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Exercise alters liver mitochondria phospholipidomic profile and mitochondrial activity in non-alcoholic steatohepatitis

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ABSTRACT

Mitochondrial membrane lipid composition is a critical factor in non-alcoholic steatohepatitis (NASH). Exercise is the most prescribed therapeutic strategy against NASH and a potential modulator of lipid membrane. Thus, we aimed to analyze whether physical exercise exerted preventive (voluntary physical activity-VPA) and therapeutic (endurance training-ET) effect on NASH-induced mitochondrial membrane changes. Sprague-Dawley rats (n=36) were divided into standard-diet sedentary (SS, n=12), standard-diet VPA (SVPA, n=6), high-fat diet sedentary (HS, n=12) and high-fat diet VPA (HVPA, n=6). After 9 weeks of diet-specific feeding, half of SS and HS group were engaged in an ET program for 8 weeks/5d/week/1h/day (SET, HET). Liver mitochondria were isolated for oxygen consumption and transmembrane-electric potential (ΔΨ) assays. Mitochondrial phospholipid classes and fatty acids were quantified through thin layer chromatography and gas chromatography, respectively, while cardiolipin (CL), phosphatidylcholine (PC) phosphatidylethanolamine (PE) and phosphatidylinositol (PI) molecular profile was determined by electrospray mass spectrometry. In parallel with histological signs of NASH, high-fat diet decreased PI, CL and PC/PE ratio, whereas PE and phosphatidic acid content increased in sedentary animals (HS vs. SS). Moreover, a decrease in linolelaidic, monounsaturated fatty acids content and an increase in saturated fatty acids (SFAS) were observed. Along with phospholipidomic alterations, HS animals showed a decrease in respiratory control ratio (RCR), ΔΨ and FCCP-induced uncoupling respiration (HS vs. SS). Both phospholipidomic (PC/PE, SFAS) and mitochondrial respiratory alterations were counteracted by exercise interventions. Exercise used as preventive (VPA) or therapeutic (ET) strategies preserved liver

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mitochondrial phospholipidomic profile and maintained mitochondrial function in a model of NASH.

Keywords: physical activity; membrane; phospholipids; fatty acids; NAFLD.

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1. INTRODUCTION

Western lifestyle, characterized by fat-rich diets and physical inactivity, resulted in a growing incidence of metabolic disorders such as obesity, diabetes and non-alcoholic fatty liver disease (NAFLD). In NAFLD spectrum, NASH is recognized as the most dangerous disease-stage since it is associated with increased risk of cirrhosis development, and consequent liver-associated morbidity and mortality (Matteoni et al. , 1999). Although the pathogenesis of NASH is not completely understood, mitochondrial alterations are crucial for the development and progression of the disease (Grattagliano et al. , 2012). Recently, some authors suggested that the perturbation of mitochondrial membrane lipid homeostasis influences mitochondrial function and induces liver damage (Aoun et al. , 2012a, Aoun et al. , 2012b, Arendt et al. , 2013, Li et al. , 2006). In fact, lipid-homeostasis has an essential role on mitochondrial physiological status due to its involvement in membrane structure and fluidity, cellular energy production and signaling (Modi and Katyare, 2009, Patil and Greenberg, 2013, Shi et al. , 2013). Among phospholipid classes, cardiolipin (CL) is the "signature phospholipid" of mitochondria with a central role in organelle bioenergetics (Claypool and Koehler, 2012), whereas phosphatidylcholine (PC) and phosphatidylethanolamine (PE) amounts regulate membrane integrity (Li, Agellon, 2006). Moreover, both CL and PE play a crucial role in mitochondrial morphology maintenance (Joshi et al. , 2012, Tasseva et al. , 2013). Aoun et al. (Aoun, Fouret, 2012b), observed that rats fed a fish oil-rich diet containing 30% of fat have reduced liver CL content and increased PE content with a consequent decrease of mitochondrial complex I activity. These results provide a novel insight regarding NASH-related mechanisms and therapeutic approaches. Actually, since no approved drugs exist for NASH treatment, lifestyle modifications are still the cornerstone

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strategy. Therefore, physical activity has been considered as an important component of lifestyle interventions, with promising systemic (Promrat et al. , 2010), hepatic (Kistler et al. , 2011) and mitochondrial beneficial effects (Ascensao et al. , 2013, Goncalves et al. , 2013). In fact, voluntary exercise or high aerobic capacity improve hepatic mitochondrial function at multiple levels, including the increase of cytochrome c content, increased fatty acid oxidation and mitochondrial enzyme activities, therefore preventing liver steatosis progression (Rector et al. , 2008, Rector et al. , 2011, Thyfault et al. , 2009). Moreover, Kalofoutis (Kalofoutis, 1984) demonstrated that endurance training (ET) modulates liver mitochondrial phospholipid profile decreasing PE and increasing PC content. However, to the best of our knowledge, data regarding the effects of exercise on liver mitochondrial membrane characteristics in a NASH phenotype are so far unknown.

Therefore, our hypothesis for the present work is that both ET and voluntary physical activity (VPA) used as therapeutic and preventive approaches, respectively, decrease liver mitochondria phospholipidomic alterations induced by high-fat diet model of NASH, thus contributing to the maintenance of mitochondrial function.

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2. METHODS

2.1. Animal and diet treatment

The local Institutional Ethics Committee approved this study, which was in compliance with the Guidelines for Care and Use of Laboratory Animals in research advised by the Federation of European Laboratory Animal Science Associations (FELASA). Male Sprague-Dawley rats (n=36) with 5-6 weeks old (125-150g) purchased from Charles River (L´Arbresele, France), were housed in individual cages under controlled environmental conditions (21-22 ºC; 50-60% humidity, with 12light/dark cycles) and free access to water and food. Animals were then randomly assigned into six groups (n=6/group): standard-diet sedentary (SS), standard-diet voluntary physical activity (SVPA), standard-diet endurance-trained (SET), high-fat diet sedentary (HS), high-fat diet voluntary physical activity (HVPA) and high-fat diet endurance-trained (HET). As diets were liquid, in the first week of feeding protocol a standard diet was given to all animals to promote adaptation. Thereafter, considering the previously described groups, the animals were fed by a standard or high-fat diet for 17 weeks. The isocaloric standard (containing 35% energy from fat, 47% from carbohydrates, and 18% from protein) or high-fat diets (71% energy derived from fat, 11% from carbohydrate, and 18% from protein) were purchased from Dyets Inc. (Bethlehem, USA).

2.2. Exercise protocol

Animals from voluntary exercised groups (SVPA and HVPA) were housed in individual cages equipped with running wheels and a digital counter from which running distance was daily obtained between 08.00 and 10.00h. The access to VPA was given during the 17 weeks of the feeding protocol (preventive perspective), while

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ET only began after 8 weeks, when metabolic-related disorders were already evident (therapeutic perspective). In the first week of the exercise protocol, the animals (SET and HET) were adapted to the treadmill for 5 days at 15m/min and 0% grade until 30min was achieved. Thereafter, endurance exercise was performed for 8 weeks, 5days/week, 60min/day at a starting speed of 15/min that was gradually increased until 25m/min was reached. In parallel, the sedentary groups (SS and HS) were placed on a non-moving treadmill in order to expose the animals to the same environmental and handling conditions.

2.3. Blood and Tissue preparation

Rats were anaesthetized (Ketamine 90mg/Kg and Xylazine 10 mg/Kg) after 17 weeks of diet and exercise. The blood of each animal was drawn from the heart, centrifuged (3000*xg*, for 10min at 4ºC) and stored for further analysis (-80ºC). Thereafter, the organs were perfused (0.9% NaCl) and the liver, heart, and visceral fat were excised and weighed. Liver right lobule was used for mitochondrial isolation and light and electron microscopy. The adiposity index was calculated as (100·(sum of fat pad weights)/(body weight)) (Taylor and Phillips, 1996).

2.4. Histology

2.4.1. Light microscopy

Liver tissues were quickly removed after animal sacrifice and fixed with 4% buffered paraformaldehyde, processed routinely and embedded in paraffin. The embedded samples were then sectioned (5μm), deparaffinized with xylene and re-hydrated with decreasing concentrations of ethanol. Finally, the samples were stained with haematoxylin and eosin and examined in a light microscope (Olympus BX61). The

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histological examination was blindly performed by two pathologists in three sections per slide based on NAFLD activity score (NAS score). In brief, the proposed NASH score was achieved by summing the scores of steatosis (0-3), lobular inflammation (0-2) and hepatocellular ballooning (0-2). A total score of 0-2 represents no steatohepatitis, 3-4 questionable and 5 or more, definite steatohepatitis (Kleiner et al. , 2005).

2.4.2. Electron microscopy

Liver tissues were immediately fixed in a 2.5% of glutaraldehyde solution, post-fixed for 2 hours in 1% osmium tetroxide and dehydrated in graded ethanol. The dehydrated samples were then embedded in Epon resin blocks. Thereafter, specimen blocks were sectioned (50-60nm), collected on cooper grids and stained with uranyl acetate, lead citrate. Finally, each grad was examined under a transmission electron microscope (JEM-1400) and the acquire images were blindly semi-quantified by a single subject, using a previously described criteria (Ahishali et al. , 2010).

2.5. Liver Mitochondrial Isolation

Liver mitochondria were daily prepared by a conventional differential centrifugation method. The right liver lobule was harvested and minced in an ice-cold isolation buffer containing 250mM sucrose, 10mM Hepes, 1mM EGTA, pH 7.4 and 0.1% defatted BSA. Minced blood-free tissue was then mechanically homogenized with a glass Potter-Elvejhem in the presence of isolation medium (7g/50ml). The homogenate was centrifuged at 800x*g* for 10min at 4ºC and the resulting supernatant was centrifuged at 10,000x*g* for 10min at 4ºC. Mitochondrial pellet was ressuspended using a paintbrush and centrifuged twice at 10.000x*g* for 10min to obtain a final

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mitochondrial suspension. Both EGTA and BSA were omitted from the medium (250mM sucrose, 10mM Hepes pH 7.4) in the two last centrifugations. After isolation proceedings, mitochondrial protein was determined by the Biuret method (Van Norman, 1909) using BSA as standard. The fresh mitochondrial suspensions (≈40-50mg protein/ml) were maintained in ice (0-4ºC) during the oxygen consumption and transmembrane electrical potential assays, which were carried out within 4h.

2.6. Bioenergetics

2.6.1. Mitochondrial oxygen consumption assays

Oxygen consumption of isolated mitochondria was determined polarographycally at 25ºC with a Biological Oxygen Monitor System (Hansatech Instruments) and a Clark-Type oxygen electrode (Model DW1, Hansatech). Liver mitochondria (0.8 mg) and substrates for the different assays, namely glutamate-malate (5mM and 2.5mM) and succinate (5mM) were added into a reaction medium containing 130mM sucrose, 50mM KCl, 2.5mM KH_2PO_4, 5mM HEPES, 2mM $MgCl_2$ (pH 7.4) under constant stirring. For complex II-assays, succinate was added with rotenone (3μM) to inhibit complex I. The respiratory parameters included: state 3 determined after adding ADP (156 nmol/mg) and state 4 measured as the rate of oxygen consumption after ADP phosphorylation. The state 4 with complex II substrates was also measured in the presence of oligomycin (2μg) and carbonylcyanide-*p*-trifluromethoxyphenylhydrazon – (FCCP, 0.5μM). Mitochondrial inner membrane integrity was estimated as the ratio between uncoupled respiration induced by FCCP addition and the respiratory rate in the presence of oligomycin (FCCP/oligomycin). The respiratory control ratio (RCR) was calculated as the ratio between state 3/state 4, while the ADP/O was calculated as the number of nmol ADP phosphorylated by natom O_2 consumed (Estabrook, 1967).

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2.6.2. Mitochondrial transmembrane electrical potential assays

Mitochondrial transmembrane electric potential (ΔΨ) was indirectly monitored based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+), using a TPP^+ - selective electrode prepared in our laboratory as described by Kamo et al. (Kamo et al. , 1979) in combination with a silver chloride reference electrode (Tacussel, Model MI 402, France). The TPP^+ and the reference electrode were inserted in an open chamber, connected to a pH meter (Jenway, Model 30505, UK). The obtained signals were fed to a potentiometric recorder (Kipp & Zonen, Model BD 112, Holland).ΔΨ was estimated as indicated by Kamo et al. (1979) through the equation: $\Delta\Psi = 59\text{x}\log(\nu/V) - 59\text{x}\log(10\,\Delta E/59\text{-}1)$, where ν, V, and Δ*E* stand for mitochondrial volume, volume of the incubation medium, and deflection of the electrode potential from the baseline, respectively. A matrix volume of 1.1 µl/mg of protein was assumed and no correction was made for the "passive" binding to the mitochondrial membranes, since the purpose of the experiments was to show relative changes in ΔΨ rather than absolute values. Liver mitochondria (0.8mg) were incubated in a reaction medium containing 130mM sucrose, 50mM KCl, 2.5mM KH_2PO_4, 5mM HEPES, 2mM $MgCl_2$ (pH 7.4), supplemented with TPP^+ (3µM). The measurement of ΔΨ with complex-I and complex II-linked substrates energization was performed with glutamate-malate (5mM and 2.5mM, respectively) or succinate (5mM) plus rotenone (3µM) and depolarization was achieved by adding ADP (156nmol/mg).

2.7. Lipid extraction

The lipids were extracted from mitochondrial polls through Bligh and Dyer method (Bligh and Dyer, 1959). In brief, 3.75 mL chloroform–methanol 1:2 (v/v) was added to each sample, vortexed, and incubated on ice for 30 min. Follow the incubation

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period, 1.25 ml of chloroform plus 1.25ml of doubly distillated water were added, and the samples were vortexed and centrifuged at 153x*g* for 5 min at 22ºC to obtain a two-phase system (aqueous top phase and organic bottom phase). The lipid extracts, recovered from the bottom phase, were dried using a nitrogen flow and stored at −20 °C.

2.7.1. Mitochondrial phospholipid separation

The different classes of phospholipids present in mitochondrial lipid extracts were separated by thin layer chromatography (TLC). Silica gel plates (Merck 2.5 x 20 cm) were washed with chloroform/methanol (1:1) and sprayed with 2.3% of boric acid. The lipid extracts (30ug) were applied in the TLC plates, which were developed with chloroform–ethanol–water–triethylamine (30:35:7:35 (v/v)) as mobile phase. In order to observe lipid spots, TLC plates were sprayed with primuline and exposed to ultraviolet light. The different phospholipid classes were identified by comparison with lipid standards. Finally, the spots were scraped from the TLC plates and lipid classes were extracted for further assays (Ferreira et al. , 2013).

2.7.2.Quantification of mitochondrial membrane phospholipids

To evaluate the phospholipid content of silica-gel scrapings from TLC plates, the phosphorus assay was performed by a method described elsewhere (Fuchs et al. , 2011). Briefly, perchloric acid (70%) was added to phosphate standards and samples, which were then incubated for 1 h at 180 °C. Thereafter, doubly distillated water, ammonium molybdate, and ascorbic acid were added to the standards and samples, which were then incubated for 5 min at 100 °C. The samples were centrifuged for 5 min at 2451x*g* to separate phospholipids from silica. Finally, the standards and sample

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solutions were measured at 800 nm in a plate reader.

2.7.3. Characterization of phospholipids profile

Characterization of individual molecular species of PE, PC and CL classes were achieved through mass spectrometry (MS) using a linear ion trap (LXQ; Thermo Finnigan, San Jose, CA, USA). The MS analysis was performed after separation of CL by TLC (point 2.7.1) and after separation of PE and PC by liquid chromatography (LC). HPLC system (Waters Alliance 2690) was used with a Ascentis®Si column (15 cm × 1 mm, 3 μm) and a precolumn split (Acurate, LC Packings, USA) in order to obtain a flow rate of 30 μL min−1. The solvent system consisted in two mobile phases as follows: mobile phase A (acetonitrile:methanol:water; 50:25:25 (v/v/v) with 1 mM ammonium acetate) and mobile phase B (acetonitrile:methanol 60:40 (v/v) with 1 mM ammonium acetate). Initially, 0% mobile phase A was held isocratically for 8 min followed by linear increase to 60% of A within 7 min and maintained for 22 min. The phospholipids were separated by HPLC, which was coupled to a linear ion trap (LXQ; Thermo Finnigan, San Jose, CA, USA) mass spectrometer. The LXQ were operated in negative (electrospray voltage −4.7 kV) with 275 ºC capillary temperature and the sheath gas flow of 8 U. Normalized collision energy™ (CE) varied between 20 and 27 (arbitrary units) for MS/MS. Data acquisition was carried out on an Xcalibur data system (V2.0) (Ferreira, Guerra, 2013). Relative quantitation of individual phospholipid species were determined by the ratio between the area of reconstructed ion chromatogram of a given *m*/*z* value against the area of the reconstructed ion chromatogram of the internal standard of the respective class (dimyristoyl-phosphatidylethanolamine, dimyristoylphosphatidylcholine, dimyristoyl-phosphatidylserine, all phospholipid standard were purchased from Avanti Polar

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Lipids).

2.7.4. Fatty acid analysis

The fatty acid content of the total lipid extract was evaluated by measuring the fatty acid methyl esters through gas chromatography (GC), according to a previously described method (Aued-Pimentel et al. , 2004). Briefly, a methanolic solution of potassium hydroxide (2M) and a saturated solution of NaCl were added to lipid extracts (60µg), vortexed and centrifuged for 5min at 613x*g* to obtain a two-phase system. The fatty acid methyl esters recovered from the bottom phase were dried using a nitrogen flow and ressuspended in hexane. The C17 (7.5µg) fatty methyl ester was used as internal standard. The GC injection port was programmed at 523.15 K and the detector at 543.15 K. Oven temperature was programmed as follows: initially stayed 3 min at 323.15 K, raised to 453.15 K (25K.min^{-1}), held isothermal for 6 min, with a subsequent increase to 533.15 K (40K.min^{-1}) and maintained there for 3 min, resulting in a total of 19 minutes. The carrier gas was hydrogen flowing at 1.7ml.min^{-1}. The gas chromatograph (Clarus 400, PerkinElmer, Inc. USA) was equipped with DB-1 column with 30m lengths, 0.25mm internal diameter and 0.15µm film thickness (J&W Scientific, Agilent Technologies, Folsom, CA, USA) and a flame ionization detector.

3. STATISTICAL ANALYSIS

The results were expressed as means and standard error means (mean±SEM) for each experimental group. 2-way ANOVA independent-measures test followed by Bonferroni post hoc test was used for comparisons between groups and differences

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were considered significant at $p < 0.05$. Statistical Package for Social Sciences (SPSS Inc, version 20) was used for all comparisons.

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4. RESULTS

4.1. Animal characteristics

Seventeen weeks of high-fat diet treatment significantly increased adiposity index (AI) and HDL and decreased VLDL and TG serum levels (Figure 1 and 2) in sedentary animals (HS vs. SS). Both exercise regiments improved all anatomic measures (body weight, heart/body weight and AI) and TG levels, in standard diet groups. The anatomic alterations were also observed in high-fat diet groups for ET, while VPA only diminished AI. No alterations were observed in ALT serum levels and energy intake *per* group.

Insert Figure 1 and 2

4.2. Histological Analysis

The histological analysis of liver tissue (Panel D, E, F) showed an accumulation of microvesicular and macrovesicular lipid droplets and hepatocyte ballooning (Figure 3) in the high-fat diet groups. Moreover, the NAFLD activity scoring (NAS) system revealed that both HS and HV animals developed a NASH typical phenotype (Table 1). These histological features were attenuated in ET group being the score classification of "definitive NASH" downgraded to "questionable NASH".

Insert Table 1 and Figure 3

Ultrastructural analysis performed in liver tissue demonstrated that high-fat feeding in sedentary and voluntary-exercised animals (HS vs. SS and HVPA vs. SVPA) increased the number of abnormal mitochondria, with loss of cristae and

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intramitochondrial granules, rarefied matrix and swelling. These mitochondrial alterations were clearly prevented by ET (Figure 4, Panel F).

Insert Figure 4

4.3. Mitochondrial phospholipid class content

Following the characterization of mitochondrial structural features, we next aimed to measure the content of mitochondrial phospholipids. High-fat diet treatment increased the relative amount of phosphatidylethanolamine (PE) and phosphatidic acid (PA) whereas phosphatidylinositol (PI), cardiolipin (CL) and PC/PE ratio were reduced in sedentary animals (Table 2). Both exercise regimens increased PC relative content and thus PC/PE ratio in high-fat diet group. Moreover, VPA increased the amount of PI and decreased PA and PE in high-fat fed animals.

To determine the profile of major classes of phospholipids, analysis of the total lipid extracts were performed by LC-MS and MS/MS and off- line TLC-MS and MS/MS. Data obtained from LC-MS allowed us to determine the relative abundance of each molecular species within each class. The PE and PC molecular profiles were affected by diet and/or exercise treatment or their interactions. As shown in Figure 5, the molecular species bearing linoleic acid (PE (16:0/18:2) and PC (16:0/18:2)) increased in HET group. The diet affected the abundances of the molecular species PE (16:0/20:4) and PC (16:0/18:1), inducing a decrease of these species for all high-fat fed animals. However, VPA and ET decreased the species PE (16:0/22:6 and 18:0/22:6) and PC (16:0/20:4 and 16:0/22:6) in both diets. No differences were observed in PC (18:0/22:6) molecular specie as well as in PE (16:0/18:1) molecular

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specie for all treatments. Regarding PI, one major specie (m/z 885, PI (18:0/20:4)) was observed and no differences were found between groups (data not shown).

In the ESI-MS spectra obtained from CL extract of control animals, previously separated by TLC (the first three spectra of Figure 6), two major $[M-H]^-$ ions at *m/z* 1449.7 attributed to the (CL (C18:1) $(C18:2)_3$) and at *m/z* 1451.7 attributed to the (CL $(C18:1)_2$ $(C18:2)_2$) were observed. Both correspond to the most abundant CL molecular species in mitochondria and no changes were observed among the 3 groups. However, alterations in liver mitochondrial CL molecular profile were observed in the extracts of animals fed the high-fat diet. In the ESI-MS spectra obtained from HS rats, a small decrease of *m/z* 1451.7 ionic species (CL $(C18:1)_2$ $(C18:2)_2$) compared with ion at *m/z* 1449.7(CL (C18:1) $(C18:2)_3$) was found. This tendency was even more evident in HT animals (Figure 5).

Insert Table 2 and Figure 5 and 6

4.3.1. Mitochondrial fatty acid content

As shown in Table 3, liver mitochondrial contents of saturated fatty acids (SFAs) (C18:0) and linoleic acid (C18:2n6c) were increased after high-fat diet treatment, while linolelaidic acid (C18:2n6t) and monounsaturated fatty acids (MUFAs) (C18:1n9c) were diminished (HS vs. SS). ET was able to reduce SFAS (C16:0 and C18:0) and increase polyunsaturated fatty acids (PUFAs) (C18:2n6c) in high-fat diet group, whereas VPA only decreased SFAs (C16:0).

Insert Table 3

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4.4. Liver mitochondrial bioenergetics

Following the characterization of mitochondrial lipid membrane components, we next evaluated mitochondrial bioenergetics. Figure 7 shows that RCR and maximal ΔΨ with glutamate-malate were significantly diminished by high-fat diet feeding (HS vs. SS), however only RCR was improved by exercise regiments. No changes were observed with substrates for complex II-driven respiration (data not shown).

As expected, high-fat diet decreased FCCP-uncoupling respiration in sedentary animals (HS vs. SS), however no changes were observed in the presence of oligomycin or in FCCP/Oligomycin ratio (Table 4). ET and VPA improved FCCP-uncoupling respiration and FCCP/Oligomycin ratio in high-fat diet groups. Nevertheless, in standard diet groups these alterations were only observed for VPA (SVPA vs. SS).

Insert Figure 7

Insert Table 4

5. DISCUSSION

5.1. Overview of findings

High-fat rich diets and sedentary behaviours are the major risk factors for metabolic diseases, among which NAFLD is the most common hepatic disorder. In this sense, dietary modifications and physical activity has been described as the "golden standard" preventive and/or therapeutic strategy against NAFLD/NASH. However, few studies have addressed the effect of exercise on NASH-related mechanisms, namely those involving mitochondria. To our knowledge, this is the first study analysing the preventive (VPA) and therapeutic (ET) effect of physical exercise on

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NASH-induced liver mitochondrial phospholipidomic alterations. The major findings of this study suggest that both ET and VPA were able to modulate mitochondrial membrane integrity and fluidity, through the modulation of PC/PE ratio, which may have contributed to preserve bioenergetic function (RCR and FCCP-uncoupling respiration and FCCP/oligomycin ratio). Despite both exercises mitigated NASH-induced liver mitochondrial membrane and bioenergetic disruption, ET was more efficient in the modulation of anatomic and histological measures.

5.2. Characterization of the animal model

Studies suggest that patients with normal weight but high levels of visceral adiposity have a great risk to develop NAFLD (Chen et al. , 2006, Kim et al. , 2004). In accordance with previous data (Lieber et al. , 2004, Wang et al. , 2009, Wang et al. , 2008), in the present study no changes were observed in body weight, while adiposity was significantly increased in the HS animals. In parallel with the anatomical alterations, high-fat feeding induced a decrease in VLDL levels, which is suggestive of hepatic damage. This suspicion was confirmed by light microscopic data, which shown that HS animals developed the typical histopathological features of NASH (Abdelmegeed et al. , 2011, Lieber, Leo, 2004, Wang, Ausman, 2009, Wang, Ausman, 2008). Although exercise has been proposed as a promising strategy against obesity and NASH (Ascensao, Martins, 2013, Goncalves, Oliveira, 2013), only ET was able to mitigate all the obesity-related measures and the NASH-related histological variables in high-fat diet animals. The distinct effects of the exercise preventive and therapeutic approaches may be explained, at least in part, by the high intensity achieved in ET programs compared to that accomplished during voluntary physical activity. In fact, previous studies in obese patients showed evidences for more

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favorable effects obtained with high- than moderate-intensity exercise on body weight or fat mass loss (for references see De Feo, 2013). Furthermore, the positive effect of ET on the structural features corroborates the study of Kistler et al. (Kistler, Brunt, 2011), which shown that vigorous, but not moderate exercise, nor total duration or volume is related to the decreased odds of having NASH.

5.3. Exercise prevented NASH-induced phospholipidomic alterations in mitochondrial membranes and mitochondrial bioenergetics dysfunction

Mitochondrial phospholipid composition varies little among cells, suggesting that major changes are unsustainable and result in several disorders (Monteiro et al. , 2013a, Osman et al. , 2011). In accordance with a previous report (Aoun, Fouret, 2012b), we observed that CL was reduced, while PE content was improved in the HS group. Moreover, as expected (Arendt, Ma, 2013, Li, Agellon, 2006), PC/PE ratio, a key regulator of membrane integrity/fluidity, was significantly decreased in HS animals.

Studies suggest that membranes rich in PE are less fluid than membranes rich in PC, due to PE smaller head group, lower hydration and reactivity with other phospholipid (Fajardo et al. , 2011). Kalofoutis (Kalofoutis, 1984), showed that ET increased liver mitochondria membrane PC and decreased PE. These membrane structural alterations were also observed in high-fat exercised groups (HVPA and HET). Hypothetically, an increased activity of phosphatidylethanolamine N-methyltransferase pathway, by which PE is converted to PC (Sundler and Akesson, 1975), would be responsible for the observed increase in PC content; however no changes were found in the species composition of these phospholipid classes.

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Diminished CL content represents another possible consequence of NASH (Aoun, Fouret, 2012b, Petrosillo et al. , 2007), as this phospholipid exerts a key role in the control of mitochondrial inner membrane proteins involved in oxidative phosphorylation (Paradies et al. , 2009). In fact, along with CL depletion HS animals showed a decrease in RCR, $\Delta\Psi$ and uncoupled-respiration, suggesting electron transport chain and respiratory coupling impairments in substrate oxidation. Given that the RCR is the best general measure of mitochondrial dysfunction (Brand and Nicholls, 2011), it is clear that both VPA and ET had a positive impact on mitochondrial bioenergetics. These results corroborate the recent study of Flectcher et al. (2013), which shown that both voluntary wheel running and forced treadmill exercise positively modulate hepatic mitochondrial respiration.

Nevertheless, no alterations were observed in CL content of exercised animals, which is in contrast to the increased CL content previously reported in skeletal muscle and cardiac tissues (Chicco et al. , 2008, Menshikova et al. , 2007). These contradictory data may be due to tissue-specific mitochondrial physiological diversity in the control of oxidative phosphorylation (OXPHOS) (Benard et al. , 2006). Exercise-induced protective functional phenotype was also confirmed by the increase of FCCP-uncoupled respiration and FCCP/oligomycin ratio. The observed improvement of inner membrane integrity (FCCP/oligomycin) was probably a result of phospholipid changes (PC/PE ratio) described previously. In fact, this ratio can affect membrane properties including membrane curvature elastic energy and fluidity and can modulate protein assembly and function, including enzymes involved in phospholipids biosynthesis (Dymond et al. , 2013), thus influencing mitochondrial function.

Although increased mitochondrial proton leak has been proposed as a protective mechanism against NASH-induced fat accumulation (Serviddio et al. , 2008), no

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alterations were observed in mitochondrial respiration rates after oligomycin addition. This unexpected result could be explained by the higher amount of linoleic acid in membrane composition, which has been negatively correlated with proton leak (Brookes et al. , 1998, Porter et al. , 1996). Despite the present and previously reported evidences (Monteiro et al. , 2013b, Monteiro et al. , 2013c) showing that mitochondrial function is affected by membrane lipid composition, is important to highlight that under physiological temperatures (37ºC) this interaction is not observed (Lemieux et al. , 2008).

Besides phospholipid classes', fatty acid composition of biological membranes have also a strong influence on its properties. Among fatty acid categories, PUFAs have been described as important components of membrane due to its role on membrane fluidity and thus protein function and trafficking (Schmitz and Ecker, 2008). Studies showed that dietary fat composition significantly influences the incorporation of fatty acids into membranes (Innis and Clandinin, 1981, Tahin et al. , 1981). Our results agree with these data, as higher levels of linoleic acid present in the high-fat diet (C18:2nc) resulted into a higher expression of this fatty acid in mitochondrial membranes (HS vs. SS). This effect was even evident in CL molecular species, whereas CL molecular species containing more oleic acid [CL $(C18:1)_2$ $(C18:2)_2$] showed a diminished incorporation level in comparison with CL (C18:1) $(C18:2)_3$.

The increase of linoleic *cis* configuration and decrease linoleic *trans* configuration (C18:2nt) observed in HS group seems to be a condition that increases membrane fluidity (Roach et al. , 2004). In turn, the increase in saturated fatty acids (SFAS) and decreased in unsaturated fatty acids (MUFAs) present in membranes, may had an opposite effect on HS membrane properties.

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The effect of exercise on fatty acid membrane composition is also controversial; with studies suggesting either increased PUFAs (Fiebig et al. , 2002, Quiles et al. , 1999) or unchanged profile (Fiebig et al. , 1998). In the present study, ET increased PUFAS (linoleic *cis* configuration) and decreased SFAS (C16:0 and C18:0), which may have contributed to increase membrane fluidity. Interestingly, the results from fatty acid analysis were in agreement with the results from phospholipid profile analysis by LC-MS, where an increase of PC and PE species bearing linoleic acid (PC) were seen in HET group. In contrast, it seems that VPA was unable to modulate membrane characteristics, as some of SFAs decreased (C16:0) while others increased (C18:0).

6. CONCLUSIONS

In summary, our results suggest that both ET and VPA counteracted NASH-induced membrane structural changes, which may contribute to improved mitochondrial function. This protective mechanism allows mitochondria to increase substrate oxidation, which in turn limit fat accumulation and thus decrease histological and ultrastructural signs of NASH. However, only ET was able to modulate these morphological parameters, which emphasize the major role of exercise intensity against NASH deleterious effects.

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Figure 1. Animal anatomic characteristics and food intake.

Values are means ± SEM, (n = 6). Different letter indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained.

[a] vs. SS, [b] vs. HS, [c] vs. SVPA, [d] vs. SET, [e] vs. HVPA ($p < 0.05$)

[1] Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT x ET) or the absence of differences (NS) is shown.

Figure 2. Serum biochemical analysis at 17 weeks of diet and exercise treatment.

Values are means ± SEM, (n = 6). Different letter indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained.

[a] vs. SS, [b] vs. SVPA, [c] vs. SET, [d] vs. HVPA ($p < 0.05$)

[1] Significant ($p<0.05$) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT x ET) or the absence of differences (NS) is shown.

Figure 3. Representative light photographs (x100) of liver tissue from all groups: (A), SS; (B) SVPA; (C) SET; (D) HS; (E) HVPA and (F) HET. The presence of ballooning (asterisk), macro (white arrows) and microvesicular (black arrows) lipid droplets in high-fat diet groups.

Figure 4. Semi-quantitative analysis of liver mitochondrial structural damage (based on Ahishali et al. (Ahishali, Demir, 2010)) and representative electron micrographs from all groups: SS (Panel A, x25,000); SVPA (Panel B, x30,000); SET (Panel C, x20,000); HS (Panel D, x50,000); HVPA (Panel E, x30,000) and HET (Panel F, 30,000).

Values are means ± SEM, (n = 6). Different letter indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained.

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[a] vs. SS, [b] vs. HS [c] vs., SVPA, ($p < 0.05$).

[1] Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences (NS) is shown.

Figure 5. ESI-MS spectra obtained in negative mode of CL extracts from animals fed a control diet (SS - standard diet sedentary, SVPA - standard diet voluntary physical activity and ST - standard diet endurance trained) and for extracts from animals fed high-fat diet (HS - high-fat diet sedentary, HVPA - high-fat diet voluntary physical activity and HT - high-fat diet endurance trained). In the inset, the CL species corresponding to each [M-H]- ion are presented.

Figure 6. Phosphatidylethanolamine (PE) (A) and phosphatidylcholine (PC) (B) molecular species relative composition. Values are means ± SEM, (n = 6) expressed as the percentage of species *per class*. For the different groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained [a] vs. SS, [b] vs. HS, [c] vs. SVPA, [d] vs. SET, [e] vs. HVPA ($p < 0.05$).

[1] Significant ($p<0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences (NS) is shown.

Figure 7. Respiratory control ratio (RCR) and maximal membrane potential ($\Delta\Psi$) of glutamate-malate energized mitochondria.

Values are means ± SEM, (n = 6) expressed in percentage of control (SS) values. Different letter superscript indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained.

[a] vs. SS, [b] vs. HS ($p < 0.05$).

[1] Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences (NS) is shown.

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Table 1. NAS scores recorded from livers of all experimental groups, observed blindly under light microscopy.

NAS scores: 0-2 No NASH; 3-4 Questionable NASH; > 5 Definitive NASH (according to Kleiner et al. (2005))

Diet treatment	Exercise type	NAS score	*P* value[1]
Standard diet	Sedentary	1.5±0.5	DT x ET
	Voluntary	2.1±0.5	
	Trained	2.2±0.3	
High fat diet	Sedentary	6.3±0.4[a]	
	Voluntary	6.1±0.3[c]	
	Trained	4.0±0.7[b]	

Values are means ± SEM (n = 6). Different letter superscript indicates significant differences between groups: standard diet sedentary – SS; standard diet voluntary physical activity – SVPA; standard diet endurance trained –SET; high-fat diet sedentary – HS; high-fat diet voluntary physical activity – HVPA; high-fat diet endurance trained – HET.

[a] vs. SS, [b] vs. HS, [c] vs. SVPA ($p < 0.05$)

[1]Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) and their interaction (DT x ET) or the absences of differences (NS) are shown

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Table 2. The effect of diet and exercise on liver mitochondria phospholipid classes composition.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[1]
% LPC	11.36±0.05	10.96±0.18	12.55±3.57	11.64±1.70	9.04±0.31	9.22±0.75	NS
% PC	33.86±1.32	33.35±1.85	31.82±1.06	32.72±2.72	39.67±0.85[b,c]	40.27±2.52[b,d]	DT x ET
% PI	12.61±0.81	19.17±2.42[a,d]	12.03±1.76	7.82±1.37[a]	15.66±0.63[b]	11.23±0.97	DT, ET
% PE	20.47±1.03	14.85±0.37	19.08±1.36	32.02±3.06[a]	17.26±2.36[b]	21.80±2.46	DT, ET
% PA	3.52±0.39	6.00±0.54	3.87±0.35	9.17±2.37[a]	4.17±0.49[b]	7.03±0.69[d]	DT x ET
% CL	18.18±1.36	15.68±2.49	20.66±4.06	10.33±1.90[a]	14.46±1.21	10.44±0.54[d]	DT
PC/PE	1.67±0.10	2.26±0.15	1.70±0.16	1.06±0.16[a]	2.41±0.28[b]	1.92±0.25[b]	ET

Values are means ± SEM, (n = 6) expressed as the percentage of phospholipid per class. Different letter superscript indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained. Legend: LPC – lysophosphatidylcholine; PC – phosphatidylcholine; PI-phosphatidylinositol; PE-phosphatidylethanolamine; PA-phosphatidic acid and CL – cardiolipin.

[a] vs. SS, [b] vs. HS, [c] vs. SVPA, [d] vs. SET ($p < 0.05$).

[1] Significant ($p<0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences (NS) is shown.

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Table 3. The effect of diet and exercise treatments on liver mitochondria fatty acid content.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[1]
C16:0	18.22±0.46	19.36±0.07	15.74±0.43[a]	18.14±0.34	16.53±0.23[b]	15.54±0.36[b]	DT x ET
C18:2n6c	12.03±0.19	12.06±0.17	13.28±0.08[a]	12.58±0.22[a]	11.92±0.08	17.46±0.25[b]	DT x ET
C18:2n6t	6.49±0.11	6.45±0.14	6.51±0.08	4.59±0.08[a]	4.37±0.05[c]	4.27±0.07[d]	DT
C18:1n9c	3.82±0.08	4.30±0.08 [a]	3.56±0.07	2.47±0.11[a]	2.20±0.05[c]	2.37±0.06[d]	DT x ET
C18:0	28.83±0.32	27.70±0.23[a,d]	29.08±0.18	31.09±0.21[a]	33.10±0.24[b,c]	29.67±0.06[b]	DT x ET
C20:4n6	30.53±1.16	30.02±0.52	31.58±0.81	31.71±0.26	31.92±0.27	30.45±0.34	NS

Values are means ± SEM, (n = 6), expressed as the percentage of total fatty acid content. Different letter superscript indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained.

[a] vs. SS, [b] vs. HS, [c] vs. SVPA, [d] vs. SET ($p < 0.05$).

[1] Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences is (NS) are shown.

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Table 4. Respiratory rates with oligomycin and FCCP of succinate energized liver mitochondria and FCCP/oligomycin ratio.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[1]
Oligomycin (% SS)	100.0±2.9	96.3±4.9	96.3±3.0	98.2±3.5	92.5±4.7	90.9±4.3	NS
FCCP (% SS)	100.0±1.7	120.4±3.2[a]	103.5±1.7[c]	89.6±1.2[a]	112.9±3.4[b]	102.9±2.6[b,d]	DT, ET
FCCP/oligomycin (%SS)	100.00±3.8	125.0±3.7[a]	107.3±4.0	91.6±4.8	122.1±3.4[b]	113.8±3.1[b]	ET

Values are means ± SE, (n = 6) expressed in percentage of control values (SS). Different symbols indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained.

[a] vs. SS, [b] vs. HS, [c] vs. SVPA, [d] vs. HVPA ($p < 0.05$).

[1] Significant ($p<0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences (NS) is shown.<ABS-P>

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Table 2. The effect of diet and exercise on liver mitochondria phospholipid classes composition.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[1]
% LPC	11.36±0.05	10.96±0.18	12.55±3.57	11.64±1.70	9.04±0.31	9.22±0.75	NS
% PC	33.86±1.32	33.35±1.85	31.82±1.06	32.72±2.72	39.67±0.85[b,c]	40.27±2.52[b,d]	DT x ET
% PI	12.61±0.81	19.17±2.42[a,d]	12.03±1.76	7.82±1.37[a]	15.66±0.63[b]	11.23±0.97	DT, ET
% PE	20.47±1.03	14.85±0.37	19.08±1.36	32.02±3.06[a]	17.26±2.36[b]	21.80±2.46	DT, ET
% PA	3.52±0.39	6.00±0.54	3.87±0.35	9.17±2.37[a]	4.17±0.49[b]	7.03±0.69[d]	DT x ET
% CL	18.18±1.36	15.68±2.49	20.66±4.06	10.33±1.90[a]	14.46±1.21	10.44±0.54[d]	DT
PC/PE	1.67±0.10	2.26±0.15	1.70±0.16	1.06±0.16[a]	2.41±0.28[b]	1.92±0.25[b]	ET

Values are means ± SEM, (n = 6) expressed as the percentage of phospholipid per class. Different letter superscript indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained. Legend: LPC – lysophosphatidylcholine; PC – phosphatidylcholine; PI-phosphatidylinositol; PE-phosphatidylethanolamine; PA-phosphatidic acid and CL – cardiolipin.

[a] vs. SS, [b] vs. HS, [c] vs. SVPA, [d] vs. SET ($p < 0.05$).

[1] Significant ($p<0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences (NS) is shown.

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Table 3. The effect of diet and exercise treatments on liver mitochondria fatty acid content.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[1]
C16:0	18.22±0.46	19.36±0.07	15.74±0.43[a]	18.14±0.34	16.53±0.23[b]	15.54±0.36[b]	DT x ET
C18:2n6c	12.03±0.19	12.06±0.17	13.28±0.08[a]	12.58±0.22[a]	11.92±0.08	17.46±0.25[b]	DT x ET
C18:2n6t	6.49±0.11	6.45±0.14	6.51±0.08	4.59±0.08[a]	4.37±0.05[c]	4.27±0.07[d]	DT
C18:1n9c	3.82±0.08	4.30±0.08 [a]	3.56±0.07	2.47±0.11[a]	2.20±0.05[c]	2.37±0.06[d]	DT x ET
C18:0	28.83±0.32	27.70±0.23[a,d]	29.08±0.18	31.09±0.21[a]	33.10±0.24[b,c]	29.67±0.06[b]	DT x ET
C20:4n6	30.53±1.16	30.02±0.52	31.58±0.81	31.71±0.26	31.92±0.27	30.45±0.34	NS

Values are means ± SEM, (n = 6), expressed as the percentage of total fatty acid content. Different letter superscript indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained.

[a] vs. SS, [b] vs. HS, [c] vs. SVPA, [d] vs. SET ($p < 0.05$).

[1] Significant ($p < 0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences is (NS) are shown.

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Table 4. Respiratory rates with oligomycin and FCCP of succinate energized liver mitochondria and FCCP/oligomycin ratio.

	SS	SVPA	SET	HS	HVPA	HET	*P* value[1]
Oligomycin (% SS)	100.0±2.9	96.3±4.9	96.3±3.0	98.2±3.5	92.5±4.7	90.9±4.3	NS
FCCP (% SS)	100.0±1.7	120.4±3.2[a]	103.5±1.7[c]	89.6±1.2[a]	112.9±3.4[b]	102.9±2.6[b,d]	DT, ET
FCCP/oligomycin (%SS)	100.00±3.8	125.0±3.7[a]	107.3±4.0	91.6±4.8	122.1±3.4[b]	113.8±3.1[b]	ET

Values are means ± SE, (n = 6) expressed in percentage of control values (SS). Different symbols indicates significant differences between groups: SS – standard diet sedentary; SVPA – standard diet voluntary physical activity; SET – standard diet endurance trained; HS – high-fat diet sedentary; HVPA - high-fat diet voluntary physical activity; HET – high-fat diet endurance trained.

[a] vs. SS, [b] vs. HS, [c] vs. SVPA, [d] vs. HVPA ($p < 0.05$).

[1] Significant ($p<0.05$) main effect of diet treatment (DT), exercise treatment (ET) their interaction (DT x ET) or the absence of differences (NS) is shown.

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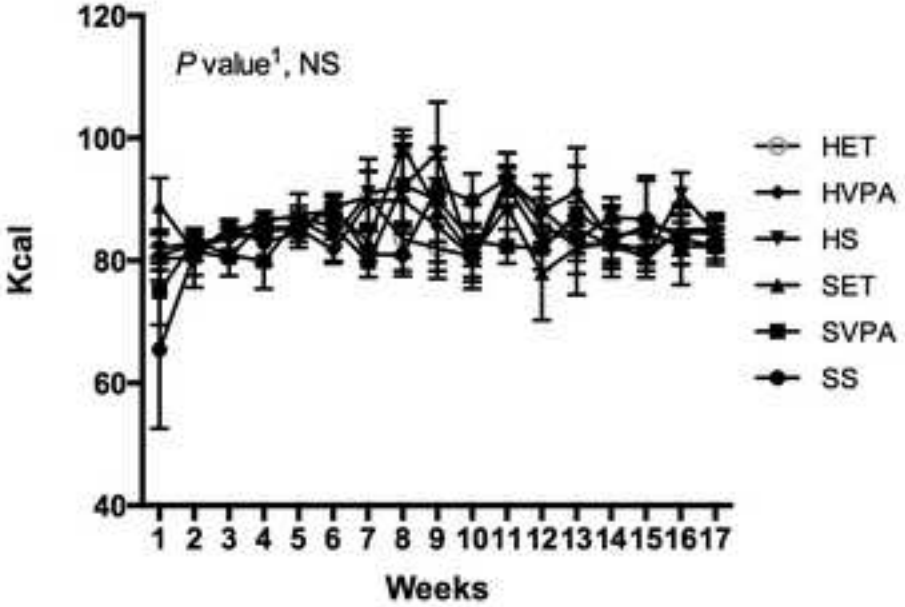
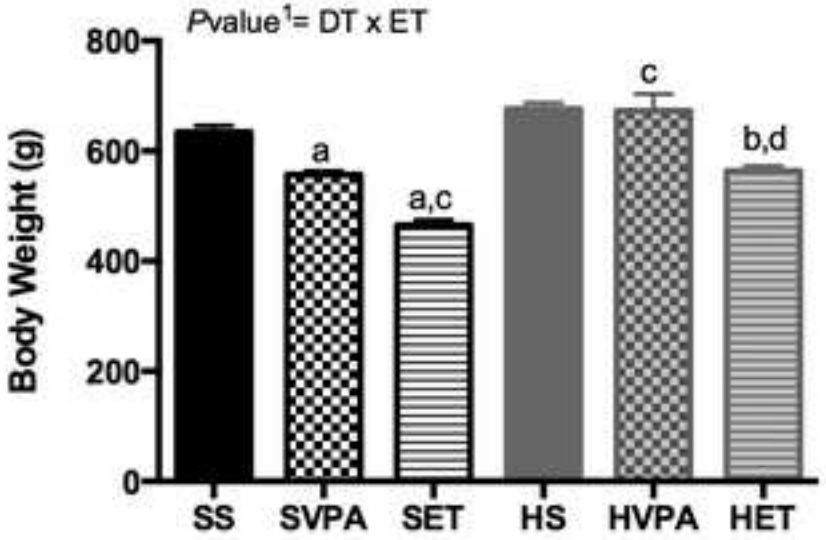
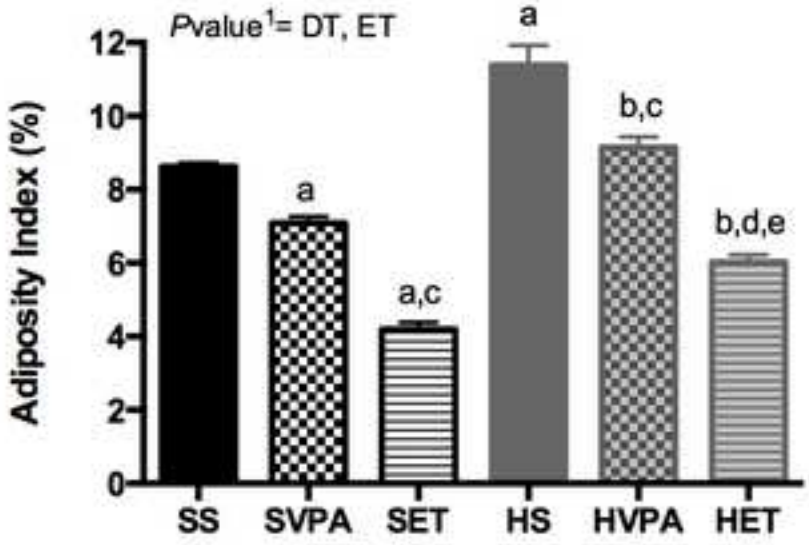
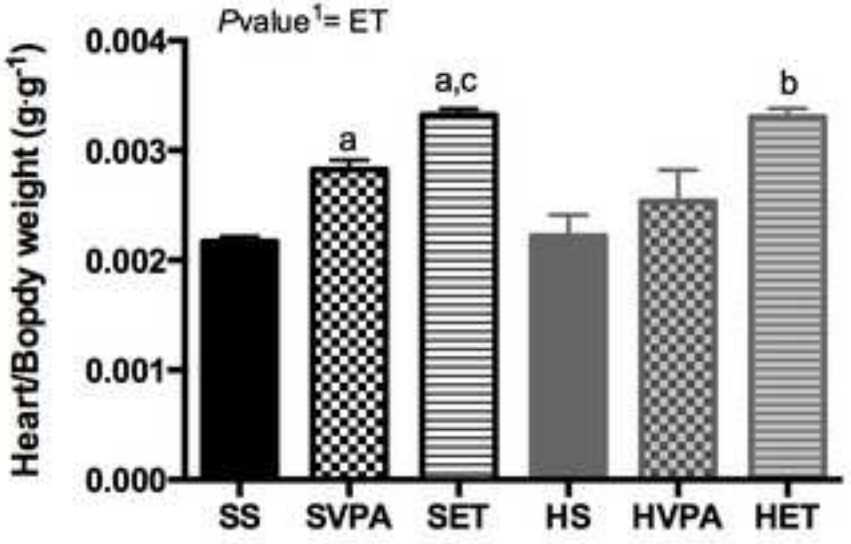
Figure(s)

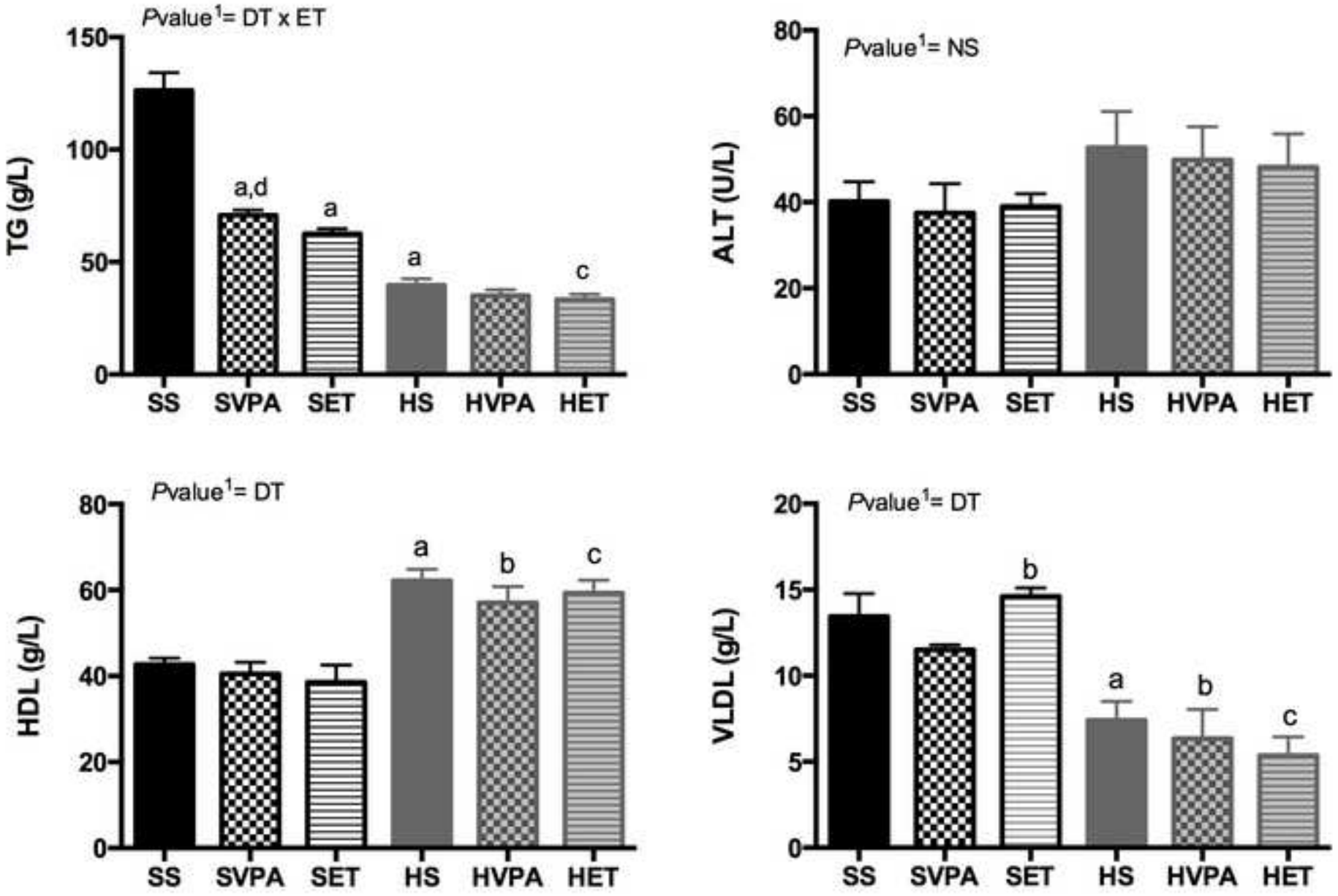

Pvalue[1], NS
Kcal
Weeks
HET
HVPA
HS
SET
SVPA
SS


Pvalue[1]= DT x ET
Body Weight (g)
SS
SVPA
SET
HS
HVPA
HET

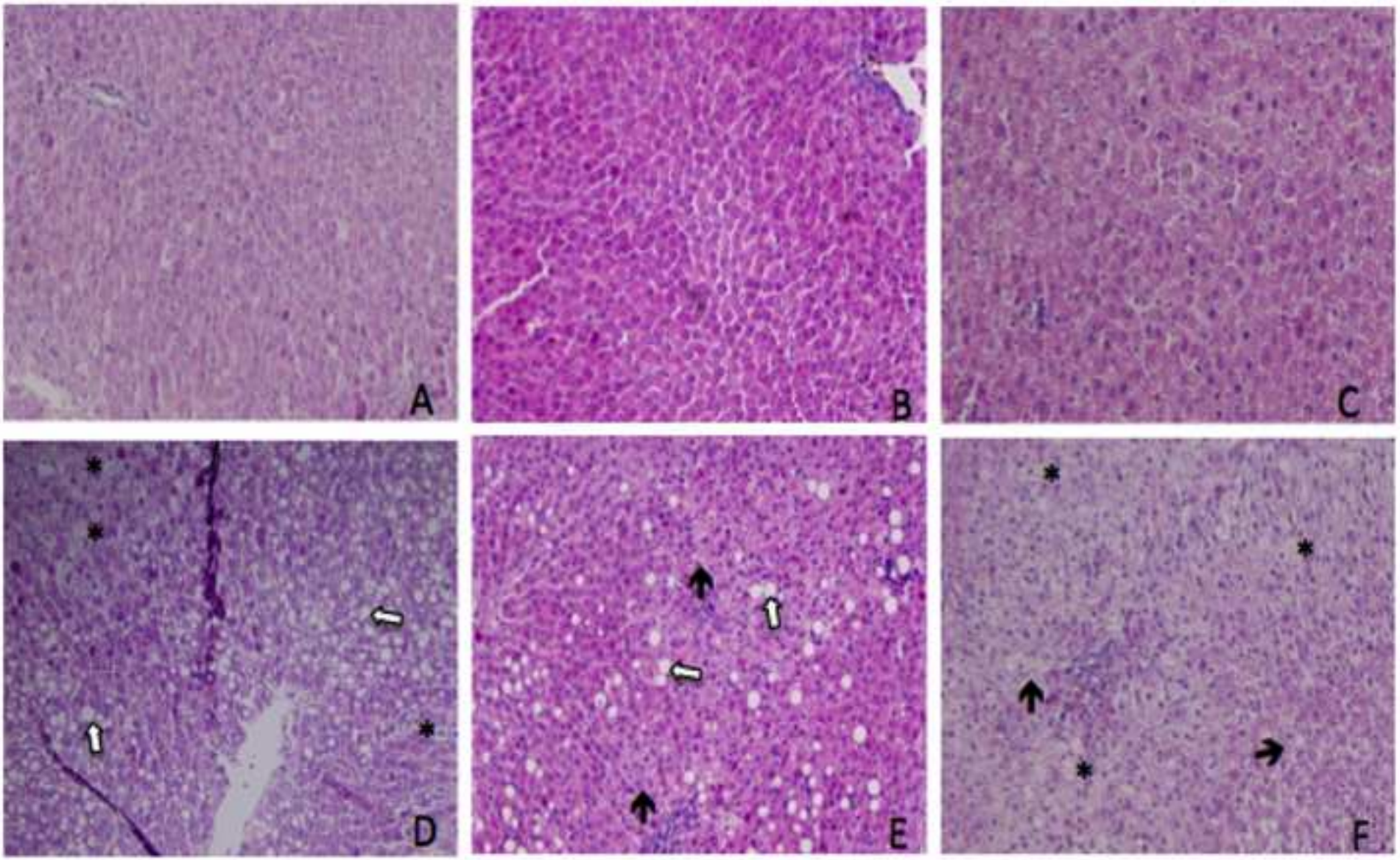

Pvalue[1]= DT, ET
Adiposity Index (%)
SS
SVPA
SET
HS
HVPA
HET


Pvalue[1]= ET
Heart/Bopdy weight (g·g-1)
SS
SVPA
SET
HS
HVPA
HET

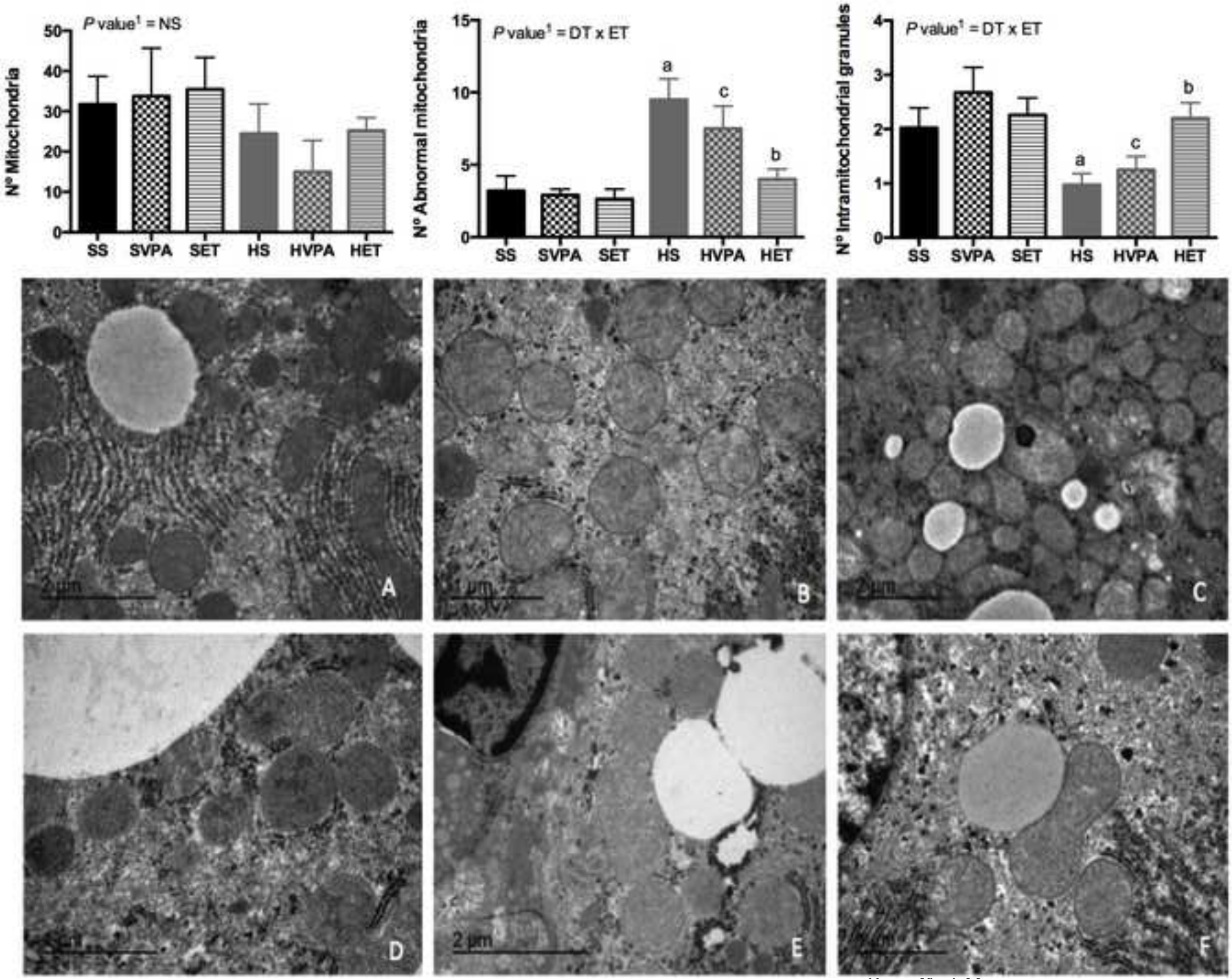
Figure(s)


Pvalue[1]= DT x ET
TG (g/L)
150
100
50
0
a,d
a
a
c
SS
SVPA
SET
HS
HVPA
HET
Pvalue[1]= NS
ALT (U/L)
80
60
40
20
0
SS
SVPA
SET
HS
HVPA
HET
Pvalue[1]= DT
HDL (g/L)
80
60
40
20
0
a
b
c
SS
SVPA
SET
HS
HVPA
HET
Pvalue[1]= DT
VLDL (g/L)
20
15
10
5
0
b
a
b
c
SS
SVPA
SET
HS
HVPA
HET

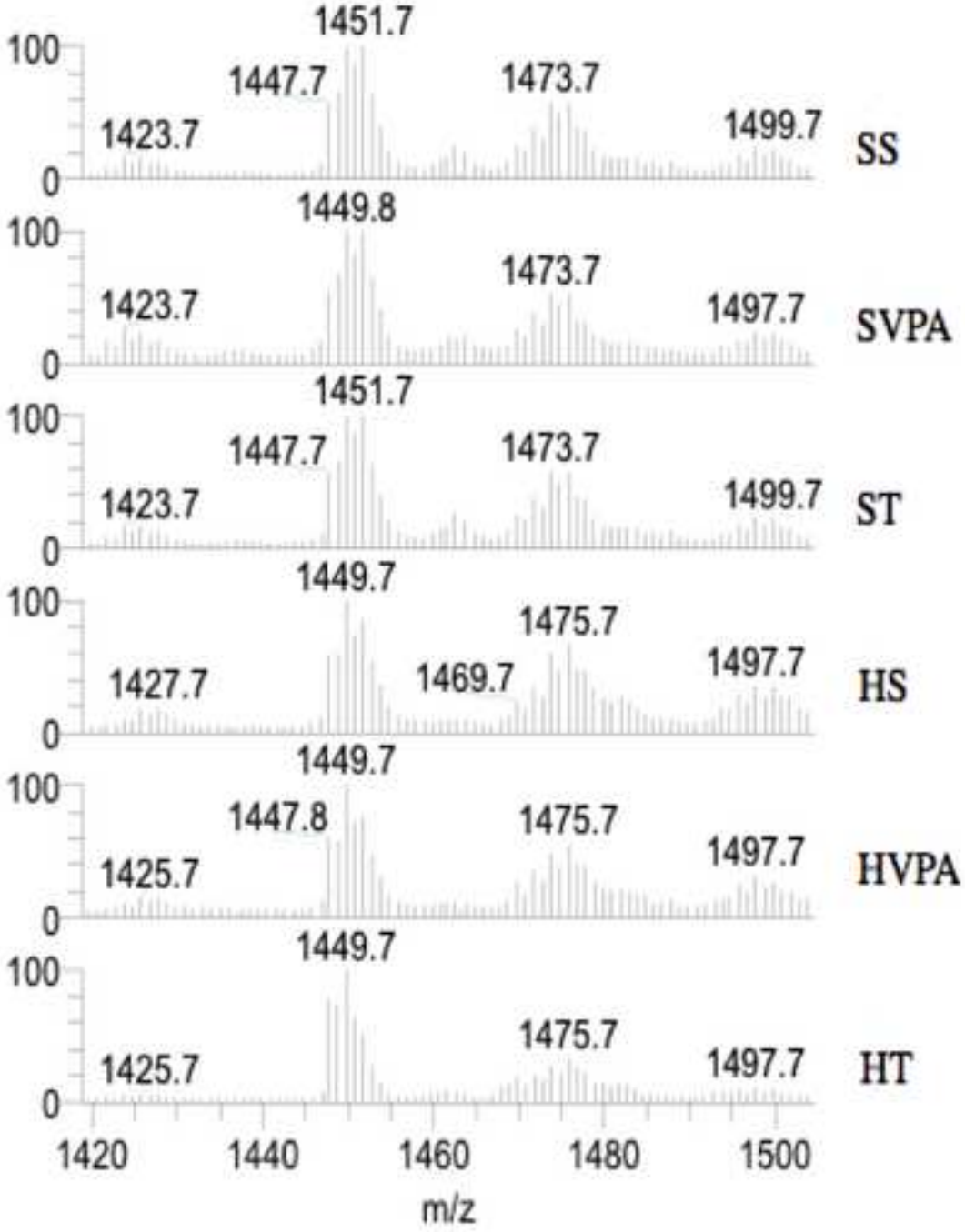
Figure(s)



Figure(s)

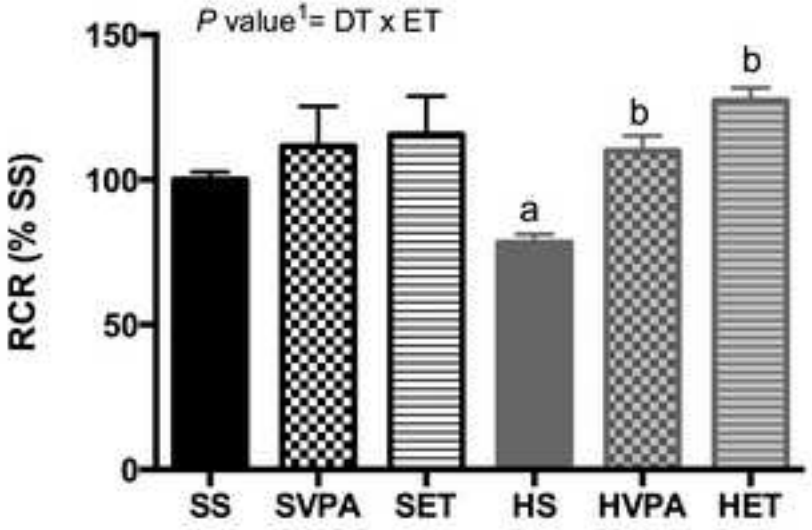

Nº Mitochondria
P value[1] = NS
50
40
30
20
10
0
SS
SVPA
SET
HS
HVPA
HET
Nº Abnormal mitochondria
P value[1] = DT x ET
15
10
5
0
a
c
b
Nº Intramitochondrial granules
P value[1] = DT x ET
4
3
2
1
0
a
c
b
2 µm
A
B
C
D
E
F

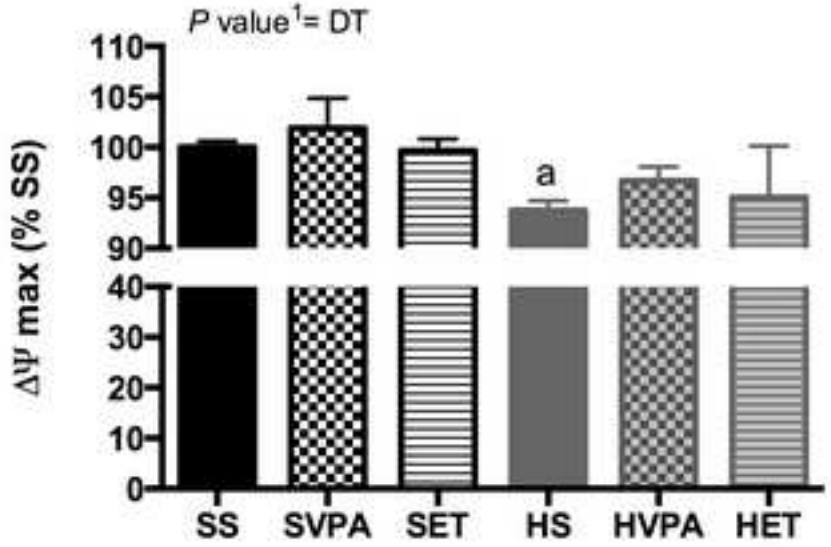
Figure(s)



$[M-H]^-$	$[M-2H]^{2-}$	Fatty acids
1423.7	711.5	$(16:1)(18:1)(18:2)_2$ $(16:0)(18:2)_3$
1425.7	712.6	$(16:0)(18:1)(18:2)_2$ $(16:1)(18:1)_2(18:2)$
1427.7	713.6	$(16:1)(18:2)(18:1)_2$
1447.7	723.6	$(18:2)_4$
1449.7	724.6	$(18:1)(18:2)_3$
1451.7	725.6	$(18:1)_2(18:2)_2$
1473.7	736.6	$(18:2)_3(20:3)$
1475.7	737.6	$(18:1)(18:2)_2(20:3)$
1477.7	738.6	$(18:1)_2(18:2)(20:3)$
1497.7	748.6	$(18:1)(18:2)_2(22:6)$
1499.7	749.6	$(18:1)_2(18:2)(22:6)$

Figure(s)





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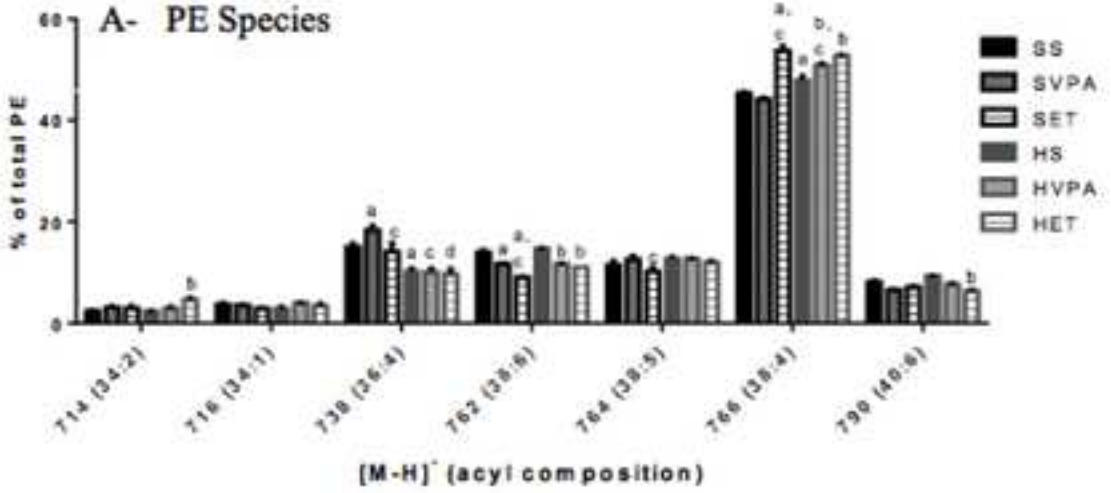
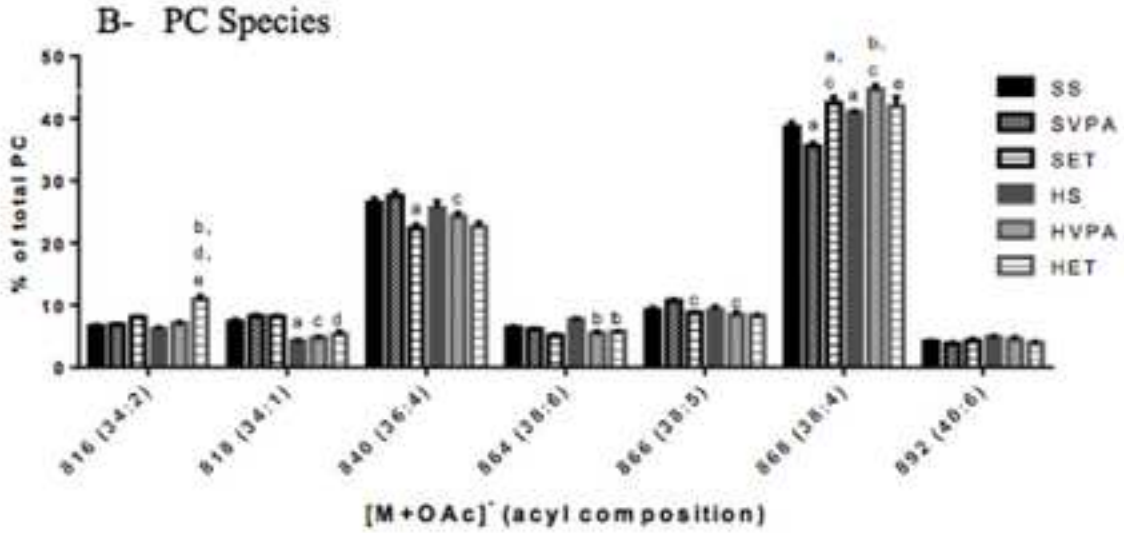
Figure(s)



m/z	Acyl composition	*P* value[1]
714.4	PE(16:0/18:2)	DT x ET
716.4	PE(16:0/18:1)	NS
738.4	PE(16:0/20:4)	DT
762.4	PE(16:0/22:6) PE(18:2/20:4)	ET
764.4	PE(16:0/22:5) PE(18:1/20:4)	ET
766.4	PE(18:0/20:4)	ET, DT
790.4	PE(18:0/22:6)	ET



m/z	Acyl composition	*P* value[1]
816.3	PC(16:0/18:2)	DT x ET
818.3	PC(16:0/18:1)	DT
840.3	PC(16:0/20:4)	ET
864.3	PC(16:0/22:6)	ET
866.3	PC(16:0/22:5)	ET
868.3	PC(18:0/20:4)	ET, DT
892.3	PC(18:0/22:6)	NS