Diastolic dysfunction in prediabetes: role of mitochondrial oxidative stress

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1. Abstract:

Although incidence and prevalence of prediabetes are increasing, little is known on its cardiac effects. Therefore, our aim was to investigate the effect of prediabetes on cardiac function and to characterize parameters and pathways associated with deteriorated cardiac performance. Long-Evans rats were fed with either control or high-fat chow for 21 weeks and treated with a single low dose (20 mg/kg) streptozotocin at week 4. High-fat and streptozotocin treatment induced prediabetes as characterized by slightly elevated fasting blood glucose, impaired glucose- and insulin tolerance, increased visceral adipose tissue and plasma leptin levels, as well as sensory neuropathy. In prediabetic animals a mild diastolic dysfunction was observed, the number of myocardial lipid droplets increased, and left ventricular mass and wall thickness were elevated, however, no molecular sign of fibrosis or cardiac hypertrophy was evidenced. In prediabetes, production of reactive oxygen species was elevated in subsarcolemmal mitochondria. Expression of mitofusin-2 was increased while the phosphorylation of phospholamban and expression of Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3, a marker of mitophagy) decreased. However, expression of other markers of cardiac auto- and mitophagy, mitochondrial dynamics, inflammation, heat shock proteins, Ca\(^{2+}\)/calmodulin-dependent protein kinase II, mTOR or apoptotic pathways were unchanged in prediabetes. This is the first comprehensive analysis of cardiac effects of prediabetes indicating that mild diastolic dysfunction and cardiac hypertrophy are multifactorial phenomena which is associated with early changes in mitophagy, cardiac lipid accumulation and elevated oxidative stress, and that prediabetes-induced oxidative stress originates from the subsarcolemmal mitochondria.

**Keywords:** obesity, type 2 diabetes, high-fat diet, ROS, diabetic cardiomyopathy
New and Noteworthy

In prediabetes induced by chronic high-fat diet and a low single dose of streptozotocin in rats, mild diastolic dysfunction and ventricular hypertrophy are observed. Elevated cardiac lipid accumulation, subsarcolemmal mitochondrial reactive oxygen species production, and early changes in cardiac mitophagy may be responsible for cardiac effects of prediabetes.
1. **Introduction:**

Type 2 diabetes mellitus is a common civilization disease with a growing prevalence worldwide (1, 66, 79). It is well established that type 2 diabetes mellitus is a risk factor of cardiovascular diseases such as heart failure and myocardial infarction contributing to their increased morbidity and mortality (6, 66). However, before the development of overt diabetes, a period of prediabetic state (*i.e.*, impaired glucose and insulin tolerance, insulin and leptin resistance, oscillations of normo- and hyperglycemic states, mild to moderate obesity) occurs (54), which may also promote cardiovascular complications (21, 30, 45). Although cardiac pathophysiological alterations are relatively well characterized in fully developed diabetes (*i.e.*, diabetic cardiomyopathy), information about prediabetes is quite limited. It has been reported that prediabetes induced mild diastolic dysfunction in OLETF rats, which is a genetic model for spontaneous long-term hyperglycemia (51), however, cardiac consequences of prediabetes and their molecular mechanism is unknown in non-genetic prediabetic settings.

Contractile dysfunction in diabetic cardiomyopathy has been attributed to numerous factors and pathways (*i.e.*, increased oxidative stress, or activated apoptosis (13, 76), of which could be connected to an impaired mitochondrial function (24), autophagy (41, 76) or to an imbalance in the calcium homeostasis (59)). Although these pathways are well studied in diabetes, their role in prediabetes has not been uncovered. Furthermore, since mitochondrial function is heavily influenced by mitochondrial dynamics including mitochondrial biogenesis, fusion, fission, and autophagy-mitophagy, and since these processes have been linked to the development of diabetic cardiomyopathy (13, 32, 41, 76), we hypothesized that altered mitochondrial dynamics might be involved in the mechanism of deteriorated cardiac functions.
in prediabetes. Moreover, development of diabetes leads to systemic sensory neuropathy that has been shown to result in diastolic dysfunction in the rat heart (7, 84).

Therefore, here we aimed to systematically characterize the cardiac effect of prediabetes on functional, morphological and molecular levels in a non-genetic rodent model.
2. Materials and Methods:

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and was approved by the animal ethics committee of the Semmelweis University, Budapest, Hungary (registration numbers: XIV-I-001/450-6/2012). Chemicals were purchased from Sigma, St. Louis, MO unless otherwise noted.

Animal model and experimental design

Male Long-Evans rats of 5-7 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in a room maintained at 12 h light-dark cycles and constant temperature of 21°C. Animals were allowed to food and water ad libitum. After one week of acclimatization rats were divided into two groups: control (CON; n=20) and prediabetic group (PRED; n=20) (Fig. 1). The control group was fed control chow, while the prediabetic group was fed a chow supplemented with 40% lard as a high-fat diet. Body weights were measured weekly. Blood was taken and fasting blood glucose levels were measured from the saphenous vein every second week with a blood glucose monitoring system (Accu-Check, Roche, Basel, Switzerland). To facilitate the development of prediabetes and to avoid hypoinsulinemia, animals on high-fat diet received 20 mg/kg streptozotocin (STZ, Santa Cruz Biotechnology, Dallas, TX) intraperitoneally (i.p.) at the fourth week of the diet according to Mansor et al. (50), while the control group was treated with same volume of ice-cold citrate buffer as vehicle. At the 20th week oral glucose tolerance test (OGTT) was performed in overnight fasted rats with per os administration of 1.5 g/kg glucose and measurements of plasma glucose levels at 15, 30, 60 and 120 minutes. Insulin tolerance test (ITT) was also performed at week 20 in overnight fasted rats. Insulin (0.5
IU/kg, Humulin R, Ely Lilly, Netherlands) was injected i.p. and plasma glucose levels were checked at 15, 30, 45, 60, 90 and 120 minutes. At week 21 of the diet, animals were anesthetized with pentobarbital (60 mg/kg, i.p., Euthasol, Produlab Pharma, Raamsdonksveer, Netherlands). Echocardiography and cardiac catheterization were performed, then hearts were excised, shortly perfused with oxygenated Krebs-Henseleit buffer in Langendorff mode as described earlier and heart weights were measured. Epididymal and interscapular brown fat tissue, which are the markers of adiposity (9, 34), were isolated and their weights were measured. Blood and tissue samples were collected and stored at -80°C.

**Assessment of sensory neuropathy**

To test if sensory neuropathy develops in prediabetes, plantar Von Frey test was performed. At week 15 of the diet, rats were placed in a plastic cage having a wire mesh bottom to allow full access to the paws. After 5-10 min acclimation time, mechanical hind paw withdrawal thresholds were measured by a dynamic plantar aesthesiometer (UGO-Basile, Monvalle, Italy) as previously described (55).

**Evaluation of body fat content**

At week 20 of the diet, computer tomography (CT) measurements were performed on NanoSPECT/CT PLUS (Mediso, Budapest, Hungary). The semicircular CT scanning was acquired with 55 kV tube voltage, 500 ms of exposure time, 1:4 binning and 360 projections in 18 minutes 7s. During the acquisitions, rats were placed in prone position in a dedicated rat bed, and were anesthetized with 2% isoflurane in oxygen. Temperature of the animals was kept at 37.2±0.3°C during imaging. In the reconstruction, 0.24 mm in-plane resolution and slice thickness were set and Butterworth filter was applied (volume size: 76.8*76.8*190 mm). Images were further analyzed with VivoQuant (inviCRO LLC) dedicated image analysis software products by placing appropriate Volume-of-Interests (VOI) on the whole body fat of
animals. The aim of segmentation was to separate the fat from other tissues. The connected
threshold method helped to choose the adequate attenuated pixels for fat tissue analysis, then
the isolated points were detected by erode 4 voxel and dilate 4 voxel steps. After the
measurements animals recovered from anesthesia.

Cardiac function by echocardiography

Before euthanasia, to measure cardiac function, echocardiography was performed as
previously described (42, 62). Briefly, anesthetized animals were placed on a controlled
heating pad, and the core temperature, measured via rectal probe, was maintained at 37°C.
Transthoracic echocardiography was performed in supine position by one investigator blinded
to the experimental groups. Two dimensional and M-mode echocardiographic images of long
and short (mid-papillary muscle level) axis were recorded, using a 13 MHz linear transducer
(GE 12L-RS, GE Healthcare), connected to an echocardiographic imaging unit (Vivid i, GE
Healthcare). The digital images were analyzed by a blinded investigator using an image
analysis software (EchoPac, GE Healthcare). On two dimensional recordings of the short-axis
at the mid-papillary level, left ventricular (LV) anterior (LVA WT) and posterior (LVPWT)
wall thickness in diastole (index: d) and systole (index: s), left ventricular end-diastolic
(LVEDD) and end-systolic diameter (LVESD) were measured. In addition, end-diastolic and
end-systolic LV areas were planimetered from short and long axis two dimensional
recordings. End-systole was defined as the time point of minimal left ventricular dimensions,
and end-diastole as the time point of maximal dimensions. All values were averaged over
three consecutive cycles. The following parameters were derived from these measurements
(63). Fractional shortening (FS) was calculated as ((LVEDD-LVESD)/LVEDD)×100. LV
mass was calculated according to the following formula:

\[ \text{LV mass} = \left( \text{LVEDD} + \text{AWTd} + \text{PWTd} \right) - \text{LVEDD} \times 1.04 \times 0.8 + 0.14. \]
Hemodynamic measurements, left ventricular pressure-volume analysis

After echocardiographic measurements, hemodynamic measurement was performed as previously described (60, 61). Briefly, rats were tracheotomized, intubated and ventilated, while core temperature was maintained at 37°C. A median laparotomy was performed. A polyethylene catheter was inserted into the left external jugular vein. A 2-Fr microtip pressure-conductance catheter (SPR-838, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the ascending aorta. After stabilization for 5 min, mean arterial blood pressure (MAP) was recorded. After that, the catheter was advanced into the LV under pressure control. After stabilization for 5 min, signals were continuously recorded at a sampling rate of 1,000/s using a Pressure-Volume (P-V) conductance system (MPVS-Ultra, Millar Instruments, Houston, TX) connected to the PowerLab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO), stored and displayed on a personal computer by the LabChart5 Software System (AD Instruments). After positioning the catheter baseline P-V loops were registered. With the use of a special P-V analysis program (PVAN, Millar Instruments), LV end-systolic pressure (LVESP), LV end-diastolic pressure (LVEDP), the maximal slope of LV systolic pressure increment (dP/dtmax) and diastolic pressure decrement (dP/dtmin), time constant of LV pressure decay (τ; according to the Glantz method), ejection fraction (EF) stroke work (SW) and LV maximal power were computed and calculated. Stroke volume (SV) and cardiac output (CO) were calculated and corrected according to in vitro and in vivo volume calibrations using the PVAN software. Total peripheral resistance (TPR) was calculated by the following equation: TPR=MAP/CO. In addition to the above parameters, P-V loops recorded at different preloads can be used to derive other useful systolic function indexes that are less influenced by loading conditions and cardiac mass (37, 57). Therefore, LV P-V relations were measured by transiently compressing the inferior vena cava (reducing preload) under the diaphragm with a cotton-tipped applicator.
The slope of the LV end-systolic P-V relationship (ESPVR; according to the parabolic curvilinear model), preload recruitable stroke work (PRSW), and the slope of the dP/dtmax-end-diastolic volume relationship (dP/dtmax-EDV) were calculated as load-independent indexes of LV contractility. The slope of the LV end-diastolic P-V relationship (EDPVR) was calculated as a reliable index of LV stiffness (37). At the end of each experiment, 100 µL of hypertonic saline were injected intravenously, and from the shift of P-V relations, parallel conductance volume was calculated by the software and used for the correction of the cardiac mass volume. The volume calibration of the conductance system was performed as previously described (37).

**Adipokine array from rat plasma**

Adipokine array was performed from 1 mL rat plasma according to manufacturer’s instructions (Proteome Profiler Rat Adipokine Array Kit, R&D Systems, Abingdon, UK).

**Biochemical measurements**

Serum cholesterol, high density lipoprotein (HDL) and triglyceride levels were measured by colorimetric assays (Diagnosticum, Budapest, Hungary) as previously described (19). Plasma leptin (Invitrogen, Camarillo, CA), TIMP metallopeptidase inhibitor 1 (TIMP-1; R&D System, Minneapolis, MN) and angiotensin-II (Phoenix pharmaceuticals, Karlsruhe, Germany) were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s instructions. Urea, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), low density lipoprotein (LDL), C-reactive protein (CRP), cholesterol, uric acid and creatinine were measured by automated clinical laboratory assays (Diagnosticum, Budapest, Hungary).

**Histology**
Heart, liver and pancreas samples were fixed in 4% neutral-buffered formalin. After 24 hours, samples were washed with phosphate buffered saline (PBS) and stored in 70% ethanol in PBS until embedded in paraffin. Samples were stained with hematoxylin-eosin (HE) and Masson’s trichrome (MA) staining. Left ventricle samples were analyzed to examine histopathological differences and evaluate cardiomyocyte hypertrophy and fibrosis. The level of fibrosis was measured on MA-stained LV sections, and transverse transnuclear width (cardiomyocyte diameter) was assessed on longitudinally oriented cardiomyocytes on HE-stained LV sections by a Zeiss microscope (Carl Zeiss, Jena, Germany). Digital images were acquired using an imaging software (QCapture Pro 6.0, QImaging, Canada) at 20× magnification. Quantification of cardiomyocyte diameter and fibrosis was performed with ImageJ Software (v1.48, NIH, Bethesda). Liver samples were evaluated for hepatic steatosis/fibrosis and scored as previously described (40).

**Nitrotyrosine immunostainig of left ventricular samples**

After embedding and cutting 5 μm thick sections, heat-induced antigen epitope retrieval was performed (95°C, 10 min, in citrate buffer with a pH of 6.0). Sections were stained with rabbit polyclonal anti-nitrotyrosine antibody (5 µg/mL, Cayman Chemical, Ann Arbor, MI) by using the ABC-kit of Vector Laboratories (Burlingame, CA) according to the manufacturer’s protocol. Nitrotyrosine-stained sections were counterstained with hematoxylin. Specific staining was visualized and images were acquired using a BX-41 microscope (Olympus, Tokyo, Japan).

**Quantitative RT-PCR**

Total RNA was isolated from LV tissue with Reliaprep™ RNA Tissue Miniprep kit (Promega, Madison, WI) according to the manufacturer’s instructions. cDNA was synthesized using Tetro cDNA Synthesis Kit (Bioline, London, UK) according to the
manufacturer’s protocol. PCR reaction was performed with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), or TaqMan Universal PCR MasterMix (Thermo Fisher Scientific, Waltham, MA) and 3 nM forward and reverse primers for collagen type 1 and 3 (COL1 and COL3), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) (Integrated DNA Technologies, Leuven, Belgium), assay mixes for α-myosin heavy chain (α-MHC, assay ID: Rn00691721_g1), β-myosin heavy chain (β-MHC, assay ID: Rn00568328_m1), tumor necrosis factor α (TNF-α, assay ID: Rn99999017_m1) and interleukin-6 (IL-6, assay ID: Rn01410330_m1, Thermo Fisher Scientific, Waltham, MA) were used. Beta-2 microglobulin (B2M) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; reference gene; assay ID: Rn01775763_g1) were used as reference genes. Quantitative real-time PCR was performed with the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). Expression levels were calculated using the CT comparative method (2^ΔCT).

Measurement of pancreatic insulin

Freeze clamped and pulverized pancreas samples were used to determine pancreatic insulin content. Analysis was performed with Insulin (I-125) IRMA Kit (Izotop Kft, Budapest, Hungary) according to the manufacturer’s instructions.

Electron microscopy

Left ventricular tissue samples (1×1 mm) were placed in modified Kranovsky fixative (2% paraformaldehyde, 2.5 % glutaraldehyde, 0.1 M Na-cacodylate buffer, pH 7.4 and 3mM CaCl₂). After washing in cacodylate buffer, samples were incubated in 1% osmium tetroxide in 0.1 M PBS for 35 min. Then samples were washed in buffer several times for 10 minutes and dehydrated in an ascending ethanol series, including a step of uranyl acetate (1%) solution in 70% ethanol to increase contrast. Dehydrated blocks were transferred to propylene oxide
before being placed into Durcupan resin. Blocks were placed in thermostat for 48 h at 56 °C.

From the embedded blocks, 1 µm-thick semithin and serial ultrathin sections (70 nm) were cut with a Leica ultramicrotome, and mounted either on mesh, or on Collodion-coated (Parlodion, Electron Microscopy Sciences, Fort Washington, PA) single-slot copper grids. Additional contrast was provided to these sections with uranyl acetate and lead citrate solutions, and they were examined with a JEOL1200EX-II electron microscope. Areas of subsarcolemmal (SSM), interfibrillar mitochondria (IFM) and lipid droplets were measured by free hand polygon selection in iTEM Imaging Platform.

**Mitochondrial enzyme activity measurements**

Fresh myocardial samples were homogenized in 1/30 weight per volume Chappel-Perry buffer (100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 50 mM Tris, pH: 7.5) supplemented with 15 mg/L trypsin-inhibitor, 15.5 mg/L benzamidine, 5 mg/L leupeptin and 7 mg/L pepstatin A. All enzyme activities were measured as duplicates with a photometer (Cary 50 Scan UV-Visible Spectrophotometer, Varian). Before adding substrate or cofactor, the reaction mix was incubated at 30°C for 10 min (except for cytochrome c oxidase). Enzyme activities were expressed relative to citrate synthase activity or total protein levels (measured with Bicinchoninic Acid assay). The activity of rotenone-sensitive NADH:ubiquinone-oxidoreductase (Complex I) was measured at 340 nm in the presence of 1 mM EDTA, 2.5 mM KCN, 1 µM antimycin A and 20 µM rotenone after adding coenzyme Q and NADH to a final concentration of 60 µM. The activity of NADH:cytochrome c-oxidoreductase (Complex I+III) was measured at 550 nm as the antimycin A- and rotenone-sensitive fraction of total NADH-cytochrome c oxidoreductase in the presence of 0.1 mM EDTA, 3 mM KCN and 0.1% cytochrome c after adding NADH to a final concentration of 0.2 mM. The activity of succinate:cytochrome c-oxidoreductase (Complex II+III) was measured at 550 nm in the presence of 0.1 mM EDTA, 2.5 mM KCN, 0.1% bovine serum albumin and 4 mM succinate.
after adding cytochrome c to a final concentration of 0.1%. The activity of succinate-dehydrogenase was measured at 600 nm in the presence of 0.1 mM EDTA, 2.5 mM KCN, 0.1% bovine serum albumin and 2 mM succinate after adding 2,6-dichloroindophenol and phenazine-methosulfate to a final concentration of 34.9 µM and 1.625 mM, respectively. The activity of cytochrome c-oxidase was measured at 550 nm in the presence of 0.08% reduced cytochrome c. The activity of citrate-synthase was measured at 412 nm in the presence of 0.1% triton-X 100, 0.1 mM 5,5'-dithiobis (2-nitrobenzoic acid), and 0.1 mM acetyl-coenzyme A after adding oxalacetate to a final concentration of 0.5 mM.

**Preparation of isolated mitochondria**

SSM and IFM fractions were isolated according to a protocol described previously (72). Using homogenization buffer (buffer A) containing (mM): 100 KCl, 50 MOPS, 5 MgSO₄, 1 EGTA and pH 7.4 (Tris-HCl). Isolation buffer (buffer B) containing (mM): 250 sucrose, 10 HEPES, 1 EGTA and pH 7.4 (Tris-HCl). Before the isolation, 1 mM ATP was added freshly to the homogenization buffer. All steps were carried out on ice. After Langendorff perfusion of the heart LV samples were cut to small species with scissors and washed in buffer A, then homogenized with five strokes of teflon pistils in a glass potter. The homogenate was centrifuged for 10 min at 800×g, 4°C. For isolation of SSM the supernatant was centrifuged for 10 min at 8,000×g. This pellet was suspended in buffer A and centrifuged for 10 min at 8,000×g, and the resulting sediment was resuspended in a small volume of buffer A. The pellet of the first centrifugation was used for isolation of IFM fraction and resuspended in buffer B (10 mL/g tissue) and after addition of 8 U/g of bacterial protease incubated for 1 min on ice and then homogenized with five strokes of teflon pistil in a glass potter and centrifuged for 10 min at 800×g. The supernatant was centrifuged for 10 min at 8,000×g, the resulting mitochondrial pellet was finally resuspended in buffer A and used for mitochondrial respiration, membrane potential, H₂O₂ production and Ca²⁺ uptake measurements. For
Western blots, the resulting SSM and IFM pellets were finally resuspended in 200 µL volume of Buffer B which were layered on 30% Percoll Solution and were ultracentrifuged (Rotor type: Beckman Type 70.1 Ti) for 30 min at 18,700×g at 4°C. After ultracentrifugation, lower rings were collected (100 µL/tube) and filled with 1 mL Buffer B and centrifuged for 10 min at 12,200×g, 4°C. After washing, pellets were stored at -80°C.

**Measurement of mitochondrial respiration**

Protein concentration of SSM and IFM samples was determined by biuret method (11). Mitochondrial oxygen consumption was measured by high-resolution respirometry with Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) a Clark-type O₂ electrode for 40 min. The mitochondrial protein content was 0.1 mg/mL in the measurements. Measuring mitochondrial respiration followed the substrate-uncoupler-inhibitor titration (SUIT) protocol. Mitochondria were energized with 5 mM glutamate and 5 mM malate. Mitochondrial respiration was initiated with 2 mM adenosine diphosphate (ADP). Cytochrome c (4 µM), succinate (5 mM), rotenone (1 µM) and carboxyatractyloside (CAT; 2 µM) were used as indicated. Measurements were performed in an assay medium containing 125 mM KCl, 20 mM HEPES, 100 µM EGTA, 2 mM K₂HPO₄, 1 mM MgCl₂ and 0.025% BSA. Data were digitally recorded using DatLab4 software.

**Measurement of mitochondrial membrane potential**

To detect mitochondrial membrane potential, we used the fluorescent, cationic dye, safranine O (2 µM) which can bind to the protein possessing negative charge in the inner mitochondrial membrane depending on the mitochondrial membrane potential. The excitation/emission wavelengths were 495/585 nm. Fluorescence was recorded at 37°C by Hitachi F-4500 spectrofluorimeter (Hitachi High Technologies, Maidenhead, UK). The reaction medium was
the following: 125 mM KCl, 20 mM HEPES, 100 µM EGTA, 2 mM K$_2$HPO$_4$, 1 mM MgCl$_2$ and 0.025% BSA.

**Detection of H$_2$O$_2$ formation in mitochondria**

H$_2$O$_2$ production of SSM and IFM was assessed by Amplex UltraRed fluorescent dye method (52). Horseradish peroxidase (2.5 U/mL) and Amplex UltraRed reagent (1 µM), then 0.05 mg/mL mitochondria were added to the incubation medium. H$_2$O$_2$ formation was initiated by the addition of 5 mM glutamate and 5 mM malate or 5 mM succinate and fluorescence was detected at 37°C with Deltascan fluorescence spectrophotometer (Photon Technology International, PTI, Lawrenceville, NJ). The excitation wavelength was 550 nm and the fluorescence emission was detected at 585 nm. A calibration signal was generated with known quantities of H$_2$O$_2$ at the end of each experiment.

**Measurement of Ca$^{2+}$ uptake in mitochondria**

The free Ca$^{2+}$ concentration at each added concentration of Ca$^{2+}$ was calculated and measured. Ca$^{2+}$ uptake by mitochondria was followed by measuring Calcium-Green-5N (100 nM) fluorescence at 505 nm excitation and 535 emission wavelengths at 37°C using a Hitachi F-4500 spectrofluorimeter (Hitachi High Technologies, Maidenhead, UK). The reaction medium was the following: 125 mM KCl, 20 mM HEPES, 100 µM EGTA, 2 mM K$_2$HPO$_4$, 1 mM MgCl$_2$ and 0.025% BSA.

**Western blot of left ventricle lysates and isolated mitochondria fractions**

Freeze clamped left ventricles were pulverized under liquid nitrogen and homogenized in homogenization buffer containing (in mmol/L): 20 Tris-HCl, 250 sucrose, 1.0 EGTA, 1.0 dithiothreitol, or in radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA), supplemented with 1 mM phenylmethylsulphonylfluoride (PMSF; Roche,
Basel, Switzerland), 0.1 mM sodium fluoride, 200mM sodium orthovanadate and complete
protease inhibitor cocktail (Roche, Basel, Switzerland) with TissueLyser LT (Qiagen, Venlo,
Netherlands) to obtain LV soluble protein fraction or LV whole cell lysate. Previously
isolated mitochondrial samples were resuspended in ice-cold 1x cell lysis buffer (Cell
Signaling Technology, Danvers, MA). Concentration of proteins was assessed with Lowry’s
assay or Bicinchoninic Acid Assay kit (Thermo Fisher Scientific, Waltham, MA).
For tropomyosin oxidation analysis, tissue samples were homogenized in ice-cold PBS, pH
7.2 containing an antiprotease mixture (Complete, Roche, Basel, Switzerland) and 5 mM
EDTA. Just before use, the protein samples were stirred under vacuum and bubbled with
argon to maximally reduce the oxygen tension. The protein suspension was centrifuged at
12,000×g for 10 min at 4°C. The resulting pellet was resuspended in sample buffer (2% SDS,
5% glycerol, 1% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8) and denatured by 10 min
boiling. This procedure referred to as reducing condition was compared with the non-reducing
condition obtained without the addition of β-mercaptoethanol. To avoid artifacts due to the
oxidation of thiol groups in vitro, non-reducing conditions were performed in the presence of
1 mM N-ethylmaleimide.
Protein samples were resolved on precast 4–20% Criterion TGX gels (Bio-Rad, Hercules,
CA) or bis-tris gels depending on the protein of interest and transferred to nitrocellulose or
Immun-Blot PVDF membranes (Bio-Rad, Hercules, CA). Quality of transfer was verified
with Ponceau S staining. Membranes were blocked with 5% nonfat milk (Bio-Rad, Hercules,
CA) or 2-5% bovine serum albumin (BSA; Santa Cruz Biotechnology, Dallas, TX) in Tris-
buffered saline with 0.05% Tween 20 (TBS-T) for 0.5-2 hours. Membranes were incubated
with primary antibodies in 1-5% nonfat milk or BSA in TBS-T: anti-tropomyosin (Tm; 1:250)
anti-phospho-phospholamban (PLB-Ser16; 1:5,000), p-PLB (Thr17; 1:5,000), anti-
sarco/endoplasmic reticulum Ca^{2+}-ATPase II (SERCA2A; 1:5,000, Badrilla, Leeds, UK),
anti-heat shock protein-60 (HSP-60; 1:500), anti-HSP-70 (1:500), anti-HSP-90 (1:500), anti-B-cell lymphoma 2 (Bcl-2; 1:500), anti-caspase-3 (1:500), anti-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIδ; 1:2,000), anti-Parkin (1:5,000, Santa Cruz Biotechnology, Dallas, TX), anti-Shc (1:1,000), anti-dynamin-related/like protein 1 (DRP1/DLP1; 1:5,000), anti-optic atrophy 1 protein (OPA1; 1:2,500, BD Biosciences, Franklin Lakes, NJ), anti-mitofusin-2 (MFN2; 1:2,500, Abcam, Cambridge, UK), anti-phospho-CaMKIIδ (Thr^{287}; 1:2,000) anti-phospho-HSP-27 (Ser^{82}; 1:1,000), anti-HSP-27 (1:1,000), anti-Bax (1:1,000), anti-sequestosome 1 (SQSTM1/p62; 1:1,000), anti-microtubule-associated protein 1 light chain 3 A/B (LC3 A/B; 1:5,000), anti-Beclin-1 (1:1,000), anti-Bcl-2 adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3; 1:5,000), anti-phospho-Akt (Ser^{473}; 1:1,000), anti-Akt (1:1,000), anti-phospho-AMP-activated protein kinase α (AMPKα-Thr^{172}; 1:1,000), anti-AMPKα (1:1,000), anti-phospho-ribosomal S6 (Ser^{235/236}; 1:1,000), anti-ribosomal S6 (1:1,000), anti-phospho-glycogen synthase kinase-3 beta (GSK3β-Ser^{9}; 1:1,000), anti-GSK3β (1:1,000) and anti-GAPDH (1:5,000) as loading control (Cell Signaling Technology, Danvers, MA). For isolated mitochondria the following primary antibodies were used in 5% nonfat milk in TBS-T: anti-OPA1 (1:2,500) from BD Bioscience (Franklin Lakes, NJ), anti-SQSTM1/p62 (1:1,000), anti-LC3 A/B (1:1,000) and anti-cytochrome c oxidase subunit 4 (COX4; 1:5,000) as loading control from Cell Signaling Technology (Danvers, MA). After three washes with TBS-T, horseradish peroxidase conjugated secondary antibody was added for 2 hours at room temperature (1:5,000 in 5% nonfat milk in TBS-T). Signals were detected with an enhanced chemiluminescence kit (Bio-Rad, Hercules, CA) by Chemidoc XRS+ (Bio-Rad, Hercules, CA). For the analysis of tropomyosin oxidation, the density of the additional band with higher molecular weight reflecting the formation of disulfide crossbridges was normalized to densitometric values of the respective tropomyosin monomer. Antibodies against
phosphorylated epitopes were removed with Pierce Stripping Buffer (Thermo Fisher Scientific, Waltham, MA) before incubation with antibodies detecting the total protein.

**Statistical analysis**

Values are expressed as mean±standard error of mean (SEM). Statistical analysis was performed between groups by unpaired two-tailed T-test or by Mann-Whitney U-test by using GraphPad Prism 6 software. A p<0.05 value was considered significant.
3. **Results**

**Moderately increased adiposity in prediabetic animals**

To determine the effect of high-fat diet and the single, low dose STZ injection, we measured body weight, fat tissue volumes and plasma lipid parameters. We found that body weights of the prediabetic animals were moderately but statistically significantly elevated from week 9 as compared to the control group, and that this difference reached 18% at the end of the diet period (Fig. 2, *A*-*B*). At week 20, plasma leptin level was significantly increased in prediabetes, however, CRP level was decreased, plasma cholesterol, HDL cholesterol and triglyceride levels, and parameters of liver and kidney function were unchanged (Table 1). To characterize prediabetes-induced changes in further obesity-related molecules, we performed an adipokine array measurement, which revealed that the circulating level of tissue inhibitor of matrix metalloprotease-1 (TIMP-1) might be influenced by prediabetes, however, we could not confirm these results by ELISA (data not shown). CT scan showed that body fat volume of prediabetic rats was substantially increased at the end of the diet (Fig. 2, *C*-*D*). Epididymal fat tissue weight, which is an indicator of total body adiposity, was increased in the prediabetic group, however, the weight of interscapular brown adipose tissue was not changed (Fig. 2, *G*-*I*). Histological score analysis of HE and MA-stained liver samples evidenced the development of hepatic steatosis in the prediabetic group (CON: 0.5±0.3 vs. PRED: 2.25±0.5; p<0.05), however, no signs of hepatic fibrosis was detected (Fig. 2*H*). Furthermore, electron microscopy showed an increased number of lipid droplets in the myocardium of prediabetic animals as compared to controls (Fig. 2, *E*-*F*). These results demonstrated a moderately increased adiposity, hepatic and cardiac fat deposits without signs of hyperlipidemia in the prediabetic group.
Impaired glucose tolerance, insulin resistance, and sensory neuropathy evidence disturbed carbohydrate metabolism in prediabetes

We aimed to characterize the glucose homeostasis in our rat model of prediabetes. At week 20 of the diet, fasting blood glucose levels were slightly elevated in prediabetes from week 10, however, remained in the normoglycemic range (Fig. 3, A-B). OGTT and ITT demonstrated impaired glucose tolerance and insulin resistance in the prediabetic group (Fig. 3, C-F), however, there was no difference in pancreatic insulin content (Fig. 3 G), or in pancreatic islet morphology (data not shown) between groups. These results demonstrate prediabetic conditions in the present model and evidence that type 1 diabetes did not develop due to the STZ treatment. Sensory neuropathy is a well-accepted accompanying symptom of diabetes (78). Accordingly, here we have found a decrease in the mechanical hind limb withdrawal threshold at week 15 (CON: 48±1g vs. PRED: 42±2g; p<0.05) of diet in the PRED, which indicates a moderate sensory neuropathy in this model of prediabetes.

Diastolic dysfunction and hypertrophy in prediabetes with no sign of fibrosis

To determine the cardiac effect of prediabetes, we measured morphological and functional parameters of the hearts. Heart weights were significantly increased (Fig. 4A), however, heart weight/body weight ratio was decreased in prediabetes (CON: 0.27±0.01% vs. PRED: 0.24±0.01%; p<0.05), plausibly due to obesity. Left ventricular (LV) mass, left ventricular anterior wall thickness, systolic (LVAWTs), left ventricular posterior wall thickness, systolic (LVPWTs) and left ventricular posterior wall thickness, diastolic (LVPWTd) were increased in prediabetic group as assessed with echocardiography, however, other cardiac dimensional parameters were unchanged (Table 2). The slope of end-diastolic pressure-volume relationship (EDPVR), which is a very early and sensitive marker of diastolic dysfunction, was significantly elevated in prediabetes, although other hemodynamic parameters, including
blood pressure, were unchanged evidencing the lack of systolic dysfunction or hypertension (Table 3, Fig. 4B). To uncover the molecular background of the observed mild diastolic dysfunction, we performed measurements on the common mechanistic contributors of heart failure (38). On hematoxylin-eosin-stained LV sections increased cardiomyocyte diameter was detected in prediabetes (Fig. 4 C-D). To characterize components affecting diastolic function, we analyzed MHC expression. Interestingly, the gene expression of β-MHC was decreased, and α-MHC also showed a tendency of decrease (p=0.17), the ratio of which resulted in a strong tendency to decrease in prediabetes. No increase in ANP or BNP gene expressions (Fig 4. G-H) or in angiotensin-II level (data not shown) was detected in prediabetes. To evaluate the extent of fibrosis, MA-stained LV sections were analyzed, which revealed no difference between groups (Fig. 4E). Similarly, we found that gene expression of type I (COL1) and III (COL3) collagen isoforms were unchanged in the left ventricle (Fig. 4F). These results indicate that mild diastolic dysfunction developed in prediabetic animals which was associated with a mild hypertrophy (increased LV mass and anterior and posterior LV wall thickness, increased cardiomyocyte diameter) without signs of fibrosis.

**Elevated reactive oxygen species formation in cardiac subsarcolemmal mitochondria in prediabetic rats**

To investigate whether cardiac mitochondrial disturbances contribute to the observed diastolic dysfunction, mitochondrial morphology and enzyme activity were analyzed from left ventricles of prediabetic rats. Our electron microscopy results showed that there is no major difference in the number of IFM between the groups (Fig. 5, A-B). However, area (CON: 0.43±0.01 vs. PRED: 0.39±0.01 µm²; p<0.05), perimeter (CON: 2.69±0.02 vs. PRED: 2.63±0.03 µm; p<0.05) and sphericity (CON: 0.35±0.01 vs. PRED: 0.31±0.01; p<0.05) of IFM are decreased in PRED group. Previous studies indicated that IFM and SSM are affected by diabetes differentially (35, 80). Therefore, we analyzed our EM imagery containing SSM
and found no difference in SSM size, perimeter, or sphericity (data not shown), although, the statistical power of these analyses was not high enough (n=2 for CON and n=4 for PRED).

Furthermore, we have not seen any major difference in mitochondrial oxygen consumption, enzyme activities (Table 4-5), Ca-uptake, or membrane potential (data not shown). However, we have found that hydrogen-peroxide production was increased in the cardiac SSM fraction with glutamate-malate as a substrate (Fig. 5C), although, there was no difference when succinate was used as substrate. Interestingly, there was no increase in reactive oxygen species (ROS) production of the IFM isolated from LV supported either with glutamate-malate or with succinate (Fig. 5, D-F). As leukocytes are one of the main sources of ROS, inflammatory mediators were measured. We could find no significant difference in TNF-α (CON: 1±0.27 vs. PRED: 0.59±0.07; ratio normalized to GAPDH; p>0.05) and IL-6 (CON: 1±0.27 vs. PRED: 0.69±0.14; ratio normalized to GAPDH; p>0.05) mRNA expressions between groups, which evidence that in our model prediabetes does not elicit cardiac or systemic inflammation. Furthermore, we have not seen any difference in other markers of oxidative stress: the expression of p66Shc and tropomyosin oxidation between groups (Fig. 6 B-E). It is known that reactive nitrogen species have important role in deteriorated contractile- and endothelial function in diabetes (16, 58), therefore, we analyzed whether nitrative stress is influenced in prediabetes. Nitrotyrosine immunohistology indicated that protein nitrosylation is increased in prediabetes (Fig. 6A). As CaMKIIδ has been proposed to be activated in oxidative stress-associated conditions (46), we measured the levels of the active forms of the kinase which might affect the contractility and relaxation capacity of the heart (49). The phosphorylation of CaMKIIδ and of its target PLB on Thr17 was not changed by prediabetes (Fig. 6, F-H). Similarly, there was no change in the protein expression of SERCA2A in our model of prediabetes as compared to control animals (Fig. 6J). On the other hand, the level of p-Ser16-PLB showed a tendency for downregulation in prediabetes (p=0.08; Fig. 6J).
Alterations in cardiac mitofusin-2 expression and mitophagy in prediabetes

To investigate the effect of cardiac mitochondrial dynamics, auto- and mitophagy in prediabetes, we analyzed protein expression changes. Cardiac expression of the mitophagy-related protein, BNIP3 was decreased in the prediabetic group in left ventricle lysates, however, other auto- and mitophagy-related proteins such as Beclin-1, LC3-II, SQSTM1/p62 and Parkin were unchanged (Fig. 7A; Table 6). Upstream modulators of autophagy such as Akt, AMPKα, GSK3β and ribosomal S6 protein (a surrogate marker of mTOR complex activity) were also measured, however, expression or phosphorylation of these proteins were not different between groups (Fig. 7A; Table 6). Furthermore, the expression of a mitochondrial fusion-related protein, MFN2 was elevated, however, expression of DRP1/DLP1 and OPA1 proteins were unchanged in whole left ventricle lysates in the prediabetic group (Fig. 7B; Table 6). Nonetheless, we measured the expression of mitochondrial dynamics- and mitophagy-related proteins from SSM and IFM isolated from left ventricles. No difference was found in the expression of OPA1, LC3-II, SQSTM1/p62 in isolated cardiac SSM and IFM between groups (Fig. 7, C-D; Table 6). Our results indicate that mitochondrial dynamics and autophagy/mitophagy were not modulated substantially by prediabetes, however, the upregulation of MFN2 (increased mitochondrial fusion, tethering to endoplasmic reticulum) and the downregulation of BNIP3 (decreased mitophagy) may implicate early changes in mitochondrial homeostasis, which might lead to the accumulation of dysfunctional mitochondria.

Expression of cardiac Bcl-2 decreases in prediabetes

Our study also aimed to explore the effect of prediabetes on apoptosis in the heart. Prediabetes did not affect the expression of pro-apoptotic caspase-3 and Bax in left ventricles.
On the other hand, the anti-apoptotic Bcl-2 was downregulated in prediabetic animals. However, the Bcl-2/Bax ratio was unchanged (Fig. 7F; Table 6).

No changes in cardiac HSPs in prediabetes

We also characterized the effect of prediabetes on the expression and/or phosphorylation of heat shock proteins in the left ventricle. Our results showed no differences in the expression of HSP-60, HSP-70 and HSP-90 or in either phosphorylation or expression of HSP-27 (Fig. 7E; Table 6).
4. Discussion

This is the first comprehensive analysis of the cardiac effects of prediabetes in a non-genetic rodent model, where we assessed cardiac functions, parameters of hypertrophy, fibrosis, oxidative and nitrative stress, inflammation, mitochondrial dynamics, autophagy, mitophagy, markers of myocardial calcium handling, apoptosis, expression of HSPs. In this model of prediabetes, we evidenced an impaired glucose and insulin tolerance, increased adiposity and myocardial lipid accumulation, a mild diastolic dysfunction and sensory neuropathy despite normal fasting plasma glucose and lipid levels. We also observed elevated ROS production in the SSM, nitrative stress, elevated expression of MFN2, decreased expression of β-MHC, and phosphorylation of PLB. Furthermore, here we found early signs of dysregulated mitophagy and decreased mitochondrial size in prediabetes, however, other major markers of mitochondrial dynamics, auto- and mitophagy, inflammation, or myocardial expression of apoptotic proteins or HSPs were not modulated by prediabetes.

In this study, we used high-fat chow-fed Long-Evans rats treated with a single, low dose STZ. This setting allowed us to investigate cardiac consequences of a moderate metabolic derangement, prediabetes, rather than of a severely disturbed glucose and lipid homeostasis, such as seen in genetically modified models of diabetes, e.g. in db/db or ob/ob mice (36, 65). Since it has been reported that LV hypertrophy had a higher prevalence in patients with diabetes, and that 40-75% of patients with type 1 or type 2 diabetes mellitus presented with diastolic dysfunction (12, 71), we aimed to investigate whether cardiac function is affected by prediabetes. Previously it has been shown that diastolic dysfunction was developed in several pathological condition, however, the underlying mechanisms are still not clearly understood (38). Here we demonstrated that the deterioration of diastolic function and sensory neuropathy occurs well before overt diabetes develops, which is accompanied by early signs of cardiac
hypertrophy. These findings are in agreement with previous reports showing that neuropathy might precede the development of a full-fledged diabetes (48), and that high-fat diet-induced prediabetes increased heart weights and decreased contractile function, as assessed by a diminished aortic output (23, 29). However, in contrast to our report, plasma triglycerides and insulin levels were elevated in these studies, highlighting that substantial difference can be observed between different diet-induced models and stages of prediabetes (29). Furthermore, it has been described that obesity promoted the hypertrophy-inducing effect of diabetes regardless of hypertension (26), which could be attributed to adipokines, such as leptin and resistin (5, 39). Similarly, here we evidenced that even mild obesity (only 18% increase in body weight was observed in the present study) with an elevated leptin level is sufficient to induce hypertrophy even without impairment of fasting plasma glucose and lipid levels or hypertension, which is in agreement with previous reports (23, 29). However, clinical data seem to contradict these findings, since no increase in the prevalence of LV hypertrophy was observed in overweight prediabetic patients with impaired fasting glucose and impaired glucose tolerance (64). Mechanistic studies on how obesity abrogates cardiac function are scarce. Increased myocardial triglyceride content is associated with diastolic dysfunction in ob/ob mice (18), which is well in line with our findings that the number of lipid particles increased in the myocardium in prediabetes. Although microRNA-451 has been demonstrated to promote cardiac hypertrophy and diminished contractile reserves in mice on high-fat diet (44), further studies are warranted to describe the relationship between cardiac dysfunction and the disturbed cardiac lipid metabolism in prediabetes. Interestingly, unlike in genetic models of prediabetes (20), diet-induced prediabetes did not result in an elevation in classical molecular markers of hypertrophy or conventional signs of fibrosis in the heart as expected in case of hypertrophy. Moreover, this is the first evidence on decreased β-MHC in prediabetes. Although the vast majority of publications evidence an increase in β-MHC in diabetes (4, 81),
a small number of studies indicate a downregulation of MHC expression in animals with
diverse cardiac or metabolic challenges. For instance, in cardiomyocytes from STZ-treated
rats, total MHC expression was significantly decreased (25). These results indicate that
although in most cardiometabolic derangements expression of the slow MHC isoform
increases, in certain conditions, such as in prediabetes, a general suppression of MHC
expression might be present. The reduction in MHC expression might also contribute to the
observed cardiac dysfunction in prediabetes, however, to uncover its significance and
mechanism, further experiments are warranted.

Oxidative stress has a major role in the development of diabetic cardiomyopathy (10, 31),
however, it has not been well described whether it is responsible for the decreased cardiac
function in prediabetes. Here we found an elevated hydrogen peroxide production in SSM,
increased nitrotyrosine formation and an elevated cardiac expression of MFN2. These
findings are in agreement with previous reports, where elevated oxidative stress, such as seen
in our model of prediabetes, leads to an increase in MFN2 in rat vascular smooth muscle cells
(33), and its robust overexpression induced apoptotic cell death in neonatal rat
cardiomyocytes (70). Similarly, in another study, high-fat diet induced oxidative stress and
MFN2 overexpression in the liver of C57BL/6 mice after 16 weeks (27). However, in a
previous study on diet-induced prediabetes, no sign of cardiac mitochondrial oxidative stress
was evidenced in male Wistar rats after 16 weeks (29), which may implicate that
mitochondrial oxidative stress might not be present in all models and stages of prediabetes
and that it might not be the primary driving force of prediabetes-induced cardiac functional
alterations.

It is well-established that mitochondria, especially the mitochondrial electron transport chain
is one of the main source of ROS, however, several other intracellular components can
produce ROS in mitochondria (17). For instance, it is known that p66Shc translocation to
mitochondria can increase the formation of ROS (22), and NADPH oxidase 4 (NOX4) and monoamine oxidase (MAO) also have important role in mitochondrial ROS production (8, 43). Although here we observed a moderately increased ROS production in SSM, no difference can be seen in mitochondrial oxygen consumption between normal and prediabetic mitochondria (see Table 5), evidencing no impairment in mitochondrial redox chains. It is currently unknown what mechanism leads to the increased ROS production exclusively in SSM in prediabetes. In mice on high-fat diet, cardiac mitochondrial ROS production was elevated, and similarly to our results, mitochondrial oxygen consumption did not change substantially, while a significant amount of cardiac lipid accumulation was observed (2), however, the source of ROS has not been identified in this study either. Thus, to reveal the direct connection between elevated ROS production, mitochondrial-, and cardiac dysfunction, further studies are warranted.

Molecular mechanisms that contribute to hypertrophy and cardiac dysfunction in prediabetes has not been investigated in detail. In our previous studies on diet-induced hypercholesterolemia or metabolic syndrome in ZDF rats, we have shown by DNA- and miRNA microarrays that a multitude of cardiac cellular processes is modulated by these conditions (67, 77). Similarly, in this study we evidenced changes in several cellular processes, suggesting that hypertrophy and deteriorated diastolic function in prediabetes maybe consequences of numerous concurrent alterations in the cardiac homeostasis (see Figure 8). Characterizing active components of the contractile apparatus and Ca\(^{2+}\) homeostasis, here we observed a tendency to decrease in the Ser\(^{16}\) phosphorylation of PLB in prediabetes. In previous studies, decreased phosphorylation of PLB on Ser\(^{16}\) was demonstrated to be associated with abnormalities in contraction and relaxation in the diabetic heart (56, 83). This notion is further supported by the findings of Abdurrachim et al, who demonstrated that phosphorylation of PLB was reduced in the heart of mice with diastolic
dysfunction induced by a high-fat diet (2). Therefore, decreased phosphorylation of PLB may also contribute to the development of early diastolic dysfunction we uncovered in prediabetes. Increased activity and expression of CaMKIIδ and reduced phosphorylation of PLB by CaMKIIδ have been found to be associated with contractile dysfunction, diabetes (47, 49), and fructose-rich diet-induced prediabetes (73). In our model, expression and phosphorylation of CaMKIIδ, and phosphorylation of PLB on Thr^{17} were unchanged. This is in contrast with previous findings which have reported the phosphorylation of CaMKIIδ being increased in the heart of STZ-treated diabetic rats (69, 74), although, in these reports, a significant hyperglycemia was present, which was shown to facilitate the activation of CaMKIIδ (28).

Apoptosis is considered to be one of the hallmarks of diabetic cardiomyopathy and it is induced by oxidative stress in diabetes (10, 76). It has been described that experimental diabetes induces upregulation of pro-apoptotic, and downregulation of anti-apoptotic proteins (3, 82), however, no data has been available on the cardiac apoptosis in prediabetes. In the present study, we show a modest downregulation of Bcl-2, however, no change in Bcl-2/Bax ratio, and in caspase-3 expression was detected in prediabetic animals. Thus, our data suggest an early dysregulation of pro- and antiapoptotic proteins in prediabetes, however, they do not evidence a gross induction of apoptosis in prediabetes. Tropomyosin is prone to loss of function by oxidative modifications that are associated with the severity of heart failure in humans (14, 15). In this study, oxidized tropomyosin content of the heart was not modulated by prediabetes. These data suggest that neither the CaMKIIδ pathway, apoptosis induction, nor tropomyosin oxidation are responsible for the diastolic dysfunction observed in prediabetes.

Here we also demonstrate an early dysregulation of mitochondrial fusion and mitophagy first in the literature, as evidenced by an elevated MFN2 and an attenuated BNIP3 expression in prediabetes, however, other canonical markers of autophagy, mitophagy and apoptosis were
unaffected. Similarly, right atrial myocardial samples of type-2 diabetic patients presenting no
signs of overt cardiomyopathy the expression of the majority of mitochondrial dynamics- and
autophagy-related proteins was not elevated, except for that of ATG5 and MFN1 (53). Therefore, we can assume that only major disturbances in glucose and lipid homeostasis, such
as seen in untreated patients or in genetic models of diabetes, might be a powerful enough
signal to extensively modulate cardiac autophagy, mitophagy, or mitochondrial dynamics,
which might result in grossly deteriorated cardiac function. Moreover, experimental systemic
sensory neuropathy by itself has been previously shown to cause diastolic dysfunction and
global gene expression changes in the rat heart (7, 84). Therefore, prediabetes-induced
sensory neuropathy observed in the present study might also contribute to the diabetic cardiomyopathy.

Furthermore, this is the first report to show that prediabetes does not modulate cardiac
expression of HSP-60, HSP-70 and HSP-90 and phosphorylation or expression of HSP-27. In
contrast, in STZ-induced diabetes, increased levels of HSP-70 has been detected in the rat
heart (75), and increased level of circulating HSP-60 found in diabetic patients (68),
suggesting that in advanced stages of diabetes HSPs might be involved in the development of
cardiac dysfunction. However, our data suggests no role of HSPs in prediabetes in the heart.

Conclusions

Taken together, this study emphasizes that parallel occurrence of several abnormalities of
metabolic, oxidative and contractile functions might trigger cardiac pathological changes
characteristic to prediabetes well before hyperglycemia or major metabolic derangements
occur, and that preventing these abnormalities might be of importance for future therapies of
cardiac pathologies observed in early metabolic diseases such as prediabetes.
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Disclosures

All authors read and approved the final manuscript. The authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Author contributions

GK, ZVV, TB, MB, TR, TK, MA performed experiments, assisted with analyses of results and interpretation and writing. KB, RT, AO and LD assisted with analyses of results, interpretation and writing the manuscript. AA, BM, ZSH, DM, SZK, PH, SR, LT and PP contributed to experiments, analysis, and interpretation of data. GK, ZVV, TB, RT, AO,
CSM, LA, and ST performed experiments with animals. PF, RS, ZVV and ZG designed the experiments, drafted and edited the manuscript.
6- References:


46. **Luczak ED, and Anderson ME.** CaMKII oxidative activation and the pathogenesis of cardiac disease. *J Mol Cell Cardiol* 73: 112-116, 2014.


2. **Figure captions:**

**Fig. 1. Experimental protocol.** Long-Evans rats were fed with either control (CON) diet for 21 weeks, or with high-fat diet and treated with 20 mg/kg streptozotocin (STZ) at week 4 (PRED) to induce prediabetes. Body weights were measured weekly and blood samples were taken from the saphenic vein every second week. Sensory neuropathy was measured at week 15. Oral glucose tolerance test (OGTT), insulin tolerance test (ITT) and computer tomography.
(CT) were performed at week 20. Echocardiography, hemodynamic analysis and parameters of mitochondrial function were measured at week 21 of diet. Tissue sampling was performed after terminal procedures.

**Fig. 2. High-fat feeding with a single low dose STZ treatment increase adiposity.** Changes in body weight during the experiment (A) and body weight data after 21 weeks (B). Axial representative CT slice from the middle of 4th lumbar spine. Red color indicates the segmented Volume-of-Interests (VOIs) showing the volume of fat in the control (CON) and prediabetic (PRED) rats (C). Whole body fat volume to body weight ratio at week 20 (D). Representative transmission electron micrographs of myocardial lipid droplets (E; white arrows) and number of lipid droplets in CON and PRED cardiomyocytes (F). Magnification 7,500×; scale bar 1 µm. Epididymal fat tissue (G) and interscapular brown adipose tissue (BAT) (I) weight to body weight ratios. Hematoxylin-Eosin (HE) and Masson’s trichrome (MA) staining of liver sections (H). Magnification 200×; scale bar 100 µm. Data are means±SEM N= 3-19 per group (*p<0.05).

**Fig. 3. Alterations in glucose homeostasis indicate the development of a prediabetes in streptozotocin-treated and high-fat-fed rats at week 21.** Fasting blood glucose levels during the experiment (A) and at week 20 (B). Oral glucose tolerance test (OGTT) (C-D) and insulin tolerance test (ITT) (E-F) results at week 20 of the diet. Insulin content of pancreas at week 21 (G). Data are means±SEM, n=6-19 per group (*: p<0.05).

**Fig. 4. Characterization of cardiac function, myocardial morphology and fibrosis in prediabetic rats.** Quantification of heart weights after 21 weeks (A). Representative pressure-volume loops and slope of EDPVR in CON and PRED group (B). HE and MA staining of myocardial sections (C) and quantification of cardiomyocyte diameter (D) and level of fibrosis (E) in control (CON) and prediabetic (PRED) rats. Magnification 200×; scale bar 100
μm. Quantification of COL1, COL3 (F), ANP, BNP (G), α-MHC, β-MHC gene expressions and α- to β-MHC ratio (H) in CON and PRED group. Abbreviations: EDPVR: end diastolic pressure-volume relationship; HE: Hematoxylin-Eosin; MA: Masson's trichrome; COL1: collagen type I; COL3: collagen type III; ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide; α-MHC: alpha-myosin heavy chain; β-MHC: beta-alpha-myosin heavy chain. Data are means±SEM, n=5-19 per group (*: p<0.05).

**Fig. 5. Mitochondrial morphology and function in prediabetes at week 21.** Representative transmission electron micrographs (A) and number of IFM (B) in the left ventricle. Magnification 12,000×, scale bar 1 μm. Quantification of H₂O₂ production in SSM (C) and IFM (D) with glutamate-malate as substrate (GM). Quantification of H₂O₂ production in SSM (E) and IFM (F) with succinate as substrate. Abbreviations: IFM: interfibrillar mitochondria; SSM: subsarcolemmal mitochondria; ADP: adenosine diphosphate. Data are means±SEM, n=5-9 per group (*: p<0.05).

**Fig. 6. Characterization of oxidative and nitrative stress in prediabetes.** Representative immunostaining of nitrotyrosine in the left ventricle (A), magnification 200×; scale bar 200 μm. Representative Western blots (B) and quantification (C) of tropomyosin oxidation. Representative Western blots (D) and quantification (E) of cardiac p66Shc expression. Representative Western blots (F) and quantification of CaMKIIδ (G) and PLB phosphorylation on Thr¹⁷ (H) and Ser¹⁷ (I), and SERCA2A (J) expression. Abbreviations: Tm: tropomyosin; Ox. Tm: oxidized tropomyosin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; CaMKIIδ: Ca²⁺/calmodulin-dependent protein kinase II; SERCA2A: sarco/endoplasmic reticulum Ca²⁺ ATPase II; PLB: phospholamban. Data are means±SEM, n=6-8 per group (*: p<0.05).
Fig. 7. Cardiac expression of mitochondrial dynamics, autophagy/mitophagy, HSPs, and apoptosis-related proteins in prediabetes. Representative Western blots of autophagy/mitophagy-related proteins and upstream modulators of autophagy (A), mitochondrial fission- and fusion-related protein (B) in whole left ventricles. Representative Western blots of mitochondrial dynamics- and mitophagy-related proteins in isolated SSM (C) and IFM (D). Representative Western blots of HSP- (E), and apoptosis-related (F) proteins in whole left ventricle. Abbreviations: SSM: subsarcolemmal mitochondria; IFM: interfibrillar mitochondria; HSP: heat shock protein; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; DRP1/DLP1: dynamin-related/like protein 1; MFN2: mitofusin-2; OPA1: optic atrophy 1; COX4: cytochrome c oxidase subunit 4, mitochondrial; LC3: 1 microtubule-associated protein 1 light chain 3; SQSTM1/p62: sequestosome 1, BNIP3: Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3; AMPKα: AMP-activated protein kinase α; GSK3β: glycogen synthase kinase-3 beta.

Fig. 8. Schematic representation of the cardiac effects of prediabetes.
### 3. Tables

#### Table 1.

<table>
<thead>
<tr>
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<th>CON</th>
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<td>Plasma leptin (ng/mL)</td>
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<td>5.91 ± 0.60*</td>
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<td>Plasma cholesterol (mmol/L)</td>
<td>1.88 ± 0.06</td>
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<td>2.75 ± 0.14</td>
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<td>Creatinine (μmol/L)</td>
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<td>CRP (mg/L)</td>
<td>109.56 ± 1.24</td>
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### Table 2.

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<tr>
<td>LV mass (g)</td>
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<td>LVAWTd (mm)</td>
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<td>2.07 ± 0.12</td>
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<td>LVAWTS (mm)</td>
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<td>3.42 ± 0.11*</td>
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<td>LVPWTd (mm)</td>
<td>1.86 ± 0.07</td>
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<td>LVPWTs (mm)</td>
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<td>LVESD (mm)</td>
<td>4.93 ± 0.22</td>
<td>4.96 ± 0.31</td>
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<tr>
<td>FS (%)</td>
<td>36.0 ± 2.2</td>
<td>37.4 ± 3.8</td>
</tr>
<tr>
<td>HR (1/min)</td>
<td>335 ± 13</td>
<td>348 ± 10</td>
</tr>
</tbody>
</table>
Table 3.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>CON</th>
<th>PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>110.1 ± 7.3</td>
<td>113.6 ± 6.1</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>116.6 ± 5.6</td>
<td>120.0 ± 6.8</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4.4 ± 0.4</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>LVEDV (µL)</td>
<td>292.8 ± 14.5</td>
<td>280.2 ± 9.6</td>
</tr>
<tr>
<td>LVESV (µL)</td>
<td>130.7 ± 6.9</td>
<td>127.5 ± 4.5</td>
</tr>
<tr>
<td>SV (µl)</td>
<td>162.1 ± 9.1</td>
<td>152.8 ± 7.7</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>59.5 ± 3.2</td>
<td>56.6 ± 2.3</td>
</tr>
<tr>
<td>EF (%)</td>
<td>55.3 ± 1.3</td>
<td>54.4 ± 1.3</td>
</tr>
<tr>
<td>SW (mmHg·mL)</td>
<td>14.5 ± 0.5</td>
<td>13.6 ± 0.6</td>
</tr>
<tr>
<td>dP/dtmax (mmHg/s)</td>
<td>7226 ± 487</td>
<td>7387 ± 401</td>
</tr>
<tr>
<td>dP/dtmin (mmHg/s)</td>
<td>-8198 ± 680</td>
<td>-8551 ± 545</td>
</tr>
<tr>
<td>τ (Glantz) (ms)</td>
<td>12.6 ± 0.3</td>
<td>12.1 ± 0.4</td>
</tr>
<tr>
<td>TPR [(mmHg·min)/mL]</td>
<td>1.90 ± 0.19</td>
<td>2.00 ± 0.12</td>
</tr>
<tr>
<td>Slope of ESPVR (mmHg/µL)</td>
<td>2.68 ± 0.12</td>
<td>2.71 ± 0.06</td>
</tr>
<tr>
<td>Slope of EDPVR (mmHg/µL)</td>
<td>0.026 ± 0.001</td>
<td>0.037 ± 0.004*</td>
</tr>
<tr>
<td>PRSW (mmHg)</td>
<td>100.5 ± 5.2</td>
<td>98.9 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>Slope of dP/dtmax-EDV [(mmHg/s)/μL]</td>
<td>34.3 ± 2.3</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>Maximal power (mW)</td>
<td>91.8 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>PRED</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Citrate-synthase activity (U/mg protein)</td>
<td>223.12 ± 9.98</td>
<td>220.44 ± 8.32</td>
</tr>
<tr>
<td>NADH:Ubiquinone-Oxidoreductase activity (U/mg protein)</td>
<td>40.52 ± 2.55</td>
<td>36.48 ± 2.99</td>
</tr>
<tr>
<td>NADH: Cytochrome c- Oxidoreductase activity (U/mg protein)</td>
<td>7.85 ± 1.18</td>
<td>8.47 ± 1.31</td>
</tr>
<tr>
<td>Succinate: Cytochrome-c-Oxidoreductase activity (U/mg protein)</td>
<td>21.09 ± 1.49</td>
<td>23.57 ± 1.61</td>
</tr>
<tr>
<td>Succinate-Dehydrogenase activity (U/mg protein)</td>
<td>84.06 ± 5.83</td>
<td>80.09 ± 3.42</td>
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<tr>
<td>Cytochrome-c-Oxidase activity (U/mg protein)</td>
<td>38.74 ± 3.15</td>
<td>40.36 ± 2.33</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>PRED</td>
</tr>
<tr>
<td>------------------</td>
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<td>-----------------------</td>
</tr>
<tr>
<td></td>
<td>Subsarcolemmal</td>
<td>Subsarcolemmal</td>
</tr>
<tr>
<td></td>
<td>(pmol/mL × sec)</td>
<td>(pmol/mL × sec)</td>
</tr>
<tr>
<td>Glutamate-malate</td>
<td>25.08 ± 3.6</td>
<td>21.06 ± 3.53</td>
</tr>
<tr>
<td>ADP</td>
<td>203.31 ± 32.57</td>
<td>194.03 ± 42.16</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>260.8 ± 28.27</td>
<td>287.06 ± 54.91</td>
</tr>
<tr>
<td>Succinate</td>
<td>306.2 ± 25.19</td>
<td>289.74 ± 23.23</td>
</tr>
<tr>
<td>Rotenone</td>
<td>143.12 ± 18.19</td>
<td>139.7 ± 23.64</td>
</tr>
<tr>
<td>CAT</td>
<td>105.95 ± 8.89</td>
<td>106.5 ± 12.22</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>PRED</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Total LV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BNIP3/GAPDH ratio</td>
<td>0.59 ± 0.03</td>
<td>0.44 ± 0.02*</td>
</tr>
<tr>
<td>MFN2/GAPDH ratio</td>
<td>0.27 ± 0.01</td>
<td>0.36 ± 0.02*</td>
</tr>
<tr>
<td>OPA1/GAPDH ratio</td>
<td>1.47 ± 0.11</td>
<td>1.63 ± 0.1</td>
</tr>
<tr>
<td>DRP1/GAPDH ratio</td>
<td>1.16 ± 0.08</td>
<td>1.34 ± 0.14</td>
</tr>
<tr>
<td>LC3-II/GAPDH ratio</td>
<td>0.56 ± 0.07</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>p62/GAPDH ratio</td>
<td>3.05 ± 0.18</td>
<td>3.45 ± 0.23</td>
</tr>
<tr>
<td>Parkin/GAPDH ratio</td>
<td>2.25 ± 0.14</td>
<td>2.43 ± 0.17</td>
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<tr>
<td>Beclin1/GAPDH ratio</td>
<td>0.81 ± 0.07</td>
<td>0.82 ± 0.07</td>
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<tr>
<td>Phospho AKT(Ser^{473})/AKT ratio</td>
<td>0.32 ± 0.04</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>Phospho AMPK(Thr^{172})/AMPK ratio</td>
<td>0.12 ± 0.02</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>Phospho S6(Ser^{235/236})/S6 ratio</td>
<td>2.62 ± 1.08</td>
<td>2.16 ± 0.61</td>
</tr>
<tr>
<td>Phospho GSK3β(Ser^{9})/GSK3β ratio</td>
<td>0.8 ± 0.09</td>
<td>0.72 ± 0.1</td>
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<tr>
<td>Bcl-2/GAPDH ratio</td>
<td>0.23 ± 0.01</td>
<td>0.20 ± 0.003*</td>
</tr>
<tr>
<td>Bcl-2/Bax ratio</td>
<td>0.15 ± 0.02</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>caspase-3/GAPDH ratio</td>
<td>0.05 ± 0.001</td>
<td>0.04 ± 0.003</td>
</tr>
<tr>
<td>P-HSP-27(Ser^{82})/T-HSP-27 ratio</td>
<td>0.34 ± 0.05</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>HSP-60/GAPDH ratio</td>
<td>0.85 ± 0.02</td>
<td>0.84 ± 0.02</td>
</tr>
<tr>
<td>HSP-70/GAPDH ratio</td>
<td>0.53 ± 0.01</td>
<td>0.51±0.01</td>
</tr>
<tr>
<td>HSP-90/GAPDH ratio</td>
<td>0.46 ± 0.01</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Subsarcolemmal mitochondria</td>
<td>Interfibrillar mitochondria</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td><strong>OPA1/COX4 ratio</strong></td>
<td><strong>CON</strong> 1.34 ± 0.07 1.32 ± 0.06</td>
<td><strong>CON</strong> 1.44 ± 0.15 1.52 ± 0.14</td>
</tr>
<tr>
<td><strong>LC3-II/COX4 ratio</strong></td>
<td>0.22 ± 0.1 0.25 ± 0.04</td>
<td>0.22 ± 0.09 0.29 ± 0.07</td>
</tr>
<tr>
<td><strong>p62/COX4 ratio</strong></td>
<td>0.13 ± 0.02 0.12 ± 0.03</td>
<td>0.35 ± 0.06 0.36 ± 0.04</td>
</tr>
</tbody>
</table>
4. Table legends

Table 1. Plasma parameters at week 21. Data are means±SEM for 12 rat per group (*: p<0.05).

Table 2. Characterization of cardiac morphology and function in prediabetes by means of echocardiography. Abbreviations: LV mass: Left Ventricular Mass; LVAWTs: Left Ventricular Anterior Wall Thickness, systolic; LVAWTd: Left Ventricular Anterior Wall Thickness, diastolic; LVPWTs: Left Ventricular Posterior Wall Thickness, systolic; LVPWTd: Left Ventricular Posterior Wall Thickness, diastolic; FS%: Fractional Shortening %; HR: Heart rate. Data are means±SEM for 10 rat per group (*: p<0.05).

Table 3. Characterization of left ventricular (LV) hemodynamics in vivo in prediabetes by means of pressure-volume analysis. Abbreviations: MAP: Mean Arterial Pressure; LVESP: Left Ventricular End-Systolic Pressure; LVEDP: Left Ventricular End-Diastolic Pressure; LVESV: Left Ventricular End-Systolic Volume; LVEDV: Left Ventricular End-Diastolic Volume; SV: Stroke Volume; CO: Cardiac Output; EF: Ejection Fraction; SW: Stroke Work; dP/dtmax: maximal slope of LV systolic pressure increment; dP/dtmin: maximal slope of LV diastolic pressure decrement; τ: time constant of LV pressure decay; TPR: Total Peripheral Resistance; ESPVR: End-Systolic Pressure-Volume Relationship; EDPVR: End-Diastolic Pressure-Volume Relationship; PRSW: Preload Recruitable Stroke Work; dP/dtmax-EDV: the slope of the dP/dtmax-end-diastolic volume relationship. Data are means±SEM for 10 rat per group (*: p<0.05).

Table 4. Quantification of cardiac mitochondria enzyme activity in left ventricle. Data are means±SEM for 5-9 rat per group (*: p<0.05).
Table 5. Quantification of mitochondrial oxygen consumption. Abbreviations: ADP: adenosine diphosphate. CAT: carboxyatractyloside. Data are means±SEM for 9 rats per group (*: p<0.05).

Table 6. Quantification of HSPs, apoptosis, mitochondrial dynamics- and mitophagy-related protein expressions in isolated mitochondrial fractions and whole left ventricles. Data are means±SEM for 8 rats per group (*: p<0.05).
Figure 1

Control (CON, n=20)

- Citrate

Prediabetic (PRED, n=20)

- STZ

- Fasting blood glucose measurement
- Sensory neuropathy test
- Oral glucose tolerance test
- Insulin tolerance test
- Computer Tomography

- Treatment
- Echocardiography
- Hemodynamic measurements
- Blood and tissue sampling
- Mitochondrial function measurements
**Figure 2**

**A**

Graph showing body weight (g) over time (week) for CON and PRED groups.

**B**

Bar graph showing body weight (g) for CON and PRED groups.

**C**

Images of the CON and PRED groups, showing different fat volume/body weight (%).

**D**

Bar graph showing fat volume/body weight (%) for CON and PRED groups.

**E**

Images of the CON and PRED groups, showing different lipid droplets/mm².

**F**

Bar graph showing number of lipid droplets/mm² for CON and PRED groups.

**G**

Graph showing epididymal fat wt/body wt (%) for CON and PRED groups.

**H**

Histological images of CON and PRED groups stained with HE.

**I**

Graph showing BAT wt/body wt (%) for CON and PRED groups.