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Abstract

Recently the attention of the scientific community has focused on the ability of polyphenols to counteract adverse epigenetic regulation involved in the development of complex conditions such as obesity. The aim of this study was to investigate the epigenetic mechanisms underlying the anti-adiposity effect of Quercetin (3,3',4',5,7-pentahydroxyflavone) and of one of its derivatives, Q2. In 3T3-L1 preadipocytes, Quercetin and Q2 treatment induce chromatin remodeling and histone modifications at the 5' regulatory region of the two main adipogenic genes, c/EBPalpha and PPARgamma. Chromatin immunoprecipitation assays revealed a concomitant increase of histone H3 di-methylation at Lys9, a typical mark of repressed gene promoters, and a decrease of histone H3 di-methylation at Lys 4, a mark of active transcription. At the same time, treatment with both compounds inhibited histone demethylase LSD1 recruitment to the 5' region of c/EBPalpha and PPARgamma genes, a necessary step for adipogenesis. The final effect is a significant reduction in c/EBPalpha and PPARgamma gene expression and attenuated adipogenesis. Q2 supplementation in rats reduced the gain in body weight and in white adipose tissue, as well as the increase in adipocyte size determined by high fat diet. Moreover, Q2 improved dyslipidemia, glucose tolerance and decreased the hepatic lipid accumulation by activating the expression of beta-oxidation related genes. Our data suggest that Q2, as well as Quercetin, has the potential to revert the unfavorable epigenomic profiles associated with obesity onset. This opens the possibility to use these compounds in targeted prevention strategies against obesity.

Keywords	Chromatin remodeling; Adipogenesis; Polyphenols; Histone modifications.

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National Research Council of Italy
Institute of Experimental Endocrinology and Oncology "G. Salvatore"

Naples, Dec 19th, 2018

Bernhard Hennig, PhD, RD
Editor-in-Chief,
The Journal of Nutritional Biochemistry
University of Kentucky, Lexington, Kentucky, USA

Dear Prof. Hennig,

My colleagues and I are pleased to submit the manuscript "Quercetin and its derivative Q2 modulate chromatin dynamics in adipogenesis and Q2 prevents obesity and metabolic disorders in rats" to be considered for publication in the Journal of Nutritional Biochemistry.

Herein we investigated the epigenetic mechanisms underlying the anti-adiposity effect of Quercetin (3,3',4',5,7-pentahydroxyflavone) and of one of its derivatives, Q2. In addition, we demonstrated that Q2 supplementation decreased lipids accumulation in adipose tissue and liver of high-fat diet fed rats.

According to the Journal instructions, we would like to suggest the following colleagues as potential reviewers:

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As Corresponding author, on behalf of all the other authors, I confirm that the manuscript has been submitted solely to Journal of Nutritional Biochemistry and is not submitted, in press, or published elsewhere. I also confirm that all the research meets the ethics guidelines, including adherence to the legal requirements of the country where the study was performed.

Finally, I confirm that all the authors have no conflict of interest to declare.

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Sincerely,

Paola Ungaro, Ph.D

Highlights

- Obesity is characterized by increased number (hyperplasia) and size (hypertrophy) of adipocytes.
- A strategic approach to prevent obesity onset is to limit the adipocyte's ability for both hypertrophic and hyperplastic growth.
- Quercetin, one of the most widespread flavonoids present in different foodstuffs, and its synthesized derivative Q2 regulate hyperplasia by reducing adipogenesis.
- Both molecules decrease the expression of the main adipogenic genes *cEBP*α and *PPAR*γ through epigenetic changes such as histone post-translational modifications.
- Q2 supplementation decreases the lipid accumulation in adipose tissue and liver of high-fat diet fed rats.

Title

Quercetin and its derivative Q2 modulate chromatin dynamics in adipogenesis and Q2 prevents obesity and metabolic disorders in rats.

Short title

Quercetin derivative Q2 in adipogenesis and obesity

Authors

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Abstract

Recently the attention of the scientific community has focused on the ability of polyphenols to counteract adverse epigenetic regulation involved in the development of complex conditions such as obesity. The aim of this study was to investigate the epigenetic mechanisms underlying the anti-adiposity effect of Quercetin (3,3',4',5,7-pentahydroxyflavone) and of one of its derivatives, *Q2*.

In 3T3-L1 preadipocytes, Quercetin and Q2 treatment induce chromatin remodeling and histone modifications at the 5' regulatory region of the two main adipogenic genes, *c/EBPα and PPARγ*. Chromatin immunoprecipitation assays revealed a concomitant increase of histone H3 di-methylation at Lys9, a typical mark of repressed gene promoters, and a decrease of histone H3 di-methylation at Lys 4, a mark of active transcription. At the same time, treatment with both compounds inhibited histone demethylase LSD1 recruitment to the 5' region of *c/EBPα and PPARγ* genes, a necessary step for adipogenesis. The final effect is a significant reduction in *c/EBPα and PPARγ* gene expression and attenuated adipogenesis.

Q2 supplementation in rats reduced the gain in body weight and in white adipose tissue, as well as the increase in adipocyte size determined by high fat diet. Moreover, Q2 improved dyslipidemia, glucose tolerance and decreased the hepatic lipid accumulation by activating the expression of beta-oxidation related genes.

Our data suggest that Q2, as well as Quercetin, has the potential to revert the unfavorable epigenomic profiles associated with obesity onset. This opens the possibility to use these compounds in targeted prevention strategies against obesity.

Keywords: Chromatin remodeling; Adipogenesis; Polyphenols; Histone modifications.

Abbreviations: Histone-acetyltransferase (HAT); Histone deacetylase (HDAC); Peroxisome proliferator-activated receptor gamma (PPARγ); CCAAT/enhancer-binding protein alpha (cEBPα); AMP-activated protein kinase (AMPK); Lysine-specific histone demethylase 1 (LSD1); 2-(3,4-Diacetoxyphenyl)-4-oxo-4*H*-chromene-3,5,7-triyl triacetate (Q2); dimethylated histone H3 at lysine 9 (H3K9me2); dimethylated histone H3 at lysine 4 (H3K4me2); Standard Diet (SD); High Fat Diet (HFD); Enhanced Chemiluminescence (ECL); Formaldehyde Assisted Isolation of Regulatory Elements (FAIRE); Chromatin Immunoprecipitation (ChIP); Body Mass Index (BMI); Cardiac Somatic Index (CSI); homeostatic model assessment estimated insulin resistance index (HOMA-IR); Fasting Plasma Glucose (FPG); Fasting Plasma Insulin (FPI).

1. Introduction

Obesity is a serious public health problem and a major risk factor associated with the development of a variety of acute and chronic diseases including type 2 diabetes, hypertension and cardiovascular disorders [1, 2]. Obesity is characterized by increased number (hyperplasia) and size (hypertrophy) of adipocytes [3]. Limiting the adipocyte's ability for both hypertrophic and hyperplastic growth can be a strategic approach to prop up weight loss-promoting dietary supplements.

Nowadays, scientific community is focusing its attention on how nutrients can regulate the metabolism and influence the consumer's health. Natural phenols are important diet components, widely present in fruits, vegetables and beverages. They are characterized by the capacity to counteract the onset of ageing-related chronic disorders, such as cancer, cardiovascular and metabolic diseases including obesity and type 2 diabetes [4]. Recent findings suggest that one of the mechanisms by which polyphenols affect metabolic traits is epigenetics [5]. The term "epigenetics" defines heritable changes in gene expression that are independent from those occurring in the genome. This evidence is of great importance also because epigenetic changes are potentially reversible and may generate metabolic memory of the nutritional hit [6]. It includes different processes such as DNA methylation, histone modifications, and post-transcriptional gene regulation by non-coding RNAs. Together, they regulate gene expression by changing chromatin organization and DNA accessibility [7].

Dietary polyphenols exhibit several of these actions, being able to influence the transcription process by altering chromatin structure through post-translational modifications of the histones.

Quercetin is a major nutraceutical compound present in the "Mediterranean diet" and it possesses both a histone-acetyltransferase (HAT) and a histone deacetylase (HDAC)-inhibitory effects [8, 9]. Quercetin has been linked to a wide range of biological and health-beneficial effects [10, 11]. In mice fed with high fat diet, Quercetin supplementation prevents the increase in body, liver and white adipose tissue weight [12]. Moreover, in 3T3-L1 cells, an established cellular model of adipogenesis, Quercetin exerts specific anti-adipogenic

effects [13] by decreasing the transcription of *PPARγ* and *c/EBPα* genes and activating the AMPK signaling pathways [14, 15].

The major limitations to the potential use of Quercetin as therapeutic agents for the treatment of several diseases (including obesity) are represented by its poor bioavailability and low stability in aqueous medium [16]. Indeed, strong differences exist between the concentrations of phytochemical applied in *in vitro* studies (usually tens of micro molars) and those found *in vivo* (human and animal sera) after fruit and vegetable consumption (usually below 1 μM) [11, 17]. Therefore, several Quercetin derivatives have been recently developed to overcome such limitations. Among these, Q2 is a Quercetin derivative in which all the OH groups have been replaced with acetyl groups (Fig. 1). These chemical modifications determine an improvement of the hydrophobicity and of the stability respect to the reference molecule [16], leading to the hypothesis that Q2 is a, *bona fide*, more bioavailable compound.

In the current work, we investigated the epigenetic mechanisms through which Quercetin and Q2 affect adipogenesis. Moreover, we evaluated the anti-obesity effect of the Q2 derivative in rats fed with a high-fat diet.

Our results demonstrated that both Quercetin and Q2 decrease adipogenesis modulating the expression of the two main adipogenic markers, PPARγ and c/EBPα, through histone post-translational modifications. In addition, we demonstrated that Q2 supplementation is able to decreases the lipid accumulation in adipose tissue and liver of high-fat diet fed rats.

Our results suggested that both compounds might potentially prevent the risk of metabolic diseases by modifying or rescuing the unfavorable epigenomic profiles associated to pathological conditions.

2. Materials and methods

2.1. Materials

Media, sera and antibiotics for cell culture were purchased from Lonza (Basel, Switzerland). PPARγ, C/EBPα, and 14-3-3ε antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal histone H3 Lys9 (H3K9) and LSD1 antibodies were purchased from Active Motif (Carlsbad, CA, USA), rabbit monoclonal histone H3 Lys4 (H3K4) antibody was from

Millipore (Darmstadt, Germany). Oligonucleotides were synthesized by Sigma (St Lous, MO, USA). Quercetin (3,5,7,3-4-pentahydroxy flavone ($C_{15}H_{10}O_7$) was a generous gift of Dr Gian Luigi Russo (ISA-CNR, Avellino, Italy). The synthesis of Q2 (2-(3,4-Diacetoxyphenyl)-4-oxo-4*H*-chromene-3,5,7-triyl triacetate) was previously described [16].

2.2. Cell culture and reagents

Mouse 3T3-L1 fibroblasts were purchased from the American Type Culture Collection (ATCC Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS), 2% glutamine, and 1% penicillin/streptomycin solution at 37°C in a humidified 95% air and 5% CO_2 atmosphere. Cells were sub cultured every 3-4 days at approximately 90% confluence.

Adipogenesis was performed as previously reported [18-20]. Briefly cells were plated onto 6-well plates at a density that allowed them to reach confluence in 3 days. At this point (day 0), cells were switched to MDI-differentiation medium (DMEM, 10% FBS, 174 nM insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX)) for 2 days. At this time, to examine the effect of Quercetin or Q2 on adipogenic differentiation, cells were treated with differentiation medium in the presence of various concentration of Quercetin or Q2 (1, 5, 10 and 25 μM).

On day 3, the dexamethasone and IBMX were removed, leaving insulin in the cell media for an additional 2 days. Thereafter, the cells were maintained in the original propagation DMEM with medium changes every 2-3 days until the mature adipocytes were obtained (day 9). Cells were collected and analyzed at days 0, 3, 5, 7 during differentiation. All the mix changes were done in parallel in control cells without Quercetin or Q2.

2.3. Sulforhodamine B colorimetric (SRB) assay

SRB assay was performed to measure cell viability. After treatment, 3T3-L1 cells were fixed with cold 10% trichloroacetic acid (TCA) and stained with SRB (Sigma). Excess dye was washed out using 1% acetic acid. The protein-bound

dye was dissolved in 10 mM unbuffered Tris-Base solution, and the OD_{595} was read on a microplate reader (TECAN).

2.4. Oil Red O staining

To measure cellular neutral lipid droplet accumulation, 3T3-L1 mature adipocytes were washed three times with iced phosphate-buffered saline (PBS) and fixed with 4% formaldehyde for 30 minutes. After fixation, cells were washed three times with PBS and stained with Oil Red O (ORO) solution (working solution 0.5 g ORO power dissolved in 60% ethanol) for 30 minutes at room temperature. Cells were washed again three times with PBS to remove excess stain. 3T3-L1 mature adipocytes were examined at microscope and the red oil lipid droplets stained in the cells were photographed. Lipid accumulation was quantified extracting the red oil droplets stained in the cells with 100% isopropanol and reading the absorbance at 510 nm [21].

2.5. Quantitative polymerase chain reaction

Total RNA extraction was performed as described under [22]. For real-time PCR (qRT-PCR) analysis, 1μg RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). qRT-PCR experiments were performed using SYBR Green mix (Bio-Rad, Hercules, CA) and relative quantification of gene expression was calculated according to the comparative $2^{-\Delta\Delta Ct}$ method [23, 24]. All reactions were performed in triplicate and ARBP (Ribosomal Protein, large, P0) was used as an internal standard. Sequences of the primers used in real time experiments are listed in Supplementary Table 1.

2.6. Western blot analysis

Protein extracts (50 or 100 μg) were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Immunoblots were performed using selective primary antibodies for PPARγ (1:1000; #sc-7196) and c/EBPα (1:1000; #166258), with 14-3-3θ (1000; #sc-732) as loading control. Immunodetected proteins were visualized using an enhanced chemiluminescence (ECL) kit (BioRad Laboratories).

2.7. Chromatin Immunoprecipitation Assay (ChIP)

Chromatin immunoprecipitation assay was performed as reported previously [25]. Sheared chromatin samples were taken as input control or used for immunuprecipitation with the following antibodies: di-methylated histone H3 at lysine 9 (H3K9me2; #39239), di-methylated histone H3 at lysine 4 (H3K4me2; #07-030), LSD1 (#61607) and normal rabbit IgG as a negative control. DNA fragments were recovered and quantified by qRT-PCR amplification using primer sequences listed in Supplementary Table 2.

2.8. Formaldehyde-assisted isolation of regulatory elements (FAIRE)

FAIRE assay was performed as previously reported [26]. Whole cells were fixed in growth medium in 1% formaldehyde for 10 min. Cells were lysed in nuclear lysis buffer containing 50mmol/l Tris-HCl (pH 8.0), 10 mmol/l EDTA, 0.8% (wt/vol.) sodium dodecyl sulfate, 1mmol/l phenyl methyl sulfonyl fluoride and inhibitors cocktail (Sigma), and then incubated on ice for 10 min. The extracts were then sonicated (Misonix 3000) and micro centrifuged at 4°C for 10 min at 16,000*g*. Protein-free DNA was then analyzed by qRT-PCR [27]. The primer sets used are shown in Supplementary Table 2. The amount of PCR product (representing nucleosome-free DNA) was plotted as a percentage of the input DNA representing total cellular DNA.

2.9. Animals

Wistar male rats (Envigo, Italy) were used in this study. Animals were housed one *per* cage under controlled light (12:12-h light-dark cycles), temperature (23-25 °C) and humidity (50-55%) conditions, and fed with a Standard Diet (SD) [Diet 2018: 6.2% fat kcal, 18.6% kcal protein, and 44.2% kcal carbohydrates (Envigo, Italy)] or with a High Fat Diet (HFD) [Teklad Diet 06414: 60.3% fat kcal, 18.3% kcal protein, and 21.4% kcal carbohydrate (Envigo, Italy)]. SD was used as normolipidic diet, while HFD, able to induce obesity as previously demonstrated [28], was used as hyperlipidic diet. The study was conducted in accordance with the Declaration of Helsinki, the Italian law (D.L. 26/2014), the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (2011) and the Directive 2010/63/EU of the European

Parliament on the protection of animals used for science. The project was approved by the Italian Ministry of Health, Rome and by the ethics review board.

2.9.1 Experimental protocols

Rats were randomly divided into 3 different experimental groups:

1) group fed with SD (indicated as SD)

2) group fed with HFD (indicated as HFD)

3) group fed with HFD and Q2 (indicated as HFD+Q2), where Q2 was orally administered every day, throughout the entire course of diets, at a dose corresponding to 0.26 mg/kg. The Q2 dose was chosen accordingly to previous studies [16, 29].

The Quercetin derivative Q2 was orally administered to each rat by using a syringe containing the daily dose, by holding the rat in one hand and letting the animal swallow the extract suspension. During the procedure, the complete ingestion of the compound was ensured, eliminating risks of variability in the intake among individual animals. In control rats, the same volume of filtered water has been equally administered [30]. For comparison, additional control group SD plus Q2 was performed.

All the experimental groups were exposed to their specific diet and treatments for 12 weeks. Food and water intake were monitored daily. Anthropometric parameters of each animal were measured weekly. Metabolic parameters and plasma analyses were performed at the end of the treatment and after the animals' sacrifice [28].

2.10 *Anthropometric variables*

The body mass index (BMI) was calculated as the ratio between the body weight and the square of the length (Kg/m^2). After the sacrifice, the liver and the abdominal fatty tissue were removed, weighed separately and stored at -80°C. The cardiac somatic index (CSI) was calculated as the ratio between the heart weight (g) and the body weight (g) multiplied by 100 [28].

2.11 Plasma biochemical measurements

At the end of treatments, rats were anesthetized with ethyl carbamate (2 g/kg body weight, i.p.) and sacrificed. The blood was drawn from each animal after

the sacrifice and centrifuged at 4°C for 15 minutes at 3000 x *g*. Plasma was collected and stored at -80°C for the subsequent analyses.

Basal blood glucose levels were determined by using a glucometer (ACCUCHEK, Roche Diagnostics, Germany). Total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were determined by using a kit from PKL® POKLER, Italy. Insulin levels were evaluated with a specific Millipore kit (St. Charles, MO, USA), while adiponectin levels were evaluated with a specific kit (Adipogen Life Sciences CH-1410, Switzerland), according to the manufacturer's protocol.

2.12 Homeostatic model assessment estimated insulin resistance index (HOMA-IR)

The insulin sensitivity was evaluated by the homeostatic model assessment estimated insulin resistance index (HOMA-IR) [31]. HOMA index was calculated as the product of the fasting plasma glucose (FPG) and fasting plasma insulin (FPI) levels, divided by a constant, assuming that control rats (SD group) have an average HOMA-IR of 1, analogous to the assumptions applied in the development of HOMA-IR in humans [31, 32]. The used equation was: HOMA-IR= (FPG (mg/dl) x FPI (μUI/ml) / 2.430 [32].

2.13 Light microscopy

Liver samples from each animal were embedded in OCT, fixed in liquid nitrogen and stored at -80°C until use. The sections (7 μm), obtained by using a cryostat (Microm HM505E), were post fixed with 4% formalin and put in distillated water. Sections were stained with OIL RED O (Bio-Optica) to detect fat vacuoles and counterstained with hematoxylin to localize nuclei.

2.14 Adipocyte size measurement

White adipose tissue was dissected and embedded in paraffin after serial ethanol dehydration. Six μm sections were stained with hematoxylin/eosin (H&E) and Cross Section Area (CSA) was measured to determine the size of the adipocytes. In particular, H&E images were captured with a color digital camera on an Olympus CX41 optical microscope at 10x magnification. For manual adipocyte-size quantification, CellF software was used and the

measurements were manual. The outline of the individual adipocyte was traced, and the total number of adipocytes was assessed. The area was calculated and expressed in μm^2.

2.15 Statistics

Statistical analyses were performed with the GraphPad Prism Software® (version 5.0; Graph Pad Software, USA). Data were expressed as mean±SEM. Comparisons were made using Student's T-test. Differences were considered statistically significant with a $p<0.05$.

3. Results

3.1. Effect of Quercetin or Q2 on cell viability

The effects of Quercetin or Q2 on 3T3-L1 adipocyte cells viability were analyzed by sulforhodamine B assay**.** Both molecules had negligible toxic effects within a concentration range of 0-10 μM. Cells treated with 25 μM of either Quercetin or Q2 showed a slight but not significant difference in viability compared to controls, while the concentration 50 μM determined a strong decrease in cell viability (Supplementary Fig. 1**)**. Based on these data, Quercetin and Q2 concentrations of 1, 5, 10 and 25 μM were used for subsequent experiments.

3.2. Effect of Quercetin or Q2 on lipid accumulation during adipogenesis

It has been previously described that Quercetin suppresses lipid accumulation in a dose-dependent manner in 3T3-L1 cells [33]. We performed the same experiment with either Quercetin or Q2 at 1, 5, 10 and 25 μM. The results indicated that concentrations up to 10 μM of Quercetin or Q2 had no effect, while the addition of 25 μM Quercetin or Q2 resulted in a reduction in lipid accumulation, compared to the controls, of 48% and 65% respectively (Fig.2A and 2B). This suggests that lipid accumulation is reduced not only by Quercetin but also by Q2, and a threshold-effect underlines this mechanism(s).

3.3. Quercetin or Q2 induce DNA remodelling and histone modifications at the cEBPα and PPARγ 5' regulatory region.

To further investigate the effects of Quercetin or Q2 on adipocytes differentiation we evaluated mRNA and protein levels of the main adipogenic markers, cEBPα and PPARγ, in 3T3-L1 cells incubated with 25μM of each molecule (Fig.2 C, D, E and F). Consistently with the morphological changes, the levels of both cEBPα and PPARγ were significantly decreased during the entire differentiation process when compared to the cells treated with vehicle alone.

Next, we assessed whether the decrease in c/EBPα and PPARγ expression in 3T3-L1 cells exposed to Quercetin or Q2, was mediated by changes in chromatin structures across the *cEBPα* and *PPARγ* genes. For this purpose, we performed a formaldehyde-assisted isolation of regulatory element assay (FAIRE) [34]: the DNA fragments were amplified using seven sets of PCR primers, covering consecutive 100 bp regions positioned at about –600/500 to 100 regions in the *cEBPα* and *PPARγ* genes (Fig.3 A and B) and FAIRE-enriched DNA was analyzed by quantitative Real-Time PCR. In 3T3-L1 cells, the amount of nucleosome-free DNA across the examined *c/EBPα* and *PPARγ* regions ranged from 40 to 20% and from 50 to 30%, respectively, for Quercetin, and was of about 20% for both *cEBPα* and *PPARγ* for Q2 compared with 3T3-L1 cells treated with vehicle alone (p<0.05 to 0.01). Figure 3 shows the results obtained at day 5 during differentiation; similar results (not shown) were obtained at days 3 and 7 of cellular differentiation. These data suggest that Quercetin or Q2 induce nucleosome remodeling in the examined regions in the proximal *cEBPα* and *PPARγ* 5' regulatory regions.

Dynamic histone modifications are well recognized mechanisms capable to mediate changes in gene expression by altering chromatin structure or by serving as a binding platform to recruit other proteins [35-38]. To further explore the hypothesis that modified histones contribute to the transcriptional repression of *cEBPα* and *PPARγ* genes by Quercetin or Q2, ChIPs analysis were performed on N-T and L-Q regions of *cEBPα* and *PPARγ* genes, respectively. The results obtained at day 5 during differentiation are shown in Figure 4. Similar results were obtained at days 3 and 7 of cellular differentiation (data not shown). Histone H3 di-methylation at lysine 4, a mark of active transcription, was reduced while the di-methylation of histone H3 at lysine 9

(H3K9me2), a typical mark of repression [39] was 1.5/2-fold higher by either Quercetin or Q2 treatment, when compared to cells incubated with vehicle alone ($p<0.001$; Fig.4 A and B). Thus, Quercetin or Q2 treatments are accompanied by an enrichment in repressive histone modifications together with a reduction of both cEBPα and PPARγ expression.

ChIP assays demonstrated that the treatment with either Quercetin or Q2 reduced also the binding of the Lysine-specific histone demethylase 1 (LSD1) to *c/EBPα* and *PPARγ* 5' regulatory element in 3T3-L1 cells ($p<0.001$; Fig. 4C). 3T3-L1 treated cells showed levels of LSD1 comparable to cells incubated with vehicle alone (data not shown). This indicated that the reduction of LSD1 binding to *c/EBPα* and *PPARγ* 5' regulatory region is not dependent on LSD1 expression.

3.4. Effects of Q2 in vivo supplementation

The beneficial effects of Quercetin against obesity in experimental models and clinical trials have been largely reported [40-42]. Here, we evaluated the efficacy of Q2 treatment on "anthropometric" and metabolic parameters in Wistar rats fed with HFD.

3.4.1 Anthropometric variables

The effects of the different diets on the anthropometric variables after 12 weeks are shown in Table 1.

Body weight of the rats in HFD group was significantly higher compared to the SD group, but significantly decreased when Q2 was simultaneously administered with the HFD.

Waist circumferences (WC) of the animals at the beginning of the experimental protocols were similar (data not shown). After 12 weeks of the different diets, a significant rise of WC, of BMI and of abdominal fat deposition was evident in the HFD respect to the SD rats. The contemporary administration of Q2 significantly reversed these effects.

Heart weight and CSI values were increased in HFD animals compared to controls as previously reported [28]. Again, the contemporary administration of Q2 was able to significantly reverse these alterations.

3.4.2 Metabolic variables

Metabolic parameters after 12 weeks of the different diets are reported in Table 2. Basal glycemia, plasma insulin levels, and HOMA index were significantly higher in HFD rats in comparison to the SD rats. Q2 supplementation to HFD was able to reverse the effects on these metabolic variables. Similarly, plasma levels of total cholesterol, LDL-cholesterol and triglycerides, were significantly higher in HFD than in SD rats, while HDL-cholesterol concentration presented an opposed trend. The supplementation with Q2 was able to significantly abolish these differences.

In addition, in comparison to SD, HFD was associated with a significant decrease in adiponectin plasma concentration, while Q2 supplementation significantly increased the circulating adiponectin.

In all anthropometric and metabolic analyses, no significant differences have been observed among SD *vs* SD plus Q2 (Supplementary Table 3).

3.5. Q2 supplementation attenuated adipocyte size in Wistar rats

The effects of Q2 on adipocyte hypertrophy were evaluated analyzing digital images of H&E-stained paraffin sections. Adipocytes obtained from rats fed with HFD had an average diameter of 5826.61±229.34 μm, while adipocytes obtained from SD rats had an average diameter of 3583.79±120.74 μm. The difference was highly significant ($p<0.001$). Consistently with the reduction of the abdominal fat mass, supplementation with Q2 determined a significant reduction in the adipocyte size (average diameter = 4813.71±202.86) in comparison to HFD ($p<0.001$) (Fig.5A and B). Moreover, the graph of adipocyte's size distribution indicated a shift toward smaller size for the HFD+Q2 group (Fig.5 C).

3.6. Q2 decreased hepatic lipid accumulation in Wistar rats

In figure 6 A are shown the liver sections derived from SD, HFD and HFD+Q2 rats. The amount of fat vacuoles (red droplets) was clearly different, with a strong increase in HFD, but not in HFD+Q2.

Next, we investigated the expression of some of the genes involved in fatty acid oxidation (*ACOX1*, *CACT*, *ACSL1* and *CPT1a*) [43]. Among these, mRNA levels of ACSL1 and CPT1a were significantly higher in the HDF+Q2 group (Fig.6 B). This suggested that the reduction in hepatic lipid accumulation induced by Q2 supplementation could be determined by an enhanced hepatic oxidation of fatty acids as well as by a reduction in adipocyte expansion.

4. Discussion

The increasing prevalence of obesity determines a major worldwide health problem since it is a primary causative factor in the development of multiple metabolic abnormalities including hypertension, insulin resistance, dyslipidemia, oxidative stress, inflammation, and fatty liver [44, 45]. The outbreak of obesity plays such a negative impact on public health to induce governments to launch a multitude of campaigns to reduce the spread of the obesity epidemic in industrialized countries. Unfortunately, these strategies get very limited effects, being introduced after that obesity is established and difficult to reverse [46-48]. Thus, prevention should be the main focus of all the intervention programs and, in this context, diet represents a valid mean in obesity management.

In recent years, circumstantial evidences suggested that phytochemicals are able to ameliorate obesity through different mechanisms including inhibition of adipogenesis, suppression of adipocyte hypertrophy, enhancement of lipolysis, or induction of fat cell apoptosis [49-52]. Therefore, polyphenols may play a significant role in the prevention of metabolic disorders, including obesity, various forms of cancer, neurodegenerative, cardiovascular and inflammatory diseases [53-55]. More recently, the attention of the scientific community has turned to the potential role of polyphenols to reverse adverse epigenetic pathways involved in pathological conditions. Therefore, dietary polyphenol-targeted epigenetics could be considered an attractive approach for disease prevention and intervention.

Unfortunately, one of the major problem of many polyphenols is their limited bioavailability due to a low degree of absorption, a high metabolization rate, a poor activity and a rapid elimination of metabolites [56-58]. Although Quercetin, one of the most widespread flavonoids present in different foodstuffs, has been

demonstrated to determine a wide range of biological and health-promoting effects, its clinical applications are limited by its poor bioavailability and low stability in aqueous medium [59].

In this work, we have investigated the anti-obesity effect of a previously synthesized Quercetin derivative, the penta-acetyl derivative Q2. This compound has been hypothesized to be more bioavailable than Quercetin, due to his chemical modification enhancing the lipophilic characteristics [16].

Previous data suggested the capacity of quercetin in regulating hepatic gene expression and lipid metabolism in a mice model of obesity [60]. Our results indicated that Quercetin and its derivative Q2 attenuated adipogenesis in 3T3-L1 preadipocytes by reducing the expression of the two main adipogenic markers, *cEBPα* and *PPARγ* genes. This is determined through a mechanism involving epigenetic modifications to the regulatory region of these genes. Indeed, using a FAIRE assay we demonstrated that both the molecules determined changes in chromatin accessibility and remodeling across the *cEBPα* and *PPARγ* proximal regions. These data confirm previous observations on 3T3-L1 cells showing that Quercetin suppressed protein levels of the key adipogenic factors cEBPβ, cEBPα, PPARγ and FABP4 [61].

Growing evidence indicates that chromatin remodeling strongly contributes to the control of gene expression [62]. Here, we demonstrated that exposure of 3T3-L1 cells to Quercetin and Q2 induce histone tails hypermethylation at H3K9 and H3K4 hypomethylation, two marks of heterochromatin causing a transcriptionally non-permissive state of *cEBPα* and *PPARγ* genes. These two transcription factors are fully expressed following the initiation of adipocytes differentiation process within approximately 5 days [63]. Their activation leads to terminal differentiation and regulates the expression of the largest part of adipocyte-specific genes, including the hormone adiponectin or the transporters FABP4 and GLUT4 [63].

Adipogenesis is a complex process tightly associated with the induction of specific chromatin regulators. A number of recent studies have demonstrated an important role of the histone lysine demethylase LSD1 in adipogenesis [64]. When adipogenesis is induced, LSD1 is recruited to the promoter of *cEBPα* gene maintaining a permissive state of the chromatin. This allows gene

expression upon stimulation of differentiation. Our results demonstrated that both Quercetin and Q2 are able to reduce LSD1 binding to the promoter regions of *cEBPα* and *PPARγ* genes, highlighting the potential role of these compounds in altering the epigenetic mechanisms involved in adipogenesis.

In this study, we also provided indications concerning the impact of Q2 compound on health. To this aim, Q2 was added to the classic hyperlipidic diet, which induces obesity and metabolic disorders in animals, mimicking the human disease [28, 65]. All the anthropometric and metabolic alterations induced in rats fed with HFD were significantly reduced by the oral supplementation of Q2. Moreover, as previously demonstrated for Quercetin in a rat model of metabolic syndrome [29], Q2 supplementation reduced also insulin resistance and dyslipidemia.

The obesity related-metabolic impairment is mitigated by Q2 treatment. In fact, HFD+Q2 animals were characterized by a decrease in total cholesterol, LDL cholesterol and triglycerides plasma levels, and by concomitant higher HDL values respect to the HFD rats. At the same time, blood glucose, insulin levels and HOMA-IR index were found to be increased in HFD compared to HFD+Q2 rats. This suggested that Q2 might represent a novel and effective dietary support in the prevention and/or treatment of obesity.

The impact of obesity and metabolic syndrome on cardiac remodeling and dysfunction is well documented. In this context, an increased interstitial fibrosis was observed in the heart of different obesity animal models [66, 67]. Studies of cardiac morphology in humans [68] and animals [66, 67] indicated that obesity determined an increase in left ventricular mass, ventricular hypertrophy and dysfunction [69, 70]. Accordingly, our study indicated that the cardiac weight and CSI were higher in obese animals fed with HFD, while Q2 supplementation reduced such parameters. This suggests a possible contribution of Q2 in the mitigation of the obesity-associated pathological heart growth.

Q2 supplementation had also evident effects on lipid metabolism in the liver. Hepatic lipid accumulation induced by HFD was reduced by oral Q2 administration through an enhanced expression of fatty acid oxidation-related genes. Hepatic steatosis, together with the presence of obesity, represents an

important aspect of metabolic syndrome [71]. Therefore, the potential role of Q2 in metabolic syndrome prevention and treatment could be determined by a reduction of lipid accumulation not only in adipose tissue but also in liver.

In conclusion, it is relevant to state that epigenetic factors may be modulated by environmental cues, including nutrition, and thus provide a mechanism by which genomes integrate signals into permanent changes of gene expression that may ultimately lead to health and disease risk [72-76]. Quercetin and its derivative Q2 may be potential therapeutic agents for the treatment of obesity by modifying unfavorable epigenomic profiles involved in the development of such complex condition.

Table 1. “Anthropometric” variables in rats fed with SD (standard diet) or HFD (high fat diet) or HFD+Q2 for 12 weeks

Anthropometric Variables	SD	HFD	HFD+Q2
Body weight (g)	439±9	501±18*	444±13§
Waist circumference (cm)	19.1±0.4	21.2±0.19*	20.3±0.3§
BMI (Kg/m^2)	6.1±0.3	7.3±0.3*	6.4±0.2§
Abdominal fat (g)	6.0±0.4	15.0±1.3*	11.0±0.6§
Hearth weight (g)	1.35±0.06	2.13±0.19*	1.36±0.09§
Cardiac Somatic Index	0.31±0.01	0.42±0.04*	0.31±0.02§

Values are mean±SEM; significance of difference: $p < 0.05$ *= HFD versus SD; §= HFD+Q2 versus HFD. (n=4 for SD and HFD groups. n=3 for HFD+Q2 group).

Table 2. Metabolic variables in rats fed with SD (Standard Diet) or HFD (High Fat Diet) or HFD+Q2 for 12 weeks

Metabolic Variables	SD	HFD	HFD+Q2
Glycemia (mg/dL)	95.2±9.9	161.7±5.4*	129.0±5.5§
Insulin (ng/mL)	2.3±0.1	7.8±0.7*	2.9±0.8§
HOMA Index	1	5.6	1.7
Triglycerides (mg/dL)	90.9±15.1	218.6±18.4*	162.3±13.9§
Total cholesterol (mg/dL)	117.0±0.3	293.0±44.3*	146.8±24.8§
HDL cholesterol (mg/dL)	51.7±3.3	22.7±3.6*	41.2±1.1§
LDL cholesterol (mg/dL)	43.4±6.2	112.1±6.0*	65.2±17.1§
Adiponectin (ng/mL)	25.1±1.0	11.0±0.5*	17.4±1.2§

Values are mean ± SEM; significance of difference: $p < 0.05$ *= HFD versus SD; §= HFD+Q2 versus HFD. (n=4 for SD and HFD groups, n=3 for HFD+Q2 group).

Figure Legends

Figure 1. Chemical Structure of Quercetin and its acetyl derivative Q2**.**

Figure 2. The effects of Quercetin or Q2 on lipid accumulation and adipogenesis in 3T3-L1 cells. 3T3-L1 adipocytes were cultured for 8 days and treated with Quercetin (Q) or Q2 (1, 5, 10 and 25 μM) during differentiation. On day 8, intracellular lipids were stained with Oil Red O and quantified. Quercetin and Q2 treatment reduced formation of adipocytes. (A) The microphotographs were obtained with an optical microscope in two original magnifications (10X and 20X), following ORO staining. (B) Lipid quantification test was expressed as optical density (OD). The data represented the mean percentage levels compared with DMSO-treated cells (Ve). 3T3-L1 induced to differentiate into adipocytes were treated with the indicated concentration of Quercetin or Q2 since start of differentiation (day 0). cEBPα and PPARγ mRNA and protein levels were assayed during adipogenesis at days 3, 5 and 7. *cEBPα* (C), *PPARγ* (D) mRNA levels were measured by quantitative RT-PCR. Data were normalized for ARBP and are presented as fold decrease relative to cells treated with vehicle alone (Ve). (E,F) Cells were solubilized, and lysates were analyzed by SDS-PAGE and subjected to western blotting with anti cEBPα, PPARγ and 14-3-3θ antibodies (cEBPα, PPARγ, 14-3-3θ), as indicated. The blots shown are representative of three indipendent experiments with similar results. Bars represent the mean±SEM of at least three independent experiments, each performed in triplicate. **p<0.01 and ***p<0.001 vs DMSO. (nt, not-treated cells; Ve, vehicle, DMSO-treated cells).

Figure 3. Enrichment of regulatory DNA across 0.6 kb of *cEBPα* and 0.5 kb of *PPARγ* genes using formaldehyde-assisted isolation of regulatory elements (FAIRE). A, B (top) Schematic representation of c*EBPα* and *PPARγ* showing the DNA fragments amplified by primer sets N to T for *cEBPα* and L to Q for *PPARγ*. A, B (down) Protein-free DNA was extracted from DMSO (black bars), Quercetin (gray bars) or Q2 (light gray bars) 3T3-L1 treated cells at day 5 of differentiation and analyzed by Real-Time PCR using the indicated primers.

Bars represent the mean±SEM of at least three independent experiments, each performed in triplicate. * p<0.05 **p<0.01.

Figure 4. Quercetin and Q2 treatment determine repressive histone modifications at *cEBPα* and *PPARγ* promoters. ChIP experiments were performed using antibodies against active histone marks, i.e. (A) H3K4me2 and repressive histone marks (B) H3K9me2 and (C) against LSD1 in DMSO (black bars), Quercetin (Q, grey bars) or Q2 (light gray bars) 3T3-L1 cells at day 5 of differentiation. ChIPs were followed by quantitative PCR amplification with primer sets for the N-T and L-Q regions of the *cEBPα* and *PPARγ* 5' regulatory region, respectively. Results are presented as enrichment relative to input DNA (%) and corrected for IgG control levels as analyzed by quantitative PCR. Bars represent the mean±SEM of three independent experiments, each performed in triplicate. ***p<0.001.

Figure 5. Q2 supplementation attenuated fat mass and adipocyte size. (A) Hematoxilin/Eosin of adipose tissue section from Wistar rats fed Standard Diet (SD), High Fat Diet (HFD) or High Fat Diet + Q2 for 12 weeks. (B) Adipocytes area was quantified on white adipose tissue sections following H&E staining with the Cell F software (Olimpus). Results are shown as the mean±SEM of the area in each group. (C) Cross section area of adipocytes was measured on the same section as in B with the CellF software (Olimpus).

Figure 6. Q2 supplementation decrease hepatic lipid accumulation. (A) Composite illustrating steatosis in the Standard Diet (SD) or High Fat Diet (HFD), or High Fat Diet + Q2 (HFD+Q2) of the rat liver stained with Red Oil O. Fat vacuoles (red); Nuclei (blu). (B) Relative mRNA levels of ACOX1, CACT, ACSL1, CPT1a in the liver by qPCR. Bars represent the mean±SEM of three separate experiments. *p<0.05.

Contributions of Authors

ICN and CR were the main contributors in terms of conception, design, acquisition and interpretation of data, and drafting the article. GM, LA, DA, FG, TS, SD, RM, DT, MD contributed to the conceptual design and the acquisition of data. AC, FB, GLR, PEM, MSS, TA contributed to the conceptual design, analysis and interpretation of data and discussion of the results. PU contributed to the conceptual design, interpretation and discussion of the results and the supervision of the overall work. All of the authors critically revised the article and approved the final version. PU is responsible for the integrity of the work as a whole.

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Disclosure Summary

The authors have nothing to disclose.

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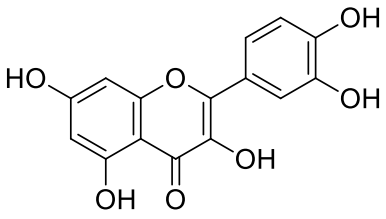
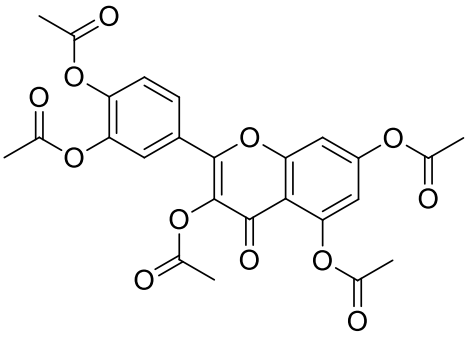
Supplementary Fig. 1. Effects of Quercetin and Q2 on cytotoxicity. 3T3-L1 cells were seeded in 24-well plates at $20x10^4$ cells/well and treated for 48 hrs with Quercetin or Q2 at the indicated concentrations, or with 0.1% DMSO (Ve) as control. Cells viability was measured using the SRB assay. Average value (±SEM) of three independent experiments are shown in graph. ***$p<0.001$



Quercetin



Q2

Fig. 1

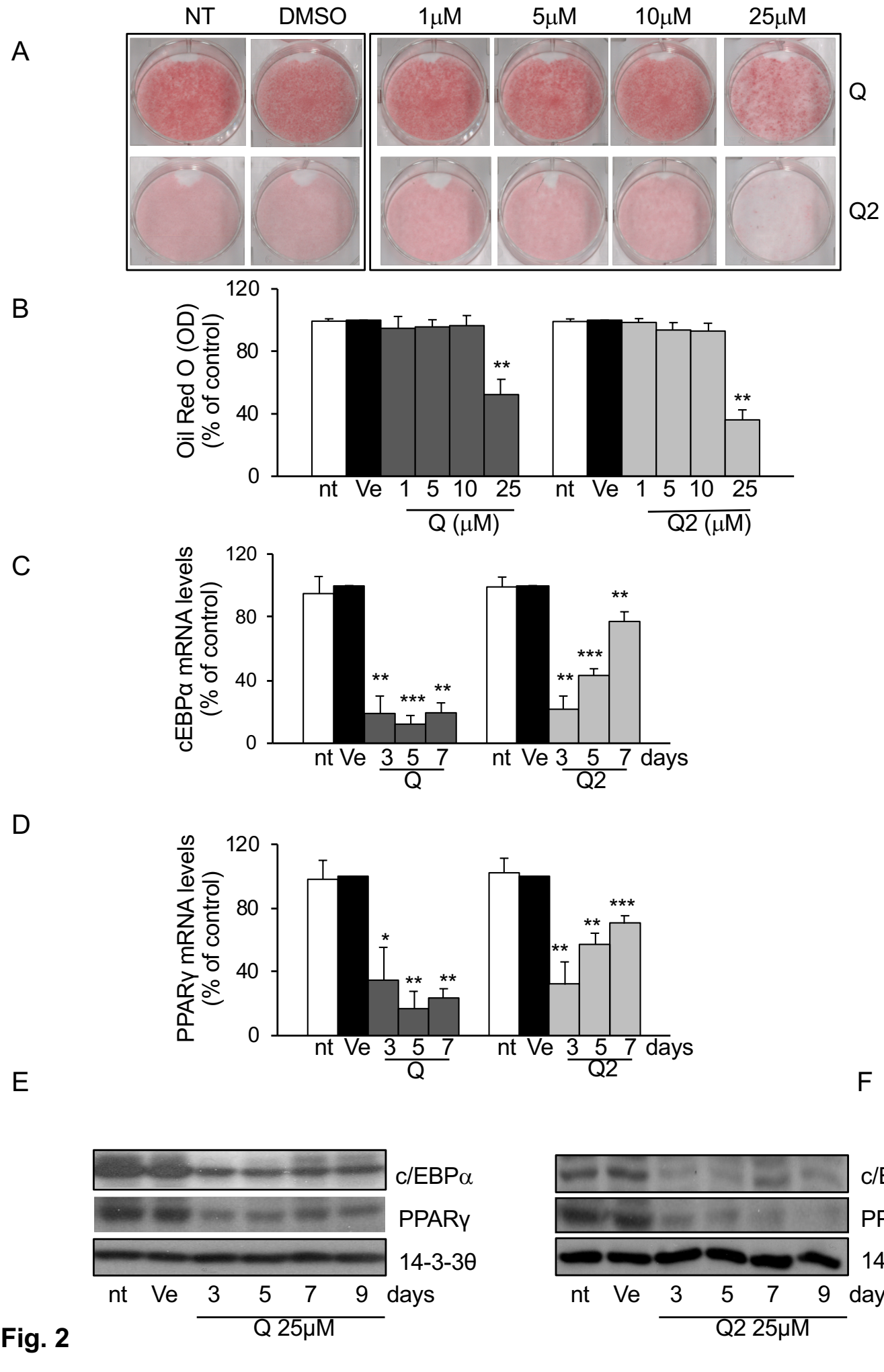

A
NT
DMSO
1μM
5μM
10μM
25μM
Q
Q2
B
Oil Red O (OD)
(% of control)
120
80
40
0
**
**
nt Ve 1 5 10 25
Q (μM)
nt Ve 1 5 10 25
Q2 (μM)
C
cEBPα mRNA levels
(% of control)
120
80
40
0
**
***
**
**
***
**
nt Ve 3 5 7
Q
nt Ve 3 5 7 days
Q2
D
PPARγ mRNA levels
(% of control)
120
80
40
0
*
**
**
**
**
***
nt Ve 3 5 7
Q
nt Ve 3 5 7 days
Q2
E
c/EBPα
PPARγ
14-3-3θ
nt Ve 3 5 7 9 days
Q 25μM
F
c/EBPα
PPARγ
14-3-3θ
nt Ve 3 5 7 9 days
Q2 25μM

Fig. 2

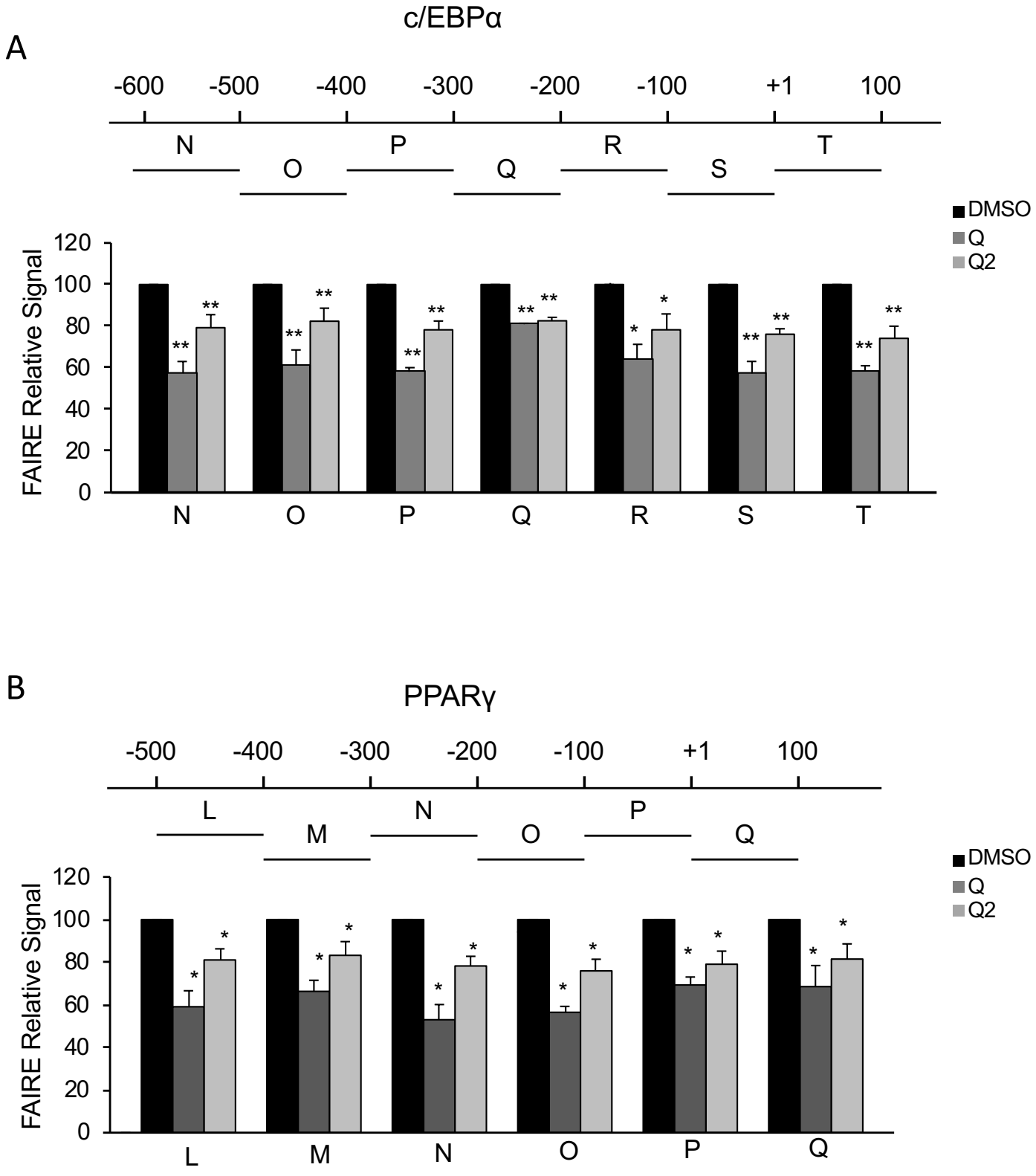

A
c/EBPα
-600
-500
-400
-300
-200
-100
+1
100
N
O
P
Q
R
S
T
DMSO
Q
Q2
FAIRE Relative Signal
120
100
80
60
40
20
0
B
PPARγ
-500
-400
-300
-200
-100
+1
100
L
M
N
O
P
Q
DMSO
Q
Q2
FAIRE Relative Signal
120
100
80
60
40
20
0

Fig. 3

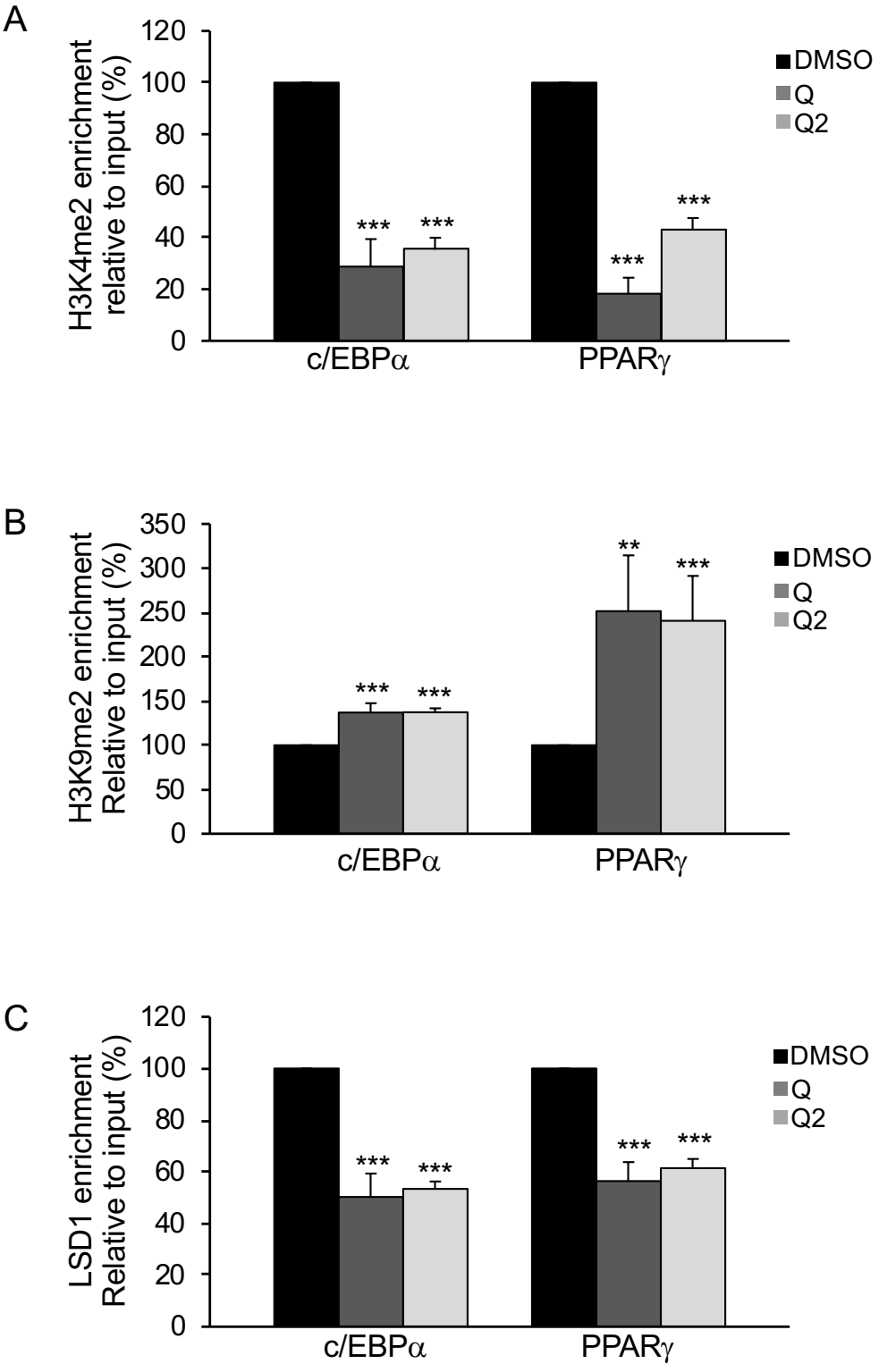
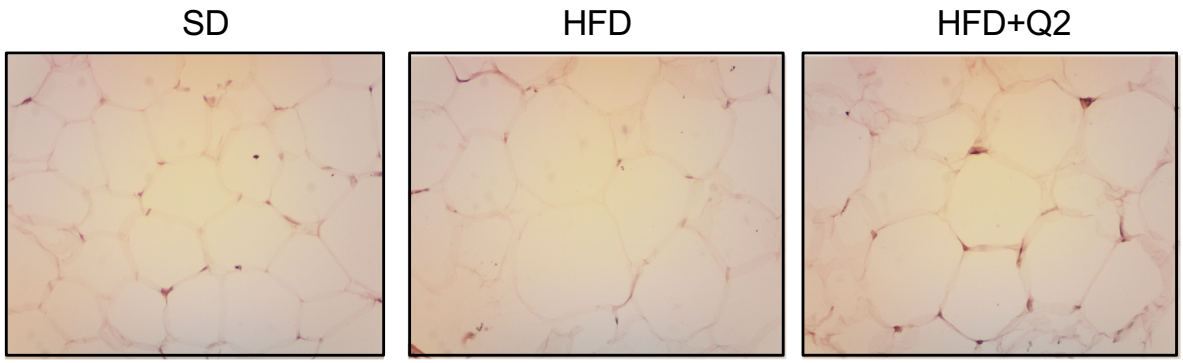
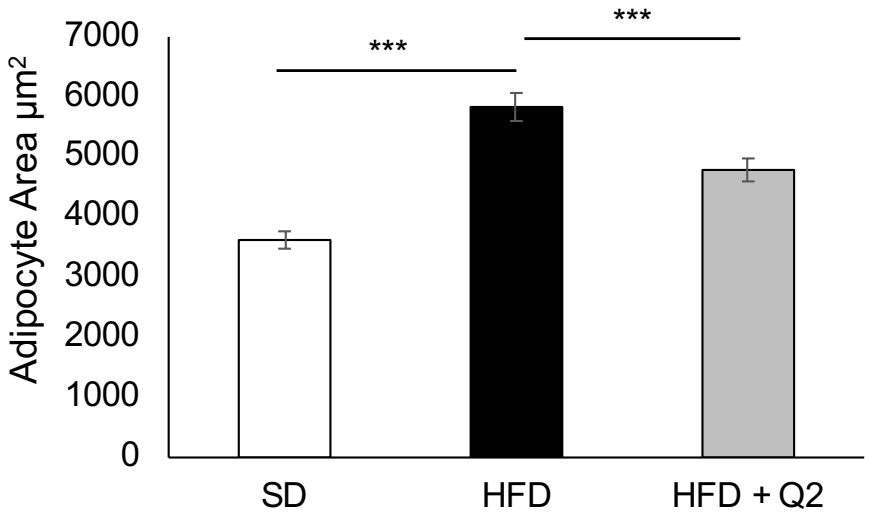

A
H3K4me2 enrichment relative to input (%)
120
100
80
60
40
20
0
***
***
***
***
DMSO
Q
Q2
c/EBPα
PPARγ
B
H3K9me2 enrichment Relative to input (%)
350
300
250
200
150
100
50
0
**
***
***
***
DMSO
Q
Q2
c/EBPα
PPARγ
C
LSD1 enrichment Relative to input (%)
120
100
80
60
40
20
0
***
***
***
***
DMSO
Q
Q2
c/EBPα
PPARγ

Fig. 4

A


SD
HFD
HFD+Q2

B


Adipocyte Area µm²
7000
6000
5000
4000
3000
2000
1000
0
***
***
SD
HFD
HFD + Q2

C

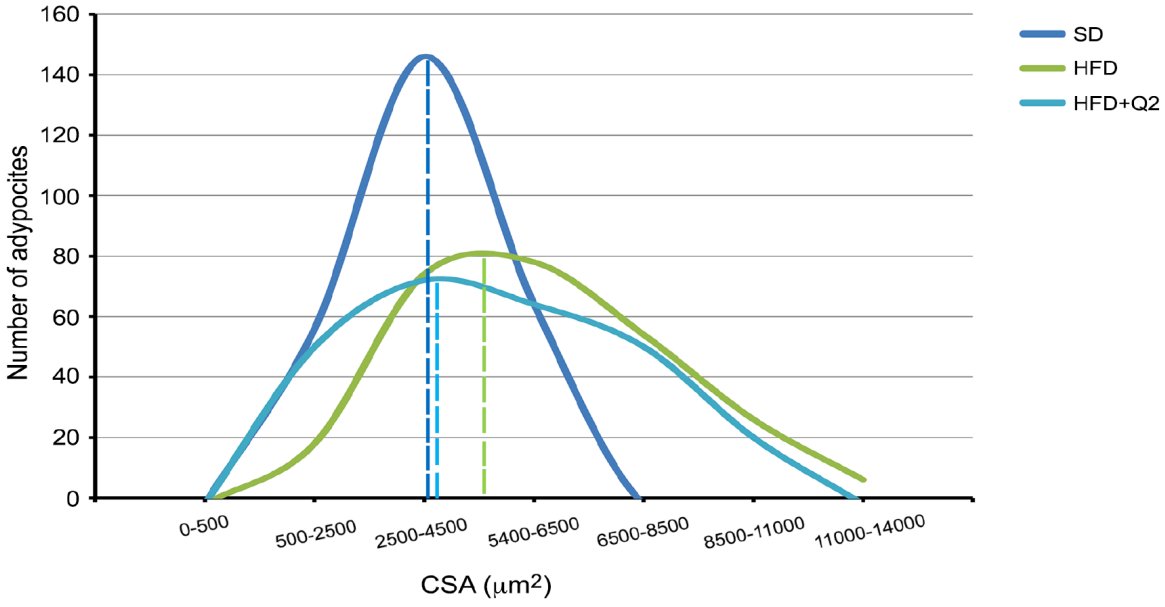
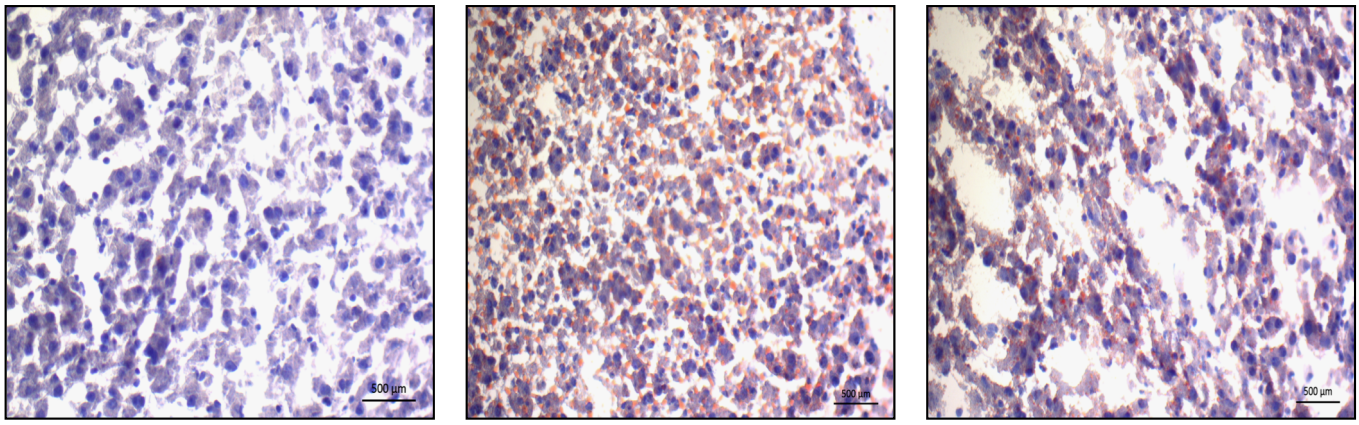

Number of adypocites
160
140
120
100
80
60
40
20
0
0-500
500-2500
2500-4500
5400-6500
6500-8500
8500-11000
11000-14000
CSA (µm²)
SD
HFD
HFD+Q2

Fig. 5

A


SD
HFD
HFD + Q2

B

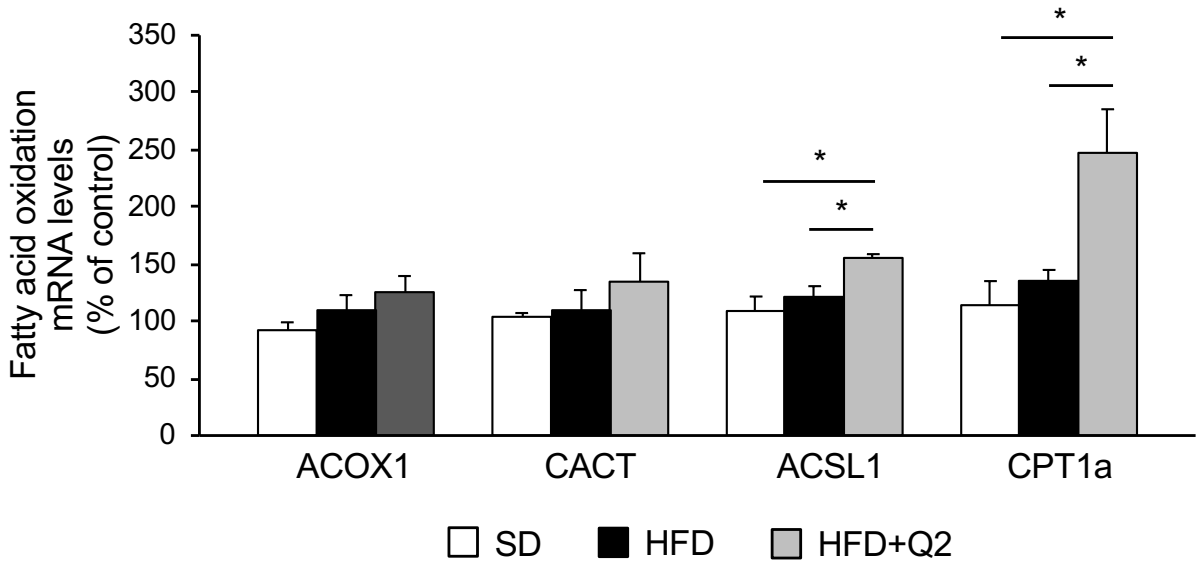
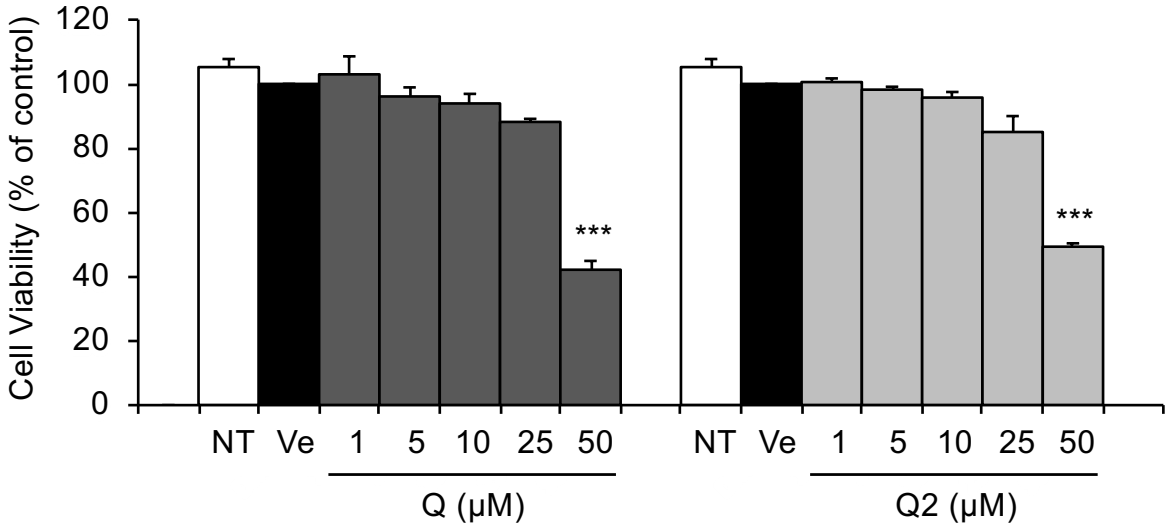

Fatty acid oxidation mRNA levels (% of control)
350
300
250
200
150
100
50
0
*
*
*
*
ACOX1
CACT
ACSL1
CPT1a
SD
HFD
HFD+Q2

Fig. 6


Cell Viability (% of control)
120
100
80
60
40
20
0
NT
Ve
1
5
10
25
50
***
Q (μM)
NT
Ve
1
5
10
25
50
***
Q2 (μM)

Suppl. Fig.1

Supplementary Table 1. Primers used for qRT-PCR assay

Gene	Primer sequences
m-cEBPα	F: 5′-ggctcgccatgccgggagaa-3′ R: 5′-ccgggtcgatgtaggcgctg-3′
m-PPARγ	F: 5′-cagtggagaccgcccaggct-3′ R: 5′-tggagcaggggtgaaggct-3′
m-LSD1	F: 5′-cgggcgaaggtagagtacag-3′ R: 5′-ctccacacccgatggttct-3′
m-ARBP	F: 5′-ccgtgatgcccagggaagacag-3′ R: 5′-ccaacagcatatcccgaatctcag-3′
m-ACOX1	F: 5′-caggaagagcaaggaagtgg-3′ R: 5′-cctttctggctgatcccata-3′
m-CPT1a	F: 5′-ctatgcgctactcgctgaagg-3′ R: 5′-ggctttcgacccgagaaga-3′
m-CACT	F: 5′-tgggatgttatctggtgtgttcac-3′ R: 5′-gaagcctgaatctgcagtaagcat-3′
m-ACSL1	F: 5′-tcctacaaagaggtggcagaact-3′ R: 5′-ggcttgaacccttctggat-3′

Abbreviations used: *cEBPα*,CCAAT/Enhancer-Binding Protein alpha; *PPARγ*, Peroxisome Proliferator-Activated Receptor gamma; *LSD1*, Lysine-Specific Histone Demethylase 1; *ARBP*, Ribosomal Protein, large, P0; *ACOX1*, Acyl-CoA oxidase 1; *CPT1a*, Carnitine Palmitoyl Transferase-1a; *CACT*, Carnitine/Acylcarnitine Translocase; *ACSL1*, Acyl-CoA Synthetase Long-Chain family member 1.

Supplementary Table 2. Primers used for FAIRE and ChIP assays

Gene	Regions	Sequences
cEBPα	N	F: 5′-tccaaagcagtctccaacct-3′ R: 5′-tgagctaccgtagtgcagac-3′
	O	F: 5′-agtctgcactacggtagctc-3′ R: 5′-ttccagccaacactagggag-3′
	P	F: 5′-ttctctctccaaacgctccc-3′ R: 5′-gcccctcctttaagcctcta-3′
	Q	F: 5′-tagaggcttaaaggaggggc-3′ R: 5′-tagtggagagagatcgtggc-3′
	R	F: 5′-cagcgcaggagtcagtgg-3′ R: 5′-gtcttagagcccgccttctc-3′
	S	F: 5′-gaaagtcacaggagaaggcg-3′ R: 5′-gcttttatagagggtcgggc-3′
	T	F: 5′-ccatcctactggcgcctt-3′ R: 5′-agcttcgggtcgcgaatg-3′
PPARγ	L	F: 5′-ggatagcagtaacattttggacct-3′ R: 5′-actcctaatcacacatctgaagaa-3′
	M	F: 5′-tgtgtgattaggagtttcaacca-3′ R: 5′-gcagtaaaatacacaccagtggc-3′
	N	F: 5′-agccactggtgtgtattttactg-3′ R: 5′-acattgtctcgccagtgacc-3′
	O	F: 5′-cactggcgagacaatgtagc-3′ R: 5′-ctgaattggctggcactgtc-3′
	P	F: 5′-gacagtgccagccaattcag-3′ R: 5′-ggcttatggtcatcgagctt-3′
	Q	F: 5′-agctcgatgaccataagcct-3′ R: 5′-catgctctgggtcaacagga-3′

Supplementary Table 3. “Anthropometric” and metabolic variables in rats fed with SD (Standard Diet) or SD+Q2 for 12 weeks

Variables	**SD**	**SD+Q2**
Body weight (g)	439±9	444±24
Waist circumference (cm)	19.1±0.4	18,8±0,4
BMI (Kg/m^2)	6.1±0.30	6,9±0,03
Abdominal fat (g)	6.0±0.4	7,0±0,9
Hearth weight (g)	1.36±0.07	1,44±0,05
Cardiac Somatic Index	0.31±0.02	0,32±0,02
Glycemia (mg/dL)	95±10	102±2
Insulin (ng/mL)	2.3±0.1	2,5±0,1
HOMA Index	1	1,15
Triglycerides (mg/dL)	105±18	66±10
Total cholesterol (mg/dL)	117±13	102±24
HDL cholesterol (mg/dL)	52±3	47±6
LDL cholesterol (mg/dL)	43±6	51±8
Adiponectin (ng/mL)	25±1.0	25±1

Values are expressed as mean ± SEM. Differences were not statistically significant (n=4 for SD, n=3 for SD+Q2)