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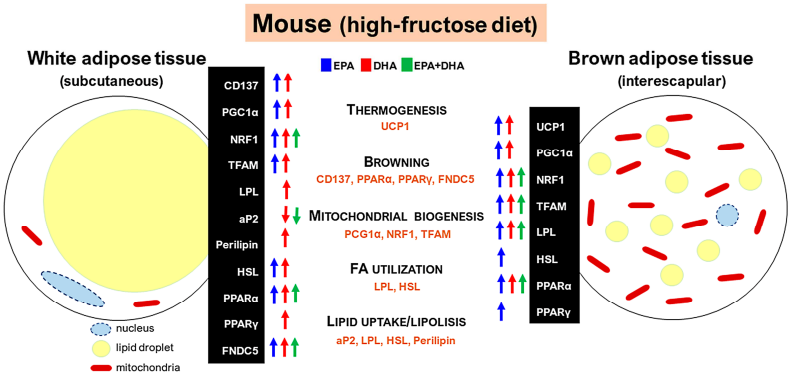

Administration of eicosapentaenoic and docosahexaenoic acids may improve the remodeling and browning in subcutaneous white adipose tissue and thermogenic markers in brown adipose tissue in mice

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Abstract

The role of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in browning and thermogenesis has not been fully elucidated. Thus, we meant to evaluate the effect of EPA and DHA, administered alone or combined, with the activation of browning markers in subcutaneous white adipose tissue (sWAT), and thermogenic markers in brown adipose tissue (BAT). C57BL/6 adult male mice received a control diet or a high-fructose diet (HFru) for eight weeks, but after the first three weeks, HFru was divided into new groups: HFru, HFru+EPA, HFru+DHA, and HFru-EPA+DHA. EPA and DHA diminished adipocyte hypertrophy, recovered markers of browning in sWAT and thermogenic factors in the BAT, and improved gene expressions linked with mitochondrial biogenesis and lipid metabolism. Importantly, EPA and DHA administrated alone showed stronger results than the combination of EPA+DHA. The results suggest that EPA and DHA might be useful as adjuvant strategies to treat metabolic-associated disorders.

Keywords: adipose tissue; fructose; browning; uncoupling protein-1; thermogenesis.

1. Introduction

Recent reports of the activation of the brown adipose tissue (BAT) in humans turned this tissue in an essential target of studies dealing with obesity and metabolic syndrome (Cypess, Chen, Sze et al., 2012,Cypess, Weiner, Roberts-Toler et al., 2015). BAT was shown to be inversely correlated with body mass index and adiposity in humans and was also associated with protective effects on glucose and lipid metabolism (Wang, Zhang, Xu et al., 2015). BAT responds to ATP production with uncoupling mitochondrial oxidation, thus generating heat in a process called thermogenesis, a characteristic of BAT conferred by the presence of uncoupling protein 1 (UCP1) (Crichton, Lee and Kunji, 2017). Together with UCP1, thermogenesis also requires the increase in mitochondrial content (i.e. mitochondrial biogenesis), which is regulated by peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1alpha) that activates the nuclear respiratory factor 1 (NRF1) that in turn controls the expression of nuclear genes involved in the process, including mitochondrial transcription factor A (TFAM) (Nadal-Casellas, Bauza-Thorbrugge, Proenza et al., 2013,Yu, Zhang, Cui et al., 2015,Bargut, Souza-Mello, Aguila et al., 2017).

However, it is questionable whether the brown adipocytes seen in humans are indeed brown, or if they are white adipocytes that under the stimulation acquire a brown-fat phenotype (Lee, Werner, Kebebew et al., 2014). Indeed, beige (or *brite*) adipocytes are white adipose tissue (WAT) that express UCP1, becoming a functional BAT (Fu, Li, Zhang et al., 2015). Beige adipocytes also express specific markers, such as the cluster of differentiation 137 (CD137) (Wu, Bostrom, Sparks et al., 2012). In humans, this browning promotes a metabolic reprogramming, favoring the use of fatty acids as energy sources for thermogenesis (Barquissau, Beuzelin, Pisani et al., 2016). In this sense, four important mediators are lipoprotein lipase (LPL), adipocyte protein 2 (aP2), perilipin 1 (PLIN1) and hormone-sensitive lipase (HSL). LPL acts on fatty acid uptake by the cell, while aP2 couples

intracellular lipids to their biological targets. PLIN and HSL are involved in lipolysis, the first protecting the lipid droplet and the second hydrolyzing triglycerides (Bartelt, Bruns, Reimer et al., 2011,Lorente-Cebrian, Mejhert, Kulyte et al., 2014,Garin-Shkolnik, Rudich, Hotamisligil et al., 2014). As BAT, the beige adipocytes are stimulated by a range of pharmacological and nutritional factors (Barquissau et al., 2016,Bonet, Mercader and Palou, 2017,Chen, Pan and Pfeifer, 2017).

The excessive consumption of fructose leads to metabolic stress since fructose acts as a substrate and as a stimulator of *de novo* lipogenesis and insulin resistance (Karise, Ornellas, Barbosa-da-Silva et al., 2017). In humans, chronic exposure to high-fructose intake leads to hepatic fat accumulation, hepatic insulin resistance, and hypertriglyceridemia that can contribute to the development of metabolic diseases (Tappy, 2018). In adolescents, fructose-rich beverages augmented insulin resistance that could be partially explained by serum uric acid and central adiposity (Lin, Chan, Huang et al., 2016). Regarding the adipose tissue, fructose induces cell hypertrophy associated with local inflammation and insulin resistance (Magliano, Penna-de-Carvalho, Vazquez-Carrera et al., 2015,Bargut, Santos, Machado et al., 2017) and reduces UCP1 expression in female, but not male mice (Dobner, Ress, Rufinatscha et al., 2017). Recent studies support the idea that high fructose consumption leads to adipose tissue inflammation with consequent increased intracellular glycerol, thus culminating in visceral adiposity (DiNicolantonio, Mehta, Onkaramurthy et al., 2018).

Contrarily, n-3 polyunsaturated fatty acids (n-3 PUFA), mainly the eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), have beneficial effects on metabolic disorders and can be recommended both as regular consumption or as supplementation (Calder, 2015). Moreover, there is evidence that EPA and fish oil (rich in EPA and DHA) may induce adipocytes to acquire a beige phenotype and may activate brown thermogenesis (Lund, Larsen and Lauritzen, 2018). Fish oil increased thermogenic markers in

the BAT (Bargut, Silva-e-Silva, Souza-Mello et al., 2016) and WAT (Bargut, Souza-Mello, Mandarim-de-Lacerda et al., 2016). EPA recruited beige adipocytes in mouse subcutaneous adipocytes (Zhao and Chen, 2014), and EPA induced beige-like markers in subcutaneous adipocytes from overweight subjects (Laiglesia, Lorente-Cebrian, Prieto-Hontoria et al., 2016). However, a comparison between the single effects of EPA and DHA still merits an update. Therefore, the study aimed to assess the impact of EPA and DHA alone or combined on the markers of browning in WAT, and of thermogenesis in BAT in mice fed a high fructose diet.

2. Materials and methods

2.1 Animals and diets

Procedures followed the standard guidelines for animal experimentation (NIH Publication number 85-23, revised 1996) and ARRIVE guidelines (Kilkenny, Browne, Cuthill et al., 2010), and were approved by the Ethics Committee for Animal Experimentation (The University of the State of Rio de Janeiro, Protocol Number CEUA/041/2015). The animals have been maintained in ventilated cages under controlled conditions (NexGen system, Allentown Inc., PA, USA, 20±2 ºC and 12 h/12 h dark/light cycle), with free access to food and water. The gene symbols were italicized, with the first letter in uppercase (Davisson, 1994).

Fifty C57Bl/6 male mice with three months of age were randomly assigned to two groups: control group (C, 76% of the total energy content of carbohydrates, n = 10) and high-fructose group (HFru, 47.43 g/100 g diet of fructose, 76% of the total energy content of carbohydrates,

n = 40). After three weeks, the animals were studied for an additional period of five weeks, totalizing eight weeks of the experiment (three weeks on diets plus five weeks of EPA and DHA treatment). EPA (Carb - FE22647; purity > 96%) and DHA (Carb - FD01734; purity > 85%) were purchased from Carbosynth (Compton, Berkshire, UK). The ingredients were incorporated into the diet preparation, and the diets were manufactured by PragSolucoes (Jau, SP, Brazil) following the American Institute of Nutrition's recommendations (AIN 93M) (Reeves, Nielsen and Fahey, 1993) (Table 1).

The C and HFru groups continue for five more weeks. The remaining animals of the HFru group were separated into three additional groups (n=10 each group):

a) HFru plus EPA (HFru-EPA, 47.43 g/100 g diet of fructose, 76% of the total energy content of carbohydrates, plus EPA as 2% of total energy content),

b) HFru plus DHA (HFru-DHA, 47.43 g/100 g diet of fructose, 76% of the total energy content of carbohydrates, plus DHA as 2% of total energy content),

c) HFru plus EPA and DHA (HFru-EPA+DHA, 47.43 g/100 g diet of fructose, 76% of the total energy content of carbohydrates, plus EPA+DHA as 2% of total energy content).

The analysis of the energy intake and adiposity, all animals were used (n=10), but we should divide tissues to microscopy and molecular analysis (n = 3-5 per group). The dose of 2% of EPA and DHA gave for five weeks was based on previous reports (LeMieux, Kalupahana, Scoggin et al., 2015,Pahlavani, Razafimanjato, Ramalingam et al., 2017). Moreover, in the HFru-EPA+DHA group, we decided to reduce by half the amount of each fatty acid to maintain the same treatment and only vary in the fatty acid composition.

Afterward, 6 h fasted animals were anesthetized (sodium pentobarbital, 150 mg/kg, intraperitoneal), and then sacrificed by exsanguination (cut of cervical vessels).

130

131 ## 2.2 Energy intake

132 Food intake was monitored daily (the difference between diet offered and the remaining
133 pellets in the cage after 24 h). The energy intake was estimated as the product of food intake
134 and the energy content of the diet.

135

136 ## 2.3 Tissue extraction and analyses

137 WAT was obtained from the epididymal, retroperitoneal and inguinal pads, while BAT was
138 obtained from the interscapular region. The inguinal fat pad was considered as subcutaneous
139 fat (sWAT), comparable to the gluteofemoral subcutaneous depot in humans. Meanwhile,
140 epididymal and retroperitoneal pads were considered as visceral fat (Chusyd, Wang, Huffman
141 et al., 2016). Fat pads were dissected and weighted, and the adiposity index was calculated
142 (the ratio between the sum of the three WAT pads divided by the body mass).

143 sWAT and BAT samples were rapidly frozen and stored at -80 °C. Alternatively, the
144 samples were kept in a freshly prepared fixative solution (4% formaldehyde w/v, 0.1 M
145 phosphate buffer, pH 7.2) for microscopy. For light microscopy, the tissues were embedded in
146 Paraplast Plus (Sigma-Aldrich, St Louis, MO, USA), the blocks were sectioned with 5 µm,
147 the slices were stained with hematoxylin and eosin, and digital photomicrographs were
148 obtained in a Nikon microscope (model 80i, and DS-Ri1 digital camera, Nikon Instruments,
149 Inc., New York, USA).

150 For UCP-1 immunofluorescence, tissue sections (5 µm thick) were submitted to citrate
151 buffer, pH 6.0 at 60 ^{o} C for 20 min for antigen retrieval, glycine 2%, and blocking buffer
152 (PBS/5% BSA). The sWAT and BAT sections were incubated overnight at the 4 ^{o}C with anti-

UCP1 antibody (SC-6529; Santa Cruz Biotechnology), diluted 1:50 in PBS/1% BSA, followed by incubation for one hour at room temperature with fluorochrome-conjugated secondary antibody anti-goat IgG-Alexa 488 (Invitrogen, Molecular Probes, Carlsbad, CA, USA), diluted 1:50 in PBS/1% BSA. After rinsing in PBS, the slides were mounted with Slow Fade Antifade (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Digital images were captured using confocal microscopy (Nikon Confocal Laser Scanning Microscopy – Model C2, Nikon Instruments, Inc., New York, USA).

2.4 Quantitative real-time PCR (qPCR)

Total RNA of sWAT and BAT was extracted using Trizol reagent (Invitrogen, CA, USA). Nanovue spectroscopy (GE Life Sciences) was used to determine RNA amount. Then, 1 µg RNA was treated with DNAse I (Invitrogen, CA, USA). Afterward, Oligo (dT) primers for mRNA and Superscript III reverse-transcriptase (both Invitrogen, CA, USA) were used for the synthesis of first strand cDNA. qPCR used a Biorad CFX96 cycler and the SYBR green mix (Invitrogen, CA, USA). The primers are described in Table 2: *Ap2*; *Cd137*; fibronectin type III domain-containing 5 (*Fndc5*); *Hsl*; *Lpl*; *Nrf1*; *Plin1*; *Pgc1 alpha*; peroxisome proliferator-activated receptor alpha (*Ppar alpha*); *Ppar gamma*; *Tfam*, and *Ucp1*. We used the endogenous beta-actin to standardize the expression of the selected genes. After the pre-denaturation and polymerase-activation program (4 min at 95 °C), 44 cycles of 95 ^{o}C for 10 s and 60 ^{o}C for 15 s were followed by a melting curve program (60 ^{o}C to 95 ^{o}C with a heating rate of 0.1 ^{o}C/s). Negative controls consisted of wells in which the cDNA was replaced for deionized water. The relative expression ratio of the mRNA was calculated using the equation $2^{-\Delta\Delta Ct}$, in which -ΔCT represents the ratio between the number of cycles (CT) of the target genes with the endogenous control.

177

178 ## 2.5 Western-blot

179 Total proteins of sWAT and BAT were extracted in homogenizing buffer containing protease
180 inhibitors. Equivalent quantities of total protein resuspended in SDS-containing sample buffer
181 were heated for 5 min at 100 °C and separated by SDS-PAGE. After electrophoresis, the
182 proteins were electroblotted onto polyvinyl difluoride transfer membranes (Amersham
183 Biosciences, Piscataway, N.J., USA). The blockade of the membrane was made with nonfat
184 dry milk. Homogenates were incubated with the primary antibody anti-UCP1 (33 kDa; SC-
185 6529; Santa Cruz Biotechnology). Beta-actin (SC81178; Santa Cruz Biotechnology) served as
186 a loading control. We used ECL for protein expression detection system and the Bio-Rad
187 Molecular Imaging ChemiDoc XRS Systems (Bio-Rad, Hercules, CA, USA). We measured
188 the chemiluminescence intensity of the bands with the ImageJ software, version 1.51 (NIH,
189 imagej.nih.gov/ij, USA).

190

191 ## 2.6 Data analysis

192 After testing the data for normal distribution (Kolmogorov-Smirnov test) and
193 homoscedasticity of the variances, the values were shown as the mean and the standard
194 deviation. The differences were analyzed with one-way analysis of variance (ANOVA) and
195 the post hoc test of Holm-Sidak (the *P*-value <0.05 was considered statistically significant,
196 GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla, CA, USA).

197

3. Results

3.1 Energy intake and adiposity

We did not observe significant differences in energy intake, adiposity index and visceral fat mass among groups (Table 3).

3.2 sWAT

3.2.1 Adipose tissue mass

The groups did not show differences in sWAT mass (Table 3).

3.2.2 Structure

In comparison with the C group, the HFru group showed hypertrophied unilocular adipocytes in sWAT, and poor browning activity (confirmed by the absence of UCP1 expression, Fig. 1). In the groups HFru-EPA, HFru-DHA, and HFru-EPA+DHA, there was sWAT remodeling with smaller adipocytes and areas presenting browning deposits (confirmed by the UCP1 expression, Fig. 1). Also, EPA and DHA ameliorated the adipocyte morphology and UCP1 expression in sWAT.

3.2.3 Browning

In comparison with the HFru group, *Ucp1* was higher in the HFru-EPA (+1057%) and HFru-DHA (+1136%). Inversely, *Ucp1* was lower in the HFru-EPA+DHA group in contrast with the HFru-EPA (-58%) and HFru-DHA (-61%) groups. The *Cd137* was increased in HFru-

EPA (+1900%) and HFru-DHA (+2300%) but reduced in HFru-EPA+DHA (HFru-EPA, -72%; HFru-DHA, -77%) (Fig. 2A). Also, UCP1 was elevated in the HFru-EPA (+252%), HFru-DHA (+200%) and HFru-EPA+DHA (+189%) compared to the HFru group (Fig. 2B and C).

3.2.4 Mitochondrial biogenesis

No significant differences were seen comparing the groups C and HFru. However, compared to the HFru group *Pgc1 alpha* was higher in HFru-EPA (+352%), HFru-DHA (+226%), and HFru-EPA+DHA (+106%). Also, *Pgc1 alpha* was lower comparing HFru-EPA vs. HFru-DHA (-28%); HFru-EPA vs. HFru-EPA+DHA (-54%), and HFru-DHA vs. HFru-EPA+DHA (-37%). All treated groups, compared to HFru, showed higher *Nrf1* (more than 1500% higher). Likewise, compared to HFru *Tfam* was higher in HFru-EPA (+655%), in HFru-DHA (+1135%), and in HFru-EPA+DHA (+450%), but there were significant differences between HFru-EPA or HFru-EPA+DHA, and HFru-EPA+DHA (Fig. 3).

3.2.5 Lipid uptake and lipolysis

The *Lpl* was augmented in HFru-DHA (+102% than HFru; +93% than HFru-EPA; +116% than HFru-EPA+DHA). In turn, the *Ap2* was higher in HFru (+224% than C), but without between HFru and HFru-EPA. Also, compared to HFru-EPA, *Ap2* was slightly diminished in HFru-DHA (-38%), and HFru-EPA+DHA (-43%) (Fig. 4).

The *Plin1* was lower in HFru (-90% than C) but augmented in HFru-DHA (+1500% than HFru; +523% than HFru-EPA; +656% than HFru-EPA+DHA); *Hsl* was higher comparing HFru vs. C (+210%). EPA and DHA augmented the expression of *Hsl*, HFru vs. HFru-EPA

(+71%), and HFru vs. HFru-DHA (+197%). EPA+DHA diminished *Hsl*, HFru vs. HFRu-EPA+DHA (-40%), HFru-EPA vs. HFru-EPA+DHA (-66%), and HFru-DHA vs. HFru-EPA+DHA (-115%) (Fig. 4).

3.2.6 Browning mediators

The *Ppar alpha* was markedly higher in the treated groups. Compared to the HFru group, *Ppar alpha* was more than 2000% higher in HFru-EPA, 1700% higher in HFru-DHA, and 1000% higher in HFru-EPA+DHA. *Ppar gamma*, in comparison to the HFu group, was 1700% higher in the HFru-DHA group, 160% higher in the HFru-EPA, and 175% higher in the HFru-EPA+DHA, but comparing HFru-EPA vs. HFru, *Ppar gamma* was +615%. Differently, *Fndc5* was substantially augmented in HFru-EPA, more than 3600% higher than HFru, 130% higher than HFru-DHA, and 155% higher than HFru-EPA+DHA. We must say that *Fndc5* was also significantly higher in both HFru-DHA (+1500%) and HFru-EPA+DHA (+1350%) than HFru (Fig. 5).

3.3 BAT

3.3.1 Adipose tissue mass

The mass of the BAT was greater in the HFru group (+30% than the C group). EPA and DHA lead to a diminished mass of the BAT compared to the HFru group (HFru-EPA, -38%; HFru-DHA, -42%, HFru-EPA+DHA, -41%) (Table 3).

3.3.2 Structure

We have the C group as a basis. Thus, the HFru group showed morphological changes in the brown adipocytes, with greater lipid accumulation and hypertrophy. However, EPA and DHA have returned the adipocytes to a phenotype like the C group. The HFru group showed immunofluorescence for UCP1 in brown adipocytes less marked than in the C group. Also, EPA and DHA were efficient in augmenting the UCP1 expression in brown adipocytes of the groups HFru-EPA, HFru-DHA, and HFru-EPA+DHA groups, a phenotype like the C group (Fig. 6).

3.3.3 Thermogenic markers

The *Ucp1* was lower in the HFru group compared to the C group (-82%). EPA most efficiently augmented *Ucp1*, compared to DHA and EPA+DHA. In comparison with the HFru group, *Ucp1* was +1465% in HFru-EPA, +980% in HFru-DHA, and +240% HFru-EPA+DHA (Fig. 7A). Also, in comparison with the HFru group, UCP1 was higher in the HFru-EPA (+351%), HFru-DHA (+303%), and HFru - EPA+DHA (+211%) (Fig. 7B).

3.3.4 Mitochondrial biogenesis

In brown adipocytes, EPA and DHA increased *Pgc1 alpha*, *Nrf1*, and *Tfam*, but in *Pgc1 alpha* the combination of EPA+DHA was not efficient, while in *Nrf1* the combination of EPA+DHA was more efficient than EPA. The HFru group showed lower *Tfam* than the C group (-70%), but there were no differences between EPA, DHA or EPA+DHA (Fig. 7C).

3.3.5 Fatty acid utilization

In brown adipocytes, the groups C and HFru did not show differences for *Lpl*, but the HFru group had lower *Hsl* than the C group. EPA and DHA augmented *Lpl* compared to the HFru group, but only EPA showed an effect increasing *Hsl* (Fig. 8A).

3.3.6 Thermogenic mediators

In brown adipocytes, *Ppar alpha* and *Ppar gamma* were different with EPA and DHA administration; both were lower in the HFru group compared to the C group. In comparison to the HFru group, EPA (+650%) and DHA (+500%) increased *Ppar alpha*, while EPA+DHA had less effect augmenting *Ppar alpha* (+270%). In comparison to the untreated HFru group, EPA increased *Ppar gamma* more than 840%, while DHA and EPA+DHA were like the HFru group (Fig. 8B).

4 Discussion

The high-fructose diet increased BAT mass and adipocyte size in sWAT and BAT, showing a reduced UCP1 expression. EPA or DHA restored the adipocyte size and UCP1 expression. The action of EPA and DHA on adipocytes seems to be linked with increased mitochondrial biogenesis markers and benefits in mediators of lipid metabolism, probably because n-3 PUFA act through PPAR and FNDC5.

The fructose dose was chosen based on our previous experience (Magliano et al., 2015, Bargut et al., 2017, Schultz, Neil, Aguila et al., 2013) and other groups (Sharma, Li and Ecelbarger, 2015). This high-fructose intake was proposed to maximize the effects of the diet, thus promoting metabolic abnormalities similar to the ones seen in humans consuming

fructose (Tappy and Le, 2010). Once the fructose-provoked alterations were established, the mice were treated with EPA and / or DHA, which dose was also based on previous reports (Depner, Philbrick and Jump, 2013,Lytle, Depner, Wong et al., 2015). The conditions we used EPA and DHA are comparable to the use for treat dyslipidemia (Barter and Ginsberg, 2008,Davidson, Stein, Bays et al., 2007), and increases plasma n-3 PUFA in mice at the same levels as patients consuming 4-6 g/day of n-3 PUFA (Depner et al., 2013,Superko, Superko, Nasir et al., 2013,Di Stasi, Bernasconi, Marchioli et al., 2004). Although it can be considered a high-intake of the fatty acids when compared to everyday human consumption (Calder, 2012), it is well correlated to the doses applied as supplementation treatments.

It is already known that fructose does not cause significant changes in body mass, but it provokes a range of metabolic disruptions, including adipocyte hypertrophy (Magliano et al., 2015). Using the same experimental protocol in a previous study of our group, we demonstrated that neither fructose nor EPA/DHA provoked changes in body mass (Bargut et al., 2017). Importantly, recent literature showed that visceral adiposity, but not body mass, is crucial in fructose-provoked abnormalities (DiNicolantonio et al., 2018) and both adolescents and overweight/obese adult humans consuming fructose showed increased visceral adiposity (Lin et al., 2016,Stanhope, Schwarz, Keim et al., 2009).

Together with adipocyte hypertrophy, fructose also promotes impairment of UCP1 (Dobner et al., 2017). On the other hand, fish oil (rich in both EPA and DHA) induced UCP1 expression in sWAT and BAT and browning of sWAT (Bargut et al., 2016,Bargut et al., 2016). Likewise, EPA recruited beige adipocytes in mouse subcutaneous adipocytes (Zhao and Chen, 2014) and induced beige markers in subcutaneous adipocytes from overweight subjects (Laiglesia et al., 2016). EPA also increased UCP1 levels in BAT from high-fat mice (Pahlavani et al., 2017), and a high-fat diet enriched with long-chain n-3 PUFA increased UCP1 expression (Worsch, Heikenwalder, Hauner et al., 2018). Herein, the fructose-induced

changes observed in adipocytes were effectively treated with EPA and DHA (especially when they were not combined), with increased expression of UCP1 in both tissues and *Cd137* in the sWAT, despite continued fructose administration.

The *Ucp1* expression designates browning in sWAT, while it cannot be considered as an indicator of BAT thermogenesis, and the UCP1 protein expression correlates better with the thermogenic capacity in the BAT (Nedergaard and Cannon, 2013). Therefore, we measured here both gene and protein expressions as thermogenic markers. The UCP1 expression was demonstrated be strongly correlated with the body temperature (Rachid, Silva-Veiga, Graus-Nunes et al., 2018,Martins, Bargut, Aguila et al., 2017), suggesting that thermogenesis is also happing in the present study, although we have not the body temperature of the animals.

Mitochondria are responsible for thermogenesis, and mitochondrial biogenesis is vital to boost mitochondrial oxidation and consequently thermogenic activity (Yu et al., 2015,Bargut et al., 2017). Mitochondrial biogenesis was diminished in WAT, liver and skeletal muscle of mice fed a high-fructose diet (Motta, Bargut, Aguila et al., 2017). In the current study, we found diminished markers of mitochondrial biogenesis in the BAT of HFru mice, supporting the reduced UCP1 expression seen in this group. Contrarily, EPA and DHA induced the mitochondrial biogenesis mediators in both sWAT and BAT. In mice fed a high-fat diet, EPA caused increased *Pgc1alpha* expression in BAT (Pahlavani et al., 2017), and in mice fed a high-fat diet enriched with long-chain n-3 PUFA, a rise in mitochondrial biogenesis markers was also confirmed (Worsch et al., 2018). Moreover, EPA improved mitochondrial content in cultured brown adipocytes (Pahlavani et al., 2017), and increased mitochondrial DNA content and *Pgc1alpha* in mouse subcutaneous adipocytes (Zhao and Chen, 2014).

Lipids are the common substrate necessary for thermogenesis (Cannon and Nedergaard, 2004). Therefore, fatty acids should be continuously supplied when intracellular lipolysis is

stimulated by thermogenic signaling (Khedoe, Hoeke, Kooijman et al., 2015). In this study, corroborating literature, fructose increased *Ap2* and mediators of lipolysis in the sWAT (Garin-Shkolnik et al., 2014,Bargut et al., 2017), and decreased these lipolytic mediators in BAT with consequent lipid accumulation, suggesting thermogenesis blockage (Boll, Weber and Stampfl, 1996,Debosch, Chen, Finck et al., 2013). EPA and DHA increased *Hsl* in primary human adipose tissue-derived stem cells (Fleckenstein-Elsen, Dinnies, Jelenik et al., 2016), and increased lipolysis with elevated expression of *Hsl* (Liu, Li, Huang et al., 2014). We observed that EPA and DHA increased genes associated with fatty acids uptake and lipolysis in sWAT and BAT, indicating that lipolysis is providing a substrate for oxidation (i.e., for thermogenesis), and stocks are being replaced. Mice fed a high-fat diet enriched with long-chain n-3 PUFA showed improvement of markers of lipid uptake (including *Lpl*) in BAT, indicating that the fatty acids supply was continued by an augmented lipid uptake instead of increased lipolysis (Worsch et al., 2018).

PPAR family activation is one of the ways through which n-3 PUFA exert their beneficial effects. PPAR alpha regulates lipid metabolism involving mitochondrial biogenesis and oxidation and fatty acid uptake, and UCP1 expression (Hondares, Rosell, Diaz-Delfin et al., 2011,Barbera, Schluter, Pedraza et al., 2001), while PPAR gamma participates in the brown fat function regulation (Lasar, Rosenwald, Kiehlmann et al., 2018). In the present study, fructose decreased *Ppar alpha* and *gamma* in the BAT, as reported in the liver (Ohashi, Munetsuna, Yamada et al., 2015) and visceral WAT (Bargut et al., 2017). Moreover, n-3 PUFA increased *Ppar* expression and were shown to be potential ligands for PPAR alpha and gamma, with beneficial effects on adipose tissue metabolism (Huang, Chien, Chen et al., 2016). Also, fish oil increased PPAR alpha and gamma expressions in the BAT in association with increased thermogenic markers (Bargut et al., 2016).

Irisin is also an essential mediator in sWAT browning. Irisin is derived from the cleavage of FNDC5 and was first demonstrated in the muscle by PGC1 alpha stimulation, being released into the bloodstream, inducing sWAT browning (Bostrom, Wu, Jedrychowski et al., 2012). Moreover, FNDC5 can be produced and secreted by the adipose tissue (Roca-Rivada, Castelao, Senin et al., 2013). In our study, EPA and DHA increased *Fndc5* expression, possibly due to increased PGC1 alpha. Fenofibrate (a PPAR alpha agonist) increased irisin circulating levels and *Fndc5* gene expression in sWAT of obese mice (Rachid, Penna-de-Carvalho, Bringhenti et al., 2015). The literature reported an association between n-3 PUFA and FNDC5/irisin levels where the supplementation with n-3 PUFA might augment the levels of serum irisin (Ansari, Djalali, Mohammadzadeh Honarvar et al., 2017). Nevertheless, EPA alone was not able to change irisin circulating levels or *Fndc5* gene expression (Huerta, Prieto-Hontoria, Fernandez-Galilea et al., 2015).

In general, EPA and DHA exert several beneficial effects, especially on the adipose tissue metabolism; however, there are significant differences between these n-3 PUFA (Martinez-Fernandez, Laiglesia, Huerta et al., 2015). The essential omega-3 fatty acid alpha-linolenic acid (ALA, 18:3n3) can be converted into EPA and DHA, but the intake of ALA is not enough for the increase of EPA+DHA in subjects on a Western diet. Specifically, a high-ALA diet results in increased EPA and declined DHA concentrations (Greupner, Kutzner, Nolte et al., 2018).

In the current study, EPA and DHA alone induced genes that indicate browning of sWAT and thermogenesis in the BAT. Recent literature has demonstrated EPA effects on browning (Zhao and Chen, 2014,Laiglesia et al., 2016), and on the thermogenesis of brown adipocytes (Kim, Okla, Erickson et al., 2016) and BAT (Pahlavani et al., 2017). EPA is the most studied n-3 PUFA, while results are scarce in the literature concerning DHA. Fish oils enriched with a high dose of EPA or a high-dose of DHA induced UCP1 expression and other thermogenic

markers in sWAT and BAT. Nevertheless, EPA showed more significant results than DHA (Kim, Goto, Yu et al., 2015), in agreement with our findings. One possible explanation is that the lipid mediators derived from EPA are more related to thermogenic activity than the ones derived from DHA (Saito, Terano, Hirai et al., 1997).

A classic study suggests a higher thermogenic activity in BAT with the combination EPA+DHA than EPA and DHA separateness (Oudart, Groscolas, Calgari et al., 1997), but our findings indicate that the mixture EPA+DHA is not ever effective than EPA and DHA. Likewise, in a murine model of myocardial infarction, EPA and DHA showed benefits ameliorating the infarct size, while the combination EPA+DHA showed no effect (Madingou, Gilbert, Tomaro et al., 2016). It is reasonable to consider that the mixture of low-dose EPA+DHA could induce competition for the same receptor (Madingou et al., 2016) reducing the efficiency of the treatment. Also, part of fatty acids effects on thermogenesis is mediated by GRP120, a member of the G protein-coupled receptors (GPCR) family (Fan, Koehler and Chung, 2018,Quesada-Lopez, Cereijo, Turatsinze et al., 2016), and GPCR was shown to present biased signaling leading to n-3 PUFA distinct cell signaling profiles (Wootten, Christopoulos, Marti-Solano et al., 2018). The finding opens possibilities for future researches.

5 Conclusions

In conclusion, in mice metabolically stressed by a high-fructose diet both EPA and DHA are useful in enhancing the expression of genes related to the browning of sWAT, and BAT thermogenic markers. The improvement of the markers of mitochondrial biogenesis and lipid metabolism seems to be the basis of the actions of EPA and DHA, partially mediated by both

PPAR and FNDC5. The findings indicate that both EPA as DHA might be suggested as an adjuvant strategy tackling metabolic-associated disorders.

Declarations of interest

None.

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References

[1] Cypess, A.M., Chen, Y.C., Sze, C., Wang, K., English, J., Chan, O., Holman, A.R., Tal, I., Palmer, M.R., Kolodny, G.M. and Kahn, C.R., 2012. Cold but not sympathomimetics activates human brown adipose tissue in vivo, Proc Natl Acad Sci U S A. 109, 10001-5 (doi: 10.1073/pnas.1207911109).

[2] Cypess, A.M., Weiner, L.S., Roberts-Toler, C., Franquet Elia, E., Kessler, S.H., Kahn, P.A., English, J., Chatman, K., Trauger, S.A., Doria, A. and Kolodny, G.M., 2015. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist, Cell Metab. 21, 33-8 (doi: 10.1016/j.cmet.2014.12.009).

[3] Wang, Q., Zhang, M., Xu, M., Gu, W., Xi, Y., Qi, L., Li, B. and Wang, W., 2015. Brown adipose tissue activation is inversely related to central obesity and metabolic parameters in adult human, PLoS One. 10, e0123795 (doi: 10.1371/journal.pone.0123795).

[4] Crichton, P.G., Lee, Y. and Kunji, E.R., 2017. The molecular features of uncoupling protein 1 support a conventional mitochondrial carrier-like mechanism, Biochimie. 134, 35-50 (doi: 10.1016/j.biochi.2016.12.016).

[5] Nadal-Casellas, A., Bauza-Thorbrugge, M., Proenza, A.M., Gianotti, M. and Llado, I., 2013. Sex-dependent differences in rat brown adipose tissue mitochondrial biogenesis and insulin signaling parameters in response to an obesogenic diet, Mol Cell Biochem. 373, 125-35 (doi: 10.1007/s11010-012-1481-x).

[6] Yu, J., Zhang, S., Cui, L., Wang, W., Na, H., Zhu, X., Li, L., Xu, G., Yang, F., Christian, M. and Liu, P., 2015. Lipid droplet remodeling and interaction with mitochondria in mouse brown adipose tissue during cold treatment, Biochim Biophys Acta. 1853, 918-28 (doi: 10.1016/j.bbamcr.2015.01.020).

[7] Bargut, T.C., Souza-Mello, V., Aguila, M.B. and Mandarim-de-Lacerda, C.A., 2017. Browning of white adipose tissue: lessons from experimental models, Horm Mol Biol Clin Investig. (doi: 10.1515/hmbci-2016-0051).

[8] Lee, P., Werner, C.D., Kebebew, E. and Celi, F.S., 2014. Functional thermogenic beige adipogenesis is inducible in human neck fat, Int J Obes (Lond). 38, 170-6 (doi: 10.1038/ijo.2013.82).

[9] Fu, J., Li, Z., Zhang, H., Mao, Y., Wang, A., Wang, X., Zou, Z. and Zhang, X., 2015. Molecular pathways regulating the formation of brown-like adipocytes in white adipose tissue, Diabetes Metab Res Rev. 31, 433-52 (doi: 10.1002/dmrr.2600).

[10] Wu, J., Bostrom, P., Sparks, L.M., Ye, L., Choi, J.H., Giang, A.H., Khandekar, M., Virtanen, K.A., Nuutila, P., Schaart, G., Huang, K., Tu, H., van Marken Lichtenbelt, W.D., Hoeks, J., Enerback, S., Schrauwen, P. and Spiegelman, B.M., 2012. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human, Cell. 150, 366-76 (doi: 10.1016/j.cell.2012.05.016).

[11] Barquissau, V., Beuzelin, D., Pisani, D.F., Beranger, G.E., Mairal, A., Montagner, A., Roussel, B., Tavernier, G., Marques, M.A., Moro, C., Guillou, H., Amri, E.Z. and Langin, D., 2016. White-to-brite conversion in human adipocytes promotes metabolic reprogramming towards fatty acid anabolic and catabolic pathways, Mol Metab. 5, 352-65 (doi: 10.1016/j.molmet.2016.03.002).

[12] Bartelt, A., Bruns, O.T., Reimer, R., Hohenberg, H., Ittrich, H., Peldschus, K., Kaul, M.G., Tromsdorf, U.I., Weller, H., Waurisch, C., Eychmuller, A., Gordts, P.L., Rinninger, F., Bruegelmann, K., Freund, B., Nielsen, P., Merkel, M. and Heeren, J., 2011. Brown adipose tissue activity controls triglyceride clearance, Nat Med. 17, 200-5 (doi: 10.1038/nm.2297).

[13] Lorente-Cebrian, S., Mejhert, N., Kulyte, A., Laurencikiene, J., Astrom, G., Heden, P., Ryden, M. and Arner, P., 2014. MicroRNAs regulate human adipocyte lipolysis: effects of miR-145 are linked to TNF-alpha, PLoS One. 9, e86800 (doi: 10.1371/journal.pone.0086800).

[14] Garin-Shkolnik, T., Rudich, A., Hotamisligil, G.S. and Rubinstein, M., 2014. FABP4 attenuates PPARgamma and adipogenesis and is inversely correlated with PPARgamma in adipose tissues, Diabetes. 63, 900-11 (doi: 10.2337/db13-0436).

[15] Bonet, M.L., Mercader, J. and Palou, A., 2017. A nutritional perspective on UCP1-dependent thermogenesis, Biochimie. 134, 99-117 (doi: 10.1016/j.biochi.2016.12.014).

[16] Chen, Y., Pan, R. and Pfeifer, A., 2017. Regulation of brown and beige fat by microRNAs, Pharmacol Ther. 170, 1-7 (doi: 10.1016/j.pharmthera.2016.10.004).

[17] Karise, I., Ornellas, F., Barbosa-da-Silva, S., Matsuura, C., Del Sol, M., Aguila, M.B. and Mandarim-de-Lacerda, C.A., 2017. Liver and Metformin: Lessons of a fructose diet in mice, Biochim Open. 4, 19-30 (doi: 10.1016/j.biopen.2017.01.002).

[18] Tappy, L., 2018. Fructose-containing caloric sweeteners as a cause of obesity and metabolic disorders, J Exp Biol. 221, (doi: 10.1242/jeb.164202).

[19] Lin, W.T., Chan, T.F., Huang, H.L., Lee, C.Y., Tsai, S., Wu, P.W., Yang, Y.C., Wang, T.N. and Lee, C.H., 2016. Fructose-Rich Beverage Intake and Central Adiposity, Uric Acid, and Pediatric Insulin Resistance, J Pediatr. 171, 90-96 e1 (doi: 10.1016/j.jpeds.2015.12.061).

[20] Magliano, D.C., Penna-de-Carvalho, A., Vazquez-Carrera, M., Mandarim-de-Lacerda, C.A. and Aguila, M.B., 2015. Short-term administration of GW501516 improves inflammatory state in white adipose tissue and liver damage in high-fructose-fed mice through modulation of the renin-angiotensin system, Endocrine. 50, 355-67 (doi: 10.1007/s12020-015-0590-1).

[21] Bargut, T.C.L., Santos, L.P., Machado, D.G.L., Aguila, M.B. and Mandarim-de-Lacerda, C.A., 2017. Eicosapentaenoic acid (EPA) vs. Docosahexaenoic acid (DHA): Effects in epididymal white adipose tissue of mice fed a high-fructose diet, Prostaglandins Leukot Essent Fatty Acids. 123, 14-24 (doi: 10.1016/j.plefa.2017.07.004).

[22] Dobner, J., Ress, C., Rufinatscha, K., Salzmann, K., Salvenmoser, W., Folie, S., Wieser, V., Moser, P., Weiss, G., Goebel, G., Tilg, H. and Kaser, S., 2017. Fat-enriched rather than high-fructose diets promote whitening of adipose tissue in a sex-dependent manner, J Nutr Biochem. 49, 22-29 (doi: 10.1016/j.jnutbio.2017.07.009).

[23] DiNicolantonio, J.J., Mehta, V., Onkaramurthy, N. and O'Keefe, J.H., 2018. Fructose-induced inflammation and increased cortisol: A new mechanism for how sugar induces visceral adiposity, Prog Cardiovasc Dis. 61, 3-9 (doi: 10.1016/j.pcad.2017.12.001).

[24] Calder, P.C., 2015. Functional Roles of Fatty Acids and Their Effects on Human Health, JPEN J Parenter Enteral Nutr. 39, 18S-32S (doi: 10.1177/0148607115595980).

[25] Lund, J., Larsen, L.H. and Lauritzen, L., 2018. Fish oil as a potential activator of brown and beige fat thermogenesis, Adipocyte. 7, 88-95 (doi: 10.1080/21623945.2018.1442980).

[26] Bargut, T.C., Silva-e-Silva, A.C., Souza-Mello, V., Mandarim-de-Lacerda, C.A. and Aguila, M.B., 2016. Mice fed fish oil diet and upregulation of brown adipose tissue thermogenic markers, Eur J Nutr. 55, 159-69 (doi: 10.1007/s00394-015-0834-0).

[27] Bargut, T.C., Souza-Mello, V., Mandarim-de-Lacerda, C.A. and Aguila, M.B., 2016. Fish oil diet modulates epididymal and inguinal adipocyte metabolism in mice, Food Funct. 7, 1468-76 (doi: 10.1039/c5fo00909j).

[28] Zhao, M. and Chen, X., 2014. Eicosapentaenoic acid promotes thermogenic and fatty acid storage capacity in mouse subcutaneous adipocytes, Biochem Biophys Res Commun. 450, 1446-51 (doi: 10.1016/j.bbrc.2014.07.010).

[29] Laiglesia, L.M., Lorente-Cebrian, S., Prieto-Hontoria, P.L., Fernandez-Galilea, M., Ribeiro, S.M., Sainz, N., Martinez, J.A. and Moreno-Aliaga, M.J., 2016. Eicosapentaenoic acid promotes mitochondrial biogenesis and beige-like features in subcutaneous adipocytes from overweight subjects, J Nutr Biochem. 37, 76-82 (doi: 10.1016/j.jnutbio.2016.07.019).

[30] Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M., Altman, D.G. and Group, N.C.R.R.G.W., 2010. Animal research: reporting in vivo experiments: the ARRIVE guidelines, Br J Pharmacol. 160, 1577-9 (doi: 10.1111/j.1476-5381.2010.00872.x).

[31] Davisson, M.T., 1994. Rules and guidelines for nomenclature of mouse genes. International Committee on Standardized Genetic Nomenclature for Mice, Gene. 147, 157-60 (doi:

[32] Reeves, P.G., Nielsen, F.H. and Fahey, G.C., Jr., 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet, J Nutr. 123, 1939-51 (doi:

[33] LeMieux, M.J., Kalupahana, N.S., Scoggin, S. and Moustaid-Moussa, N., 2015. Eicosapentaenoic acid reduces adipocyte hypertrophy and inflammation in diet-induced obese mice in an adiposity-independent manner, J Nutr. 145, 411-7 (doi: 10.3945/jn.114.202952).

[34] Pahlavani, M., Razafimanjato, F., Ramalingam, L., Kalupahana, N.S., Moussa, H., Scoggin, S. and Moustaid-Moussa, N., 2017. Eicosapentaenoic acid regulates brown adipose tissue metabolism in high-fat-fed mice and in clonal brown adipocytes, J Nutr Biochem. 39, 101-109 (doi: 10.1016/j.jnutbio.2016.08.012).

[35] Chusyd, D.E., Wang, D., Huffman, D.M. and Nagy, T.R., 2016. Relationships between Rodent White Adipose Fat Pads and Human White Adipose Fat Depots, Front Nutr. 3, 10 (doi: 10.3389/fnut.2016.00010).

[36] Schultz, A., Neil, D., Aguila, M.B. and Mandarim-de-Lacerda, C.A., 2013. Hepatic adverse effects of fructose consumption independent of overweight/obesity, Int J Mol Sci. 14, 21873-86 (doi: 10.3390/ijms141121873).

[37] Sharma, N., Li, L. and Ecelbarger, C.M., 2015. Sex differences in renal and metabolic responses to a high-fructose diet in mice, Am J Physiol Renal Physiol. 308, F400-10 (doi: 10.1152/ajprenal.00403.2014).

[38] Tappy, L. and Le, K.A., 2010. Metabolic effects of fructose and the worldwide increase in obesity, Physiol Rev. 90, 23-46 (doi: 10.1152/physrev.00019.2009).

[39] Depner, C.M., Philbrick, K.A. and Jump, D.B., 2013. Docosahexaenoic acid attenuates hepatic inflammation, oxidative stress, and fibrosis without decreasing hepatosteatosis in a Ldlr(-/-) mouse model of western diet-induced nonalcoholic steatohepatitis, J Nutr. 143, 315-23 (doi: 10.3945/jn.112.171322).

[40] Lytle, K.A., Depner, C.M., Wong, C.P. and Jump, D.B., 2015. Docosahexaenoic acid attenuates Western diet-induced hepatic fibrosis in Ldlr-/- mice by targeting the TGFbeta-Smad3 pathway, J Lipid Res. 56, 1936-46 (doi: 10.1194/jlr.M061275).

[41] Barter, P. and Ginsberg, H.N., 2008. Effectiveness of combined statin plus omega-3 fatty acid therapy for mixed dyslipidemia, Am J Cardiol. 102, 1040-5 (doi: 10.1016/j.amjcard.2008.05.056).

[42] Davidson, M.H., Stein, E.A., Bays, H.E., Maki, K.C., Doyle, R.T., Shalwitz, R.A., Ballantyne, C.M., Ginsberg, H.N. and Investigators, C.O.o.p.O.-w.S., 2007. Efficacy and tolerability of adding prescription omega-3 fatty acids 4 g/d to simvastatin 40 mg/d in hypertriglyceridemic patients: an 8-week, randomized, double-blind, placebo-controlled study, Clin Ther. 29, 1354-67 (doi: 10.1016/j.clinthera.2007.07.018).

[43] Superko, H.R., Superko, S.M., Nasir, K., Agatston, A. and Garrett, B.C., 2013. Omega-3 fatty acid blood levels: clinical significance and controversy, Circulation. 128, 2154-61 (doi: 10.1161/CIRCULATIONAHA.113.002731).

[44] Di Stasi, D., Bernasconi, R., Marchioli, R., Marfisi, R.M., Rossi, G., Tognoni, G. and Tacconi, M.T., 2004. Early modifications of fatty acid composition in plasma phospholipids, platelets and mononucleates of healthy volunteers after low doses of n-3 polyunsaturated fatty acids, Eur J Clin Pharmacol. 60, 183-90 (doi: 10.1007/s00228-004-0758-8).

[45] Calder, P.C., 2012. Mechanisms of action of (n-3) fatty acids, J Nutr. 142, 592S-599S (doi: 10.3945/jn.111.155259).

[46] Stanhope, K.L., Schwarz, J.M., Keim, N.L., Griffen, S.C., Bremer, A.A., Graham, J.L., Hatcher, B., Cox, C.L., Dyachenko, A., Zhang, W., McGahan, J.P., Seibert, A., Krauss, R.M., Chiu, S., Schaefer, E.J., Ai, M., Otokozawa, S., Nakajima, K., Nakano, T., Beysen, C., Hellerstein, M.K., Berglund, L. and Havel, P.J., 2009. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans, J Clin Invest. 119, 1322-34 (doi: 10.1172/JCI37385).

[47] Worsch, S., Heikenwalder, M., Hauner, H. and Bader, B.L., 2018. Dietary n-3 long-chain polyunsaturated fatty acids upregulate energy dissipating metabolic pathways conveying anti-obesogenic effects in mice, Nutr Metab (Lond). 15, 65 (doi: 10.1186/s12986-018-0291-x).

[48] Nedergaard, J. and Cannon, B., 2013. UCP1 mRNA does not produce heat, Biochim Biophys Acta. 1831, 943-9 (doi: 10.1016/j.bbalip.2013.01.009).

[49] Rachid, T.L., Silva-Veiga, F.M., Graus-Nunes, F., Bringhenti, I., Mandarim-de-Lacerda, C.A. and Souza-Mello, V., 2018. Differential actions of PPAR-alpha and

PPAR-beta/delta on beige adipocyte formation: A study in the subcutaneous white adipose tissue of obese male mice, PLoS One. 13, e0191365 (doi: 10.1371/journal.pone.0191365).

[50] Martins, F.F., Bargut, T.C.L., Aguila, M.B. and Mandarim-de-Lacerda, C.A., 2017. Thermogenesis, fatty acid synthesis with oxidation, and inflammation in the brown adipose tissue of ob/ob (-/-) mice, Ann Anat. 210, 44-51 (doi: 10.1016/j.aanat.2016.11.013).

[51] Motta, V.F., Bargut, T.L., Aguila, M.B. and Mandarim-de-Lacerda, C.A., 2017. Treating fructose-induced metabolic changes in mice with high-intensity interval training: insights in the liver, white adipose tissue and skeletal muscle, J Appl Physiol (1985). jap 00154 2017 (doi: 10.1152/japplphysiol.00154.2017).

[52] Cannon, B. and Nedergaard, J., 2004. Brown adipose tissue: function and physiological significance, Physiol Rev. 84, 277-359 (doi: 10.1152/physrev.00015.2003).

[53] Khedoe, P.P., Hoeke, G., Kooijman, S., Dijk, W., Buijs, J.T., Kersten, S., Havekes, L.M., Hiemstra, P.S., Berbee, J.F., Boon, M.R. and Rensen, P.C., 2015. Brown adipose tissue takes up plasma triglycerides mostly after lipolysis, J Lipid Res. 56, 51-9 (doi: 10.1194/jlr.M052746).

[54] Boll, M., Weber, L.W. and Stampfl, A., 1996. Nutritional regulation of the activities of lipogenic enzymes of rat liver and brown adipose tissue, Z Naturforsch C. 51, 859-69 (doi:

[55] Debosch, B.J., Chen, Z., Finck, B.N., Chi, M. and Moley, K.H., 2013. Glucose Transporter-8 (GLUT8) Mediates Glucose Intolerance and Dyslipidemia in High-Fructose Diet-Fed Male Mice, Mol Endocrinol. 27, 1887-96 (doi: 10.1210/me.2013-1137).

[56] Fleckenstein-Elsen, M., Dinnies, D., Jelenik, T., Roden, M., Romacho, T. and Eckel, J., 2016. Eicosapentaenoic acid and arachidonic acid differentially regulate adipogenesis, acquisition of a brite phenotype and mitochondrial function in primary human adipocytes, Mol Nutr Food Res. 60, 2065-75 (doi: 10.1002/mnfr.201500892).

[57] Liu, P., Li, C., Huang, J. and Ji, H., 2014. Regulation of adipocytes lipolysis by n-3 HUFA in grass carp (Ctenopharyngodon idellus) in vitro and in vivo, Fish Physiol Biochem. 40, 1447-60 (doi: 10.1007/s10695-014-9939-2).

[58] Hondares, E., Rosell, M., Diaz-Delfin, J., Olmos, Y., Monsalve, M., Iglesias, R., Villarroya, F. and Giralt, M., 2011. Peroxisome proliferator-activated receptor alpha (PPARalpha) induces PPARgamma coactivator 1alpha (PGC-1alpha) gene expression and contributes to thermogenic activation of brown fat: involvement of PRDM16, J Biol Chem. 286, 43112-22 (doi: 10.1074/jbc.M111.252775).

[59] Barbera, M.J., Schluter, A., Pedraza, N., Iglesias, R., Villarroya, F. and Giralt, M., 2001. Peroxisome proliferator-activated receptor alpha activates transcription of the brown fat uncoupling protein-1 gene. A link between regulation of the thermogenic and lipid oxidation pathways in the brown fat cell, J Biol Chem. 276, 1486-93 (doi: 10.1074/jbc.M006246200).

[60] Lasar, D., Rosenwald, M., Kiehlmann, E., Balaz, M., Tall, B., Opitz, L., Lidell, M.E., Zamboni, N., Krznar, P., Sun, W., Varga, L., Stefanicka, P., Ukropec, J., Nuutila, P., Virtanen, K., Amri, E.Z., Enerback, S., Wahli, W. and Wolfrum, C., 2018. Peroxisome Proliferator Activated Receptor Gamma Controls Mature Brown Adipocyte Inducibility through Glycerol Kinase, Cell Rep. 22, 760-773 (doi: 10.1016/j.celrep.2017.12.067).

[61] Ohashi, K., Munetsuna, E., Yamada, H., Ando, Y., Yamazaki, M., Taromaru, N., Nagura, A., Ishikawa, H., Suzuki, K., Teradaira, R. and Hashimoto, S., 2015. High fructose consumption induces DNA methylation at PPARalpha and CPT1A promoter regions in the rat liver, Biochem Biophys Res Commun. 468, 185-9 (doi: 10.1016/j.bbrc.2015.10.134).

[62] Huang, C.W., Chien, Y.S., Chen, Y.J., Ajuwon, K.M., Mersmann, H.M. and Ding, S.T., 2016. Role of n-3 Polyunsaturated Fatty Acids in Ameliorating the Obesity-Induced Metabolic Syndrome in Animal Models and Humans, Int J Mol Sci. 17, (doi: 10.3390/ijms17101689).

[63] Bostrom, P., Wu, J., Jedrychowski, M.P., Korde, A., Ye, L., Lo, J.C., Rasbach, K.A., Bostrom, E.A., Choi, J.H., Long, J.Z., Kajimura, S., Zingaretti, M.C., Vind, B.F., Tu, H., Cinti, S., Hojlund, K., Gygi, S.P. and Spiegelman, B.M., 2012. A PGC1-alpha-

dependent myokine that drives brown-fat-like development of white fat and thermogenesis, Nature. 481, 463-8 (doi: 10.1038/nature10777).

[64] Roca-Rivada, A., Castelao, C., Senin, L.L., Landrove, M.O., Baltar, J., Belen Crujeiras, A., Seoane, L.M., Casanueva, F.F. and Pardo, M., 2013. FNDC5/irisin is not only a myokine but also an adipokine, PLoS One. 8, e60563 (doi: 10.1371/journal.pone.0060563).

[65] Rachid, T.L., Penna-de-Carvalho, A., Bringhenti, I., Aguila, M.B., Mandarim-de-Lacerda, C.A. and Souza-Mello, V., 2015. Fenofibrate (PPARalpha agonist) induces beige cell formation in subcutaneous white adipose tissue from diet-induced male obese mice, Mol Cell Endocrinol. 402, 86-94 (doi: 10.1016/j.mce.2014.12.027).

[66] Ansari, S., Djalali, M., Mohammadzadeh Honarvar, N., Mazaherioun, M., Zarei, M., Agh, F., Gholampour, Z. and Javanbakht, M.H., 2017. The Effect of n-3 Polyunsaturated Fatty Acids Supplementation on Serum Irisin in Patients with Type 2 Diabetes: A Randomized, Double-Blind, Placebo-Controlled Trial, Int J Endocrinol Metab. 15, e40614 (doi: 10.5812/ijem.40614).

[67] Huerta, A.E., Prieto-Hontoria, P.L., Fernandez-Galilea, M., Sainz, N., Cuervo, M., Martinez, J.A. and Moreno-Aliaga, M.J., 2015. Circulating irisin and glucose metabolism in overweight/obese women: effects of alpha-lipoic acid and eicosapentaenoic acid, J Physiol Biochem. 71, 547-58 (doi: 10.1007/s13105-015-0400-5).

[68] Martinez-Fernandez, L., Laiglesia, L.M., Huerta, A.E., Martinez, J.A. and Moreno-Aliaga, M.J., 2015. Omega-3 fatty acids and adipose tissue function in obesity and metabolic syndrome, Prostaglandins Other Lipid Mediat. 121, 24-41 (doi: 10.1016/j.prostaglandins.2015.07.003).

[69] Greupner, T., Kutzner, L., Nolte, F., Strangmann, A., Kohrs, H., Hahn, A., Schebb, N.H. and Schuchardt, J.P., 2018. Effects of a 12-week high-alpha-linolenic acid intervention on EPA and DHA concentrations in red blood cells and plasma oxylipin pattern in subjects with a low EPA and DHA status, Food Funct. 9, 1587-1600 (doi: 10.1039/c7fo01809f).

[70] Kim, J., Okla, M., Erickson, A., Carr, T., Natarajan, S.K. and Chung, S., 2016. Eicosapentaenoic Acid Potentiates Brown Thermogenesis through FFAR4-dependent Up-regulation of miR-30b and miR-378, J Biol Chem. 291, 20551-62 (doi: 10.1074/jbc.M116.721480).

[71] Kim, M., Goto, T., Yu, R., Uchida, K., Tominaga, M., Kano, Y., Takahashi, N. and Kawada, T., 2015. Fish oil intake induces UCP1 upregulation in brown and white adipose tissue via the sympathetic nervous system, Sci Rep. 5, 18013 (doi: 10.1038/srep18013).

[72] Saito, J., Terano, T., Hirai, A., Shiina, T., Tamura, Y. and Saito, Y., 1997. Mechanisms of enhanced production of PGI2 in cultured rat vascular smooth muscle cells enriched with eicosapentaenoic acid, Atherosclerosis. 131, 219-28 (doi:

[73] Oudart, H., Groscolas, R., Calgari, C., Nibbelink, M., Leray, C., Le Maho, Y. and Malan, A., 1997. Brown fat thermogenesis in rats fed high-fat diets enriched with n-3 polyunsaturated fatty acids, Int J Obes Relat Metab Disord. 21, 955-62 (doi:

[74] Madingou, N., Gilbert, K., Tomaro, L., Prud'homme Touchette, C., Trudeau, F., Fortin, S. and Rousseau, G., 2016. Comparison of the effects of EPA and DHA alone or in combination in a murine model of myocardial infarction, Prostaglandins Leukot Essent Fatty Acids. 111, 11-6 (doi: 10.1016/j.plefa.2016.06.001).

[75] Fan, R., Koehler, K. and Chung, S., 2018. Adaptive thermogenesis by dietary n-3 polyunsaturated fatty acids: Emerging evidence and mechanisms, Biochim Biophys Acta Mol Cell Biol Lipids. (doi: 10.1016/j.bbalip.2018.04.012).

[76] Quesada-Lopez, T., Cereijo, R., Turatsinze, J.V., Planavila, A., Cairo, M., Gavalda-Navarro, A., Peyrou, M., Moure, R., Iglesias, R., Giralt, M., Eizirik, D.L. and Villarroya, F., 2016. The lipid sensor GPR120 promotes brown fat activation and FGF21 release from adipocytes, Nat Commun. 7, 13479 (doi: 10.1038/ncomms13479).

[77] Wootten, D., Christopoulos, A., Marti-Solano, M., Babu, M.M. and Sexton, P.M., 2018. Mechanisms of signalling and biased agonism in G protein-coupled receptors, Nat Rev Mol Cell Biol. 19, 638-653 (doi: 10.1038/s41580-018-0049-3).

Figure legends

Figure 1. sWAT: structure and immunofluorescence. The HFru group, when compared to the C group, showed hypertrophy of the unilocular adipocytes and reduction in the browning deposit, confirmed by the absence of uncoupling protein 1 (UCP1) expression (Fig. 1A-B, F-G). The HFru-EPA, HFru-DHA and HFru-EPA+DHA groups showed improvement in sWAT remodeling, exhibiting smaller adipocytes and areas with browning deposits, confirmed by UCP1 expression (Fig. 1C-E, H-J). **A-E**: hematoxylin and eosin staining. **F-J**: labeling for UCP1. Scale bars: 10 µm. Groups: C, control; HFru, high-fructose; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 2. sWAT: gene and protein expressions of browning markers. Values are the mean ± SD (n = 5). Significant differences were tested with one-way ANOVA and posthoc test of Holm Sidak: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Groups: C, control; HFru, high-fructose; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Abbreviations: CD137, cluster of differentiation 137; UCP1, uncoupling protein 1.

Figure 3. sWAT: gene expressions of mitochondrial biogenesis. Values are the mean ± SD (n = 5). Significant differences were tested with one-way ANOVA and posthoc test of Holm Sidak: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Groups: C, control; HFru, high-fructose; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Abbreviations: NRF1, nuclear respiratory factor 1; PGC1, peroxisome proliferator-activated receptor gamma coactivator 1; TFAM, mitochondrial transcription factor A.

Figure 4. sWAT: gene expressions of lipid metabolism and lipolysis markers. Values are the mean ± SD (n = 5). Significant differences were tested with one-way ANOVA and posthoc test of Holm Sidak: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Groups: C, control; HFru, high-fructose; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Abbreviations: aP2, adipocyte protein 2; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; PLIN, perilipin.

Figure 5. sWAT: gene expressions of browning mediators. Values are the mean ± SD (n = 5). Significant differences were tested with one-way ANOVA and posthoc test of Holm Sidak: *P < 0.05, **P < 0.01, and ***P < 0.001. Groups: C, control; HFru, high-fructose; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Abbreviations: FNDC5, fibronectin type III domain containing 5 (precursor of irisin); PPAR, peroxisome proliferator-activated receptor.

Figure 6. BAT: structure and immunofluorescence. The HFru group, when compared to the C group, showed cytoplasmic changes exhibiting greater lipid accumulation - adipocyte hypertrophy (Fig. 5A-B). The HFru-EPA, HFru-DHA and HFru-EPA+DHA groups presented recovery and morphological improvement of the adipocytes, whose histological structure remained like the C group (Fig. 5C-E). The immunofluorescence for uncoupling protein 1 (UCP1) showed that the HFru group when compared to the C group, presented a significant decrease in the expression of this protein (Fig. 5F-G). The HFru-EPA, HFru-DHA and HFru-EPA+DHA groups filed UCP1 expression like the C group (Fig; 5H-J). **A-E**: hematoxylin and eosin staining. **F-J**: labeling for UCP1. Scale bars: 10 µm. Groups: C, control; HFru, high-fructose; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 7. BAT: gene and protein expressions of thermogenic markers and mitochondrial biogenesis markers. Values are the mean ± SD (n = 5). Significant differences were tested with one-way ANOVA and posthoc test of Holm Sidak: *P < 0.05, **P < 0.01, and ***P < 0.001. Groups: C, control; HFru, high-fructose; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Abbreviations: PGC1, peroxisome proliferator-activated receptor gamma coactivator 1; NRF1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; UCP1, uncoupling protein 1.

Figure 8. BAT: gene expressions of lipid metabolism markers and thermogenic mediators. Values are the mean ± SD (n = 5). Significant differences were tested with one-way ANOVA and posthoc test of Holm Sidak: *P < 0.05, **P < 0.01, and ***P < 0.001. Groups: C, control; HFru, high-fructose; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Abbreviations: HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor.

Table 1. Composition and energy content of the diets (AIN 93M based diets).

Ingredients (g/kg)	**C**	**HFru**	**HFru+EPA**	**HFru+DHA**	**HFru+EPA+DHA**
Casein (≥ 85% of protein)	140.0	140.0	140.0	140.0	140.0
Cornstarch	620.7	146.4	146.4	146.4	146.4
Sucrose	100.0	100.0	100.0	100.0	100.0
Fructose	-	474.3	474.3	474.3	474.3
Soybean oil	40.0	40.0	31.53	31.53	31.53
EPA	-	-	8.47	-	4.235
DHA	-	-	-	8.47	4.235
Fiber	50.0	50.0	50.0	50.0	50.0
Vitamin mix[a]	10.0	10.0	10.0	10.0	10.0
Mineral mix[a]	35.0	35.0	35.0	35.0	35.0
L-Cystin	1.8	1.8	1.8	1.8	1.8
Choline	2.5	2.5	2.5	2.5	2.5
Total mass	1000	1000	1000	1000	1000
Proteins (% Energy)	14	14	14	14	14
Carbohydrates (% Energy)	76	76	76	76	76
Fructose (% Energy)	-	50	50	50	50
Lipids (% Energy)	10	10	10	10	10
EPA (% Energy)	-	-	2	-	1
DHA (% Energy)	-	-	-	2	1
Energy content (kcal/kg)	3811	3811	3811	3811	3811

[a] Mineral and vitamin mixtures are in accordance with AIN 93M

Abbreviations: control (C), high-fructose (HFru), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA).

Table 2. Primes.

Primers	**FW (5'-3')**	**RV**
Ap2	TGGAAGCTTGTCTCCAGTGA	AATCCCCATTTACGCTGATG
β-Actin	CTCCGGCATGTGCAA	CCCACCATCACACCCT
Cd137	CCCACATATTCAAGCAACCA	GCTCATAGCCTCCTCCTCCT
Fndc5	GGTGCTGATCATTGTTGTGG	CGCTCTTGGTTTTCTCCTTG
Hsl	GACAGAGGCAGAGGACCATT	TGAGGAACAGCGAAGTGTCT
Lpl	TTCAACCACAGCAGCAAGAC	TTCTCTCTTGTACAGGGCGG
Nrf1	GTTGGTACAGGGGCAACAGT	GTAACGTGGCCCAGTTTTGT
Plin1	ACGACCAGACAGACACAGAG	GGCTGTAACCTCTCTGAGCA
Pgc1α	AACCACACCCACAGGATCAGA	TCTTCGCTTTATTGCTCCATGA
Pparα	CAAGGCCTCAGGGTACCACTAC	GCCGAATAGTTCGCCGAAA
Pparɣ	CACAATGCCATCAGGTTTGG	GCTGGTCGATATCACTGGAGATC
Tfam	GAAGAACGCATGGAGGAGAG	TTCTGGGGAGAGTTGCAGTT
Ucp1	TCTCAGCCGGCTTAATGACT	TGCATTCTGACCTTCACGAC

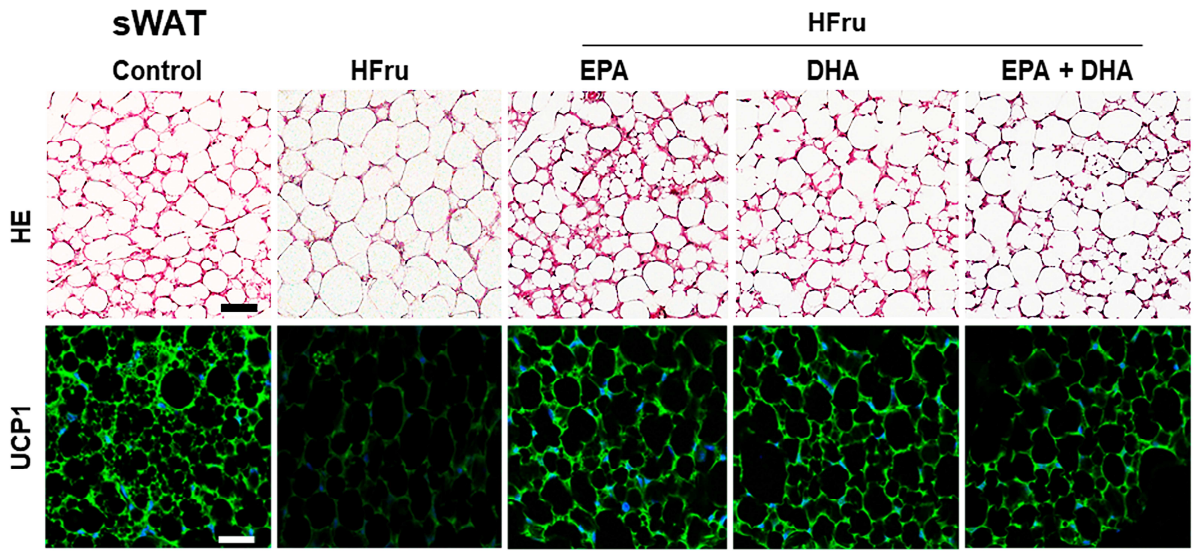
Abbreviations: aP2, adipocyte protein 2; CD, cluster of differentiation; FNDC5, fibronectin type III domain containing 5 (precursor of irisin); HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; NRF1, nuclear respiratory factor 1; PLIN, perilipin; PGC1, peroxisome proliferator-activated receptor gamma coactivator 1; PPAR, peroxisome proliferator-activated receptor; TFAM, mitochondrial transcription factor A; and UCP1, uncoupling protein 1.

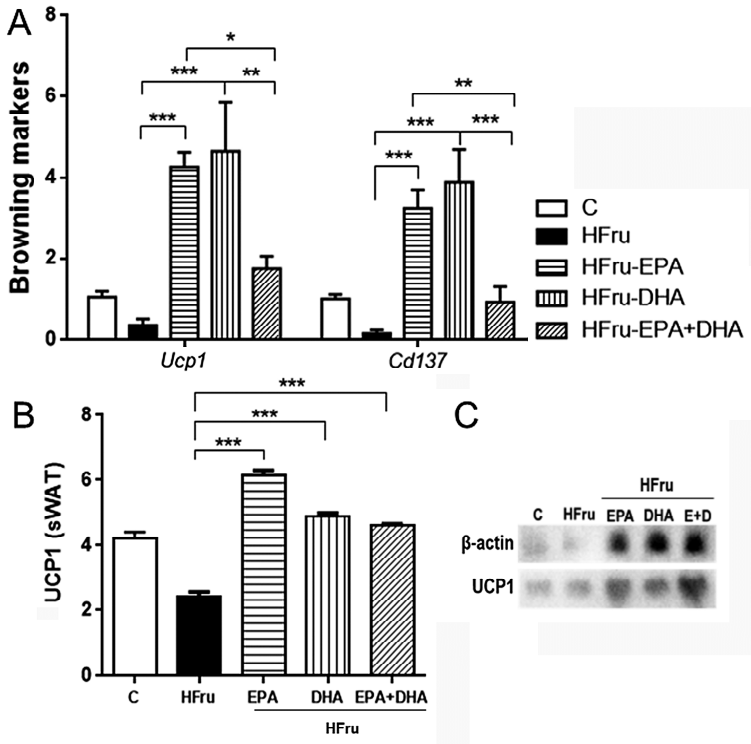
Table 3. Energy intake and adiposity.

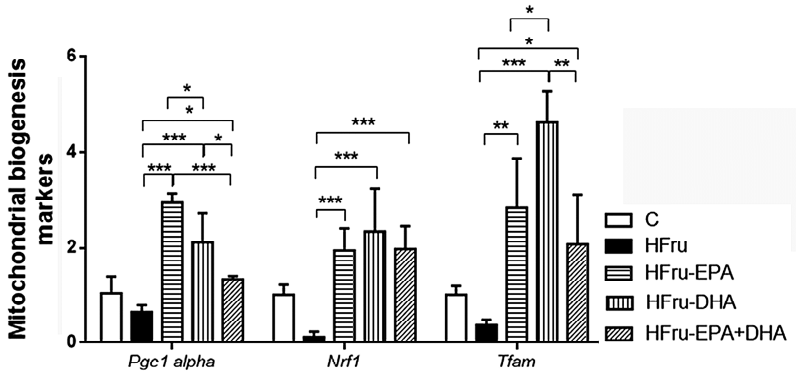
Data	C	HFru	HFru+EPA	HFru+DHA	HFru+EPA+ DHA
EI (kJ)	43.74 ± 8.20	44.93 ± 9.90	44.01 ± 7.19	42.37 ± 5.50	43.26 ± 5.98
AI (%)	3.55 ± 0.97	2.97 ± 0.73	2.81 ± 0.41	3.25 ± 0.56	3.07 ± 0.67
Visceral fat (g; epididymal plus retroperitoneal pads)	0.76 ± 0.22	0.63 ± 0.16	0.57 ± 0.09	0.68 ± 0.12	0.59 ± 0.07
sWAT mass (g)	0.17 ± 0.05	0.17 ± 0.02	0.14 ± 0.03	0.17 ± 0.04	0.14 ± 0.04
BAT mass (g)	0.09 ± 0.01	0.12 ± 0.02[a]	0.07 ± 0.01[b]	0.07 ± 0.02[b]	0.07 ± 0.01[b]

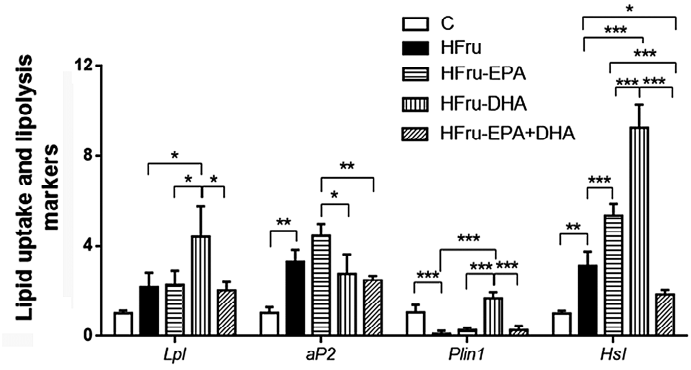
Legend: Control (C), high-fructose (HFru), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA). Values are the means ± SD, n=10/group. Significant differences are indicated ($P < 0.05$), one-way ANOVA and post hoc test of Holm Sidak: a ≠ C and b ≠ HFru.

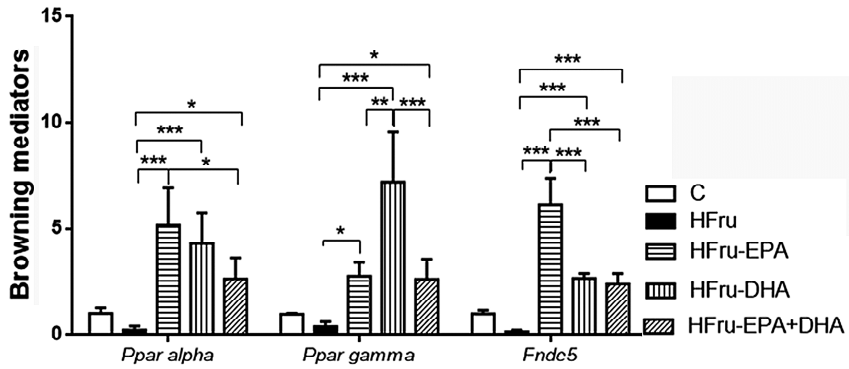
Abbreviations: AI, adiposity index; BAT, brown adipose tissue; EI, energy intake; sWAT, subcutaneous white adipose tissue.

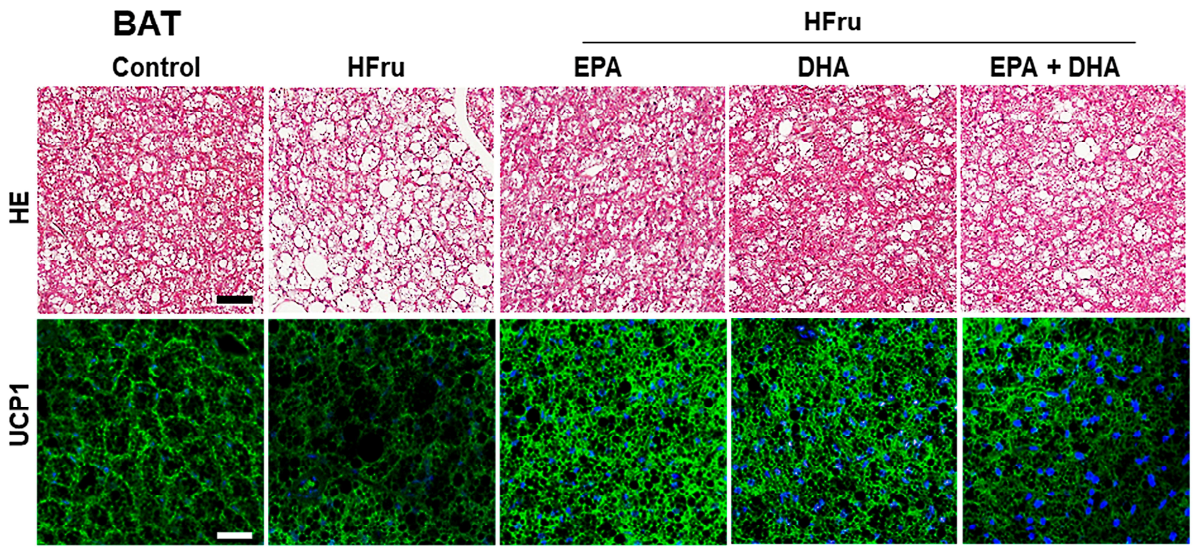


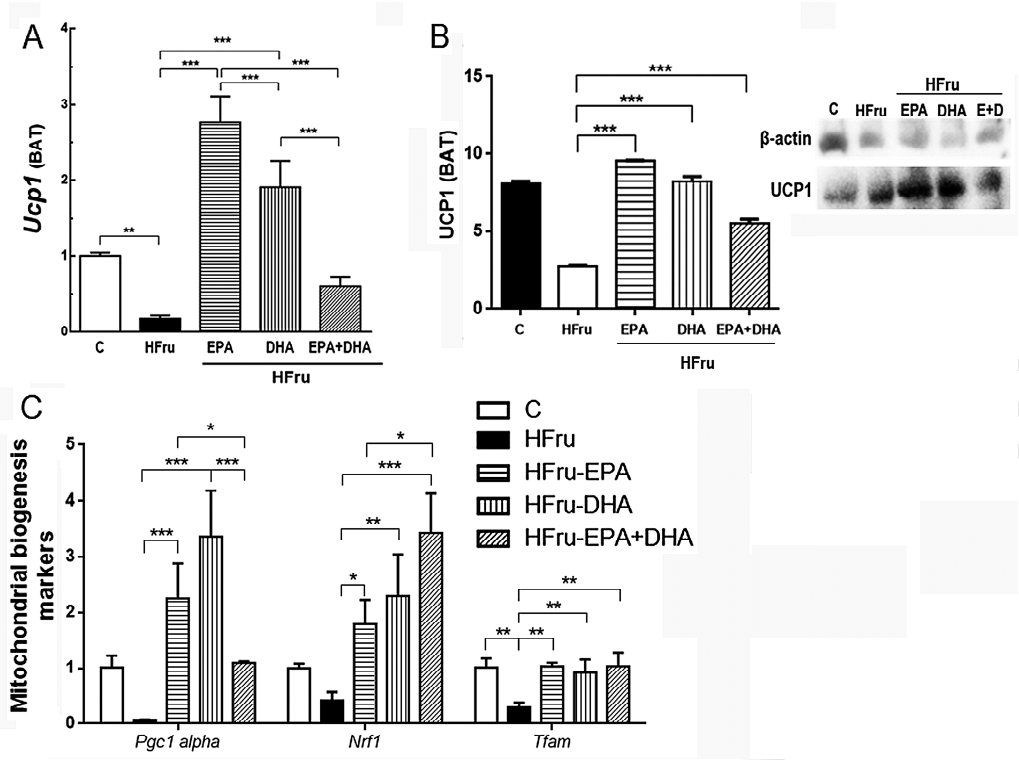


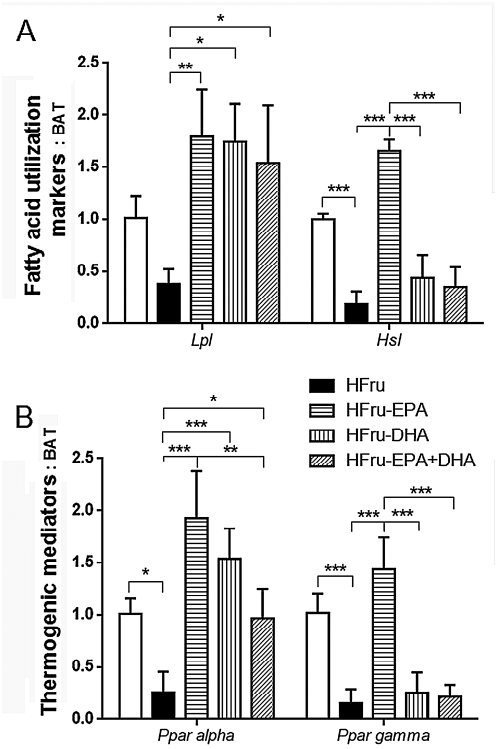












Highlights

- N-3 polyunsaturated fatty acids have beneficial effects on metabolic disorders;
- We studied the subcutaneous white adipose tissue and brown adipose tissue;
- EPA and DHA may enhance the gene expressions related to browning and thermogenesis;
- EPA and DHA may improve mitochondrial biogenesis and lipid metabolism.