A PPAR-alpha agonist and DPP-4 inhibitor mitigate adipocyte dysfunction in obese mice


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Abstract

Obesity causes white and brown adipocyte dysfunction, reducing browning and stimulating whitening. Drugs that tackle adipocyte dysfunction through thermogenesis stimulation could be used to treat obesity. This study sought to address whether a combination of the PPAR-alpha agonist (WY14643) and DPP4i (linagliptin) potentiates browning and mitigates adipose tissue dysfunction, emphasizing the pathways related to browning induction and the underlying thermogenesis in high-fat-fed mice. Adult male C57BL/6 mice were randomly assigned to receive a control diet (C, 10% lipids) or a high-fat diet (HF, 50% lipids) for 12 weeks. Experiment 1 aimed to evaluate whether 5 weeks of combined therapy was able to potentiate browning using a five-group design: C, HF, HFW (monotherapy with WY14643, 2.5 mg/kg body mass), HFL (monotherapy with linagliptin, 15 mg/kg body mass), and HFC (a combination of both drugs). Experiment 2 further addressed the pathways involved in browning maximization using a four-group study design: C, CC (C diet plus the drug combination), HF, and HFC (HF diet plus the drug combination). The HF group showed overweight, oral glucose intolerance, sWAT adipocyte hypertrophy, and reduced numerical density of nuclei per area of BAT confirming whitening. Only the combined treatment normalized these parameters in addition to body temperature increase, browning induction, and whitening rescue. The high expression of thermogenic marker genes parallel to reduced expression of inflammatory and endoplasmic reticulum stress genes mediated the beneficial findings. Hence, the PPAR-alpha agonist and DPP-4i combination is a promising target for obesity control by inducing functional brown adipocytes, browning of sWAT, and enhanced adaptive thermogenesis.

Introduction

Obesity damages the white adipose tissue (WAT) and brown adipose tissue (BAT) structure and function (Strissel et al. 2007, Shimizu et al. 2014). White adipocyte dysfunction is reflected in inflammation, increased lipolysis, and lipogenesis, resulting in adipocyte hypertrophy and ectopic fat accumulation (Gustafson & Smith 2015, Saponaro et al. 2015). In BAT, insulin resistance and inflammation induce white-like adipocytes with impaired thermogenesis, a phenomenon called whitening (Kotzbeck et al. 2018).
Endoplasmic reticulum (ER) stress, a condition triggered by the accumulation of unfolded proteins, precedes adipocyte dysfunction and inhibits browning of s.c. WAT (sWAT). Hence, decreased thermogenesis and maximized inflammation aggravate adipocyte dysfunction in a vicious cycle (Hummasti & Hotamisligil 2010, Fernandes-da-Silva et al. 2021). Therefore, pharmacological targets that induce beige adipocytes (browning) in the sWAT reservoir while reducing whitening in BAT could treat obesity by increasing the conversion of chemical energy into heat (Nedergaard et al. 2007, Miranda et al. 2020).

In this context, peroxisome proliferator-activated receptors (PPARs) are transcription factors highly relevant in counter metabolic disorders (Souza-Mello 2015), and the PPAR-alpha isoform is essential to lipid homeostasis through oxidative metabolism and thermogenesis (Li et al. 2005, Seale 2015). Concerning linagliptin, its action encompasses increased plasma GLP1 concentrations by dipeptidyl peptidase 4 (DPP-4) inhibition (Drucker & Nauck 2006). DPP4 is the catalytic site of CD26 and is also an adipokine whose levels are augmented with adipocyte hypertrophy (Lamers et al. 2011). A high dose of DPP4i has recently increased adaptive thermogenesis and macrophage polarization to the M2 state in HFD-fed mice (de Oliveira Correia et al. 2019). Furthermore, in high-fructose-fed mice, the PPAR-alpha and linagliptin combination had beneficial effects on the gut-liver axis, mitigating fatty liver (Silva-Veiga et al. 2020). This result could imply favored thermogenesis as gut–adipose axis impairments precede liver alterations due to metabolic diseases (Poekes et al. 2017). However, there are no reports in the literature about this drug combination on adipose tissue function in obese mouse models. Therefore, we hypothesized that a combined therapy (PPAR-alpha agonist + DPP4i) could restore adipose tissue thermogenic function by mitigating BAT whitening while eliciting browning in the sWAT of obese mice.

This study sought to address whether a combination of the PPAR-alpha agonist (WY14643) and DPP4i (linagliptin) potentiates browning and mitigates adipose tissue dysfunction, emphasizing the pathways related to browning induction and the underlying thermogenesis in high-fat-fed mice.

**Materials and methods**

The study protocol, approved by the Ethical Committee in Animal Experimentation of the State University of Rio de Janeiro (CEUA 041/2018), followed the Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised in 1996).

**Animals and diets**

Sixty-five male C57BL/6 mice were group-housed in ventilated cages (n = 5 per cage, reusable IVC housing, NexGen, Allentown, Pennsylvania, USA) under controlled luminosity (12 h light:12 h darkness cycle), humidity (60 ± 10%), and temperature (21 ± 2°C) conditions with free access to food and water.

At 3 months of age, the mice were randomly allocated into two nutritional groups for 12 weeks: an HFD (HF, n = 40, 50% energy as lipids, 14% energy as protein, and 36% energy as carbohydrates, 20.90 kJ/g) and a control diet (C, n = 25, 10% energy as lipids, 14% energy as protein, and 76% energy as carbohydrates, 15.88 kJ/g). PragSoluções (Jau, São Paulo, Brazil) produced the diets according to the recommendations of AIN-93 M for rodents (Reeves et al. 1993). These animals were used in two different experiments:

Experiment 1 addressed whether the PPAR-alpha agonist and DPP-4i combination was necessary to induce the browning of sWAT. After the first 12 weeks of obesity induction, a 5-week treatment started, with the randomly divided following groups: C (n= 5), control diet, untreated; HF (n= 5), high-fat diet, untreated; HFW (n= 5), high-fat diet + PPAR-alpha agonist (WY14643, Cayman Chemical); HFL (n= 5), high-fat diet + DPP4i (linagliptin, Tradjenta, Boehringer Ingelheim, Germany); HFC (n = 5), high-fat diet, treated with PPAR-alpha agonist + DPP4i.

Experiment 2 further explored the main pathways related to the expressive browning found in the group that received the combination of PPAR-alpha agonist with DPP4i. After the same 12-week dietary protocol to induce obesity, animals were randomly assigned to four groups: C (n = 10), control diet, untreated; CC (n= 10), control diet, treated with PPAR-alpha agonist (WY14643, Cayman Chemical) + DPP4i (linagliptin, Tradjenta, Boehringer Ingelheim) combination; HF (n = 10), high-fat diet, untreated; HFC (n= 10), high-fat diet, treated with PPAR-alpha agonist + DPP4i combination. The treatment also lasted for 5 weeks, and the inclusion of a control group treated with the newly proposed drug combination aimed to evaluate possible side effects.

The drugs were mixed with the diets at a dose of 2.5 mg/kg of body mass (BM) for WY14643 and 15 mg/kg BM for
linagliptin in both experiments (Kern et al. 2012, Rachid et al. 2018).

**Thermography**

At the end of both experiments, a thermographic camera (Flir C2, Flir Systems, Wilsonville, Oregon, EUA) was used to measure the conscious animals' body surface temperature.

**Sacrifice**

After the 17-week protocol, animals were sacrificed under i.p. anesthesia (ketamine, 240 mg/kg, and xylazine 30 mg/kg) after a 6-h fast. Blood samples, obtained through cardiac puncture, had the plasma separated by centrifugation (712 g, 15 min) at room temperature. These samples were used to perform ELISA.

The sWAT from the inguinal region (located between the inferior part of the rib cage and the middle of the lower limb) and the interscapular region (iBAT) were carefully dissected, weighed, fixed in 4% w/v formaldehyde, 0.1 M phosphate buffer, and pH 7.2 Millonig formalin for observation by light microscopy. The sWAT was also frozen at –80°C for molecular analyses. In addition, other compartments (the retroperitoneal and epididymal fat pads) had their masses determined and comprised the intra-abdominal fat to evaluate the fat distribution.

**Experiment 1**

Food intake, energy intake, and body mass

Food intake was evaluated daily as the difference between the amount of food offered and the remaining food after 24 h. Energy intake is the food intake in kilogram multiplied by the energy density per gram of each diet in kilojoule. In addition, body mass was measured weekly.

Oral glucose tolerance test (OGTT)

In the last week of the treatment, the animals had their OGTT measured after a 6-h fast. All glucose measurements used a manual glucometer (Accu-Chek, Roche) and blood samples from the caudal vein. Initially, glucose evaluation at time 0 (fasting glycemia) occurred before administering a glucose solution (25% in sterile saline (0.9% NaCl)) by orogastric gavage at a dose of 2 g glucose/kg BM. Follow-up glucose measurements occurred at 15, 30, 60, and 120 min following oral glucose overload. The area under the curve (AUC) was calculated (GraphPad Prism version 8.4.0).

**Indirect calorimetry**

Before sacrifice, the animals underwent a 48-h protocol in the Oxylet system (Panlab/Harvard, Barcelona, Spain), with the first 24 h discarded as the acclimatization time (Penna-de-Carvalho et al. 2014). The respiratory exchange ratio (RER) complied with the ratio of carbon dioxide production (VCO₂) to oxygen consumption (VO₂).

**Plasma analysis**

Plasma concentrations of total GLP1 and insulin were measured in duplicate with commercially available ELISA (Multispecies GLP-1 ELISA Kit Cat. #EZGLP1T-36K; Rat/ Mouse Insulin ELISA kit Cat. #EZRMI-13K, using Fluostar Omega equipment (BMG LABTECH GmbH, Germany)).

**Light microscopy, stereology, and immunohistochemistry**

sWAT sWAT obtained by dissection, after 48 h of fixation, was embedded in Paraplast plus (Sigma–Aldrich Corp.). Tissue cut in 5-μm sections was stained with hematoxylin–eosin and analyzed under a light microscope (Olympus BX51, and the digital camera Infinity 1-5 c, Lumenera Corp., Ottawa, ON, Canada). Random microscopic fields were photographed to blindly estimate the cross-sectional area of adipocytes: volume density (Vv) of the adipocytes divided by twice the numerical density of the adipocytes per area (Qₙ) (Mandarim-de-Lacerda 2003). A 16-point test system was used to estimate the Vv of the adipocytes (points that hit adipocytes, Pp, divided by the total number of points within the test system, Pₚ). Conversely, Qₙ estimation was carried out considering the number of adipocytes within a test area (except for the adipocytes that touched the forbidden lines) divided by the test system area (Bargut et al. 2016).

For immunohistochemistry, the sWAT slides were deparaffinized, and after antigen retrieval (citrate buffer, pH 6.0 at 60°C for 20 min), peroxidase and nonspecific binding blockade, they were incubated with primary antibodies against UCP1 (Cusabio, PA025554ESR2HU, Houston, Tx, USA, dilution 1:150) or β3-AR (Santa Cruz, sc-1473, 1:100) for 2 h at room temperature. Both antibodies were diluted in 1.5% horse serum (Vector Laboratories, CA, USA). Then, the slides were incubated with a biotinylated pan-specific secondary antibody for 10 min (Vector Laboratories), followed by incubation with streptavidin and peroxidase for 5 min. The staining was developed with DAB (incubation for 5 min, Vectastain Universal Quick HRP kit, peroxidase, PK-7800, Vector Laboratories). The slides were counterstained with hematoxylin and mounted with Entellan (Merck).
Concerning UCP1 immunodensity estimation, random images of five animals per group were evaluated using ImageJ (ImageJ bundled with Java 1.8.0, National Institute of Health, NIH). After normalization of the images using a plugin to remove the background, color deconvolution was performed. The image with the isolated DAB signal was analyzed with the histogram tool to determine the percentage occupied by the DAB, which was considered the UCP1 immunodensity (%).

**BAT** BAT followed the same routine described for sWAT in the previous section to obtain the slides, staining protocol, and photomicrographs. Digital images of random microscopic fields were obtained to blindly estimate the numerical density of nuclei per area \(Q_n\) (nuclei, BAT). Briefly, a frame of the known area was superimposed on photomicrographs (produced by Stepanizer) (Tschanz et al. 2011), and BAT nuclei within the frame but not intersected by exclusion lines of the frame were counted (Gundersen 1977). Then, \(Q_n\) (nuclei, BAT) = nuclei counted/test area (Mandarim-de-Lacerda 2003).

**Statistical analysis**

Data are presented as the mean and s.d. The statistical analysis comprised Welch’s t-test during the first 12 weeks and Brown–Forsythe and Welch ANOVA followed by a Dunnett T3 post hoc test for the treatment phase. In addition, one-way repeated measures ANOVA was used to evaluate the duration of the glucose peak in the OGTT as it evaluates whether the means within the same group are different.

**Experiment 2**

**Metabolic and plasma analysis**

Body mass was monitored weekly, and energy intake was evaluated daily. Plasma concentrations of leptin and total adiponectin were determined by ELISA (Rat/Mouse Leptin ELISA kit Cat. #EZML-82K; Mouse adiponectin ELISA kit Cat. #EZMADP-60K, Millipore).

**UCP1 immunofluorescence**

After paraffin removal, antigen retrieval with citrate buffer (pH 6.0 at 60°C) and nonspecific bond blockade (2% glycine and 5% BSA in PBS), 5 µm sWAT slides were incubated with the anti-UCP1 primary antibody (1:50 CSB-PA025554ESR2HU, Cusabio Technology LLC, Houston, TX, USA) overnight, followed by incubation with the secondary antibody conjugated with Alexa Fluor 488 (rabbit anti-mouse IgG H+L, Thermo Fisher Scientific). SlowFade (Invitrogen, Molecular Probes) was used to mount the slides to maintain fluorescence until analyses under a fluorescence microscope (Nikon Confocal Laser Scanning Microscopy – Model C2; Nikon Instruments, Inc.).

**RT-qPCR**

In the analysis, 50 µg of sWAT was transferred to autoclaved microtubes containing lysis solution, and mRNA was extracted and isolated. Nanovue spectroscopy (GE Life Sciences) was applied to determine the RNA amount, followed by obtaining the first strand of cDNA synthesis from the mRNA of the samples. Real-time PCR (RT-qPCR) used a StepOne plus cycler, a SYBR Green (Invitrogen) mixture, and Primer3web online software version 4.0.0 for primer design. Table 1 shows the primers used in this study.

The endogenous control adopted was beta-actin, which was used to normalize the expression of the selected gene. RT-qPCR efficiency, calculated from a series of cDNA dilutions, was approximately equal to the endogenous control for the target gene. The relative mRNA expression ratio (RQ) was calculated using the equation \(2^{-\Delta\Delta Ct}\), which expresses the difference between the number of cycles (CT) of the target genes and the endogenous control. Throughout the text, gene nomenclature followed the international standard for rodents, with the first letter in capital letters and italics and protein acronyms in capital letters (Davison 1994).

**Statistical analysis**

Data are presented as the mean and s.d. Brown–Forsythe and Welch ANOVA followed by a Dunnett T3 post hoc test was used to determine the significant differences at the end of the experiment. Two-way ANOVA tested the isolated influence of diet or treatment and possible interactions between these variables on the evaluated parameters. A significance level of \(P < 0.05\) was considered statistically significant (GraphPad Prism, version 8.4.0).

**Results**

**Experiment 1: PPAR-alpha agonist plus DPP-4i is necessary to induce browning of sWAT**

All proposed treatments decreased BM without altering food intake

The high energy density of the HF diet elicited BM gain and large fat pads in different compartments. The C and
HF groups began the experiment without differences in BM (week 0). After 1 week of diet intake, the HF group showed a higher BM than the C group (+5%, Fig. 1A), which steadily increased throughout the remaining weeks of obesity induction (+12%, week 12, Fig. 1A) until the end of the experiment (+12%, Fig. 1A). All treatments caused a significant BM reduction from the second week. However, the BM of the HFW group remained at intermediate values between the C and HF groups (Fig. 1A), whereas both the HFL and HFC groups showed BM values similar to the C group at the end of treatment (Fig. 1A).

**Table 1** Detailed forward and reverse primer sequences of RT-qPCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Atf4</td>
<td>CCGAGATGAGCTTCCTGAAAC</td>
<td>ACCCATGAGTTGTTCACTGCA</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>TGTTTCCACCTGGAGCACA</td>
<td>GGGGTGTTGAAAGGTCTCACA</td>
</tr>
<tr>
<td>Bmp8b</td>
<td>CTATGCAAGGCGCTGTTGACAT</td>
<td>AGGCCTGGACTACCATGTTG</td>
</tr>
<tr>
<td>Chop</td>
<td>CGACCTTTCACTTTGGAGAC</td>
<td>CGTTTCTGGAGGATGAGATA</td>
</tr>
<tr>
<td>Cidea</td>
<td>CGGCCGCTGCTCTGCAATGCA</td>
<td>GAAACTGCCACACACAGT</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>GGGCTGGCGGTTGGCAGATT</td>
<td>GGAACGTCCCGTCATCCTG</td>
</tr>
<tr>
<td>Dpp4</td>
<td>TGGTAGCAGCTGTTGGAATTGACCA</td>
<td>AGGAGGCGAGGGTTTGTGAG</td>
</tr>
<tr>
<td>Gadd45</td>
<td>GCGAGAAGACACATCACAATC</td>
<td>GATCGTACCCACAGCACAGT</td>
</tr>
<tr>
<td>Nlpr3</td>
<td>ATGCTGCTCCTGACATCCTCC</td>
<td>GTTTCTGGAGGTTGCAAGCC</td>
</tr>
<tr>
<td>Pdk4</td>
<td>ACCACATGCTTCTGGAGACCA</td>
<td>AAGGAGGCGAGGGTTTGTGAG</td>
</tr>
<tr>
<td>Pgc1-a</td>
<td>GCACGACAGAAGAACACAAAGC</td>
<td>GTGTGAGGAGGGTCTCAGTT</td>
</tr>
<tr>
<td>Plin1</td>
<td>ACGACCAAGCAGCAGCAGAG</td>
<td>GGGCTGCAACCTTGAGACCA</td>
</tr>
<tr>
<td>Ppar-a</td>
<td>TCGGACTCTGCGTCTCAGT</td>
<td>TCTTCCCAAAGCCTCTTCA</td>
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<tr>
<td>Prdm16</td>
<td>AGGGCAAGAACATCAGACAGC</td>
<td>GAGGAGGTTTTGTGCTTCCA</td>
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<tr>
<td>Tlr4</td>
<td>GCCGGAAGGGTTTATTGCTGGA</td>
<td>GAAACTGCCATGTTGAGCA</td>
</tr>
<tr>
<td>Ucp1</td>
<td>TCTCACGGGGCGCTTAATGACT</td>
<td>TGCAATGCTGCTCTCAGCAG</td>
</tr>
<tr>
<td>β3ar</td>
<td>ACAGGAATGCGCCTCACTACT</td>
<td>TTAGCCACACGGAAACACTCG</td>
</tr>
</tbody>
</table>

**Figure 1**
Weekly body mass (A); Epididymal fat pad (B); s.c. fat pad (C). Brown–Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± s.d., n = 5). Significant differences (P < 0.05) are indicated: a ≠ C; b ≠ HF; c ≠ HFW; d ≠ HFL. Groups: C, control diet; HF, high-fat diet; HFW, high-fat diet plus WY14643 (PPAR-alpha agonist); HFL, high-fat diet plus linagliptin (DPP-4i); HFC, high-fat diet plus WY14643 (PPAR-alpha agonist) and linagliptin (DPP-4i).
Furthermore, none of the groups differed regarding food intake throughout the experiment (Table 2). Notwithstanding, all HF-fed animals (untreated and treated) showed higher energy intake than the C group during the treatment (Table 2), as expected due to the high energy density of the HF diet.

The combined treatment countered insulin resistance while increasing GLP1 concentrations

The HF group had a 27% increase in fasting glycemia compared to the C group (Fig. 2A). Only the combined

Table 2  Food behavior, thermography, BAT mass/body mass, and indirect calorimetry.

<table>
<thead>
<tr>
<th>Data</th>
<th>C</th>
<th>HF</th>
<th>HFW</th>
<th>HFL</th>
<th>HFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (g/day/animal)</td>
<td>3.07 ± 0.03</td>
<td>3.08 ± 0.01</td>
<td>3.03 ± 0.05</td>
<td>3.03 ± 0.06</td>
<td>3.06 ± 0.02</td>
</tr>
<tr>
<td>Cumulative energy intake (kJ/day/animal)</td>
<td>48.80 ± 0.59</td>
<td>64.29 ± 0.30</td>
<td>63.42 ± 1.10</td>
<td>63.31 ± 1.246</td>
<td>64.04 ± 0.59</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>31.70 ± 0.27</td>
<td>33.64 ± 0.11</td>
<td>34.05 ± 0.26</td>
<td>34.30 ± 0.55</td>
<td>34.10 ± 0.23</td>
</tr>
<tr>
<td>BAT / body mass (%)</td>
<td>0.27 ± 0.02</td>
<td>0.36 ± 0.04</td>
<td>0.29 ± 0.02</td>
<td>0.27 ± 0.03</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Respiratory Exchange ratio (CO$_2$/O$_2$)</td>
<td>0.84 ± 0.02</td>
<td>0.91 ± 0.01</td>
<td>0.79 ± 0.04</td>
<td>0.70 ± 0.05</td>
<td>0.82 ± 0.02</td>
</tr>
</tbody>
</table>

Data presented as mean ± s.d. Significant differences (P < 0.05) are indicated: a ≠ C; b ≠ HF; d ≠ HFL as determined by Brown–Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test. Groups: C, control diet; HF, high-fat diet; HFW, high-fat diet plus WY14643 (PPAR-alpha agonist); HFL, high-fat diet plus linagliptin (DPP-4i); HFC, high-fat diet plus WY14643 (PPAR-alpha agonist) and linagliptin (DPP-4i), brown adipose tissue (BAT).

**Figure 2**
Oral glucose tolerance test curve (A); area under the curve (B); plasma insulin concentrations (C); plasma GLP-1 concentrations (D). Brown–Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± s.d., n = 5). Significant differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Groups: C, control diet; HF, high-fat diet; HFW, high-fat diet plus WY14643 (PPAR-alpha agonist); HFL, high-fat diet plus linagliptin (DPP-4i); HFC, high-fat diet plus WY14643 (PPAR-alpha agonist) and linagliptin (DPP-4i).
treatment caused a significant decrease in fasting glycemia compared to the HF group (−13%, Fig. 2A), suggesting a complete rescue of this parameter, as glycemic values in the HFC did not differ from those of the C group. Moreover, the area under the curve (AUC) revealed that the HF group had oral glucose intolerance through increased AUC values (+38% for HF vs C, Fig. 2B). In line with the AUC results, repeated-measures ANOVA showed that all treated groups reestablished their baseline glucose levels 15 min after the peak, except in the C group.

In agreement with the OGTT results, the HF group showed hyperinsulinemia (+119% for HF vs C, Fig. 2C). In contrast to the insulin results, the HFC group showed increased GLP1 concentrations (−65% for HFW vs HF and −64% for HFC vs HF, Fig. 2C). In contrast to the insulin results, the HFC group showed increased GLP1 concentrations compared to the HF (+23%), HFW (+22%), and HFL (+18%) groups (Fig. 2D), suggesting a possible interaction between PPAR-alpha activation and DPP-4 inhibition regarding this endpoint.

Only the combined treatment caused marked browning in the sWAT, in addition to rescuing the white adipocyte size

The C group showed standard-sized unilocular adipocytes. In contrast, there were hypertrophied unilocular adipocytes in the HF group (Fig. 3A). The HFW and HFL groups showed standard-sized unilocular adipocytes but without significant browning. Conversely, the HFC group had abundant multilocular adipocytes within sparse standard-sized unilocular adipocytes, characterizing the browning of sWAT (Fig. 3A). The HF group showed a larger cross-sectional area of adipocytes than the C group (+148%, Fig. 3B), indicating adipocyte hypertrophy. However, all treated groups exhibited a decreased cross-sectional area of adipocytes (−54% for HFW vs HF; −55% for HFL vs HF, and −75% for HFC vs HF, Fig. 3B). The HFC group exhibited

Figure 3
sWAT histology and immunohistochemistry for β3AR and UCP1 (A); cross-sectional area of sWAT adipocytes (B); UCP1 immunodensity (C).

(A) Photomicrographs of s.c. white adipose tissue (sWAT) stained with hematoxylin-eosin showing hypertrophied adipocytes in the HF group and normal-sized white adipocytes intertwined with beige adipocytes in the HFC group. Representative images of β3AR immunostaining revealed a positive immunoreaction in the HFC group, reinforcing the abundant browning observed. UCP1 immunostaining followed the same pattern, and the immunodensity confirmed the enhanced UCP1 expression in the HFC group, suggesting increased thermogenesis. All scale bars = 50 μm.

(B and C) Brown-Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± s.d., n = 5). Significant differences (P < 0.05) are indicated as follows: **P < 0.01; ***P < 0.001; ****P < 0.0001. Groups: C, control diet; HF, high-fat diet; HFW, high-fat diet plus WY14643 (PPAR-alpha agonist); HFL, high-fat diet plus linagliptin (DPP-4i); HFC, high-fat diet plus WY14643 (PPAR-alpha agonist) and linagliptin (DPP-4i). A full color version of this figure is available at https://doi.org/10.1530/JME-21-0084.
adipocytes smaller than the C group due to abundant browning (−37%, Fig. 3B).

To further confirm the browning phenomenon in sWAT, immunohistochemistry revealed a positive immunoreaction for β3-AR (thermogenesis initiator) in the HFC group (Fig. 3A). Moreover, the HFC group showed expression augmentation of the thermogenesis effector UCP1 compared to the HF (+468%), HFW (+768%), and HFL (+502%) groups (Fig. 3A and C).

The combined treatment was unique to counter BAT whitening

The BAT photomicrographs (Fig. 4A) show brown adipocytes that resemble unilocular white adipocytes in the HF group, and the whitening phenomenon contrasts with the multilocular brown adipocytes in the C group. The HF group showed increased BAT mass/g BM (+33%, Table 2) coupled with a marked reduction in the numerical density of nuclei per area (Q₄ nuclei) in comparison with the C group (−50% Fig. 4B), consistent with the presence of large, unilocular adipocytes, in line with a whitening phenotype. The groups treated with the monotherapies had a mild whitening improvement, as both HFW and HFL showed plentiful whitened adipocytes within the BAT (Fig. 4A). The HFL group showed a significant reduction in BAT mass/g BM (−25% for HFL vs HF, Table 2), and the HFW group had a discrete increase in Q₄ nuclei (+27% for HFW vs HF, Fig. 4B) but neither rescued brown adipocyte adverse remodeling. Conversely, only the HFC group showed multilocular adipocytes similar to the C group, without whitening (Fig. 4A). Of note, the HFC group had a lessened BAT mass/g BM (−24%, Table 2) coupled with greater Q₄ nuclei than the HF group (+71%, Fig. 4B), suggesting a restoration of BAT cytoarchitecture after the combined treatment.

The HF group showed a higher RER than the C group (+8%, Table 2). In comparison, all treated groups exhibited a significant reduction in RER (−13% for HFW vs HF; −23% for HFL vs HF; and −10% for HFC vs HF, Table 2) without any difference from the C group.
Final body mass (A); energy intake (B); plasma leptin concentrations (C), plasma adiponectin concentrations (D); UCP1 immunofluorescence (upper panel), and infrared thermography (lower panel) (E). Brown–Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± s.d., n = 5). Significant differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Positive UCP1 areas appear green, showing positive immunoreactions for both treated groups and the absence of UCP1 expression in the HF group (upper panel). Infrared thermography showed increased body temperature in the treated groups (lower panel). Both results suggest increased thermogenesis after the treatment. Groups: C, control diet; CC, control diet plus WY14643 and linagliptin; HF, high-fat diet; HFC, high-fat diet plus WY14643 (PPAR-alpha agonist) and linagliptin (DPP-4i). A full color version of this figure is available at https://doi.org/10.1530/JME-21-0084.

Figure 6
sWAT gene expression of Ppar-a (A), Pgc1-a (B), β3ar (C), Ucp1 (D), Prdm16 (E) and Bmp8b (F). Brown–Forsythe and Welch one-way ANOVA and Dunnett T3 post hoc test (mean ± s.d., n = 5). Significant differences (P < 0.05) are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001. Groups: C, control diet; CC, control diet plus WY14643 and linagliptin; HF, high-fat diet; HFC, high-fat diet plus WY14643 (PPAR-alpha agonist) and linagliptin (DPP-4i).
Experiment 2: The combined treatment elicited UCP1+ beige adipocytes by countering ER stress and inflammation

The combined treatment enhanced body temperature and UCP1 expression in sWAT and normalized the adipokine profile

At the end of the treatment, the HFC rescued the BM without any difference compared to the C group and a significant reduction compared to the HF group (−11%, Fig. 5A). Interestingly, the CC group presented a lower BM than the C group (−3%, Fig. 5A). The BM reduction can be attributed to the treatment, as animals fed the same diet did not have different energy intakes (Fig. 5B).

The high BM found in the HF group complied with hyperleptinemia (+136% for HF vs C, Fig. 5C), while the combined treatment countered this metabolic impairment (−56% for HFC vs HF, Fig. 5C). In contrast, the adiponectin concentrations were lower in the HF group than in the C group (−16%, Fig. 5D), while the combined treatment increased the adiponectin levels in both treated groups when compared to their counterparts (+18% for CC vs C and +38% for HFC vs HF, Fig. 5D).

UCP1 showed positive immunostaining in both treated groups, confirming the presence of functional beige adipocytes in the sWAT of the CC and HFC groups (Fig. 5E). Moreover, the average body temperature increased significantly after the treatment (+3% for CC vs C and +2% for HFC vs HF, Fig. 5E), implying increased thermogenesis and energy release as heat.

PPAR-alpha agonism plus DDP4 inhibition induced thermogenic genes in sWAT

The relative mRNA expression of Ppar-alpha, which has thermogenic markers as its target genes, was lower in the HF group than in the C group (−50%, Fig. 6A) but it was higher in both treated groups than in their counterparts (+189% for CC vs C and +296% for HFC vs HF, Fig. 6A).

The mitochondrial biogenesis regulator Pgc1-a had reduced expression in the HF group (−74% for HF vs C, Fig. 6B). In contrast, the HFC group showed higher Pgc1-a expression than the HF group (+430%, Fig. 6B). Significantly, the HFC group did not differ from the C group or the CC group.

β3-ar expression also increased in the HFC group (+396% for HFC vs HF, Fig. 6C), implying that the combined treatment activated thermogenesis in response to the beta-adrenergic stimulus. Consistent with this, the HF group had lower Ucp1 expression than the C group (−69%, Fig. 6D), while the HFC group showed enhanced Ucp1 expression (+227% for HFC vs HF, Fig. 6D).

Prdm16, an essential gene to maintain the beige phenotype, had higher expression in the HFC group than in the HF and C groups (+478% and +122%, Fig. 6E). Bmp8b, a batokine involved in energy homeostasis, also had higher expression in the HFC group (+569% for HFC vs HF, Fig. 6F), consistent with enhanced thermogenesis.
Table 3  Detailed two-way ANOVA results.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Interaction % of total variance</th>
<th>P-value</th>
<th>Treatment % of total variance</th>
<th>P-value</th>
<th>Diet % of total variance</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body mass</td>
<td>37.44</td>
<td>&lt;0.0001</td>
<td>44.27</td>
<td>&lt;0.0001</td>
<td>14.23</td>
<td>&lt;0.0001</td>
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<td>Energy intake</td>
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<td>0.0023</td>
<td>NS</td>
<td>0.0268</td>
<td>25.71</td>
<td>0.0003</td>
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<tr>
<td>sWAT</td>
<td>32.38</td>
<td>0.0001</td>
<td>22.20</td>
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<td>83.00</td>
<td>&lt;0.0001</td>
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<td>eWAT</td>
<td>22.39</td>
<td>0.0036</td>
<td>16.45</td>
<td>0.0099</td>
<td>21.15</td>
<td>0.0001</td>
</tr>
<tr>
<td>BAT/g body mass</td>
<td>NS</td>
<td>0.0642</td>
<td>28.15</td>
<td>0.0036</td>
<td>23.47</td>
<td>0.0067</td>
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<td>Body temperature</td>
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<td>11.45</td>
<td>&lt;0.0001</td>
<td>7.22</td>
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<td>RER</td>
<td>45.13</td>
<td>&lt;0.0001</td>
<td>23.67</td>
<td>&lt;0.0001</td>
<td>5.53</td>
<td>0.0138</td>
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<tr>
<td>AUC for OGTT</td>
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<td>&lt;0.0001</td>
<td>53.79</td>
<td>&lt;0.0001</td>
<td>14.60</td>
<td>0.0006</td>
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<td>GLP1</td>
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<td>42.37</td>
<td>&lt;0.0001</td>
<td>21.15</td>
<td>0.0001</td>
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<td>Insulin</td>
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<td>&lt;0.0001</td>
<td>73.96</td>
<td>&lt;0.0001</td>
<td>21.15</td>
<td>0.0001</td>
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<td>Adiponectin</td>
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<td>0.0028</td>
<td>25.48</td>
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<td>Leptin</td>
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<td>48.28</td>
<td>&lt;0.0001</td>
<td>13.85</td>
<td>&lt;0.0001</td>
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<td>sWAT adipocytes area</td>
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<td>24.49</td>
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<td>58.50</td>
<td>&lt;0.0001</td>
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<td>Qₐ nuclei (BAT)</td>
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<td>Chop</td>
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<td>Ptim1</td>
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<td>Cpt1b</td>
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<td>0.0009</td>
<td>NS</td>
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<td>Pdk4</td>
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<td>0.0160</td>
<td>39.94</td>
<td>0.0009</td>
<td>NS</td>
<td>0.2455</td>
</tr>
</tbody>
</table>

The most powerful factor influencing total variance is in bold for each parameter. Whenever a factor or the interaction influence on the parameter was not significant, it is described as NS.
expression in the HFC group than in the HF group (+117%, Fig. 6F).

Treatment mitigated local inflammation within the sWAT
The relative mRNA expression of Dpp4 did not differ between the C and HF groups. However, the treatment caused a significant reduction in Dpp4 expression in the HFC group (−59% for HFC vs HF, Fig. 7A).

Tlr4, a proinflammatory receptor, showed higher expression in the HF group than in the C group (+183%, Fig. 7B). Nevertheless, the combined treatment elicited reduced Tlr4 expression (−66%, for HFC vs HF, Fig. 7B), suggesting enhanced thermogenesis. The expression level of Tlr4 in the HFC group did not differ from that of the C group.

Similarly, Nlrp3, an inflammasome, showed higher expression in the HF group than in the C group (+197%, Fig. 7C). Conversely, the HFC group had a marked reduction in Nlrp3 expression (−44% for HFC vs HF, Fig. 7C).

The treatment alleviated endoplasmic reticulum stress
The expression of Atf4, a transcription factor that regulates several genes involved in the UPR during ER stress, was augmented in the HF group compared with the C group (+61%, Fig. 7D). In contrast, the HFC group showed lower Atf4 expression than the HF group (−34%, Fig. 7D).

Chop, a transcription factor that mediates apoptosis, followed the same pattern as Atf4: the HF group had higher expression than the C group (+91%, Fig. 7E), whereas the CC and HFC groups showed lower expression than their counterparts (−42% for CC vs C and -76% for HFC vs HF, Fig. 7E). Similarly, Gadd45 showed higher expression in the HF group than in the C group (+70%, Fig. 7F), parallel to lower values in the HFC group than in the HF group (-59%, Fig. 7F). These findings reinforce the beneficial effects of PPAR-alpha agonism and DPP4 inhibition association on ER function, attenuating the expression of genes linked to UPR and apoptosis.

PPAR-alpha agonism plus DPP4 inhibition caused the cellular metabolism to shift toward lipid oxidation
Cidea, whose expression correlates with fat accumulation in WAT, showed the lowest expression in the treated groups. However, only the HFC was significantly reduced (-46% for HFC vs HF, Fig. 8A). Likewise, Plin1, related to lipid droplet instability, showed significantly lower expression in the HFC group than in the HF group (-58%, Fig. 8B).

The relative expression of Cpt1b, responsible for beta-oxidation in adipose tissue, was enhanced in the HFC group compared to the HF group (+128%, Fig. 8C). Furthermore, Pdk4 expression increased in the HFC group compared to the HF group (+64%, Fig. 8D). These findings suggested that lipids were the preferential fuel for thermogenesis induced by the treatment.

Two-way ANOVA
The treatment exerted the most significant influence on the final body mass, BAT/g BM, GLP1, insulin and adiponectin concentrations, cross-sectional sWAT adipocyte area, thermogenic genes (Ppar-a, Pgc1-a, Ucp1, Prdm16, Bmp8b), inflammatory and ER stress genes (Dpp4, Chop, Gadd45), lipolysis, beta-oxidation, and fuel preference-related genes (Plin1, Cidea, Cpt1b, Pdk4).

Diet was the most significant factor in determining energy intake, sWAT mass, body temperature, Qc, nuclei, thermogenic genes (β3ur), inflammasome genes (Nlrp3), and ER stress genes (Atf4). Both factors (diet and treatment) interact to exert the most significant influence on sWAT mass, AUC for OGTT, leptin concentrations, RER, and Tlr4 gene expression. The two-way ANOVA details are described in Table 3.

Discussion
The present findings showed that the HF diet for 17 weeks caused overweight, insulin resistance, white adipocyte hypertrophy, BAT whitening, increased proinflammatory and ER stress markers, and decreased thermogenesis. Both monotherapies (PPAR-alpha agonist or DPP-4 inhibitor) yielded a reduced body mass and better glucose tolerance. Even though the HFW and HFL white adipocytes exhibited a normal size, significant browning did not occur and BAT whitening persisted. Conversely, animals treated with a combination of PPAR-alpha agonist and DPP4i benefited showing a complete rescue of BM, glucose tolerance, white adipocyte size, and brown adipocyte multilocularity, in addition to inflammation and ER stress alleviation. Furthermore, these positive outcomes complied with UCP1+ beige adipocyte induction and small and multilocular brown adipocytes, confirming browning of sWAT and alleviation of BAT whitening.

Due to the diet’s high saturated fatty acid content, HF-fed mice developed an obesity phenotype with increased fat pad distribution in different compartments, as
Huang 2017, resulted in increased thermogenesis. Increased adrenergic. To further explore. Monotherapy with WY14643 increased BAT. Thus, the high. This scenario complies with their increased adipocyte cross-sectional area and their poor performance on the OGTT, with a significant AUC and oral glucose intolerance. Monotherapies with WY14643 or linagliptin alleviated oral glucose intolerance and adipocyte hypertrophy but did not revert the adipoinasular axis disruption or induce browning of sWAT. Conversely, the combined treatment restored the adipoinasular axis function and normalized the plasma insulin and leptin concentrations, as reflected by their standard-sized white adipocytes and robust browning induction in the sWAT, associated with metabolic benefits (Gray et al. 2010, Bartelt & Heeren 2014).

Only the combined treatment produced increased plasma total GLP-1 concentrations, which potentialize glucose-stimulated insulin secretion concomitant with glucagon inhibition (Drucker & Nauck 2006, Campbell & Drucker 2013). Interestingly, HFL and HFC showed similar performances on OGTT, but HFL had higher insulin concentrations than HFC. These results imply that although this dose of linagliptin (15 mg/kg body mass) for 5 weeks did not increase the total GLP1 concentration like the high dose (Santos et al. 2020), it increased insulin release in the HFL group. The HFC group may benefit from a possible additive effect of PPAR-alpha activation increasing the total GLP1 levels (Silva-Veiga et al. 2020) suggesting that this combined treatment is a promising approach to treat obesity by maximizing the insulin-sensitizing and anti-inflammatory effects of both agents (Zhuge et al. 2016, Takahashi et al. 2017), resulting in increased thermogenesis.

A stereological tool aimed to address brown adipocyte dysfunction (whitening) for the first time, along with metabolic data. Chronic HF feeding caused enlargement of brown adipocytes with whitened unilocular adipocytes resulting in a decreased numerical density of nuclei per tissue area. BAT whitening is triggered by proinflammatory signals and hypoxia, causing mitochondrial loss, and reducing BAT thermogenic function (Shimizu et al. 2014). Monotherapy with WY14643 increased BAT nuclei density, but it was not enough to rescue BAT whitening. In contrast, the combined treatment elicited an increased BAT nuclei density, equal to the C group, consistent with small brown adipocytes, a restoration of their multilocular characteristics, and the competence to perform thermogenesis. Furthermore, the increased body temperature and adiponectin concentrations in animals treated with the combined therapy agree with these results, suggesting energy dissipation as heat due to enhanced BAT activity (Spiegelman 2013, Sun et al. 2020).

Concerning the sWAT reservoir, only the sWAT of the HFC group expressed β3AR and UCP1, ensuring that the browning phenomenon occurred. The thermogenic pathway in sWAT relies on sympathetic stimulation for initiation, and UCP1 acts as an alternative channel to proton gradient return from the mitochondrial intermembrane space, uncoupling energy utilization from ATP synthesis and releasing energy as heat instead (Spiegelman 2013, Ricquier 2017). To further explore the browning phenomenon observed in the HFC group, immunofluorescence confirmed the observed multilocular adipocytes as UCP1+, in addition to β3-ar and Ucp1 upregulation coupled with increased Bmp8b implying a greater sWAT thermogenic responsiveness to the adrenergic stimulus (Whittle et al. 2012).

It is noteworthy that the temperature used in the present study cannot induce browning of sWAT itself (Kalinothic et al. 2017) and that the browning phenomenon is reversible, with increased Prdm16 expression being necessary to maintain the beige phenotype (Cohen et al. 2014). The combined treatment yielded the most pronounced Prdm16 expression supporting the hypothesis that this drug combination induced beige adipocytes competent to perform thermogenesis (Nedergaard et al. 2007).

Regarding cellular metabolism, the WAT of obese and insulin-resistant mice benefits from Pdk4 upregulation (Wan et al. 2012), which means inhibition of glucose uptake by the cells (White et al. 2007). Thus, the high Pdk4 expression complied with lipid utilization as fuel to the beige adipocytes induced by the combined treatment in HF-fed animals. However, the Pdk4 pathway in adipose tissue from HF-fed rodents is not like that in adipose tissue from lean rodents (Zhang et al. 2014). Increased adrenergic stimulation and PPAR-alpha activation by WY14643 can induce Pdk4 in white adipose tissue and emerge as a surrogate of sWAT browning (Huang et al. 2002, Zhang et al. 2014). In agreement with the preference for lipids as fuel for thermogenesis, the HFC group showed Plin1 and Cidea downregulation, implying more stability (Sztalryd...
et al. 2003) and smaller lipid droplets (Barneda et al. 2013, Chen et al. 2020) within the observed beige adipocytes. These observations suggest that lipid overload from the HF diet supplied cell metabolism in the sWAT.

Linagliptin association with a PPAR-alpha agonist is promising, as both mechanisms of action may overlap and interact. Recently, mice fed a high-fructose diet and treated with WY14643 showed increased GLP1 concentrations (Silva-Veiga et al. 2020) implying that PPAR-alpha anti-inflammatory effects can also trigger DPP4 inhibition (Takahashi et al. 2017). On the other hand, DPP4 inhibition by a high dose of linagliptin provoked browning in sWAT through PPAR-alpha induction (de Oliveira Correia et al. 2019). This transcription factor has many thermogenic markers as target genes (Hondares et al. 2011). Hence, the present results endorse this challenging combination as suitable to treat obesity in this experimental model by combining the highest Ppar-alpha expression and GLP1 concentrations parallel to the lowest Dpp4 and Tlr4 in the sWAT.

The PPAR-alpha agonist combined with DPP4i normalized Pgc1α expression in the HF-treated group, resembling the C and CC groups. This result indicates increased mitochondrial biogenesis, a viable strategy to counter obesity by sustaining the new beige adipocyte physiology favoring thermogenesis over lipogenesis (Tiraby et al. 2003, Puigserver 2005). Anti-inflammatory effects also mediated the present findings. This drug combination has previously been shown to counter endotoxemia by rescuing the gut ultrastructure and mitigating hepatic steatosis via reduced TLR4 expression in the liver of high-fructose-fed mice (Silva-Veiga et al. 2020). Previous findings indicate that linagliptin inhibits lipopolysaccharide-induced inflammation, causing TLR4 suppression (Sato et al. 2019) in addition to yielding cardioprotective effects by attenuating inflammasomes (Birnbaum et al. 2019). The combined treatment has driven similar findings in sWAT, showing reduced Dpp4 expression, an adipokine involved in obesity metainflammation (Lamers et al. 2011). The reduced Dpp4 and Tlr4 expression levels comply with Nlrp3 downregulation in sWAT, which protects UCP1 and favors beta-adrenergic stimulated beige adipocyte formation (Okla et al. 2018).

Figure 9
Summary of the main findings. The HF diet caused white adipocyte hypertrophy coupled with whitening of brown adipocytes. Obesity and insulin resistance cause endoplasmic reticulum stress, inflammation, and impaired thermogenesis. Conversely, the conjugation of PPAR-alpha agonism with DPP-4 inhibition reversed brown adipocyte whitening, inflammation, and ER stress in s.c. white adipose tissue, resulting in the induction of beige adipocytes and enhanced thermogenesis. These results complied with the normalization of body mass in treated animals fed a high-fat diet. A full color version of this figure is available at https://doi.org/10.1530/JME-21-0084.
On the other hand, Tlr4 induction by an HF diet blocks the browning of sWAT through mitochondrial functional impairment and inflammasome activation, which reduces Ucp1 in a paracrine fashion (Okla et al. 2018). Chronic HF diet intake triggers glucolipotoxicity by driving ectopic lipid accumulation (lipotoxicity) concomitant with hyperglycemia (glucotoxicity) (Veiga et al. 2017). These conditions underlie ER stress, as this organelle uses glucose as the primary fuel. ER stress, in turn, aggravates inflammation and triggers apoptosis (Humasti & Hotamisligil 2010). When ER homeostasis is compromised, UPR activation aims to counter ER stress (Zhao et al. 2013). Herein, a chronic HF diet intake caused inflammation through Tlr4 and Nlrp3, followed by increased expression of UPR genes. Atf4 is a transcription factor that regulates other UPR genes, such as Chop (Fusakio et al. 2016), blocking the cell cycle by Gadd45 transcription (Oyadomari & Mori 2004). Atf4, Chop, and Gadd45 upregulation in the HF group aimed to protect the adipocytes from the UPR toxic effects (Galligan et al. 2012), consistent with the results of a previous study that used an HF diet (40% energy as fat) for 12 weeks (Luo et al. 2016). Instead, the combined treatment managed to suppress the UPR-related genes, alleviating ER stress, complying with better insulin sensitivity and mitochondrial enhancement toward the beige phenotype in sWAT. Figure 9 summarizes the main results from the present study.

This study has some limitations, as we could not evaluate gene expression in BAT due to a limited sample. We plan to establish a new stereological tool to address BAT whitening. Therefore, future research may focus on the pathways related to BAT whitening alleviation, in addition to evaluating the active GLP1 and high molecular weight adiponectin concentrations.

Conclusion
In conclusion, a PPAR-alpha agonist and DPP-4i combination were necessary to drive expressive browning in the sWAT of high-fat-fed mice as the monotherapies did not elicit browning. In BAT, the combined treatment yielded an increased nuclear density and a restoration of multilocularity suggesting the measurement of nuclei density as a complementary tool to address whitening. The present results point to a browning stimulation parallel to a complete whitening alleviation after combining a PPAR-alpha agonist with a DPP-4i treatment suggesting promising anti-obesity effects of the proposed treatment. Furthermore, this combined treatment rescued inflammation and ER stress in the sWAT, increasing thermogenesis fueled by excessive dietary lipids in the induced beige adipocytes.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
D A S-O and A S-F: conceptualization, methodology, formal analysis, investigation, writing – original draft, project administration. C S M and F F M: formal analysis, investigation, visualization, writing – review and editing. C A M L: writing – review and editing, visualization, funding acquisition. V S-M: conceptualization, supervision, visualization, writing – review and editing, methodology, funding acquisition.

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