Aerobic exercise training regulates serum extracellular vesicle miRNAs linked to obesity to promote their beneficial effects in mice

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Running title: Metabolic changes in EV miRNAs due to aerobic exercise

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Abstract

There is growing body of evidence that extracellular vesicles (EVs) and their cargo of RNA, DNA, and protein are released into the circulation with exercise and might mediate inter-organ communication. C57BL6/J male mice were diet-induced obesity and subjected to aerobic training on a treadmill for 8 weeks. The effect of aerobic training was evaluated in the liver, muscle, kidney and white/brown adipose tissue. In order to provide new mechanistic insight, we profiled miRNA from serum EVs of obese and obese trained mice. We demonstrate that aerobic training changes circulating EVs miRNA profile of obese mice, including decreases in miR-122, miR-192 and miR-22 levels. Circulating miRNA levels were associated with miRNA levels in mouse liver white adipose tissue (WAT). In WAT, aerobically-trained obese mice showed reduced adipocyte hypertrophy and increased the number of smaller adipocytes and the expression of *Cebpa*, *Pparg*, *Fabp4* (adipogenesis markers) and ATP-citrate lyase enzyme activity. Importantly, miR-22 levels negatively correlated with the expression of adipogenesis and insulin sensitivity markers. In the liver, aerobic training reverted obesity-induced steatohepatitis, and steatosis score and *Pparg* expression were negatively correlated with miR-122 levels. The pro-metabolic effects of aerobic exercise in obesity possibly involves EV miRNAs, which might be involved in communication between liver and WAT. Our data provide significant evidence demonstrating that aerobic training exercise-induced EVs mediate effect of exercise on adipose tissue metabolism.

Keywords: physical exercise, extracellular vesicles, steatosis, adipose tissue, noncoding RNAs

Introduction

The epidemiological characteristics of obesity has made it a major public health problem in several countries, since it is associated with a diversity of comorbidities (35, 58). The main trigger of most of the damages promoted by obesity in other tissues is ectopic fat deposition, which is caused by a dysregulation of preadipocyte and adipocyte functions, facilitating the lipid accumulation in skeletal muscle, kidney, liver and many other tissues (13, 44).

Skeletal muscle and liver both play important metabolic roles in obesity. Skeletal muscle has an important metabolic role, accounting for approximately 80% of insulin-stimulated glucose uptake via glucose transporter type 4 (GLUT4) (31, 32). Obesity-induced insulin resistance in this tissue, promoted mainly by intramyocellular lipids accumulation, is the major defect involved in the development of type 2 diabetes, affecting systemic insulin sensitivity (8). The liver is an essential metabolic organ responsible for endogenous glucose production, so increased lipid accumulation on this organ may lead to the development of non-alcoholic fatty liver disease (NAFLD) (37); NAFLD comprises a spectrum of disorders characterized by liver steatosis and affects a third of the world's population and its rapid rise parallels the increase in hepatocellular carcinoma (46).

Regular practice of physical exercise increases muscle endurance (14) and strength (40), spends calories and leads to a decrease in the mass of adipose tissue, resulting on important beneficial effects on the prevention of obesity and type 2 diabetes (33). Despite the known benefits of physical exercise in preventing type 2 diabetes and reduced insulin sensitivity, the molecular mechanisms for exercise-induced improvements in obesity are not fully understood. However, losing weight can improve or prevent obesity and obesity-related diseases, and dietary and behavioral changes and physical activity are effective nonpharmacological interventions in the prevention and management of obesity and obesity-related diseases (26, 29).

Recently, using quantitative proteomics, it has been demonstrated that EVs are released into the circulation with exercise and might be involved in inter-organ communication as pulse-chase and intravital imaging experiments suggested EVs liberated by exercise have a propensity to localize in the liver and can transfer their protein cargo (56).

EVs, in particular exosomes, are generated in late-endosomal compartments. Besides proteins, exosomes carry nucleic acid (DNA, messenger RNAs (mRNAs), microRNAs, and other non-coding RNAs) signatures. The levels of different components in exosomes depend largely on the functional states of cells producing them, that is, whether they are stimulated, transformed, rested, or stressed (17). EVs have been shown to be mediators of intercellular communication and they transfer their cargo from a donor cell to a recipient cell (36).

MicroRNAs (miRs or miRNAs) are a class of small noncoding RNAs that negatively regulate post-transcriptionally gene expression (4). Circulating or extracellular microRNAs are detectable in almost all biofluids (48) and can travel into microvesicles such as exosomes, apoptotic bodies or membrane-free carriers, such as HDL or associated with argonaute protein (53). Circulating miRNAs have been found to change in response to exercise in humans (1–3, 54). More importantly, secreted miRNAs, especially those in EVs, have paracrine and endocrine effects in different tissues and thus modulate gene expression and the function of distal cells (6, 28, 51). However, these studies have evaluated serum/plasma miRNAs and there is a lack of relation between miRNA abundance in whole plasma/serum and circulatory exosomes, thus, one measurement cannot be replaced by the other (57). As EV miRNA content reflects the functional state of a cell/organ, it might be more reliable as biomarkers.

The potential changes in circulating EV miRNA profile in response to aerobic training in obese mice was investigated in the present study. Circulating miRNA levels were correlated with miRNA tissue levels and more importantly positively demonstrate the benefits of physical exercise on NAFLD and adipose tissue hypertrophy and insulin sensitivity.

Research Design and Methods

Ethical Approval

Male wild-type C57BL/6J mice (Facility for SPF mice production at USP Medical School, Sao Paulo, Brazil) were maintained at 12:12-h light–dark cycle and 23°C±2°C. The animals were housed in cages (2-4 animals/cage) and received standard diet (Nuvilab-Nuvital Nutrients Ltd., Parana, Brazil) and water *ad libitum* until to the beginning of the experimental period. The Experimental Animal Ethics Committees of the Institute of

Biomedical Sciences (ICB), University of Sao Paulo (USP), approved the experimental procedure of this study (Protocol Number: 54/2017).

Experimental Design

Eight weeks old male mice (n=60) were randomly divided into two groups: control diet (C) (76% Kcal carbohydrates, 9% Kcal fat and 15% Kcal proteins) or high-fat diet (26% Kcal carbohydrates, 59% Kcal fat and 15% Kcal proteins) supplemented with condensed milk (68% Kcal carbohydrates, 23% Kcal fat and 9% Kcal proteins) (H) (ad libitum, with 1% vitamin AIN-93 Rhoster). After 4 weeks, the physical exercise protocol was initiated with part of the animals (C) and (H) for 8 weeks, resulting in 2 new groups: control diet + physical training (CT) and high-fat diet supplemented with condensed milk + physical training (HT). During the week before euthanasia, indirect calorimetry, VO2max, glucose, insulin and pyruvate tolerance tests were performed in mice from all groups. By the end of 12 weeks, the animals were euthanized using the anesthetic isoflurane (5%). Blood, brown, epididymal, inguinal and retroperitoneal adipose tissues, gastrocnemius skeletal muscle, liver and kidneys were collected.

Physical training protocol and treadmill exercise tolerance test

Initially the animals were placed on the unplugged treadmill for 10 minutes (min) per day for 5-day acclimatization period two weeks prior to the start of the test. After the acclimatization period, animals were adapted to the treadmill (10 m/min/day) for 5 days for an additional week before the test. The mice started the aerobic physical training protocol after 4 weeks of feeding with control diet or high-fat diet. The test started at a speed of 10 m/min for 5 min, then every 1 min velocity was increased 3 m/min until complete exhaustion of the animal. The test was repeated in the middle of the protocol (at week 4) in order to correct for physiological adaptations. Physical training was carried out on a treadmill for a total of 8 weeks, with 60 min physical exercise sessions for five consecutive days during the week (Mon-Fri 5pm-6pm). The speed used in the training was 50% of the maximum speed reached in the treadmill exercise tolerance test (Speed max= CT: 29.8 ± 3.3 m/min *vs* HT: 30.2 ±2.4) (15).

During the last week of protocol, animals were submitted to a VO2 max test, an exercise tolerance test performed in a calorimetric treadmill. The test started at a speed of 6

m/min, then every 1 min velocity was increased 3m/min. This is different from an exercise tolerance test, as in VO2 max testing, mice were not motivated to run on the treadmill via touching them with a hand. Maximum speed, time to exhaustion, rates of oxygen consumption (VO2) were measured.

Serum Parameters

After 6h of fasting (7am-1pm), the animals were euthanized using the anesthetic isoflurane (5%) and the blood collected by cardiac puncture for biochemical determinations in the serum. Blood was allowed to clot for 30 min at room temperature before centrifuging at 2,000 x g for 10 min. Haemolysis was observed visually, and samples without significant haemolysis had their serum transferred to a new tube. Total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, triglycerides were evaluated by enzymatic assays using specific kits according to the manufacturer's instructions (Bioclin, Minas Gerais, Brazil). Insulin levels were measured with rat/mouse insulin ELISA kit (EZRMI-13K, EMD Millipore).

Glucose tolerance test (GTT), Insulin tolerance test (ITT) and Pyruvate tolerance test (PTT)

For GTT, mice (n=10) received a dose of glucose (2g/kg b.w.) by intraperitoneal injection after a 6 hour fast (7am-1pm). 5µL of caudal capillary blood was used to determine the glucose concentration using glucose kit, at the following times: 0 (pre-injection), 15, 30, 60 and 90 minutes after glucose administration.

For ITT, animals (n=10) received an insulin dose (0.75 UI/kg b.w) by intraperitoneal injection after a 6 hour fast (7am-1pm). The glycemia was then measured and monitored every 4 minutes. Blood glucose level was measured using glucometer (Accu-Chek, Roche, USA) by caudal capillary blood collection at the following times: 0 (pre-injection), 4, 8, 12, 16 and 20 min after insulin administration. In the event of hypoglycemic shock, minimal doses of glucose were administered intraperitoneally and post-glucose injection data were discarded from the analysis.

For PTT, animals (n=10) received a pyruvate dose (2 g/kg b.w) by intraperitoneal injection after a 12 hour fast (8pm-8am). Blood glucose level was measured using glucometer (Accu-Chek) by caudal capillary blood collection at the following times: 0 (pre-

injection), 10, 20, 30, 40,60, 75 and 90 min after pyruvate administration.

Indirect calorimetry

Mice (n=4) were housed individually in the calorimeter chambers at 12:12-h light-dark cycle, 23± 2°C and *ad libitum* access to water and diet. Data was collected using the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, EUA) and analyzed for 60h after an acclimatization period of 12h. Rates of oxygen consumption (VO2), carbon dioxide production (VCO2) and ambulatory locomotor activity were measured for each chamber every 32 minutes. Heat (Kcal/h) and respiratory exchange ratio (RER; RER = VCO2/VO2) were calculated by Oxymax software (Columbus Instruments, Columbus, OH, EUA) to estimate energy expenditure and the relative oxidation of carbohydrate versus fat, respectively.

EV Isolation and Characterization

For exosome isolation, 150µL pre-cleared mouse serum by centrifugation at 2,000xg for 10 min and at 20,000xg for 10 min at 4ºC, to remove apoptotic bodies and large vesicles, was mixed with 30 µL of ExoQuick-TC reagent (System Biosciences, Palo Alto, CA, EUA), incubated overnight at 4ºC and centrifuged at 1,500xg for 30 min. Supernatant was collect and centrifuged again at 1,500 x g for 5 min and the pellet resuspended in 1 x PBS. Random samples (n=3-4) were used to characterize EV (50) by western blot, dynamic light scattering, nanoparticle and transmission electron microscopy, as described below.

Detection of positive EV markers (Cd9, Cd63, Alix and TSG101) and a negative small EV marker (Grp94) was performed by Western blot. 100uL samples of EV diluted in PBS were extracted for total protein in RIPA buffer with protease inhibitors. Total protein was quantified by Bradford Assay (BioRad) and 50ug was resolved by 12% SDS-PAGE gels and transferred to a nitrocellulose membrane (BioRad). Anti-CD9 (cat. #C993, Sigma-Aldrich, 1:500 dilution), anti-CdD63 (cat. 556019 BD Pharmingen, 1:1000), Alix (cat. #2171, CST), Anti-TSG101 (cat. 612697, BD Transduction Laboratories, 1:1000) and Grp94 (cat. #2104, CST, 1:1000) were diluted in TBS (20mM Tris, 150mM NaCl, pH7.5) and membrane was incubated overnight at 4°C. Following primary antibody incubation,

membrane was incubated with HRP-conjugated secondary anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Chemiluminescence was detected by using Clarity™ Western ECL Substrate (Bio-Rad), in a C-Digit imager (LI-COR Biosciences).

Presence of albumin in the EV preparation was evaluated by adding 50μg of EV protein or serum to 10% SDS-PAGE and staining with SilverQuest™ Silver Staining (Thermo Fisher Scientific) following manufacturer's instructions. Bovine serum albumin was used as a positive control.

Polydispersity index (PDI) and size determination of the EVs were performed by dynamic light scattering using Zetasizer Nano ZS90 (Malvern Instruments). EVs were diluted 1/10 in filtered 0.22um saline phosphate buffer and read in the equipment.

Vesicle morphology and size were analyzed after negative staining using transmission electron microscopy. Briefly, isolated EVs were suspended and fixed in 2% glutaraldehyde for 2 h at 4 °C. Following fixation, samples were allowed to dry on top of Formvar-carbon coated grids for 20 min and contrasted with 2% uranyl acetate for 3min. Preparations were examined in a transmission electron microscope Tecnai FEI G20 at 200kV (Thermo ScientificTM, Waltham, MA, EUA).

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV200060) (12). EV metric for C and H were 50% and for CT and HT samples 38%.

EV miRNA profiling

miRNA-seq profiling and bioinformatic analysis were performed by Proteimax NGS Service (Proteimax, SP, Brazil). Briefly, libraries were generated with Clontech SMARTer smRNA kit and qualified libraries were sequenced on an Illumina X ten sequencer using paired end 150 bp sequencing configuration after pooling according to its effective concentration and expected data volume. Differential expression was performed using edgeR software and $|\log2(FC)| \geq 1.00$ and $FDR \leq 0.01$ were set as screening criteria. A heatmap was constructed by plotting on the y-axis the expression fold change (log2) between C and H groups and H and HT groups.

Quantification of mRNA expression and miRNA expression

Total RNA was extracted from serum EVs, liver, epididymal, retroperitoneal and inguinal adipose tissue, gastrocnemius muscle using miRVana RNA Isolation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The concentration and quality of total RNA were measured using a BioDrop µLITE spectrophotometer (BioDrop, Cambridge, UK).

For mRNA expression, the cDNA synthesis was performed using the High Capacity kit (Thermo Fisher Scientific, CA, USA) from 500 ng of total RNA extracted from the liver, gastrocnemius, inguinal, retroperitoneal and epididymal fat pads. All PCR reactions were performed using diluted (1/10) cDNA template, forward and reverse primers (200 nM each) (**Table 1**) and Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). For normalization of expression the constitutive genes *Rpl* or *Hprt1* were used.

For miRNA expression, universal miRNA cDNA was synthesized using TaqMan advanced miRNA cDNA synthesis kit (Thermo Fisher Scientific), following the manufacturer instructions. The following TaqMan advanced assays were used for miRNA quantification: miR-15b-3p, 477929_mir; miR-22-3p, mmu481004_mir; miR-29c-3p, 479229_mir; miR-122-5p, 477855_mir; miR-192-5p, 478262_mir; miR-802-5p, mmu482544_mir.

RT-qPCR was performed on a QuantStudio 3 equipment using universal PCR protocol (Thermo Fisher Scientific). For the quantification of gene expression, the relative quantification $2^{-\Delta\Delta CT}$ method was used (25).

Histological analyses

For light microscopy, inguinal WAT (iWAT) and interscapular brown adipose tissue (iBAT) were fixed in 4% paraformaldehyde (PFA) for 24 hours at 4ºC. After fixation, sections of iWAT, iBAT were incubated in 30% sucrose and embedded in Tissue-Plus Optimal Cutting Temperature (OCT) compound (Fisher HealthCare, Houston, TX, USA). Frozen tissue blocks were cut into 10-µm-thick sections using Leica CM3050 cryostat (Leica Biosystems, Wetzlar, Germany) and stained with HE for histological analysis or used for Ucp1 detection by immunofluorescence. For each tissue, 3 sections per animal (n=5) were stained with HE and 5 random fields per section were photographed at 10x and 20x magnification. iWAT images were analyzed according to (Parlee *et al*., 2014). For Ucp1 detection, BAT sections were stained with anti-Ucp1 antibody at 1:400 dilution

(Abcam ab10983) and donkey anti-rabbit Alexa fluor 488 (Jackson ImmunoResearch).

Five-µm-thick liver sections (3 sections/animal) were paraffin-embedded and stained with HE for histological analysis. Liver histology was evaluated and blind-scored by two independent observers using a non-alcoholic fatty liver disease (NAFLD) scoring system for rodents (23). The NAFLD score is based on a semi-quantitative analysis of steatosis (macrovesicular steatosis, microvesicular steatosis and hypertrophy) and inflammation (number of inflammatory foci/field) where each subcategory of steatosis and inflammation may receive a score ranging from 0 to 3. Thus, steatosis total scores range from 0 to 9 and inflammation, from 0 to 3.

For Oil Red-O (ORO) staining, fragments of liver were fixed with 4% paraformaldehyde overnight and cryoprotected in 20% sucrose. ORO stock solution (350 mg ORO powder dissolved in 100 ml isopropyl alcohol) and working solution (3:2 stock solution: distilled water) were prepared. 10-µm-thick cryosections were air dried onto a slide drying bench, washed with tap water for 3 minutes and then fixed in formalin 10% for 20 minutes. Next, slides were rinsed with 60% isopropanol (10 minutes) and then stained with a filtered ORO solution for 20 minutes. After Oil Red O staining, slides were washed with tap water for 3 minutes, stained with filtered hematoxylin for 5 minutes and washed again with tap water (3 minutes). The ORO positive area of 5 random fields/3 sections/animal (n=5) were quantified using an ImageJ (NIH, Bethesda, MD) plugin for color deconvolution (41).

Four-µm kidney sections paraffin-embedded were stained with periodic acid–Schiff (PAS) and photographed at 400X magnification. From each mouse (n=5), images of 30 randomly selected glomeruli in the outer cortex of each kidney section were evaluated in a blinded manner. The glomerular area was quantified by ImageJ software.

All photographs were obtained using a digital camera (Nikon DMX1200 - Nikon Instruments, Inc. (Melville, USA) coupled to a microscope (Leica DMIRB -Leica Biosystems).

ATP-citrate lyase and malic enzymes activities

Samples of retroperitoneal WAT were rapidly collected, placed in liquid nitrogen and kept as -70ºC until activity analysis of ATP-citrate lyase and malic enzymes. Each sample was subdivided in two aliquots of 50 mg and were separately homogenized (1:5;

w/v) in malic extraction buffer (pH - 7,6; 20 mM Tris-HCL, 1 mM $MgCl_2$, 100 mM KCl and Sucrose 250 mM) and ACLY extraction buffer (pH - 8,0; 20 mM $MgCl_2$, 1 mM EDTA and 100 mM Tris aminomethane). Homogenates were centrifuged at 18.000 G for 2 minutes at 4ºC and the resulting supernatants were used in the following procedures. Malic and ACLY enzyme activities were measured according to the methods described by Newsholme and Williams 1978(30) and Corrigan and Rider 1983 (7, 30), respectively. Tissue protein content was determined by the Bradford method (BioRad).

Statistical analyses

The results presented as mean ± S.D. were analyzed by two-way ANOVA for diet (D), Training (T) and interaction between the two factors. When interaction between the factor (DxT) was significant, Tukey's posttest for pairwise comparison was performed. The power of the test for exercise parameters were calculated for our sample (n=4) and was 0.97. Correlation analyses were performed using the Pearson correlation test. The significance level was set at $p<0.05$.

Results

Diet-induced obesity changes profile of circulating EV microRNAs which is reversed by physical exercise

To explore the role EVs play in the beneficial effects promoted by physical training, we used a diet-induced obesity mouse model, which was subjected to an 8-week protocol of aerobic training. To address if aerobic endurance was established successfully, at the last week of exercise protocol mice from sedentary and trained groups were submitted to a VO_2 max test (exercise tolerance test in a calorimetric treadmill). Maximum oxygen uptake (VO2 peak) was higher in trained control mice when compared to control group and, more importantly, trained obese mice had a higher VO2 peak when compared to obese group (**Figure 1J).** Trained obese mice obtained a higher maximum speed, consequently increasing the amount of time to achieve the exhaustion when compared to obese mice (**Figure 1K and 1L).** Altogether, these data suggest that in obese mice the exercise protocol promoted an adaptation which resulted in better exercise tolerance test

performance. This is directly related to benefits of physical exercise in the treatment of obesity and obesity-related diseases described herein.

Obesity features and metabolic parameters are described in **Table 2**. Obese mice had greater weight gain compared to control mice, a reflex of increased fat mass as indicated by increased visceral (retroperitoneal, epididymal and mesenteric fat) and subcutaneous (inguinal fat) WAT and iBAT in obese mice (**Table 2**). After only 2 weeks of physical training, increased weight gain induced by HFD was stopped, and fat pads weights were also lower in HT group (**Figure 1A, Table 2**). Increased fat mass in mice of H group resulted in increased fasting insulinemia and glycemia (**Table 2**), glucose intolerance and insulin resistance measured by GTT and ITT, respectively, as indicated by increased AUC after GTT and reduced K_{ITT} during ITT (**Table 2**, **Figure 1B and 1C**). Obese mice have also shown increased blood glucose rise, as measured by PTT, consistent with increased gluconeogenesis (**Table 2**, **Figure 1D**). Physical training lowered plasma glucose levels, likely as a result of increased glucose uptake by the cells and inhibition of gluconeogenesis. During GTT, trained animals (HT group) had a similar AUC compared to control group (**Table 2**) and a reduction in K_{ITT} during ITT, confirming better insulin sensitivity compared to untrained obese animals (**Table 2**). Corroborating with reduced gluconeogenesis, the HT group also had decreased blood glucose rise after pyruvate injection, resulting in a lower AUC compared to H group (**Table 2**).

No differences were found in kidney weight, triglycerides and total cholesterol contents and kidney morphology, as indicated by measurements of glomerular area (**Table 2**).

One week prior to harvest, indirect calorimetry was performed in trained mice fed a control or a HFD (**Figure 1E-H**). RER indicated that fat was the main fuel source in animals fed a HFD (**Figure 1F**, 0.93 *vs* 0.78, $p<0.0001$). Energy expenditure was higher in mice fed an obesogenic diet compared to those fed a control diet (**Figure 1H**). Locomotor activity was also recorded, and an obesogenic diet reduced locomotor activity of mice. Training had no effect, even though training *per se* increases ambulatory locomotor activity of control diet-fed mice (**Figure 1I**).

EVs were isolated from equal amounts of serum from control and obese mice - both sedentary and trained. EV total protein estimation by Bradford revealed the amount of EV isolated from serum of the different groups was not different (C:20605 ± 3625 μg/mL; H:

224425 ± 3046 µg/mL; CT: 21904 ± 2573 µg/mL; HT: 22815 ± 2879 µg/mL; p>0.05). First, circulating EVs were characterized by light scattering (**Figure 2A**), transmission electronic microscopy (**Figure 2B**), western blot with EV markers (**Figure 2C-D**) and albumin detection by SDS-PAGE for purity (**Figure 2D, top**). As expected, the EVs measurements range from 30 to 150 nm in diameter and were positive to Cd9, Cd63, Alix, TSG101 and negative to Grp94 (large EVs marker) (**Figures 2C-D**). Albumin was also detected in our preparation, as previously shown for precipitation-based methods (22, 47) (**Figure 2D**).

Next, EVs obtained from the pooled serum of 5 control, obese and obese trained mice were profiled for microRNA expression using miRNA-seq. Among the miRNAs increased in obesity and reestablished by aerobic training, we identified eleven (**Figure 2E and 2F**). After filtering for conserved miRNAs between mouse and humans, 6 miRNAs remained (miR-15b, miR-22, miR-29c, miR-122, miR-192 and miR-802) that were validated by RT-qPCR in individual samples from another cohort of control and obese mice, both sedentary and trained. We found miR-22, miR-122 and miR-192 were increased in circulating EVs of obese mice, and their expression returned to control levels after aerobic training (**Figure 2G**). Circulating exosomal miR-22 and miR-122 positively correlated with insulin levels (miR-22 vs Insulin r=0.45, p=0.0143; miR-122 vs Insulin r=0.47, p=0.0108) (**Figure 2I**).

Next, we measured miR-22, miR-122 and miR-192 expression in epididymal WAT (epiWAT), liver and gastrocnemius muscle (**Figure 2H**) because tissue expression of these miRNAs has been shown a role in glucose intolerance and dyslipidemia (6, 9). No correlation was found between EV and tissue miRNAs levels when all samples were included in the analysis. However, when we isolated the effect of obesity, EV miR-22 positively correlates with epiWAT miR-22 levels (r=0.75, p=0.0103). Interestingly, when only trained animals independent of diet were added into the analysis was a correlation observed between EV and gastrocnemius muscle for miR-192 (EV vs gastro r=0.65, p=0.049) and for miR-122 (r=-0.72, p=0.0369). In addition, miR-192 gastro is negatively correlated with fat mass (r=-0.71, p= 0.0268) in an exercise context.

There was a positive correlation between expression of miR-122, miR-22 and miR-192 (**Figure 2I**). miR-22 was not affected by diet or training but miR-122 expression was increased by training in obese mice compared to sedentary obese and miR-192 was

decreased by diet *per se* (**Figure 2 H**). In epiWAT, miR-22 was increased in obese mice and its expression reversed by aerobic training. miR-122 was decreased in obese mice and training had no effect and miR-192 was increased by training *per se* (**Figure 2H**). Expression of miR-22 was positively correlated with insulin levels and fat mass, and negatively correlated with liver miR-22 expression (**Figure 2I**). In gastrocnemius muscle, all three microRNAs were positive correlated and only miR-22 expression was reduced by diet (**Figure 2H and I**). Gastrocnemius miR-122 expression was negative correlated with fat mass and liver miR-122 expression.

These data indicate circulating miRNA patterns are reflected by miRNA expression at tissue level in obesity; lifestyle strategies such as physical exercise can regulate miRNA expression to promote its beneficial action. Moreover, EV miRNAs might be mediating communication occurring between adipose tissue, liver and gastrocnemius muscle.

Improvements in liver steatosis promoted by physical exercise correlates with miRNAs expression

NAFLD activity score was calculated as the sum of steatosis and inflammation scores, two key features of steatohepatitis, obtained from H&E staining analysis (**Figures 3A and 3B**). Mice fed a high-fat diet supplemented with condensed milk had increased ectopic triglycerides (TG) accumulation oil red staining **(Figure 3C**) accompanied by increased inflammatory foci (**Figure 3A and 3B**). Macro and microvesicular steatosis and hepatocellular hypertrophy were features of the steatosis induced by this kind of diet (**Figure 3B**). A positive correlation was observed between TG content and steatosis score in the liver of these animals (r=0.71; p=0.0031, **Figure 3B**). Physical exercise was able to reverse the accumulation of lipids (**Figures 3A and 4A**), mainly macro and microvesicular steatosis, and inflammation promoted by the high-fat diet supplemented with condensed milk (**Figures 3A)**.

Liver expression of miR-22, miR-122 and miR-192 were all negatively correlated with steatosis score (miR-22: r= -0.47, p= 0.0254; miR-122: r= -0.63, p= 0.0017; miR-192: r= -0.45, p= 0.0367). Targets of these microRNAs such as *Ppara*, *Pparg, Pgc1a* and *Fasn* were measured in mouse liver. Obesity increased *Pparg* expression in the liver which was decreased by 2.2-fold after aerobic training of obese mice. No difference was observed in gene expression of *Pgc1a. Ppara* expression was increased was reduced by high-fat diet

(**Figure 3D**). *Fasn* expression was reduced by aerobic training, suggesting that *de novo* lipogenesis is decreased (**Figure 3D**). Increased *Pparg* expression was correlated with liver steatosis ($r= 0.84$, $p < 0.0001$) and miR-122 expression ($r = -0.61$, $p=0.0149$) (**Figure 3E**). Liver expression of pro-inflammatory cytokines *Tnfa* was increased by high-fat diet (**Figure 3D**). *Il-6* was positively correlated with steatosis score ($r=0.65$, $p=0.0078$) but not with inflammation score ($r=-0.27$, $p=0.29$) (**Figure 3E**). Training *per se* decreased the inflammatory markers *Il-6* and *Tnfa* expression (**Figure 3D**).

miR-22 and WAT effects promoted by physical training

In H group, H&E staining shows that adipocyte cross sectional area is larger than in C group, indicating that the diet promoted fat cell hypertrophy in inguinal adipose tissue, and aerobic training attenuated the hypertrophy of the adipocytes (**Figure 4A and 4B**). Interestingly, we observed a higher proportion of smaller adipocytes (adipocyte area <600 μm^2) in HT group (HT: 27% *vs* H: 4%) whereas H group has a higher proportion of larger adipocytes (>1000 μm^2) (HT: 49% vs H:73%) (**Figure 4C**). Enzymatic activity of ATP-citrate lyase and malic enzymes activities in rWAT suggests that physical exercise induced lipogenesis, as aerobic exercise increased their activity. This data accompanied by the higher proportion of smaller adipocytes in CT and HT groups suggests that training may have induced adipogenesis (**Figure 4D**). In addition, *Atgl* expression, a lipolysis marker, was decreased by diet and increased by training in all three adipose tissue depots, suggesting stimulation of lipolysis in adipose tissue (**Figure 4F**), which contributes to fat mass reduction.

miR-22 has been shown to promote adipogenesis, and its levels decreases during adipocyte differentiation (16). Corroborating with the increased levels of miR-22 in epiWAT of obese mice, the expression of key markers genes of adipocyte differentiation *Pparg*, *Cebpa, Fabp4* and *Fasn* was decreased in H group suggesting in obese state hypertrophy, but not hyperplasia, of fat cells is predominant (**Figure 4E**). *Cebpa* and *Pparg* expression were increased by training, suggesting differentiation was restored in WAT (**Figure 4E**).

The presence of smaller adipocytes in HT group compared to H group (**Figure 4C**), suggests insulin sensitivity is improved in adipose tissue (20). We measured *Glut4* and *Irs1* expression in WAT as indicators of insulin sensitivity and found a decreased expression of

Glut4 in all three fat depots evaluated, Additionally, decreased expression of *Irs1* in epiWAT and rWAT depots occurred, indicating those mice are insulin resistant. This effect was reversed by aerobic training of obese mice (**Figure 4E**).

Finally, we performed a correlation between miRNAs and mRNA levels in epididymal adipose tissue to further show the relationship between miRs and its targets. As hypothesized, miR-22 negatively correlates with adipogenesis, insulin sensitivity and fatty acid oxidation (*Ppara* and *Pgc1a)* markers (**Figure 4G**). miR-122 positively correlated with lipogenesis markers (**Figure 4G**) and miR-192 positively correlated with *Ppara* and *Pgc1a* (**Figure 4G**).

Regarding BAT, a high-fat diet promoted fat cell hypertrophy in BAT, a phenomenon known as whitening (**Figure 5A**). Aerobic exercise was able to reverse this whitening process in HT group (**Figure 5A**). Corroborating with H&E staining, UCP1 positive area was decreased in H group compared to C group (C: 43.37 ± 0.67% vs H: 32.00 ± 1.21%, $p<0.0001$) and training reverted this effect (H: 32.00 ± 1.21% vs HT: 52.84 ± 1.06%, $p<0.0001$) (**Figures 5B**). In addition, lipid droplet size was altered by diet and aerobic exercise (**Figure 5B**).

We also assessed the effect of physical exercise on genes related to lipid and glucose metabolism in gastrocnemius muscle of obese mice. We found *Glut4* and *Irs1* expression was both significantly decreased in obese mice gastrocnemius muscle and training increased *Glut4* and *Irs1* expression in obese mice, suggesting an increase in insulin sensitivity (**Figure 5C**).

Discussion

The majority of circulating exosomal microRNAs are derived from adipose tissue and can travel to the liver, resulting in functional changes in the recipient cells (51). Further, alterations in fat mass and function, such as those seen in obesity, may affect miRNA content in EVs, as recently showed by Castaño *et al*., 2018 (6).

Aerobic exercise is a nonpharmacological approach to treat obesity and obesity-related diseases, and many studies using different exercise protocols have shown physical exercise can stabilize weight gain and reverse insulin resistance and glucose intolerance (5, 21, 52). Exercise induces the release of EVs into the circulation, which may be one of the mechanisms by which skeletal muscle promotes organ crosstalk communication (42, 57).

Although it is plausible to implicate the skeletal muscle as a source of circulating miRNAs after exercise, D'Souza *et al*., 2018 (10) have shown circulatory exosomal miRNAs upregulated after acute high-intensity interval exercise were unrelated to muscle-observed miRNA changes in humans.

The lack of related changes between muscle and EVs may be attributable to non-muscle tissue in the turnover of miRNAs. Our research provides new mechanistic insights on the effect of aerobic training in the treatment of obesity. We demonstrated that miR-22 possibly reflects adipose tissue expression in obese subjects, however other tissues contribute for the pool of circulating miRNAs, as EV miR-122 and miR-192 levels were not correlated with liver and muscle levels. Physical training regulates circulatory EV miRNAs (such as miR-22, miR-122 and miR-192) that were related to the gastrocnemius, liver and adipose tissue miRNA changes. Moreover, regulation of mainly miR-22 seems to counteract the hypertrophy of adipocytes and insulin resistance in adipose tissue promoted by obesity. Interestingly, a crosstalk between liver and gastrocnemius was found for miR-122, which is reduced in obesity and upregulated in a physical exercise context.

Although studies have shown the majority of miRNAs present in serum are contained within exosomes, it is not currently possible to separate and characterize each of the EVs and have a pure exosome fraction (50). Furthermore, we have limited knowledge on which mechanisms drive and regulate RNA incorporation into EV, so possibly, circulating miRNAs may be derived from microvesicles or may be associated to Argonaute or lipoproteins (27). The origin of circulating EV miRNAs is beyond this study and remains to be determined. Herein, by measuring circulatory EV miRNAs, we provide more evidence to literature that the circulatory miRNAs that are dysregulated in obesity probably are liver/adipose tissue-derived as previously shown for miR-22, miR-122 and miR-192 (18, 24, 51, 55). In addition, we demonstrated that physical training regulates circulatory EV miRNAs.

Plasma miR-122 and miR-192 levels have been previously correlated with visceral fat mass in humans as well as TG/HDL ratio (45) and positively correlated with severity of NAFLD (34). Importantly, treatment of lean mice with exosome enriched in miR-122 and miR-192 caused a significant enlargement of the epididymal adipose depot, glucose intolerance and hepatic steatosis (6). Accordingly, we have shown miR-122 and miR-192 are increased in serum EVs in obese mice, and these mice develop the features of NAFLD.

Aerobic training of obese mice increased miR-122, improved hepatic steatosis and importantly were negatively correlated with *Pparg* levels. Increased circulating miR-122 and miR-192 is correlated with a decrease in liver levels in a model of hepatoxicity induced by acetaminophen (55) or induced by high-fat diet (6). We also observed a reduced expression of miR-192 in mice fed a high-fat diet.

Regarding miR-22, some reports have shown a role of miR-22 in adipogenesis (16), gluconeogenesis (19) and dyslipidemia (9). In this study, we found miR-22 in increased in circulating EVs of obese mice and aerobic training reduces its levels to control levels. miR-22 expression in epididymal white adipose tissue correlated with serum levels and suggests that adipose tissue is the main source of circulating miR-22 in obesity. Interestingly, miR-22 correlates negatively with liver miR-22 levels, suggesting a crosstalk between these organs.

In our study a negative correlation was found between fat mass and circulating/epididymal fat miR-22 levels. Supporting a role of miR-22 in fat mass increase, the loss of miR-22 in mice attenuates the gain of fat mass and dyslipidemia induced by high-fat diet (9). The mechanism by which miR-22 decreases fat mass was not evident within this study, but miR-22 has a role of miR-22 in adipogenesis.

Previous *in vitro* findings have shown miR-22 impairs adipogenesis (16). Herein, a negative correlation between miR-22 and adipogenesis markers was described, suggesting *in vivo*, miR-22 also impairs adipogenesis. In the adipose tissue of obese trained mice compared to sedentary mice, we reported reduced miR-22 levels, increased number of small adipocytes and reduced number of larger adipocytes, increased expression of adipogenesis markers and increased activity of lipogenesis enzymes. This effect was also observed in trained control diet fed mice, demonstrating that exercise *per se* is a positive regulator of adipogenesis, which includes morphological changes, cessation of cell growth, expression of many lipogenic enzymes, extensive lipid accumulation, and the establishment of sensitivity to most or all of the key hormones that impact on this cell type, including insulin (39).

It is well established that in contrast with smaller adipocytes that are associated with insulin sensitivity, larger adipocytes are associated with insulin resistance and hyperglycemia (11, 20). Accordingly, in this study improvement in adipocyte hypertrophy was also associated with increased insulin sensitivity and may have contributed to

peripheral and skeletal muscle reversal of insulin resistance. Corroborating with this hypothesis of increased adipogenesis, (49) it has been demonstrated that subcutaneous adipose tissue has an important role in glucose homeostasis. They transplanted subcutaneous adipose tissue from trained mice in obese mice and observed improvements in glucose tolerance and insulin sensitivity, and increased glucose uptake in skeletal muscle.

Brown fat is classically known as a thermogenic tissue that, through non-shivering thermogenesis, participates in the regulation of body temperature. Although brown fat is increased in the obese group, this does not imply a healthy process of expansion of this tissue; the phenomenon known as browning, and implies just the opposite. High-fat diets promote the expansion of this tissue associated with the whitening process, in which the tissue becomes more characteristic of white adipose tissue, thus losing its main function of thermogenesis, and is also responsible for maintaining body temperature (38, 43). Corroborating with these results, our data shows the brown fat of obese mice has phenotypic characteristics of a white fat and exercise in the obese exercised group prevented this whitening process.

A potential limitation of this study is the small sample size used in the estimation of correlation coefficients, so our data needs to be interpreted cautiously.

In summary, physical exercise modulates circulatory exosomal miRNAs in obesity. Particularly in adipose tissue, aerobic training regulates miR-22 to counteracts effect of obesity such as adipocyte hypertrophy and insulin resistance. Our data provide significant evidence demonstrating that aerobic training exercise-induced exosomes mediate effect of exercise on adipose tissue metabolism. Other microRNAs are possibly involved in these effects and may contribute for the overall effect of aerobic training and should be further investigated.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

Author contributions

Conceived and designed the research: AR, MM. Acquired, analyzed or interpreted data: MM, AR, KR ES, BP, LMO. Wrote the manuscript: MM and AR. Final revision: MM, AR, KR ES, BP, LMO.

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Figure Legends

Figure 1: Effect of physical exercise energy metabolism and locomotor activity of C57BL/6J mice fed for 12 weeks with control or high-fat diet with condensed milk. (A) Time-course of weight gain; **(B)** Glucose tolerance test curve; **(C)** Insulin tolerance test Curve; **(D)** Pyruvate tolerance test curve. **(E)** VO2; **(F)** VCO2; **(G)** Respiratory exchange ratio (RER); **(H)** Heat; **(I)** locomotor activity; (**J-L**) VO_2 max test: VO2 peak (**J**), speed max (**K**) and time to exhaustion (**L**). For VO_2 max test, exercise tolerance test was performed in a calorimetric treadmill, thus mice could not be motivated to run on the treadmill via touching them with a hand, for instance. Testing then ended when mice meet the criterion for exhaustion, i.e. spending five continuous seconds laying on top of the shock grid. Results are presented as mean ± SD. *$p<0.05$ as analyzed by Two-Way ANOVA for effect of diet (D), training (T) or interaction DxT. When interaction was significant, Tukey's posttest was applied and superscript letters were used to shown statistically significant differences between the groups; $^{a, b}$ $p<0.05$: (a) vs C; (b) vs. H. From A-D: n± 10 mice; From E-I and J-L: n=4 mice. C: mice fed a control diet; H: mice fed a high-fat diet with condensed milk; CT: C trained; HT: H trained.

Figure 2: Circulating exosomal microRNAs profile after aerobic training of obese mice. (**A-D**) Characterization of EVs: Size distribution by dynamic light Scattering (**A**); Representative image of transmission electron microscopy (50,000X) (**B**); Western blot for Cd9, Cd63, TSG1 and Ali (**C**); Albumin detected by SDS-PAGE (top); Western blot for Grp94, large EVs marker (bottom) (**D**). (**E-G**) Profiling of miRNAs from serum EVs: heatmap of log2(FC) of known differentially expressed miRNAs between C and H and H and HT. In red are upregulated miRNAs and green downregulated miRNAs (**E**); Venn diagram indicates the number of differentially expressed known microRNAs in common between C *vs* H and H *vs* HT (**F**); To validated miRNAs differentially expressed in pooled samples, RT-qPCR of individual samples of serum EVs was performed (n=8) (**G**). (**H**) miRNA expression in the liver, epididymal adipose tissue (epiWAT) and gastrocnemius muscle (gastro) performed by RT-qPCR (n=13-14). (**I**) Significant Pearson correlation for miRNAs miR-22, miR-122, miR-192. (**G and H**) Results are presented as mean ± SD. *$p<0.05$ as analyzed by Two-Way ANOVA for effect of diet (D), training (T) or interaction DxT. When interaction was significant, Tukey's posttest was applied and superscript letters were used to shown statistically significant differences between the groups; $^{a, b}$ $p<0.05$: (a)

915 vs C; (b) vs. H. C: mice fed a control diet; H: mice fed a high-fat diet with condensed milk;
916 CT: C trained; HT: H trained.
917
918 **Figure 3: Effect of exercise on liver of C57BL/6J mice fed a control or high-fat diet**
919 **with condensed milk for 12 weeks. (A)** Representative images of liver sections after
920 H&E staining; **(B)** Macrovesicular steatosis, microvesicular steatosis and hepatocytes
921 hypertrophy, inflammation and NAFLD activity scores and correlation between steatosis
922 score and triglyceride content. (**C**) Representative images of liver section after oil red O
923 staining and quantification of the images (n=5); **(D)** Relative expression of liver mRNA
924 expression of genes related to lipid homeostasis and inflammation (n= 5-7); (**E**)
925 Statistically significant Pearson correlation. Results are presented as mean ± SD. *p<0.05
926 as analyzed by Two-Way ANOVA for effect of diet (D), training (T) or interaction DxT.
927 When interaction was significant, Tukey's posttest was applied and superscript letters were
928 used to shown statistically significant differences between the groups; [a, b] p <0.05: (a) vs C;
929 (b) vs. H; (n ± 6 animals). C: fed a control diet; H: fed a high-fat diet supplemented with
930 condensed milk; CT: C trained; HT: H trained.
931
932 **Figure 4: Effect of exercise on white adipose tissue of C57BL/6J mice fed for 12 weeks**
933 **with control or high-fat diet supplemented with condensed milk.** (**A**) Representative
934 images of inguinal adipose tissue sections after H&E staining; (**B**) Average adipocyte cross
935 sectional area; (**C**) Frequency of distribution of areas of adipocyte section; (**D**) Activity of
936 lipogenesis enzymes; (**E**) Relative expression of mRNA in inguinal (iWAT),
937 retroperitoneal (rWAT) and epididymal (epiWAT) white adipose tissues; (**F**) Statistically
938 significant Pearson correlation. Results are presented as mean ± SD. *p<0.05 as analyzed
939 by Two-Way ANOVA for effect of diet (D), training (T) or interaction DxT. When
940 interaction was significant, Tukey's posttest was applied and superscript letters were used
941 to shown statistically significant differences between the groups; [a, b] p <0.05: (a) vs C; (b)
942 vs. H; (n ± 6 animals). C: fed a control diet; H: fed a high-fat diet supplemented with
943 condensed milk; CT: C trained; HT: H trained.
944
945 **Figure 5: Aerobic training reverses whitening of brown adipose tissue and insulin**
946 **resistance of skeletal muscle in obese mice.** (**A**) Representative images of brown adipose

947 tissue (BAT) sections after H&E staining and (**B**) Representative images of Ucp1
948 immunofluorescence. Lipid droplet size and UCP1 positive (UCP1+) area for BAT sections
949 were calculated and are represented. DAPI we stained in blue. **(C)** Relative mRNA
950 expression in soleus muscle of genes related to glucose and lipid metabolism. Results are
951 presented as mean ± SD. *$p<0.05$ as analyzed by Two-Way ANOVA for effect of diet (D),
952 training (T) or interaction DxT. When interaction was significant, Tukey's posttest was
953 applied and superscript letters were used to shown statistically significant differences
954 between the groups; $^{a, b}p<0.05$: (a) vs C; (b) vs. H (n ± 6 animals).
955

Table 1: Sequences of primers used to quantify mRNA expression by RT-qPCR

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
36b4	TAAAGACTGGAGACAAGGTG	GTGTACTCAGTCTCCACAGA
Acc	CCAGCAGATTGCCAACATC	ACTTCGGTACCTCTGCACCA
Atgl	GGTCCTCTGCATCCCTCCTT	AGACATTGGCCTGGATGAGC
Cebpa	TAGGTTTCTGGGCTTTGTGG	TAGGTTTCTGGGCTTTGTGG
Fabp3	ATGACCGGAAGGTCAAGTCAC	CCCGTTCCACTTCTGCACAT
Fabp4	TGAAATCACCGCAGACGACA	ACACATTCCACCACCAGCTT
Fasn	GATTCGGTGTATCCTGCTGTC	CATGCTTTAGCACCTGCTGT
Glut4	CATTCCCTGGTTCATTGTGG	GAAGACGTAAGGACCCATAGC
Hprt1	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Il6	TGATGCACTTGCAGAAAACA	ACCAGAGGAAATTTTCAATAGGC
Irs1	CCCTCTCAACAGCAGTCCCT	TTACGCTATTGACGATCCTC
Pgc1a	CACCAAACCCACAGAAAACAG	GGGTCAGAGGAAGAGATAAAGTTG
Ppara	TCGAATATGTGGGGACAAGG	TCTTGCAGCTCCGATCACAC
Pparg	CAAACCTGATGGCATTGTGAG	ATCTTAACTGCCGGATCCAC
Tnfa	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT

Table 2: Obesity features and metabolic parameters from C57BL/6J mice in control diet (C); high-fat diet supplemented with condensed milk (H); control diet + physical training (CT) and high-fat diet supplemented with condensed milk + physical training (HT).

	C (10)	H (10)	CT (9)	HT (9)	D	T	DxT
Initial BW (g)	21.2 ± 2.0	21.3 ± 2.4	21.8 ± 2 .0	20.4 ± 1.5	ns	ns	ns
Final BW (g)	29.3 ± 1.6	**39.0 ± 4.2**[a]	26.9 ± 2.6	**30.1 ± 2.7**[b]			**0.0027**
BW gain (g)	8.3 ± 2.0	**17.2 ± 34**[a]	**5.1 ± 1.6**[a]	**9.9 ± 25**[b]			**0.0172**
Energy Intake (Kcal/day/animal)	10.3 ± 0.4	15.1 ± 1.5	9.8 ± 0.8	13.0 ± 1.2	ns	ns	ns
Total Cholesterol (mg/dL)	162 ± 29	**419 ± 96**	167 ± 74	**363 ± 71**	**<0.0001**	ns	ns
Triglycerides (mg/dL)	82 ± 22	88 ± 15	97 ± 23	88 ± 21	ns	ns	ns
HDL-C (mg/dL)	82 ± 14	**112 ± 10**[a]	59 ± 14	**115 ± 12**[a]			**0.0365**
LDL-C (mg/dL)	63 ± 19	**268 ± 85**	89 ± 87	**230 ± 72**	**0.0002**	ns	ns
Inguinal fat pad (g)	0.76 ± 0.31	**1.73 ± 0.60**[a]	0.69 ± 0.24	**0.95 ± 0.41**[b]			**0.0144**
Retroperitoneal fat pad (g)	0.32 ± 0.12	**0.70 ± 0.22**[a]	0.29 ± 0.09	**0.40 ± 0.12**[b]			**0.0088**
Epididymal fat pad (g)	0.87 ± 0.25	**1.89 ± 0.22**[a]	0.80 ± 0.17	**1.16 ± 0.48**[b]			**0.0019**
Mesenteric fat pad (g)	0.44 ± 0.11	**1.30 ± 0.40**[a]	0.36 ± 0.10	**0.46 ± 0.21**[b]			**0.0009**
Brown adipose tissue (g)	0.10 ± 0.02	**0.15 ± 0.05**[a]	0.10 ± 0.02	**0.11 ± 0.01**[b]			**0.0197**
Liver weight (g)	1.20 ± 0.14	**1.57 ± 0.39**[a]	1.03 ± 0.09	**0.99 ± 0.12**[b]			**0.0099**
Liver total cholesterol (mg/g.mg^{-1})	5 ± 3	6 ± 2	5 ± 1	7 ± 1	ns	**0.0140**	ns
Liver Triglycerides (mg/g.mg^{-1})	11 ± 2	**15 ± 2**[a]	**16 ± 2**[a]	15 ± 3			**0.0141**
Gastrocnemius weight(g)	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	**0.12 ± 0.01**[a.b]			**0.0006**
Kidney weight (g)	0.31 ± 0.02	0.33 ± 0.01	**0.28 ± 0.02**	**0.28 ± 0.03**	ns	**0.0001**	ns

Kidney total cholesterol (mg/g.mg^{-1})	2.4 ± 0.2	2.8 ± 0.4	2.7 ± 0.5	2.7 ± 0.4	ns	ns	ns
Kidney Triglycerides (mg/g.mg^{-1})	2.1 ± 0.9	3.2 ± 2.0	2.5 ± 1.0	2.8 ± 0.8	ns	ns	ns
Glomerular área (µm^2)	1164 ± 109	1159 ± 137	**942 ± 84**	**910 ± 47**	ns	**0.0004**	ns
Fasting Insulin levels (ng/mL)	4.02 ± 1.48	**10.77 ± 3.53**[a]	3.06 ± 0.46	**4.87 ± 1.36**[b]			**0.0077**
Fasting Serum Glucose (mg/dl)	115 ± 15	**210 ± 50** [a]	123 ± 30	**125 ± 11** [b]			**0.0027**
AUC GTT (mg/dL.min^{-1})	1107 ± 200	1841 ± 213	975 ± 127	1209 ± 110			**0.0043**
AUC PTT (mg/dL.min^{-1})	9955 ± 429	**15118 ± 1679**[a]	12448 ± 496	**11786 ± 772**[b]			**0.0313**
K_{ITT} (%/min)	6.6 ± 1.7	1.8 ± 1.5	6.5 ± 1.6	5.7 ± 1.4			**0.0025**

Note: In parenthesis number of mice used. ns= not statistically significant, p>0.05. BW= body weight; AUC= area under curve; K_{ITT}= constant of glucose disappearance. Results are presented as mean ± SD. Data was analyzed by Two-Way ANOVA for effect of diet (D), training (T) or interaction DxT. When interaction was significant, Tukey's posttest was applied and superscript letters were used to shown statistically significant differences between the groups; [a, b] p <0.05: (a) vs C; (b) vs. H. C: fed a control diet; H: fed a high-fat diet supplemented with condensed milk; CT: C trained; HT: H trained