extracting genes that may be putatively targeted by miR-34a. Thus, we performed genome-wide Quant-Seq mRNA sequencing on the same samples used for miR-34a measures. We found 378 differentially expressed genes (DEGs) (adjusted $p < .10$) between RSD and CTR rats, of which 214 were down- and 164 were up-regulated, respectively, in RSD rats (Supplementary Tables 1 and 2). A subset of down- and up-regulated genes in RSD rats with higher statistical significance (adjusted $p < .05$) and their fold changes are illustrated in the heatmaps of Figure 2 and Figure 3, respectively.

To unravel the physiological functions that are mostly represented by these altered transcripts, we performed Gene Ontology analysis and found several significantly enriched biological functions (adjusted $p < .001$) that were grouped in 3 main annotation clusters (Table 1). The first cluster included the “mitochondrion” as the main keyword, the second cluster comprised several neurodegenerative diseases and mitochondrial-related components or pathways like oxidative phosphorylation, mitochondrial respiratory chain complex I, mitochondrial inner membrane and cytochrome-c oxidase activity, and the third cluster included the cellular main signaling component phosphatidylinositol triphosphate.

Furthermore, among the 378 DEGs, we aimed at identifying transcripts that may be directly regulated by miR-34a. With the aid of bioinformatic web-based predictor tool “Target Scan” (http://www.targetscan.org/vert_72/) we overlapped the list of DEGs between RSD and CTR rats with web-predicted miR-34s targets and found 61 matching genes (Figure 4A), which are reported in the heatmap of Figure 4B. Interestingly, Gene Ontology analysis performed on these 61 genes revealed several significantly enriched biological functions ($p < .05$), including the molecular function “zinc ion binding”, the cellular component “mitochondrial outer membrane”, and the biological process “apoptotic mitochondrial changes” (Figure 4C).

Experiment 2

Repeated social defeat promotes adverse electromechanical remodeling of the heart

In this experiment we aimed at documenting the effects of repeated social defeat stress on cardiac function. Following potent sympathetic stimulation with isoproterenol, RSD rats displayed a significantly higher arrhythmic burden compared with CTR rats (+65%, $p < .05$) (Figure 5). No group differences were observed in mean heart rate values after isoproterenol administration (RSD: 475 ± 8 bpm vs CTR: 466 ± 10 bpm).

We could not assess hemodynamic parameters in one RSD rat, which died just after the insertion of the catheter into the right carotid artery. RSD and CTR rats showed similar mean heart rate values under anesthesia (RSD: 242 ± 9 bpm vs CTR: 227 ± 8 bpm). Signs of systolic ventricular dysfunction were found in RSD rats compared with CTRs, as indicated by the significant reduction in the maximal rate of LV pressure rise during isovolumic contraction ($+dP/dt_{max}$) (-12%; $p < .05$), the significant prolongation of IVCT (+17%; $p < .05$), and the significant increase in MPI (+20%, $p < .05$) (Figure 6). In addition, a decrease in ET (-9%) was observed in RSD rats compared with CTRs, although the difference did not reach statistical significance ($p = .09$) (Table 2). No significant differences between the two groups were found for $-dP/dt_{max}$ (Figure 6), systolic and diastolic blood pressures, LVSP, LVEDP, and LVRT (Table 2).

RSD and CTR rats had similar body weight prior to the first social defeat/control procedure (RSD: 348 ± 7 g vs CTR: 355 ± 7 g). However, RSD rats gained significantly less weight than CTR rats during the experimental protocol (RSD: + 15 ± 3 g vs CTR: + 22 ± 2 g, $p < .05$). No differences in heart weights were observed between RSD and CTR rats (Table 3).

DISCUSSION

In this work we explored the molecular and functional consequences of repeated social defeat stress in the rat heart, with a specific focus on miRs-34a as known modulators of both neurochemical and behavioral stress responses and cardiac pathophysiology. The major finding of this study is that repeated social defeat stress increases miR-34a expression and alters transcriptomic pattern in the left ventricular tissue. These molecular modifications are associated with increased vulnerability to pharmacologically-induced arrhythmias and signs of systolic left ventricular dysfunction. In the next sections, we will discuss the molecular and functional effects observed in the socially stressed rat heart as well as potential pathophysiological pathways linking miR-34a up-regulation to adverse cardiac remodeling.

Up-regulation of miR-34a expression and transcriptomic profile changes in the socially stressed heart

The miR-34 family has recently been implicated in the regulation of stress responses (Andolina *et al.*, 2016; Andolina *et al.*, 2018; Lo Iacono *et al.*, 2020). Specifically, we have shown in mice that the expression of the miR-34a isoform is augmented upon chronic stress exposure in the raphe nuclei, the primary brain region for the production of the neurotransmitter serotonin, and that miRs-34a in this brain region play a key role in mediating chronic stress-induced adverse behavioral effects (Andolina *et al.*, 2018; Lo Iacono *et al.*, 2020). To the best of our knowledge, this is the first study to document a larger relative expression of miRs-34a in the heart of rats exposed to repeated social defeat stress. Of note, clear signs of cardiac miR-34a overexpression were found well beyond (24h after) the last defeat, but not after a single defeat episode. Notably, several studies have

implicated miR-34a regulatory activity in the pathogenesis of CVD (Fan *et al.*, 2013; Zhang *et al.*, 2018; Hu *et al.*, 2019; Toshima *et al.*, 2020). For example, miR-34a expression is elevated in cardiac tissue from patients with diabetic ischemic heart and heart failure (Thum *et al.*, 2007; Greco *et al.*, 2012; Dehaini *et al.*, 2019), and following cardiac damage in preclinical models (Lin *et al.*, 2010; Bernardo *et al.*, 2012; Boon *et al.*, 2013; Bernardo *et al.*, 2014; Yang *et al.*, 2015). Consistent with the pathogenic role of miR-34-mediated regulation in the heart, inhibition of the miR-34 family improved cardiac function in preclinical models of cardiac pathology (Bernardo *et al.*, 2012; Ooi *et al.*, 2017). Based on these premises, increased expression of miR-34a in the heart of rats exposed to repeated social defeat stress might be interpreted as a first molecular sign of an altered homeostasis that ultimately leads to stress-related cardiac disorders. Supporting this view, Gene Ontology analysis on over 300 differently expressed genes upon repeated social defeat revealed clusters of genes associated with morphological (e.g., mitochondrial inner membrane) and functional (e.g., oxidative phosphorylation) properties of the mitochondria, the predominant energy generators in the heart. This is in line with the view that psychosocial stressors may affect cardiac mitochondria, altering their morphology and decreasing their ability to produce energy via oxidative phosphorylation (Picard & McEwen, 2018). A recent study reported changes in gene expression associated with endothelial cell migration, mesenchymal development, and extracellular matrix organization in rats exposed to a predator model of PTSD, which were coupled with multifocal cardiac lesions characterized by myofiber necrosis, fibrosis, and infiltration by mononucleated immune cells (Rorabaugh *et al.*, 2020). Differences in gene expression profile of stressed rats from the study by Rorabaugh and colleagues (Rorabaugh *et al.*, 2020) and the current investigation may be ascribed to the different nature of the stress protocols, cat exposure versus repeated social defeat, respectively. The latter, as we will also detail below, is known to induce severe alterations in the structural and functional properties of the heart compared to other stressors (Sgoifo *et al.*, 2014). Nevertheless, we must acknowledge that in studies of social defeat, the control group is often placed into novel cages, whereas in this study control rats were handled and

remained in their home cages. This could magnify the social defeat effect which includes novel cage exposure.

MiRNAs act through complementary binding with mRNAs to interfere with the translation process. Due to the short size of the complementary sequence, a single miRNA can potentially bind to hundreds of target mRNAs, resulting in the fine-tuning of gene expression profile. Therefore, we searched within the list of differentially expressed genes for those which represent putative targets of miR-34a. Similar to what was observed with the entire list of genes, many of the gene targets of miR-34a are linked to "mitochondrial outer membrane" and "mitochondrial apoptotic changes". Of note, out of several hundreds of miRs expressed in all cell types, only 41 miRs (mitomiRs) have been detected in mitochondria, including the miR-34a isoform (Rippo *et al.*, 2014). Based on this and the results of our study, we hypothesize that repeated social defeat stress may induce morphological and/or functional alterations in cardiac mitochondria and that miR-34a regulatory activity may be, at least partially, responsible for this effect.

Adverse electromechanical remodeling in the socially stressed heart: is compromised mitochondrial efficiency involved?

Rats exposed to repeated social defeat showed signs of both electrical and mechanical alterations that may predispose to cardiac dysfunction. Specifically, we found a larger vulnerability to cardiac arrhythmias following β-adrenergic stimulation with isoproterenol and signs of compromised ventricular contractility. Of note, heart rate under isoproterenol was similar between the two groups, suggesting that repeated social defeat did not affect β-adrenergic receptor sensitivity. This finding is in line with previous studies showing that repeated exposure to social defeat alters the electrophysiological properties of the ventricular myocardium, lowering the threshold for cardiac arrhythmias (Carnevali *et al.*, 2013; Sgoifo *et al.*, 2014). Notably, the increased susceptibility to arrhythmias and the reduced hemodynamic efficiency found in repeatedly stressed rats are consistent with the molecular data indicating a potential impairment of cardiac mitochondrial efficiency

associated with miR-34a up-regulation. Indeed, studies have reported links between miR-34a expression levels and mitochondrial content, morphology and function. Specifically, miR-34a over-expression has been reported to (i) alter mitochondrial morphology and decrease mitochondrial content (Wen *et al.*, 2018), (ii) favor mitochondrial-mediated increase in ROS production and cell apoptosis (Rippo *et al.*, 2014), and (iii) reduce mitochondrial oxidative phosphorylation (Bukeirat *et al.*, 2016). In contrast, inhibition of miR-34a was found to significantly restore the mitochondrial transmembrane potential (Wen *et al.*, 2018).

The normal functioning heart requires coordinated electrical activity and contractile action. The energy demand to meet this unceasing action is satisfied by ATP predominantly produced by the mitochondria through oxidative phosphorylation (Harris & Das, 1991). Thus, cardiac function is particularly dependent on the efficiency of mitochondria performance. Mitochondria are also critically involved in the homeostatic regulation of cellular cations such as Ca^{2+}, Na^+ and K^+, which is essential for normal electrical activity and cardiac contractility (Murphy & Eisner, 2009; Williams *et al.*, 2013). Emerging evidence indicates that mitochondrial dysfunction can adversely impact cardiac electrical functioning by impairing intracellular ion homeostasis and membrane excitability through reduced ATP production and excessive reactive oxidative species (ROS) generation, resulting in increased propensity to cardiac arrhythmias (Yang *et al.*, 2014). Relatedly, mitochondrial dysfunction is prevalent in arrhythmogenic cardiac diseases including cardiac hypertrophy, heart failure, and myocardial ischemia (Yang *et al.*, 2014). Reduced ATP synthesis and increased ROS production associated with mitochondrial dysfunction, besides promoting apoptotic cell death (Dehaini *et al.*, 2019), can also affect several cardiomyocyte molecular mechanisms, leading to impaired cardiac contractility (Bocchi *et al.*, 2019). In addition, mitochondrial dysfunction has a profibrotic activity via TGF-β1-WNT signalling, resulting in fibroblast differentiation into myofibroblasts with collagen accumulation in the extracellular matrix (Yousefi *et al.*, 2020). All these processes can lead to the development of an arrhythmogenic substrate and worsen hemodynamic performance. Therefore, based on the results of our study, we speculate that overexpression of miRs-34a in the heart of rats exposed to repeated social defeat may

contribute to impaired mitochondrial efficiency, thereby favoring a larger vulnerability to pharmacologically-induced arrhythmias and signs of systolic left ventricular dysfunction.

Conclusion

miRNAs have emerged as one of the most promising areas for earlier prevention and novel treatment of stress-related disorders (Dwivedi, 2014; Scott *et al.*, 2015; Issler & Chen, 2017). Our results suggest the presence of an association between miR-34a overexpression and signs of adverse electromechanical remodeling in the heart of rats exposed to repeated social defeat, and point to compromised mitochondrial efficiency as a potential mediator of this link.

In interpreting these results, we must acknowledge several limitations: first, this study provides indirect correlations between molecular (miR-34a expression) and cardiac functional data, and does not investigate the events in a dependent manner; second, no experiments have been conducted to assess the effects of repeated social defeat on cardiac mitochondrial function. Therefore, future studies are required to detail the causal relationship (for instance by antimiR-34 treatment) between miR-34a expression, mitochondrial (dys)function and cardiac alterations under stressful conditions, which could have important implications in the context of stress-related CVD. Moreover, given the documented role of miR34-a in mediating the adverse behavioral sequelae of chronic stress in mice (Andolina *et al.*, 2016; Andolina *et al.*, 2018; Lo Iacono *et al.*, 2020), it would be interesting to test whether miR-34a up-regulation could represent a common epigenetic mechanism underlying the comorbidity between stress-related psychopathologies and CVD.

Declarations of interest: none.

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Table 1. Gene Ontology analysis performed on the 378 differentially expressed genes between repeated social defeat stress rats and control rats.

Annotation Cluster 1	Enrichment score: 7.52	P value	BENJAMINI
UP_KEYWORDS	Mitochondrion	3.3E-13	4.2E-11*
UP_KEYWORDS	Transit Peptide	7.5E-6	3.8E-3*
Annotation Cluster 2	Enrichment score: 4.7	P value	BENJAMINI
KEGG_PATHWAY	Parkinson's disease	1.6E-8	3.1E-6*
KEGG_PATHWAY	Alzheimer's disease	3.3E-8	3.2E-6*
KEGG_PATHWAY	Oxidative phosphorylation	5.2E-8	3.4E-6*
KEGG_PATHWAY	Huntington's disease	1.7E-7	8.0E-6*
KEGG_PATHWAY	Non-alcoholic fatty liver disease	1.5E-6	5.8E-5*
GOTERM_CC_DIRECT	Mitochondrial inner membrane	3.8E-5	3.4E-3*
UP_KEYWORDS	Mitochondrion inner membrane	4.0E-5	1.3E-3*
GOTERM_CC_DIRECT	Mitochondrial respiratory chain complex I	2.9E-3	9.1E-2
KEGG_PATHWAY	Cardiac muscle contraction	1.4E-2	2.7E-1
GOTERM_MF_DIRECT	Cytochrome-c oxidase activity	1.7E-2	4.8E-1
GOTERM_BP_DIRECT	Hydrogen ion trans membrane transport	2.6E-2	9.1E-1
Annotation Cluster 3	Enrichment score: 2.67	P value	BENJAMINI
GOTERM_MF_DIRECT	Phosphatidylinostil-3,4,5-triphosphate binding	4.2E-5	1.0E-2*
GOTERM_MF_DIRECT	Phosphatidylinostil-3,4-biphosphate binding	4.6E-3	3.6E-1
GOTERM_MF_DIRECT	Phosphatidylinostil-3,5-biphosphate binding	5.0E-2	7.5E-1

Table 2. Hemodynamic parameters in rats exposed to repeated social defeat (RSD) or control (CTR) condition

	CTR (n=7)	RSD (n=6)
SBP (mmHg)	150.3 ± 1.1	138.6 ± 5.4
DBP (mmHg)	100.1 ± 1.3	101.9 ± 4.1
LVSP (mmHg)	150.4 ± 1.9	140.9 ± 5.4
LVEDP (mmHg)	12.0 ± 0.5	8.0 ± 1.6
LVRT (s)	0.029 ± 0.001	0.030 ± 0.001
ET (s)	0.102 ± 0.002	0.093 ± 0.004

Values are reported as means ± SEM. Abbreviations: SBP = systolic blood pressure; DBP = diastolic blood pressure; LVSP = left ventricular systolic pressure; LVEDP = left ventricular end-diastolic pressure; LVRT = left ventricular relaxation time; ET = ejection time. $P > .05$ for all comparisons (unpaired Student's t tests).

Table 3. Myocardial weights in rats exposed to repeated social defeat (RSD) or control (CTR) condition

	CTR (n=7)	RSD (n=7)
HW (g)	0.967 ± 0.025	0.945 ± 0.017
HW/BW	0.00260 ± 0.0003	0.00260 ± 0.0010
LVW (g)	0.698 ± 0.016	0.733 ± 0.027
LVW/HW	0.722 ± 0.006	0.776 ± 0.0
LVW/BW	0.00185 ± 0.0002	0.00200 ± 0.0010
RVW (g)	0.204 ± 0.007	0.164 ± 0.023
RVW/HW	0.211 ± 0.005	0.173 ± 0.023

Values are reported as means ± SEM. Abbreviations: HW = heart weight; BW = body weight; LVW = left ventricular weight; RVW = right ventricular weight. $P > .05$ for all comparisons (unpaired Student's t tests).

FIGURE CAPTIONS

Figure 1. Relative expression of miR-34a in the left ventricle of control (CTR, n = 5), single social defeat (SSD, n = 5), and repeated social defeat (RSD, n = 5) rats. The CTR group served as a reference group for both the SSD and RSD groups. Values are reported as means ± SEM. * indicates a significant difference ($p < .05$, Bonferroni post-hoc test).

Figure 2. Heatmap showing a subset of down-regulated genes between control (CTR, n = 5) and repeated social defeat stress (RSD, n = 5) rats with the highest statistical significance (adjusted $p < .05$). Color intensity is proportional to the individual level of expression (normalized reads count).

Figure 3. Heatmap showing a subset of up-regulated genes between control (CTR, n = 5) and repeated social defeat stress (RSD, n = 5) rats with the highest statistical significance (adjusted $p < .05$). Color intensity is proportional to the individual level of expression (normalized reads count).

Figure 4. Panel A reports the number of matching genes (n = 61) that resulted from the comparison between the 378 differentially expressed (DEGs) found in control (CTR, n = 5) *vs* repeated social defeated (RSD, n = 5) rats (black circle), and those genes (n = 3530) that were predicted as putative miR-34 targets by TargetScan (red circle). Panel B shows the heatmap with the list of the 61 matching genes; color intensity is proportional to the to the individual level of expression (normalized reads count). Panel C reports the results of the Gene Ontology analysis on the 61 matching genes.

Figure 5. Top and middle panels report electrocardiographic examples of the most common forms of arrhythmias observed and classified in the rat. Classification criteria are defined in the methods according to (Grippo *et al.*, 2012). Arrows denote arrhythmic events. Bottom panel represents the arrhythmic burden following isoproterenol injection in rats exposed to

repeated social defeat (RSD, n = 7) or control procedure (CTR, n = 7). Values are reported as means ± SEM. * indicates a significant difference ($p < .05$, unpaired Student's t test).

Figure 6. Hemodynamic parameters in rats exposed to repeated social defeat (RSD, n = 6) or control procedure (CTR, n = 7). Values are reported as means ± SEM. Abbreviations: $+dP/dt_{max}$ = maximal rate of ventricular pressure rise; $-dP/dt_{max}$ = maximal rate of ventricular pressure decline; IVCT = isovolumic contraction time; MPI = myocardial performance index. * indicates a significant difference ($p < .05$, unpaired Student's t test).

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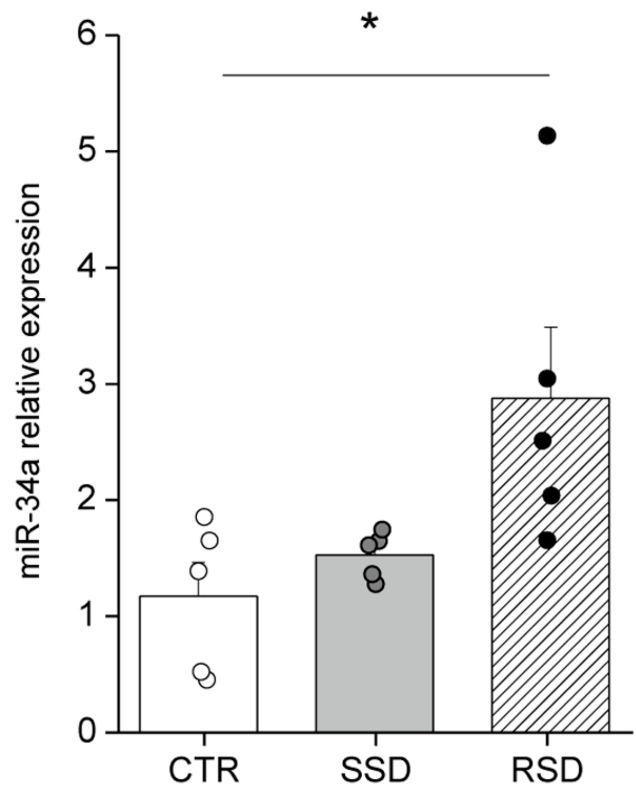


Figure 1

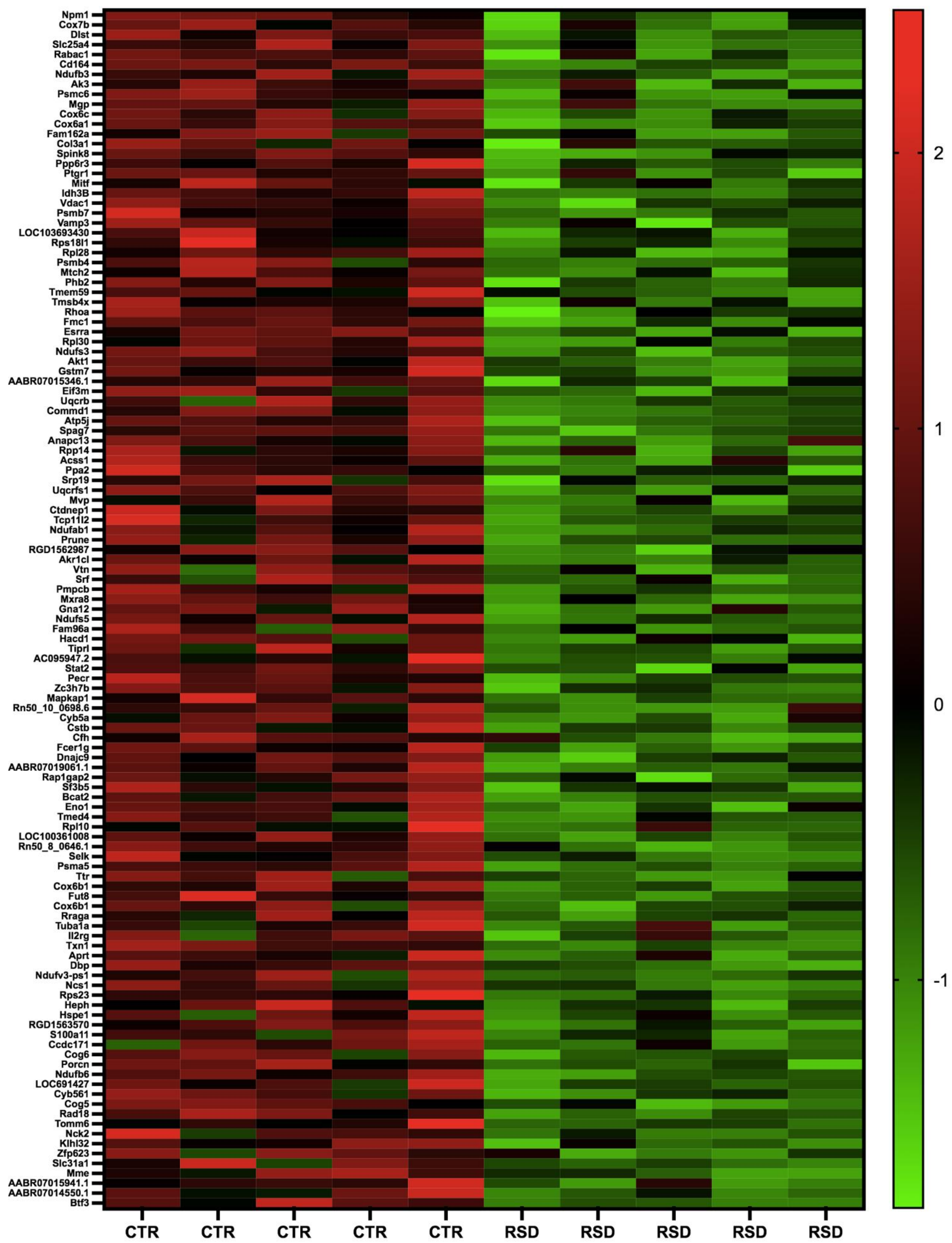


Figure 2

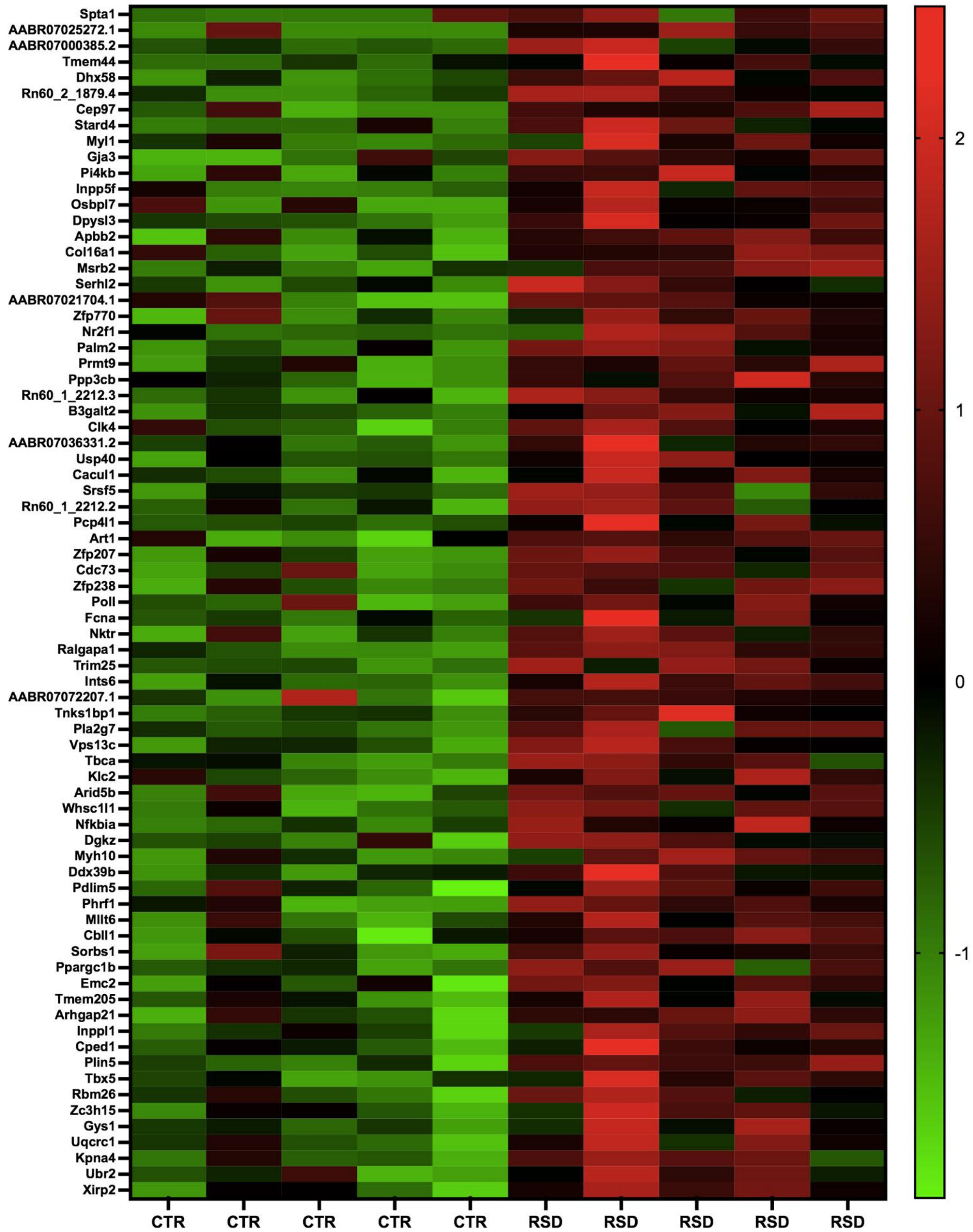


Figure 3

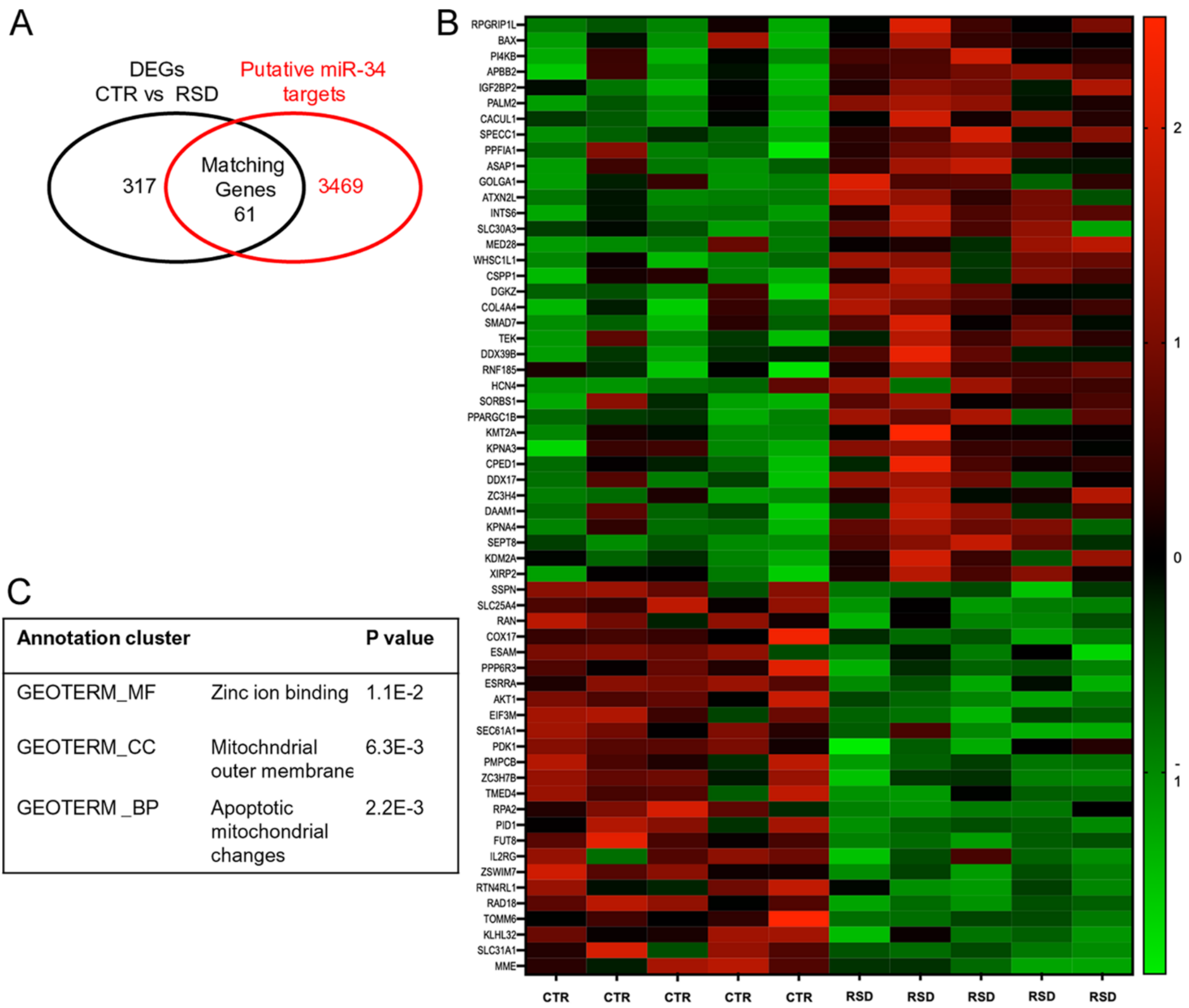
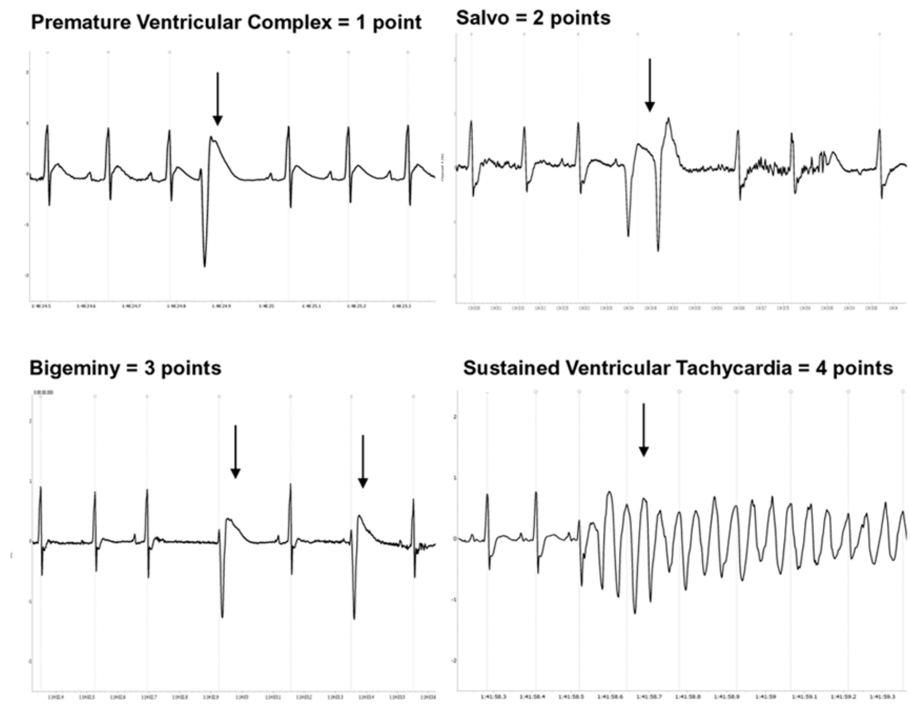


Figure 4



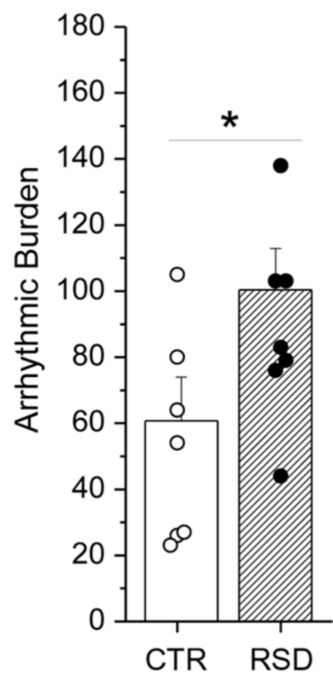


Figure 5

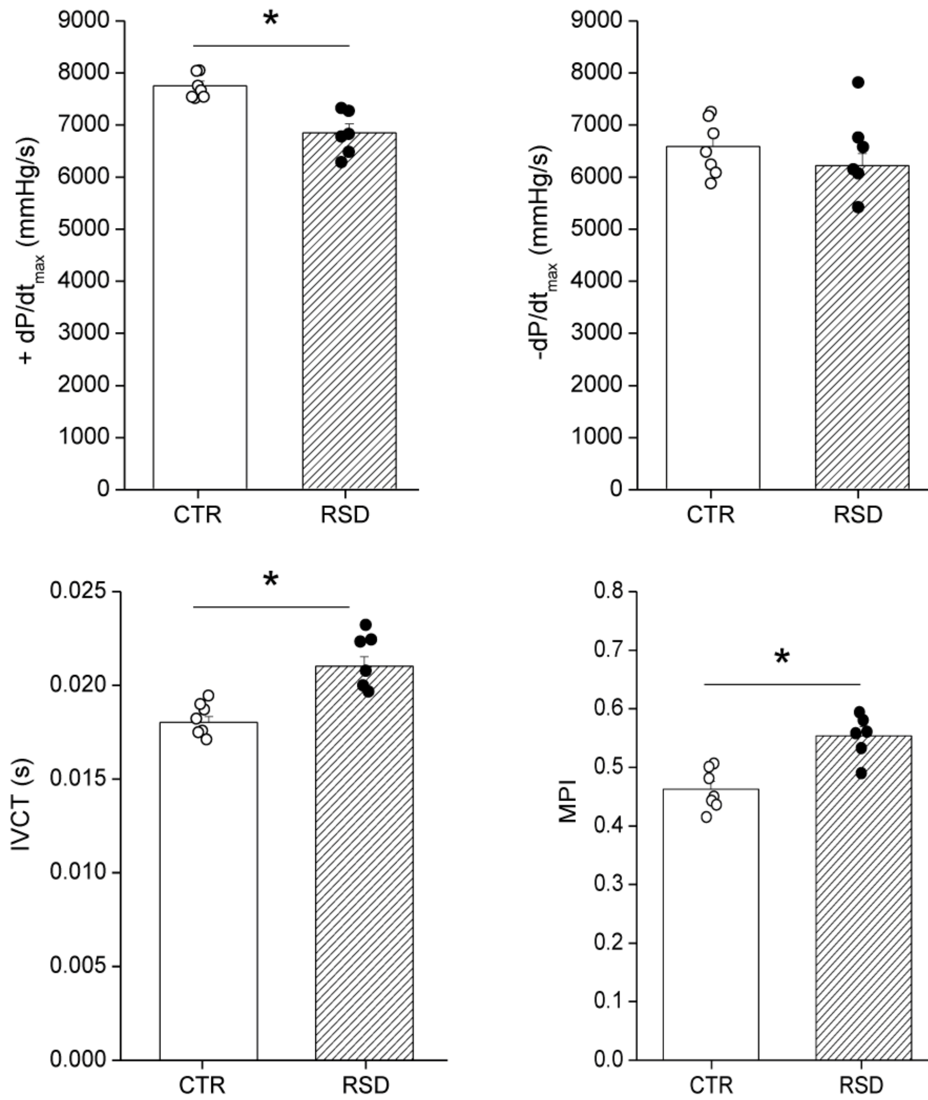


Figure 6