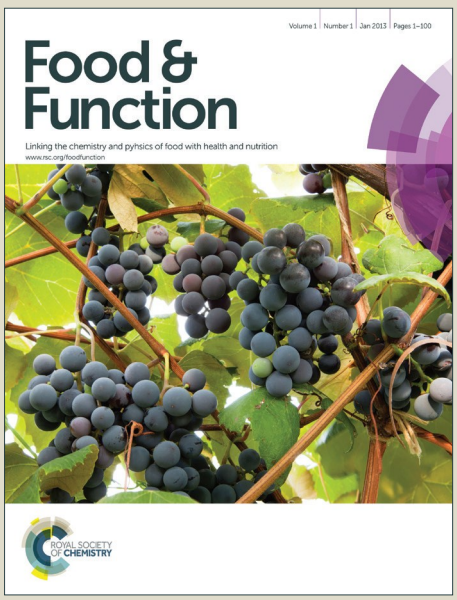
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Fish oil diet modulates epididymal and inguinal adipocyte metabolism in mice

Thereza Cristina Lonzetti Bargut,

Vanessa Souza-Mello,

Carlos Alberto Mandarim-de-Lacerda[1],

Marcia Barbosa Aguila

Laboratory of Morphometry, Metabolism, and Cardiovascular Disease, Biomedical Center, Institute of Biology, State University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

[1] Corresponding author: Laboratory of Morphometry, Metabolism and Cardiovascular Disease, Biomedical Center, Institute of Biology, State University of Rio de Janeiro, Av 28 de Setembro 87 fds, 20551-030 Rio de Janeiro, RJ, Brazil. Phone: (+55.21) 2868.8316. FAX: 2868.8033. E-mail: mandarim@uerj.br, Website: www.lmmc.uerj.br.

Abstract

We aimed to investigate the impact of different high-fat diets containing fish oil on adiposity and white adipose tissue (WAT) function in mice, comparing the effects on epididymal (eWAT) and subcutaneous (sWAT) depots. For this, we used C57BL/6 male mice fed four types of diets for eight weeks: standard chow (SC), high-fat lard (HF-L), high-fat lard plus fish oil (HF-L+FO), and high-fat fish oil (HF-FO). The HF-L group had a greater body mass (BM) gain, insulin resistance, an increased gene expression related to lipogenesis (CD36, aP2, SREBP1c, and FAS) and a decreased gene expression of perilipin in both eWAT and sWAT, and reduced genes related to beta-oxidation (CPT-1a) and to mitochondrial biogenesis (PGC1alpha, NRF1, and TFAM) in eWAT and sWAT. On the other hand, the HF-L+FO and HF-FO groups showed a smaller BM gain and adiposity, and normalization of insulin resistance and lipogenic genes in both eWAT and sWAT. These animals also showed a decreased perilipin gene expression and an elevated beta-oxidation and mitochondrial biogenesis genes in eWAT and sWAT. 'Beige' adipocytes were identified in sWAT of the HF-FO animals. In conclusion, fish oil intake has anti-obesity effects through modulation of both eWAT and sWAT metabolism in mice and is relevant in diminishing the BM gain, adiposity, and insulin resistance even in concomitance with a high-fat lard diet intake in mice.

Keywords: n-3 polyunsaturated fatty acids; fish oil; adipose tissue; browning; beige cells.

Introduction

Mammals have depots of white adipose tissue (WAT) in their body containing large unilocular cells, the adipocytes, which have the primary function of store fat as triacylglycerol. The adipocytes release fatty acids, in response to nutritional and hormonal stimuli. The WAT also functions as an endocrine organ that secretes a range of mediators that act controlling glucose metabolism and insulin signaling. [1, 2]

Visceral white adipose tissue dysfunction underlies insulin resistance and the majority of obesity comorbidities. [3] Conversely, subcutaneous white adipocytes exert a protective role. Recently, the intra-abdominal transplant of subcutaneous white adipose tissue reverted glucose intolerance, hepatic steatosis and systemic inflammation in mice fed a high-fat diet. [4, 5] Also, the transdifferentiation of white-to-brown adipocytes, generating the beige cells, occurs in subcutaneous WAT. White adipocytes acquire a multilocular appearance and high levels of uncoupling protein (UCP) type 1. [6, 7] These changes lead to elevated thermogenic activity in this tissue with the improvement of obesity and insulin resistance. [8-10]

Fish oil has high amounts of the n-3 PUFA eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). Much attention has been given to the beneficial effects of fish oil (and n-3 PUFA) on dyslipidemia, cardiovascular diseases, and inflammatory diseases, [11] and we know now that these fatty acids are also important in the prevention of obesity in humans [12, 13] and animal models. [14-16] However, little is known about the mechanisms involved in the anti-obesity effects of fish oil. [17, 18]

The present study aimed to investigate the impact of different high-fat diets containing fish oil on adiposity and WAT function in mice, comparing the epididymal (visceral) and the inguinal (subcutaneous) adipose tissue.

Materials and Methods

Animals and diet

All procedures were approved by the local Ethics Committee for Animal Experimentation (Protocol Number CEUA/018/2013) in accordance with the conventional guidelines for animal experimentation (NIH Publication number 85-23, revised 1996). The mice were maintained under controlled conditions, group housed (five animals per cage) in ventilated cages (Nexgen system, Allentown Inc., PA, USA), 20 ± 2º C, 12 h/12 h dark/light cycle, and free access to food and water. Forty 3-mo-old C57Bl/6 male mice were randomly assigned to four groups (n = 10/group):

a) Standard-chow group (SC): 40 g soybean oil/Kg diet, 10 % of the total energy content of lipids;
b) High-fat lard group (HF-L): (40 g soybean oil + 238 g lard)/Kg diet, 50 % of the total energy content of lipids;
c) High-fat lard plus fish oil group (HF-L+FO): (40 g soybean oil + 119 g lard + 119 g FO)/Kg diet, 50 % of the total energy content of lipids; and
d) High-fat fish oil group (HF-FO): (40 g soybean oil + 238 g FO)/Kg diet, 50 % of the total energy content of lipids.

The diets were elaborated with purified nutrients by PragSolucoes (Jau, SP, Brazil) based on the American Institute of Nutrition's recommendations (AIN 93M). [19] The diets were offered over an eight-week period (Table 1).

Energy intake and body mass gain

Energy intake was monitored daily through the difference between what was offered and what was left in the cage. The body mass (BM) gain was calculated as the difference between BM at the end of the experiment and BM at baseline.

Blood analyses

At sacrifice, animals were deprived of food for 6 h, the fasting glycemia was measured (glucometer Accu-Check, Roche, SP, Brazil), and then the animals were deeply anesthetized (150 mg/kg sodium pentobarbital intraperitoneal). Blood samples were obtained, and the serum was separated by centrifugation (120 g, 15 min) and stored individually at -80° C for further analyzes. Insulin was measured using the SinglePlex kit EZRMI-13K (Millipore, Billerica, MA, USA). The homeostasis model assessment of insulin resistance index (HOMA-IR) was calculated (fasting glucose in mmol/L multiplied by the fasting insulin level in μIU/L, divided by 22.5). [20]

White adipose tissue

Both fat pads, epididymal (eWAT) and subcutaneous inguinal (sWAT) were carefully removed, weighed and prepared for analyzes. Samples were rapidly frozen and stored at -80° C for molecular analyzes. Alternatively, samples were kept in freshly made fixative solution (4 % formaldehyde w/v, 0.1 M phosphate buffer; PH 7.2).

Adipocyte morphometry

The distribution of the adipocytes based on their size was studied in eWAT and sWAT. The tissues were embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA), the blocks were sectioned at a nominal thickness of five micrometers, and the slices were stained with

hematoxylin and eosin. The analysis was made considering ten non-consecutive slices and at least 50 adipocytes per animal had their small diameters measured, at random, in a blinded manner (Leica DMBRE light microscope, Wetzlar, Germany; Infinity 1-5c camera Lumenera Co., Ottawa, ON, Canada; Image Pro Plus software v. 7.01, Media Cybernetics, Silver Spring, MD, USA).

In addition, the average cross-sectional area of the adipocytes was evaluated by stereology as the ratio between the volume density of adipocytes (Vv[adipocyte]) and twice the numerical density per area of adipocytes (Q_A[adipocyte]). Vv[adipocyte] was estimated by point counting on a test system, and Q_A[adipocyte] was estimated as the ratio between the number of adipocytes counted into a frame (without hit the "forbidden line" [21]) and the test area of the frame. [22]

Immunofluorescence

For UCP-1 immunofluorescence, tissue sections were submitted to citrate buffer, pH 6.0, at 60^{o} C for 20 min for antigen retrieval, glycine 2 %, and blocking buffer (PBS/ 5 % BSA). The sWAT sections were incubated overnight at 4^{o} C with anti-UCP1 antibody (SC-6529; Santa Cruz Biotechnology), diluted 1:50 in PBS/ 1 % BSA, followed by an incubation for 1 h 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 SlowFade Antifade (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Digital images were kept with the confocal laser scanning microscopy (Nikon Model C2; Nikon Instruments, Inc., New York, USA).

RT-qPCR

Total RNA was extracted from approximately 50 mg of eWAT and sWAT using Trizol reagent (Invitrogen, CA, USA). The quantity of RNA was determined using Nanovue spectroscopy (GE Life Sciences). Then, 1 μg RNA was treated with DNAse I (Invitrogen). First-strand cDNA was synthesized using Oligo (dT) primers for mRNA and Superscript III reverse transcriptase (both Invitrogen). Quantitative real-time PCR was performed using a BioRad CFX96 cycler and SYBR Green mix (Invitrogen). The primers are described in Table 2. The endogenous expression of *beta*-actin was used to normalize the expression of the selected genes.

In the eWAT we evaluated: cluster of differentiation 36 (CD36), adipocyte protein 2(aP2), sterol regulatory element-binding transcription factor 1c (SREBP1c), fatty acid synthase (FAS), perilipin, carnitine palmitoyiltransferase-1a (CPT-1a), peroxisome proliferator-activated receptor *gamma* coactivator 1 *alpha* (PGC1*alpha*), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (TFAM).

In the sWAT, we evaluated: SREBP1c, FAS, perilipin, CPT-1a, PGC1*alpha*, NRF1, TFAM, *beta*3-adrenergic receptor (*beta*3-AR), UCP1, and a cluster of differentiation 137 (CD137).

After a pre-denaturation and polymerase-activation program (4 min at 95° C), 44 cycles of 95º C for 10 s and 60º C for 15 s were followed by a melting curve program (60º C to 95º C with a heating rate of 0.1º C/s). Negative controls consisted of wells in which the cDNA was substituted for deionized water. The relative expression ratio of the mRNA was calculated using the equation $2^{-\Delta\Delta Ct}$, in which -ΔCT represents the difference between the number of cycles (CT) of the target genes and the endogenous control.

Data analysis

Values are presented as the mean and the standard deviation. We tested the data for normal distribution and homoscedasticity of the variances, and then the groups were compared with one-way analysis of variance (ANOVA) and the posthoc test of Holm-Sidak. A P-value<0.05 was considered statistically significant (GraphPad Prism version 6.05 for Windows, GraphPad Software, La Jolla CA USA).

Results

Energy intake and body mass gain

The three HF groups presented elevated energy intake (+27%; P=0.0003, for the HF-L group; +33%; P<0.0001, for the HF-L+FO group; +35%; P<0.0001, for the HF-FO group). There were no differences among these three groups (Table 3).

The BM gain was greater in the HF-L group (+181 %; P<0.0001), and in the HF-L+FO group (+112 %; P=0.0011) in comparison with the SC group. There was no difference in the BM gain between the HF-FO and the SC groups (Table 3).

Blood analyses

The HF-L group had a fasting glycemia higher than the SC group (+18 %; P=0.013) (Table 3). However, the HF-L+FO group (-28 %; P<0.0001), and the HF+FO group (-35 %; P<0.0001) had lower glycemia than the HF-L group.

The plasma insulin levels were 209 % higher in the HF-L group than in the SC group (P=0.0013). Conversely, plasma insulin was normalized in both groups HF-L+FO and HF-FO

groups (insulinemia was around 45 % lower in these FO groups than in the HF-L group; P=0.018). The HOMA-IR index accompanied the insulin levels (Table 3).

Fat pads

Epididymal fat pad mass

The HF-L group showed the heaviest epididymal fat pad mass, 53 % heavier than the SC group (P<0.0001). The epididymal fat pad mass was 43 % heavier than the SC group (+112 %, P=0.0026), but 20 % lighter than the HF-L group (P=0.0059). The HF-FO group showed the slightest epididymal fat pad mass, similar to the SC group (Table 3).

Inguinal fat pad mass

The HF-L group showed the heaviest inguinal fat pad mass, 80 % heavier than the SC group (P<0.0001). In the HF-FO group, which showed the lightest inguinal fat pad mass, it was 33 % smaller than the SC group (P=0.0047), 57 % smaller than the HF-L group (P<0.0001), and 40 % smaller than the HF-L+FO group (P=0.0036) (Table 3).

Adipocytes

The adipocytes (diameter) in eWAT were 23 % bigger in the HF-L group than in the SC group (P=0.0002). Compared to the HF-L group, the adipocytes were 8 % smaller in the HF-L+FO group (P=0.047), and 20 % smaller in the HF-FO group (P<0.0001). Compared to the SC group, the adipocytes were 13 % bigger in the HF-L+FO group (P=0.013), but equivalent (no difference) in the HF-FO group. Similarly, the distribution per size of the adipocytes showed larger adipocytes in the HF-L group and a fair number of large adipocytes in the HF-L+FO group. The SC and the HF-FO group had a similar pattern of distribution per size of the adipocytes (Fig. 1).

In eWAT, the adipocytes showed a greater sectional area in the HF-L group (+175%; $P<0.0001$), and a greater area in the HF-L+FO group (+66%, $P=0.0218$) than in the SC group. The adipocyte sectional area had no difference between the HF-FO group and the SC group (Table 3).

In sWAT, the HF-L group showed bigger adipocytes than the SC group (+20 %; $P=0.0464$). The HF-L+FO group and the SC group showed similar adipocytes size. Lastly, the HF-FO group showed smaller adipocytes than the other three groups (-39%, $P=0.0011$, in comparison with the SC group; -49%, $P<0.0001$, compared with the HF-L group; and -39%, $P=0.0007$, as compared with the HF-L+FO group) (Fig. 1).

The average sectional area of the adipocytes in sWAT was greater in the HF-L group (+73%, $P<0.0001$), but in the HF-L+FO group it was similar to the SC group. The smallest sectional area of adipocytes was seen in the HF-FO group: -55% ($P<0.0001$) than the SC group; -74% ($P<0.0001$) than the HF-L group; and -54% ($P=0.0001$) than the HF-L+FO group (Table 3).

eWAT

a) Gene expression of lipogenesis

Compared to the SC group, the CD36 mRNA expression was higher in the groups HF-L, HF-L+FO and HF-FO. The CD36 mRNA expression was +166 % in the HF-L group ($P=0.0031$), +209 % in the HF-L+FO group ($P=0.0011$), and +228 % in the HF+FO group. The gene expression of aP2 was higher in the groups HF-L (+370 %; $P<0.0001$) and HF-L+FO (+124 %; $P=0.026$). In addition, the gene expression of aP2 was lower in the HF-L+FO group than in the HF-L group (-52 %; $P=0.0006$) and in-between in the groups HF-FO and SC (Fig. 2).

In comparison with the SC group, the SREBP1c and FAS mRNA expressions were higher in the HF-L group (SREBP1c +78 %; $P=0.022$; FAS +56 %; $P=0.026$). The HF-L+FO and

HF-FO groups showed SREBP1c and FAS mRNA expressions equivalent to the SC group (Fig. 2).

b) Gene expression of lipolysis and beta-oxidation

Compared to the SC group, the perilipin gene expression was lower in the groups HF-L (-39 %; P=0.0034), HF-L+FO (-36 %; P=0.0045), and HF+FO (-57 %; P=0.0002). The CPT-1a mRNA expression was lower in the HF-L group (-70 %; P=0.017), but higher in the groups HF-L+FO (+297 %; P<0.0001) and HF-FO (+539 %; P<0.0001) than in the SC group (Fig. 2).

c) Gene expression of mitochondrial biogenesis

Compared to the SC group, the PGC1*alpha* and TFAM mRNA expressions were lower in the HF-L group (PGC1*alpha* -51 %, P=0.026; TFAM -48 %, P=0.030).

Relative to the SC group, the PGC1*alpha*, NRF1 and TFAM gene expressions were higher in the HF-L+FO group (PGC1*alpha*: +53 %; P=0.026; NRF1: +176 %; P=0.0090; TFAM: +45 %; P=0.030), and were higher in the HF-FO group (PGC1*alpha*: +271 %; P<0.0001; NRF1: +514 %; P<0.0001; TFAM: +184 %; P<0.0001) (Fig. 2).

sWAT

a) Gene expression of lipogenesis

Compared to the SC group, the SREBP1c and FAS gene expressions were higher in the HF-L group (SREBP1c +89 %; P=0.0003; FAS +212 %; P=0.0002), but normalized in both FO groups (Fig. 3).

b) Gene expression of lipolysis and beta-oxidation

Having the SC group at baseline, the perilipin gene expression was lower in the three HF groups: HF-L group, -88 % (P=0.010); HF-L+FO group, -66 % (P=0.041); HF-FO group, -79 % (P=0.030). On the other hand, CPT-1a was lower in the HF-L group (-78 %; P=0.0021), but higher in the HF-FO group as compared with the other three groups (+361 %; P<0.0001 in comparison with the SC group) (Fig. 3).

c) Gene expression of mitochondrial biogenesis

Compared to the SC group, the gene expressions of mitochondrial biogenesis were lower in the HF-L group (PGC1*alpha* -51 %, P=0.039; NRF1 -34 %, P=0.029; TFAM -37 %, P=0.014). PGC1*alpha* was higher in the HF-L+FO group (+61 %; P=0.036) and the three gene expressions were higher in the HF-FO group (PGC1*alpha* +228 %, P<0.0001; NRF1 +42 %, P=0.011; TFAM +73 %, P=0.0002) (Fig. 3).

d) Gene expression of thermogenesis

Relative to the SC group, the gene expressions of *Beta*3-AR and UCP1 were lower in the HF-L group (*Beta*3-AR, -45 %, P=0.0092; UCP1, -55 %, P=0.0032), normalized in the HF-L+FO group and higher in the HF-FO group (*Beta*3-AR, +227 %, P<0.0001; UCP1, +237 %, P<0.0001) (Fig. 3D). The gene expression of CD137 was only higher in the HF-FO group than in the SC group (+525 %; P<0.0001) (Fig. 3).

Adipose tissue structure and Immunofluorescence

The HF-L group showed larger lipid droplets within sWAT. The fish oil intake was able to restore the adipose tissue structure in the HF-L+FO inducing a brown adipocyte-like

phenotype in the HF-FO group. In addition, the HF-FO group, as compared to the other three groups, showed a more intense UCP1 staining in sWAT (Fig. 4).

Discussion

The HF diets containing fish oil might diminish or prevent body adiposity, adipocyte dysfunction, and insulin resistance in mice. These anti-obesity effects of fish oil are associated with a reduction of genes related to lipogenesis and a decrease in perilipin gene expression, a stimulation of genes related to beta-oxidation, and increased mitochondrial biogenesis in both eWAT and sWAT. Additionally, the findings suggest that fish oil may induce browning in sWAT.

As expected, the HF-L group presented a significant BM gain linked with an increased epididymal and inguinal fat pad masses. Of note, we also found hypertrophied adipocytes (i.e. increased diameter and sectional area) in the HF-L group, as already shown by previous literature. [23] Even though we did not measure adipocyte number in WAT depots, it is known that a high-fat feeding promotes adipose tissue hyperplasia, [24] and we believe that this might be occurring in our HF-L group.

Interestingly, both diets HF-L+FO and HF-FO yielded a diminished BM gain, epididymal and inguinal fat pad masses. The partial substitution of a HF diet for n-3 PUFA resulted in a reduced BM gain, mostly accounted for a decrease in the epididymal fat mass. [25] Animals treated with a mix of EPA/DHA (both of which are present in fish oil), also showed a small epididymal fat accumulation. [18] In addition, EPA was able to reduce lipid accumulation in 3T3-L1 cells. [1]

In the HF-L group, we also observed hyperglycemia, hyperinsulinemia and elevated HOMA-IR, indicating insulin resistance in these animals. On the contrary, in the HF-L+FO and HF-FO groups, these data were normalized even in the HF-L+FO group (that consumed lard). In a study in mice fed a HF diet partially substituted by EPA, although the animals showed an increase in the BM and greater epididymal fat pad mass, the glycemia, insulinemia, and HOMA-IR were alike the control group. [26]

Adipose tissue dysfunction included alterations in lipogenesis and lipolysis, the two primary metabolic activities of WAT, and was associated with insulin resistance. Once fish oil reduced/prevented the BM gain and associated adipocyte hypertrophy, it became necessary to evaluate if these fatty acids were acting in WAT lipogenesis and lipid oxidation. The first step to lipogenesis is the entrance of fatty acids into adipose tissue, through transport proteins, such as CD36 and aP2. [27] CD36 is crucial to long chain fatty acids uptake and processing before mitochondrial entry. [28] The aP2, also known as fatty acid binding protein 4 (FABP4), acts transporting fatty acids to the site where triacylglycerol synthesis occurs. [27, 29] Alternatively, fatty acids can derive from *de novo* lipogenesis, in which SREBP1c and FAS play decisive roles. [30, 31] Since the gene expressions of these proteins were found elevated in the HF-L group, we hypothesize that lipogenesis was in these animals in both WAT depots. Accordingly, CD36 is increased in obese animals, [32] as well as aP2, SREBP1c and FAS are related to the fat accumulation and insulin resistance. [29-31] In contrast, the groups fed fish oil showed different results, with a reduction of aP2, SREBP1c and FAS mRNA expressions in eWAT and sWAT, suggesting that lipogenesis was normalized. In the inguinal adipocytes, EPA might increase gene expression of CD36, indicating greater metabolization of the available long chain fatty acids (present in fish oil). [8] In human WAT, supplementation with n-3 PUFA reduced aP2 gene expression. [2] In addition, in porcine adipocytes DHA was able to

reduce FAS mRNA expression, [33] and rats fed a diet with alpha-linolenic acid (rich in n3-PUFA) showed diminished mRNA expression of SREBP1c and FAS. [30]

The other primary metabolic activity of WAT, lipolysis, is also elevated in adipocyte dysfunction. Perilipin is present on the surface of lipid droplets, preventing them from suffering lipolysis. When perilipin is suppressed, the action of lipases is facilitated, and triacylglycerol hydrolysis begin. Obesity and saturated fatty acids are associated with decreased perilipin in adipose tissue, [2, 34] indicating that lipolysis was probably activated in the HF-L group. Interestingly, perilipin is also found diminished in adipose tissue of humans supplemented with n-3 PUFA, [2] and 3T3-L1 cells treated with n-3 PUFA showed suppressed gene expression of perilipin, [35] as we saw in both WAT depots of our HF-L+FO and HF-FO groups.

Increased CPT-1 is associated with fatty acid utilization and oxidation capacity. [36, 37] Although our HF-L group showed enhanced expression of CD36 mRNA, the animals also had lower levels of CPT-1a mRNA. The intracellular fatty acids resulting from the action of CD36 may not enter the beta-oxidation pathway and, consequently, are deviated to lipogenesis. [36] On the contrary, our groups HF-L+FO and HF-FO had increased CD36, perilipin, and CPT-1a, indicating that lipid metabolism was activated towards lipolysis and beta-oxidation in both WAT depots. In addition, 3T3-L1 adipocytes treated with EPA suffer increased lipolysis, with a concomitant increase in mRNA expression of CPT-1. [1] Moreover, in inguinal adipocytes, EPA was able to increase CPT-1a expression, [8] as we saw in our HF-L+FO and HF-FO groups.

Sustained activation of beta-oxidation, clearly demonstrated in our FO groups in both eWAT and sWAT, means a significant mitochondrial content in adipocytes and indicates a high expression of PGC1*alpha*. [38] PGC1*alpha* is a key transcription factor that regulates mitochondrial fatty acid oxidation enzymes, and PGC1*alpha* inhibition causes lipid

accumulation. [39] NRF1 is a transcription factor that is the main target of PGC1*alpha* while TFAM is required for mitochondrial DNA replication and maintenance, and, together, they are considered markers of mitochondrial biogenesis. [40] In addition, an EPA/DHA quantity increased PGC1*alpha* and NRF1expressions together with an elevated WAT beta-oxidation in mice. [41] Similarly, inguinal adipocytes treated with EPA showed upregulation of PGC1*alpha* and NRF1, [8] corroborating with our results and indicating mitochondrial biogenesis in eWAT and sWAT of both FO groups.

Besides the above-mentioned anti-obesity effects related to lipogenesis and beta-oxidation, a relevant subject of the present study was to analyze the browning induction by fish oil in sWAT. 'Beige' adipocytes are cells located in WAT and are characterized by an elevated adaptive thermogenic activity (i.e. expression of UCP1) that can be induced by several regulatory factors, such as cold exposure and hormonal stimuli. [42] We observed that only the HF-FO group showed 'beige' cells with their consequent thermogenic capacity, as demonstrated by the elevated expression of CD137 in this group, a specific marker of browning. [7, 42] However, an issue to be clarified is if the 'beige' cells observed are explained by the elevated quantities of fish oil in the HF-FO diet or by the absence of lard intake of the animals.

In a culture of human subcutaneous adipocytes, it was found a reduction of the UCP1 gene expression in obese individuals compared with lean individuals, [43] which suggests an impairment of the thermogenic capacity of this tissue, as we saw in our HF-L group. On the other hand, in the HF-FO group the thermogenic markers (*beta*3-AR and UCP1) were upregulated indicating a shift in the metabolic profile of the sWAT cells towards a brown phenotype. Recent reports announced that fish oil induces *beta*3-AR and UCP1 overexpression in brown adipose tissue of mice, [44] and subcutaneous adipocytes treated with EPA show thermogenic activity with increased gene expression of UCP1. [8]

Despite our results of gene expression in sWAT, we cannot affirm that these changes culminated in functional relevance. Zhao and Chen demonstrated that cultured subcutaneous adipocytes treated with EPA showed increased uptake of glucose and fatty acids, suggesting increased activity of these cells. [8] A recent report showed that DHA increases glycerol release to the media in adipocytes, indicating increased lipolysis. [35] Another study demonstrated, in mice supplemented with n-3 PUFA and submitted to caloric restriction, that mitochondrial oxidative capacity was enhanced along with elevated palmitate oxidation in isolated epididymal adipocytes, indicating a stimulation of energy expenditure. [45] These studies allow us to speculate that the alterations of sWAT gene expression in the current study might have resulted in enhanced functionality of this tissue.

The present study also aimed to compare the effects of fish oil on eWAT and sWAT. Aside from browning, the changes seen in both WAT depots were quite similar. Hypertrophied eWAT release a great influx of free fatty acids and adipokines that ultimately will provoke insulin resistance and dyslipidemia, among other effects. On the contrary, sWAT appears to have a protective role in insulin resistance and dyslipidemia, acting, for example, as anti-inflammatory. [5, 23] Besides, sWAT is also associated with augmented thermogenic capacity, which can be one of the reasons for its beneficial effect. [8] In the current study, as expected, the HF-L diet lead to dysfunction in both eWAT and sWAT that agree with recent literature describing that both eWAT and sWAT are expanded in obesity, leading to tissue dysfunction [10, 23]. In the case of HF-L+FO and HF-FO, the fish oil intake was beneficial in blocking the hypertrophy, and consequently the dysfunction, of both WAT depots, and was also capable of inducing beneficial changes in sWAT. These results were expected and are in agreement with other studies that showed improvement of eWAT dysfunction with fish oil intake in mice [46] and improvement of insulin sensitivity in sWAT of humans with metabolic syndrome

supplemented with n-3 PUFA. [47] Moreover, it was also already proven that EPA can induce the protective role of browning in subcutaneous adipocytes. [8]

In conclusion, fish oil intake has anti-obesity effects through modulation of both eWAT and sWAT metabolism in mice and is relevant in diminishing the BM gain, adiposity, and insulin resistance even in concomitance with a high-fat lard diet intake in mice. The mechanism of action of fish oil is associated with a reduction of lipogenesis, an increase of lipolysis, beta-oxidation and mitochondrial biogenesis in both eWAT and sWAT, and upregulation of thermogenic markers in sWAT. Interestingly, while the effects on eWAT were seen as a result of both fish oil diets, in sWAT only the HF-FO diet was able to lead to beneficial effects, especially in inducing browning.

Funding

This research was supported by the Brazilian agencies CNPq (Brazilian Council of Science and Technology, www.cnpq.br, grant #302.154/2011-6 to CAML, and #306.077/2013-2 to MBA), FAPERJ (Rio de Janeiro Foundation for Research, www.faperj.br, grant #102.944/2011 to CAML, and #103.062/2011), and CAPES (Coordination for Perfectionnement of Superior Personal, www.capes.br), scholarship to TLB.

Acknowledgements

The authors would like to thank Ms. Aline de Carvalho, Ms. Michele Soares and Ms. Gezileia Lau for their technical assistance.

References

1. M. S. Lee, I. S. Kwun and Y. Kim, *Genes Nutr*, 2008, **2**, 327-330.
2. A. Camargo, M. E. Meneses, P. Perez-Martinez, J. Delgado-Lista, Y. Jimenez-Gomez, C. Cruz-Teno, F. J. Tinahones, J. A. Paniagua, F. Perez-Jimenez, H. M. Roche, M. M. Malagon and J. Lopez-Miranda, *Eur J Nutr*, 2014, **53**, 617-626.
3. B. Gustafson and U. Smith, *Atherosclerosis*, 2015, **241**, 27-35.
4. M. E. D'Alessandro, D. Selenscig, P. Illesca, A. Chicco and Y. B. Lombardo, *Food Funct*, 2015, **6**, 1299-1309.
5. S. L. Hocking, R. L. Stewart, A. E. Brandon, E. Suryana, E. Stuart, E. M. Baldwin, G. A. Kolumam, Z. Modrusan, J. R. Junutula, J. E. Gunton, M. Medynskyj, S. P. Blaber, E. Karsten, B. R. Herbert, D. E. James, G. J. Cooney and M. M. Swarbrick, *Diabetologia*, 2015.
6. A. Giordano, A. Smorlesi, A. Frontini, G. Barbatelli and S. Cinti, *Eur J Endocrinol*, 2014, **170**, R159-171.
7. J. Wu, P. Bostrom, L. M. Sparks, L. Ye, J. H. Choi, A. H. Giang, M. Khandekar, K. A. Virtanen, P. Nuutila, G. Schaart, K. Huang, H. Tu, W. D. van Marken Lichtenbelt, J. Hoeks, S. Enerback, P. Schrauwen and B. M. Spiegelman, *Cell*, 2012, **150**, 366-376.
8. M. Zhao and X. Chen, *Biochemical and biophysical research communications*, 2014, **450**, 1446-1451.
9. T. L. Rachid, A. Penna-de-Carvalho, I. Bringhenti, M. B. Aguila, C. A. Mandarim-de-Lacerda and V. Souza-Mello, *Cell Biochem Funct*, 2015, **33**, 249-256.
10. T. L. Rachid, A. Penna-de-Carvalho, I. Bringhenti, M. B. Aguila, C. A. Mandarim-de-Lacerda and V. Souza-Mello, *Mol Cell Endocrinol*, 2015, **402**, 86-94.
11. P. C. Calder, *J Nutr*, 2012, **142**, 592S-599S.

12. C. Couet, J. Delarue, P. Ritz, J. M. Antoine and F. Lamisse, *Int J Obes Relat Metab Disord*, 1997, **21**, 637-643.
13. M. Kabir, G. Skurnik, N. Naour, V. Pechtner, E. Meugnier, S. Rome, A. Quignard-Boulange, H. Vidal, G. Slama, K. Clement, M. Guerre-Millo and S. W. Rizkalla, *Am J Clin Nutr*, 2007, **86**, 1670-1679.
14. L. Lionetti, M. P. Mollica, R. Sica, I. Donizzetti, G. Gifuni, A. Pignalosa, G. Cavaliere and R. Putti, *Int J Mol Sci*, 2014, **15**, 3040-3063.
15. X. Liu, Y. Xue, C. Liu, Q. Lou, J. Wang, T. Yanagita, C. Xue and Y. Wang, *Lipids Health Dis*, 2013, **12**, 109.
16. H. Q. Liu, Y. Qiu, Y. Mu, X. J. Zhang, L. Liu, X. H. Hou, L. Zhang, X. N. Xu, A. L. Ji, R. Cao, R. H. Yang and F. Wang, *Nutr Res*, 2013, **33**, 849-858.
17. J. D. Buckley and P. R. Howe, *Nutrients*, 2010, **2**, 1212-1230.
18. J. Ruzickova, M. Rossmeisl, T. Prazak, P. Flachs, J. Sponarova, M. Veck, E. Tvrzicka, M. Bryhn and J. Kopecky, *Lipids*, 2004, **39**, 1177-1185.
19. P. G. Reeves, F. H. Nielsen and G. C. Fahey, Jr., *J Nutr*, 1993, **123**, 1939-1951.
20. D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher and R. C. Turner, *Diabetologia*, 1985, **28**, 412-419.
21. H. J. G. Gundersen, *J Microsc*, 1977, **111**, 219-227.
22. C. A. Mandarim-de-Lacerda, *Ann Brazil Acad Sci*, 2003, **75**, 469-486.
23. S. Barbosa-da-Silva, J. C. Fraulob-Aquino, J. R. Lopes, C. A. Mandarim-de-Lacerda and M. B. Aguila, *PLoS One*, 2012, **7**, e39837.
24. J. Jo, O. Gavrilova, S. Pack, W. Jou, S. Mullen, A. E. Sumner, S. W. Cushman and V. Periwal, *PLoS computational biology*, 2009, **5**, e1000324.
25. P. Flachs, V. Mohamed-Ali, O. Horakova, M. Rossmeisl, M. J. Hosseinzadeh-Attar, M. Hensler, J. Ruzickova and J. Kopecky, *Diabetologia*, 2006, **49**, 394-397.

458 26. N. S. Kalupahana, K. Claycombe, S. J. Newman, T. Stewart, N. Siriwardhana, N.
459 Matthan, A. H. Lichtenstein and N. Moustaid-Moussa, *J Nutr*, 2010, **140**, 1915-1922.
460 27. A. R. Proenca, R. A. Sertie, A. C. Oliveira, A. B. Campana, R. O. Caminhotto, P.
461 Chimin and F. B. Lima, *Braz J Med Biol Res*, 2014, **47**, 192-205.
462 28. J. Pohl, A. Ring, U. Korkmaz, R. Ehehalt and W. Stremmel, *Mol Biol Cell*, 2005, **16**, 24-
463 31.
464 29. T. Garin-Shkolnik, A. Rudich, G. S. Hotamisligil and M. Rubinstein, *Diabetes*, 2014, **63**,
465 900-911.
466 30. B. S. Muhlhausler, R. Cook-Johnson, M. James, D. Miljkovic, E. Duthoit and R. Gibson,
467 *J Nutr Metabol*, 2010, **2010**.
468 31. J. Berndt, P. Kovacs, K. Ruschke, N. Kloting, M. Fasshauer, M. R. Schon, A. Korner,
469 M. Stumvoll and M. Bluher, *Diabetologia*, 2007, **50**, 1472-1480.
470 32. L. Cai, Z. Wang, A. Ji, J. M. Meyer and D. R. van der Westhuyzen, *PloS one*, 2012, **7**,
471 e36785.
472 33. B. H. Liu, C. F. Kuo, Y. C. Wang and S. T. Ding, *J Anim Sci*, 2005, **83**, 1516-1525.
473 34. H. Ray, C. Pinteur, V. Frering, M. Beylot and V. Large, *Lipids Health Dis*, 2009, **8**, 58.
474 35. E. Barber, A. J. Sinclair and D. Cameron-Smith, *Prostaglandins, leukotrienes, and*
475 *essential fatty acids*, 2013, **89**, 359-366.
476 36. D. C. Magliano, T. C. Bargut, S. N. de Carvalho, M. B. Aguila, C. A. Mandarim-de-
477 Lacerda and V. Souza-Mello, *PLoS One*, 2013, **8**, e64258.
478 37. Z. H. Yang, H. Miyahara, Y. Iwasaki, J. Takeo and M. Katayama, *Nutr Metab (Lond)*,
479 2013, **10**, 16.
480 38. S. Bijland, S. J. Mancini and I. P. Salt, *Clin Sci (Lond)*, 2013, **124**, 491-507.
481 39. R. B. Vega, J. M. Huss and D. P. Kelly, *Mol Cell Biol*, 2000, **20**, 1868-1876.
482 40. D. P. Kelly and R. C. Scarpulla, *Genes Dev*, 2004, **18**, 357-368.

41. P. Flachs, O. Horakova, P. Brauner, M. Rossmeisl, P. Pecina, N. Franssen-van Hal, J. Ruzickova, J. Sponarova, Z. Drahota, C. Vlcek, J. Keijer, J. Houstek and J. Kopecky, *Diabetologia*, 2005, **48**, 2365-2375.

42. T. Shan, X. Liang, P. Bi, P. Zhang, W. Liu and S. Kuang, *J Lipid Res*, 2013, **54**, 2214-2224.

43. A. L. Carey, C. Vorlander, M. Reddy-Luthmoodoo, A. K. Natoli, M. F. Formosa, D. A. Bertovic, M. J. Anderson, S. J. Duffy and B. A. Kingwell, *PloS one*, 2014, **9**, e91997.

44. T. C. Bargut, E. S. A. C. Silva, V. Souza-Mello, C. A. Mandarim-de-Lacerda and M. B. Aguila, *Eur J Nutr*, 2015 (doi: 10.1007/s00394-015-0834-0).

45. P. Flachs, R. Ruhl, M. Hensler, P. Janovska, P. Zouhar, V. Kus, Z. Macek Jilkova, E. Papp, O. Kuda, M. Svobodova, M. Rossmeisl, G. Tsenov, V. Mohamed-Ali and J. Kopecky, *Diabetologia*, 2011, **54**, 2626-2638.

46. T. C. Bargut, C. A. Mandarim-de-Lacerda and M. B. Aguila, *J Nutr Biochem*, 2015, **26**, 960-969.

47. Y. Jimenez-Gomez, C. Cruz-Teno, O. A. Rangel-Zuniga, J. R. Peinado, P. Perez-Martinez, J. Delgado-Lista, A. Garcia-Rios, A. Camargo, R. Vazquez-Martinez, M. Ortega-Bellido, F. Perez-Jimenez, H. M. Roche, M. M. Malagon and J. Lopez-Miranda, *Mol Nutr Food Res*, 2014, **58**, 2177-2188.

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

Ingredients (g/kg)	SC	HF-L	HF-L+FO	HF-FO
Casein (≥ 85% of protein)	140.0	175.0	175.0	175.0
Cornstarch	620.692	347.692	347.692	347.692
Sucrose	100.0	100.0	100.0	100.0
Soybean oil	40.0	40.0	40.0	40.0
Lard	-	238.0	119.0	-
Fish oil	-	-	119.0	238.0
Fiber	50.0	50.0	50.0	50.0
Vitamin mix[a]	10.0	10.0	10.0	10.0
Mineral mix[a]	35.0	35.0	35.0	35.0
L-Cystin	1.8	1.8	1.8	1.8
Choline	2.5	2.5	2.5	2.5
Antioxidant	0.008	0.060	0.060	0.060
Total mass	1,000.0	1,000.0	1,000.0	1,000.0
Proteins (% Energy)	14	14	14	14
Carbohydrates (% Energy)	76	36	36	36
Lipids (% Energy)	10	50	50	50
Energy content (kcal/kg)	3811	5000	5000	5000

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

Abbreviations: standard-chow (SC), high-fat lard diet (HF-L), high-fat lard plus fish oil diet (HF-L+FO), and high-fat fish oil diet (HF-FO).

Table 2 – RT-qPCR primers

Primer	5'-3'	Anti-sense
aP2	TGGAAGCTTGTCTCCAGTGA	AATCCCCATTTACGCTGATG
CD36	TGCATTTGCCAATGTCTAGC	CCCTCCAGAATCCAGACAAC
CD137	CCCACATATTCAAGCAACCA	GCTCATAGCCTCCTCCTCCT
CPT-1a	AAGGAATGCAGGTCCACATC	CCAGGCTACAGTGGGACATT
FAS	CACCCACTGGAAGCTGGTAT	TCGAGGAAGGCACTACACCT
NRF1	GTTGGTACAGGGGCAACAGT	GTAACGTGGCCCAGTTTTGT
Perilipin	AATATGCACAGTGCCAACCA	CGATGCTTCTCTTCCACTCC
PGC1α	GTGTGAGGAGGGTCATCGTT	GTCAACAGCAAAAGCCACAA
SREBP1c	AGCAGCCCCTAGAACAAACA	TCTGCCTTGATGAAGTGTGG
TFAM	GAAGAACGCATGGAGGAGAG	TTCTGGGGAGAGTTGCAGTT
UCP1	TCTCAGCCGGCTTAATGACT	TGCATTCTGACCTTCACGAC
β3-AR	ACAGGAATGCCACTCCAATC	AAGGAGACGGAGGAGGAGAG
β-actin	CTCCGGCATGTGCAA	CCCACCATCACACCCT

Abbreviations: aP2, adipocyte protein 2; CD36, cluster of differentiation 36; CD137, cluster of differentiation 137; CPT-1a, carnitine palmitoyltransferase-1a; FAS, fatty acid synthase; IL-6, interleukin-6; MCP1, monocyte chemotactic protein 1; NRF1, nuclear respiratory factor 1; PGC1α, peroxisome proliferator-activated receptor *gamma* coactivator 1 *alpha*; SREBP1c, sterol regulatory element-binding transcription factor 1c; TFAM, mitochondrial transcription factor A; UCP1, uncoupling protein type 1; β3-AR, *beta*3 adrenergic receptor.

Table 3 – Food behavior, adiposity, and insulin resistance

Data	Groups			
	SC	**HF-L**	**HF-L+FO**	**HF-FO**
Food behavior				
Energy intake (KJ)	47.8±1.3	60.8±3.3[a]	63.5±5.9[a]	64.6±7.9[a]
Adiposity				
Body mass gain (g)	2.3±0.9	6.5±2.3[a]	4.9±0.8[a,b]	3.4±1.4[b,c]
Epididymal fat pad (g)	0.30±0.03	0.54±0.11[a]	0.43±0.04[a,b]	0.25±0.05[b,c]
Inguinal fat pad (g)	0.15±0.02	0.23±0.05[a]	0.17±0.04[b]	0.10±0.02[a,b,c]
Adipocyte sectional area				
eWAT adipocyte (μm^2)	371.1±91.7	1020.5±250.1[a]	618.0±299.1[a,b]	356.6±93.1[b,c]
sWAT adipocyte (μm^2)	305.8±109.2	530.5±92.3[a]	294.5±58.8[b]	136.6±30.6[a,b,c]
Insulin resistance				
Fasting glycemia (mmol/L)	8.1± 0.6	9.6±1.2[a]	6.8±1.6[a,b]	6.2±0.8[a,b]
Insulin (µUI/L)	15.9±8.5	49.1±9.3[a]	26.7±9.8[b]	28.2±10.5[b]
HOMA-IR	5.6±3.8	18.5±7.2[a]	8.5±3.3[b]	8.0±3.5[b]

Legend: Standard-chow group (SC); high-fat lard group (HF-L); high-fat lard plus fish oil group (HF-L+FO), and high-fat fish oil group (HF-FO). In the signaled cases, $P \leq 0.05$ when [a] compared to the SC group, [b] compared to the HF-L group, [c] compared to the HF-L+FO group (one-way ANOVA and posthoc test of Holm-Sidak). Values are the means ± SD, n = 5 per group.

Abbreviations: HOMA-IR, homeostasis model assessment of insulin resistance index.

Figure legends

Figure 1. Adipocyte size distribution in eWAT and sWAT. Horizontal black bars are medians, n = 5 per group. When indicated, $P<0.05$ (one-way ANOVA and the posthoc test of Holm-Sidak): [a] ≠ SC, [b] ≠ HF-L, [c] ≠ HF-L+FO.

Groups: standard-chow group (SC), high-fat lard group (HF-L), high-fat lard plus fish oil group (HF-L+FO), and high-fat fish oil group (HF-FO).

Figure 2. Gene expression of CD36, aP2, SREBP1c, FAS, perilipin, CPT-1a, PGC1*alpha*, NRF1, and TFAM in eWAT. Values are the means ± SD, n = 5 per group. When indicated, $P<0.05$ (one-way ANOVA and the posthoc test of Holm-Sidak): [a] ≠ SC, [b] ≠ HF-L, [c] ≠ HF-L+FO.

Groups: standard-chow group (SC), high-fat lard group (HF-L), high-fat lard plus fish oil group (HF-L+FO), and high-fat fish oil group (HF-FO).

Figure 3. Gene expression of SREBP1c, FAS, perilipin, CPT-1a, PGC1*alpha*, NRF1, TFAM, *beta*3-AR, UCP1, and CD137 in sWAT. Values are the means ± SD, n = 5 per group. When indicated, $P<0.05$ (one-way ANOVA and the posthoc test of Holm-Sidak): [a] ≠ SC, [b] ≠ HF-L, [c] ≠ HF-L+FO.

Groups: standard-chow group (SC), high-fat lard group (HF-L), high-fat lard plus fish oil group (HF-L+FO), and high-fat fish oil group (HF-FO).

Figure 4. Analysis of the sWAT (in the upper line, hematoxylin and eosin stain; in the lower line, immunofluorescence for UCP1 (all photomicrographs have the same magnification, bar = 50 μm). The SC group showed typical white adipocytes, whereas the HF-L group showed larger lipid droplets within sWAT. FO restored white adipocyte cytoarchitecture in the HF-L+FO group and induced a brown adipocyte-like phenotype in the HF-FO group. Likewise, tissues were labeled for UCP1 and showed the different intensity of positive immunoreactions. The three groups SC, HF-L, and HF-L+FO, showed less intense immunostaining. The HF-FO group showed the higher intensity of UCP1 labeling.

Groups: standard-chow group (SC), high-fat lard group (HF-L), high-fat lard plus fish oil group (HF-L+FO), and high-fat fish oil group (HF-FO).