Expression enhancement in brown adipose tissue of genes related to thermogenesis and mitochondrial dynamics after administration of pepsin egg white hydrolysate

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Acknowledgements: The authors want to acknowledge the financial support from the Spanish Ministry of Economy, Industry and Competitiveness (MINECO) (under the project AGL2012-32387, AGL2017-89213, CSIC – Intramural 201570I028 and under the grant numbers BES-2013-065684 and

EEBB-I-16-11349). The authors are grateful as well to Raquel Franco, Julio Paredes and Antonio Márquez for their technical assistance.

Abstract

Purpose: Nutritional compounds could be a safe and less expensive treatment for complications associated to obesity and metabolic syndrome (MetS). The aim of this study was to investigate the mechanism of action and the target tissues of a pepsin egg white hydrolysate (EWH) which had previously demonstrated to improve some obesity-related disorders on high-fat/high-glucose rat model.

Methods: Wistar rats were used and divided in 3 groups: Control group (C), High-fat/high-glucose diet (MS) and high-fat/high-glucose diet + EWH (MSH). The rats were fed during 20 weeks and the EWH was administrated from week 9^{th}. At the end of the study, white adipose tissue (WAT), brown adipose tissue (BAT) and muscle samples were collected for RT-qPCR analyses and immunohistochemistry.

Results: Our results showed a gene expression enhancement in BAT of genes related to thermogenesis and mitochondrial dynamics. Mitochondrial DNA quantification and immunohistochemistry results showed an increase of mitochondrial content in this tissue.

Conclusions: In conclusion, our results show the potential metabolic effect of this pepsin EWH by enhancing mitochondria proliferation and gene expression related to thermogenesis in BAT. EWH could be used as functional food ingredient able to increase energy expenditure and counteract obesity-related MetS in a chronically obese society.

Keywords

Bioactive peptides; Egg white hydrolysate; Metabolic syndrome; Pathway; Rat model.

1. Introduction

Metabolic syndrome (MetS) refers to a cluster of metabolic risk factors including central obesity, insulin resistance, impaired glucose tolerance, hypertension and dyslipidemia. This pathology increases the risk to develop other diseases such as type 2 diabetes mellitus or non-alcoholic fatty liver disease [1]. In the last decades, the prevalence of this pathology has increased fast, reaching epidemic rates. This increase is especially important in developed areas, due to important changes in our lifestyle and dietary patterns [2, 3]. Sedentary habits and overnutrition both combined, lead to an imbalance between energy intake and energy expenditure that cause an excessive fat accumulation and thus obesity, main risk factor to develop MetS [1, 2].

The current treatment used in MetS complications are, firstly, lifestyle change interventions in order to balance the caloric intake, through changing the dietary patterns and reducing the sedentary habits on MetS patients. However, it is difficult for most people to maintain hypocaloric diets and lifestyle changes over a long term [2]. Due to this, in almost all cases, it is necessary a pharmacotherapy intervention and, sometimes, surgery. Such treatments seriously increase public health care expenses [4]. There is therefore an imperative need for new weight-loss treatments.

Nutritional compounds are nowadays attracting much attention because, compared to drugs, they could be a safe and less expensive treatment for complications associated to obesity and MetS [5]. There is a large and growing list of food-derived compounds that have demonstrated to control metabolic complications related to MetS [2, 6-8], being several of them potential stimulators of thermogenesis and lipids oxidation, both potential targets on weight loss treatments [5, 9, 10]. Among the different food-derived products used to treat MetS, bioactive peptides released from both animal and plant proteins, have already shown

to have potential interest as treatment for numerous chronic diseases and risk factors. These bioactive peptides have shown different biological activities such as antihypertensive, antioxidant or anti-inflammatory activities which separately or combined could be useful for MetS treatment [11-18].

Adipose tissue acts as the body's energy reservoir by storing excess of fatty acids in the form of triglycerides. Its main function is to buffer variations in energy supply and demand by storing and releasing fatty acids, and avoiding lipid accumulation in peripheral organ, thus preventing metabolic diseases such as non-alcoholic fatty liver disease [19]. In addition, adipose tissue is recognized as an endocrine organ which regulates and modulates several biological processes including immune response, inflammation, glucose metabolism or regulation of appetite and satiety [20]. Since white adipose tissue (WAT) main function is fatty acid storage, brown adipose tissue (BAT) is a key site of non-shivering thermogenesis and it has been considered an attractive target to promote weight loss [4, 21]. BAT is activated by cold exposure through sympathetic signaling in rodents and it has been shown to improve insulin sensitivity [21]. BAT is definitively present and functional in adult humans, and fluorodeoxyglucose positron emission tomography (FDG-PET) application has demonstrated that BAT in adults is also stimulated by cold and by insulin (89-92), but the activity of BAT is lower in obese than lean individuals (88-90,93,94). Recently, reduced activity in BAT has ~~recently~~ linked to the development of the metabolic syndrome (99, 101). Therefore, biomedical interest in BAT has focused on the potential capacity of this tissue to counteract MetS, including obesity and type 2 diabetes [4]. However, cold exposure is not enough to activate BAT in humans and mediate weight loss. It is hence needed to develop compounds able to efficiently differentiate human BAT and enhance nutrient oxidation and energy expenditure [1, 21]. In this context, whey and salmon protein hydrolysates have

already shown to enhance thermogenesis and energy expenditure in interscapular BAT [5, 9].

Our research group has recently obtained a pepsin egg white hydrolysate (EWH) which simultaneously possess antioxidant, hypocholesterolemic and DPP-IV inhibitory activities *in vitro* [22] and have also shown to improve some MetS alterations in Zucker Fatty rats as a genetic model of obesity [23] and in a diet induced obesity (DIO) experimental model using high-fat/high-glucose fed Wistar rats [24]. However, the pathway by which this EWH exerts its effects *in vivo* is still unclear.

The aim of our study was to investigate the specific pathways and target tissues involved in the beneficial effects observed after administration of this previously characterized pepsin EWH [22] on high-fat/high-glucose fed Wistar rats.

2. Materials and Methods

2.1. Preparation of egg white hydrolysate

The EWH was carried out according to the method of Garcés-Rimón *et al*. Briefly, pasteurized egg white (Huevos Guillén S.L., Spain) was hydrolysed with food grade pepsin from pork stomach (E.C. 3.4.23.1. BC PEPSIN 1:3000 Biocatalysts, United Kingdom). The egg white was acidified with concentrated food grade HCl 37 % (Panreac Quimica S.L.U., Spain) to pH 2. The samples were incubated at 37 °C under constant stirring in a thermostatic water bath for 8 hours. Inactivation of pepsin was achieved by increasing the pH to 7.0 with food grade NaOH 10 M (Panreac Quimica S.L.U.). The hydrolysate was centrifuged for 15 min at 4500 g, and the supernatant was freeze-dried and stored at -20 °C until use [22].

2.2. General protocol in animals

The experiments were designed to minimize the number of animals used and performed in accordance with the European and Spanish legislation on care and use of experimental animals (210/63/UE; Real Decreto 53/2013), and were approved by the Ethics Committee at University Rey Juan Carlos (URJC).

Thirty-four 8-week old Wistar male rats weighting 280-310 g purchased from Harlan Laboratories (Harlan Ibérica, Spain) were used in this study. During the experimental period the animals were maintained in a temperature-controlled room (23 °C), 12 h light/dark cycles and *ad libitum* access to water and feed.

The rats were randomly divided into 3 groups which were fed, for 20 weeks, with standard chow diet (A04, SAFE, France) + tap water as drinking fluid (Control group, C, n=7), high-fat diet (Purified Diet 235 HF, SAFE, France) + a solution of tap water with 25% glucose as drinking fluid (MetS group, MS, n=10) and high-fat diet with + a solution of tap water with 25% glucose and 1g/kg/day EWH as drinking fluid (MetS + EWH group, MSH, n=10). The EWH was provided from the week 9^{th} until the week 20^{th} of the study. The daily dose of EWH was selected according to the good results obtained from previous *in vivo* studies using different doses of EWH after short and long term treatments to evaluate the antihypertensive and antioxidants properties of the hydrolysate in spontaneously hypertensive rats [13, 14, 17].

At the end of the study, rats were anesthetized with an intraperitoneal injection of ketamin (87 mg/kg) and xilacin (13 mg/kg), and sacrificed by decapitation. Epididymal adipose tissue, BAT and soleus muscle were immediately excised and kept frozen at -80ºC until analysis. Samples of BAT were also obtained, fixed in buffered 10% formalin and embedded in paraffin. The tissues were then prepared for immunohistochemistry analysis in order to stain mitochondria.

141 *2.3. RNA isolation and qPCR*

142 Total RNA was extracted using TRIzol reagent (Life Technologies, France) according to
143 the manufacturer's instructions. The cDNA was synthetized from 1µg of total RNA using
144 random primers (Life Technologies) and Moloney murine leukemia virus reverse
145 transcriptase (Life Technologies) as previously described [6]. Real-time quantitative PCR
146 analyses (qPCR) were performed using the Mx3005P Real-Time PCR System (Stratagene,
147 USA). For each condition, expression was quantified in duplicate, and 18S rRNA was used as
148 the endogenous control in the comparative cycle threshold (C_T) method. Data were
149 expressed as relative expression ratio [25]. The sequences of the primers used for qPCR
150 determination of gene expression are displayed in Table S1.

151 *2.4. Mitochondrial DNA quantification*

152 Total DNA was extracted from cells using DNAzol (Euromedex, France). The content
153 of mitochondrial DNA (mtDNA) was calculated using real-time quantitative PCR by
154 measuring the threshold cycle ratio (ΔC_T) of a mitochondrial-encoded gene Cox1 versus a
155 nuclear-encoded gene cyclophilin A [10].

156 *2.5. Immunohistochemistry*

157 5 µm sections were washed with phosphate buffered saline (PBS) with 0.05% Tween
158 20 (Calbiochem, Darmstadt, Germany). Thereafter sections were incubated for 10 min in 3%
159 (v/v) in hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with fetal
160 bovine serum (FBS) for 30 min to minimize non-specific binding of the primary antibody.
161 Sections were then incubated overnight at 4°C with monoclonal mouse anti-SDHA antibody
162 (1:5000) (Abcam, United Kingdom). After incubation, samples were washed with PBS-Tween.
163 The peroxidase-based kit Masvision (Master Diagnostica, Spain) was used as secondary

antibody. Samples were counterstained with hematoxylin and coverslips mounted with Eukitt mounting media (O. Kindler GmbH & Co., Germany). To determine the level of non-specific staining, the preparations were incubated without the primary antibody as a negative control.

Samples were studied under a Zeiss Axioskop 2 (Zeiss, Germany) microscope equipped with the image analysis software package AxioVision 4.6 (Zeiss). The analysis was made under a 40× objective; the experimenter was blind to the treatment received by the rat from which the sample under analysis was obtained.

2.6. Statistical analysis

Data were expressed as the mean ± SEM. Significant differences between groups were determined using the non-parametric test Mann-Whitney using GraphPad Prism 6 software (Graph pad, USA). Differences between the means were considered to be significant when $P < 0.05$.

3. Results

3.1. Effects of EWH on white adipose tissue

Fig. 1 shows relative expression of genes implicated on synthesis, oxidation and mobilization of fatty acids in WAT. No differences were shown between experimental groups on Sterol Regulatory Element-Binding Protein 1c (SREBP1c) or Cell Death Activator CIDE-A (CIDEA). Gene expression of both Fatty Acid Synthase (FAS) and Acyl Co-A Carboxylase (ACC) were significantly lower in MS and MSH animals when compared to C group. No significant differences were observed between MS and MSH animals on gene expression of FAS and ACC. Gene expression of Adipocyte Protein 2 (AP2) was significantly higher in MS rats when compared to C and MSH rats. No differences were observed in this parameter between C

and MSH groups. No differences were observed on Acyl Co-A Oxidase (ACO) gene expression between MS and C animals. This parameter was significantly lower in MSH animals, when compared to MS and C groups.

No significant differences were observed between groups on Leptin or Leptin Receptor (LeptR) gene expression. Both MS and MSH animals showed significantly lower adiponectin gene expression when compared to C animals. Resistin gene expression was significantly lower on MSH group when compared to C and MS groups. No differences were observed between C and MS animals on this parameter. Insulin Receptor (InsR) gene expression was significantly lower on MS and MSH animals when compared to C, and no significant differences were observed on this parameter between MS and MSH. MS showed significantly lower Glutathione Peroxidase 3 (GPX3) expression when compared to C. Inducible Nitric Oxide Synthase (iNOS) showed to be increased in MSH animals when compared to MS animals, but this increase did not show to be significant when compared to C animals (Table 1).

3.2. Effects of EWH on fatty acid oxidation and metabolism

As shown in Fig. 2A, genes implicated on oxidative metabolism in muscle, such as Peroxisome Proliferator-Activated Receptor alpha (PPARα) and Medium-Chain Acyl-CoA Dehydrogenase (Mcad) were significantly downregulated in MS animals when compared to control animals. No differences were observed between MS and MSH animals in these genes. Although no changes were observed in Long-Chain Acyl-CoA Dehydrogenase (Lcad) expression when MS was compared to C animals, MSH animals showed a significant downregulation of Lcad gene when compared to MS and C animals. Regarding to Pyruvate Dehydrogenase Kinase 4 (Pdk4) and PPARγ Coactivator 1β (PGC1β), the same results as those obtained on Lcad were observed. Carnitine Palmytoiltransferase 1B (Cpt1b) was

significantly downregulated in MSH animals when compared to C animals, while MS animals stayed unchanged when compared to C. PPARγ Coactivator 1α (PGC1α) was significantly downregulated on MS animals when compared to C, while no differences were observed in MSH animals when compared to C and MS animals.

As shown in Fig 2B, all genes implicated on fatty acid and glucose oxidation (PPARα, Mcad, Lcad, Cpt1b, Pdk4, PGC1α, PGC1β) were significantly upregulated on MSH when compared to MS animals. PPARα, Mcad, Lcad, Cpt1b and PGC1β did not show expression changes in MS animals when compared to C animals. Pdk4 and PGC1α were significantly downregulated in MS animals when compared to C animals.

3.3. Effects of EWH on thermogenesis and mitochondrial dynamics on BAT

Although no significant differences were observed when compared C and MS animals, both UCP1 and PRDm16 showed a slight downregulation in MS group, MSH group showed a significant overexpression of both genes (Fig. 3**Fig. 3**A, Fig. 3B), when compared to C and MS groups.

Genes related with mitochondrial biogenesis and dynamics (Tfam, MFN2, Tfb2m, NRF1, OPA1) showed a significant downregulation on MS group when compared to C group (Fig. 3C). This downregulation was significantly reverted on MSH animals, and gene expression reached similar values to C animals, even surpassing them as on Tfb2m gene. Dynamin Like 1 (Dnm1L) expression was significantly higher on MSH animals when compared to MS animals, although no differences were observed when we compared both groups with respective controls.

Regarding to relative mtDNA quantity (Fig. 4A), a significant reduction of this parameter was observed in MS animals when compared to C animals. This reduction was

234 significantly reverted on MSH animals, which even surpass C values. Regarding to
235 immunohistochemistry analysis of BAT (Fig. 4B), it can be observed a significant reduction of
236 mitochondria density and larger lipid droplets on MS tissues when compared to C group.
237 There was as well an important reduction of lipid droplets and a notable increase of
238 mitochondria density on MSH tissues, when compared to MS tissues.

239 **4. Discussion**

240 In spite of the extensive current research on food-derived compounds and energy
241 expenditure stimulation [5, 9, 21], just a few studies are focused on food peptides and
242 proteins [9]. The aim of the present study was to determine the mechanisms of action by
243 which pepsin EWH exerts *in vivo* effect. This EWH had been previously characterized
244 showing to possess *in vitro* biological multi-activities [22], and have shown to improve MetS
245 related-pathologies in both Zucker Fatty rats and DIO Wistar rats [23, 24]. To carry out this
246 study, high-fat/high-glucose-fed Wistar rats were used as experimental MetS model. This
247 model has already shown to develop obesity, abdominal fat deposition, increased oxidative
248 stress biomarkers, impaired glucose tolerance and low-grade dyslipidemia [24].

249 In previous *in vivo* studies [23, 24], we had demonstrated that the consumption of
250 EWH significantly decreased the epididymal adipose tissue, improved hepatic steatosis, and
251 lowered plasmatic concentration of free fatty acids in the Zucker obese animals. It also
252 decreased plasma levels of tumor necrosis factor-alpha and reduced oxidative stress in this
253 experimental model. Recently, EWH consumption also normalized body weight gain, the
254 abdominal obesity and the peripheral neuropathy developed in high fat/high glucose
255 experimental model, reduced adipose tissue and liver weight, as well as plasma glucose.
256 Oxidative stress and inflammation biomarkers were normalized in these animals. Therefore,
257 we considered of great interest to clarify the mechanisms of action of EWH on obesity

condition to measure gene expression on tissues and organs involved in energetic metabolism (WAT, BAT, Muscle). Regarding gene expression on epididymal adipose tissue, genes related with *de novo* fatty acids synthesis (ACC, ACC) are strongly downregulated on MS animals. This is an expected result since *de novo* lipogenesis is usually reduced in obese individuals [26]. However, MSH animals did not show a reversion of such downregulation. On the contrary we observed a strong induction of AP2 in MS animals, which was not observed on MSH animals. Floresta *et al.* recently reviewed the important role of this protein in the development of major components of MetS [27].

Oxidative stress is strongly related to obesity and has been shown to precede MetS manifestation. As mentioned above oxidative stress was reduced after administration of EWH in several experimental models (citas). The impairment of mitochondrial function and an excessive ROS production leads to dysfunctional adipose tissue, contributing to a pro-inflammatory signaling and glucose metabolism impairment [28, 29]. Indeed, a continuous reduction of mitochondrial function is followed by the overproduction of oxidants from non-mitochondrial sources [29], such as the induction of peroxisomal oxidative metabolism [29, 30]. The MetS model used in this study has already shown an increase of malondialdehyde (MDA), an oxidative stress biomarker, and reduced plasma antioxidant activity [24]. In our study, the reduction observed on ACO gene expression of MSH animals seems to reflect a reduction peroxisomal oxidative metabolism thus reducing this source of oxidative stress [30, 31]. In previous works we observed an important reduction of oxidative stress biomarkers on high-fat/high-glucose-fed rats when consumed EWH [24]. Garcés-Rimón *et al.* also described the potential antioxidant activity of the studied EWH both *in vitro* [22] and *in vivo* [23], showing a significant decrease of lipid peroxidation (plasmatic MDA decreased), and increased levels of reduced glutathione in the liver of obese Zucker rats. It is well known

282 the role of nitric oxide (NO) during cardiovascular disease. Perivascular adipose tissue
283 dysfunction produced in obese individuals is strongly related with a lower NO availability and
284 a reduced vasodilation leading eventually to hypertension [32]. NO is an important mediator
285 of angiogenesis and vasodilation in hypoxia events [33]. In addition, Wang and Hai already
286 reviewed the importance of NO levels during adipocyte differentiation and modulation [34].
287 Although the pathways are complex and many factors are implied in these processes, our
288 result on iNOS gene expression could suggest an activation of adipocytes differentiation and
289 maturation combined with a stimulation of angiogenesis and adipose tissue vascularization
290 in MSH animals. Adipose tissue expansion is an important step on obese individuals, since
291 obesity-associated diseases, such as MetS could occur if adipose tissue storage capacity is
292 exceeded. Adipocyte differentiation and proliferation increases adipose tissue storage
293 capacity thus avoiding the release of free fatty acids to systemic circulation and then
294 avoiding ectopic lipid accumulation [1]. The stimulation of angiogenesis in this tissue would
295 besides avoid hypoxia and related adipose tissue dysfunction by a correct vascularization
296 and oxygen supply [35].

297 Resistin is a cytokine specifically produced on WAT that, in rodents, is positively
298 related with the development of insulin resistance [36]. Since our previous results suggested
299 an improvement of insulin sensitivity on MetS induced animals fed with pepsin EWH [24,
300 37], we decided to evaluate the expression of this cytokine. Our results showed a reduction
301 on the expression of this cytokine, in agreement with our previous results and the insulin
302 sensitizing effect of the studied EWH.

303 Although our results on WAT showed an effect of EWH on MetS development and
304 adiposity, we did not observe a strong trend which could suggest its mechanism of action.

Due to this, we suggest that the studied pepsin EWH could exert its action in other tissues, thus improving WAT markers and metabolism.

In previous studies, we had observed an increased body weight and abdominal circumference on MetS-induced animals which were reverted, at least partially, on EWH-treated animals [24]. These results were surprising considering that MetS-induced animals treated with EWH showed the highest caloric intake during the experimental period. Those results suggested a possible stimulation of energy expenditure. A large amount of food-derived compounds, including peptides and hydrolysates have already shown to induce energy expenditure by muscle or BAT stimulation [5, 6, 9], which led us to suggest that EWH could act stimulating energy expenditure. However, this idea should be investigated performing functional experiments using indirect calorimetry or FDG-PET.

For that reason, we decided to focus our experiments on energy expenditure specialized organs, such as muscle and BAT. On Fig. 2, it can be observed an important increase of oxidative genes on BAT of MSH rats (PPARα, Mcad, Lcad, Cpt1b, Pdk4), suggesting a strong activation of this tissue after EWH consumption, which could lead to an increased energy expenditure. To the contrary, these genes seemed to be downregulated in muscle of MSH animals even when this downregulation is not observed in MS rats. The counter-regulatory activity between BAT and muscle has been largely discussed [38-40]. It is been described that an over-activation of BAT by non-shivering thermogenesis leads to a reduction in the thermogenesis produced in muscle, probably leading to a reduction y energy expenditure in this tissue [39]. It is important as well the activation observed of PGC1β expression, which is considered one of the most important regulators of mitochondrial biogenesis [4, 29], suggesting an increase of mitochondria content on MSH rats BAT tissues. This result is of great importance as mitochondrial dynamics and biogenesis

329 are critical processes for the maintenance of mitochondrial function and the oxidative
330 balance in the cell. Our results suggest therefore that EWH could activate BAT metabolism
331 directly, but further studies are necessary to conclude that the enhancing thermogenesis as
332 mechanism of thIS activation.

333 BAT is specialized in regulated energy dissipation as heat through UCP1 and is been
334 suggested as a target for new anti-obesity treatments due to its capacity to counteract
335 metabolic diseases associated to obesity, such as MetS [4, 21]. These treatments are focused
336 on stimulating the mitochondrial proliferation as well as increasing the uncoupling capacity
337 of mitochondria in adipose tissues through PRDm16 and thus UCP1 activation [4]. In this
338 way, both UCP1 and PRDm16 showed to be overexpressed on MSH animals when compared
339 to C and MS animals, suggesting an increase of thermogenic capacity in BAT after
340 hydrolysate consumption. In addition, genes related with mitochondrial dynamics and
341 biogenesis (Tfam, MFN2, Tfb2m, NRF1, OPA1) showed to be less expressed in MS animals
342 when compared to controls. These alterations were counteracted with EWH consumption,
343 as MSH animals showed results similar to C animals, or even higher for some genes (Tfb2m).
344 Since these results suggested an important increase of mitochondrial content in BAT, mtDNA
345 quantification and mitochondria-specific immunohistochemistry were performed.
346 Accordingly with the mitochondrial dynamics observed, a significant decrease of mtDNA was
347 observed in BAT of MS animals. This implies not only a lower ability of these animals to
348 expend energy, but a major risk to develop oxidative stress and then metabolic disease.
349 Otherwise, MSH animals showed a significant increase of mtDNA in BAT, even when
350 compared to C animals. Regarding immunohistochemistry, BAT sections showed a lower
351 density of mitochondria on MS samples as well as an observable hypertrophy and larger lipid

352 droplets, while MSH animals showed an important reduction of lipid droplets, even when
353 compared to controls, as well as a higher density of mitochondria.

354 Altogether, our results showed a potential metabolic effect of our pepsin EWH by
355 enhancing mitochondria proliferation on BAT. As a consequence, An enhancing
356 thermogenesis could be happening and consequently a reduction in body weight and
357 adiposity could be observed. In addition, inflammation and oxidative stress biomarkers were
358 less expressed. To confirm whether the potential benefits observed after consumption of
359 pepsin EWH to counteract MetS in genetic and diet induced obesity animals could be due to
360 an increase in energy expenditure, more studies are needed to clarify the complete pathway
361 and the peptides implied in the observed activity.

362

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

5. References

1 Grundy SM (2016) Overnutrition, ectopic lipid and the metabolic syndrome. J Investig Med 64:1082-1086.

2 de la Iglesia R, Loria-Kohen V, Zulet MA, Martinez JA, Reglero G, Ramirez de Molina A (2016) Dietary strategies implicated in the prevention and treatment of metabolic syndrome. Int J Mol Sci. https://doi.org/10.3390/ijms17111877.

3 Rodríguez-Monforte M, Sánchez E, Barrio F, Costa B, Flores-Mateo G (2017) Metabolic syndrome and dietary patterns: a systematic review and meta-analysis of observational studies. Eur J Nutr 56:925-947.

4 Harms M, Seale P (2013) Brown and beige fat: development, function and therapeutic potential. Nat Med 19:1252-1263.

5 Bonet ML, Mercader J, Palou A (2017) A nutritional perspective on UCP1-dependent thermogenesis. Biochimie 134:99-117.

6 Marcotorchino J, Tourniaire F, Astier J, Karkeni E, Canault M, Amiot MJ, Bendahan D, Bernard M, Martin JC, Giannesini B, Landrier JF (2014) Vitamin D protects against diet-induced obesity by enhancing fatty acid oxidation. J Nutr Biochem 25:1077-1083.

7 Rühl R, Landrier JF (2016) Dietary regulation of adiponectin by direct and indirect lipid activators of nuclear hormone receptors. Mol Nutr Food Res 60:175-184.

8 Fenni S, Hammou H, Astier J, Bonnet L, Karkeni E, Couturier C, Tourniaire F, Landrier JF (2017) Lycopene and tomato powder supplementation similarly inhibit high-fat diet induced obesity, inflammatory response, and associated metabolic disorders. Mol Nutr Food Res 61. https://doi.org/10.1002/mnfr.201601083.

9 Dulloo AG (2011) The search for compounds that stimulate thermogenesis in obesity management: from pharmaceuticals to functional food ingredients. Obes Rev 12:866-883.

10 Tourniaire F, Musinovic H, Gouranton E, Astier A, Marcotorchino J, Arreguin A, Bernot D, Palou A, Bonet ML, Ribot J, Landrier JF (2015) All-trans retinoic acid induces oxidative phosphorylation and mitochondria biogenesis in adipocytes. J Lipid Res 56:1100-1109.

11 Miguel M, Recio I, Gomez-Ruiz JA, Ramos M, Lopez-Fandiño R (2004) Angiotensin I-converting enzyme inhibitory activity of peptides derived from egg white proteins by enzymatic hydrolysis. J Food Prot 67:1914-1920.

12 Dávalos A, Miguel M, Bartolomé B, López-Fandiño R (2004) Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. J Food Prot 67:1939-1944.

13 Miguel M, López-Fandiño R, Ramos M, Aleixandre A (2005) Short-term effect of egg-white hydrolysate products on the arterial blood pressure of hypertensive rats. Br J Nutr 94:731-737.

14 Miguel M, López-Fandiño R, Ramos M, Aleixandre A (2006) Long-term intake of egg-white hydrolysate products on the arterial blood pressure of hypertensive rats. Life Sci 78:2960-2966.

15 Miguel M, Alvarez Y, López-Fandiño R, Alonso MJ, Salaices M (2007) Vasodilator effects of peptides derived from egg white proteins. Regul Pept 140:131-135.

16 Miguel M, Manso M, Aleixandre A, Alonso MJ, Salaices M, López-Fandiño R (2007) Vascular effects, angiotensin I-converting enzyme (ACE)-inhibitory activity, and antihypertensive properties of peptides derived from egg white. J Agric Food Chem 55:10615-10621.

17 Manso MA, Miguel M, Even J, Hernández R, Aleixandre A, López-Fandiño R (2008) Effect of the long-term intake of an egg white hydrolysate on the oxidative status and blood lipid profile of spontaneously hypertensive rats. Food Chem 109:361-367.

18 Cicero AFG, Fogacci F, Colletti A (2017) Potential role of bioactive peptides in prevention and treatment of chronic diseases: a narrative review. Br J Pharmacol 174:1378-1394.

19 Lee YH, Mottillo EP, Granneman JG (2014) Adipose tissue plasticity from WAT to BAT and in between. Biochim Biophys Acta 1842:358-369.

20 Fasshauer M, Blüher M (2015) Adipokines in health and disease. Trends Pharmacol Sci 36:461-470.

21 Scheele C, Nielsen S (2017) Metabolic regulation and the anti-obesity perspectives of human brown fat. Redox Biol 12:770-775.

22 Garcés-Rimón M, López-Exposito I, López-Fandiño R, Miguel M (2016) Egg white hydrolysates with in vitro biological multiactivities to control complications associated with the metabolic syndrome. Eur Food Res Technol 242:61-69.

23 Garcés-Rimón M, González C, Uranga JA, López-Miranda V, López-Fandiño R, Miguel M (2016) Pepsin egg white hydrolysate ameliorates obesity-related oxidative stress inflammation and steatosis in Zucker Fatty rats. PLoS One 11. https://doi.org/10.1371/journal.pone.0151193.

24 Moreno-Fernández S, Garcés-Rimón M, González C, Uranga JA, López-Miranda V, Vera G, Miguel M (2018) Pepsin egg white hydrolysate ameliorates metabolic syndrome in high-fat/high-dextrose fed rats. Food Funct 9:78-86.

25 Livak KJ, Shmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402-408.

26 Tang Y, Wallace M, Sanchez-Gurmaches J, Hsiao WY, Li H, Lee PL, Vernia S, Metallo CM, Guertin DA (2016) Adipose tissue mTORC2 regulates ChREBP-driven de novo lipogenesis and hepatic glucose metabolism. Nat Commun 7. https://doi.org/10.1038/ncomms11365.

27 Floresta G, Pistarà V, Amata E, Dichiara M, Marrazzo A, Prezzavento O, Rescifina A (2017) Adipocyte fatty acid binding protein 4 (FABP4) inhibitors. A comprehensive systematic review. Eur J Med Chem 138:854-873.

438 28 Castro JP, Grune T, Speckman B (2016) The two faces of reactive oxygen species (ROS) in
439 adipocyte function and dysfunction. Biol Chem 397:709-724.
440 29 Wada J, Nakatsuka A (2016) Mitochondrial dynamics and mitochondrial dysfunction in
441 diabetes. Acta Med Okayama 70:151-158.
442 30 Zeng J, Deng S, Wang Y, Li P, Tang L, Pang Y (2017) Specific inhibition of acyl-CoA oxidase-1
443 by an acetylenic acid improves hepatic lipid and reactive oxygen species (ROS) metabolism
444 in rats fed a high-fat diet. J Biol Chem 292:3800-3809.
445 31 Dansen TB, Wirtz KWA (2001) The peroxisome in oxidative stress. IUBMB Life 51:223-230.
446 32 Xia N, Weisenburger S, Koch E, Burkart M, Reifenberg G, Förstermann U, Li H (2017)
447 Restoration of perivascular adipose tissue function in diet-induced obese mice without
448 changing bodyweight. Br J Pharmacol 174:3443-3453.
449 33 Kane AJ, Barker JE, Mitchell GM, Theile DRB, Romero R, Messina A, Wagh M, Fraulin FOG,
450 Morrison WA, Stewart A (2001) Inducible nitric oxide synthase (iNOS) activity promotes
451 ischaemic skin flap survival. Br J Pharmacol 132:1631-1638.
452 34 Wang X, Hai C (2015) Redox modulation of adipocyte differentiation: hypothesis of “redox
453 chain” and novel insights into intervention of adipogenesis and obesity. Free Radic Biol
454 Med 89:99-125.
455 35 Engin A (2017) Adipose tissue hypoxia in obesity and its impact on preadipocytes and
456 macrophages: hypoxia hypothesis. Adv Exp Med Biol 960:305-326.
457 36 Huang X, Yang Z (2016) Resistin’s, obesity and insulin resistance: the continuing disconnect
458 between rodents and humans. J Endocrinol Invest 39:607-615.
459 37 Garcés-Rimón M, González C, Vera G, Uranga JA, López-Fandiño R, López-Miranda V,
460 Miguel M (2018) Pepsin Egg White Hydrolysate Improves Glucose Metabolism
461 Complications Related to Metabolic Syndrome in Zucker Fatty Rats. Nutrients 10:441.

462 38 Sanchez-Delgado G, Martinez-Tellez B, Olza J, Aguilera CM, Gil A, Ruiz JR (2015) Role of
463 exercise in the activation of Brown adipose tissue. Ann. Nutr Metab 67:21-32.
464 39 Palmer BF, Clegg DJ (2017) Non-shivering thermogenesis as a mechanism to facilitate
465 sustainable weight loss. Obes Rev 18:819-831.
466 40 Aldiss P, Betts J, Sale C, Pope M, Budge H, Symons ME (2018) Exercise-induced “browning”
467 of adipose tissues. Metabolism 81:63-70.
468

Figures and tables captions

Fig. 1 Relative gene expression in epididymal adipose tissue (WAT) of (A) Sterol Regulatory Binding Protein 1c (SREBP1c), (B) Fatty Acid Synthase (FAS), (C) Acetyl CoA Carboxylase (ACC), (D) Adipocyte Protein 2 (AP2), (E) Acyl CoA Oxidase (ACO) and (F) Cell Death Activator CIDE-A (CIDEA). Experimental groups: Control group (☐), MetS group (▒) and MetS + EWH (■). Values are expressed as mean ± SEM (n ≥ 6). Different letters mean that values are significantly different ($p<0.05$) among groups

Fig. 2 Relative gene expression in (A) muscle and (B) brown adipose tissue (BAT) of Peroxisome Proliferator-Activated Receptor α (PPARα), Medium-Chain Acyl-CoA Dehydrogenase (Mcad), Long-Chain Acyl-CoA Dehydrogenase (Lcad), Carnitine Palmitoyltransferase 1B (Cpt1b), Pyruvate Dehydrogenase Kinase 4 (Pdk4), PPARγ Coactivator 1α (PGC1α) and PPARγ Coactivator 1β (PGC1β). Experimental groups: Control group (☐), MetS group (▒) and MetS + EWH (■). Values are expressed as mean ± SEM (n ≥ 6). Different letters mean that values are significantly different ($p<0.05$) among groups

Fig. 3 Relative gene expression in brown adipose tissue (BAT) of (A) Uncoupling Protein 1 (UCP1), (B) PR domain containing 16 (PRDm16) and (C) mitochondrial dynamics related genes. Experimental groups: Control group (C) (☐), MetS group (MS) (▒) and MetS + EWH (MSH) (■). Values are expressed as mean ± SEM (n ≥ 6). Different letters mean that values are significantly different ($p<0.05$) among groups

Fig. 4 (A) Relative quantity of mitochondrial DNA in brown adipose tissue (BAT). (B) Representative images of brown adipose tissue after mitochondria-specific stain, taken at 40x magnification (Scale bar, 50 μm). Experimental groups: Control group (C) (☐), MetS

491 group (MS) (▒) and MetS + EWH (MSH) (■). Values are expressed as mean ± SEM (n ≥ 6).

492 Different letters mean that values are significantly different ($p<0.05$) among groups

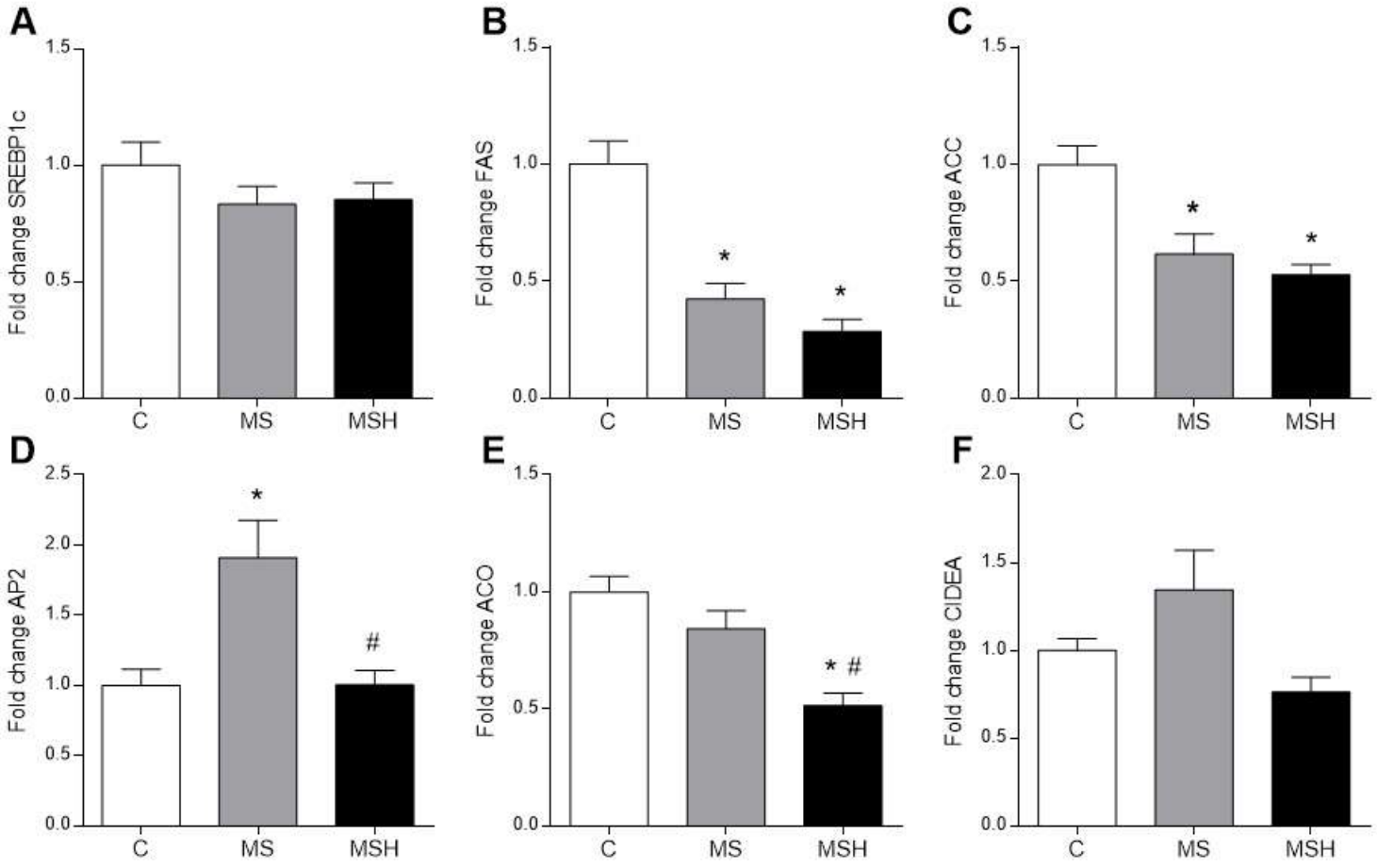
493 **Table 1** Relative gene expression in epididymal adipose tissue (WAT) of Leptin, Leptin

494 Receptor (LeptR), Adiponectin, Resistin, Insulin Receptor (InsR), Glutathione Peroxidase 3

495 (GPX3) and Inducible Nitric Oxide Synthase (iNOS). Experimental groups: Control group (C),

496 MetS group (MS) and MetS + EWH (MSH). Values are expressed as mean ± SEM (n ≥ 6).

497 Different letters mean that values are significantly different ($p<0.05$) among groups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0.0
C
MS
MSH
B
Fold change FAS
*
*
C
Fold change ACC
*
*
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Fold change ACO
* #
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Fold change CIDEA
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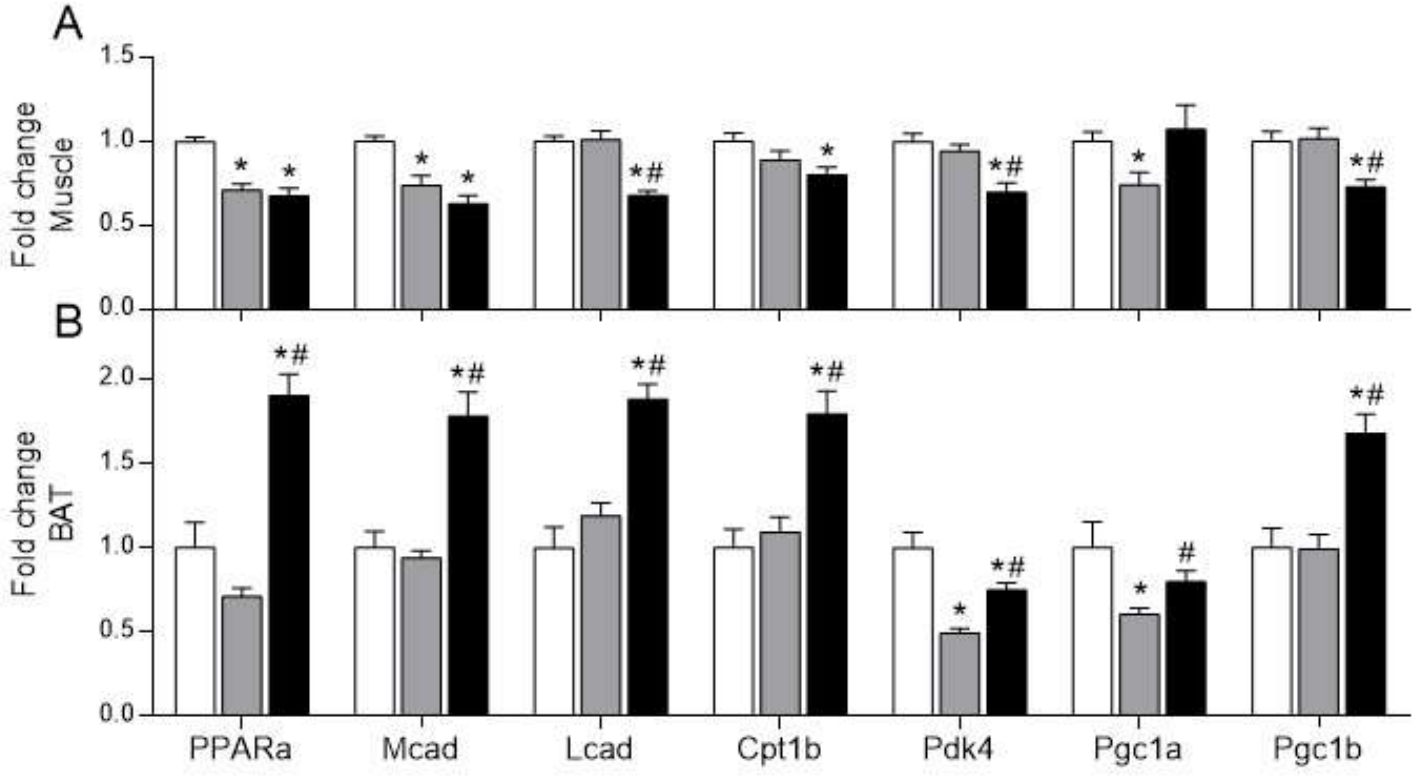
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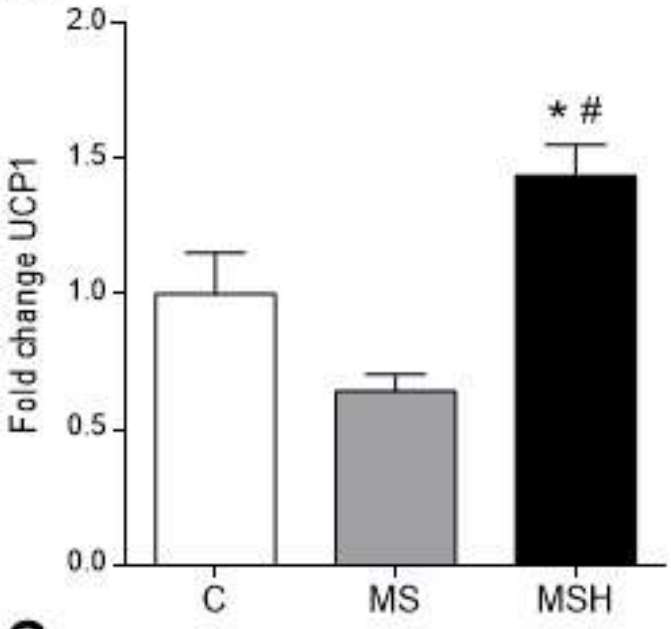
A



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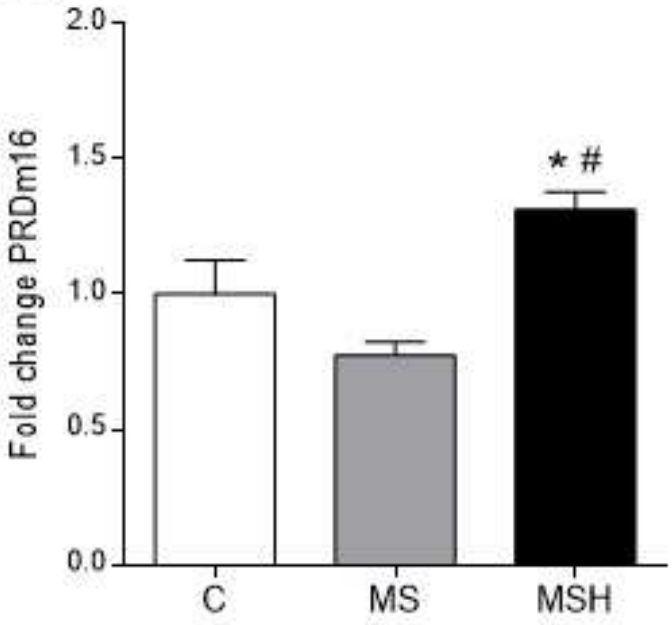
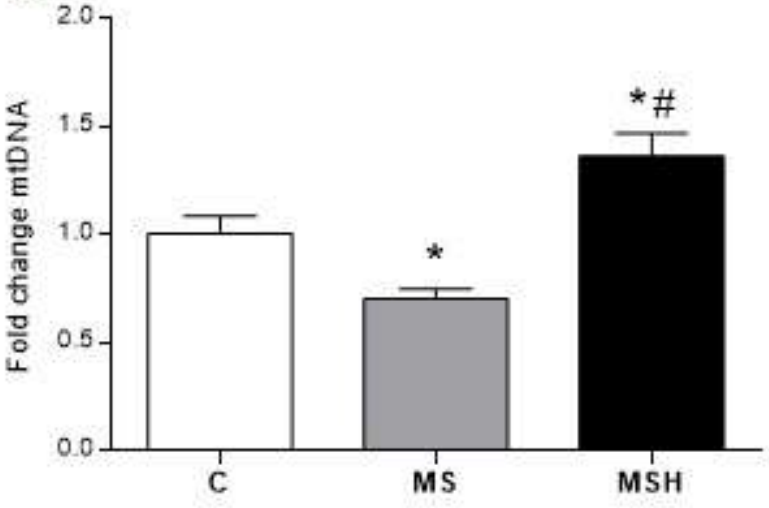


C

	Experimental group		
Measured gene (n≥6)	C	MS	MSH
Tfam	1.00 ± 0.08	0.52 ± 0.05*	1.05 ± 0.11#
MFN2	1.00 ± 0.11	0.72 ± 0.03*	1.22 ± 0.09#
Tfb2m	1.00 ± 0.08	0.69 ± 0.06*	1.25 ± 0.08*#
NRF1	1.00 ± 0.08	0.63 ± 0.04*	0.90 ± 0.04#
OPA1	1.00 ± 0.12[a]	0.53 ± 0.11*	1.12 ± 0.11#
Dmn1L	1.00 ± 0.10	0.86 ± 0.07	1.33 ± 0.11#

A



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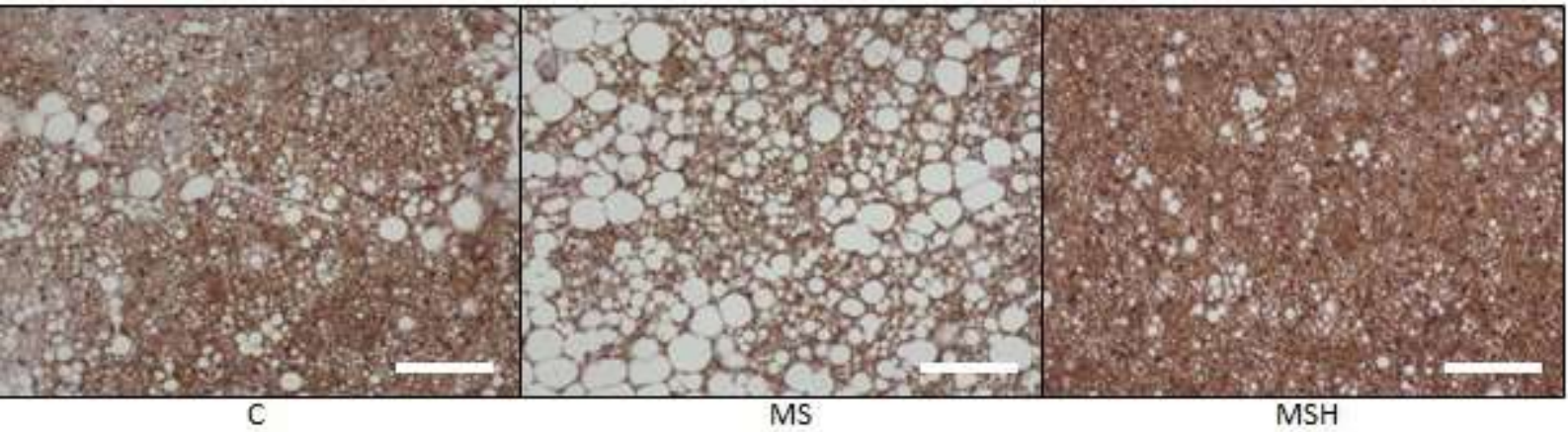

	Experimental group		
Measured gene (n≥6)	C	MS	MSH
Leptin	1.00 ± 0.15	0.76 ± 0.09	0.63 ± 0.07
LeptR	1.00 ± 0.17	0.98 ± 0.10	1.08 ± 0.11
Adiponectin	1.00 ± 0.09	0.53 ± 0.07*	0.41 ± 0.05*
Resistin	1.00 ± 0.14	0.79 ± 0.11	0.47 ± 0.04*#
InsR	1.00 ± 0.07	0.60 ± 0.06*	0.77 ± 0.07*
GPX3	1.00 ± 0.08	0.61 ± 0.07*	0.81 ± 0.04
iNOS	1.00 ± 0.15	0.83 ± 0.11	1.23 ± 0.07#