Effects of food bioactives on lipid metabolism: applications in obesity and related metabolic alterations.

Andrea Mosqueda Solís

TESIS DOCTORAL

Departamento de Farmacia y Ciencia de los Alimentos
Vitoria-Gasteiz
22 de Enero del 2018

ACAT: acyl coenzyme A cholesterol O-acyltransferase
ACC: acetyl-CoA carboxylase
ACRP30: adipocyte complement related protein
ADD1: adipocyte determination and differentiation-dependent factor 1
AMPK: AMP-activated protein kinase
Ap2: adipocyte protein 2
ATGL: adipose triglyceride lipase
BAT: brown adipose tissue
CAP: capsaicin
C/EBPs: CCAAT/ enhancer binding proteins
C/EBPβ: CCAAT/ enhancer binding protein β
C/EBPδ: CCAAT/ enhancer binding protein δ
CACT: carnitine/acylacarnitine translocase
cAMP: 3´,5´-cyclic adenosine monophosphate
CD36: cluster of differentiation 36
CGI-58: comparative gene identification-58
ChREBP: carbohydrate response element binding protein
CIDE-A: cell death-inducing DNA fragmentation factor like effector A
CPT I: carnitine palmitoyltransferase I
CPT II: carnitine palmitoyltransferase II
CPT1a: carnitine palmitoyltransferase 1a, liver
CPT1b: carnitine palmitoyltransferase 1b, muscle isoform
DAG: diacylglycerols
eNOS: endothelial nitric oxide synthase
FA: fatty acids
FABP: fatty acid binding protein
FAP: fatty acid translocase
FAS: fatty acid synthase
FASN: fatty acid synthase
FATP: fatty acid transport proteins
FGF21: fibroblast growth factor 21
G6PHD: glucose-6-phosphate dehydrogenase
GADPH: glyceraldehyde 3-phosphate dehydrogenase
GCK: Glucokinase
GDI: guanosine Diphosphate Dissociation Inhibitor
GPAT: glycerol-3-phosphate acyltransferase
HK2: hexokinase II
HMG-CoA: 3-hydroxy-3methyl-glutary-coenzyme A

HESP: hesperidin
HESP + CAP: hesperidin and capsaicin
HSL: hormone-sensitive lipase
INSR: insulin receptor
IRS1: insulin receptor substrate 1
KLF2: Kruppel Like Factor 2
LEPR: leptin receptor
LIPE: hormone-sensitive lipase
LPL: lipoprotein-lipase
LXRα: liver X receptor α
MAG: monoacylglycerol
ME: malic enzyme
MGL: monoacylglycerol lipase
Myf5: non-myogenic factor
NADPH: nicotinamide adenine dinucleotide phosphate
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
PCR: Polymerase chain reaction
PGC1-α: proliferator-activated receptor gamma coactivator 1-alpha
PKA: protein kinase A
PKLR: pyruvate kinase
PNPLA2: patatin-like phospholipase domain containing 2
PPARα: peroxisome proliferator-activated receptor *α*
PPARγ: peroxisome proliferator-activated receptor γ
PPARδ: peroxisome proliferator-activated receptor δ
RXR: retinoid X receptor
SCD1: sterol coenzyme A desaturase
SLC2A4: glucose transporter 4
SREBP1c: sterol response element binding protein 1c
TG: triglycerides
TRPV1: transient receptor potential vanilloid type-1
UCP1: uncoupling protein 1
UCP2: uncoupling protein 2
VLDL: very light density lipoproteins
WD: western diet

Hace cuatro años inició este camino, es un trabajo que además de lo académico, te enseña a valorar cada momento, a la gente que te rodea y te hace aprender de ello. Pese a momentos difíciles, cuando me preguntaban qué es lo que más me gustaba de la tesis, la respuesta era siempre la misma: que no ha pasado ni un día en que no aprenda algo nuevo, un aprendizaje interminable del cuál me siento muy afortunada.

En primer lugar quiero agradecer la realización de esta Tesis Doctoral a mis directoras. A la Dra. María del Puy Portillo, por abrirme las puertas a la investigación, por tu cercanía, hacerme parte de tu grupo de trabajo, enseñarme, aconsejarme y guiarme en todo momento. A la Dra. Catalina Picó, por la oportunidad de realizar la mitad de esta tesis en su grupo de investigación y hacerme ver que para hacer algo bien, hay que cuidar todos los detalles. Por su apoyo y confianza, muchas gracias a las dos.

Agradezco también al Gobierno de México, a el CONACYT, por la concesión de una beca en el extranjero que me ha proporcionado la infraestructura necesaria para la consecución del presente trabajo.

A la Universidad del País Vasco, a Leixuri, Pili, Maite, Alfredo, Iñaki, Marian, Itziar Txurruca, Laura y Bittor por hacer mis días en el laboratorio agradables, siempre tener una sonrisa y por todos sus conocimientos. Jona, ¿cómo haces para siempre lo más complicado hacerlo lo más fácil? Gracias por tener ese don y compartirlo, gracias por despertar mi interés científico con el trabajo fin de máster y mira ahora. Arrate, por enseñarme a no darle vueltas a las cosas, hacerlas bien y disfrutar de ello, gracias por siempre estar ahí.

A ustedes: Ana Gracia, Saioa Gómez, Noemí Arias, Itziar Eseberri e Idoia Larretxi, desde el primer día hasta el último, gracias por su amistad y compañerismo, ustedes sí que han *entrado dentro* de mi vida para hacerla mejor. Ana, porque de todo lo que me has enseñado y hemos vivido escribiría una tesis y no podría ser mejor. Saio, porque ni 1000 kms por monte serían

suficientes para agradecerte lo que hemos recorrido juntas (más todo lo que nos queda). Noe, por ser incondicional y bondadosa. Itzi, porque sabes el qué y el cómo de las cosas. Ido, por tus interminables ganas de siempre hacer algo nuevo. Simplemente chicas, porque son de lo que no hay.

Marta, Leire, Nagore y Javier, por su apoyo y todos los buenos momentos que hemos compartido junto a risas interminables.

A todas las personas de LBNB de la Universidad de las Islas Baleares, por sacar mi lado más valiente. Joana, porque tenías la mejor respuesta antes de preguntar, por enseñarme a ser práctica y objetiva, por siempre tener algo positivo para mí, porque eres una gran investigadora y persona ¡moltes gràcis! . Alberto, estoy segura que no te haces una idea de lo importante que fue encontrarte. Pep, me recomendaron acercarme a ti y por supuesto no hubo equivocación, encontré esa persona que con decir dos palabras o sin ellas decía todo lo que necesitaba escuchar, gracias. “San” Miguel, mi vasquito en Mallorca, un amigo auténtico desde el día 0. Andre, porque sin tu alegría y amistad mi vida no sería igual de divertida. Nara, por vivir cada día como si fuera el último.

Neus, sin duda eres el tesoro que la isla me regaló, no tengo palabras para agradecerte TODO lo que compartimos y aprendí de ti. Mary, porque la vida nos juntó cuando más necesitábamos una buena amiga cerca.

I would like to thank Kirsty Spalding for the opportunity to do the doctoral stay in the Cell and Molecular Biology Department from the Karolinska Institute, a unique place, where the slogan “Improving health through education & research” is a daily way of life. Thanks for everything, for every technique and knowledge that improved my motivation to continue in the scientific field. Specially, thank you Firoozeh, Annitta, Bea, Carolina, Christina, Qian, Fu, María and Helena for make me feel part of the KI and teach me all that you do. Ismael, por compartir conmigo el síndrome de Estocolmo.

José y Marian, no hay suficiente espacio para agradecerles hacerme parte de su hogar, su familia, sus amigos, su país y su cariño, por hacerme sentir con un poco de sangre euskalduna y aportar mucho a mi formación personal y académica.

A más de 9000 kms de distancia: Angie, Lore, Rafa, Rorras, Mau, Quique, Diego L., Arturo, Jorge, Susana, Shair, Josele y Juan David, porque pese a que seguramente no sabían de lo que les hablaba siempre ponían el mayor interés, los he tenido lejos pero he sentido cerca, por no dudar nunca de mí. Angie, por darme ánimos cuando más lo he necesitado y seguir creciendo juntas. Lore, porque con dos minutos en una llamada arreglas el mundo. Diego V., por aparecer en mi vida, compartir momentos, lugares indescriptibles y hacer que la distancia sea pequeña cuando se quiere de verdad. A ustedes, por escuchar mis problemas y compartir mis alegrías.

Tía Ana y tío Memo, porque siempre estuvieron pendientes de mí. A mis abuelos, porque donde estén, sé que estarán orgullosos de ver la familia que han formado. Abuelita Josefina, porque ves el mundo diferente.

Y por último y más importante: mi familia, que he extrañado cada segundo. Má y Pá, este trabajo es fruto de la educación y cariño que me han dado, sin ustedes nada de esto sería posible. De corazón, gracias infinitas por su confianza y siempre estar ahí, darme todo lo que soy, nunca dejarme caer, quererme, cuidarme, aconsejarme, consentirme y darme el mejor ejemplo a seguir todos los días. A mis hermanos: Augus y Cane (y mi cuñado Rodrigo), gracias por hacerme creer que la ciencia es fácil, tener un SÍ para todo y darme lo mejor de ustedes. A mis sobrinos, Anelí y Braulio, porque con una sonrisa logran desconectarme de la realidad y llevarme a su mundo mágico e inocente.

Gracias Eskerrik asko Gràcis Thank you tack så mycket

Introduction

1. Obesity

Obesity is a pathology defined as an excessive fat accumulation in white adipose tissue that appears when there is a chronic unbalance between energy intake and energy expenditure, whose components are resting metabolic rate (the energy needed to maintain life at rest), thermogenesis and physical activity (Hill JO. *et al.,* 2013). Thus, fat accumulation occurs if energy intake is greater than energy expenditure over time (Prieto-Hontoria *et al*., 2011; Jung *et al.,* 2014; Apalasamy and Mohamed, 2015) (Figure 1). Although this is a simple way to explain the origin of obesity, it is important not to forget that obesity has a multifactorial etiology and that it results from the interaction among biological, genetic, environmental and psychosocial factors.

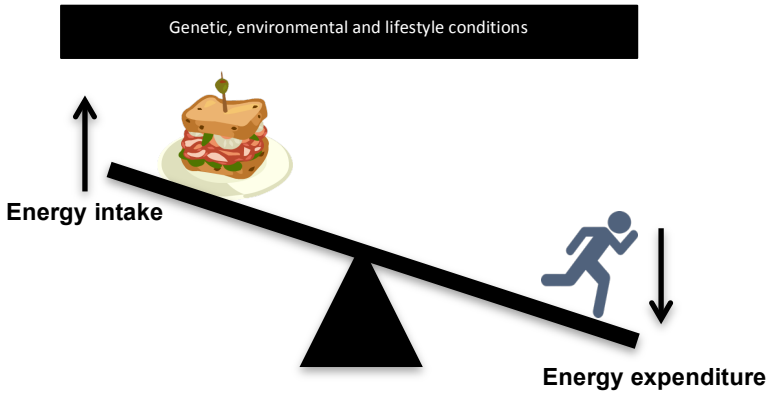


Figure 1. Energy unbalance as a cause of obesity.

The increase in adipose tissue size when obesity is developed results from increased adipocyte number (hyperplasia), increased adipocyte size (hypertrophy) or both (Couillard *et al*., 2000; Virtue and Vidal-Puig, 2010; Rutkowski *et al.,* 2015). According to the anatomical location, two types of obesity are defined. In peripheral obesity the excess of fat, mainly in the

subcutaneous area, is located around the hip and the thigh. It is also called pear-like body shape or gynoid obesity because it is more common in young women. In central obesity, fat is concentrated in the abdominal region. It is also called apple-like body shape or android obesity and it can be subcutaneous, visceral or both. Visceral obesity is more pernicious because it is closely correlated to increased risk of developing cardiovascular and metabolic alterations, such as hypertension, dyslipidaemias, atherosclerosis, and type 2 diabetes (Kotani *et al.,* 1994; González-Muniesa *et al.,* 2017).

When energy balance is positive, excessive fat accumulation can be observed not only in adipose tissue, but also in other tissues and organs, such as liver and skeletal muscle. This ectopic fat accumulation results in steatosis and insulin resistance, which are co-morbidities of obesity. In addition to the mentioned cardiovascular and metabolic alterations, other co-morbidities, such as respiratory problems (sleep apnea) and cancer (endometrial, liver and kidney cancers) are also quite common in obese subjects (Renehan *et al*., 2010; Prieto-Hontoria *et al.,* 2011; Wang *et al*., 2011; Nordestgaard *et al.,* 2012; Park *et al.,* 2013; Saltiel and Olefsky, 2017). Finally, obesity could have a negative impact on mood issues and cognitive functions (Jauch-Chara *et al*., 2014). All these co-morbilities contribute to premature death in obese individuals (Cornier *et al.,* 2011, WHO Technical Report Series 894, 2000).

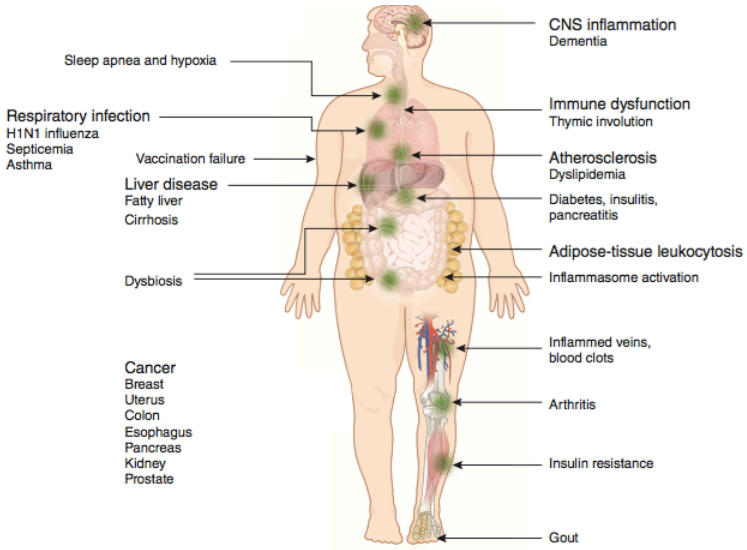


Figure 2. Obesity- related complications (from Kanneganti and Dixit 2012).

During the past few decades the prevalence of obesity has reached epidemic proportions. It is now recognized as one of the most important public health problems, and is increasing not only in industrialized countries, but also in non-industrialized ones, particularly in those undergoing economic transition (Scully, 2014). In 2015, the World Health Organization reported that more than one billion adults were overweight, and at least 300 million of them were clinically obese (WHO, 2015). In addition, overweight and obesity are leading risks for worldwide deaths. It is estimated that 2.8 million adults die each year as a result of being overweight or obese (Barcelo-Batllori *et al.,* 2009; Popkin *et al.,* 2012).

Since the complications of obesity have an economic impact on public health and decrease quality of life, the need of implementing effective, therapeutic and preventive strategies to manage obesity and its co-morbidities

have acquired extreme importance in the scientific community (Seidell and Halberstadt, 2015).

2. Adipose tissue

2.1. Function, location and classification

In mammals, two types of adipose tissue are well distinguished: white adipose tissue (WAT) and brown adipose tissue (BAT) that has essentially antagonistic functions (Hassan *et al.,* 2012). Both of them have the capacity to store lipids but have distinct cell structure, location, colour, vascularization and functional function. The main function of WAT is energy storage, and that of BAT is thermogenesis (Cinti *et al.,* 2012). Remarkably, a third type of adipocytes that is known as beige or "brite" (brown in white) has been identified (Petrovic *et al*., 2010, Bartelt *et al*., 2014). Beige adipocytes are co-located within specific WAT depots, but display comparable functional and molecular features, as brown adipocytes. Also, their development seems to be induced by similar stimuli as brown adipocytes (Wu *et al.,* 2012). The main morphological and functional characteristics of the three types of adipocytes are summarized in Figure 3.

White adipose tissue

WAT is a loose connective tissue containing lipid-laden cells, known as adipocytes, which comprise approximately 35% to 70% of adipose mass (Frühbeck *et al*., 2008). The remaining cell types are found in the stroma-vascular fraction and include macrophages, fibroblasts, pericytes, blood cells, endothelial cells, and adipose precursor cells, among others (Frühbeck *et al*., 2008).

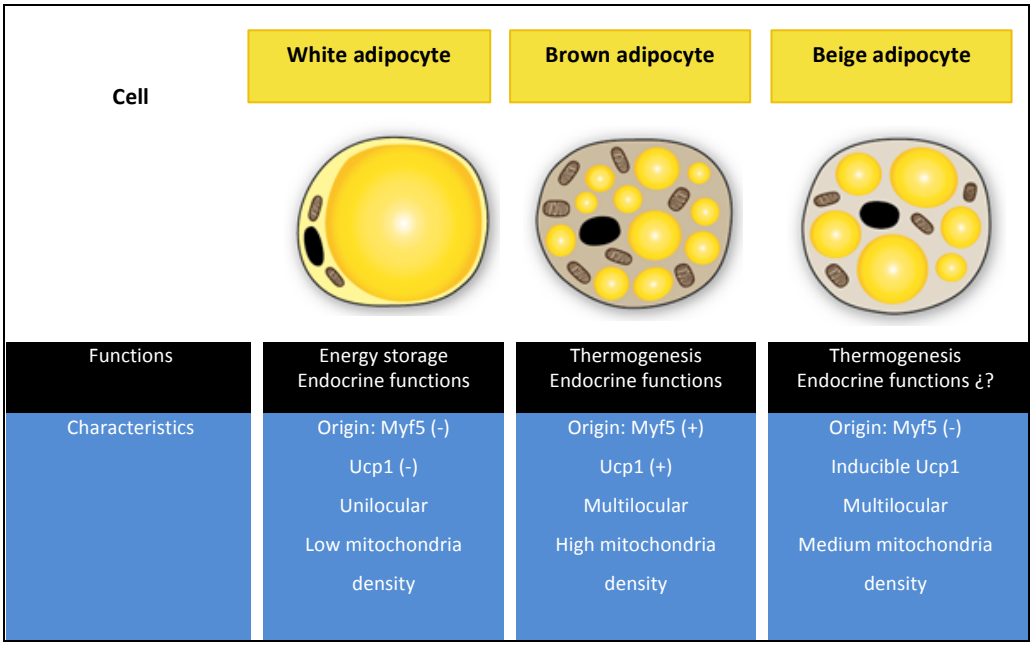


Figure 3. Main functions and characteristics of adipocytes. Myogenic factor (Myf5), uncoupling protein 1 (UCP1). Modified from Bartelth *et al*., 2014.

The morphology of WAT reflects its central role in energy storage; white adipocytes principally derivate from non-Myf5 lineage progenitors, although a subpopulation in WAT depots can be derived from the Myf5 lineage (Shan *et al.,* 2013) (Figure 4). They have a typical morphology, with a prominent lipid droplet that occupies almost the entire cytoplasm (90%) and a semilunar nucleus, which is thus pushed to the cell periphery against the lipid membrane, giving adipocytes a characteristic appearance (Arner and Spalding, 2010). They have a thin ring of cytoplasmic matrix and few mitochondria without uncoupling protein 1 (UCP1) (Crichton *et al.,* 2017).

WAT has been recognized as the major reservoir for energy storage (Sethi *et al*., 2007), which plays an essential function in the regulation of whole-body lipid homeostasis (Galic *et al.,* 2010). It was originally considered as a passive

storage tissue, with the main function of accumulating the excess of energy as triglycerides (TG), and allowing a rapid mobilization under energy deprivation. Currently, it is know that WAT constitutes a complex and dynamic endocrine organ with an important role in the development of obesity and its complications (Frühbeck *et al.,* 2001; Frühbeck, 2008).

Brown adipose tissue

BAT also stores lipid droplets, but it has a different role than that of WAT. BAT is an energy-dissipating tissue whose main function is to generate heat in order to maintain body temperature through a process called "adaptive thermogenesis" due to the presence in this tissue of UCP1 (Cannon *et al.,* 2004; Townsend *et al.,* 2012).

The principal cell type of BAT is the brown adipocyte, with entirely different characteristics and morphology from white adipocytes. Brown adipocytes are multilocular and they store TG in several small lipid droplets. They have an abundant cytoplasm containing numerous mitochondria (responsible of the brown adipose tissue colour), a round centrally located nucleus, and they are highly vascularized with sympathetic innervations (Cinti *et al.,* 2009). Brown adipocytes derive from a Myf5-expressing cell lineage, different from white adipocytes (Seale *et al.,* 2008) (Figure 4).

Beige adipocytes and browning process

Beige adipocytes have mixed characteristics of both white and brown adipocytes. They are also multilocular and have thermogenic properties due to increased mitochondrial function and expression of inducible UCP1, similarly to

brown adipocytes (Wu *et al.,* 2012). Nevertheless, beige adipocytes do not derive from the Myf5 linage, but from Myf5 negative progenitors, as white adipocytes do (Figure 4). However, the origin of brown-like or inducible adipocytes has not been clearly established so far. Two main theories exist, which propose that brite adipocytes could originate from proliferation and *de novo* differentiation of a specific pool of precursor cells contained in WAT depots (Petrovic *et al.,* 2010; Wu *et al.,* 2012) or from "transdifferentiation" from pre- existing white adipocytes (Figure 4) (Barbatelli *et al.* 2010; Cinti *et al*., 2011; Barneda *et al*., 2013; Rosenwald *et al.,* 2013).

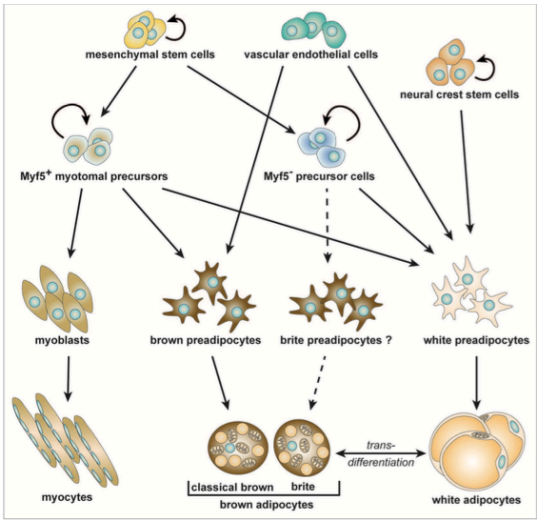


Figure 4. Developmental lineages of adipocytes (Rosenwald *et al.,* 2013).

Recent research has evidenced that different stimuli, such as β-adrenergic stimulation, chronic cold exposure and certain pharmacological and dietary compounds, can induce the appearance of brown-like adipocytes in WAT depots (Bonet *et al.,* 2013). This process has been named "browning" and has been observed in different animal models, mainly rodents. The "brite"

adipocytes express UCP1, although at lower levels than classic brown adipocytes. With an appropriate stimulation they can switch on a tough program of mitochondrial respiration and energy expenditure equivalent to that of "classic" brown adipocytes (Waldén *et al.,* 2012). In this sense, WAT browning and BAT activation could be useful in strategies to prevent and manage obesity and related diseases. In fact, WAT browning process has already been described to confer protection against obesity in many rodents studies (Bonet *et al.,* 2013; Bonet *et al.,* 2017).

3. Main processes involved in adiposity and triglyceride metabolism in adipose tissue and/or liver

Various organs are involved in the regulation of systemic TG metabolism. Adipose tissue plays an important role in this process because it is the main organ for energy storage as TG (Frayn *et al.,* 2006). The amount of this lipid species within adipocytes is mainly regulated by the build-up of fatty acids (FA) pathway called lipogenesis, their mobilization through lipolysis, and FA oxidation within the mitochondria (Rutkowski *et al.,* 2015). Moreover, an important process is the differentiation of multipotential cells into mature adipocytes to fulfil metabolic and endocrine functions.

3.1. Adipogenesis

Adipogenesis is a two-step process in which undifferentiated mesenchymal cells differentiate into pre-adipocytes, which then undergo a secondary differentiation step to become lipid filled adipocytes (mature adipocytes) (Ali *et al*., 2013) (Figure 5). This process involves six defined stages: mesenchymal precursors, committed pre-adipocytes, growth-arrested pre-adipocytes, mitotic

clonal expansion, terminal differentiation and mature adipocytes (Lefterova and Lazar, 2009). In order to achieve a successful transformation into mature adipocytes, pre-adipocytes undergo marked changes in morphology and gene expression.

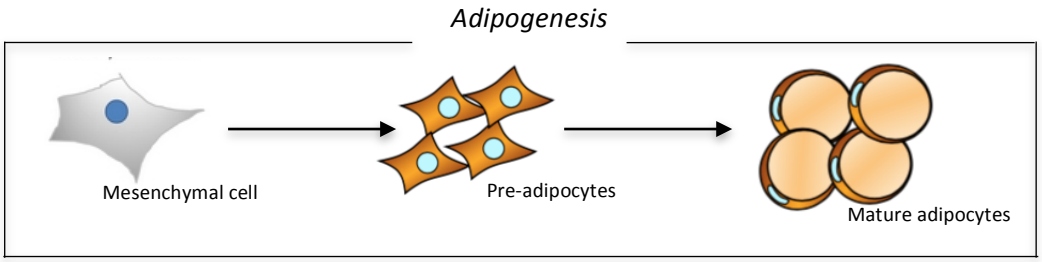


Figure 5. Morphological changes during adipogenesis (Modified from Leffternova *et al.*, 2014).

In the last years, a relation between adipocyte differentiation and several physiological and pathological processes like energy balance, obesity, diabetes and hyperlipidaemia, has been revealed. Thus, it is important to study the mechanisms involved in the adipocyte differentiation in order to better understand the aetiology of diseases to found out new anti- obesity therapies.

In vitro adipocyte cell differentiation is a good reference to analyse the process that takes place during the *in vivo* adipogenesis, such as stop cell growth, enzymatic lipogenic expression and lipid accumulation. The most extensively characterized and used pre-adipocyte cell lines are 3T3-L1 and 3T3-F442A, which derive from disaggregated 17-19 day old Swiss 3T3 mouse embryos (Green and Meuth 1974; Green and Kehinde 1976).

Once 3T3-L1 cells reach the confluence state, their growth ends because it is inhibited by cellular contact. At this moment, the differentiation could be induced by hormonal stimuli with methylisobutylxanthine, dexamethasone and insulin (Smith, 1978). Approximately 24 hours later, adipocytes start the cell cycle and undergo a process called "clonal expansion" which consists in several DNA replications and cell duplication, and finally the growth stops (Bernlohr *et al*., 1985). Along with the clonal expansion, activation of the transcriptional cascade of specific adipocyte genes begins. Changes in gene expression, biochemistry and morphology lead to the acquisition of adipocyte phenotype. Guo Y. *et al.,* associated this phenotype with changes in the expression of more than 200 genes (Guo *et al.,* 2008*)*. During the terminal phase of differentiation, there is an increase in lipogenesis *de novo* and insulin sensibility because of the proliferation of insulin receptors and glucose transporters (GLUT 4). Finally, synthesis of adipocyte-secreted products including perilipin, adipocyte protein 2 (Ap2), leptin, adipsin, adiponectin, resistin, angiotensin, apelin, visfatin and adipocyte complement related protein (Acrp30) begins, producing a highly specialized endocrine cell that will play key roles in various physiological processes (Moreno-Aliaga y Martínez, 2002).

Adipogenesis regulation

Several transcription factors regulate adipogenesis; three classes have been identified that directly influence fat cell development. These include peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/ enhancer binding proteins (C/EBPs), and sterol response element binding protein 1c (SREBP1c). First, C/EBPβ and C/EBPδ are detected, followed by PPARγ (the master of adipogenesis), which in turn activates C/EBPα. C/EBPα exerts positive feedback

on PPARγ to maintain the differentiated state. SREBP1 can activate PPARγ by inducing its expression, as well as by promoting the production of an endogenous PPARγ ligand. All of these factors supply to the expression of genes that characterize the terminally differentiated phenotype (Rosen and Spiegelman, 2000).

3.2. *De novo* lipogenesis

In adipocytes, TG storage is the consequence of the re-esterification of FA circulating in blood, from serum TG, or from FA synthesized in *de novo* lipogenesis. Dietary TG transported in blood by chylomicrons and liver TG transported in very light density lipoproteins (VLDL) are hydrolysed by lipoprotein-lipase (LPL) located in the vascular endothelium of adipose tissue and stimulated by insulin. Circulating FA can be up-taken into adipocytes through specific transporters, including fatty acid binding protein (FABP), fatty acid transport proteins (FATP), fatty acid translocase (FAP) among others.

De novo lipogenesis specifically refers to the metabolic pathway that synthesizes FA from acetyl-CoA. It mainly takes place in the liver and the adipose tissue (Pearce *et al.,* 1983). Acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC). Fatty acid synthase (FAS) is the key rate-limiting enzyme that is in charge of the conversion of malonyl-CoA into palmitate. Besides acetyl-CoA, nicotinamide adenine dinucleotide phosphate (NADPH), a substrate with reducing power, is also necessary for fatty acid synthesis. NADPH comes from biochemical reactions catalyzed by the enzymes glucose-6-phosphate dehydrogenase (G6PHD), malate dehydrogenase, malic enzyme (ME) and NADP isocitrate dehydrogenase. After several reactions, other

FA could be formed from palmitate (Ameer *et al.,* 2014). The acetyl-CoA needed for fatty acid synthesis is present in the mitochondria, whereas biosynthesis takes place in the cytosol. The citrate shuttle system is an essential transport mechanism, which allows acetyl-CoA effectively to move into the cytosol

Regulation of lipogenesis

ACC and FAS are mostly controlled by the modulation of their transcription, but they also can be regulated by post-transcriptional mechanisms. The main transcriptional regulators of these enzymes, present in adipose tissue and liver, are SREBP1c and carbohydrate response element binding protein (ChREBP). SREBP1c is an isoform of the SREBP family induced in response to insulin. The nutritional status is also and a regulator of SREBP. The expression of this transcription factor decreases with fasting and increases after feeding high-carbohydrate diets, as a result of increased blood glucose and insulin. This transcription factor promotes the expression of genes encoding lipogenic enzymes, due to the interaction with sterol response elements located in the gene promoter. It is known that SREBP 1c is negatively regulated by AMP-activated protein kinase (AMPK), which is an energy sensor that regulates cellular metabolism. Activated AMPK stimulates ATP-producing catabolic pathways, such as fatty acid oxidation, and inhibits ATP consuming processes, like lipogenesis. Therefore, AMPK activation suppresses the expression of ACC and FAS via down-regulation of SREBP 1c (Kohjima *et al*., 2008). In addition to this regulation, activation of AMPK also inhibits directly ACC expression by the phosphorylation of a serine residue (mostly SER_{79}) in the N-terminal enzyme region. ChREBP is another transcription factor; it was identified as a glucose responsive element, which regulates glycolytic, gluconeogenic and lipogenic

gene expression (Xu *et al.,* 2013). Transcriptional targets of ChREBP encode enzymes in lipogenic pathway such as FAS and ACC. Liver X receptor (LXR) is also a key regulator of the lipogenic pathway. In rodents, there are two isoforms, which form heterodimers with the retinoid X receptor (RXR) to activate their target genes. The effect of this nuclear factor on liver lipogenesis involves direct and indirect mechanisms. LXR can bind gene promoter of FAS, or regulate gene expression via the insulin-mediated activation of SREBP 1c. Transcription factors implicated in the lipogenesis regulation are schematized in Figure 6.

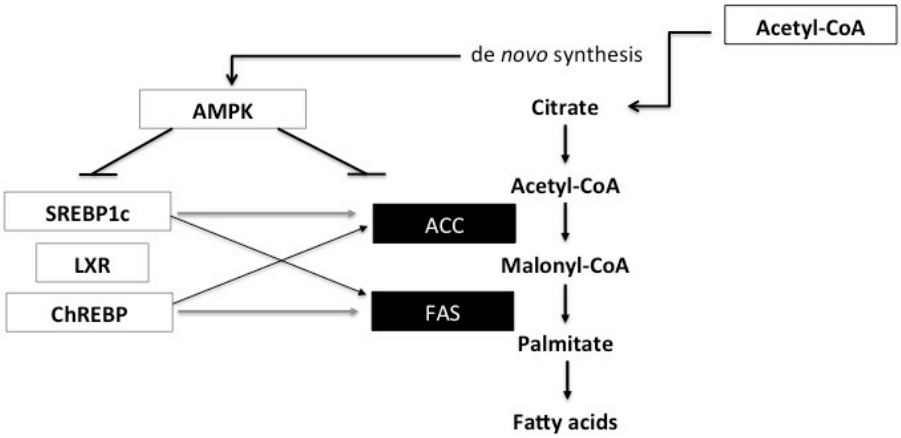


Figure 6. *De novo* synthesis of fatty acids and regulation of lipogenic genes by AMPK, SREBP1c, LXR and ChREBP (Letexier *et al*., 2003).

Additionally, the principal transcriptional regulator of LPL is PPARγ, this receptor is stimulated by the fatty acid concentration (and inhibited in fasted state). PPARγ is important for the lipogenic-genes transcription and activates enzymes responsible of carrying out lipogenesis. Two isoforms of PPARγ are

known, PPARγ$_1$ and PPARγ$_2$, the γ$_2$ isoform is mostly found in adipose tissue and key regulator in the adipocyte differentiation (Mueller *et al*., 2002).

3.3. Lipolysis

Lipolysis is the process that allows TG stored in the adipocyte to be hydrolysed into one molecule of glycerol and three fatty acids, which are delivered to the plasma. During lipolysis, an intracellular TG breakdown occurs by the actions of three lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (Zechner *et al.,* 2012).

ATGL catalyzes the first step of TG hydrolysis, converting TG to diacylglycerols (DAG). HSL is mainly responsible for the hydrolysis of DAG, and produce monoacylglycerols (MAG) and FA (Schweiger *et al.,* 2006). In the last step of lipolysis, MAG are released from the lipid droplet into the cytosol and eventually cleaved by MGL to generate glycerol and FA (Figure 7) (Gaidhu *et al*., 2010; Gizem *et al.,* 2017). Both, ATGL and HSL are considered responsible for 95% of triglyceride lipase activity due to complementary actions between them (Zechner *et al.,* 2009; Thompson *et al.,* 2010).

Lipolysis regulation

The lypolitic process is regulated by hormonal factors such as catecholamines. In basal conditions, ATGL is located in lipid droplet surface, and even tough is partially inactivated it could carry out the basal lipolysis (Zimmerman *et al*., 2004). In this situation, HSL is located in the cytoplasm with no access to the internal lipid droplet TG.

Also, in the lipid droplet surface, there are located two proteins: perilipin-A and CGI-58, the protein activator of ATGL. In basal conditions, both are interacting and minimizing the interaction ATGL-CG1-58, which maintains ATGL inactive, thus preventing TG breakdown (Tansey *et al.,* 2001). After the union of catecholamine to their β receptors (adrenergic β-stimulation) they bind the G-proteins, which leads to the increase of 3´,5´-cyclic adenosine monophosphate (cAMP) produced by adenylate cyclase. This cAMP activates the protein kinase A (PKA), which in turn activates HSL by phosphorylation (Holm, 2003) and promotes its translocation from cytosol to the lipid droplet surface (Strissel *et al*., 2007). There, HSL binds perilipin-A and it starts working. PKA also phosphorylates perilipin A, thus resulting in the separation of this protein from protein CGI-58. Later on protein CGI-58 binds ATGL in the lipid droplet surface and activates it (Watt and Steinberg, 2008).

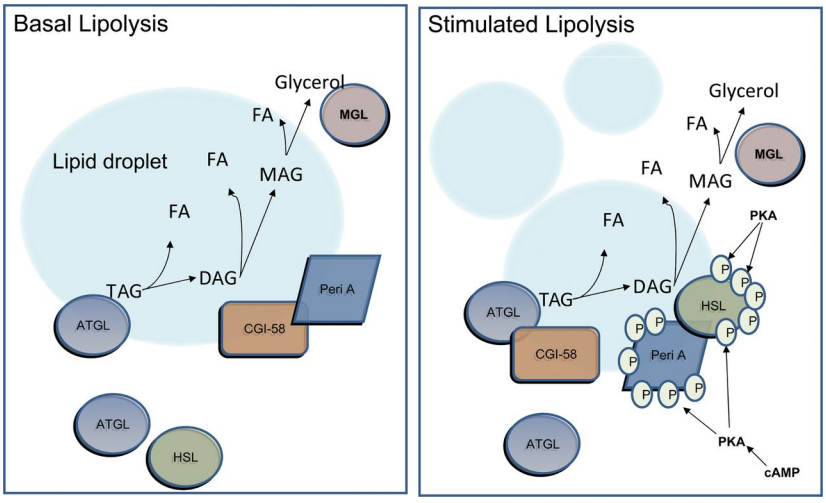


Figure 7. The emerging view of regulated lipolysis process (Watt *et al., 2008*).

3.4. Fatty acid oxidation

The main pathway for degradation of plasma FA is fatty acid oxidation (β-oxidation). This process can take place in both mitochondria and peroxisomes. Due to the fact that FA are found in the cytoplasm and fatty acid oxidation takes place in the mitochondria, a membrane transporter is needed to introduce FA inside mitochondria. The initial conversion of acyl-CoA to an acylcarnitine ester, followed by the transport of the acylcarnitine across the inner mitochondria membrane into the mitochondrial matrix, and the reformation of acyl-CoA constitutes a carnitine shuttle that requires the concreted action of three proteins: carnitine palmitoyltransferase I (CPT I), carnitine/acylacarnitine translocase (CACT) and carnitine palmitoyltransferase II (CPT II). Mitochondrial oxidation may be either complete, generating acetyl-CoA, or incomplete with the development of ketone bodies (Nguyen *et al*., 2008; Kompare *et al*., 2008).

Regulation of fatty acid oxidation

Fatty acid oxidation in liver is transcriptionally regulated by two main systems under the control of either liver X receptors (LXRs) or PPARs. LXRs are ligand-activated transcription factors of the nuclear receptor superfamily. In the recent years, they have been considered as key regulators of lipid metabolism. In rodents, there are two isoforms, which form heterodimers with the retinoid X receptor (RXR) to activate their target genes (Baranowski, 2008). PPARs from heterodimers with RXRs regulate transcription of various genes. PPARα is highly expressed in the liver and regulates the expression of genes involved in mitochondrial and peroxisomal β-oxidation. Also, it increases transcription and

expression of proteins and enzymes necessary to catabolise fatty acids (Giby *et al.,* 2014).

3.5. Thermogenesis

Thermogenesis is defined, as the capacity to generate heat by metabolic processes. It is the principal function of BAT. The thermogenic process is managed by UCP1, a protein located in the inner mitochondrial membrane of brown adipocytes and whose principal activity is to dissipate the proton gradient generated by the respiratory chain by simplifying proton reentry into the mitochondrial matrix, circumventing ATP synthase (Bonet *et al.,* 2017).

As a result of FA oxidation, the Krebs cycle and the respiratory chain are switched on, generating a gradient of protons across the inner mitochondrial membrane that activates UCP1 thermogenesis (Kajimura *et al.,* 2014). In brown adipocytes, this proton gradient is dissipated through UCP1 and this energy is mainly liberated as heat, instead of generating ATP through ATP-synthase (Palou *et al.,* 1998; Cannon *et al.,* 2004). A crucial activator of UCP1 gene expression and function is adrenergic stimulation (Kozak *et al.,* 2008), which is mediated by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), a co-activator of PPARγ (Barbera *et al*., 2001). PGC1-α activation regulates oxidative metabolism increasing in a simultaneous way the mitochondrial function and transcriptional PPARγ activity.

Cold exposure is a key regulator of BAT thermogenesis that works by activating the sympathetic nervous system. “Cold-induced thermogenesis” is crucial for body temperature (Cannon *et al*. 2004; Townsend *et al*. 2012). In addition, adaptive thermogenesis can be activated by excessive caloric intake, it

is called “diet-induced thermogenesis” (Rothwell *et al.,* 1979; Rothwell *et al.,* 1997). For this reason, in addition to its thermoregulatory role, BAT has been linked to maintenance of energy balance and body weight control.

4. Bioactive molecules with potential anti-obesity effects

The most frequently used strategies for the treatment and prevention of obesity, such as energy restriction and increased physical activity, sometimes are not as efficient as expected. This fact, and the reduced number of pharmacological tools available in the market, increases the interest to search new alternatives to manage this disease. In recent years, natural bioactive molecules present in some vegetal foods have been discovered for their potential health benefits by reducing the risk of chronic disorders such as inflammatory diseases, cardiovascular diseases, diabetes, cancer and obesity (Meydani, *et al.,* 2010).

The development of functional foods for the prevention and treatment of obesity, acting on appetite, energy expenditure and metabolism, represent an opportunity for the research community. Functional compounds with anti-obesity potential include bioactive fatty acids, phenolic compounds, plant sterols, calcium, capsaicinoids and fiber among others (Trigueros *et al*., 2013). Some of them, concretely phenolic compounds and capsaicinoids will be revised here.

4.1. Phenolic compounds

Phenolic compounds are produced in plants in response to stress as a defence mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation (Bradamante *et al.,* 2004; Cucciolla *et al.,* 2007). More than 8000 molecules with different chemical structures and activities have been described (Harborne *et al*., 2000). We can found them in vegetables, seeds, fruits, nuts, red wine, tea, and many other food sources.

Structurally, phenolic compounds are secondary plant metabolites characterized by at least one aromatic ring with one or more hydroxyl groups attached (Figure 8), which confer important characteristics that determine their functional activities (i.e. the capacity to capture free radicals) (Zdunczyk *et al*., 2002). Plant phenolics are synthesized from carbohydrates via the shikimate and phenyl propanoid pathways, the position and number of the hydroxyl groups on the aromatic ring creates the variety.

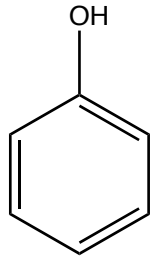


Figure 8. Phenol chemical structure.

Generally, phenolic compounds are found in conjugated form with one or more sugar moieties linked through OH groups (O-glycosides) or through carbon-carbon bonds (C-glycosides). The sugar bounds could be monosaccharide, disaccharides or even oligosaccharides, being glucose the most common. Other sugars found in these molecules are galactose, rhamnose, arabinose, xylose or glucuronic acid (Manach *et al*., 2004).

Phenolic compounds are divided into several classes, according to the number of phenol rings and to the structural element that binds these rings to one another. They are classified as flavonoids and non-flavonoids, which in turn have different subclasses as shown in Figure 9.

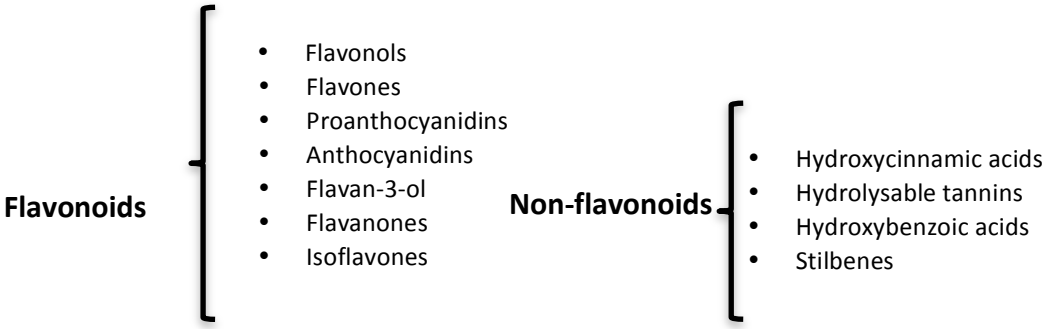


Figure 9. Phenolic compounds classification.

There is mounting evidence an accumulating number of studies that report physiological health effects of dietary polyphenols (Figure 10). These effects depend on both their respective intakes and their bioavailability, which can vary greatly (Manach *et al*., 2004).

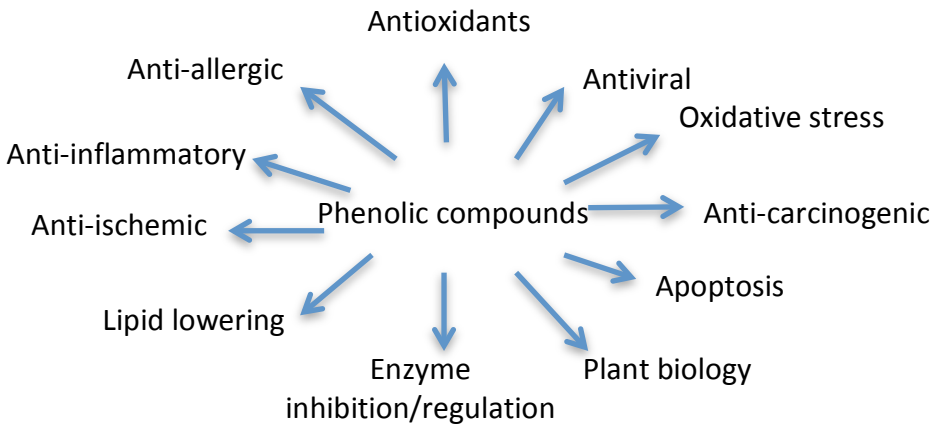


Figure 10. Phenolic compound activities (Modified from Ramos and Kampa *et al*., 2007).

Flavonoids

Flavonoids are important natural compounds with diverse biological activities. They are involved in many organoleptic properties of fruits and vegetables, such as the colour and flavour. It has been reported that they have anti-oxidant, anti-proliferative, anti-carcinogenic, anti-inflammatories, anti-hyperlipidemic and anti-hypertensive activities (Del Rio *et al*., 2013).

Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C_6-C_3-C_6 configuration. Basically, the structure consists of two aromatic rings, A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C (Figure 11). They have also been subdivided into subclasses, depending on the position of the B ring relative to the C ring, as well as the functional group and the presence or absence of a double bond in the C ring (Beecher, 2003).

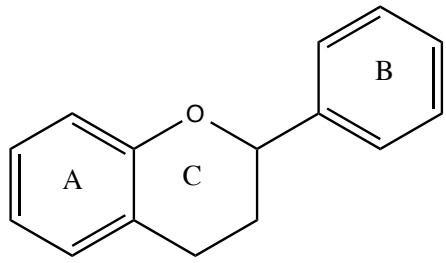
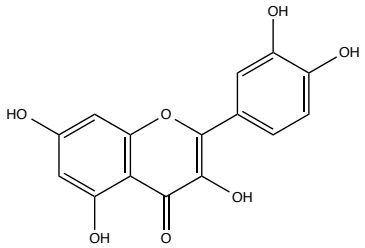
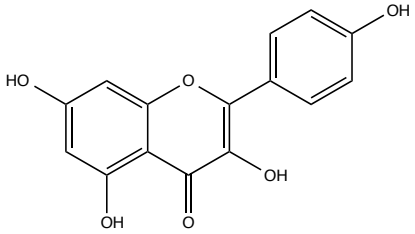
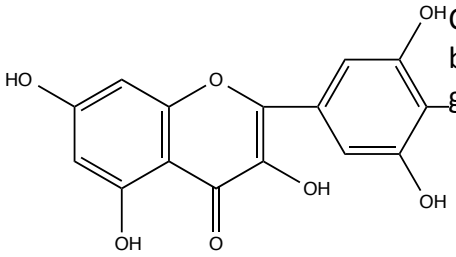
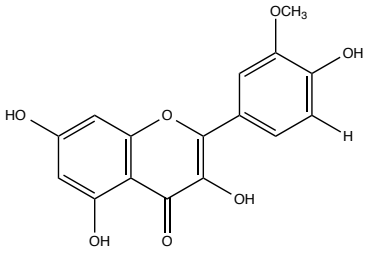
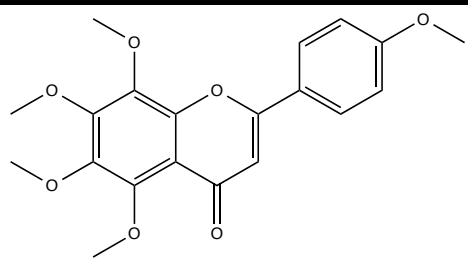
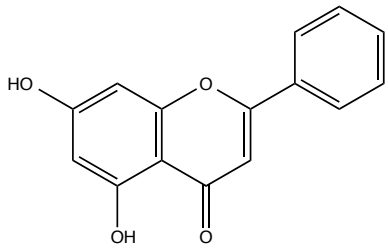
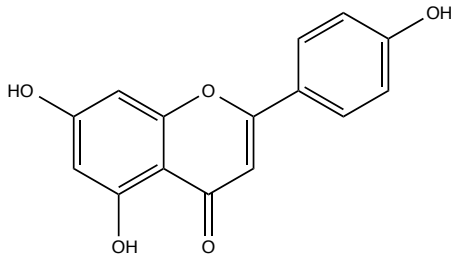
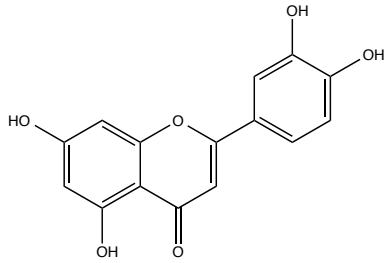


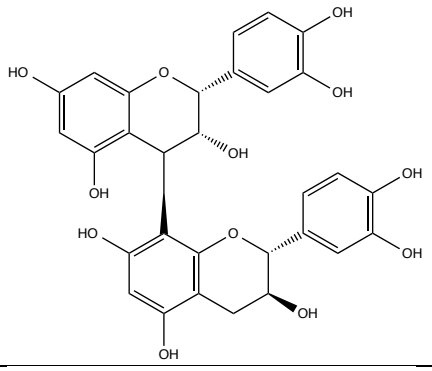
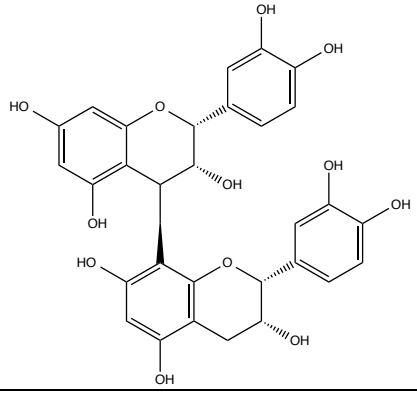
Figure 11. Flavonoid general chemical structure.

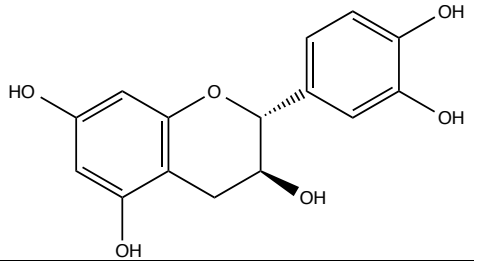
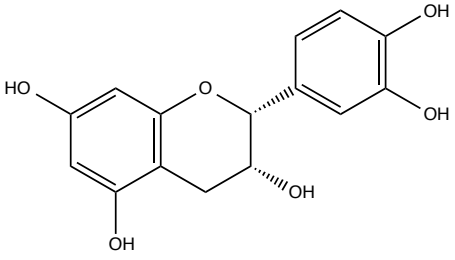
Flavonoids subclasses, chemical structures and food sources are summarised in table 11.

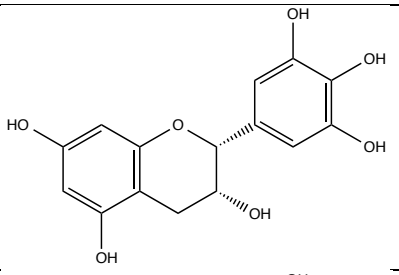
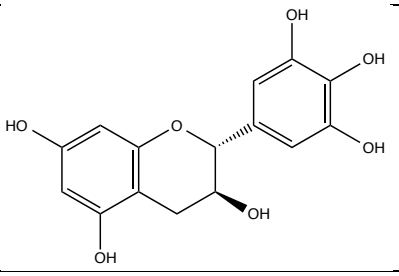
Table 11. Flavonoids subclasses, chemical structure and food sources (http://phenol-explorer.eu).

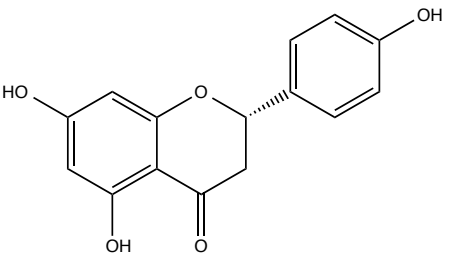
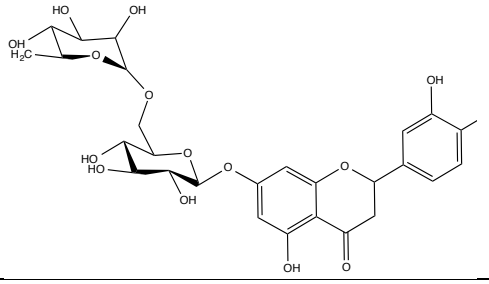
Flavonols	Quercetin		Red onions, apples, lettuce, berries, tomato, broccoli, green and black tea
	Kaempferol		Grapes, Brussels sprouts, apple, green and black tea, leek
	Myricetin		Cranberries, berries, red onion, grapes
	Isorhamnetin		Yellow onion, cherry tomato, green and black tea, cranberries

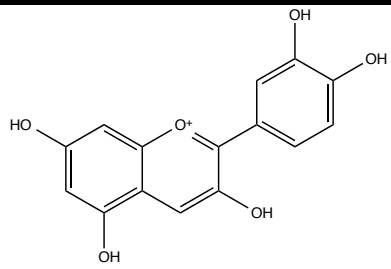
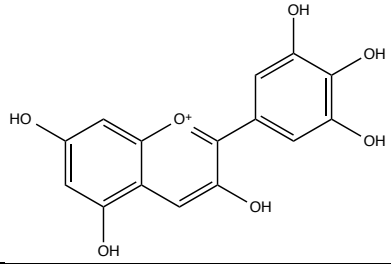
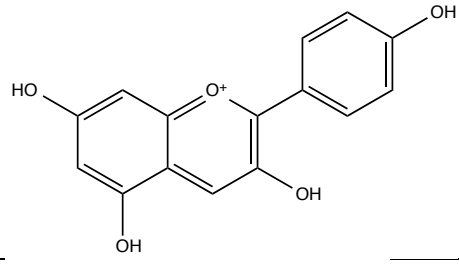
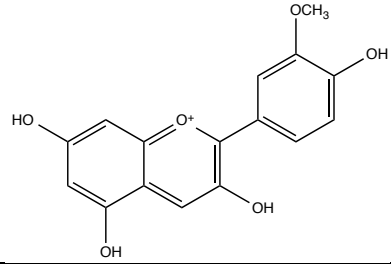
Group	Compound	Chemical structure	Food source
Flavones	Tangeretin		Green pepper, apple, onion, thyme, celery, parsley, grapefruit, tea, fruit peels
	Chrysin		
	Apigenin		
	Luteolin		

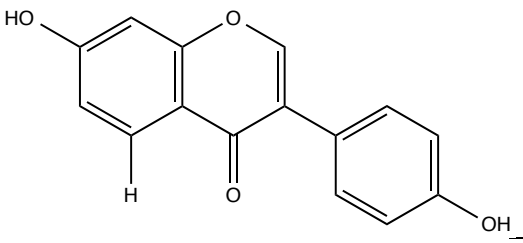
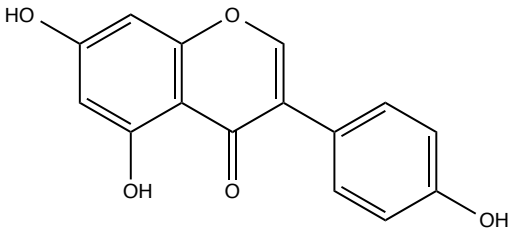
Group	Compound	Chemical structure	Food sources
Proanthocyanidins	Procyanidin B1		Grapes, cocoa, red wine
	Procyanidin B2		

Group	Compound	Chemical structure	Food sources
Flavan-3-ol	Catechin		Nuts, cocoa beans, grapes, berries, tea, wine
	Epicatechin		

	Epigalocatechin	
	Gallocatechin	

Group	Compound	Chemical structure	Food sources
Flavanones	Naringenin		Orange, lemon, grapefruit
	Hesperidin		

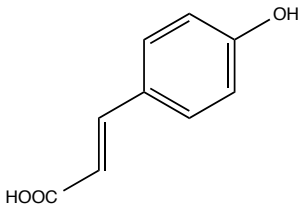
Group	Compound	Chemical structure	Food sources
Anthocyanidins	Cyanidin		Berries, blackberries, raspberries, cherries, cranberries, red corn, grapes, redcurrant
	Delphinidin		
	Pelargonidin		
	Peonidin		

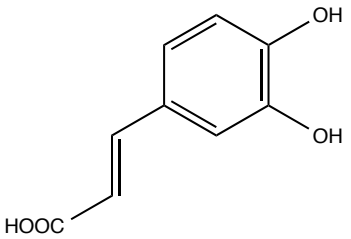
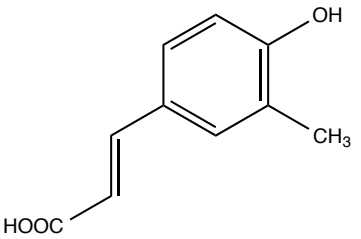
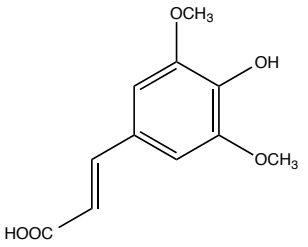
Group	Compound	Chemical structure	Food sources
Isoflavones	Daidzein		Soy, medical plants, coffee
	Genistein		

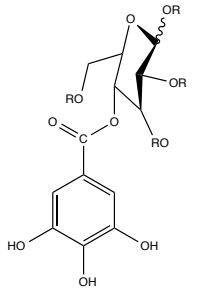
Non-flavonoids

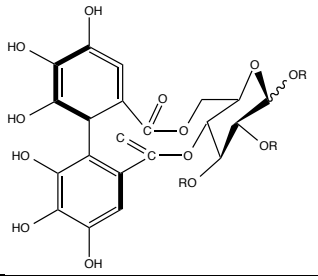
Non-flavonoids have been identified in plants, and several are found in edible plants, such as vegetables, fruits and seeds. In Table 12 non-flavonoids subclasses, chemical structure and food sources are shown.

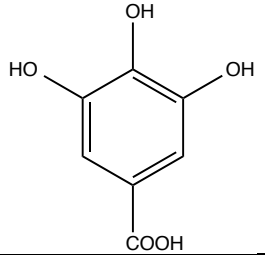
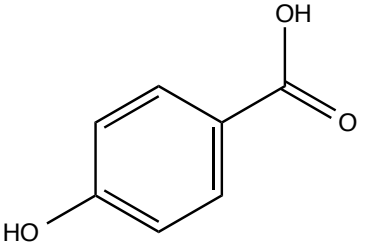
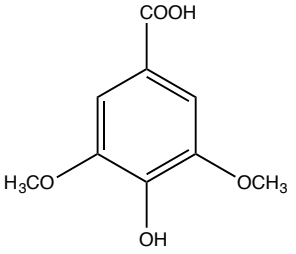
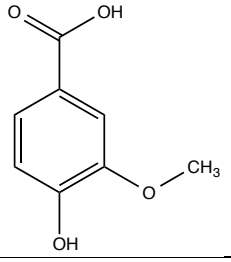
Table 12. Non-flavonoids subclasses, chemical structure and food sources

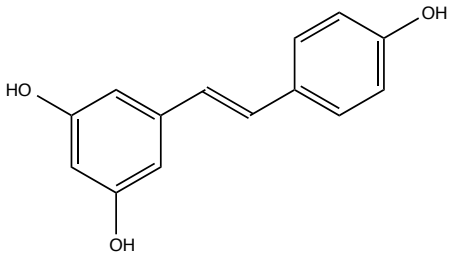
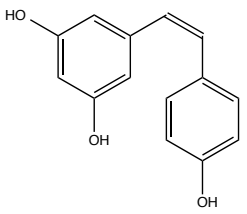
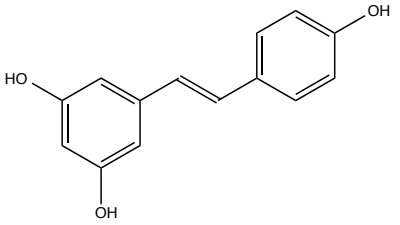
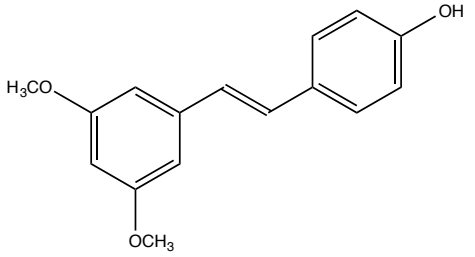
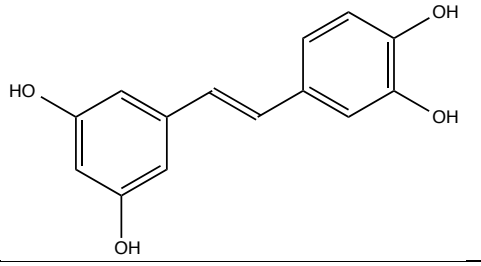
Group	Compound	Chemical structure	Food sources
Hydroxycinnamic acids	*p*-Coumaric		Lettuce, apple, artichoke, lemon, pineapple, plum, broccoli

	Caffeic acid	
	Ferulic acid	
	Sinapinic acid	

Group	Compound	Chemical structure	Food sources
Hydrolysable tannins	Gallotannin		Raspberry, pomegranate, raspberries, blackberries, nuts, acorn

	Ellagitannins	

Group	Compound	Chemical structure	Food sources
Hydroxybenzoic acids	Gallic acid		Cherries, melon, grapes, redcurrant, raspberries, blackberries, raspberries, plum
	p-hydroxybenzoic		
	Syringic		
	Vanillic acid		

Group	Compound	Chemical structure	Food sources
Stilbenes	Resveratrol		Grapes, red wine, peanuts, pistachio
		 *cis*-resveratrol	
		 *trans*-resveratrol	
	Pterostilbene		
	Piceatannol		

4.2. Hesperidin

Hesperidin was first isolated in 1828 by the chemist Lebreton from the spongy inner portion of the oranges peel (albedo) (Manthey and Grohmann, 1988). It is the major flavanone present in citrus fruit and has been under continuous investigation since it was discovered (Fluckiger and Hanbury, 1986; Barthe *et al*., 1988).

4.2.1. Structure

Hesperidin (3',5,7-trihydroxy-4'-methoxy-flavanone-7-rhamnglucoside), a flavanone glycoside, belonging the group of flavonoids, with the molecular formula $C_{28}H_{34}O_{15,}$ is the food-bound from hesperitin (Figure 12) and is one of the molecules which were erroneously named "Vitamin P" (Garg *et al*., 2001).

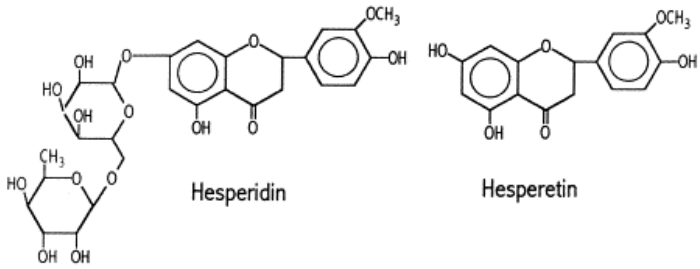


Figure 12. Hesperitin and hesperidin chemical structure.

4.2.2. Food sources and intake

In addition to citrus fruits, several food sources contain different amounts of hesperidin, as it is described in the following table (Table 13).

Table 13. Food sources and content of Hesperidin (http://phenol-explorer.eu).

Food source	Mean content (mg/100g)
Dry peppermint	480.65
Orange blond juice (from concentrated)	52.68
Orange blood juice (from concentrated)	51.30
Orange blood (pure juice)	43.61
Tangerine juice (from concentrated)	36.11
Orange blond (pure juice)	25.85
Lemon juice (from concentrated)	24.99
Lemon juice	17.81
Lime pure juice	13.41
Grapefruit juice (from concentrated)	1.55
Grapefruit juice (pure juice)	0.65
Welsh onion (fresh)	0.02

The intake of phenolic compounds depends on dietary habits, availability and preferences. To date, limited data exist on intake of phenolic compounds in populations. In 2016, Zamora-Ros *et al.,* carried out a cross-sectional analysis aimed to estimate dietary intakes of all currently known polyphenols and total intake in 10 European countries (Denmark, France, Germany, Greece, Italy, Norway, Spain, Sweden, the Netherlands, and the UK). The average polyphenol consumption in Mediterranean countries was 449 mg/day. Also, it was analysed the flavanones percentage intake, and the results were 3.3% and 3.0% men and woman respectively. Specifically, hesperidin intake in Europe population is 27 mg/day (Zamora-Ros *et al.,* 2016).

4.2.3. Bioavailability

Phenolic compounds are highly metabolized via a common pathway. After absorption, they are conjugated in the phase II metabolism, in small intestine and liver. Those phenolic compounds not absorbed reach the colon and are either excreted in faeces or metabolized by microbiota. Phenolic compounds that are absorbed, and then metabolized by phase II enzymes are more hydrophilic and subsequently they are excreted in urine.

Also, phenolic compounds that are absorbed, metabolized in the liver and excreted in the bile or directly from the enterocyte back to the small intestine will also reach the colon in a different chemical form, such as glucoronide (Manach *et al*., 2004; Kumar *et al.,* 2013; Scalbert *et al*., 2000) (Figure 13).

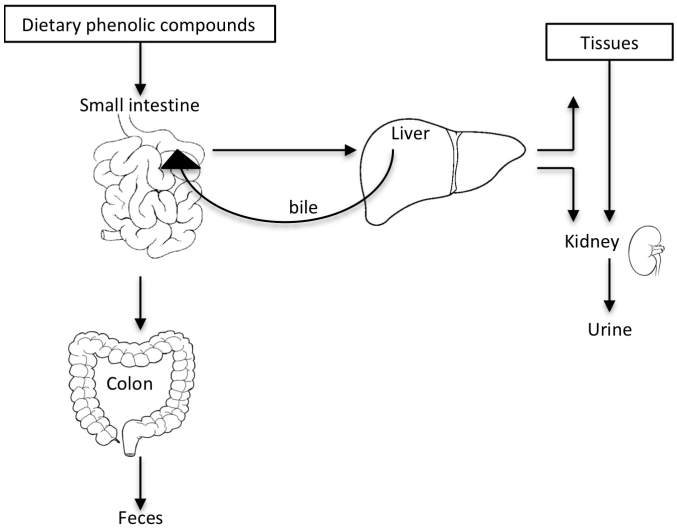


Figure 13. Possible routes for ingested phenolic compounds in humans (Modified from Scalbert A., *et al*., 2000).

Focusing on hesperidin, it is known that it has a limited bioavailability (Erlund *et al.,* 2001; Kanaze *et al*., 2007; Ross *et al*., 2002) due to a low water solubility and disposition via phase II conjugating enzymes (UDP-glucuronosyltransferases, sulfotransferases, N-acetyltransferases, glutathione S-transferases and methyltransferases) (Jancova *et al*., 2010; Yamada *et al*., 2006; Jeong, *et al*., 2005; Li *et al.,* 2008). Furthermore, hesperidin must pass onto the colon and be fermented by intestinal microbiota to an alternate form that is more readily absorbed (Kim *et al*., 1998; Lee *et al.,* 2004). Although hesperidin is poorly absorbed and rapidly eliminated, it has a reasonable half-life of 6 hours, which is likely due to the prolonged absorption phase (Chaoyun Li and Hermann Schluesene, 2015).

4.2.4. Health benefits

In the past decade, several *in vivo* and *in vitro* studies have been performed to evaluate the effects of hesperidin. It has been proposed that hesperidin could be effective in treating a great variety of diseases, such as diabetes, hypertension, hypercholesterolemia and fatty liver disease, Alzheimer's disease and rheumatoid arthritis. Specific information has been summarized in Table 14.

In addition, several authors have shown positive effects of hesperidin against obesity-related alterations, mainly due to its TG-lowering effects (Assini *et al*., 2013). As an example, Bok *et al.,* found that dietary supplementation of hesperidin and naringin lowered hepatic serum cholesterol levels via the inhibition of 3-hydroxy-3methyl-glutary-coenzyme A (HMG-CoA) and acyl coenzyme A cholesterol O-acyltransferase (ACAT) activities in rats fed a high-

cholesterol diet (Bok *et al.,* 1999). However, the mechanisms by which hesperidin could affect fat accumulation and TG metabolism need further research.

Table 14. Effects of Hesperidin.

Disease	Experimental model	Dose	Results	Reference
Diabetes	White male albino rats High-fat diet Streptozotocin treatment	50 mg/kg body weight	Attenuates hyperglycaemia-mediated oxidative stress and proinflammatory cytokine production	Mahmoud *et al.,* 2012.
	Male C57BL/KsJ-db/db mice	0.2 g/kg diet	Prevents the progression of hyperglycaemia by increasing hepatic glycolysis and glycogen concentration and/or by lowering hepatic gluconeogenesis	Jung *et al*., 2004.
Hypertension	Male spontaneously hypertensive rats (SHRs)	10, 30 and 50 mg/kg body weight	Lowers blood pressure	Yamamoto *et al*., 2008.
Hypercholesterolemia and fatty liver	Male Wistar rats High- cholesterol diet	20 mg/kg body weight	Improves hypercholesterolemia and fatty liver by inhibiting both the synthesis and absorption of cholesterol and	Wang *et al*., 2011.

			regulating the expression of mRNA for RBP, C-FABP, and H-FABP	
Alzheimer's disease	PC12 rat pheochromocytoma cell line $A\beta_{25-35}$ treatment	10, 25 and 50 µM	Protects against Aβ- induced neurotoxicity via VDAC1-regulated mitochondrial apoptotic pathway	Wang *et al*., 2013.
Rheumatoid arthritis	Male Wistar rats Collagen treatment	160 mg/kg body weight	Inhibits collagen-induced arthritis through suppression of free radical load and reduction in neutrophil activation and infiltration	Umar *et al*., 2013.

Modified from Li and Schluesener, 2015.

4.3. Capsaicinoids and capsaicin

Capsaicinoids are a group of molecules distinctive in fruits and plants from the genus *Capsicum* found in chilli peppers. They are responsible for the pungent sensation, which occurs when they bind to the same group of nociceptors that also leads to the sensation of pain from heat and acid (Tanaka *et al.,* 2009). Capsaicinoids display potentially valuable pharmacological and bioactive properties (Thiele *et al.,* 2008).

The basic chemical structure of capsaicinoids is an acid amide of vanillylamine combined with a fatty acid (Aza-Gonzalez *et al.,* 2011). It is known

that more than 10 structures exist, nevertheless, the most prominent forms are capsaicin and dihydrocapsaicin, accounting for almost 90% of capsaicinoids (Figure 14). (Meghvansi *et al.,* 2010). An independent group of compounds named capsinoids (naturally occurring from the pepper CH-19 sweet) have also been subject of different research trials and seems to have similar effects to capsaicinoids but without the pungency (Hursel *et al.,* 2010). The fundamental structure of capsinoids is an ester of vanillyl alcohol with a fatty acid.

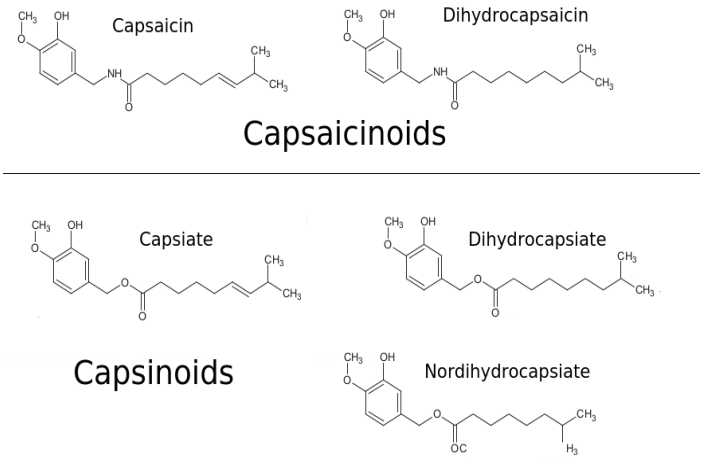


Figure 14. Basic capsaicinoids and capsinoids chemical structure.

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is the main compound in plants from the genus *Capsicum* and is responsible for their unique potency upon ingestion. In addition to it use as a major spice and food additive in most cuisines around the world, capsaicin has been also used for its medicinal and therapeutic potential in human health since the 16th century (Bode and Dong, 2011).

4.3.1. Structure

Capsaicin is a naturally occurring alkaloid. The molecular formula for capsaicin is $C_{18}H_{27}NO_3$ (Figure 15). At room temperature, pure capsaicin is a highly volatile, pungent, hydrophobic, and odourless white crystalline powder. It is synthesized in the chilli pepper by addition of a branched-chain fatty acid to vanillylamine (Fujiwake *et al*., 1979). Commercially, it is manufactured by the reaction of vanillylamine with 7-methyloct-5-ene- 1-carboxylic acid chloride or isolated from paprika or obtained by grinding dried ripe fruits of *Capsicum frutescens L.* (chilli peppers) into a fine powder. The formulation types registered are dry powder, liquid formulation and liquid spray ground. Recently, it has been reported that the production of capsaicin can also take place using the fungus Alternaria alternata (Devari *et al*., 2014). Capsaicin binds to the vanilloid receptor with the highest affinity, making it the most pungent and potent agent in the capsicum family (Reilly and Yost 2006).

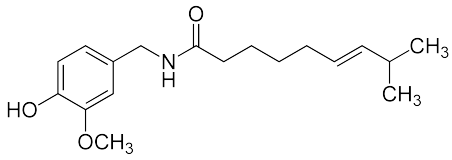


Figure 15. Capsaicin chemical structure.

4.3.2. Food sources and intake

Capsaicin contents of diverse peppers range from 0.1 to 4.25 mg/g (Al Othman *et al.,* 2011). In 1912, the pharmacist Scoville created the Scoville scale to measure the pungency of chilli peppers reported in Scoville heat units (SHU) (Gmyrek, 2013). The quantity of units is different depending on the type of

peppers as shows in the Table 15. The chilli pepper plant is one of the most widely grown spices in the world (Kim *et al*., 2014).

Table 15. Summary of the Scoville scale.

Pepper type	Scoville heat units
Pure Capsaicin	15,000,000
Naga Jolokia	>1,000,000
Habanero /Scotch Bonnet	100,000-350,000
Thai Green	50,000-100,000
Tabasco	30,000-50,000
Chipotle	5,000-10,000
Jalapeño pepper	2,500-8,000
Bell pepper	0

The global consumption of capsaicin is not well documented in the literature. It is particularly consumed through food-products and spices at high levels in certain areas of the world, particularly Mexico, South America, Ethiopia, India, Indonesia, Korea, Laos, Malaysia, Pakistan, Southwest China, Sri Lanka and Thailand (López-Carrillo, *et al.,* 1994; Perry, Dickau *et al.,* 2007; Al Othman *et al.,* 2011; Bode and Dong 2011).

Orally consumed capsaicin, usually in the form of chilli pepper extracts or supplements, has been commercially used as a weight management tool

through appetite suppression and activation of the thermoregulatory system (Chaiyasit *et al.,* 2009).

4.3.3. Bioavailability

Nearly all the research focusing on the bioavailability and metabolism of capsaicin is limited to animal studies. In rodents, orally administered capsaicin is absorbed in the stomach and undergoes the first pass effect reducing its bioavailability (Reily and Yost, 2006). It is transported to the portal vein through the gastrointestinal (GI) tract by a non-active process and it is partially metabolized during absorption (Reily and Yost, 2006). The total absorption capacity is around 50-90% (Leelahuta *et al.,* 1983; Kawada *et al*., 1984; Donnerer et al., 1990). In the liver, capsaicin undertakes some modifications by P450 enzymes to decrease their ability to activate the transient receptor potential vanilloid type-1 (TRPV1) receptors, thus reducing their pharmacological and toxicological potency. Furthermore, capsaicin is believed to be metabolized by dehydrogenation, producing unique macrocyclic, -diene, and -imide metabolites. The metabolism is mediated by the following enzymes: cytochrome P 1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 (Reilly *et al*., 2003; Sharma *et al*., 2013).

Human bioavailability studies have found that subjects had serum concentrations of capsaicin ranging from 13.4-16.3 ng/mL after consuming a chilli meal (Hartley *et al.,* 2013). Whether the bioavailability is enough to achieve therapeutic effectiveness is not clear. Thus, this issue needs further investigation.

4.3.4. Health benefits

The effects of capsaicin on human body have been studied for more than a century. In 1878, for the first time was observed the burning sensation and hyperemia produced by an extract of Capsicum when applied on human skin (Toh *et al.,* 1955). Later on, numerous studies revealed that capsaicin was an exciting pharmacological agent useful in different clinical conditions (Table 16).

Table 16. Beneficial effects of capsaicin.

Role of capsaicin	Administration way	Beneficial effects
Pain	Dermal patch	Reduces neuropathic pain (Maihofner and Heskamp, 2013)
Body weight	Diet	Increases diet-induced thermogenesis and lipid oxidation (Yoshioka *et al*., 1995, 1998), increases lipid oxidation (Josse *et al*., 2010; Lee *et al.,* 2011; Lejeune *et al.,* 2003), decreases appetite (Ludy and Mattes, 2011), and decreases serum triglyceride level in genetically obese mice (Kang *et al*., 2011)
Cancer	Diet	Anti-cancer effects in pancreas (Zhang *et al*., 2013), stomach (Wang *et al*., 2011), colon (Lu *et al*., 2010), breast (de-Sa-Junior *et al.,* 2013), prostate (Mori *et al*., 2006), lung (Anandakumar *et al*., 2012), leukaemia (Tsou *et al*., 2006), and liver (Moon *et al*., 2012)
Cardiovascular system	Diet	Smaller infarct sizes (Wang, 2005), protection against reperfusion injury (Sexton *et al.,* 2007), and platelet anti-aggregating effect (Adams *et al.,* 2009; Mittelstadt *et al*., 2012; Raghavendra and Naidu, 2009; Sylvester and LaHann, 1989)

Gastrointestinal system	Diet	Increases absorptive surface of small intestine (Prakash and Srinivasan, 2010)
Urinary bladder	Intravesicle instillation	Painful bladder syndrome, hyperactive bladder (Cruz *et al.,* 1997)
Dermatological conditions	Topical	Psoriasis (Yu, 2011), and histamine mediated itch (Sekine *et al.,* 2012)

Modified from Sharma S.K., *et al.,* 2013.

Evidence suggests that capsaicin decreases body weight by increasing energy expenditure (Kawada *et al.,* 1986; Watanabe *et al.,* 1987; Sherriffs *et al.,* 2010; Lee *et al.,* 2010; Ludy and Mattes 2011), stimulating adipose tissue lipid mobilization and fat oxidation (Kawada *et al.,* 1986; Lejeune *et al*., 2003; Sherriffs *et al*. 2010; Lee *et al.,* 2010), and reducing energy intake (Watanabe *et al.,* 1987; Yoshioka *et al.,* 1999; Westerterp-Plantenga *et al.,* 2005; Ludy and Mattes 2011). However, its mechanisms of action have not been completely described.

Capsaicin has the potential to regulate metabolism via activation of TRPV1 receptors, which belong to the family of non-selective cation channels with high calcium permeability. These receptors are found not only on nociceptive sensory neurons, but also in a range of other cells (endothelial cells, hepatocytes, adipocytes, smooth muscle cells, fibroblasts, various epithelia, T cells, mast cells and astrocytes in the brain and spinal column (Gunthorpe and Szallasi, 2008). TRPV1 activation induces calcium influx, and in certain tissues this is associated with increased activation or expression of key proteins such as uncoupling protein 2 (UCP2), endothelial nitric oxide synthase (eNOS), Kruppel

Like Factor 2(KLF2), PPARδ, PPARγ, and liver X receptor α (LXRα) (McCarty *et al.,* 2015). Nevertheless, the mechanism of action is not presently fully understood.

In general, the basis of obesity treatment is energy restriction and increased physical activity. But, in several cases these treatments are not efficient enough to reach their goal. For this reason, the scientific community is been searching for new molecules (dietary ingredients or drugs), which could be effective in avoiding excessive body fat accumulation and its associated complications.

The aim of the present Doctoral Thesis was to analyse potential interesting effects of bioactive molecules, mainly phenolic compounds on obesity and several co-morbilities. For this propuse, two different approaches were proposed. First, carried out using cellular (*in vitro* studies) performed at the Department of Pharmacy and Food Science of the Basque Country University, and secondly using animal models (*in vivo* studies) took place at the University of the Balearic Islands.

The specific objectives of each of these studies are described below.

SPECIFIC OBJECTIVES

The specific objectives of this experimental work were:

In vitro study

1. To analyse the anti-adipogenic effects of fifteen phenolic compounds belonging to various chemical groups, on adipogenesis in 3T3-L1 adipocytes **(Manuscript 1)**

2. To determine the mechanism of action of the most active ones by measuring their effects on the main genes involved in adipogenesis regulation **(Manuscript 1)**

In vivo study

1. To assess the potential effects of hesperidin and capsaicin, individually and in combination, to prevent the development of obesity and its related metabolic alterations in rats fed an obesogenic diet **(Manuscript 2)**

2. To analyse whether Hesperidin, Capsaicin or a combination of these compounds would induce white adipose tissue browning in rats feed with obesogenic diet **(Manuscript 3)**

1. *In vitro* study

An in vitro study in 3T3-L1 pre-adipocytes supplied by American Type Culture Collection (Manassas, VA, USA) was performed. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS). Two days after confluence (day 0), the cells were stimulated to differentiate with DMEM containing 10% FBS, 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 µM dexamethasone for 2 days. On day 2, the differentiation medium was replaced by FBS/DMEM medium (10%) containing 0.2 µg/mL insulin. This medium was changed every two days until cells were harvested (day 8). All media contained 1% Penicillin/Streptomycin (10,000 U/mL), and the medium for differentiation contained 1% (v/v) of Biotin and Panthothenic Acid. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

In a first experiment (Figure 16), pre-adipocytes grown in 6-well plates were incubated with the following phenolic compounds: apigenin, catechin, daidzein, epicatechin, epigallocatechin, genistein, vanillic acid, hesperidin, kaempferol, luteolin, naringenin, piceatannol, pterostilbene, quercetin and resveratrol, all of them at 25 µM (diluted in 95% ethanol), during the adipogenic stage, from day 0 to day 8 of differentiation. Control cells received the same amount of ethanol dilution without phenolic compounds. The medium was changed every 2 days. On day 8, cells were harvested, culture supernatant was removed and cells were used for triacylglycerol determination and RNA extraction. Each experiment was performed in triplicate.

In a second experiment (Figure 17), those phenolic compounds that showed a significant anti-adipogenic activity at 25µM (apigenin, luteolin,

genistein, daizein, naringenin, hesperidin, quercetin, kaempferol, piceatannol, pterostilbene, resveratrol and vanillic acid) were selected for new treatments at lower doses (1 and 10 µM). Incubation conditions were exactly the same as in experiment 1. Each experiment was performed in triplicate.

In order to analyse the influence of the effective phenolic compounds at 25 µM on the regulation of adipogenesis a third experiment was carried out. In general terms, two stages of adipogenesis can be distinguished: eary stage of differentiation: 60 hours post-confluence and the post-mitotic stage (late stage).

To asses the effects of luteolin, hesperidin, kaempferol, pterostilbene, genistein and vanilic acid at 25 µM in early and late stage of adipogenic differentiation, 3T3-L1 cells were incubated in 6-well plates with each phenolic compound. From day 0 to 60 hours after the induction of differentiation to elucidate the effects of those phenolic compounds on the early stage and the effect on late stage was measured by incubating cells from 60 hours after differentiation to day 8. Control cells received the same amount of ethanol dilution as treated cells, without polyphenols. Incubation media was changed every two days. Each experiment was performed in triplicate.

Experimental design

3T3-L1 ADIPOCYTES

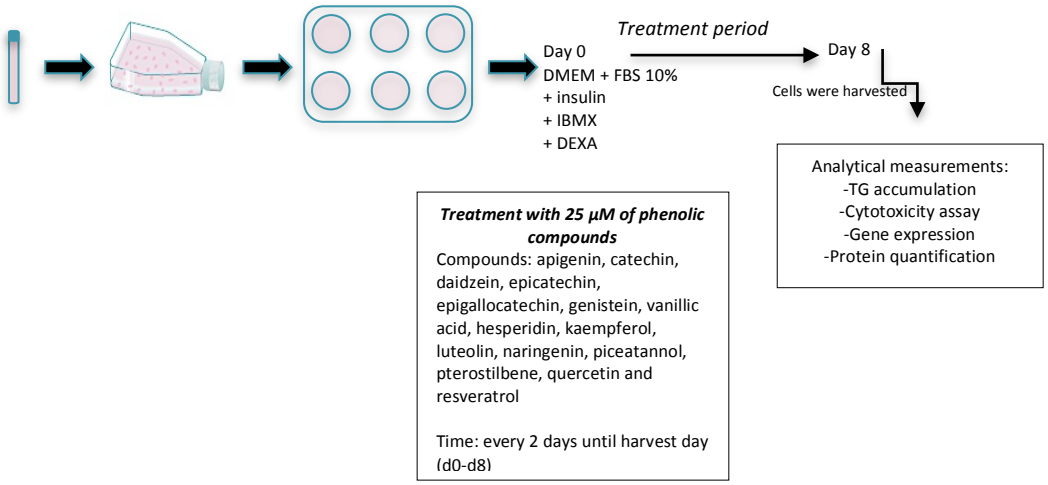


Figure 16. Schematic diagram of experiment 1.

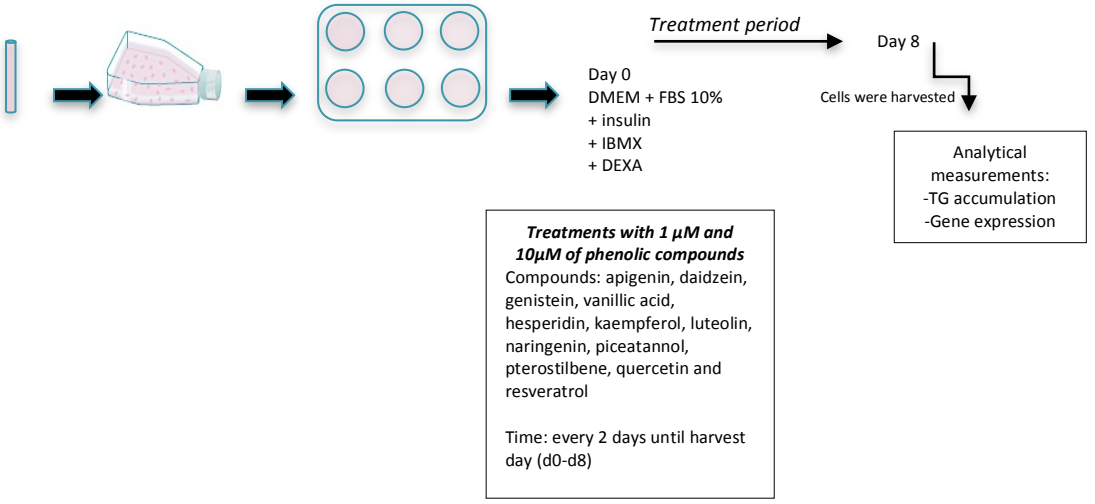


Figure 17. Schematic diagram of experiment 2.

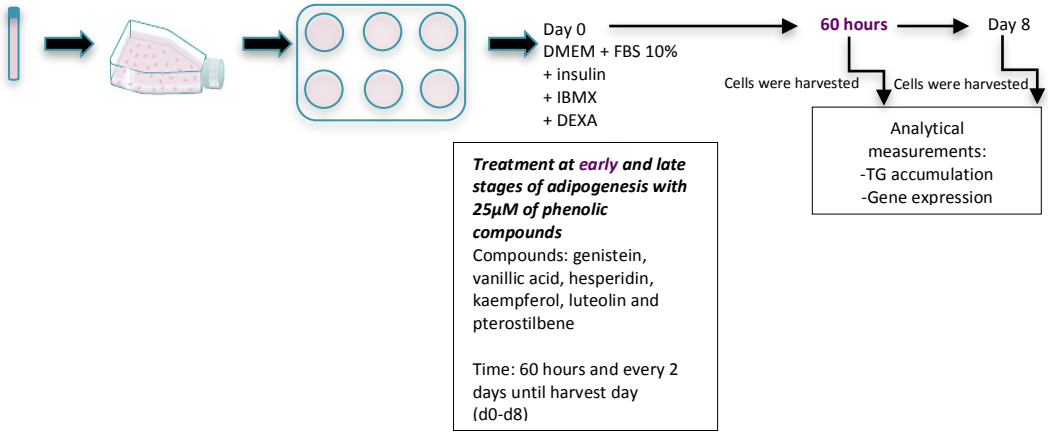


Figure 18. Schematic diagram of experiment 3.

In this experiment, the following analyses were carried out:

- Triglyceride content was quantified in maturing pre-adipocytes and mature adipocytes.
- Cell viability was assessed by using the neutral red assay (TOX4 kit).
- Gene expression: CCAAT/enhancer-binding protein beta *(c/ebpβ)*, CCAAT/enhancer-binding protein beta *(c/ebpα)*, peroxisome proliferator activated receptor gamma (*pparγ)*, sterol regulatory element binding transcription factor 1c (*srebp1c)*, lipoprotein lipase (*lpl)* and acetyl-CoA carboxylase *(acc)* were quantified using Real-Time PCR.
- Protein expression: PPARγ was measured by Western Blotting in 3T3-L1 maturing adipocytes.

The detailed protocols are included in manuscript 1.

***In vivo* Study**

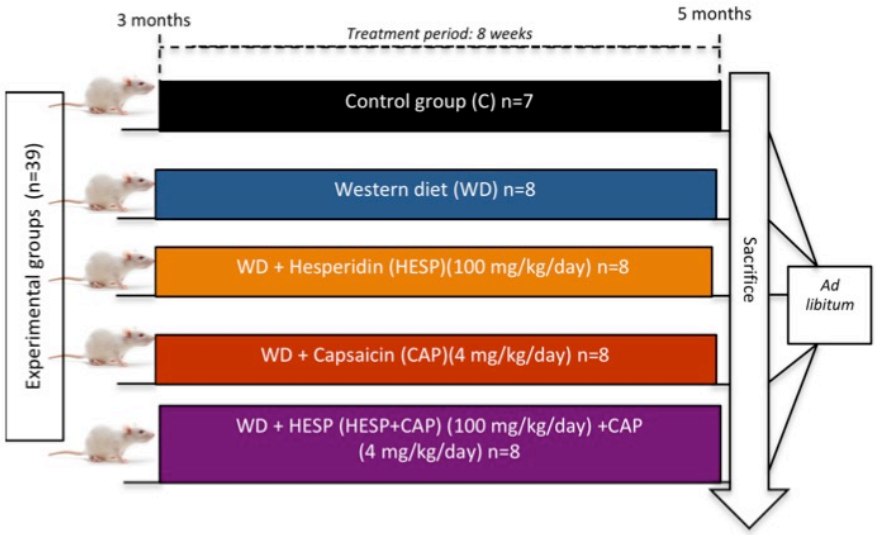


Figure 19. Diagram of the *in vivo* experimental design.

To fulfil our objectives, we design an *in vivo* experiment (Figure 19), in which 39 three-months-old male Wistar rats randomly divided into 5 groups. Control (n=7): animals fed with a standard chow diet; WD (n=8): animals fed with a high-fat, high-sucrose diet ("western diet), HESP (n=8): animals fed a western diet and treated with hesperidin (100 mg/kg body weight/day); CAP (n=8): animals fed a western diet and treated with capsaicin (4 mg/kg body weight/day), and Hesp+Cap (n=8): animals fed a western diet and treated with the combination of hesperidin (100 mg/kg body weight/day) and capsaicin (4 mg/kg body weight/day). All the treatments were administered by gavage. The animals were kept with this treatment for 8 weeks, until they were aged 5 month-old. All rats were individually housed under controlled temperature (22°C) and a 12 hours light dark cycle. They had unlimited access to tap water and a Western Diet (4.7 Kcal/g, with 17% Kcal from protein, 43% Kcal from

carbohydrate, and 41% Kcal from fat) (Research Diets, Inn, New Brunswick, NJ, USA) or a Standard Diet (3.3 Kcal/g, with 19 % Kcal from protein, 73 % Kcal from carbohydrate, and 8% Kcal from fat) (Pan-lab, Barcelona, Spain). The animal protocol followed was approved by the Bioethical Committee of the University of the Balearic Islands (Resolution Number 3513. March, 2012) and guidelines for the use and care of laboratory animals of the University were followed.

At baseline and every 2 weeks until the end of the treatment (week 8), body weight and body composition (EchoMRI-700TM, Echo Medical Systems, LLC., TX, USA) were measured. Blood samples were obtained after 12 hours of fasting from saphenous vein on week 7 and also under feeding conditions (truncal blood) at sacrifice. Blood samples were collected in heparinized containers, then centrifuged at 1000 x g for 10 min to obtain the plasma, and stored at -20°C until analysis. After 8 weeks of treatment animals were sacrificed by decapitation under feeding conditions. Retroperitoneal white adipose tissue (rWAT), inguinal white adipose tissue (iWAT) and liver were rapidly removed, weighted, frozen in liquid nitrogen, and stored at -80°C until further studies. Other fat depots (mesenteric and gonadal) were also dissected and weighted.

In this experiment the following analyses were carried out:

- Indirect calorimetry and locomotive activity was measured by using the LabMaster-CalSys-Calorimetry System (TSE Systems, Bad Homburg, Germany).
- Systolic blood pressure (SBP) was measured using a tail-cuff sphygmomanometer with a photoelectric sensor (Niprem 546,Cibertec

S.A., Spain) and Niprem software V1.8 was used to establish the SBP value.

- Circulating parameters (glucose, insulin, leptin, non-esterified fatty acids (NEFA), triglycerides and cholesterol) were measured with specific commercial kits.
- Hepatic lipid content was quantified by the Folch's method.
- Histological analysis in liver and adipose tissue samples was carried out by fixing them by immersion in 4% paraformaldehyde and embedding them in paraffin blocks for light microscopy. Liver sections were classified into four grades depending on fat accumulation following Burnt's classification.
- Immunostaining in rWAT for uncoupling protein 1 (UCP1) and cell death-inducing DNA fragmentation factor, alpha subunit- like effector A (CIDE-A).
- The area of white adipocytes was measured in hematoxylin/eosin-stained sections of rWAT and iWAT depots and the images from light microscopy were digitized and the area was determined using Axio Vision software.

Gene expression in liver: carnitine palmitoyltransferase 1a, liver (*cpt1a*), fatty acid synthase (*fasn*), fibroblast growth factor 21 (*fgf21*), glyceraldehyde 3-phosphate dehydrogenase (*gadph*), insulin Receptor (*Insr),* insulin receptor substrate 1 (*irs1*), leptin receptor (*lepr*), pyruvate kinase (*pirK*), transient receptor potential cation channel, peroxisome proliferator activated receptor alpha (*pparα*), sterol coenzyme A desaturase (*scd1*), sterol regulatory element binding transcription factor 1 (*srebf1*), transient receptor potential cation

channel subfamily V member 1 (*trpv1*) and uncoupling protein 2 (*ucp2*) were quantified using Real-Time quantitative PCR.

- Gene expression in rWAT: adipose triglyceride lipase (*atgl*), cluster of differentiation 36 (*Cd36)*, Cell death-inducing DNA fragmentation factor, alpha subunit- like effector A *(cide-a),* carnitine palmitoyltransferase 1b, muscle isoform (*cpt1b*), fatty acid synthase (*fasn*), glucose transporter 4 (*glut4*), glycerol-3-phosphate acyltransferase (*gpat*), hexokinase II (*hk*), *homebox C9 (hoxc9)*, hormone-sensitive lipase (*hsl*), insulin Receptor (*Insr*), lipoprotein lipase (*lpl*), peroxisome proliferator-activated receptor c coactivator 1 *(pgc1α)*, peroxisome proliferator activated receptor gamma (*pparγ*), PRD1-BF1-RIZ1 homologous domain-containing protein-16 (*prdm16*), sterol regulatory element binding transcription factor 1 (*srebf1*) and uncoupling protein 1 (*ucp1)* were quantified using Real-Time quantitative PCR.
- Gene expression in iWAT: Cell death-inducing DNA fragmentation factor, alpha subunit- like effector A *(cide-a)*, *homebox C9 (hoxc9),* peroxisome proliferator-activated receptor c coactivator 1 *(pgc1α)*, PRD1-BF1-RIZ1 homologous domain-containing protein-16 (*prdm16*), transient receptor potential cation channel subfamily V member 1 (*trpv1*) and uncoupling protein 1 (*ucp1)* were quantified using Real-Time PCR.
- Protein expression in BAT: UCP1 was determined by western blot.
- Protein expression in iWAT: CIDE-A was determined by western blot.

The detailed protocols are included in manuscripts 2 and 3.

Manuscript 1

Screening of potential anti-adipogenic effects of phenolic compounds showing different chemical structure in 3T3-L1 preadipocytes

Andrea Mosqueda-Solís, Arrate Lasa, Saioa Gómez-Zorita, Itziar Eseberri, Catalina Picó, María P. Portillo

Food and Function, 2017 Oct; 8(10): 3576-86

http://pubs.rsc.org/en/Content/ArticleLanding/2017/FO/C7FO00679A#!divAbstract

Food & Function

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c7fo00679a

Screening of potential anti-adipogenic effects of phenolic compounds showing different chemical structure in 3T3-L1 preadipocytes†

Andrea Mosqueda-Solís,[a] Arrate Lasa,[a,c] Saioa Gómez-Zorita,[a,c] Itziar Eseberri,[a,c] Catalina Picó[b,c] and María P. Portillo*[a,c]

This study was designed to analyze the anti-adipogenic effect of fifteen phenolic compounds from various chemical groups in 3T3-L1 pre-adipocytes. Cells were treated with 25 μM, 10 μM or 1 μM of apigenin, luteolin, catechin, epicatechin, epigallocatechin, genistein, daizein, naringenin, hesperidin, quercetin, kaempferol, resveratrol, vanillic acid, piceatannol and pterostilbene for 8 days. At 25 μM lipid accumulation was reduced by all the compounds, with the exception of catechin, epicatechin and epigallocatechin. At a dose of 10 μM apigenin, luteolin, naringenin, hesperidin, quercetin and kaempferol induced significant reductions, and at 1 μM only naringenin, hesperidin and quercetin were effective. The expression of *c/ebpα* was not. *C/ebpβ* was significantly reduced by genistein and kaempferol, *pparγ* by genistein and pterostilbene, *srebp1c* by luteolin, genistein, hesperidin, kaempferol, pterostilbene and vanillic acid, and *lpl* by kaempferol. In conclusion, the most effective phenolic compounds are naringenin, hesperidin and quercetin. Differences were found in terms of effects on the expression of genes involved in adipogenesis among the analyzed compounds.

Received 8th May 2017,
Accepted 2nd September 2017
DOI: 10.1039/c7fo00679a
rsc.li/food-function

Introduction

In recent years a great deal of attention has been paid by the scientific community to phenolic compounds due to their beneficial effects on human health: prevention of cardiovascular diseases, cancer, neurodegenerative diseases, diabetes and obesity among others.[1–4] Consequently, their study has become an increasingly important area of research.

Phenolic compounds are members of a very large family of plant-derived molecules exhibiting an extensive variety of chemical structures. They are classified as flavonoids and non-flavonoids. Various groups can be distinguished among flavonoids: flavanols, flavones, flavanones, falvan-3-ols and anthocyanidins. Non-flavonoids include stilbenes, hydrolysable tannins and phenolic acids.[3]

It is known that the chemical structure of phenolic compounds has an influence, not only on their bioavailability, but also on their biological actions.[5,6] Several examples can illustrate this issue. For instance, when the PPARα agonism of two stilbenes (resveratrol and its methoxy derivative pterostilbene) was compared, pterostilbene showed significantly greater affinity for this nuclear receptor than resveratrol.[7] In a study devoted to analyzing the anti-tubercular and antibacterial effects of polyphenols belonging to flavanons, flavones, isoflavones or stilbenes, authors observed important differences in terms of activity among these phenolic compound groups.[8] Another example refers to the anti-carcinogenic effect on polyphenols. Du *et al.*[9] evaluated the chemopreventive effects of ten tea polyphenols belonging to flavan-3-ols and phenolic acids on human colorectal cancer cells. They observed that, in general terms, phenolic acids showed relatively lower anti-proliferative effects compared to active flavan-3-ols.

Bearing all this in mind, and the major health problem that obesity represents, our interest focused on the effects of phenolic compounds on obesity, and more specifically on

[a]*Nutrition and Obesity Group, Department of Nutrition and Food Science, University of the Basque Country (UPV/EHU) and Lucio Lascaray, Vitoria, Spain. E-mail: mariapuy.portillo@ehu.es; Fax: +34-945-013014; Tel: +34-945-013067*
[b]*Laboratory of Molecular Biology, Nutrition and Biotechnology (Nutrigenomics), University of the Balearic Islands, Palma de Mallorca, Illes Balears*
[c]*Spanish Biomedical Research Centre in Physiopathology of Obesity and Nutrition (CIBERobn), Institute of Health Carlos III, Spain*

†**Chemical compounds studied in this article:** Apigenin (PubChem CID: 5280443); Luteolin (PubChem CID: 5280445); Catechin (PubChem CID: 73160); Epicatechin (PubChem CID: 72276); Epigallocatechin (PubChem CID: 72277); Genistein (PubChem CID: 5280961); Daidzein (PubChem CID: 5281708); Naringenin (PubChem CID: 932); Hesperidin (PubChem CID: 10621); Kaempferol (PubChem CID: 5280863); Resveratrol (PubChem CID: 445154); Vanillic acid (PubChem CID: 8468); Quercetin (PubChem CID 5280343); Piceatannol (PubChem CID: 667639); Pterostilbene (PubChem CID: 5281727).

adipogenesis, process which allows adipose tissue expansion by the transformation of adipocyte precursors (pre-adipocytes) into mature adipocytes.[10]

Adipogenesis is a complex process that involves the coordinated interplay of several transcription factors, CCAAT/enhancer-binding protein δ (*c/ebp δ*), CCAAT/enhancer-binding protein β (*c/ebp β*), sterol regulatory element-binding protein 1c (*srebp1c*), peroxisome proliferator-activated receptor γ (*pparγ*), CCAAT/enhancer-binding protein α (*c/ebpα*) in a programmed manner.[11] Whereas *c/ebpδ* alone possesses minimal adipogenic activity,[12,13] *c/ebpβ* and *srepb1c* trigger high-level expression of *c/ebpα* and *pparγ*, which is considered the master coordinator of adipocyte differentiation. Once activated, *pparγ* and *c/ebpα* cross-regulate each other to maintain their gene expression. Finally, during the late stage, *pparγ*, *srebp1c* and *c/ebpα* induce the expression of late markers of differentiation, such as acetyl-CoA carboxylase (*acc*), *perilipin* and lipropotein lipase (*lpl*). When these genes are expressed, the cells round-up, accumulate fat droplets and become terminally differentiated adipocytes.[5–7,14–18]

Several authors have reported the anti-adipogenic effects of various phenolic compounds.[3] When comparisons are made among them, in order to know which chemical structures are more potent, clear conclusions cannot be drawn because important differences in the experimental design (type of cell, phenolic compound doses, length of the experimental period, *etc.*) exist among these studies, which complicate their comparison.

Thus, the aim of the present study was to analyze, under the same experimental conditions, the anti-adipogenic effects of fifteen phenolic compounds belonging to various chemical groups, on adipogenesis in 3T3-L1 adipocytes. A second aim of our study was to analyze the effects of these phenolic compounds on the main genes involved in adipogenesis regulation. The information provided by this study may be useful for choosing the best phenolic compounds in *in vivo* studies devoted to preventing or treating obesity Nevertheless, taking into account that these molecules suffer an intensive metabolism in intestine and liver,[3] further studies are needed to analyze the potential effects of phenolic compounds metabolites on adipogenesis.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (BRL Life Technologies, Grand Island, NY). Phenolic compounds, apigenin, luteolin, catechin, epicatechin, epigallocatechin, genistein, daizein, naringenin, hesperidin, kaempferol, resveratrol and vanillic acid were supplied by Extrasynthese (Genay, France). Quercetin, piceatannol and pterostilbene were provided by Sigma-Aldrich (St Louis, MO). Purity of phenolic compounds was as follows: quercetin, kaempferol, apigenin, luteolin, catechin, epicatechin, and naringenin (≥99%), epigallocatechin and piceatannol (≥98%), hesperidin (≥98.5%), pterostilbene (≥97%), daidzein, genistein, resveratrol and vanillic acid (≥95%).

Experimental design

3T3-L1 pre-adipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10% fetal bovine serum (FBS). Two days after confluence (day 0), the cells were stimulated to differentiate with DMEM containing 10% FBS, 10 μg mL^{-1} insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone for 2 days. On day 2, the differentiation medium was replaced by FBS/DMEM medium (10%) containing 0.2 μg mL^{-1} insulin. This medium was changed every two days until cells were harvested (day 8). All media contained 1% penicillin/streptomycin (10 000 U mL^{-1}), and the media for differentiation contained 1% (v/v) of Biotin and Panthothenic Acid. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Cell treatment

In a first experiment, pre-adipocytes grown in 6-well plates were incubated with the phenolic compounds listed in the "Reagents" paragraph, all of them at 25 μM (diluted in 95% ethanol), during the adipogenic stage, from day 0 to day 8 of differentiation. Control cells received the same amount of ethanol dilution without polyphenols. The medium was changed every 2 days. On day 8, culture supernatant was removed and cells were used for triacylglycerol determination and RNA extraction. Each experiment was performed in triplicate.

In a second experiment, those phenolic compounds which showed a significant anti-adipogenic activity at 25 μM (apigenin, luteolin, genistein, daizein, naringenin, hesperidin, quercetine, kaempferol, piceatannol, pterostilbene, resveratrol and vanillic acid), were selected for a new treatment at lower doses (1 and 10 μM). Incubation conditions were exactly the same as in experiment 1. Each experiment was performed 3 times.

To assess the effects of luteolin, hesperidin, kaempferol, pterostilbene, genistein and vanilic acid at 25 μM in early and late stage of adipogenic differentiation the following treatments were carried out in 6-well plates. Cells were incubated with them from day 0 to 60 hours after the induction of differentiation to elucidate the effects of those phenolic compounds on the early stage. The effect on late stage was measured by incubating cells from 60 hours after differentiation to day 8, as previously reported by Tang *et al.* (2003).[18] In the case of the control group control cells received the same amount of ethanol dilution as treated cells, without polyphenols. Incubation media was changed every two days. Cells were used for triacylglycerol determination. Each experiment was performed 3 times.

Measurement of triacylglycerol content in maturing pre-adipocytes

For triacylglycerol extraction, cells were washed extensively with phosphate-buffered saline (PBS) and incubated 3 times

with 800 μL of hexane/isopropanol (2 : 1). The total volume was then evaporated by nitrogen gas and the pellet was re-suspended in 200 μL of Triton X-100 in 1% distilled water. Afterwards, triacylglycerols were disrupted by a sonicator and the content was measured by Infinity Triglycerides reagent (Thermo Scientific, Rockford, IL, USA). For protein determinations, cells were lysed in 0.3 N NaOH, 0.1% SDS. Protein measurements were performed using the BCA reagent (Thermo Scientific, Rockford, IL, USA).

Citotoxicity assay

Cell viability was assessed in cells treated with 25 μM of each phenolic compound by using the neutral red assay (TOX4 kit, Sigma-Aldrich, St Louis, MO, USA).

Extraction and analysis of RNA and quantification by reverse transcription-polymerase chain reaction (real time RT-PCR)

RNA samples were extracted from cells by using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using a RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with DNase I kit (Applied Biosystems, Foster City, CA, USA) to remove any contamination with genomic DNA.

One μg of total RNA in a total reaction volume of 20 μL was reverse transcribed using the iScript cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocols. Reactions were incubated initially at 25 °C for 10 min and subsequently at 37 °C for 120 min and 85 °C for 5 min.

Relative mRNA levels of genes that play an important role in adipogenesis, *c/ebpβ*, *c/ebpα*, *pparγ*, *srebp1c*, *lpl* and *acc* were quantified using Real-Time PCR with an iCycler™-MyiQ™ Real Time PCR Detection System (BioRad, Hercules, CA, USA). 18S mRNA levels were similarly measured and served as the reference gene. The PCR reagent mixture consisted of 1 μL of each cDNA (10 pmol μL^{-1}), SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the upstream and downstream primers (300 nM each, in the case of *pparγ*, *lpl* and *acc* and 600 nM each in the case of *srebp1c*, *c/ebp1β and c/ebpα*). Specific primers were synthesized commercially (TibMolbiol, Berlin, Germany) (Table 1).

PCR parameters were as follows: initial 2 min at 50 °C, denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s in the case of *pparγ*, *lpl*, *srebp1c* and *acc*. In the case of *c/ebpβ* and *c/ebpα* the annealing was at 68.4 °C and 66.4 °C respectively, and extension at 60 °C for 30 s. All sample mRNA levels were normalized to the values of 18S (housekeeping) and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method.[19]

Western blotting

Total protein was isolated from maturing 3T3-L1 adipocytes using 200 μL of lysis buffer (2 nM Tris-HCl, 0.1 M sodium chloride (NaCl), 1% Triton, 10% glycerol, 1 mM sodium orthovanadate (OvNa), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium fluoride (FNa) and 1% protease inhibitor) and centrifuged (12 000*g*, 15 minutes, 4 °C) to remove membranes and other proteic residues. Protein concentration was determined by BCA protein assay kit (Thermo Scientific, Wilmington, DE, USA). Total protein (30 μg) was subjected to 4–15% Mini-PROTEAN® TGX Precast gels, electroblotted onto PVDF membranes (BioRad, CA, USA and Millipore, Bradford, MA, USA). The membranes were then blocked with 5% casein PBS-Tween buffer for 2 hours at room temperature. Subsequently, they were incubated with polyclonal mouse anti-PPARγ (1 : 1000) and monoclonal rabbit anti-β-actin (1 : 5000) (Santa-Cruz Biotech, CA, USA) overnight at 4 °C. Afterward, polyclonal goat anti-mouse IgG-HRP for PPARγ (1 : 5000) and goat anti-rabbit IgG-HRP for β-actin (1 : 5000) (Santa-Cruz Biotech, CA, USA) were incubated for 2 hours at room temperature. The bound antibodies were visualized by an ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) and quantified by Chemi-Doc MP imaging system (BioRad, CA, USA).

Statistical analysis

Results are presented as mean ± standard error of the mean. Statistical analysis was performed using SPSS 23.0 (SPSS Inc. Chicago, IL, USA). Data from each phenolic compound-treated cells were compared with control cells by using Student's *t* test. Statistical significance was set-up at the $P < 0.05$ level.

Table 1 Primers for PCR amplification of each gene studied

	Sense primer	Antisense primer
acc	5′-GGA CCA CTG CAT GGA ATG TTA A-3′	5′-TGA GTG ACT GCC GAA ACA TCT C-3′
c/ebpα	5′-TGG ACA AGA ACA GCA ACG AG-3′	5′-TCA CTG GTC AAC TCC AGC AC-3′
c/ebpβ	5′-CAA GCT GAG CGA CGA GTA CA-3′	5′-CAG CTG CTC CAC CTT CTT CT-3′
lpl	5′-CAG CTG GGC CTAACT TTG AG-3′	5′-CCT CTC TGC AAT CAC ACG AA-3′
pparγ	5′-ATT CTG GCC CAC CAA CTT CGG-3′	5′-TGG AAG CCT GAT GCT TTA TCC CCA-3′
srebp1c	5′-GCT GTT GGC ATC CTG CTA TC-3′	5′-TAG CTG GAA GTG ACG GTG GT-3′
18S	5′-GTG GGC CTG CGG CTT AAT-3′	5′-GCC AGA GTC TCG TTC GTT ATC-3′

acc = acetyl-CoA carboxylase, *c/ebpα* and *cebpβ* = CCAAT/enhancer-binding protein α and β, *lpl* = lipoprotein lipase, *pparγ* = peroxisome proliferator-activated receptor γ, *srebp1c* = sterol regulatory element-binding protein 1c.

Results

Triacylglycerol content

Three doses of phenolic compounds were used for cell treatments, 25, 10 and 1 μM. As described in the following lines, the number of effective compounds reduced while doses decreased.

When cell were treated from day 0 to day 8 at a dose of 25 μM flavan-3-ols (catechin, epicatechin and epigallocatechin) were ineffective. By contrast, significant reductions in triacylglycerol content were induced by the other phenolic compounds. In order to simplify the figure, only the effective compounds are shown in Fig. 1. The percentages of reduction were as follows: apigenin (23.3 ± 6.3%), luteolin (43.3 ± 8.3%), genistein (41.0 ± 10.5%), daizein (31.4 ± 7.3%), naringenin (38.9 ± 6.8%), hesperidin (53.9 ± 6.5%), quercetin (46.2 ± 7.1%), kaempferol (43.5 ± 10.3%), piceatannol (31.6 ± 8.6%), pterostilbene (57.1± 5.6%), resveratrol (33.0 ± 1.6%) and vanillic acid (38.7 ± 7.2%).

At a dose of 10 μM only apigenin, luteolin, naringenin, hesperidin, quercetin and kaempferol, in other words flavones, flavanones and flavonols, induced significant reductions in triacylglycerol content (Fig. 2). The percentages of triacylglycerol content reduction were 26.7 ± 2.7, 24.4 ± 2.9%, 3.4 ± 1.0%, 28.3 ± 3%, 12.7 ± 3.2% and 26.6 ± 8.6% respectively. Finally, at the lowest dose (1 μM) just naringenin, hesperidin and quercetin were effective (Fig. 3), showing percentages of triacylglycerol content reduction of 24.8 ± 5.0%, 19.6 ± 3.2% and 26.6 ± 8.6%, respectively.

When cultures were carried out at early stage of differentiation (0 to 60 hours), with phenolic compounds at 25 μM, the accumulation of triacylglcyerols was reduced in cells treated with luteolin (52.5.7 ± 5.6%), genistein (22.6 ± 8.9%), hesperidin (21.3 ± 8.6%), kaempferol (50.2 ± 9.2%), pterostilbene (29.7 ± 8.7%) and vanillic acid (19.9 ± 7.0%) (Fig. 4). During late stages of differentiation (60 hours to day 8) triacylglycerols were not reduced (Fig. 5).

In Table 2 chemical structures of the phenolic compounds used in the present study, and a summary of their anti-adipogenic effect at different doses is presented in order to facilitate the analysis of the relationship between both features.

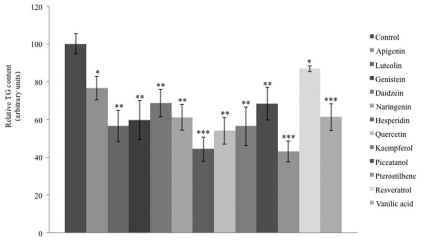


Fig. 1 Triacylglycerol content in 3T3-L1 pre-adipocytes treated from day 0 to day 8 with those phenolic compounds which showed delipidating effects: apigenin, luteolin, catechin, epicatechin, epigallocatechin, genistein, daizein, naringenin, hesperidin, quercetine, kaempferol, piceatannol, pterostilbene, resveratrol, vanillic acid, at a dose of 25 μM. Control cells are pre-adipocytes not treated with phenolic compounds. Data are means ± SEM (standard error of the mean) of three independent experiments carried out in sextuplicates. Student's *t* test was used for the analysis of comparisons between each phenolic compound and the control group (**P* < 0.05, ***P* < 0.01, ****P* < 0.01).

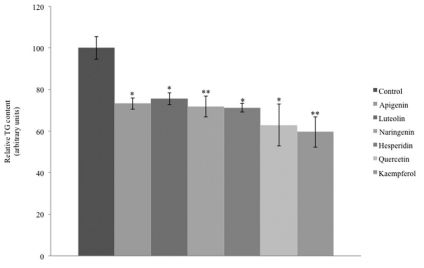


Fig. 2 Triacylglycerol content in 3T3-L1 pre-adipocytes treated from day 0 to day 8 with which showed delipidating effects: apigenin, luteolin, catechin, epicatechin, epigallocatechin, genistein, daizein, naringenin, hesperidin, quercetine, kaempferol, piceatannol, pterostilbene, resveratrol, vanillic acid, at a dose of 10 μM. Control cells are pre-adipocytes not treated with phenolic compounds. Data are means ± SEM (standard error of the mean) of three independent experiments carried out in sextuplicates. Student's *t* test was used for the analysis of comparisons between each phenolic compound and the control group (**P* < 0.05, ***P* < 0.01).

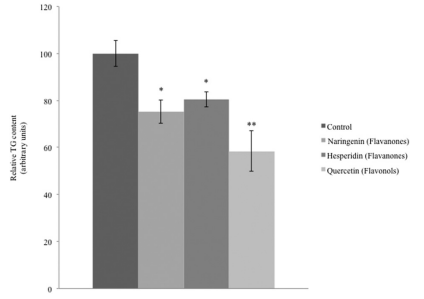


Fig. 3 Triacylglycerol content in 3T3-L1 pre-adipocytes treated from day 0 to day 8 with those phenolic compounds which showed delipidating effects: apigenin, luteolin, catechin, epicatechin, epigallocatechin, genistein, daizein, naringenin, hesperidin, quercetine, kaempferol, piceatannol, pterostilbene, resveratrol, vanillic acid, at a dose of 1 μM. Control cells are pre-adipocytes not treated with phenolic compounds. Data are means ± SEM (standard error of the mean) of three independent experiments carried out in sextuplicates. Student's *t* test was used for the analysis of comparisons between each phenolic compound and the control group (**P* < 0.05, ***P* < 0.01).

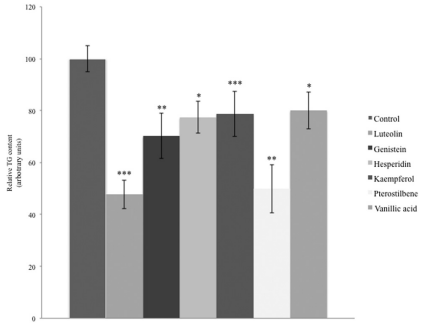


Fig. 4 Triacylglycerol content in 3T3-L1 preadipocytes in early stage of adipogenesis treated from day 0 to 60 hours with luteolin, genistein, hesperidin, kaempferol, pterostilbene and vanillic acid at a dose of 25 μM. Control cells are pre-adipocytes not treated with phenolic compounds. Data are means ± SEM (standard error of the mean) of three independent experiments carried out in sextuplicates. Student's *t* test was used for the analysis of comparisons between each phenolic compound and the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

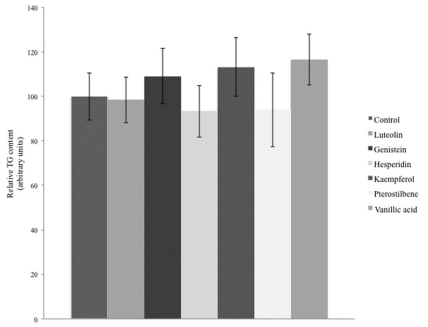


Fig. 5 Triacylglycerol content in 3T3-L1 preadipocytes in late stage of adipogenesis treated from 60 hours to day 8 with luteolin, genistein, hesperidin, kaempferol, pterostilbene and vanillic acid at a dose of 25 μM. Control cells are pre-adipocytes not treated with phenolic compounds. Data are means ± SEM (standard error of the mean) of three independent experiments carried out in sextuplicates. Student's *t* test was used for the analysis of comparisons between each phenolic compound and the control group. No significant differences were observed.

Cell viability

3T3-L1 pre-adipocytes exposed from day 0 to day 8 of differentiation to each phenolic compound at 25 μM showed no loss of viability (Fig. 6).

Gene expression

Gene expression was analyzed in adipocytes treated with phenolic compounds which were effective at a dose of 25 μM. For this purpose, the compound in each group showing the highest percentage of triacylglycerol reduction was selected. In the case of flavonols, this percentage was greater for quercetin than for kaempferol, but we selected the latter because, unlike quercetin, little information on the anti-adipogenic effect of this molecule is available in the literature. As a result, the compounds finally analyzed were luteolin, hesperidin, kaempferol, pterostilbene, genistein and vanillic acid.

The expression of *c/ebpβ* was significantly reduced by genistein, kaempferol and pterostilbene (Fig. 7A). By contrast, *srebp1c* was decreased by luteolin, genistein, hesperidin, kaempferol, pterostilbene and vanillic acid (Fig. 7B) and *pparγ* by genistein and pterostilbene (Fig. 7C). The expression of *c/ebpα* was not modified by the phenolic compounds analyzed (Fig. 7D). Concerning *acc*, luteolin, genistein, kaempferol and pterostilbene decreased mRNA levels (Fig. 7E). Finally, only kaempferol reduced *lpl* gene expression (Fig. 7F). In Fig. 8 a summary of the effects on phenolic compounds on the expression of adipogenesis-related genes is presented.

Western blotting

PPARγ2 protein levels were measured in 3T3-L1 using a dose of 25 μM of genistein and pterostilbene in 3T3-L1 maturing preadipocytes treated from day 0 to day 8. No significant changes were induced by experimental treatments compared to control group ($P = 0.792$ for pterostilbene and $P = 0.620$ for genistein) (Fig. 9).

Discussion

The increasing prevalence of obesity and related metabolic diseases in both developed and developing countries has encouraged the search for new treatment strategies. Besides feeding control and energy efficiency, regulation of other processes such as adipogenesis can be considered as important targets for obesity control.[20] Research in this field aimed at the identification of bioactive compounds with anti-adipogenic effects may be of great interest for the development of functional foods or nutritional supplements.

Several studies have reported the anti-adipogenic effects of various phenolic compounds, but the vast majority of them have used far higher doses than the serum concentrations reached when these compounds are administered to subjects.[3] Consequently, the extrapolation of these results to the *in vivo* situation is rather limited. Moreover, important differences in terms of experimental design exist among the studies, making comparisons among these compounds difficult.

In this context, the present study aimed to analyze the anti-adipogenic effects of fifteen phenolic compounds, belonging to various chemical groups, in 3T3-L1 pre-adipocytes under the same experimental conditions, in order to know which chemical structures show the greatest potency in inhibiting this

Table 2 Chemical structure of phenolic compounds and summary of the anti-adipogenic effect at the three doses used

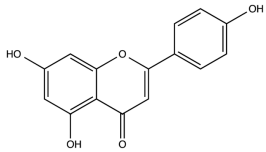
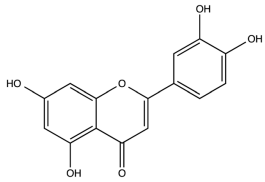
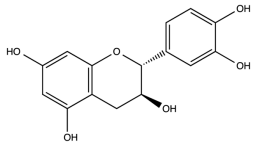
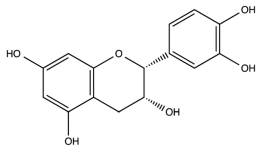
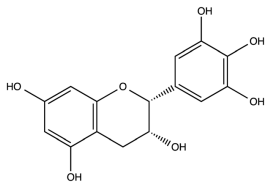
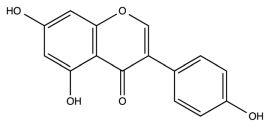
General group	Sub-group	Compound	Chemical structure	25 μM	10 μM	1 μM
Flavonoids	Flavones	Apigenin		Yes	Yes	No
	Flavones	Luteolin		Yes	Yes	No
	Flavan-3-ols	Catechin		No	No	No
	Flavan-3-ols	Epicatechin		No	No	No
	Flavan-3-ols	Epigallocatechin		No	No	No
	Isoflavones	Genistein		Yes	No	No

Table 2 *(Contd.)*

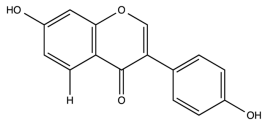
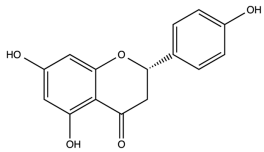
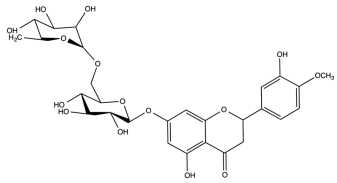
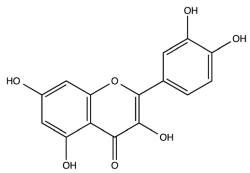
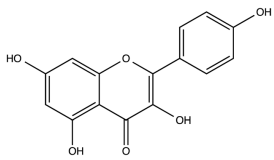
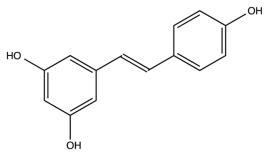
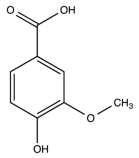
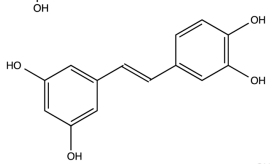
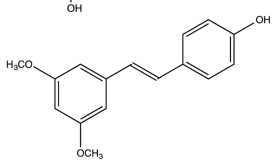
General group	Sub-group	Compound	Chemical structure	25 μM	10 μM	1 μM
	Isoflavones	Daidzein		Yes	No	No
	Flavanones	Naringenin		Yes	Yes	Yes
	Flavanones	Hesperidin		Yes	Yes	Yes
	Flavonols	Quercetin		Yes	Yes	Yes
	Flavonols	Kaempferol		Yes	Yes	No
Non flavonoids	Stilbenes	Resveratrol		Yes	No	No

Table 2 *(Contd.)*

General group	Sub-group	Compound	Chemical structure	25 μM	10 μM	1 μM
	Hydroxybenzoic acids	Vanillic acid		Yes	No	No
	Stilbenes	Piceatannol		Yes	No	No
	Stilbenes	Pterostilbene		Yes	No	No

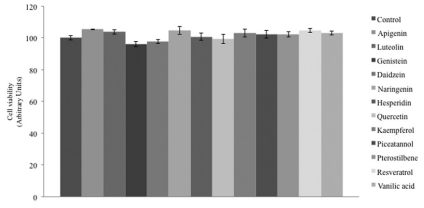


Fig. 6 Cell viability assessed in cells treated with 25 μM of phenolic compounds from day 0 to day 8 of differentiation. Control cells are pre-adipocytes not treated with phenolic compounds. Data are means ± SEM (standard error of the mean) of three independent experiments carried out in triplicates. Student's *t* test was used for the analysis of cytotoxicity between phenolic groups and control group. No significant differences were observed.

process. For the screening we used commercially available phenolic compounds: apigenin and luteolin (flavones showing a basic structure based on the backbone of 2-phenyl-1-benzopyran-4-one), catechin, epicatechin and epigallocatechin (flavan-3-ols showing a basic structure based on the backbone of 2-phenyl-3,4-dihydro-2*H*-chromen-3-ol), genistein and daizein (isoflavones showing a basic structure based on 3-aryl-1-benzopyran-4-one (3-aryl-4*H*-chromen-4-one)), naringenin and hesperidin (flavanones showing a basic structure based on the backbone of 3,4-dihydro-2-aryl-2*H*-1-benzopyran-4-one), quercetine and kaempferol (flavonols showing a basic structure based on the backbone of 3-hydroxy-2-phenylchromen-4-one), piceatannol, pterostilbene and resveratrol (stilbenes showing a basic structure based on 1,1′-(ethene-1,2-diyl)dibenzene), and vanillic acid (hydroxybenzoic acids showing a basic structure based on the backbone of hydroxybenzoic acid). A range of doses from 25 μM to a physiological dose of 1 μM was studied. The use of this low dose is one of the main novelties of this study.

The first experiment, carried out at a dose of 25 μM, showed that the majority of the compounds analyzed, with the exception of catechin, epicatechin, and epigallocatechin, induced a significant reduction in triacylglycerol content. For a second experiment, lower doses, namely 10 μM and 1 μM (physiological dose), were selected. At a dose of 10 μM, phenolic compounds showing a chemical structure of flavones, flavanones and flavanols, but not isoflavones, stilbenes and benzoic acids, were able to inhibit adipogenesis. When the dose was reduced to 1 μM only the two flavanones studied (naringenin and hesperidin) and quercetin, belonging to the group of flavones, induced a significant reduction in triacylglycerol content in pre-adipocytes. These results show that the chemical structure of flavanones is the most potent one in the inhibition of adipogenesis, at least under our experimental conditions, because both compounds showing this structure were able to reduce triacylglycerol accumulation in pre-adipocytes at the three doses studied, including 1 μM, the dose which is in the range of biological concentrations.

When the effects of phenolic compounds belonging to the same chemical group were compared, we observed that both apigenin and luteolin flavones were active at 25 and 10 μM,

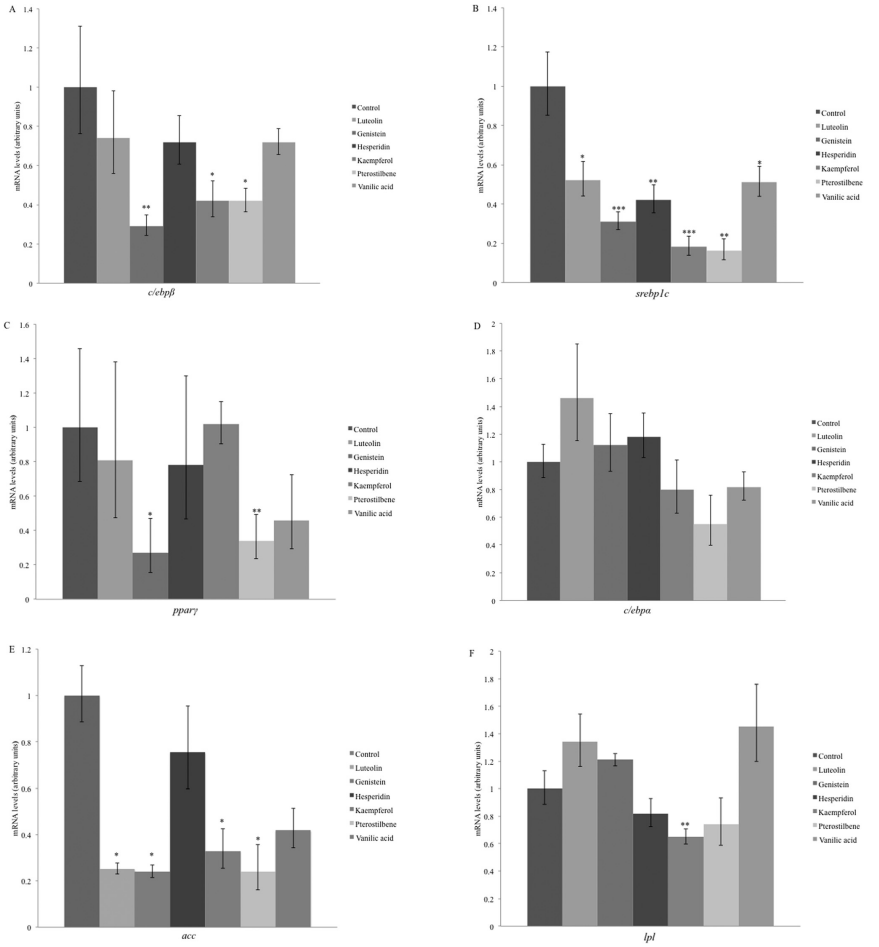


Fig. 7 Gene expression of *c/ebpβ* (A), *srebp1c* (B) *pparγ* (C), *c/ebpα* (D), *acc* (E) and *lpl* (F) in 3T3-L1 pre-adipocytes treated from day 0 to day 8 with luteolin, genistein, hesperidin, kaempferol, pterostilbene, or vanillic acid at a dose of 25 μM Data are means ± SEM (standard error of the mean) of three independent experiments carried out in triplicates. Student's *t* test was used for the analysis of comparisons between each phenolic compound and the control group (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

both genistein and daizein isoflavones were active at 25 μM, both naringenin and hesperidin flavones were active at the three doses and the three stilbenes were active at 25 μM. These results suggest that small differences, mainly based on substituent groups (Table 2), among compounds showing a similar general chemical structure are not crucial for the anti-adipogenic activity of these molecules. In the case of flavonols, the pattern of response was different because while quercetine was effective at the three doses, kaempferol only showed an anti-adipogenic effect at 25 and 10 μM. This difference could be

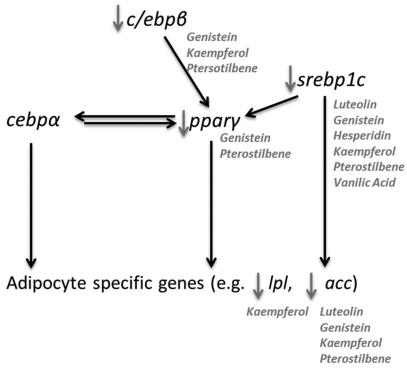


Fig. 8 Summary of the effects on phenolic compounds on the expression of adipogenesis-related genes.

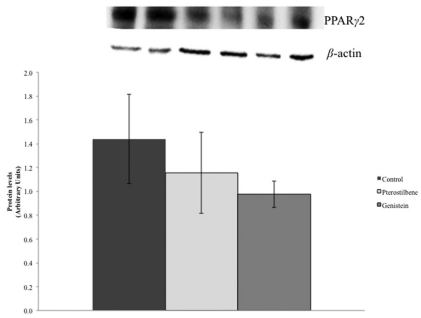


Fig. 9 Effects of 25 μM of pterostilbene and genistein on PPARγ2 protein expression in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are mean ± SEM. Student's *t* test was used for the analysis of comparisons between each phenolic compound and the control group. No significant differences were observed.

due to the presence of an extra hydroxyl group in quercetin structure (Table 2).

When we compared our results with those reported in the literature, using these phenolic compounds at the same doses as those used in the present study, we found a good agreement with several authors.[21–28] Nevertheless, discrepancies with other authors were also found, probably due to differences in various aspects of the experimental design.[24,29–34]

To determine whether phenolic compounds reduced adipogenesis through cytotoxic effects, we measured cell viability when 3T3-L1 cells were incubated with the highest dose of these molecules (25 μM). This analysis revealed that these molecules showed a real anti-adipogenic effect.

A second aim of our study was to analyze the effects of these phenolic compounds on the regulation of adipogenesis. Since the majority of the compounds studied in the present work were effective at 25 μM, we chose this dose in order to compare as many molecules as possible. As indicated in the Results section, the compound in each group which showed the highest percentage of triacylglycerol reduction was selected for this molecular approach. In the case of flavonols, kaempferol was selected instead of quercetin because their anti-adipogenic effects have been much less studied.

As explained in the Introduction, adipogenesis is regulated by an elaborate network of transcription factors. In general terms, two stages can be distinguished, the pre-mitotic stage (early stage of differentiation; 60 hours post-confluence), regulated by *c/ebpβ* and *srebp1c*, and the post-mitotic stage (late stage), regulated by *pparγ and c/ebpα*.[14,16–18] Nevertheless, the influence of *pparγ* on these two stages is difficult to be established. Ntambi *et al.*[15] reported that this transcription factor starts its expression around 48 hours and reaches its maximal expression around 3–4 days of differentiation. In the present study, after analyzing the effects of the phenolic compounds on the expression of these genes, it can be proposed that all of them acted by inhibiting the early stage of adipogenesis because they significantly reduced gene expression of *srebp1c*, or both *c/ebpβ* and *srebp1c* (genistein and kaempferol). This proposal is confirmed by the reduction in triacylglycerols observed when cells were treated from 0 to 60 hours with all the phenolic compounds analyzed in this second experiment (luteolin, genistein, hesperidin, kaempferol, pterostilbene, vanilic acid).

None of the tested compounds modified *c/ebpa* gene expression. In the case of *pparγ* gene expression, both genistein and pterostilbene induced significant reductions, but this effect was not accompanied by the expected reduction in triglyceride content when cells were incubated from 60 hours to day 8. In order to gain more insight concerning this issue, PPARγ2 protein expression was measured and no significant differences were found when cells treated with these phenolic compounds were compared with the control cells. These results are in good accordance with the lack of delipidating effect showed in the latest stage of differentiation.

As far as more lately expressed genes are concerned, luteolin, genistein, kaempferol and pterostilbene reduced *acc* gene expression and kaempferol that of *lpl*, which suggests a reduced capacity of mature adipocytes previously treated with these compounds for triglyceride accumulation. Finally, it should be pointed out that the reduction observed in the expression of genes from the early stage of adipogenesis should have led to a reduction in the expression of regulator genes of the late stage. Nevertheless, in the present study, not all the compounds demonstrated this effect. This situation has also been found in other studies from our group[35] and by other authors.[15,17,18,36–39]

In conclusion, the present results show that the most potent phenolic compounds in terms of anti-adipogenic effects, of those studied in the present work, are flavanones (naringenin and hesperidin) and the flavonol quercetin. As far as the effects of these molecules on genes involved in the adipogenic process are concerned, differences among chemical groups can be observed.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

This study was supported by grants from the Instituto de Salud Carlos III (CIBERObn), Government of the Basque Country (IT-572-13) and University of the Basque Country (UPV/EHU) (ELDUNANOTEK UFI11/32). A. Mosqueda-Solís is a recipient of a doctoral fellowship from the CONACYT (Mexico).

References

1 A. Crozier, I. B. Jaganath and M. N. Clifford, *Nat. Prod. Rep.*, 2009, **26**, 1001–1043.
2 M. Meydani and S. T. Hasan, *Nutrients*, 2010, **2**, 737–751.
3 D. Del Rio, A. Rodriguez-Mateos, J. P. Spencer, M. Tognolini, G. Borges and A. Crozier, *Antioxid. Redox Signaling*, 2013, **18**, 1818–1892.
4 A. Leiherer, A. Mündlein and H. Drexel, *Vasc. Pharmacol.*, 2013, **58**, 3–20.
5 A. S. Mayhoub, L. Marler, T. P. Kondratyuk, E. J. Park, J. M. Pezzuto and M. Cushman, *Bioorg. Med. Chem.*, 2012, **20**, 7030–7039.
6 L. Lipińska, E. Klewicka and M. Sójka, *Acta Sci. Pol., Technol. Aliment.*, 2014, **13**, 289–299.
7 R. Mikstacka, A. M. Rimando and E. Ignatowicz, *Plant Foods Hum. Nutr.*, 2010, **65**, 57–63.
8 D. Sun, J. G. Hurdle, R. Lee, M. Cushman and J. M. Pezzuto, *ChemMedChem*, 2012, **7**, 1541–1545.
9 G. J. Du, Z. Zhang, X. D. Wen, C. Yu, T. Calway, C. S. Yuan and C. Z. Wang, *Nutrients*, 2012, **4**, 1679–1691.
10 A. T. Ali, W. E. Hochfeld, R. Myburgh and M. S. Pepper, *Eur. J. Cell Biol.*, 2013, **92**, 229–236.
11 A. Palou, F. Serra, M. L. Bonet and C. Picó, *Eur. J. Nutr.*, 2000, **39**, 127–144.
12 Z. Wu, Y. Xie, N. L. Bucher and S. R. Farmer, *Genes Dev.*, 1995, **9**, 2350–2363.
13 Z. Wu, N. L. Bucher and S. R. Farmer, *Mol. Cell. Biol.*, 1996, **16**, 4128–4136.
14 S. R. Farmer, *Cell Metab.*, 2006, **4**, 263–273.
15 J. Ntambi and K. Young-Cheul, *J. Nutr.*, 2000, **130**, 3122S–3126S.
16 M. T. Bengoechea-Alonso and J. Ericsson, *Curr. Opin. Cell Biol.*, 2007, **19**, 215–222.
17 L. Fajas, J. C. Fruchart and J. Auwerx, *Curr. Opin. Cell Biol.*, 1998, **10**, 165–173.
18 Q. Q. Tang, T. C. Otto and M. D. Lane, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 44–49.
19 K. Livak and T. Schmittgen, *Methods*, 2001, **25**, 402–408.
20 A. Palou, C. Picó and M. L. Bonet, *Nutr. Rev.*, 2004, **62**, S169–S181.
21 S. Rayalam, M. Della-Fera, J. Yang, H. Park, S. Ambati and C. Baile, *J. Nutr.*, 2007, **137**, 2668–2673.
22 S. Rayalam, J. Yang, S. Ambati, M. Della-Fera and C. Baile, *Phytother. Res.*, 2008, **22**, 1367–1371.
23 J. Ahn, H. Lee, S. Kim, J. Park and T. Ha, *Biochem. Biophys. Res. Commun.*, 2008, **373**, 545–549.
24 X. K. Fang, J. Gao and D. N. Zhu, *Life Sci.*, 2008, **82**, 615–622.
25 N. Sakurai, K. Mochizuki, H. Kameji, M. Shimada and T. Goda, *Nutrition*, 2009, **25**, 1047–1056.
26 C. Y. Chan, L. Wei, F. Castro-Muñozledo and W. L. Koo, *Life Sci.*, 2011, **89**, 779–785.
27 M. Ono and K. Fujimori, *J. Agric. Food Chem.*, 2011, **59**, 13346–13352.
28 J. Y. Kwon, S. G. Seo, Y. S. Heo, S. Yue, J. X. Cheng, K. W. Lee and K. H. Kim, *J. Biol. Chem.*, 2012, **287**, 11566–11578.
29 J. Yang, M. Della-Fera, S. Rayalam, S. Ambati, D. Hartzell, H. Park and C. Baile, *Life Sci.*, 2008, **82**, 1032–1039.
30 C. L. Hsu, Y. J. Lin, C. T. Ho and G. C. Yen, *Food Funct.*, 2012, **3**, 49–57.
31 A. J. Richard, Z. Amini-Vaughan, D. M. Ribnicky and J. M. Stephens, *Evidence-Based Complementary Altern. Med.*, 2013, **2013**, 549750.
32 H. J. Jeon, M. J. Seo, H. S. Choi, O. H. Lee and B. Y. Lee, *Phytother. Res.*, 2014, **28**, 1701–1709.
33 A. Nishina, M. Ukiya, M. Fukatsu, M. Koketsu, M. Ninomiya, D. Sato, J. Yamamoto, K. Kobayashi-Hattori, T. Okubo, H. Tokuoka and H. Kimura, *Biol. Pharm. Bull.*, 2015, **38**, 1794–1800.
34 B. Poudel, S. Nepali, M. Xin, H. H. Ki, Y. H. Kim, D. K. Kim and Y. M. Lee, *Mol. Med. Rep.*, 2015, **12**, 3139–3145.
35 I. Eseberri, J. Miranda, A. Lasa, I. Churruca and M. P. Portillo, *Oxid. Med. Cell. Longevity*, 2015, **2015**, 480943.
36 M. D. Lane, Q. Q. Tang and M. S. Jiang, *Biochem. Biophys. Res. Commun.*, 1999, **266**, 677–683.
37 R. L. Erickson, K. A. Longo, S. E. Ross, N. Hemati and O. A. MacDougald, Structure and function of C/EBP, in *Adipocyte Biology and Hormone Signaling*, ed. J. M. Ntambi, IOS Press, Inc., Burke, VA, 2000.
38 P. Dowell, C. Flexner, P. O. Kwiterovich and M. D. Lane, *J. Biol. Chem.*, 2000, **275**, 41325–41332.
39 S. Allender and M. Rayner, *Obes. Rev.*, 2007, **8**, 467–473.

Manuscript 2

Hesperidin and capsaicin, but not the combination, prevent hepatic steatosis and other metabolic syndrome-related alterations in western diet fed rats

Mosqueda-Solís A., Sánchez J., Portillo MP., Palou A., Picó C

Abstract

Scope: We aimed to assess the potential effects of hesperidin and capsaicin, independently and in combination, to prevent the development of obesity and its related metabolic alterations in rats fed an obesogenic diet.

Methods and Results: Three-month-old male Wistar rats were divided into 5 groups: Control (animals fed a standard diet), WD (animals fed a high fat/sucrose (western) diet), HESP (animals fed a western diet + hesperidin (100 mg/kg/day)), CAP (animals fed a western diet + capsaicin (4 mg/kg/day)), and HESP+CAP (animals fed a western diet + hesperidin (100 mg/kg/day) + capsaicin (4 mg/kg/day)). Capsaicin decreased body fat gain and prevented insulin resistance, whereas hesperidin showed little effect on body fat gain and no apparent effects on insulin resistance. No additive effects were observed with the combination. Capsaicin and hesperidin, separately, improved blood lipid profile, diminished hepatic lipid accumulation, and prevented non-alcoholic steatohepatitis in western diet-fed rats, but the combination showed lower effects. Hesperidin alone, and to a lesser extent capsaicin or the combination of both bioactives, displayed hypotensive effects in western diet-fed rats.

Conclusion: Capsaicin and hesperidin, separately, exhibit different health beneficial effects on metabolic syndrome-related alterations in western diet-fed rats, but the effects are mitigated with the combination.

Key words: Obesity, NASH, hesperidin, capsaicin, bioactive compounds.

Introduction

Obesity has reached epidemic proportions globally. In 2016, more than 1.9 billion adults worldwide were overweight, and of these over 600 million were clinically obese [1] . Obesity is the consequence of a prolonged disruption in energy homeostasis, in which the energy gain exceeds the energy expenditure. This condition is considered as a multifactorial disease that is influenced by lifestyle, cultural, environmental, genetic, physiological and metabolic factors. Notably, the intake of western diets, characterized especially in high consume of simple carbohydrates and saturated fats, are leading to an increase in the prevalence of obesity and its related alterations, such as insulin resistance, hyperlipemia and non-alcoholic fatty liver, among others [2]. Attention of the scientific community is focused on the implementation of innovative and effective strategies for the prevention and treatment for this pathology and its comorbidities [3].

Nowadays the use of natural bioactive compounds is trending as alternative methods for the treatment and management of obesity and related diseases, but the efficacy of such approaches depends on the absorption, metabolism and bioavailability of such compounds, which may be influenced by disease state [4]. In addition, possible interactions between bioactive agents, leading to additive or synergistic effects or, on the contrary, to a decrease in their efficacy, should be considered. These aspects may have important implications for functional food development and assessment. However, no much information is available regarding this issue. The study is of interest since the level of a single natural compound may be too low to exert sufficient beneficial effects. By contrasts, the combination of compounds acting via an additive and/or

synergistic mode to either the same or diverse targets may be of interest in preventing a pathological process.

For hesperidin ($C_{28}H_{34}O_{15}$), a flavanone present in citrus fruit, diverse biological activities of therapeutic interest have been described, including the capacity to lower serum and liver triacylglycerols as well as anti-adipogenic, anti-inflammatory, antioxidant, insulin-sensitizing properties [5-8]. Therefore, hesperidin may be of interest to improve obesity-related disorders. In fact, several studies, including preclinical and clinical trials, have demonstrated that hesperidin may have therapeutic effects on a great variety of diseases, such as cardiovascular diseases, diabetes, cancer, and neurological and psychiatric disorders, among others [9].

Other compounds of potential interest are the capsaicinoids (also known as capsinoids), a group of molecules naturally present in chilli peppers. The most abundant and studied is the capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide ($C_{18}H_{27}NO_3$), which is responsible for the pungent sensation [10, 11]. Capsaicin is recognized for its potential anti-inflammatory, antioxidant, antimicrobial, anticancer, and antiobesity properties among others [12]. Several studies have demonstrated that capsaicin decreases body weight gain, hepatic lipid accumulation and insulin resistance induced by high-fat diet feeding [13, 14]. The antiobesity effects of capsaicin have been related in part to its capacity to stimulate the sympathetic nervous system and thus to reduce energy intake and increase energy expenditure and fat oxidation, through the effects of catecholamines [12]. However, it is not clear whether the long-term effects of capsaicin on obesity may be explained by this mechanism. It is accepted that much of the effects of capsaicin on metabolic health, particularly linked to its fat-lowering action, are caused by stimulation of the transient receptor

potential cation channel subfamily V member 1 (TRPV1) [15, 16]. TRPV1, also known as capsaicin receptor, belongs to the family of non-selective cation channels with high calcium permeability [17]. This is highly expressed in sensory neurons and in vasculature, adipose, and liver tissues [18, 19]. TRPV1 activation has been described to result in recruitment of catecholaminergic neurons in the rostral ventrolateral medulla of the brain [20]. Capsaicin-induced calcium influx through TRPV1 channels has been shown to prevent adipogenesis and obesity in wild-type mice under high-fat diet feeding but not in TRPV1 knockout mice, indicating that TRPV1 is directly involved in these effects in vivo [16].

Considering that hesperidin and capsaicin can affect lipid metabolism and induce triglyceride-lowering effects by different mechanisms, the combined effects of both compounds on obesity and related metabolic alterations is of interest. In this context, the aim of this study was to screen the potential effects of dietary hesperidin and capsaicin, separately, and the combination of both compounds (hesperidin + capsaicin) to prevent the development of obesity and its related metabolic alterations, particularly insulin resistance, dyslipidemia, fatty liver disease and hypertension, induced in rats by feeding a western diet.

Material and Methods

Animals and experimental design

The animal protocol followed was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands (Resolution Number 7619, October, 2015) and guidelines for the use and care of laboratory animals of the University were followed.

The study was performed in 39 three-month-old male Wistar rats randomly divided into 5 groups: Control (n=7), animals fed with a standard chow diet (3.3

kcal/g, with 19% Kcal from protein, 73% from carbohydrate, and 8% from fat) (Pan-lab, Barcelona, Spain); WD (n=8), animals fed with a high-fat, high-sucrose diet (western diet, 4.7 kcal/g, with 17% Kcal from protein, 43% from carbohydrate, and 41% from fat) (Research Diets, Inn, New Brunswick, NJ, USA); HESP (n=8), animals fed with a western diet and treated with hesperidin (100 mg/kg/day); CAP (n=8), animals fed a western diet and treated with capsaicin (4 mg/kg/day); and HESP+CAP (n=8), animals fed with a western diet and treated with the combination of hesperidin (100 mg/kg/day) and capsaicin (4 mg/kg/day). Hesperidin and capsaicin were purchased from Aldrich Co. LLC. (St. Louis, MO). They were dissolved in 0.9% saline and administered orally, by gavage, once a day after the beginning of the light cycle (8:00h). Control and WD groups received the same volume of saline (1 ml/Kg). The animals were kept with this treatment for 8 weeks, until they were 5 months old. All rats were individually housed under controlled temperature (22°C) and a 12 hours light-dark cycle, and had unlimited access to tap water and standard diet or western diet, depending on the group.

Body weight and body composition (by EchoMRI-700TM, Echo Medical Systems, LLC., TX, USA) were measured at baseline and at the end of the treatment (week 8). Blood samples were obtained on week 7 after 12 hours fasting from saphenous vein and at sacrifice under feeding conditions (truncal blood). Blood samples were collected in heparinized containers, then centrifuged at 1000 x g for 10 min to obtain the plasma, and stored at 20°C until analysis. After 8 weeks of treatment, animals were sacrificed by decapitation under fed condition. Retroperitoneal white adipose tissue (rWAT) and the liver were rapidly removed, weighted, frozen in liquid nitrogen, and stored at -80°C until subsequent studies.

Indirect calorimetry and locomotive activity measurements

Animals were monitored for 24 hours to assess energy expenditure by indirect calorimetry and locomotive activity by using the LabMaster-CalSys-Calorimetry System (TSE Systems, Bad Homburg, Germany) after 6 weeks of treatment. In order to reduce potential stress, animals were individually housed and acclimated to the respiratory cages for 24 hours before the measurement began. Data on gas exchanges (VO_2; ml kg $^{-1}h^{-1}$ and VCO_2; ml kg $^{-1}h^{-1}$) were measured via an open circuit indirect calorimetry system for 24 hours Rates of oxygen consumption and carbon dioxide production were monitored for 5 min every 45 min for each animal or reference cage (our system can handle 8 animal cages and 1 reference cage, simultaneously). Mean energy expenditure (kcal/h) and respiratory exchange ratio (RER) values were calculated over 24 hours. Locomotive activity (counts/h) was measured continuously by an infrared beam system integrated in the LabMaster System for 24 hours.

Blood pressure measurement

Systolic and diastolic blood pressure was measured after 5 weeks of treatment. This was determined without anesthesia using non-invasive blood pressure methodology. It consists in using a tail-cuff sphygmomanometer with a photoelectric sensor (Niprem 546,Cibertec S.A., Spain) placed on the animal´s tail to occlude the blood flow. Niprem software V1.8 was used. For each animal, systolic and diastolic blood pressure values were calculated as the mean of five measurements.

Measurement of circulating parameters

Fresh blood glucose concentration was measured with an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Commercial rat ELISA kits were used for the quantification of circulating plasma levels of insulin

(Mercodia AB, Uppsala, Sweden), and leptin (R&D Systems, Minneapolis, MN, USA. Commercial enzymatic kits were used for determination of plasma levels of triglycerides (TG) (Triglyceride (INT) 20, (Sigma-Aldrich Co., LLC, Madrid, Spain), and non-esterified fatty acid (NEFA) (Wako Chemicals GmbH, Neuss, Germany), each one according to the manufacturer´s instructions. The homeostatic model assessment for insulin resistance (HOMA-IR) was used to assess insulin resistance according to the formula described by Matthews and collaborators [21].

Quantification of hepatic lipid content

Total lipids were extracted from about 600 mg of hepatic tissue and quantified by the method of Folch *et al*. [22].

Histological analysis

Liver samples of 5 animals per group were used for histological analysis. Liver tissue samples were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C. Following, they were washed in phosphate buffer, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin blocks. Five-micrometer-thick sections of tissues were cut with a microtome and mounted on slides.

Liver sections were classified into four grades depending on fat accumulation following Burnt *et al.* classification [23]: grade 0 was assigned when there was no fat accumulation; grade 1 when fat vacuoles were observed in less than 33% of hepatocytes; grade 2 when 33-66% of hepatocytes contained fat vacuoles; and grade 3 when they were found in more than 66% of hepatocytes.

RNA Extraction

Total RNA was extracted from the liver by Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) and from retroperitoneal white adipose tissue (rWAT) by an E.Z.N.A. RNA purification system (Omega Biotek, Inc., Norcross, GA) each one according to the manufacturer´s instructions. Isolated RNA was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE, USA). Its integrity was confirmed using agarose gel electrophoresis.

Real-time quantitative PCR analysis

Real-time polymerase chain reaction was used to measure mRNA expression levels of selected genes in the liver and rWAT. Precisely, in liver: sterol regulatory element binding transcription factor 1 (*Srebf1*), fatty acid synthase (*Fasn*), sterol coenzyme A desaturase (*Scd1*), glyceraldehyde 3-phosphate dehydrogenase (*Gadph),* carnitine palmitoyltransferase 1a, liver (*Cpt1a*), peroxisome proliferator activated receptor alpha (*Ppara*), fibroblast growth factor 21 (*Fgf21*), Glucokinase (*Gck*), pyruvate kinase (*Pklr*), insulin receptor (*Insr*), insulin receptor substrate 1 (*Irs1*), leptin receptor (*Lepr*), transient receptor potential cation channel, subfamily V, member 1 (*Trpv1*), and uncoupling protein 2 (*Ucp2*). In WAT: peroxisome proliferator activated receptor gamma (*Pparg*), *Srebf1, Fasn*, glycerol-3-phosphate acyltransferase (*Gpat*), lipoprotein lipase (*Lpl*), CD36 molecule (*Cd36*), patatin-like phospholipase domain containing 2 (*Pnpla2*), hormone-sensitive lipase (*Lipe*), carnitine palmitoyltransferase 1b, muscle isoform (*Cpt1b*), glucose transporter 4 (*Slc2a4*), hexokinase II (*HK2*), and *Insr.* Guanosine Diphosphate Dissociation Inhibitor (*Gdi*) was used as a reference gene. All primers were obtained from Sigma Genosys (Sigma Aldrich Co., LLC, Madrid, Spain).

Total RNA (0.25 µg, in a final volume of 5 µL) was denatured at 65 °C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain) at 20 °C for 15 min and 42 °C for 30 min with a final step of 5 min at 95 °C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems). Each polymerase chain reaction (PCR) was performed from diluted (1/20) cDNA template, forward and reverse primers (1 µM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the Applied Biosystems StepOnePlus real-time PCR system with the following profile: 10 min at 95 °C, followed by a total of 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C). To verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.2.2), and the relative expression of each mRNA was calculated as a percentage of male control rats using de $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All data are expressed as the mean ± Standard Error of the Mean (SEM.). Data were checked for normality using Shapiro-Wilks normality test. For multi-group comparisons Levene´s test was performed to assess whether the variance is equal between groups; if the variance was heterogeneous, data were log-transformed before analysis. Differences among groups were assessed by one-way ANOVA followed by least significances difference (LSD) *post-hoc* comparison. The effect of fasting versus *ad libitum* feeding in blood parameters was assessed with a paired *t* test. The analyses were performed with IBM SPSSP Statistics 21. Threshold of significance was defined at $P < 0.05$.

Results

Body weight, food intake and circulating parameters

Results on body weight and body fat, liver weight, and food intake of the 5 groups of animals are summarized in Table 1.

At the age of 5 months, after 8 weeks of treatment, no significant differences in body weight were found among the 5 experimental groups. However, the WD group, but not the CAP group, showed higher body weight gain than controls. Animals of the HESP and HESP+CAP groups showed intermediate values ($P<0.05$, LSD *post-hoc* analysis). Capsaicin-treated animals also displayed, at the end of the intervention period, lower body fat percentage than animals of the WD group, but higher than the control group. Body fat content in HESP and the HESP+CAP groups was slightly higher than that of the CAP group, and not significantly different from the WD group ($P<0.05$, LSD post-hoc analysis). Animals in the WD group, but not animals in the CAP group, also showed higher liver weight than the controls, whereas animals treated with hesperidin or with the combination of both compounds showed intermediate values ($P<0.05$, LSD *post-hoc* analysis). Regarding food intake, animals of the WD group showed greater cumulative energy intake than the controls, whereas WD-fed animals treated with hesperidin, capsaicin or the combination of both compounds showed intermediate values ($P<0.05$, LSD *post-hoc* analysis).

Circulating levels of glucose, insulin, leptin, TG and NEFA under *ad libitum* and fasting conditions are showed in Table 2. Non-significant differences were found among groups in glucose and insulin levels in both conditions. However, the HOMA-IR was different among groups. Capsaicin-treated animals showed lower values with respect to the animals from the WD and HESP groups

(P<0.05, LSD *post-hoc* analysis). Animals of the control and HESP+CAP groups showed intermediate levels. Glucose and insulin levels were lower under fasting conditions with respect to levels in the fed state in all groups except in the HESP+CAP group (for both glucose and insulin) and in the HESP group (for insulin) (P<0.05, Paired *t* test). Animals on the WD group showed greater plasma leptin levels (both under fed and fasting conditions) than control animals (P<0.05, LSD *post-hoc* analysis). Notably, animals of the CAP and HESP+CAP groups showed lower fed-state leptin levels than animals on the WD group and intermediate levels between control and WD animals under fasting conditions. All groups showed lower leptin levels under fasting conditions compared to levels under feeding conditions (P<0.05, Paired *t* test). Animals on the WD group showed higher circulating TG levels under feeding conditions than the control animals (P<0.05, LSD *post-hoc* analysis). TG levels were partially normalised to control levels in the HESP and CAP groups, but not in the HESP+CAP group (P<0.05, LSD post-hoc analysis). No significant differences were found among groups regarding TG levels under fasting conditions. However, fasting TG levels were significantly lower than fed-state TG levels in the WD and HESP+CAP groups.

Regarding NEFA, animals on the WD group showed higher circulating levels under feeding conditions than the control animals (P<0.05, LSD *post-hoc* analysis). HESP, CAP and HESP+CAP groups showed lower levels than animals on the WD group, but higher than controls (P<0.05, LSD *post-hoc* analysis). No significant differences were found among groups regarding NEFA levels under fasting conditions. NEFA levels increased under fasting conditions in the control and HESP groups (p<0.05, paired *t* test).

Energy expenditure, locomotive activity and respiratory exchange ratio

No differences were found concerning energy expenditure or locomotive activity in the 5 experimental groups at the age of 5 months (Figure 1A). Respiratory exchange ratio (RER) was decreased in all western diet-fed groups with respect to controls ($P<0.05$, LSD *post-hoc* analysis), but no differences were found among them (Figure 1A).

Blood pressure

Animals fed a western diet and treated with hesperidin showed lower systolic and diastolic blood pressure than WD-fed animals and even the control animals ($P<0.05$, LSD *post-hoc* analysis) (Figure 1B). The treatment with capsaicin or the combination of hesperidin and capsaicin was also associated with decreased values of diastolic blood pressure, and a trend to lower systolic blood pressure, reaching values not significantly different from controls.

Hepatic lipid content and histological analysis

Figure 2A shows the hepatic lipid content of animals after the intervention period. Animals of the WD group displayed a significant increase (112%) in the lipid content with respect to controls. Notably, WD-fed animals treated with either hesperidin or capsaicin separately and, to a lesser extent with the combination, showed significantly lower hepatic lipid content with respect to the WD group (39%, 38%, and 15% decrease, respectively). Nevertheless, all western diet-fed groups displayed higher hepatic lipid content than controls ($P<0.05$, LSD *post-hoc* analysis). Liver histological analysis (Figure 2B-F) revealed no signs of steatosis in the control animals. However, WD-fed groups presented different degrees of hepatic steatosis: grade 1 (HESP and CAP groups), grade 2 (HESP+CAP group), and grade 3 (WD group). Moreover, the WD and HESP+CAP

groups exhibited the presence of hepatocyte ballooning, necrotic hepatocytes and infiltrated lymphocytes, which are indicative characteristics of non-alcoholic steatohepatitis (NASH), and distinguished from simple steatosis [23]. These results suggest that hesperidin and capsaicin separately, but not the combination, can prevent the development of NASH induced by western diet feeding.

Expression of energy metabolism-related genes in liver

Expression levels of selected genes related to energy metabolism in liver in the different groups of animals are summarized in Figure 3.

WD group presented higher expression levels of the lipogenesis-related genes *Srebf1*, and *Scd1* with respect to controls ($P<0.05$, LSD *post-hoc* analysis). The increase was prevented in hesperidin-treated animals and, in the case of *Srebf1*, in animals treated with the combination of both molecules. Animals treated with capsaicin or the combination showed a trend to lower *Scd1* mRNA expression levels than WD-fed animals, but not different from the controls ($P<0.05$, LSD *post-hoc* analysis).

Animals treated with hesperidin, capsaicin or the combination also showed lower expression levels of *Fasn* with respect to animals of the WD group ($P<0.05$, LSD *post-hoc* analysis), whereas the control animals showed intermediate levels. Animals treated with the combination displayed greater expression levels of *Gadph* than the controls and heperidin-treated animals, whereas the WD and CAP groups exhibited intermediate levels ($P<0.05$, LSD *post-hoc* analysis).

Regarding fatty acid oxidation-related genes, the WD group showed increased expression levels of *Ppara* and *Cpt1a*, whereas animals of the HESP and CAP

groups showed decreased (or a trend to decreased) expression levels, reaching values not different from controls. However, this trend was not observed in animals treated with the combination, which exhibited expression levels similar to the WD group. A similar pattern was observed for mRNA expression levels of *Fgf21*, but differences among groups were not significant.

The expression levels of the glucose metabolism related gene *Pklr*, was significantly increased in the WD group with respect to controls ($P<0.05$, LSD *post-hoc* analysis). Notably, hesperidin and capsaicin, individually or the combination of both, normalised their expression to the control levels. No significant differences were found regarding *Gck* expression levels among groups.

Regarding insulin and leptin signaling, the HESP, CAP and HESP+CAP groups showed lower expression levels of *Insr* than control and WD groups, with no significant differences among them ($P<0.05$, LSD *post-hoc* analysis); however, no significant changes were observed in *Irs1* expression among groups. In turn, the HESP group showed decreased *Lepr* mRNA expression levels than the WD and the HESP+CAP groups, while the controls and animals treated with capsaicin showed intermediate levels ($P<0.05$, LSD *post-hoc* analysis).

The expression levels of capsaicin receptor *Trpv1* and of *Ucp2* were also studied to ascertain potential mechanisms involved in the hepatic health benefits shown by capsaicin treatment alone, but not by the combination of hesperidin and capsaicin (Figure 4). Animals treated with capsaicin showed greater *Trpv1* expression levels than the controls and animals treated with hesperidin. However, animals treated with the combination displayed intermediate levels, similar to those of the WD group. Regarding *Ucp2*, animals treated with

capsaicin displayed higher expression levels than the control, HESP and HESP + CAP groups (P<0.05, LSD *post-hoc* analysis).

Expression of energy metabolism-related genes in rWAT

The mRNA expression levels of selected genes related to energy metabolism in the rWAT in the different groups of animals are showed in Figure 5. The retroperitoneal depot was chosen as a representative of WAT because of its high metabolic activity compared to other depots [24] and its relationship with the development of insulin resistance and type 2 diabetes [25].

Animals treated with capsaicin or the combination of hesperidin and capsaicin displayed lower expression levels of the lipogenesis-related genes *Pparg* and *Srebf1* with respect to the WD group (P<0.05, LSD *post-hoc* analysis). Animals treated with hesperidin also showed lower expression levels of *Pparg* with respect to the WD group, but the decrease was less marked than that occurring for the CAP and HESP+CAP groups (P<0.05, LSD *post-hoc* analysis). No significant differences were found between the controls and the WD group concerning the expression levels of *Pparg* and *Srebf*. *Fasn* and *Gpat* expression levels were decreased in all groups of animals under WD feeding with respect to controls (P<0.05, LSD *post-hoc* analysis), with no significance differences among them.

Regarding fatty acid uptake-related genes, HESP, CAP and HESP+CAP displayed lower expression levels of *Lpl* than the control and WD groups. The decrease in the CAP and HESP+CAP groups was more marked and significant than that occurring for the HESP group (P<0.05, LSD *post-hoc* analysis). The CAP and HESP+CAP groups also displayed a decrease in *Cd36* mRNA expression levels with respect to the WD group (P<0.05, LSD *post-hoc* analysis) whereas no changes were observed in the HESP group. No significant differences were

found between the controls and the WD group concerning the expression levels of both genes.

The CAP and HESP+CAP groups displayed lower expression levels of the lipolysis related genes *Pnpla2* and *Lipe* with respect to the control, WD and HESP groups ($P<0.05$, LSD *post-hoc* analysis). The HESP group also showed lower mRNA expression levels of *Pnpla2* than the WD group, but the decrease was less marked than that occurring in the CAP and HESP+CAP groups ($P<0.05$, LSD *post-hoc* analysis). No significant differences were found between the control and the WD groups concerning the expression levels of both genes.

The expression of the fatty acid oxidation related gene *Cpt1b* showed a pattern of expression similar to that described for the *Lpl* in the CAP and HESP+CAP groups, and to a lesser extent the HESP group, showing lower expression levels than the control and WD groups ($P<0.05$, LSD *post-hoc* analysis).

As far as glucose uptake and metabolism are concerned, the CAP and HESP+CAP groups also showed lower expression levels of the *Slc2a4* and *Hk2* genes when compared to the control, WD and HESP groups. In turn, the HESP group showed lower expression levels of *Slc2a4* than the controls and of *Hk2* than the WD group ($P<0.05$, LSD *post-hoc* analysis). No significant differences were found between the control and the WD groups concerning the expression levels of both genes.

Regarding insulin signaling, the HESP, CAP and HESP+CAP groups showed reduced expression levels of the *Insr* gene with respect to the WD group. The decrease was more marked in the CAP and HESP+CAP groups, which also showed lower expression levels than those of the HESP and the control groups.

Discussion

The prevalence of obesity and its related metabolic complications has increased in the last decades at an epidemic rate, becoming a serious global health problem [1]. Feeding behavior, and particularly the intake of a high-fat, high-sucrose diet, the so called western diet, may be considered as one of the main determinants [2]. Besides nutritional advises, the development of food-related strategies to prevent or attenuate metabolic syndrome related consequences is of great interest.

Studies performed in last decades have pointed out the bioactive effects of the flavonoid hesperidin against obesity-related alterations, mainly because to its TG-lowering, anti-inflammatory, antioxidant, and insulin-sensitizing effects [5-9, 26]. In turn, capsaicin has a demonstrated anti-obesity effect. It has been shown to reduce body weight gain, hepatic lipid accumulation and insulin resistance induced by high-fat diet feeding in animals [13, 14]. Thus, both hesperidin and capsaicin might be promising bioactives for prevention or treatment of metabolic syndrome components, with potential complementary action. However, the potential benefits of the combination of both compounds preventing or ameliorating diet-induced alterations had not been explored yet. Here, we assessed the effects of hesperidin and capsaicin, alone and in combination, to prevent metabolic alterations related to the metabolic syndrome in rats exposed to a western diet.

As expected, the consumption of a western diet for 8 weeks in adult rats resulted in a higher body weight and body fat gain, and, thereby, the animals exhibited at the end of treatment greater fat content than animals fed standard

diet. Moreover, western diet-fed animals displayed greater leptin levels and higher TG and NEFA levels in the fed state than the controls. Interestingly, capsaicin treatment (4 mg/kg) during 2 months in western diet-fed animals effectively reduced body fat gain, as well as fed-state levels of leptin and NEFAs. In turn, hesperidin treatment (100 mg/kg/day) attenuated body fat increase, as well as the increase in circulating leptin levels associated to western diet feeding, but the effects were lower than those observed by capsaicin and did not reach statistical significance. Other authors have also found no significant effects of hesperidin on body weight in obese mice under low-fat diet, but significant effects were observed when hesperidin was combined with caffeine [27]. Notably, the combination of hesperidin and capsaicin did show lower effects on body fat gain than those observed by capsaicin; in fact, body fat percentage of these animals at the end of the treatment was similar to that of animals treated with hesperidin, and not different from that of western diet-fed animals.

The anti-obesity action of capsaicin (and non-pungent related compounds) has been previously described in both animal and human studies. Evidence suggests that capsaicin decreases body weight by increasing energy expenditure [28-32], stimulating adipose tissue lipid mobilization and fat oxidation [30, 31, 33, 34], and reducing energy intake [29, 32, 35, 36]; however, its concrete mechanism is not clear.

In the present study we show that animals receiving either hesperidin or capsaicin, separately, or the combination, along with a western diet, showed a trend to eat fewer calories than western diet-fed animals (decreases of 5.4%, 7.4% and 7.1%, respectively), reaching values not different from that of the

controls. However, no additive effects were found with the combination of both compounds. Thus, the lower intake may contribute to the lower body fat gain of animals, more marked for the animals that received capsaicin. Nevertheless, in spite of the described effects of capsaicin increasing energy expenditure, we did not observe any significant enhancement in energy expenditure induced by capsaicin, or by hesperidin, at 6 weeks of treatment. The lack of significant effects might be tentatively explained by a reduced sympathetic responsiveness to capsaicin treatment in western diet fed-rats, as described in obese subjects [37], which may impair diet-induced thermogenesis; however, the thermogenic effects of capsaicin in white and brown adipose tissues have not been directly explored here.

The effects of capsaicin in preventing fat accumulation may be explained, at least in part, by the effects on lipid metabolism in adipose tissue, particularly by its lipogenesis inhibitory effect [38]. In fact, animals treated with capsaicin displayed in rWAT decreased expression levels of lipogenesis-related genes (*Pparg* and *Srebf1*), along with decreased expression levels of genes related with fatty acid uptake (*Lpl*, *Cd36*) and oxidation (*Cpt1b*), lipolysis (*Pnpla2*, *Lipe*), and glucose uptake and metabolism (*Slc2a4*, *Hk2*). These effects may be tentatively related with a decreased insulin action in the adipose tissue, as deduced from the trend to lower insulin levels found in animals in the capsaicin group, together with the decreased expression levels of the *Insr* gene with respect to animals in the WD group. Expression levels of some of the above-mentioned genes (*Pparg*, *Lpl*, *Pnpla2*, *Cpt1b*, *Hk2*, and *Insr*) were also diminished in animals treated with hesperidin, with respect to western diet-fed animals, but the effects were generally less marked than those observed with

capsaicin, in accordance with the more modest effect of hesperidin on body fat gain. The combination of capsaicin and hesperidin also elicited no additional effects to those observed with capsaicin alone.

According to the improvement of lipid metabolism in adipose tissue, capsaicin treatment also showed interesting effects in preventing diet-induced insulin resistance. Capsaicin treatment prevented the increase in HOMA-IR in western diet-fed rats, but notably, the effects were reduced when capsaicin was combined with hesperidin. The capacity of capsaicin in ameliorating insulin resistance has been previously described in rodents [13]. Several studies in humans have also revealed that capsaicin has a modest effect in type 2 diabetes [39]. Unlike capsaicin, no significant effects were observed for hesperidin on insulin resistance in western diet-fed rats.

Treatment with either hesperidin or capsaicin alone, but not with the combination of both bioactives, attenuated the increase of circulating TG levels in the fed-state occurring in western diet-fed rats. In addition, both compounds separately, and to a lesser extent the combination, attenuated hepatic lipid increase due to western diet feeding. Moreover, histological analysis unveiled that both hesperidin and capsaicin, when administered alone, conferred protective effect on the development of NAFLD and prevented the features of NASH, such as hepatocyte ballooning, necrotic hepatocytes and infiltrated lymphocytes, found in western diet-fed animals. However, some signs of NASH were found in animals treated with the combination of both bioactives. Hesperidin has been previously described to reduce serum TG in animal models [40-42] and hypertriglyceridemic subjects [7]. The mechanisms involved in the TG-lowering effects of hesperidin have been reported to be the reduction of

hepatic TG content through inhibition of lipogenesis and induction of fatty acid oxidation [42], and the down regulation of synthesis and secretion of very-low-density lipoproteins (VLDL) [43]. According to this notion, we observed here that hesperidin-treated animals displayed decreased expression levels of three key lipogenesis-related genes, *Srebf1*, *Fasn* and *Scd1*, as well as normalization of *Pklr* gene expression to control levels. In addition, these animals exhibited decreased hepatic expression levels of the *Insr* and *Lepr* genes, compared to levels of expression in western diet-fed rats, suggesting a decreased lipogenic action of insulin, along with decreased fatty oxidation activity. Although there is no clear consensus in the literature on the effects of leptin in liver [44], it has been proposed that leptin may have insulin sensitizing effects, controlling the extent of insulin action on this tissue [45]. Notably, disruption of hepatic leptin signaling has been shown to protect mice from diet-related glucose intolerance [45]. Therefore, it is suggested that the decreased hepatic lipogenesis in hesperidin-treated animals, associated to a decreased insulin action on this tissue, may be one of the mechanisms whereby this bioactive protects against diet-induced hepatic pathologies.

In turn, capsaicin treatment elicited little effects on the expression of hepatic lipogenesis-related genes compared to changes observed with hesperidin. It has been reported that capsaicin regulates hepatic lipid metabolism and prevent lipid deposition in liver through TRPV1 activation [19]. Capsaicin action on TRPV1 involves up-regulation of UCP2, a mitochondrial membrane transporter that can provide fatty acid translocation, preventing its accumulation in the mitochondrial matrix [19]. Here, the presence of higher expression levels of *Ucp2* in capsaicin-treated animals compared to the other groups is in

accordance with the involvement of UCP2 in the protective effects of capsaicin on lipid deposition in liver, providing protective effects from hepatocellular lipotoxicity [46].

Therefore, both capsaicin and hesperidin, separately, are able to overcome the effects of western diet on hepatic lipid metabolism, and hence ameliorate hepatic steatosis and prevent NASH associated with western diet consumption. Unexpectedly, the effects were blunted or even negligible when animals were treated with both compounds simultaneously. Mechanisms underlying this interaction are unknown, but the comparison of gene expression patterns in liver has given some clues. On the one hand, capsaicin treatment blocked the decrease in the hepatic expression of *Lepr* induced by hesperidin, which may be tentatively associated with an increase in insulin-induced lipogenesis activity in this tissue. In fact, the combination of both compounds up-regulated the expression of the lipogenic gene *Gadph*, reaching levels higher than those of animals treated with hesperidin alone. On the other hand, hesperidin seems to override the effects of capsaicin through its TRPV1 receptor. In fact, induction of *Trpv1* expression by capsaicin was attenuated with the simultaneous treatment with hesperidin, suggesting that the responsiveness to the capsaicin action was blunted in animals treated with the combination of bioactives. Accordingly, animals treated with the combination of bioactives did not show the increase in *Ucp2* expression levels characteristic of capsaicin treated animals. Thus, it is suggested that both bioactives may mutually impair their ways of action on the improvement of liver health in western diet-fed animals.

Finally, regarding other components of the metabolic syndrome, results of the present study also bring evidence supporting the hypotensive effects of

hesperidin and, to a lesser extent, of capsaicin. In fact, systolic and diastolic blood pressure was decreased in hesperidin treated animals, compared to untreated western diet-fed rats, reaching levels lower than the controls. Animals treated with capsaicin or the combination of hesperidin and capsaicin also exhibited lower diastolic blood pressure than western diet-fed animals, and similar to control animals. This suggests interesting anti-hypertension effects of hesperidin, beyond their potential fat-lowering effects. The hypotensive effect of hesperidin has been previously described both at short- and long-term treatments in models of hypertensive rats [47, 48], and this has been related to the capacity to improve endothelium-dependent vasorelaxation by increasing the availability of nitric oxide [49, 50].

In summary, the results of the present study show that capsaicin and hesperidin, separately exhibit different effects on fat accumulation and metabolic syndrome related disorders in rats fed on western diet. More precisely, capsaicin induces protective phenotype against obesity, decreasing body fat gain and preventing insulin resistance under western diet feeding, whereas hesperidin has little effects on body fat gain and no apparent effects on insulin resistance. No additive effects, or even blunted with respect to those observed with capsaicin, were observed with the combination of both bioactives. However, capsaicin and hesperidin alone, improve blood lipid profile, diminish hepatic lipid accumulation, and prevent NASH in western diet-fed rats, although the effects are mitigated or even annulled with the combination of both compounds. In turn, hesperidin alone, and to a lesser extent capsaicin or the combination of hesperidin plus capsaicin, display hypotensive effects in western diet-fed rats. Therefore, these results give

additional evidence supporting that intervention with either capsaicin or hesperidin alone may be promising in populations at high risk of metabolic syndrome-related alterations, particularly fatty liver disease. However, negative results found with the combination of both compounds deserve to be taken into account when considering potential mixtures of bioactives as strategies for obesity prevention.

References

[1] WHO. Obesity and overweight. Fact sheet N°311. Updated oct 2017. http://www.who.int/mediacentre/factsheets/fs311/en/.
[2] Cordain, L., Eaton, S. B., Sebastian, A., Mann, N.*, et al.*, Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr* 2005, *81*, 341-354.
[3] Chan, R. S., Woo, J., Prevention of overweight and obesity: how effective is the current public health approach. *Int J Environ Res Public Health* 2010, *7*, 765-783.
[4] Redan, B. W., Buhman, K. K., Novotny, J. A., Ferruzzi, M. G., Altered Transport and Metabolism of Phenolic Compounds in Obesity and Diabetes: Implications for Functional Food Development and Assessment. *Adv Nutr* 2016, *7*, 1090-1104.
[5] Peng, H., Wei, Z., Luo, H., Yang, Y.*, et al.*, Inhibition of Fat Accumulation by Hesperidin in Caenorhabditis elegans. *J Agric Food Chem* 2016, *64*, 5207-5214.
[6] Assini, J. M., Mulvihill, E. E., Huff, M. W., Citrus flavonoids and lipid metabolism. *Curr Opin Lipidol* 2013, *24*, 34-40.
[7] Miwa, Y., Mitsuzumi, H., Sunayama, T., Yamada, M.*, et al.*, Glucosyl hesperidin lowers serum triglyceride level in hypertriglyceridemic subjects through the improvement of very low-density lipoprotein metabolic abnormality. *J Nutr Sci Vitaminol (Tokyo)* 2005, *51*, 460-470.
[8] Bok, S. H., Lee, S. H., Park, Y. B., Bae, K. H.*, et al.*, Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA: cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids. *J Nutr* 1999, *129*, 1182-1185.
[9] Li, C., Schluesener, H., Health-promoting effects of the citrus flavanone hesperidin. *Crit Rev Food Sci Nutr* 2017, *57*, 613-631.
[10] Whiting, S., Derbyshire, E., Tiwari, B. K., Capsaicinoids and capsinoids. A potential role for weight management? A systematic review of the evidence. *Appetite* 2012, *59*, 341-348.
[11] Thiele, R., Mueller-Seitz, E., Petz, M., Chili pepper fruits: presumed precursors of fatty acids characteristic for capsaicinoids. *J Agric Food Chem* 2008, *56*, 4219-4224.
[12] Sharma, S. K., Vij, A. S., Sharma, M., Mechanisms and clinical uses of capsaicin. *Eur J Pharmacol* 2013, *720*, 55-62.
[13] Kang, J. H., Goto, T., Han, I. S., Kawada, T.*, et al.*, Dietary capsaicin reduces obesity-induced insulin resistance and hepatic steatosis in obese mice fed a high-fat diet. *Obesity (Silver Spring)* 2010, *18*, 780-787.

[14] Leung, F. W., Capsaicin as an anti-obesity drug. *Prog Drug Res* 2014, *68*, 171-179.
[15] Zheng, J., Zheng, S., Feng, Q., Zhang, Q., Xiao, X., Dietary capsaicin and its anti-obesity potency: from mechanism to clinical implications. *Biosci Rep* 2017, *37*.
[16] Zhang, L. L., Yan Liu, D., Ma, L. Q., Luo, Z. D*., et al.*, Activation of transient receptor potential vanilloid type-1 channel prevents adipogenesis and obesity. *Circ Res* 2007, *100*, 1063-1070.
[17] Montell, C., Birnbaumer, L., Flockerzi, V., The TRP channels, a remarkably functional family. *Cell* 2002, *108*, 595-598.
[18] Fernandes, E. S., Fernandes, M. A., Keeble, J. E., The functions of TRPA1 and TRPV1: moving away from sensory nerves. *Br J Pharmacol* 2012, *166*, 510-521.
[19] Li, L., Chen, J., Ni, Y., Feng, X*., et al.*, TRPV1 activation prevents nonalcoholic fatty liver through UCP2 upregulation in mice. *Pflugers Arch* 2012, *463*, 727-732.
[20] Akabori, H., Yamamoto, H., Tsuchihashi, H., Mori, T*., et al.*, Transient receptor potential vanilloid 1 antagonist, capsazepine, improves survival in a rat hemorrhagic shock model. *Ann Surg* 2007, *245*, 964-970.
[21] Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A*., et al.*, Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985, *28*, 412-419.
[22] Folch, J., Lees, M., Sloane Stanley, G. H., A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957, *226*, 497-509.
[23] Brunt, E. M., Janney, C. G., Di Bisceglie, A. M., Neuschwander-Tetri, B. A., Bacon, B. R., Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999, *94*, 2467-2474.
[24] Palou, M., Sánchez, J., Priego, T., Rodríguez, A. M*., et al.*, Regional differences in the expression of genes involved in lipid metabolism in adipose tissue in response to short- and medium-term fasting and refeeding. *J Nutr Biochem* 2010, *21*, 23-33.
[25] Gabriely, I., Ma, X. H., Yang, X. M., Atzmon, G*., et al.*, Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process? *Diabetes* 2002, *51*, 2951-2958.
[26] Mosqueda-Solís, A., Lasa, A., Gómez-Zorita, S., Eseberri, I*., et al.*, Screening of potential anti-adipogenic effects of phenolic compounds showing different chemical structure in 3T3-L1 preadipocytes. *Food Funct* 2017.

[27] Ohara, T., Muroyama, K., Yamamoto, Y., Murosaki, S., A combination of glucosyl hesperidin and caffeine exhibits an anti-obesity effect by inhibition of hepatic lipogenesis in mice. *Phytother Res* 2015, *29*, 310-316.
[28] Kawada, T., Watanabe, T., Takaishi, T., Tanaka, T., Iwai, K., Capsaicin-induced beta-adrenergic action on energy metabolism in rats: influence of capsaicin on oxygen consumption, the respiratory quotient, and substrate utilization. *Proc Soc Exp Biol Med* 1986, *183*, 250-256.
[29] Watanabe, T., Kawada, T., Yamamoto, M., Iwai, K., Capsaicin, a pungent principle of hot red pepper, evokes catecholamine secretion from the adrenal medulla of anesthetized rats. *Biochem Biophys Res Commun* 1987, *142*, 259-264.
[30] Josse, A. R., Sherriffs, S. S., Holwerda, A. M., Andrews, R.*, et al.*, Effects of capsinoid ingestion on energy expenditure and lipid oxidation at rest and during exercise. *Nutr Metab (Lond)* 2010, *7*, 65.
[31] Lee, T. A., Li, Z., Zerlin, A., Heber, D., Effects of dihydrocapsiate on adaptive and diet-induced thermogenesis with a high protein very low calorie diet: a randomized control trial. *Nutr Metab (Lond)* 2010, *7*, 78.
[32] Ludy, M. J., Mattes, R. D., The effects of hedonically acceptable red pepper doses on thermogenesis and appetite. *Physiol Behav* 2011, *102*, 251-258.
[33] Kawada, T., Hagihara, K., Iwai, K., Effects of capsaicin on lipid metabolism in rats fed a high fat diet. *J Nutr* 1986, *116*, 1272-1278.
[34] Lejeune, M. P., Kovacs, E. M., Westerterp-Plantenga, M. S., Effect of capsaicin on substrate oxidation and weight maintenance after modest body-weight loss in human subjects. *Br J Nutr* 2003, *90*, 651-659.
[35] Westerterp-Plantenga, M. S., Smeets, A., Lejeune, M. P., Sensory and gastrointestinal satiety effects of capsaicin on food intake. *Int J Obes (Lond)* 2005, *29*, 682-688.
[36] Yoshioka, M., St-Pierre, S., Drapeau, V., Dionne, I.*, et al.*, Effects of red pepper on appetite and energy intake. *Br J Nutr* 1999, *82*, 115-123.
[37] Matsumoto, T., Miyawaki, C., Ue, H., Yuasa, T.*, et al.*, Effects of capsaicin-containing yellow curry sauce on sympathetic nervous system activity and diet-induced thermogenesis in lean and obese young women. *J Nutr Sci Vitaminol (Tokyo)* 2000, *46*, 309-315.
[38] Hong, Q., Xia, C., Xiangying, H., Quan, Y., Capsinoids suppress fat accumulation via lipid metabolism. *Mol Med Rep* 2015, *11*, 1669-1674.
[39] Effect of treatment with capsaicin on daily activities of patients with painful diabetic neuropathy. Capsaicin Study Group. *Diabetes Care* 1992, *15*, 159-165.

[40] Akiyama, S., Katsumata, S., Suzuki, K., Nakaya, Y*., et al.*, Hypoglycemic and hypolipidemic effects of hesperidin and cyclodextrin-clathrated hesperetin in Goto-Kakizaki rats with type 2 diabetes. *Biosci Biotechnol Biochem* 2009, *73*, 2779-2782.
[41] Chiba, H., Uehara, M., Wu, J., Wang, X*., et al.*, Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. *J Nutr* 2003, *133*, 1892-1897.
[42] Mitsuzumi, HY-YA, N, A., Sadakiyo*, et al.*, Jpn Pharmacol Ther 2011, pp. 727–740.
[43] Miwa, Y., Mitsuzumi, H., Yamada, M., Arai, N*., et al.*, Suppression of apolipoprotein B secretion from HepG2 cells by glucosyl hesperidin. *J Nutr Sci Vitaminol (Tokyo)* 2006, *52*, 223-231.
[44] Ceddia, R. B., Koistinen, H. A., Zierath, J. R., Sweeney, G., Analysis of paradoxical observations on the association between leptin and insulin resistance. *FASEB J* 2002, *16*, 1163-1176.
[45] Huynh, F. K., Levi, J., Denroche, H. C., Gray, S. L*., et al.*, Disruption of hepatic leptin signaling protects mice from age- and diet-related glucose intolerance. *Diabetes* 2010, *59*, 3032-3040.
[46] Baffy, G., Uncoupling protein-2 and non-alcoholic fatty liver disease. *Front Biosci* 2005, *10*, 2082-2096.
[47] Yamamoto, M., Suzuki, A., Hase, T., Short-term effects of glucosyl hesperidin and hesperetin on blood pressure and vascular endothelial function in spontaneously hypertensive rats. *J Nutr Sci Vitaminol (Tokyo)* 2008, *54*, 95-98.
[48] Ohtsuki, K., Abe, A., Mitsuzuwi, H., Kondo, M*., et al.*, Effects of long-term administration of hesperidin and glucosyl hesperidin to spontaneously hypertensive rats. *J Nutr Sci Vitaminol (Tokyo)* 2002, *48*, 420-422.
[49] Rizza, S., Muniyappa, R., Iantorno, M., Kim, J. A*., et al.*, Citrus polyphenol hesperidin stimulates production of nitric oxide in endothelial cells while improving endothelial function and reducing inflammatory markers in patients with metabolic syndrome. *J Clin Endocrinol Metab* 2011, *96*, E782-792.
[50] Yamamoto, M., Jokura, H., Hashizume, K., Ominami, H*., et al.*, Hesperidin metabolite hesperetin-7-O-glucuronide, but not hesperetin-3'-O-glucuronide, exerts hypotensive, vasodilatory, and anti-inflammatory activities. *Food Funct* 2013, *4*, 1346-1351.

Abbreviations: CAP, capsaicin: Cd36, CD36 molecule; Cpt1a, carnitine palmitoyltransferase 1a; Cpt1b, carnitine palmitoyltransferase 1b; Fasn, fatty acid synthase; Fgf21, fibroblast growth factor 21; Gadph, glyceraldehyde 3-phosphate dehydrogenase; Gck; glucokinase; Gdi, guanosine diphosphate dissociation inhibitor; Gpat, glycerol-3-phosphate acyltransferase; HESP, hesperidin; HK2, hexokinase II; HOMA-IR, homeostatic model assessment for insulin resistance; Insr, insulin receptor; Irs1, insulin receptor substrate 1; Lepr, leptin receptor; Lipe, hormone-sensitive lipase; Lpl, lipoprotein lipase; LSD, least significant difference; NEFA, non-esterified fatty acid; PCR, polymerase chain reaction; Pklr, pyruvate kinase; Pnpla2, patatin-like phospholipase domain containing 2; Ppara, peroxisome proliferator activated receptor alpha; Pparg, peroxisome proliferator activated receptor gamma; RER, respiratory exchange ratio; rWAT, retroperitoneal white adipose tissue; Scd1, sterol coenzyme A desaturase; SEM, standard error of the mean; Slc2a4, glucose transporter 4; Srebf1, sterol regulatory element binding transcription factor 1; TG, triacylglycerols; Trpv1, transient receptor potential cation channel subfamily V member 1; Ucp2, uncoupling protein 2; VLDL, very low density lipoprotein; WD, western diet.

Author contributions

CP, AP and MPP conceived and designed the experiments. AMS and JS carried out experimental determinations. AMS, JS, CP and AP, participated in the data analysis and results interpretation. AMS, JS and CP wrote the manuscript, and MPP and AP revised the definitive version. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Spanish Government (AGL2015-67019-P), and the Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición, CIBERobn. Laboratory of Molecular Biology, Nutrition and Biotechnology is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: no. FP6-506360). A. Mosqueda-Solís is a recipient of a doctoral fellowship from the CONACYT (Mexico).

Conflict of interest statement: The authors declare that they have no conflict of interest.

Figure legends

Figure 1. A) Energy expenditure, locomotive activity, and respiratory exchange ratio (RER) after 6 weeks of treatment, and B) systolic and diastolic blood pressure after 5 weeks of treatment, of control (C), western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups. Data are mean ± SEM (n=7-8). Statistical analysis between groups was performed by one-way ANOVA, followed by LSD post-hoc analysis ($P<0.05$), a≠b≠c.

Figure 2. A) Hepatic lipid content, and B-F) representative liver slides stained with hematoxylin/eosin (x20) and numerical grading of hepatic steatosis in liver of control (C), western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups at the end of treatment. In A), data are mean ± SEM (n=7-8). Statistical analysis between groups was performed by one-way ANOVA, followed by LSD post-hoc analysis (P<0.05), a≠b≠c. Symbol in images: PT: portal triad, b: ballooned hepatocytes, arrow: necrotic hepatocyte, arrowhead: lymphocytes.

Figure 3. Expression of genes related to energy metabolism (lipogenesis, fatty acid oxidation, glucose uptake and metabolism, and insulin and leptin signaling) in liver of control (C), western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) rats at the end of treatment. mRNA levels were measured by real-time PCR and expressed as a percentage of the control group. Data are mean ± SEM (n=7-8). Statistical analysis between groups was

performed by one-way ANOVA, followed by LSD post-hoc analysis (P<0.05), a≠b≠c.

Figure 4. Expression levels of *Trpv1* and *Ucp2* in liver of control (C), western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) rats at the end of treatment. mRNA levels were measured by real-time PCR and expressed as a percentage of the control group. Data are mean ± SEM (n=7-8). Statistical analysis between groups was performed by one-way ANOVA, followed by LSD post-hoc analysis (P<0.05), a≠b≠c.

Figure 5. Expression of genes related to energy metabolism (lipogenesis, fatty acid uptake, lipolysis, fatty acid oxidation, glucose uptake and metabolism, and insulin signaling) in retroperitoneal white adipose tissue of control (C), western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) rats at the end of treatment. mRNA levels were measured by real-time PCR and expressed as a percentage of the control group. Data are mean ± SEM (n=7-8). Statistical analysis between groups was performed by one-way ANOVA, followed by LSD post-hoc analysis (P<0.05), a≠b≠c.

Table 1. Weight-related parameters and food intake.

	Control	WD	HESP	CAP	HESP+CAP
nitial body weight (g)	362 ± 16	353 ± 8	351 ± 15	356 ± 11	349±16
Final body weight (g)	428 ± 19	443± 14	433 ± 14	423 ± 15	433±19
Body weight gain (3-5 months) (g)	66 ± 7[a]	91 ± 9[b]	83 ± 4[a,b]	67± 4[a]	75±8[a,b]
nitial body fat (%)	10.8±0.7	10.8±0.8	10.7±0.7	10.9±0.4	10.9±0.8
Final body fat (%)	11.5±0.5[a]	18.4±1.5[b]	15.9±0.5[b,c]	15.1±0.4[c]	15.9±1.0[b,c]
Liver (g)	12.5 ± 0.7[a]	14.6 ± 0.3[b]	13.9 ± 0.5[a,b]	13.0 ± 0.4[a,c]	14.1±0.5[b,c]
Cumulative food intake (3-5 months) (Kcal)	3970 ± 151[a]	4683 ± 356[b]	4431 ± 104[a,b]	4337± 191[a,b]	4351±242[a,b]

Data are mean ± SEM. One-way ANOVA was used to determine differences between groups followed by a least significance difference (LSD) *post hoc* analysis ($P<0.05$), a≠b≠c.

Table 2. Blood parameters.

	Control	WD	HESP	CAP	HESP+CAP
Glucose (mg dl^{-1})					
Ad libitum	127±4	123±3	119±4	119±2	120±5
14h fasting	100±5*	104±8*	100±3*	97±4*	107±5
Insulin (µg l^{-1})					
Ad libitum	1.29±0.25	1.60±0.24	1.56±0.10	1.22±0.09	1.71±0.36
14h fasting	0.83±0.24*	1.07±0.18*	1.10±0.20	0.72±0.14*	0.75±0.11
HOMA-IR					
14h fasting	3.69 ± 0.80^{a,b}	7.07 ± 1.38^{a}	6.77± 1.31^{a}	3.60 ± 0.68^{b}	4.93 ± 0.71^{a,b}
Leptin (µg l^{-1})					
Ad libitum	5.30±0.45^{a}	12.19±1.65^{b}	9.71±1.04^{b,c}	8.08±0.59^{c}	8.20±0.99^{c}
14h fasting	2.08±0.28$^{a\,*}$	5.11±0.97$^{b\,*}$	4.35±0.82$^{b\,*}$	3.37±0.51$^{a,b\,*}$	3.50±0.72$^{a,b\,*}$
TG (mg ml^{-1})					
Ad libitum	0.730±0.127^{a}	1.82±0.25^{b}	1.39±0.22^{a,b}	1.39±0.18^{a,b}	1.8±0.32^{b}
14h fasting	0.769±0.124	0.717±0.049*	0.905±0.141	0.816±0.171	0.928±0.196*
NEFA (mM)					
Ad libitum	0.498±0.036^{a}	0.984±0.084^{b}	0.781±0.046^{c}	0.740±0.046^{c}	0.765±0.063^{c}
14h fasting	1.19±0.11*	0.902±0.058	1.11±0.14*	0.978±0.089	0.938±0.194

Data are mean ± SEM. One-way ANOVA was used to determine differences between groups under ad libitum and fasting condition separately, followed by a least significance difference (LSD) *post-hoc* analysis ($P<0.05$), a≠b≠c. *, $P<0.05$ fasting *versus ad libitum* condition (Paired *t*-test).

Figure 1

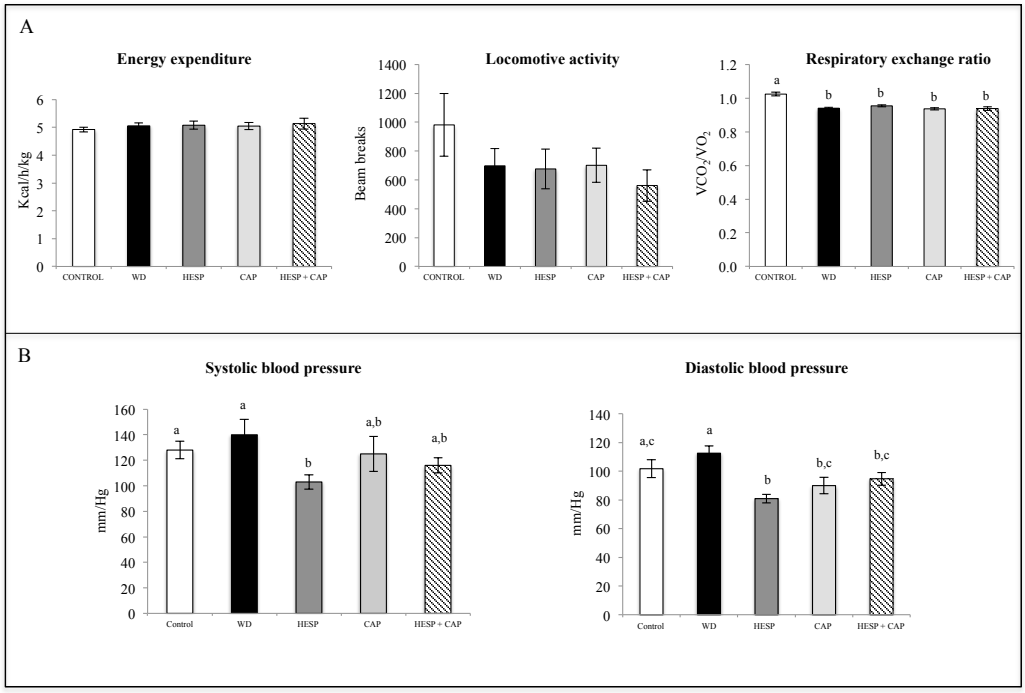

A
Energy expenditure
Kcal/h/kg
0
1
2
3
4
5
6
CONTROL
WD
HESP
CAP
HESP + CAP
Locomotive activity
Beam breaks
0
200
400
600
800
1000
1200
1400
CONTROL
WD
HESP
CAP
HESP + CAP
Respiratory exchange ratio
VCO_2/VO_2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
a
b
b
b
b
CONTROL
WD
HESP
CAP
HESP + CAP
B
Systolic blood pressure
mm/Hg
0
20
40
60
80
100
120
140
160
a
a
b
a,b
a,b
Control
WD
HESP
CAP
HESP + CAP
Diastolic blood pressure
mm/Hg
0
20
40
60
80
100
120
140
a,c
a
b
b,c
b,c
Control
WD
HESP
CAP
HESP + CAP

Figure 2

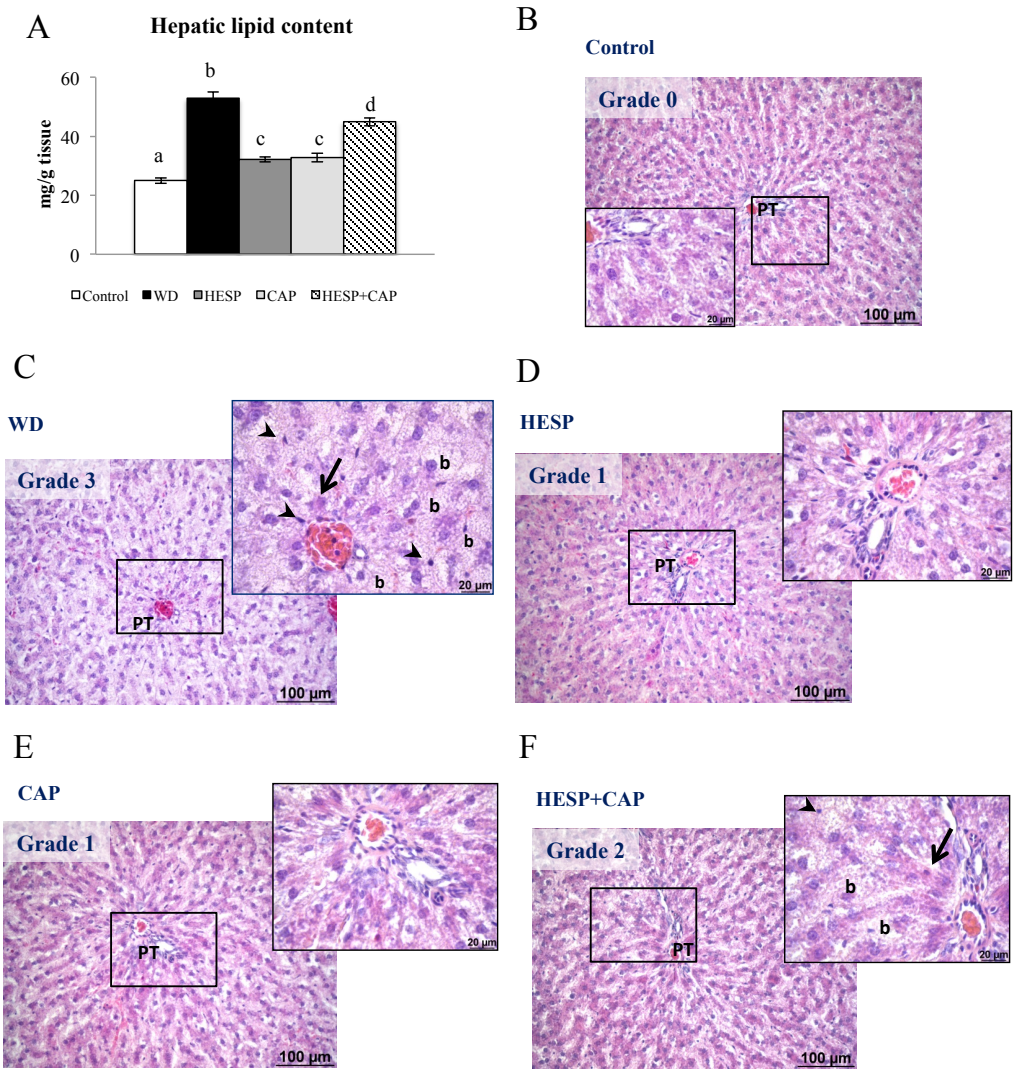

A
Hepatic lipid content
mg/g tissue
60
40
20
0
a
b
c
c
d
Control
WD
HESP
CAP
HESP+CAP
B
Control
Grade 0
PT
20 µm
100 µm
C
WD
Grade 3
PT
b
b
b
b
20 µm
100 µm
D
HESP
Grade 1
PT
20 µm
100 µm
E
CAP
Grade 1
PT
20 µm
100 µm
F
HESP+CAP
Grade 2
PT
b
b
20 µm
100 µm

Figure 3

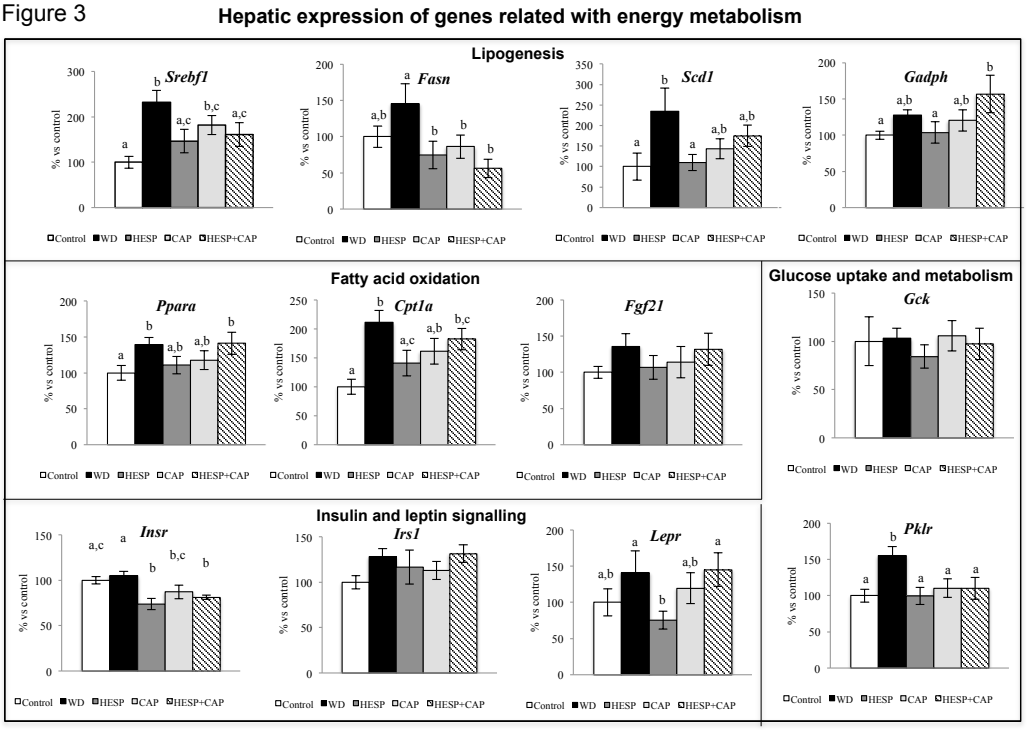


Figure 4

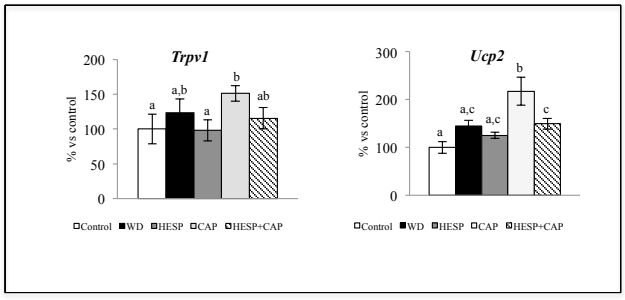
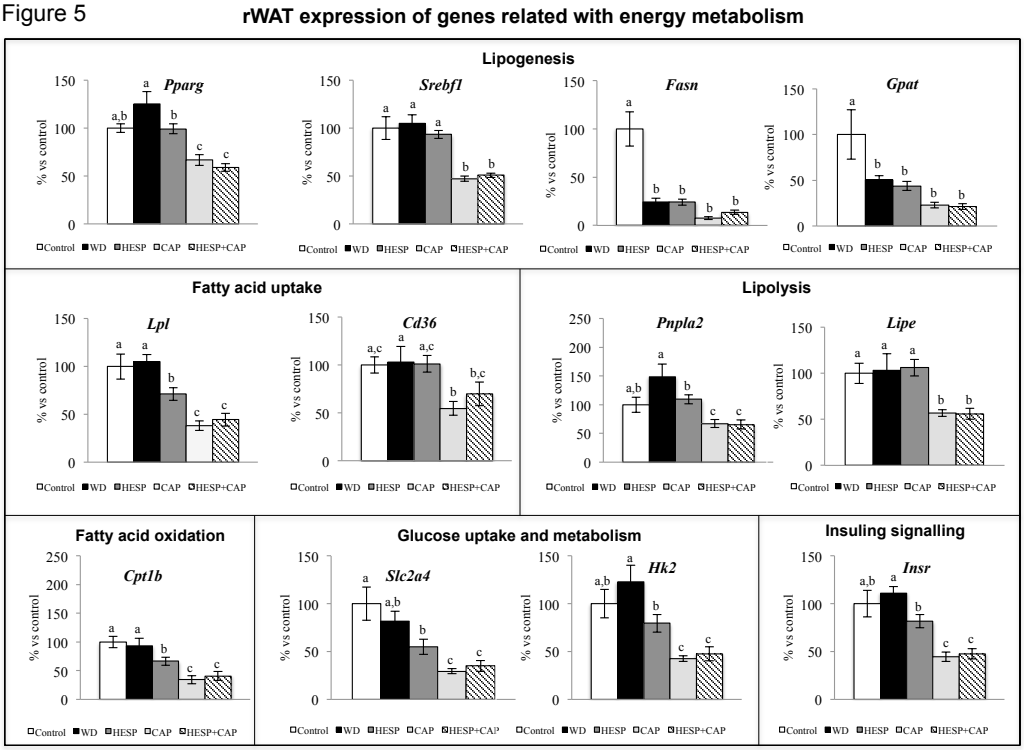


Figure 5


rWAT expression of genes related with energy metabolism
Lipogenesis
Pparg
Srebf1
Fasn
Gpat
Fatty acid uptake
Lpl
Cd36
Lipolysis
Pnpla2
Lipe
Fatty acid oxidation
Cpt1b
Glucose uptake and metabolism
Slc2a4
Hk2
Insuling signalling
Insr
% vs control
Control WD HESP CAP HESP+CAP

Manuscript 3

Effects of capsaicin and hesperidin and its combination on adipose tissue morphology and browning induction in western diet fed rats

Mosqueda-Solís A., Sánchez J., Portillo MP.,Palou A., Picó C

Abstract

Aim: To explore the potential effects of hesperidin and capsaicin separately and in combination to induce white adipose tissue (WAT) browning and help in body weight management in rats feed an obesogenic diet.

Material and methods: Three-month-old male Wistar rats were divided into 5 groups: Control (animals fed a standard diet), WD (animals fed a high fat/sucrose (western) diet), HESP (animals fed a western diet + hesperidin (100 mg/kg/day)), CAP (animals fed a western diet + capsaicin (4 mg/kg/day)), and HESP+CAP (animals fed a western diet + hesperidin (100 mg/kg/day) + capsaicin (4 mg/kg/day)). Body weight, body fat, and energy expenditure were measured. After 8 weeks of treatment, morphological and immunohistochemical studies, and gene expression and protein analysis of brown/brite adipocytes markers were performed in inguinal and retroperitoneal WAT (iWAT, rWAT). UCP1 was also determined in brown adipose tissue (BAT).

Results: Animals treated with capsaicin displayed lower body weight gain and fat content vs WD group, but no differences were detected regarding energy expenditure. Treatment with capsaicin and hesperidin separately, but not the combination, resulted in a decreased size of adipocytes and induced the appearance of multilocular UCP1- and CIDE-A-positive brown-like adipocytes in rWAT. Capsaicin treatment brought about a significant increase in gene expression levels of *Prdm16* (vs control and WD groups) and *Cidea* (vs controls) and in protein levels of

CIDE-A (vs WD group) in iWAT. Hesperidin treatment increased *Cidea* expression levels vs WD group in iWAT. Both capsaicin and hesperidin increased total UCP1 protein levels in BAT vs control.

Conclusion: Our results demonstrate the effects of capsaicin and hesperidin, separately, but not the combination, to reduce adipocyte size, and the capacity of capsaicin and, to a lesser extent, of hesperidin, to induce WAT browning in rats fed a western diet. Therefore, this combination should be avoided in formulations designed to assist body weight loss and management.

Introduction

Health problems related to body weight are major concerns worldwide. Overweight and obesity are associated with increased risks for type II diabetes, cardiovascular diseases, several types of cancer, and other diseases (Pi-Sunyer, 2009).The increase in sedentary lifestyle and consumption of calorie-dense food have notable influence on the prevalence of obesity, which has substantially rise in the last decades (Ng et al., 2014).

The most direct approach to weight control is the reduction of calorie intake and the increase of physical activity, although this strategy fails for many people because of the difficulty of being followed. It is necessary the development of innovative strategies directed towards primary prevention and treatment of overweight and obesity. In this regard, the few medical options for the treatment of obesity point to the potential interest of bioactive compounds for assisting with body weight loss and management.

Adipose tissue is a complex organ with numerous effects on physiology and pathophysiology; the most important function is as a master regulator of energy balance and nutritional homeostasis. White adipose tissue (WAT), mainly composed by unilocular white adipocytes, has a main function to storage energy and release fatty acids when fuel is required, while brown adipose tissue (BAT), with multilocular adipocytes, is specialized in dissipate stored chemical energy in the form of heat (thermogenesis) as a result of the action of uncoupling protein-1 (UCP-1), a specific protein located on the inner mitochondrial membrane (Cannon & Nedergaard, 2004; Giralt & Villarroya, 2013; Spiegelman,

2013) UCP-1 plays a major role in energy homeostasis in rodents (Palou, Pico, Bonet, & Oliver, 1998) and a deficient BAT is found in almost all animal models of obesity (Himms-Hagen, 1990). The evidence of the existence of an active BAT in adult humans has unveiled new strategies to face obesity (Nedergaard, Bengtsson, & Cannon, 2007). Moreover, the discovery of the appearance of brown-like adipocytes disseminated in WAT of human adults, termed beige or brite (brown in white) adipocytes, in a process known as browning, has further increased their interest as a therapeutic target for obesity (Harms & Seale, 2013). In addition, brown adipocytes may have beneficial metabolic effects, since they are able to utilize blood glucose and lipid and improve glucose metabolism and lipidemia independently of weight loss (Kim & Plutzky, 2016). Browning can appear in WAT in response to specific stimuli, such as cold exposure, but also in response to specific dietary components (Bonet, Oliver, & Palou, 2013) or particular diets, such as high-fat diets (García-Ruiz et al., 2015). Therefore, bioactive factors able to increase BAT and/or convert white into brite adipocytes may help correcting the energy imbalance that underlines obesity. In addition, considering that nutritional factors generally exert limited effects, the combination thereof or with other bioactive agents that target other processes related to adipocyte metabolism, such as adipogenesis and/or lipolysis, may further facilitate energy metabolism and weight management (Stohs & Badmaev, 2016). For example, the combination of resveratrol and quercetin has been reported to exert increased effects on browning in rats fed an obesogenic diet, while both compounds individually exert little effects (Arias et al., 2017).

In this regard, there is extensive research showing the potential interest of capsaicinoids (also known as capsinoids), a group of molecules naturally present in chilli peppers. The most abundant and studied is capsaicin, which has anti-obesity, anti-diabetic, and anti-inflammatory properties (Whiting, Derbyshire, & Tiwari, 2012). Their anti-obesity effects are related in part to their capacity to activate the sympathetic system and induce BAT thermogenesis(Saito, 2015) and trigger WAT browning by activating transient receptor potential cation channel subfamily V member 1 (TRPV1) (Baskaran, Krishnan, Ren, & Thyagarajan, 2016) Notably, capsaicin and its non-pungent analogs have potential interest as weight control agents because they act as thermogenic agents without causing stimulant and adverse cardiovascular effects (Stohs & Badmaev, 2016).

On the other hand, citrus flavonoids, such as hesperidin, have emerged as promising therapeutic agents for the treatment of metabolic dysregulation. Several studies have pointed out the bioactive effects of hesperidin against obesity-related alterations mainly because of its fat-lowering, antiinflammatory, antioxidant, and insulin-sensitizing effects (Li & Schluesener, 2017). Notably, in a human study, hesperidin has been shown to enhance the thermogenic effect of p-synephrine, the primary protoalkaloid in bitter orange (Stohs et al., 2011). Therefore, it is suggested that appropriate combination of thermogenic agents with flavonoids or other fat-lowering bioactives could potentially increase the effects of individual compounds and be highly valuable in the nutritional field to assist weight management.

Considering the ability of capsaicin to stimulate thermogenesis and the lipid-lowering effects of hesperidin, here we attempted to explore the potential effects of these compounds alone and in combination to induce WAT browning and help in body weight management in rats fed an obesogenic diet.

Material and Methods

Animals and experimental design

The animal protocol followed was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands (Resolution Number 3513. March, 2012) and guidelines for the use and care of laboratory animals of the University were followed.

The study was performed in three-months-old 39 male Wistar rats randomly divided into 5 groups: Control (n=7): animals fed with a standard chow diet; WD (n=8): animals fed a high sucrose and high fat diet ("western" diet); HESP (n=8): animals fed a western diet + hesperidin (100 mg/kg/day); CAP: animals fed a western diet + capsaicin (4 mg/kg/day)(n=8); and, HESP+CAP (n=8): animals fed a western diet + hesperidin (100 mg/kg/day) + capsaicin (4 mg/kg/day). The animals were kept with this treatment for 8 weeks, until animals aged 5 months. All rats were individually housed under controlled temperature (22°C) and a 12 hours light/dark cycle and had unlimited access to tap water and RD Western Diet (4.7 kcal/g, with 17% Kcal from protein, 43% from carbohydrate, and 41% from fat) (Research Diets, Inn, New Brunswick, NJ, USA) or Standard Diet (3.3 kcal/g, with 19 % Kcal from protein, 73 % from carbohydrate, and 8% from fat) (Pan-lab, Barcelona, Spain).

After 8 weeks of treatment, animals were sacrificed by decapitation under fed condition. Retroperitoneal and inguinal WAT depots (rWAT and iWAT, respectively), and interscapular brown adipose tissue (BAT) were rapidly removed, weighted, frozen in liquid nitrogen, and stored at -80°C until subsequence's studies. Other WAT depots (mesenteric and epididymal) were also weighted.

Reagents

Hesperidin and Capsaicin were purchased from Sigma-Aldrich Co., LLC, (St. Louis, MO).

Body weight parameters

Body weight was daily monitored during the 8 weeks of treatment. Body composition (by EchoMRI-700TM, Echo Medical Systems, LLC., TX, USA) was measured at baseline, at week 4 and at the end of the treatment (week 8).

Indirect calorimetry

Energy expenditure was assessed after 6 weeks of treatment by indirect calorimetry by using the LabMaster-CalSys-Calorimetry System (TSE Systems, Bad Homburg, Germany). Animals were individually housed and acclimated to the respiratory cages for 24 hours before the measurement began, to reduce potential stress. Data on gas exchanges (VO2; ml kg -1h-1 and VCO2; ml kg -1h-1) were measured via an open circuit indirect calorimetry system for 24 hours. To calculate energy expenditure (kcal/h), rates of oxygen consumption and carbon dioxide production were monitored for 5 min every 45 min for each animal or

reference cage (our system can handle 8 animal cages and 1 reference cage, simultaneously).

Morphometric and immunohistochemical analyses

Retroperitoneal and inguinal WAT samples were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4 °C, washed in phosphate buffer, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin blocks for light microscopy. Five-micrometer-thick sections of tissues were cut with a microtome and mounted on slides. The area of white adipocytes from six to seven animals was measured in hematoxylin/eosin-stained sections of inguinal and retroperitoneal WAT depots. Images from light microscopy were digitized and the area of a cells in a fixed area (around $1mm^2$) was determined using Axio Vision software (Carl Zeiss Imaging Solutions, Barcelona, Spain).

The percentage of multilocularity was determined by quantifying the area of multilocular adipocytes in relation to the entire area of the section.

Immunohistochemistry analysis

Immunohistochemistry analysis of CIDE-A (cell death-inducing DNA fragmentation factor alpha-like effector A) and UCP1 was performed in the rWAT, since it is the depot in which multilocular adipocytes were detected. Five-micrometer-thick sections of rWAT from the different experimental groups were immunostained by means of the avidin–biotin

technique (Hsu SM., 1981). Briefly, serial sections were incubated with normal goat serum 2% in phosphate-buffered saline pH 7.3 to block unspecific sites and then overnight at 4°C with primary rabbit polyclonal UCP1 antibody (GeneTex International Corporation) diluted 1:300 in phosphate-buffered saline and with CIDE-A primary rabbit polyclonal antibody (Sigma) diluted 1:150 in phosphate-buffered saline. Sections were then incubated with the corresponding biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA), diluted 1:200 and finally with ABC complex (Vectastain ABC Kit, Vector Laboratories). Peroxidase activity was revealed with Sigma Fast 3,3'-diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA) as substrate. Finally, sections were counterstained with hematoxilin and mounted in Eukitt (Kindler, Germany). Images were acquired with a Zeiss Axioskop 2 microscope equipped with AxioCam ICc3 digital camera and AxioVision 40 V 4.6.3.0 Software (Carl Zeiss, S.A., Barcelona, Spain).

RNA Extraction

Total RNA was extracted from the rWAT, iWAT and BAT depots by an E.Z.N.A. RNA purification system (Omega Biotek, Inc., Norcross, GA) according to the manufacturer´s instructions. Isolated RNA was quantified using NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Wilmington, DE, USA). Its integrity was confirmed using agarose gel electrophoresis.

Real-time quantitative PCR analysis

Real-time polymerase chain reaction was used to measure mRNA expression levels of selected genes in rWAT and iWAT. Specifically: alpha subunit- like effector A (*Cidea*), Homeobox C9 (*Hoxc9*), peroxisome

proliferator-activated receptor c coactivator 1(*Pgc1α*), PRD1-BF1-RIZ1 homologous domain-containing protein-16 (*Prdm16*), uncoupling protein 1 (*Ucp1*) and transient receptor potential cation channel, subfamily V member 1 (*Trpv1*).

Guanosine Diphosphate Dissociation Inhibitor (*Gdi*) and Low-density lipoprotein receptor related protein 10 (*Lrp10*) were used as reference genes. All primers were obtained from Sigma Genosys (Sigma Aldrich Co., LLC, Madrid, Spain). The nucleotide sequences of primers and amplicon size used for performing qRT-PCR are described in supplementary Table 1.

Summarily, a 0.25 µg of total RNA (in a final volume of 5 µL) was denatured at 65 °C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystems, Madrid, Spain) at 20 °C for 15 min and 42 °C for 30 min with a final step of 5 min at 95 °C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems). Each polymerase chain reaction (PCR) was performed from diluted (1/20) cDNA template, forward and reverse primers (1 µM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the Applied Biosystems StepOnePlus real-time PCR system with the following profile: 10 min at 95 °C, followed by a total of 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C). To verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne

Software v2.2.2), and the relative expression of each mRNA was calculated as a percentage of male control rats using de $2^{-\Delta\Delta Ct}$ method.

Western Blot analysis

UCP1 and CIDE-A protein levels were determined by western blotting in iWAT, rWAT and BAT depots. iWAT, rWAT and BAT were homogenized separately at 4 °C in 1:5 (w:v). In all, 100 µg (for iWAT), 50 µg (for rWAT) and 5 µg (for BAT) of RIPA buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher, Rockford, IL, USA). Western blot was performed in a 4–20% Criterion TGX Precast (BioRad, Madrid, Spain), and transferred to a nitrocellulose membrane. The primary antibodies used were the following: rabbit anti-UCP1 (catalogue number GTX10983, GeneTex, Inc. (Irvine, CA, USA), and rabbit anti-CIDE-A (catalogue number 7977, Sigma-Aldrich (St. Louis, MO, USA)), diluted 1:1000 in TBS-T for UCP1 and 1:1000 for CIDE-A. Afterwards, membranes were also incubated with anti-HSP90 antibody (catalogue number 4877, Cell Signaling Inc. (Danvers, MA, USA)) diluted 1:1000 in Odyssey Blocking Buffer (LI-COR Biosciences) or anti-β-actin antibody diluted 1:1000 (catalog number 3700, Cell signaling, Inc. (Danvers, MA,USA)) to ensure the equal loading. Specific infrared-dyed secondary anti-IgG antibodies (LI-COR Biosciences, Lincoln, NE, USA) were used. For infrared detection, membranes were scanned in Odyssey Imager (LI-COR); the bands were quantified using the software Odyssey V3.0 (LI-COR) with 800- and 700-nm channel.

Statistical Analysis

All data are expressed as the mean ± Standard Error of the Mean (S.E.M.). Data were checked for normality using Shapiro-Wilks normality

test. For multi-group comparisons Levene´s test was performed to assess whether the variance is equal between groups; if the variance was heterogeneous, data were log-transformed before analysis. Differences among groups were assessed by one-way ANOVA followed by least significances difference (LSD) *post hoc* comparison to assess statistical differences between the groups. Differences in body weight gain overtime were assessed by repeated measures ANOVA. The analyses were performed with IBM SPSSP Statistics 21.Threshold of significance was defined at $P < 0.05$.

Results

Body weight parameters

Initial and final body weight, body weight gain, the weight of adipose tissue depots at the end of treatment, and energy expenditure are summarized in Table 1. Body weight gain overtime and body fat percentage at weeks 0, 4 and 8 of treatment are shown in Figure 1. Animals of the WD group, but not those treated with capsaicin, showed higher body weight gain during the period of treatment compared to their controls (Table 1). Animals fed on a western diet and treated with hesperidin or the combination of capsaicin and hesperidin showed intermediate values, not different from the control and the WD groups. Curves of body weight gain during the period of treatment were different from day 21 of treatment in the WD group *versus* the control and CAP groups (Fig 1A). Animals on the different groups fed with western diet presented a higher percentage of body fat with respect to

the controls, which was already significant at 4 weeks of treatment (Fig 1B). However, animals of the CAP group showed lower body fat, after 4 and 8 weeks of treatment, compared to animals of the WD group, while animals treated with hesperidin or the combination of capsaicin and hesperidin showed not significant effect on body fat percentage. Animals of the WD group also displayed increased weight of the different WAT depots studied (inguinal, retroperitoneal, epididymal and mesenteric), as well as of the interscapular BAT (Table 1). Animals of the CAP and CAP+HESP groups showed lower weights of the iWAT and BAT compared to WD group.

No differences were found between groups concerning energy expenditure, which was measured for 24 hours after 6 weeks of treatment.

Histological analysis and UCP1 and CIDEA immunostaining in WAT

To study potential morphological changes and browning induction in WAT by effect of hesperidin and capsaicin, separately and in combination, histological analysis were performed in the inguinal and retroperitoneal depots. These depots were selected because they are those with a greater browning capacity, according to the literature (Waldén, Hansen, Timmons, Cannon, & Nedergaard, 2012; Garcia-Ruiz *et al.*,2015). In both adipose depots, western diet treatment brought about a significant increase in the size of adipocytes (Fig 2). However, in the rWAT, the treatment with HESP, CAP, and, to a lesser extent, the combination of both compounds, resulted in a decreased size of the

adipocytes to values of control animals. In the iWAT, the treatment with hesperidin resulted in a decreased size of adipocytes, whereas animals of the CAP and HESP+CAP groups showed intermediate values, not different from control and WD animals. Differences among groups were also apparent when quantifying adipose cell size distribution (Fig 3). A shift in adipocyte distribution toward a higher size was observed in both depots by effect of western diet, compared to control animals, but the distribution was apparently normalised by treatment with hesperidin, capsaicin, and, to a lesser extent, by the combination of both compounds.

The presence of brown-like adipocytes in rWAT and IWAT was also investigated in hematoxylin-eosin stained preparations. In the retroperitoneal depot, the majority of cells in all groups showed an appearance of white unilocular adipocytes. Nevertheless, in the HESP and CAP groups, multilocular adipocytes were found dispersed among white unilocular adipocytes in some of the animals. This was particularly relevant in two out of 8 animals in the CAP group, where multilocularity represented around 0.07% and 0.11%, respectively, of total area analysed. Two animals of the HESP group also showed the presence of multilocularity, but it was only relevant in one of them (0.09% of total area analysed). No apparent multilocularity was found in the Control, WD, and HESP+CAP groups. Immunostaining for UCP1 and CIDE-A conducted in the rWAT revealed that multilocular adipocytes from rats in the HESP and CAP groups showed positive staining for both UCP1 and CIDE-A (Fig 4).

Unlike the rWAT, no multilocularity was observed in any of the animals in the inguinal depot, where cells displayed an appearance of white unilocular adipocytes.

Gene expression in WAT

To further study potential browning induction by the treatment with HESP and CAP, we conducted gene expression analysis of brown/brite adipocyte markers in the selected fat depots, inguinal and retroperitoneal. Expression levels of the capsaicin receptor, *Trpv1*, were also studied.

Regarding iWAT, dietary administration of capsaicin in western diet fed rats produced a significant increase in mRNA expression levels of classical brown adipocyte genes, *Prdm16* (with respect to the control and WD groups) and *Cidea* (with respect to the control group) (Fig 5A). A similar trend was found regarding *Ucp1* and *Pgc1α* expression, but no significant differences were observed. No changes were found by the treatment with hesperidin or the combination of hesperidin + capsaicin with respect to the WD group regarding the expression of browning genes. Notably, expression levels of *Ucp1* and *Pgc1α* were lower in animals treated with the combination of hesperidin + capsaicin with respect to animals of the CAP group.

Expression levels of the brite selective marker *Hoxc9* in the iWAT did not increase by the treatment with the bioactive compounds; animals of the HESP group showed decreased expression levels of this gene compared to the control group (Fig 4B). A similar trend was found regarding *Trpv1* gene (Fig 4C).

With respect to rWAT, expression of *Ucp1* was very low and only detected in few animals. Moreover, a great of variability was present between the groups (data not shown). Regarding the other brown/brite adipocyte markers analysed, we did not observe any increase as an effect of bioactives studied (data not shown).

Western blot analysis in WAT

At protein level, we focused our attention on UCP1 and CIDE-A (Fig 6 and 7). In the iWAT (Fig 6), animals of the CAP and HESP+CAP groups displayed higher UCP1 levels than animals of the HESP group, but all they showed no differences with respect to the WD group (Fig 6A). Protein levels of CIDE-A were increased in the HESP and CAP groups compared to the WD group, but no significant effects were found with the combination (Fig 6B).

In the rWAT (Fig 7), UCP1 protein levels were higher in the CAP group compared to the HESP group, but they were not significantly different compared to the WD group (Figure 7A). Values of the control group were very variable between animals and are not included in the figure. No significant differences between groups were found regarding protein levels of CIDE-A (Fig 7B).

Western blot analysis in BAT

Protein expression of UCP1 was also determined in BAT (Fig 8). No significant differences were observed between groups regarding specific UCP1 levels. (Fig 8A). However, regarding total UCP1 (i.e. levels

corresponding to the whole BAT), HESP and CAP groups showed higher levels with respect to the control group (Fig 8B).

Discussion

The interest on therapies to combat obesity and related complications based on increased thermogenesis have re-emerged since the discovery of the presence of inducible BAT in adult humans (Nedergaard, et al., 2007). The presence of BAT has been related with a reduced risk of obesity and related diseases in adult humans (Jacene, Cohade, Zhang, & Wahl, 2011; Vijgen et al., 2011). In addition, the finding of the presence of brite, inducible brown adipocytes in WAT, which have a different origin from brown adipocytes but share many functional characteristics (such as a high mitochondria content, UCP1 expression, and thermogenic capacity) has increased the interest for the study of the browning process, besides BAT induction, as plausible targets for fighting obesity(Kim & Plutzky, 2016). In this regard, natural molecules from the diet, such as phenolic compounds derived from plants, have been studied as activators of browning(Azhar, Parmar, Miller, Samuels, & Rayalam, 2016). Besides the interest of individual compounds by themselves, the less studied combinations of bioactives is also of interest for any potential sinergistic or negative interactions. In this study, we investigated the effects of two bioactives previously proposed to act against body fat accumulation and its comorbidities, capsaicin and hesperidin respectively, administered individually and in combination, on the induction of BAT and WAT browning in rats fed an obesogenic diet.

Capsaicin has been recognized of potential interest to counter diet-induced obesity (Baskaran, et al., 2016). This has been associated to its thermogenic effects due to the capacity to trigger browning in WAT involving the induction of sirtuin-1 expression and activity via TRPV1 channel activation. Sirtuin-1 triggers the deacetylation and interaction of PRDM-16 and PPARγ (peroxisome-proliferator-activated receptor-γ) to mediate browning of WAT (Baskaran, et al., 2016). TRPV1 has been shown to be essential for the effects of capsaicin, since it does not have effects on fat depots in TRPV1-/- mice (that genetically lack TRPV1 channels) (Baskaran, et al., 2016).

Here, we have seen that capsaicin treatment decreased body weight gain and body fat accumulation in rats fed a western diet. This was associated to a decreased size of adipocytes, particularly in the retroperitoneal fat pad, where capsaicin treatment was associated to the appearance of multilocular UCP1- and CIDE-A-positive brown-like adipocytes. This suggests that the fat-lowering effect involves a browning induction in WAT. Accordingly, a trend to a higher expression of the UCP1 protein was also observed in this depot, although levels did not attain significant differences *versus* those of the WD group, probably due to the high variability among the CAP group. In the inguinal depot, although the browning process was not evident at the morphological level, capsaicin treatment brought about a significant increase in the mRNA expression levels of *Prdm16* and *Cidea,* and increased protein levels of CIDE-A with respect to the control and/or the WD group. The transcriptional regulator PRDM-16 is a determinant of brown adipocyte lineage. PRDM-16 stimulates brown adipogenesis by binding to PPAR-γ

and activating its transcriptional function (Seale et al., 2008).Thus, dietary capsaicin could induce the molecular conversion of white adipocytes to brite cells by increasing the expression of *Prdm16*. This is consistent with previous work showing increased expression of *Prdm16* and *Pgc1α* in mice due to dietary intake of capsaicin along with a high fat diet (Baskaran, et al., 2016).

CIDE-A is a lipid droplet-associated protein (Puri et al., 2008). The precise role of CIDE-A is not clearly known, but it has been shown to be implicated in lipid droplet formation and lipid storage in adipose tissue (Wu et al., 2014). Notably, transgenic mice expressing human CIDE-A display a robust increase of adipose tissue lipid storage capacity on a high-fat diet, suggesting that it is involved in protection against the metabolic complications of obesity (Abreu-Vieira et al., 2015). In humans, *Cidea* is expressed in WAT and expression levels are positively correlated with insulin sensitivity and biomarkers of good health or decreased risk factors in obese subjects; in fact, low expression levels are associated with several features of the metabolic syndrome (Nordström et al., 2005). Therefore, the increased expression of *Cidea* in capsaicin-treated animals may be indicative of improved metabolic health associated to the browning effect on WAT.

It should be mentioned the finding of a depot-specific response to the treatments, since capsaicin entailed increased expression of brown markers in the inguinal depot, whereas histological evidence of browning was only found in the retroperitoneal fat. This is consistent with previous work showing that browning induction by the intake of a cafeteria diet was only evident at the histological level in the retroperitoneal depot,

despite the inguinal depot showed increased expression levels of brown/brite markers (García-Ruiz, et al., 2015).

Besides capsaicin, citrus flavonoids, such as hesperidin, have interesting properties for the treatment of obesity-related alterations. However, unlike capsaicin, no clear effects of hesperidin have been described on body weight control, nor has it been reported to promote WAT browning or thermogenesis (Li & Schluesener, 2017), although it has been reported to potentiate the thermogenic effect of p-synephrine (Stohs, et al., 2011). Here, we describe that hesperidin treatment induced morphological changes in WAT, concretely this was associated to a decreased mean size of adipocytes in both retroperitoneal and inguinal WAT depots, as did capsaicin. Notably, hesperidin treatment also induced the appearance of multilocular adipocytes, positive for UCP1 and CIDE-A, in the retroperitoneal depot. Protein levels of CIDE-A were also increased in the inguinal depot, similarly to what observed by capsaicin treatment, but no significant induction was observed at the gene expression level in any of the two fat pads analysed. Therefore, to the best of our knowledge, it is described for the first time that hesperidin along with a western diet may have the potential to induce a certain browning in the WAT, although to a lesser extent than capsaicin. However, besides this, no significant effects on body weight gain or body fat percentage have been observed here in rats treated with hesperidin along with a western diet, beyond the effects decreasing adipocyte size. Human studies have also described that the supplementation with citrus-based juice, rich in Vitamin C and hesperidin, improved risk factors

in metabolic syndrome-patients but had no effect on anthropometric parameters (Mulero et al., 2012).

Besides browning induction in WAT, both capsaicin and hesperidin treatments led to a significant increase in total UCP1 levels in interscapular BAT, compared to control animals, suggesting that these animals may have increased thermogenic capacity than controls, but not compared to western diet fed animals.

To what extent the effects on WAT browning and BAT total UCP1 content by capsaicin and hesperidin treatments along with the western diet could be translated into increased whole-body energy expenditure is not clear. In fact, in the present study we did not observe any significant increase in daily energy expenditure by capsaicin or hesperidin, measured at 6 weeks of treatment. Other authors such as Baskaran et al have demonstrated an enhancement of energy expenditure in mice, but after 26 weeks of treatment with dietary capsaicin (Baskaran, et al., 2016). It could be possible that the potential increase in thermogenic capacity presented by the animals treated with capsaicin or hesperidin only resulted in a greater effective energy expenditure under appropriate stimuli. Actually, it has been described that combination of capsinoids with mild cold exposure (17°C) in mice synergistically promoted brite adipocyte biogenesis in inguinal WAT, and ameliorated diet-induced obesity but this antiobesity effect of capsinoids observed in vivo was completely blunted under thermoneutral conditions of 30°C (Ohyama et al., 2016).

Despite the potential of capsaicin and hesperidin, individually, to induce certain browning in western diet fed rats, it was surprising that the

combination of both bioactives did not exert apparent effects on browning, neither at the histological level, nor at the gene expression level, in any of the fat depots studied. Even, expression levels of brown adipocyte markers, such as *Ucp1* and *Pgc1α* in the inguinal depot were lower compared to expression levels achieved by capsaicin treatment alone. This is in agreement with the lesser effects exerted by the combination of both compounds vs. capsaicin alone counteracting western diet-induced increase in body weight and adiposity. Mechanisms underlying the negative interaction between both compounds are unknown. Considering the involvement of the TRPV1 channel in the browning program of WAT induced by capsaicin, it could hypothesized that hesperidin could override the effects of capsaicin through its TRPV1 receptor. In fact, rats treated with hesperidin showed lower *Trpv1* expression levels in the iWAT compared to control rats. However, the involvement of other mechanisms responsible for these interactions cannot be ruled out. The knowledge of these negative interactions and their underlying mechanisms may be of interest to design the most appropriate combinations of bioactives in the treatment of obesity and its related metabolic complications.

In conclusion, our results demonstrate the capacity of capsaicin and hesperidin, administered separately, to reduce the adipocyte size and to induce WAT browning in western diet fed rats. Besides, capsaicin counteracts the increased body weight gain associated to the obesogenic diet. However, the combination of hesperidin and capsaicin reduces the effectiveness of each compound in reducing the adipocyte size and

induction of browning and therefore should be avoided in eventual formulations trying to help in body weight control.

Author contributions

CP, AP and MPP conceived and designed the experiments. AMS and JS carried out experimental determinations. AMS, JS, CP and AP, participated in the data analysis and results interpretation. AMS wrote the first draft and CP wrote the definitive version. JS, MPP and AP revised the definitive version. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Spanish Government (AGL2015-67019-P), and the Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición, CIBERobn. Laboratory of Molecular Biology, Nutrition and Biotechnology is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: no. FP6-506360). A. Mosqueda-Solís is a recipient of a doctoral fellowship from the CONACYT (Mexico).

Conflict of interest statement: The authors declare that they have no conflict of interest.

Table 1. Body weight related parameters

	Control	WD	HESP	CAP	HESP+CAP
Initial body weight (g)	362 ± 16	353 ± 8	351 ± 15	356 ± 11	358±15
Final body weight (g)	428 ± 19	443± 14	433 ± 14	423 ± 15	433±19
Body weight gain (8 weeks) (g)	66 ± 7[a]	91 ± 9[b]	83 ± 4[a,b]	67± 4[a]	75±8[a,b]
Weight of adipose tissue depots					
Inguinal WAT (g)	6.76 ±0.61[a]	11.25 ± 1.46[b]	8.59 ±0.70[a,b]	7.22 ± 0.50[a]	7.99 ±0.93[a]
Retroperitoneal WAT (g)	7.55 ± 0.60[a]	12.59 ±1.13[b]	9.79 ± 0.61[a,c]	9.98 ± 0.75[a,b]	10.99 ± 1.38[b,c]
Epididymal WAT (g)	7.00 ± 0.54[a]	11.39 ± 1.00[b]	8.86 ± 0.67[a,b]	8.80 ± 0.75[a,b]	9.28 ± 1.50[a,b]
Mesenteric WAT (g)	3.39 ± 0.22[a]	5.64 ± 0.54[b]	4.71 ± 0.45[a,b]	4.19 ± 0.60[a,b]	4.63 ± 0.48[a,b]
Interscapular BAT (mg)	176 ± 20[a]	297 ± 25[b]	291 ± 13[b]	238 ± 7[c]	214 ± 10[c]
Energy expenditure (kcal/h/kg)	4.93±0.09	5.05±0.11	4.98±0.18	5.01±0.13	5.12±0.20
Total	5.44±0.16	5.30±0.13	5.39±0.14	5.34±0.08	5.56±0.22
Nocturnal	4.41±0.07	4.83±0.11	4.66±0.17	4.64±0.11	4.51±0.12
Diurnal					

Data are mean ± SEM. One-way ANOVA was used to determine differences between groups followed by a least significance difference (LSD) *post hoc* analysis ($P<0.05$), a≠b≠c.

Supplementary Table 1. Primers for PCR amplification of each gene studied

GENE	FORWARD PRIMER (5´to 3´)	REVERSE PRIMER(5´to 3´)	AMPLICON SIZE(pb)
Cidea	TCAGACCCTAAGAGACAACACA	CATTGAGACAGCCGAGGA	164
Hoxc9	CGGCAGCAAGCACAAAGA	AGAAACTCCTTCTCCAGTTCCA	138
Pgc1- α	CCTGCCATTGTTAAGACCGAGAA	CTGTGGGTTTGGTGTGAGGAG	141
Prdm16	ACGACCACCTCTGCTACCTC	GGGCTCCTATTTTTGACCTTCT	216
Trpv1	AATGGAGCAGATGTCCAGGC	TCTGCAGCAGGAACTTCACA	142
Ucp1	GGGCTGATTCCTTTTGGTCT	GGTGGTGATGGTCCCTAAGA	229

Figure legends

Figure 1. A) Body weight gain during the treatment period (8 weeks), and B) body fat percentage in week 0, week 4 and week 8 of control, western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups. Data are mean ± SEM (n=7-8). TxG, interactive effect between time and group, $P<0.05$, repeated measures ANOVA. a≠b≠c, one-way ANOVA followed by LSD *post hoc* analysis ($P<0.05$),

Figure 2. Adipocyte morphometry. Mean adipocyte area in retroperitoneal WAT (rWAT) (A) and inguinal WAT (iWAT) (B) and representative sections of rWAT (C) and iWAT (D) of control, western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups at the end of treatment. The area of individual adipocytes (μm^2) was measured using a quantitative morphometric method at 20 magnification with the assistance of Axio Vision software. Statistical analysis between groups was performed by one-way ANOVA, followed by LSD *post hoc* analysis ($P<0.05$), a≠b≠c.

Figure 3. Analysis of adipocyte size distribution in retroperitoneal WAT (rWAT) (A) and inguinal WAT (iWAT) (B) of control, western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups at the end of treatment. Distributions of adipocyte sizes across the tissue depot were obtained from individual data of cell area and considering all animals in each group. The number of cells analyzed (n) is shown.

Figure 4. Immunostaining of UCP1 and CIDE-A in retroperitoneal WAT (rWAT) of control, western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups at the end of treatment. Positive immunostaining for UCP1 and CIDE-A was detected in multilocular adipocytes in HESP and CAP groups. Co-localization for both proteins is evidenced by serial section immunostaining (examples of cells co-expressing both proteins are indicated with arrows). Staining for the studied proteins was not evident in the Control, WD and HESP+CAP groups.

Figure 5. Gene expression of brown (A) and brite markers (B), and of *Trpv1* (C) in inguinal WAT of control, western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups at the end of treatment. mRNA levels were measured by real-time PCR and expressed as a percentage of the control group. Data are mean ± SEM (n=7-8). Data of the control group were set to 100% and the rest of the values are referred to this. Statistical analysis between groups was performed by one-way ANOVA, followed by LSD *post hoc* analysis ($P<0.05$), a≠b.

Figure 6. Specific protein levels of UCP1 (A) and CIDE-A (B) in inguinal WAT (iWAT) from control, western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups at the end of treatment. Representative bands are shown. Data are mean ± SEM (n=7-8). Data of the control group were set to 100% and the rest of the values are referred to this. Statistical analysis between groups was performed by one-way ANOVA, followed by LSD *post hoc* analysis ($P<0.05$), a≠b≠c.

Figure 7. A) Specific protein levels of UCP1 (A) and CIDE-A (B) in retroperitoneal WAT (rWAT) from control, western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups at the end of treatment. Representative bands are shown. Data are mean ± SEM (n=7-8). Data of the WD group (in A) or the control group (in B) were set to 100% and the rest of the values are referred to them. Statistical analysis between groups was performed by one-way ANOVA, followed by LSD *post hoc* analysis ($P<0.05$), a≠b. No letter = no statistical difference.

Figure 8. Specific levels of UCP1 (A) and total protein of UCP1 (B) in brown adipose tissue (BAT) from control, western diet (WD), hesperidin (HESP), capsaicin (CAP), and hesperidin and capsaicin (HESP+CAP) groups at the end of treatment. Representative bands are shown. Data are mean ± SEM (n=7-8). Data of the control group was set to 100% and the rest of the values are referred to this. Statistical analysis between groups was performed by one-way ANOVA, followed by LSD *post hoc* analysis ($P<0.05$), a≠b. No letter = no statistical difference.

Abreu-Vieira, G., Fischer, A. W., Mattsson, C., de Jong, J. M., Shabalina, I. G., Rydén, M., Petrovic, N. (2015). Cidea improves the metabolic profile through expansion of adipose tissue. *Nat Commun, 6*, 7433. doi: 10.1038/ncomms8433

Arias, N., Picó, C., Teresa Macarulla, M., Oliver, P., Miranda, J., Palou, A., & Portillo, M. P. (2017). A combination of resveratrol and quercetin induces browning in white adipose tissue of rats fed an obesogenic diet. *Obesity (Silver Spring), 25*(1), 111-121. doi: 10.1002/oby.21706

Azhar, Y., Parmar, A., Miller, C. N., Samuels, J. S., & Rayalam, S. (2016). Phytochemicals as novel agents for the induction of browning in white adipose tissue. *Nutr Metab (Lond), 13*, 89. doi: 10.1186/s12986-016-0150-6

Baskaran, P., Krishnan, V., Ren, J., & Thyagarajan, B. (2016). Capsaicin induces browning of white adipose tissue and counters obesity by activating TRPV1 channel-dependent mechanisms. *Br J Pharmacol, 173*(15), 2369-2389. doi: 10.1111/bph.13514

Bonet, M. L., Oliver, P., & Palou, A. (2013). Pharmacological and nutritional agents promoting browning of white adipose tissue. *Biochim Biophys Acta, 1831*(5), 969-985. doi: 10.1016/j.bbalip.2012.12.002

Cannon, B., & Nedergaard, J. (2004). Brown adipose tissue: function and physiological significance. *Physiol Rev, 84*(1), 277-359.

García-Ruiz, E., Reynés, B., Díaz-Rúa, R., Ceresi, E., Oliver, P., & Palou, A. (2015). The intake of high-fat diets induces the acquisition of brown adipocyte gene expression features in white adipose tissue. *Int J Obes (Lond), 39*(11), 1619-1629. doi: 10.1038/ijo.2015.112

Giralt, M., & Villarroya, F. (2013). White, brown, beige/brite: different adipose cells for different functions? *Endocrinology, 154*(9), 2992-3000. doi: 10.1210/en.2013-1403

Harms, M., & Seale, P. (2013). Brown and beige fat: development, function and therapeutic potential. *Nat Med, 19*(10), 1252-1263. doi: 10.1038/nm.3361

Himms-Hagen, J. (1990). Brown adipose tissue thermogenesis: interdisciplinary studies. *FASEB J, 4*(11), 2890-2898.

Jacene, H. A., Cohade, C. C., Zhang, Z., & Wahl, R. L. (2011). The relationship between patients' serum glucose levels and

metabolically active brown adipose tissue detected by PET/CT. *Mol Imaging Biol, 13*(6), 1278-1283. doi: 10.1007/s11307-010-0379-9

Kim, S. H., & Plutzky, J. (2016). Brown Fat and Browning for the Treatment of Obesity and Related Metabolic Disorders. *Diabetes Metab J, 40*(1), 12-21. doi: 10.4093/dmj.2016.40.1.12

Li, C., & Schluesener, H. (2017). Health-promoting effects of the citrus flavanone hesperidin. *Crit Rev Food Sci Nutr, 57*(3), 613-631. doi: 10.1080/10408398.2014.906382

Mulero, J., Bernabé, J., Cerdá, B., García-Viguera, C., Moreno, D. A., Albaladejo, M. D.,Zafrilla, P. (2012). Variations on cardiovascular risk factors in metabolic syndrome after consume of a citrus-based juice. *Clin Nutr, 31*(3), 372-377. doi: 10.1016/j.clnu.2011.11.014

Nedergaard, J., Bengtsson, T., & Cannon, B. (2007). Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab, 293*(2), E444-452. doi: 10.1152/ajpendo.00691.2006

Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., . . . Gakidou, E. (2014). Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet, 384*(9945), 766-781. doi: 10.1016/S0140-6736(14)60460-8

Nordström, E. A., Rydén, M., Backlund, E. C., Dahlman, I., Kaaman, M., Blomqvist, L., . . . Arner, P. (2005). A human-specific role of cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A (CIDEA) in adipocyte lipolysis and obesity. *Diabetes, 54*(6), 1726-1734.

Ohyama, K., Nogusa, Y., Shinoda, K., Suzuki, K., Bannai, M., & Kajimura, S. (2016). A Synergistic Antiobesity Effect by a Combination of Capsinoids and Cold Temperature Through Promoting Beige Adipocyte Biogenesis. *Diabetes, 65*(5), 1410-1423. doi: 10.2337/db15-0662

Palou, A., Pico, C., Bonet, M. L., & Oliver, P. (1998). The uncoupling protein, thermogenin. *Int J Biochem Cell Biol, 30*(1), 7-11.

Pi-Sunyer, X. (2009). The medical risks of obesity. *Postgrad Med, 121*(6), 21-33. doi: 10.3810/pgm.2009.11.2074

Puri, V., Ranjit, S., Konda, S., Nicoloro, S. M., Straubhaar, J., Chawla, A., Czech, M. P. (2008). Cidea is associated with lipid droplets and insulin sensitivity in humans. *Proc Natl Acad Sci U S A, 105*(22), 7833-7838. doi: 10.1073/pnas.0802063105

Saito, M. (2015). Capsaicin and Related Food Ingredients Reducing Body Fat Through the Activation of TRP and Brown Fat Thermogenesis. *Adv Food Nutr Res, 76*, 1-28. doi: 10.1016/bs.afnr.2015.07.002

Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Spiegelman, B. M. (2008). PRDM16 controls a brown fat/skeletal muscle switch. *Nature, 454*(7207), 961-967. doi: 10.1038/nature07182

Spiegelman, B. M. (2013). Banting Lecture 2012: Regulation of adipogenesis: toward new therapeutics for metabolic disease. *Diabetes, 62*(6), 1774-1782. doi: 10.2337/db12-1665

Stohs, S. J., & Badmaev, V. (2016). A Review of Natural Stimulant and Non-stimulant Thermogenic Agents. *Phytother Res, 30*(5), 732-740. doi: 10.1002/ptr.5583

Stohs, S. J., Preuss, H. G., Keith, S. C., Keith, P. L., Miller, H., & Kaats, G. R. (2011). Effects of p-synephrine alone and in combination with selected bioflavonoids on resting metabolism, blood pressure, heart rate and self-reported mood changes. *Int J Med Sci, 8*(4), 295-301.

Vijgen, G. H., Bouvy, N. D., Teule, G. J., Brans, B., Schrauwen, P., & van Marken Lichtenbelt, W. D. (2011). Brown adipose tissue in morbidly obese subjects. *PLoS One, 6*(2), e17247. doi: 10.1371/journal.pone.0017247

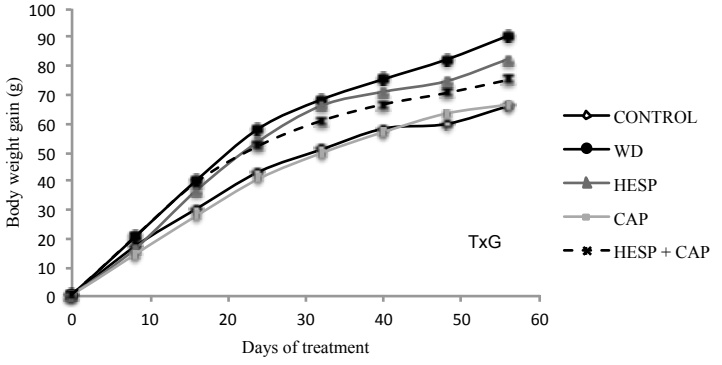
Waldén, T. B., Hansen, I. R., Timmons, J. A., Cannon, B., & Nedergaard, J. (2012). Recruited vs. nonrecruited molecular signatures of brown, "brite," and white adipose tissues. *Am J Physiol Endocrinol Metab, 302*(1), E19-31. doi: 10.1152/ajpendo.00249.2011

Whiting, S., Derbyshire, E., & Tiwari, B. K. (2012). Capsaicinoids and capsinoids. A potential role for weight management? A systematic review of the evidence. *Appetite, 59*(2), 341-348. doi: 10.1016/j.appet.2012.05.015

Wu, L., Zhou, L., Chen, C., Gong, J., Xu, L., Ye, J.,Li, P. (2014). Cidea controls lipid droplet fusion and lipid storage in brown and white adipose tissue. *Sci China Life Sci, 57*(1), 107-116. doi: 10.1007/s11427-013-4585-y

Figure 1

A.


Body weight gain (g)
Days of treatment
TxG
CONTROL
WD
HESP
CAP
HESP + CAP

B.

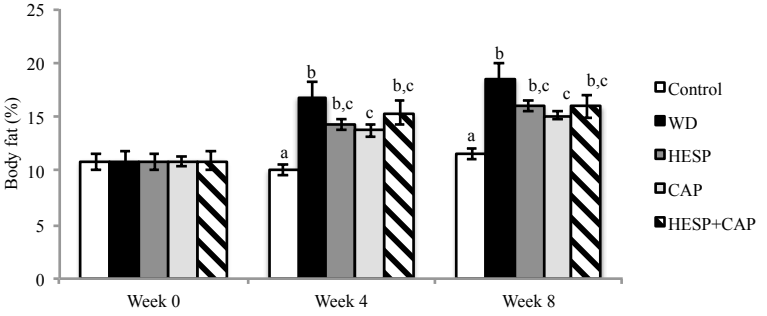

Body fat (%)
Week 0
Week 4
Week 8
Control
WD
HESP
CAP
HESP+CAP

Figure 2

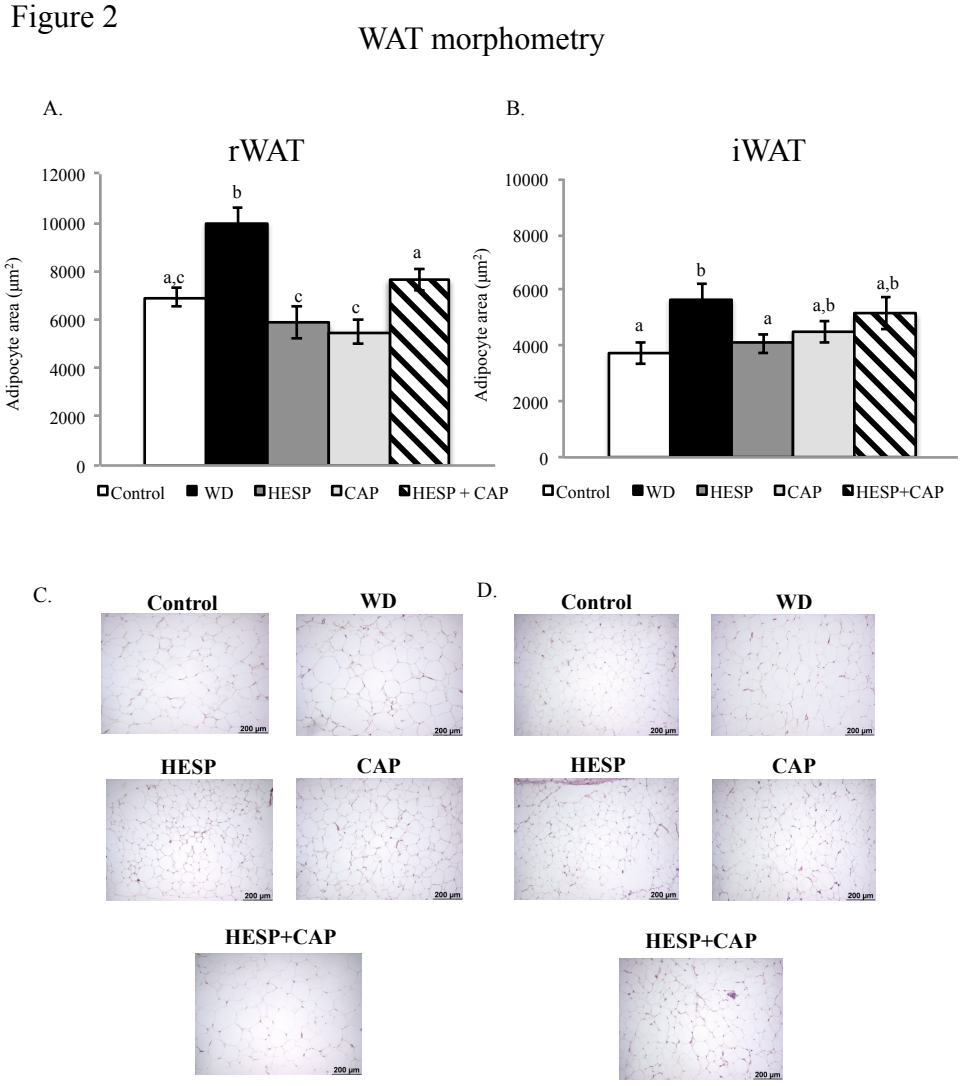

WAT morphometry
A.
rWAT
Adipocyte area (μm²)
12000
10000
8000
6000
4000
2000
0
a,c
b
c
c
a
Control
WD
HESP
CAP
HESP + CAP
B.
iWAT
Adipocyte area (μm²)
10000
8000
6000
4000
2000
0
a
b
a
a,b
a,b
Control
WD
HESP
CAP
HESP+CAP
C.
Control
WD
HESP
CAP
HESP+CAP
D.
Control
WD
HESP
CAP
HESP+CAP

Figure 3

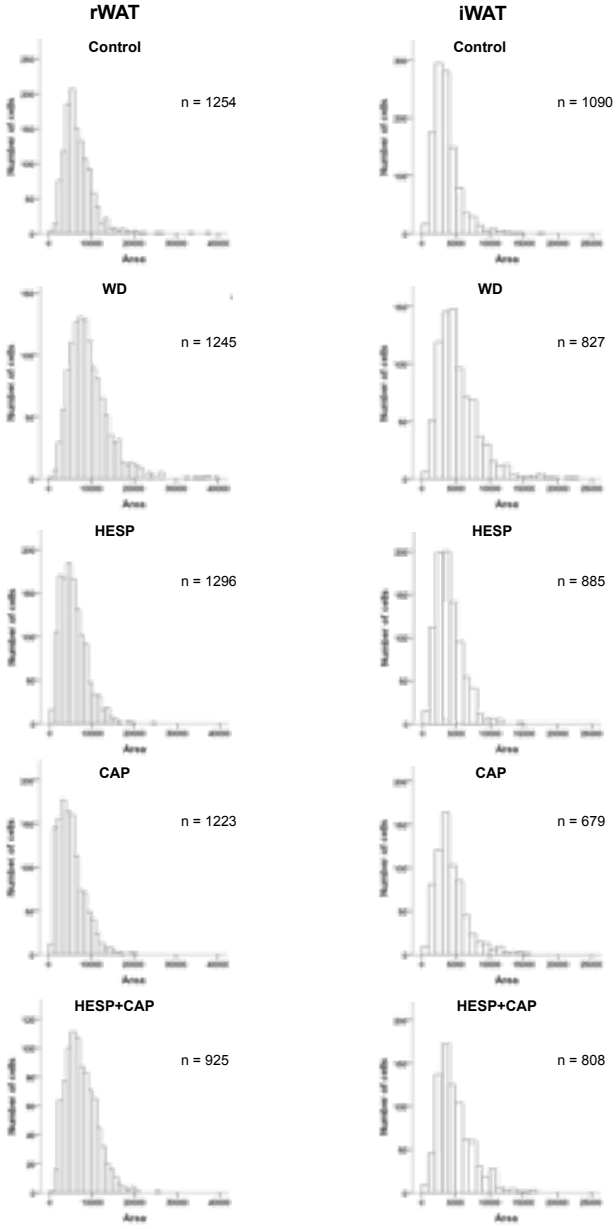

rWAT
iWAT
Control
n = 1254
Control
n = 1090
WD
n = 1245
WD
n = 827
HESP
n = 1296
HESP
n = 885
CAP
n = 1223
CAP
n = 679
HESP+CAP
n = 925
HESP+CAP
n = 808
Area

Figure 4

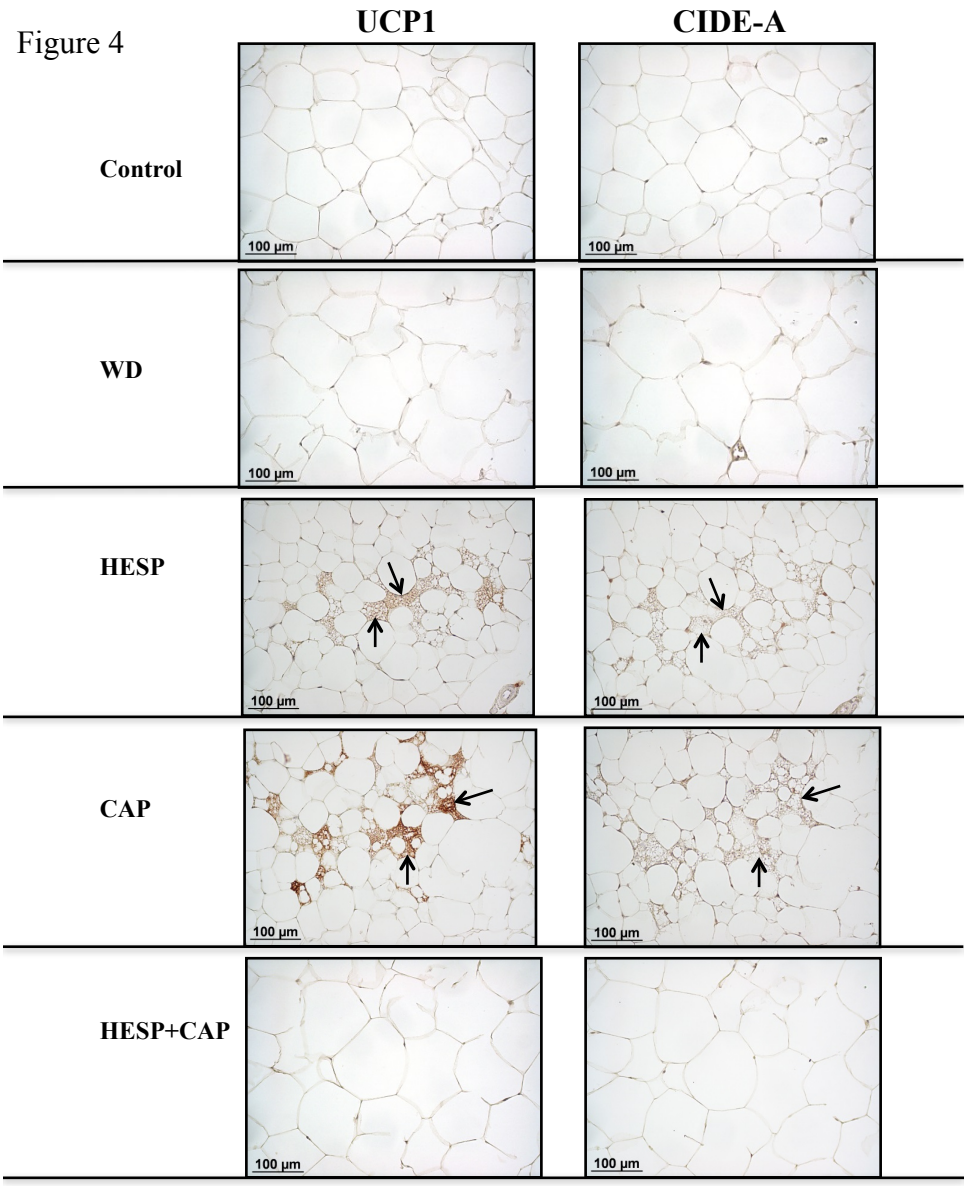


Figure 5 **Gene expression of browning and brite markers and of *Trpv1* in iWAT**

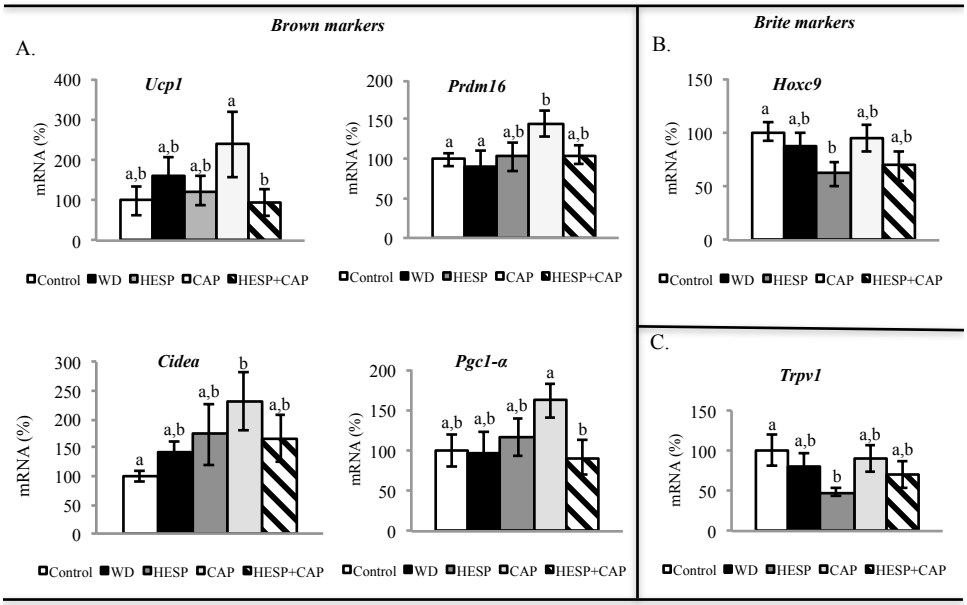


Figure 6

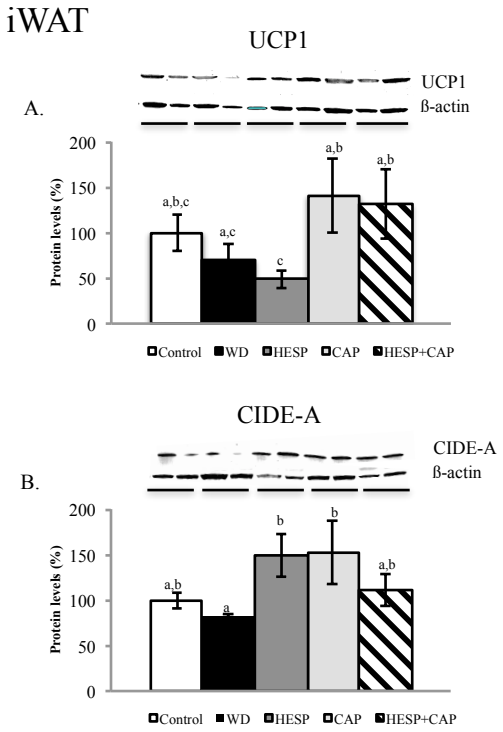

iWAT
UCP1
A.
UCP1
ß-actin
Protein levels (%)
200
150
100
50
0
a,b,c
a,c
c
a,b
a,b
Control
WD
HESP
CAP
HESP+CAP
CIDE-A
B.
CIDE-A
ß-actin
Protein levels (%)
200
150
100
50
0
a,b
a
b
b
a,b
Control
WD
HESP
CAP
HESP+CAP

Figure 7

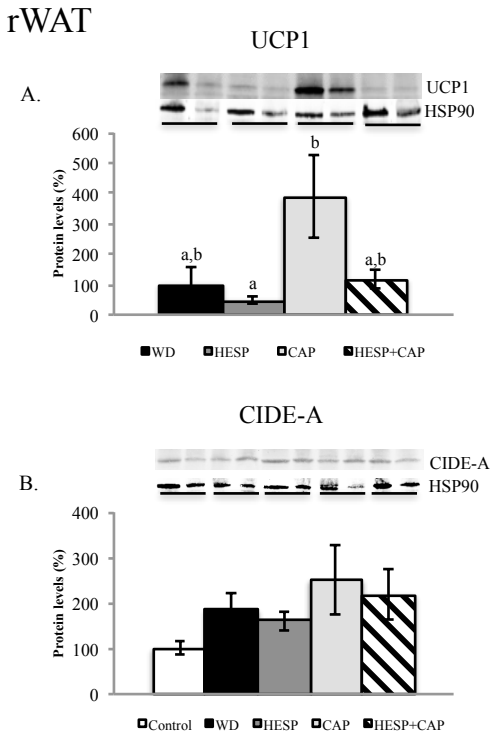
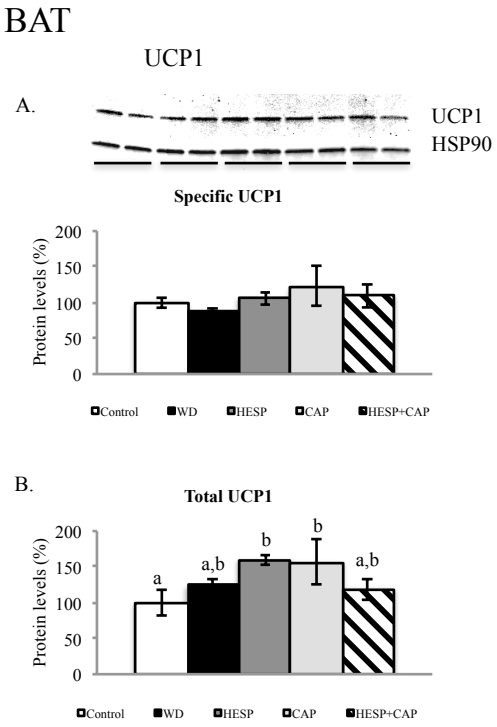

rWAT
UCP1
A.
UCP1
HSP90
600
500
400
300
200
100
0
Protein levels (%)
a,b
a
b
a,b
WD
HESP
CAP
HESP+CAP
CIDE-A
B.
CIDE-A
HSP90
400
300
200
100
0
Protein levels (%)
Control
WD
HESP
CAP
HESP+CAP

Figure 8


BAT
UCP1
A.
UCP1
HSP90
Specific UCP1
Protein levels (%)
200
150
100
50
0
Control
WD
HESP
CAP
HESP+CAP
B.
Total UCP1
Protein levels (%)
a
a,b
b
b
a,b
Control
WD
HESP
CAP
HESP+CAP

As previously mentioned in the Introduction of this Doctoral Thesis, given the increasing prevalence of obesity and its co-morbidities, worldwide scientific research is constantly looking for new molecules that would be effective in avoiding excess body fat accumulation and/or reducing it once accumulated.

My Doctoral Thesis has been developed in two research groups that focus their interest since long-time ago on this field of research. The group "Nutrition and Obesity" of the University of the Basque Country, led by Prof. María del Puy Portillo, has extensive experience in the study of the effects of different dietary components on lipid metabolism. In recent years the studies have been devoted to analysing the potential beneficial effects of phenolic compounds on the prevention and treatment of obesity. Also, the Laboratory of Molecular Biology, Nutrition and Biotechnology (LBNB) of the University of the Balearic Islands, a group led by Prof. Andreu Palou, and of which Prof. Catalina Picó is the deputy director, has extensive experience in the study of the body weight regulation system (obesity), the relationships between diet and genetics (nutrigenomics and personalized nutrition) and topics related to food effectiveness and safety or functional foods, as well as the identification of healthy properties in foods and new biomarkers that substantiate European health claims on foods.

In this Thesis, two different approaches have been carried out. First, an *in vitro* study consisting of a screening of 15 phenolic compounds to explore and compare their effects at different doses on adipogenesis. Second, an *in vivo* study where we assessed the potential effects of

hesperidin (selected from the *in vitro* study because of its anti-adipogenic effects) and of capsaicin (selected from the literature because of its potential thermogenic effects), separately and in combination, to prevent the development of obesity and metabolic alterations induced by the intake of an obesogenic diet in rats.

A) Screening of potential anti-adipogenic effects of phenolic compounds showing different chemical structure in 3T3-L1 preadipocytes

Phenolic compounds are members of a very large family of plant-derived molecules exhibiting an extensive variety of chemical structures. It is known that the chemical structure of phenolic compounds has an influence, not only on their bioavailability, but also on their biological actions. Bearing all this in mind, and considering the major health problem that obesity represents nowadays, our interest focused on the effects of phenolic compounds on obesity, and more specifically on adipogenesis, process which allows adipose tissue expansion by the transformation of adipocyte precursors (pre-adipocytes) into mature adipocytes.

In this experiment, we analysed the anti-adipogenic effect of fifteen phenolic compounds from various chemical groups in 3T3-L1 pre-adipocytes. To this end, *in vitro* models have been invaluable in determining the mechanisms involved in adipocyte proliferation, differentiation, and gene expression. The cells presently available for research purposes all have unique advantages and disadvantages that

one should be aware of when selecting cells. An established cell line, such as 3T3-L1 is widely used in biological research on adipose tissue. It is a cell line derived from mouse with a fibroblast-like morphology that under appropriate conditions can differentiate into an adipocyte-like phenotype.

The phenolic compounds used were flavones (apigenin and luteolin), flavan-3-ols (catechin, epicatechin and epigallocatechin), isoflavones (genistein and daizein), flavanones (naringenin and hesperidin), flavonols (quercetin and kaempferol), stilbenes (resveratrol, piceatannol and pterostilbene) and hydroxybenzoic acid (vanillic acid) for 8 days. A range of doses from 25µM to a physiological dose of 1µM was studied. The use of this low dose is one of the main novelties of this study.

Triglyceride quantification showed that at 25 µM all the compounds, with the exception of catechin, epicatechin and epigallocatechin, reduced lipid accumulation. At a dose of 10 µM apigenin, luteolin, naringenin, hesperidin, quercetin and kaempferol induced significant reductions, and at 1 µM only naringenin, hesperidin and quercetin were effective. These results showed that the chemical structure of flavanones was the most potent one in the inhibition of adipogenesis, at least under our experimental conditions, because both compounds showing this structure were able to reduce triacylglycerol accumulation in pre-adipocytes at the three doses studied, including 1 µM.

It is known that the chemical structure of phenolic compounds has an influence, not only on their bioavailability, but also on their biological actions. When the effects of phenolic compounds belonging to the same

chemical group were compared, we observed that both flavones were active at 25 and 10 µM, both isoflavones were active at 25 µM, both flavanones were active at the three doses and the three stilbenes were active at 25 µM. These results suggest that small differences, mainly based on substituent groups, among compounds showing a similar general chemical structure are not crucial for the anti-adipogenic activity of these molecules. In the case of flavonols, the pattern of response was different because while quercetin was effective at the three doses, kaempferol only showed an anti- adipogenic effect at 25 µM and 10 µM. This difference could be due to the presence of an extra hydroxyl group in quercetin structure.

In order to gain more insight concerning the effects of the phenolic compounds on adipogenesis, we analyzed potential changes in the expression of genes that play a key role on this process. As mentioned in the Introduction, adipogenesis is regulated by an elaborate network of transcription factors. In general terms, two stages can be distinguished, the pre-mitotic stage (early stage of differentiation; 60 hours post-confluence), regulated by C/EBP*β* and SREBP1c, and the post-mitotic stage (late stage) regulated by *PPARγ and C/EBPα* (Farmer *et al*., 2007; Fajas *et al*., 1998; Tang *et al*., 2003). Nevertheless, the influence of *PPARγ* on these two stages is difficult to be established. Ntambi *et al.* reported that this transcription factor starts its expression around 48 hours and reaches its maximal expression around 3-4 days of differentiation (Ntambi *et al.,* 2000).

In the present study, after analysing the effects of the phenolic compounds on the expression of the above mentioned genes, it can be proposed that all of them acted by inhibiting the early stage of adipogenesis because they significantly reduced gene expression of *srebp1c*, or both *c/ebpβ* and s*rebp1c* (genistein and kaempferol). This proposal is confirmed by the reduction in triacylglycerols observed when cells were treated from 0 to 60 hours with all the phenolic compounds analysed in the second experiment (luteolin, genistein, hesperidin, kaempferol, pterostilbene, vanilic acid). None of the tested compounds modified *c/ebpa* gene expression. In the case of *pparγ* gene expression, both genistein and pterostilbene induced significant reductions, but this effect was not accompanied by the expected reduction in triglyceride content when cells were incubated from 60 hours to day 8. In order to explain this fact, PPARγ2 protein expression was measured and no significant differences were found when cells treated with these phenolic compounds were compared with the control cells. These results are in good accordance with the lack of delipidating effect showed in the latest stage of differentiation.

With regard to the lately expressed genes, luteolin, genistein, kaempferol and pterostilbene reduced *acc* gene expression and kaempferol that of *lpl*, which suggests a reduced capacity of mature adipocytes previously treated with these compounds for triglyceride accumulation. The reduction observed in the expression of genes from the early stage of adipogenesis should have led to a reduction in the expression of regulator genes of the late stage. Nevertheless, in the

present study, not all the compounds demonstrated this effect. We do not have an explanation for this fact but it is not unusual because this situation has also been found in other studies from our group (Eseberri *et al.,* 2015) and by other authors (Ntambi *et al.,* 2000; Fajas *et al.,* 1998; Tang *et al.,* 2003; Lane *et al.,* 1999; Erickson *et al.,* 2000; Dowell *et al.,* 2000; Allender and Rayner, 2007). Nevertheless, we should indicate as concluding remark that, in general, the phenolic compounds tested act in the early stage of adipogenesis.

The efficacy of the use of natural health products as alternative methods for the treatment and management of obesity and related diseases depends on their bioavailability (Redan *et al*., 2016). Consequently, although as explained before in this summary, the use of cultured cells provides several advantages for obesity research, *in vivo* studies using animal models are needed before administering these products to human beings interventional studies. In addition, since the level of a single natural compound may be too low to exert sufficient beneficial effects *in vivo*, possible interactions between bioactive agents, leading to additive or synergistic effects or, on the contrary, to a decrease in their efficacy, should be considered. In this context we analysed the effects of hesperidin, one of the most active phenolic compounds in the *in vi*tro study and capsaicin, separately and in combination, on obesity and metabolic-related alterations in a model of diet-induced obesity.

B) Hesperidin and capsaicin, but not the combination, prevent hepatic steatosis and other metabolic syndrome-related alterations in western diet-fed rats.

Among all the phenolic compounds analysed in the screening previously described in Manuscript 1, we chose hesperidin for the *in vivo* study due to the interesting results obtained *in vitro* with this molecule. As previous studies showed that different combinations of phenolic compounds were able to reduce body fat and obesity co-morbidities even more than the administration of these compound individually, an interest arise in combining hesperidin with another compound (De Santi *et al*., 2000; Ohara *et al*., 2015; Arias *et al*., 2015, Arias *et al.,* 2016; Arias *et al*., 2017). In this regard, we considered capsaicin, because of the described potential anti-obesity effects associated to the capacity to induce BAT thermogenesis (Saito, 2015) and WAT browning (Baskaran *et al*., 2016).

In this context, this research focused on the potential beneficial effects of hesperidin and capsaicin to counteract diet-induced metabolic alterations. They were assessd separately and also in combination, to determine possible interactions between them, which had not been explored yet.

The *in vivo* experiment was conducted with Wistar rats randomly divided in 5 groups: Control group: fed with a standard chow diet, WD group: animals fed with a high-fat, high-sucrose diet, HESP: animals fed with a western diet and treated with hesperidin (100 mg/kg/day), CAP:

animals fed a western diet and treated with capsaicin (4 mg/kg/day) and HESP+CAP: animals fed with a western diet and treated with the combination of hesperidin (100 mg/kg/day) + capsaicin (4 mg/kg/day). The aim was to asses the potential effects of dietary hesperidin and capsaicin, independently, and the combination of both compounds to prevent the development of obesity and its related metabolic alterations.

After 8 weeks of treatment no significant differences were found between the 5 groups of animals concerning body weight. However, the WD group, but not the CAP group, showed higher body weight gain than controls. Animals of the HESP and HESP+CAP groups showed intermediate values. Capsaicin treatment also displayed at the end of the intervention period, lower body fat percentage than animals of the WD group, but higher than the control group. Body fat content in HESP and the HESP+CAP groups were slightly higher than that of the CAP group and not significantly different from the WD group. These results are in good accordance with others authors that have also found no significant effects of hesperidin on body weight in obese mice under low fat diet. However, when glucosyl hesperidin was combined with caffeine significant effects were observed (Ohara *et al.,* 2014).

The anti obesity action of capsaicin has been previously described by several authors (Watanabe *et al.,* 1986; Sherriffs *et al.,* 2010; Li *et al.,* 2010 and Mattes, 2011) suggesting that capsaicin decreases body fat. Notably, the combination of hesperidin and capsaicin did show lower effects on body fat gain than those observed when they were

administrated separately.

The expression of different genes were measured in order to found potential mechanisms underlying the effects of capsaicin preventing fat accumulation by the effects on lipid metabolism in adipose tissue. Animals treated with capsaicin displayed in rWAT decreased expression levels of lipogenesis-related genes (*Pparg* and *Srebf1*), along with decreased expression levels of genes related with fatty acid uptake (*Lpl*, *Cd36*) and oxidation (*Cpt1b*), lipolysis (*Pnpla2*, *Lipe*), and glucose uptake and metabolism (*Slc2a4*, *Hk2*). Animals treated with hesperidin also showed diminished expression levels of some of the above-mentioned genes (*Pparg*, *Lpl*, *Pnpla2*, *Cpt1b*, *Hk2*, and *Insr*) with respect to animals of the WD group, but the effects were generally less marked than those observed by capsaicin treatment, in accordance with the more modest effect of hesperidin on body fat gain. The combination of CAP and HESP also elicited no additional effects to those observed with capsaicin alone.

As described in the Introduction section, obesity is a risk factor for several metabolic complications. Regarding glucose-insulin system, the results obtained for the homeostasis model assessment of insulin resistance (HOMA-IR) indicates that capsaicin treatment prevented the increase in HOMA-IR in western diet-fed rats, but notably, the effects were reduced when capsaicin was combined with hesperidin. The capacity of capsaicin in ameliorating insulin resistance has been previously described in rodents (Kang *et al.*, 2010). Several studies in humans have also revealed that capsaicin has a modest effect in type 2

diabetes. Unlike capsaicin, no significant effects were observed for hesperidin on insulin resistance in western diet-fed rats.

Liver plays an important role in lipid accumulation and oxidation (Lazarow, 1978; Vernon, 1980). Under our experimental conditions, animals in the WD group, but not animals in the CAP group, showed higher liver weight than controls, whereas animals treated with hesperidin or with the combination of both compounds showed intermediate values. In spite of that, we measured hepatic lipid content in order to know if this organ was delipidated by effects of these bioactives. Animals of the WD group showed a significant increase (112%) in the lipid content with respect to controls. Notably, WD-fed animals treated with either hesperidin or capsaicin separately and, to a lesser extent, with the combination showed significantly lower hepatic lipid content with respect to the WD group (39%, 38%, and 15% decrease, respectively). Nevertheless, all western diet-fed groups displayed higher hepatic lipid content than controls. Histological analysis unveiled that both hesperidin and capsaicin, when administered alone, conferred protective effect on the development of NAFLD and prevented the features of NASH, such as hepatocyte ballooning, necrotic hepatocytes and infiltrated lymphocytes, found in western-diet fed animals. However, some signs of NASH were found in animals treated with the combination of both bioactives.

With regard to circulating triglycerides, treatment with either hesperidin or capsaicin alone, but not with the combination, attenuated the increase of circulating triglycerides levels in the fed-state occurring in

western diet-fed rats.

Mitsuzumi *et al.,* previously described that hesperidin reduces serum triglycerides in animal models and in hypertriglyceridemic subjects (Mitsuzumi *et al.,* 2011). Specifically, the mechanisms involved in the triglycerides-lowering effects of hesperidin have been reported to be the reduction of hepatic triglyceride content through inhibition of lipogenesis and induction of fatty acid oxidation (Mitsuzumi *et al.,* 2011), and the down-regulation of synthesis and secretion of very-low-density lipoproteins (VLDL) (Miwa *et al.,* 2006).

According to this notion, after analysing hepatic expression of selected genes, we observed that hesperidin treated animals displayed decreased expression levels of three key lipogenesis-related genes, *Srebf1*, *Fasn* and *Scd1*, as well as normalization of *Pklr* gene expression to control levels. In addition, these animals exhibited decreased hepatic expression levels of the *Insr* and *Lepr* genes, compared to levels of expression in western diet-fed rats, suggesting a decreased lipogenic action of insulin, along with decreased fatty oxidation activity. Although there is no clear consensus in the literature on the effects of leptin in liver (Ceddia *et al.*, 2002), it has been proposed that leptin may have insulin sensitizing effects, controlling the extent of insulin action on this tissue (Huynh *et al.,* 2010). Notably, disruption of hepatic leptin signalling has been shown to protect mice from diet-related glucose intolerance (Huynh *et al,.* 2010). Therefore, it is suggested that the decreased hepatic lipogenesis in hesperidin-treated animals, associated to a decreased insulin action on this tissue, may be one of the

mechanisms whereby this bioactive protects against diet-induced hepatic pathologies.

In turn, capsaicin treatment elicited little effects on the hepatic expression of lipogenesis-related genes compared to changes observed with hepseridin. It has been reported that capsaicin regulates hepatic lipid metabolism and prevents lipid deposition in liver through TRPV1 activation (Li *et al*., 2012). Capsaicin action on TRPV1 involves up-regulation of UCP2, a mitochondrial membrane transporter that can provide fatty acid translocation, preventing its accumulation in the mitochondrial matrix (Li *et al*., 2012). Here, the presence of higher expression levels of *Ucp2* in capsaicin-treated animals compared to the other experimental groups is in accordance with the involvement of UCP2 in the protective effects of capsaicin on lipid deposition in liver, providing protective effects from hepatocellular lipotoxicity (Baffy, 2005).

Therefore, both capsaicin and hesperidin, separately, are able to overcome the effects of western diet on hepatic lipid metabolism, and hence ameliorate hepatic steatosis and prevent NASH associated to western diet consumption. Unexpectedly, the effects were blunted or even negligible when animals were treated with both compounds simultaneously. Mechanisms underlying this interaction are unknown, but it has been observed that capsaicin treatment blocked the decrease in the hepatic expression of *Lepr* induced by hesperidin, which may be tentatively associated with an increase in insulin-induced lipogenesis activity in this tissue. In fact, the combination of both compounds up-

regulated the expression of the lipogenic gene *Gadph*, reaching levels higher than those of animals treated with hesperidin alone. On the other hand, hesperidin seems to override the effects of capsaicin through its TRPV1 receptor. In fact, induction of *Trpv1* expression by capsaicin was attenuated with the simultaneous treatment with hesperidin, suggesting that the responsiveness to the capsaicin action was blunted in animals treated with the combination of bioactives. Accordingly, animals treated with the combination of bioactives did not show the increase in *Ucp2* expression levels characteristic of capsaicin treated animals. Thus, it is suggested that both bioactives may mutually impair their ways of action on the improvement of liver health in western diet-fed animals.

Regarding other components of the metabolic syndrome, results of the present study also bring evidence supporting the hypotensive effects of hesperidin and, to a lesser extent, of capsaicin. In fact, systolic and diastolic blood pressure was decreased in hesperidin treated animals, compared to untreated western diet-fed rats, reaching levels lower than controls. Animals treated with capsaicin or the combination of hesperidin and capsaicin also exhibited lower diastolic blood pressure than western diet-fed animals, and similar to control animals. This suggests interesting anti-hypertension effects of hesperidin, beyond their potential fat-lowering effects. The hypotensive effect of hesperidin has been previously described both at short- and long-term treatment in models of hypertensive rats (Ohtsuki *et al*., 2002; Yamamoto *et al.,* 2008), and this has been related with the capacity to improve

endothelium-dependent vasorelaxation by increasing the availability of nitric oxide (Rizza *et al*., 2011; Yamamoto *et al,.* 2013).

Therefore, the results of the present study show that capsaicin and hesperidin, separately, exhibit different health beneficial effects ameliorating fat accumulation and metabolic syndrome-related alterations in western diet-fed rats. However, lack or lesser effects found with the combination of both compounds deserve to be taken into account when considering potential mixtures of bioactives as strategies for obesity prevention.

C) Effects of capsaicin and hesperidin and its combination on adipose tissue morphology and browning induction in western diet-fed rats

Considering the ability of capsaicin and hesperidin, separately, to exhibit the beneficial health effects mentioned above, we further explored potential properties of these bioactive compounds, separately and in combination, to affect WAT morphology and induce WAT, as a help in body weight management in rats feed an obesogenic diet. Browning can appear in WAT in response to specific stimuli, such as cold exposure, but also in response to specific dietary components (Bonet, Oliver, & Palou, 2013) or particular diets, such as high-fat diets (García-Ruiz *et al*., 2015). Therefore, bioactive molecules able to increase BAT and/or to convert white into brite adipocytes may help to correct the energy imbalance that underlines obesity. In addition, considering that nutritional factors generally exert limited effects, the combination with other bioactive agents that target other processes related to adipocyte

metabolism, such as adipogenesis and/or lipolysis, may further facilitate energy metabolism and weight management (Stohs & Badmaev, 2016). For example, the combination of resveratrol and quercetin has been reported to exert increased effects on browning in rats fed an obesogenic diet, while both compounds individually exert little effects (Arias *et* al., 2017).

In this regard, there is extensive research showing the potential interest of capsaicinoids (also known as capsinoids), a group of molecules naturally present in chilli peppers. The most abundant and studied is the capsaicin, which has anti-obesity, anti-diabetic, and anti-inflammatory properties (Whiting, Derbyshire, & Tiwari, 2012). Their anti-obesity effects are related in part to their capacity to activate the sympathetic system and induce BAT thermogenesis (Saito, 2015) and trigger WAT browning by activating transient receptor potential cation channel subfamily V member 1 (TRPV1) (Baskaran et al.,2016). Notably, capsaicin and its non-pungent analogs have potential interest as weight control agents because they act as thermogenic agents, without causing stimulant and adverse cardiovascular effects (Stohs & Badmaev, 2016). On the other hand, citrus flavonoids, such as hesperidin, have emerged as promising therapeutic agents for the treatment of metabolic dysregulation. Several studies have pointed out the bioactive effects of hesperidin against obesity-related alterations mainly because of its fat-lowering, antiinflammatory, antioxidant, and insulin-sensitizing effects (Li & Schluesener, 2017). Notably, in a human study, hesperidin has been shown to enhance the thermogenic effect of p-synephrine, the primary protoalkaloid in bitter orange (Stohs *et al*., 2011). Therefore, it is

suggested that appropriate combination of thermogenic agents with flavonoids or other fat-lowering bioactives could potentially increase the effects of individual compounds and be highly valuable in the nutritional field to assist weight management.

In the present study, animals treated with capsaicin displayed lower body weight gain and fat content *vs* WD group, but no differences were detected regarding energy expenditure. This was also associated with a decreased size of adipocytes, particularly in the retroperitoneal fat pad. The fat-lowering effect may be due, in part, to browning induction in WAT. This effect was morphologically evident in the retroperitoneal depot, where capsaicin treatment was associated with the appearance of multilocular UCP1- and CIDE-A-positive brown-like adipocytes. Accordingly, a trend to a higher expression of the UCP1 protein was also observed in this depot, although levels were not different from those of the WD group, probably due to the high variability among this group. Considering that UCP1 is the hallmark protein responsible for thermogenesis, the induction by dietary capsaicin of brown-like cells expressing this protein suggest that adipose tissue of these animals may have an increased thermogenic capacity. However, in the inguinal depot, although the browning process was not evident at the morphological level, capsaicin treatment brought about a significant increase in the mRNA expression levels of *Prdm16* and *Cidea,* and increased protein levels of CIDE-A with respect to the control and/or the WD group. The transcriptional regulator PRDM-16 is a determinant of brown adipocyte lineage. PRDM-16 stimulates brown adipogenesis by binding to PPAR-γ (peroxisome-proliferator-activated receptor-γ) and

activating its transcriptional function *(Seale et al.,* 2008). Thus, dietary capsaicin could induce the molecular conversion of white adipocytes to brite cells by increasing the expression of *Prdm-16*. This is consistent with previous work showing increased expression of *Prdm-16* and *Pgc-1α* in mice due to dietary intake of capsaicin along with a high fat diet (Baskaran *et al.*, 2016). The mechanism by which capsaicin induces browning involves the induction of sirtuin-1 expression and activity via TRPV1 channel activation. In turn, sirtuin-1 triggers the deacetylation and interaction of PRDM-16 and PPARγ to mediate browning of WAT (Baskaran *et al*., 2016). TRPV1 has been shown to be essential for the effects of capsaicin, since it does not have effects on fat depots in TRPV1-/- mice (that genetically lack TRPV1 channels) (Baskaran *et al*., 2016).

The precise role of cell death-inducing DNA fragmentation factor alpha-like effector A (CIDE-A) is not clearly known, but it has been shown to be implicated in lipid droplet formation and lipid storage in adipose tissue (Wu *et al*., 2014). Particularly, transgenic mice expressing human CIDE-A display a robust increase of adipose tissue lipid storage capacity on a high-fat diet, suggesting that it is involved in protection against the metabolic complications of obesity (Abreu-Vieira *et al*., 2015). In humans, *Cidea* is expressed in WAT and expression levels are positively correlated with insulin sensitivity and healthy obesity; in fact, low expression levels are associated with several features of the metabolic syndrome (Nordström *et al*., 2005). Therefore, the increased expression of *Cidea* in capsaicin-treated animals may indicative of improved metabolic health.

Present results showing browning induction by capsaicin are in accordance with previous studies (Baboota *et al*., 2014; Baskaran *et al*., 2016), although the effects are depending on the animal model and conditions of treatment. For example, combination of capsinoids with mild cold exposure (17°C) in mice synergistically promoted brite adipocyte biogenesis in inguinal WAT, and ameliorated diet-induced obesity (Ohyama *et al.*, 2016).

It should be mentioned the finding of a depot-specific response to the treatments, since capsaicin entailed increased expression of brown markers in the inguinal depot, whereas histological evidence of browning was only found in the retroperitoneal fat. This is consistent with previous work showing that browning induction by the intake of a cafeteria diet was only evident at the histological level in the retroperitoneal depot, despite the inguinal depot showed increased expression levels of brown/brite markers (García-Ruiz *et al.*, 2015).

Besides capsaicin, hesperidin has interesting properties for the treatment of obesity-related alterations, mainly because of its fat-lowering effect. However, unlike capsaicin, no clear effects of hesperidin have been described on body weight control, nor has it been reported to promote WAT browning or thermogenesis (Li & Schluesener, 2017), although it has been shown to potentiate the thermogenic effect of p-synephrine (Stohs *et al.*, 2011). Here, we describe that hesperidin treatment induced morphological changes in WAT, concretely this was associated with a decreased mean size of adipocytes in both retroperitoneal and inguinal WAT depots, as did capsaicin. Notably, hesperidin treatment also induced the appearance of multilocular

adipocytes, positive for UCP1 and CIDE-A, in the retroperitoneal depot. Protein levels of CIDE-A were also increased in the inguinal depot, similarly to what observed by capsaicin treatment, but no significant induction was observed at the gene expression level in any of the two fat pads analysed. Therefore, hesperidin along with a western diet may have the potential to induce a certain browning in WAT, although to a lesser extent compared with capsaicin. However, besides this, no significant effects on body weight gain or body fat percentage have been observed here in rats treated with hesperidin along with a western diet, beyond the effects decreasing adipocyte size. Human studies have also described that the supplementation with citrus-based juice, rich in Vitamin C and hesperidin, improved risk factors in metabolic syndrome-patients but had no effect on anthropometric parameters (Mulero *et al*., 2012).

Besides browning induction, it is important to mention that both capsaicin and hesperidin treatments led to a significant increase in total UCP1 levels in interscapular BAT, compared to control animals, suggesting that these animals may have increased thermogenic capacity versus the control group, but not compared to western diet-fed animals. However, in spide of the effects of capsaicin and hesperidin on WAT browning and BAT total UCP1 content, we did not observe any significant increase in daily energy expenditure by capsaicin or hesperidin, measured at 6 weeks of treatment. Other authors such as Baskaran *et al.* have demonstrated an enhancement of energy expenditure in mice, but after 26 weeks of treatment with dietary capsaicin (Baskaran *et al.*, 2016). It could be possible that the potential increase in thermogenic capacity presented by the animals treated with capsaicin or hesperidin

only resulted in a greater effective energy expenditure under appropriate stimuli. In fact, Ohyama *et al.* have described that dietary supplementation of capsinoids promotes cold-induced thermogenesis *in vivo*, but the anti-obesity effect of capsinoids was completely blunted under thermoneutral conditions of 30°C (Ohyama *et al.*, 2016). Regarding hesperidin, there are no previous studies showing its potential thermogenic effects *in vivo*.

Despite the potential of capsaicin and hesperidin, individually, to induce certain browning in western diet-fed rats, it was surprising that the combination of both bioactives did not exert apparent effects on browning, neither at the histological level, nor at the gene expression level, in any of the fat depots studied. This is in agreement with the lesser effects exerted by the combination of both compounds *vs* capsaicin alone counteracting western diet-induced increase in body weight and adiposity. Mechanisms underlying the negative interaction between both compounds are unknown. Considering the involvement of the TRPV1 channel in the browning program of WAT induced by capsaicin, it could hypothesized that hesperidin could override the effects of capsaicin through its TRPV1 receptor. In fact, rats treated with hesperidin showed lower *Trpv1* expression levels in the iWAT compared to control rats, suggesting that the responsiveness to the capsaicin action was impaired in animals treated with the combination of bioactives. This is in accordance with the results described in liver (manuscript 2), where induction of *Trpv1* expression by capsaicin was attenuated with the simultaneous treatment with hesperidin, and hence

the beneficial effects of capsaicin on liver health were not evident with the combination of both bioactives. However, we cannot rule out the involvement of other mechanisms, but have not been explored here. The knowledge of these negative interactions may be of interest to design appropriate combinations of bioactives in the treatment of obesity and its related metabolic complications.

In conclusion, our results demonstrate the capacity of capsaicin and hesperidin, separately, to reduce the adipocyte size and of capsaicin and, to a lesser extent, of hesperidin, to induce WAT browning, and counteract, in the case of capsaicin, the increased body weight gain associated to western diet feeding in rats. However, the combination of hesperidin and capsaicin reduces the effectiveness of each compound in reducing the adipocyte size and induction of browning and therefore should be avoided in formulations designed to assist body weight loss and management.

***In vitro* study**

1. The most potent phenolic compounds in terms of anti-adipogenic effect are flavanones (naringenin and hesperidin) and the flavonol quercetin.

2. The mechanism underlying the anti-adipogenic effect is not exactly the same for all the compounds.

***In vivo* study**

3. Capsaicin treatment in western-diet fed rats induces a protective phenotype against obesity, decreasing body fat gain and preventing insulin resistance, whereas hesperidin has little effects on body fat gain and no apparent effects on insulin resistance. No additive effects, or even blunted with respect to those observed with capsaicin, were observed with the combination of both bioactives.

4. The effects of capsaicin in preventing fat accumulation may be explained, at least in part, by the effects on lipid metabolism in adipose tissue, particularly by its lipogenesis inhibitory effect. The combination of capsaicin and hesperidin also elicited no additional effects to those observed with capsaicin alone.

5. Treatment with capsaicin and hesperidin separately reduces the adipocyte size and induce WAT browning in western diet-fed rats. However the combination of both molecules shows lower effectiveness.

6. Capsaicin and hesperidin, separately, improve blood lipid profile, diminish hepatic lipid accumulation, and prevent non-alcoholic steatohepatitis in western diet-fed rats, although the effects are mitigated or even blunted with the combination of both compounds.

7. Hesperidin alone, and to a lesser extent capsaicin or the combination of hesperidin plus capsaicin, display hypotensive effects in western diet-fed rats.

8. All in all, capsaicin and hesperidin, separately, exhibit different effects on fat accumulation and metabolic syndrome related disorders in western diet-fed rats, but a combination of both bioactives attenuates the beneficial effects produced by each molecule when administered individually.

Adams MJ, Ahuja KD, Geraghty DP. (2009). Effect of capsaicin and dihydrocapsaicin on in vitro blood coagulation and platelet aggregation. Thromb Res. 124(6):721-3.

Al Othman ZA, Ahmed YB, Habila MA, Ghafar AA. (2011).Determination of capsaicin and dihydrocapsaicin in Capsicum fruit samples using high performance liquid chromatography. *Molecules*.16(10):8919-29.

Ali AT, Hochfeld WE, Myburgh R, Pepper MS. (2013).Adipocyte and adipogenesis. Eur J Cell Biol. 92(6-7):229-36.

Ameer F, Scandiuzzi L, Hasnain S, Kalbacher H, Zaidi N. (2014).De novo lipogenesis in health and disease. Metabolism. 63(7):895-902.

Anandakumar P, Kamaraj S, Jagan S, Ramakrishnan G, Asokkumar S, Naveenkumar C, *et al.* (2012).Capsaicin inhibits benzo(a)pyrene-induced lung carcinogenesis in an in vivo mouse model. *Inflamm Res.* 61(11):1169-75.

Apalasamy, Y.D. and Z. Mohamed (2015). Obesity and genomics: role of technology in unraveling the complex genetic architecture of obesity Hum Genet 134(4): 361-374.

Arner P, Spalding KL. (2010). Fat cell turnover in humans. Biochem Biophys Res Commun.396(1):101-4.

Assini JM, Mulvihill EE, Huff MW. (2013).Citrus flavonoids and lipid metabolism. Curr Opin Lipidol. 24(1):34-40.

Aza-González C, Núñez-Palenius HG, Ochoa-Alejo N.(2011).Molecular biology of capsaicinoid biosynthesis in chili pepper (Capsicum spp.). *Plant Cell Rep*.;30(5):695-706.

Baranowski M. (2008).Biological role of liver X receptors. J Physiol Pharmacol.59 Suppl 7:31-55.

Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, et al. (2010).The emergence of cold-induced brown adipocytes in mouse white

fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Am J Physiol Endocrinol Metab.* 298(6):E1244-53.

Barbera MJ, Schluter A, Pedraza N, Iglesias R, Villarroya F, Giralt M (2001). Peroxisome proliferator-activated receptor alpha activates transcription of the brown fat uncoupling protein-1 gene. A link between regulation of the thermogenic and lipid oxidation pathways in the brown fat cell. *J Biol Chem* 276(2): 1486-1493.

Barcelo-Batllori, S. and R. Gomis (2009). “Proteomics in obesity research”. Proteomics Clin Appl 3(2):263-278.

Barneda D, Frontini A, Cinti S, Christian M.(2013). Dynamic changes in lipid droplet-associated proteins in the "browning" of white adipose tissues. Biochim Biophys Acta. 1831(5):924-33.

Bartelt, A. and J. Heeren (2014). "Adipose tissue browning and metabolic health." Nat Rev Endocrinol 10(1): 24-36.

Barthe G., Pablo S., Jourdan Cecilia A, McIntosh Richard, L Mansell. (1988). Radioimmunoassay for the quantitative determination of hesperidin and analysis of its distribution in Citrus sinensis. Phytochemistry. 27(1):249-254.

Beecher GR. (2003).Overview of dietary flavonoids: nomenclature, occurrence and intake. J Nutr. 133(10):3248S-54S.

Bernlohr DA, Bolanowski MA, Kelly TJ, Lane MD. (1985). Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. J Biol Chem. 260(9):5563-7.

Bode AM, Dong Z. (2011).The two faces of capsaicin. Cancer Res. 71(8):2809-14.

Bok, S. H., S. H. Lee, Y. B. Park, K. H. Bae, K. H. Son, T. S. Jeong and M. S. Choi (1999). "Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA: cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids." *J Nutr* 129(6): 1182-1185.

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

Bonet ML, Oliver P, Palou A. (2013).Pharmacological and nutritional agents promoting browning of white adipose tissue. Biochim Biophys Acta.1831(5):969-85.

Bouchard, C., Despres, J.P.(2000). Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia. International Journal of Obesity and Related Metabolic Disorders 24 (6): 782–788.

Bradamante S, Barenghi L, Villa A.(2004). Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev. 22(3):169-88.

Cannon B, Nedergaard J. (2004).Brown adipose tissue: function and physiological significance. Physiol Rev.84(1):277-359.

Chaiyasit K, Khovidhunkit W, Wittayalertpanya S. (2009).Pharmacokinetic and the effect of capsaicin in Capsicum frutescens on decreasing plasma glucose level. J Med Assoc Thai.92(1):108-13.

Cinti S. (2009).Transdifferentiation properties of adipocytes in the adipose organ. Am J Physiol Endocrinol Metab.;297(5):E977-86.

Cinti S. (2011).Between brown and white: novel aspects of adipocyte differentiation. Ann Med. 43(2):104-15.

Cinti S. (2012)The adipose organ at a glance. Dis Model Mech.5(5):588-94.

Cornier MA, Marshall JA, Hill JO, Maahs DM, Eckel RH. (2011). Prevention of overweight/obesity as a strategy to optimize cardiovascular health. Circulation. 124(7):840-50.

Couillard, C., Mauriege, P., Imbeault, P., Prud'homme, D., Nadeau, A., Tremblay, A.,

Crichton PG, Lee Y, Kunji ER. (2017). The molecular features of uncoupling protein 1 support a conventional mitochondrial carrier-like mechanism. Biochimie. 134:35-50.

Cruz F, Guimarães M, Silva C, Rio ME, Coimbra A, Reis M. (1997).Desensitization of bladder sensory fibers by intravesical capsaicin has long lasting clinical and urodynamic effects in patients with hyperactive or hypersensitive bladder dysfunction. *J Urol*. 157(2):585-9.

Cucciolla V, Borriello A, Oliva A, Galletti P, Zappia V, Della Ragione F. (2007).Resveratrol: from basic science to the clinic. *Cell Cycle*. 6(20):2495-510.

De-Sá-Júnior PL, Pasqualoto KF, Ferreira AK, Tavares MT, Damião MC, de Azevedo RA, *et al.* (2013).RPF101, a new capsaicin-like analogue, disrupts the microtubule network accompanied by arrest in the G2/M phase, inducing apoptosis and mitotic catastrophe in the MCF-7 breast cancer cells. *Toxicol Appl Pharmacol.* 266(3):385-98.

Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. (2013).Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal. 18(14):1818-92.

Devari S, Jaglan S, Kumar M, Deshidi R, Guru S, Bhushan S, *et al*. (2014).Capsaicin production by Alternaria alternata, an endophytic fungus from Capsicum annum; LC-ESI-MS/MS analysis. *Phytochemistry.* 98:183-9.

Donnerer J, Amann R, Schuligoi R, Lembeck F. (1990).Absorption and metabolism of capsaicinoids following intragastric administration in rats. *Naunyn Schmiedebergs Arch Pharmacol.* 342(3):357-61.

Erlund, I.; Meririnne, E.; Alfthan, G.; Aro, A. (2001). Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. *J. Nutr. 131* (2), 235–41.

Fluckiger FA, Hanbury D. (1986). In PharmacographiaÐA History of the Principle Drugs of Vegetable Origin. *International Book Distributors: Delhi, India*; 104±105.

Frayn KN, Arner P, Yki-Järvinen H. (2006). Fatty acid metabolism in adipose tissue, muscle and liver in health and disease. Essays Biochem. 42:89-103.

Frühbeck G, Gómez-Ambrosi J, Muruzábal FJ, Burrell MA. (2001). The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. Am J Physiol Endocrinol Metab. 280(6):E827-47.

Frühbeck G. (2008). Overview of adipose tissue and its role in obesity and metabolic disorders. Methods Mol Biol.456:1-22.

Fujiwake H, Iwai K, Suzuki T, Oka S. (1979).Simultaneous microdetermination of capsaicin and its four analogues by using high-performance liquid chromatography and gas chromatography--mass spectrometry. J Chromatogr. 172:303-11.

Gaidhu MP, Anthony NM, Patel P, Hawke TJ, Ceddia RB. (2010).Dysregulation of lipolysis and lipid metabolism in visceral and subcutaneous adipocytes by high-fat diet: role of ATGL, HSL, and AMPK. Am J Physiol Cell Physiol. 298(4):C961-71.

Galic S, Oakhill JS, Steinberg GR. (2010). Adipose tissue as an endocrine organ. Mol Cell Endocrinol.316(2):129-39.

Garg A, Garg S, Zaneveld LJ, Singla AK. (2001). Chemistry and pharmacology of the Citrus bioflavonoid hesperidin. Phytother Res.;15(8):655-69.

Giby VG, Ajith TA. (2014). Role of adipokines and peroxisome proliferator-activated receptors in nonalcoholic fatty liver disease. World J Hepatol. 6(8):570-9.

Gil-Izquierdo, A.; Gil, M. I.; Ferreres, F.; Tomas-Barberan, F. A.(2001). In vitro availability of flavonoids and other phenolics in orange juice. *J. Agric. Food Chem. 49* (2), 1035–41.

Gizem Onal *et al.* (2017). Lipid Droplets and Health and Disease. *Lipids in Health and Disease.*16(1)28.

Gmyrek DP. (2013).Wilbur Lincoln Scoville: the prince of peppers. Pharm Hist. 55(4):136-56.

González-Muniesa P, Mártinez-González MA, Hu FB, Després JP, Matsuzawa Y, Loos RJF, *et al*. (2017).Obesity. Nat Rev Dis Primers.3:17034.

Green H, Kehinde O. (1976). Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell.7(1):105-13.

Green H, Meuth M. (1974). An established pre-adipose cell line and its differentiation in culture. Cell.3(2):127-33.
Gunthorpe MJ, Szallasi A. (2008).Peripheral TRPV1 receptors as targets for drug development: new molecules and mechanisms. *Curr Pharm Des.* 14(1):32-41.

Guo, Y., Walther, T.C., Rao, M., Stuurman, N., Goshima, G., Terayama, K., Wong, J.S., Vale, R.D., Walter, P., and Farese, R.V. (2008). Functional genomic screen reveals genes involved in lipid-droplet formation and utilization. Nature *453*, 657–661

Harborne, J.B.; Williams, C.A. (2000).Advances in flavonoid research since 1992. *Phytochemistry* 55, 481-504.

Hartley T, Stevens B, Ahuja KD, Ball MJ. (2013). Development and experimental application of an HPLC procedure for the determination of capsaicin and dihydrocapsaicin in serum samples from human subjects. *Indian J Clin Biochem*. 28(4):329-35.

Hassan SJ, O'Shea D. (2012).Weight management services for adults--highlighting the role of primary care. Ir Med J.105(10):344.

Hill JO, Wyatt HR, Peters JC. (2012).Energy balance and obesity. Circulation. 126(1):126-32.

Holm C. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. (2003).*Biochem Soc Trans.* 31(Pt 6):1120-4.

Hursel R, Westerterp-Plantenga MS. (2010).Thermogenic ingredients and body weight regulation. *Int J Obes* (Lond). 34(4):659-69.

Jancova P, Anzenbacher P, Anzenbacherova E. (2010).Phase II drug metabolizing enzymes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 154(2):103-16.

Jauch-Chara, K. & Oltmanns, K. M. (2014). Obesity a neuropsychological disease? Systematic review and neuropsychological model. *Prog. Neurobiol.* 114, 84–101.

Jeong, E. J.; Liu, X.; Jia, X.; Chen, J.; Hu, M. (2005).Coupling of conjugating enzymes and efflux transporters: Impact on bioavailability and drug interactions. *Curr. Drug Metab.6* (5), 455– 68.

Josse AR, Sherriffs SS, Holwerda AM, Andrews R, Staples AW, Phillips SM. (2010). Effects of capsinoid ingestion on energy expenditure and lipid oxidation at rest and during exercise. *Nutr Metab (Lond*). 7:65.

Jung J, Shin JS, Lee SY, Hwang SW, Koo J, Cho H, *et al.* (2004).Phosphorylation of vanilloid receptor 1 by Ca2+/calmodulin-dependent kinase II regulates its vanilloid binding. *J Biol Chem.* 279(8):7048-54.

Jung, U. J. and M.-S. Choi (2014). Obesity and Its Metabolic Complications: The Role of Adipokines and the Relationship between Obesity, Inflammation, Insulin Resistance, Dyslipidemia and Nonalcoholic Fatty Liver Disease. International Journal of Molecular Sciences 15(4): 6184-6223.

Kajimura S, Saito M. (2014).A new era in brown adipose tissue biology: molecular control of brown fat development and energy homeostasis. Annu Rev Physiol. 76:225-49.

Kampa M, Nifli AP, Notas G, Castanas E. (2007).Polyphenols and cancer cell growth. *Rev Physiol Biochem Pharmacol*. 159:79-113.

Kanaze FI, Bounartzi MI, Georgarakis M, Niopas I. (2007).Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. *Eur J Clin Nutr.* 61(4):472-7.

Kang JH, Tsuyoshi G, Le Ngoc H, Kim HM, Tu TH, Noh HJ, *et al.* (2011).Dietary capsaicin attenuates metabolic dysregulation in genetically obese diabetic mice. *J Med Food*. 14(3):310-5.

Kanneganti, T.D. and V.D.Dixit (2012). "Immunological complications of obesity" Nat Immunol 13(8):707-712.

Kawada T, Suzuki T, Takahashi M, Iwai K. (1984). Gastrointestinal absorption and metabolism of capsaicin and dihydrocapsaicin in rats. Toxicol Appl Pharmacol. 72(3):449-56.

Kawada T, Watanabe T, Takaishi T, Tanaka T, Iwai K. (1986).Capsaicin induced beta-adrenergic action on energy metabolism in rats: influence of capsaicin on oxygen consumption, the respiratory quotient, and substrate utilization. Proc Soc Exp Biol Med.183(2):250-6.

Kim DH, Jung EA, Sohng IS, Han JA, Kim TH, Han MJ (1998). Intestinal bacterial metabolism of flavonoids and its relation to some biological activities. Arch Pharm Res 21(1): 17-23.

Kim S, Park M, Yeom SI, Kim YM, Lee JM, Lee HA, *et al.* (2014). Genome sequence of the hot pepper provides insights into the evolution of pungency in Capsicum species. *Nat Genet.* 46(3):270-8.

Kobayashi, S.; Tanabe, S.; Sugiyama, M.; Konishi, Y. (2007(. Transepithelial transport of hesperetin and hesperidin in intestinal Caco-2 cell monolayers. Biochim. Biophys. Acta 2007.

Kohjima M, Higuchi N, Kato M, Kotoh K, Yoshimoto T, Fujino T, *et al.* (2008).SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. Int J Mol Med. 21(4):507-11.

Kompare M, Rizzo WB. (2008).Mitochondrial fatty-acid oxidation disorders. *Semin Pediatr Neurol.* 15(3):140-9.

Kotani K, Tokunaga K, Fujioka S, Kobatake T, Keno Y, Yoshida S, et al. (1994). Sexual dimorphism of age-related changes in whole-body fat distribution in the obese. *Int J Obes Relat Metab Disord*.18(4):207-2.

Kozak LP, Anunciado-Koza R. (2008). UCP1: its involvement and utility in obesity. Int J Obes (Lond).32 Suppl 7:S32-8.

Kumar S, Pandey AK. (2013). Chemistry and biological activities of flavonoids: an overview. *ScientificWorldJournal.* 2013:162750.

Lee MS, Kim CT, Kim IH, Kim Y. (2011). Effects of capsaicin on lipid catabolism in 3T3-L1 adipocytes. *Phytother Res.* 25(6):935-9.

Lee, N. K.; Choi, S. H.; Park, S. H.; Park, E. K.; Kim, D. H.(2004). Antiallergic activity of hesperidin is activated by intestinal microflora. *Pharmacology 71* (4), 174–80.

Leelahuta Y, Glinsukon T, Wangpanish W. (1983) In vitro capsaicin metabolism in the rat, mouse and hamster. *Toxicon 21* (Suppl 3):245–248.

Lefterova MI, Lazar MA. (2009). New developments in adipogenesis. Trends Endocrinol Metab. 20(3):107-14.

Lejeune MP, Kovacs EM, Westerterp-Plantenga MS. (2003).Effect of capsaicin on substrate oxidation and weight maintenance after modest body-weight loss in human subjects. *Br J Nutr.* 90(3):651-59.

Letexier D, Pinteur C, Large V, Fréring V, Beylot M. (2003).Comparison of the expression and activity of the lipogenic pathway in human and rat

adipose tissue. J Lipid Res. 44(11):2127-34.

Li JJ, Huang CJ, Xie D (2008). Anti-obesity effects of conjugated linoleic acid, docosahexaenoic acid, and eicosapentaenoic acid. Mol Nutr Food Res 52(6): 631-645

Liu, Y.; Liu, Y.; Dai, Y.; Xun, L.; Hu, M. (2003). Enteric disposition and recycling of flavonoids and ginkgo flavonoids. *J. Altern. Complement. Med. 9* (5), 631–40.

López-Carrillo L, Hernández Avila M, Dubrow R. (1994).Chili pepper consumption and gastric cancer in Mexico: a case-control study. *Am J Epidemiol*. 139(3):263-71.

Lu HF, Chen YL, Yang JS, Yang YY, Liu JY, Hsu SC, *et al.* (2010).Antitumor activity of capsaicin on human colon cancer cells in vitro and colo 205 tumor xenografts in vivo. *J Agric Food Chem.* 58(24):12999-3005.

Ludy MJ, Mattes RD. (2011).The effects of hedonically acceptable red pepper doses on thermogenesis and appetite. Physiol Behav. 102(3-4):251-8.

Mahmoud AM, Ashour MB, Abdel-Moneim A, Ahmed OM.(2012). Hesperidin and naringin attenuate hyperglycemia-mediated oxidative stress and proinflammatory cytokine production in high fat fed/streptozotocin-induced type 2 diabetic rats. *J Diabetes Complications.* 26(6):483-90.

Maihofner C, Heskamp ML. (2013).Prospective, non-interventional study on the tolerability and analgesic effectiveness over 12 weeks after a single application of capsaicin 8% cutaneous patch in 1044 patients with peripheral neuropathic pain: first results of the QUEPP study. *Curr Med Res Opin.*29(6):673-83.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. (2004). Polyphenols: food sources and bioavailability. *Am J Clin Nutr*. 79(5):727-47.

Manach, C., Morand, C., Gil-Izquierdo, A., Bouteloup-De- mange, C., Remesy, C.(2003). Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *Eur. J. Clin. Nutr. 57* (2), 235–42.

Manthey JA, Grohmann K. (1998).Flavonoids of the orange subfamily Aurantioideae. Adv Exp Med Biol. 439:85-101.

Matthew J. Watt, Gregory R. Steinberg (2008). *Regulation and function of triacylglycerol lipases in cellular metabolism. Biochemical Journal*.414 (3) 313-325.

McCarty MF, DiNicolantonio JJ, O'Keefe JH. (2015).Capsaicin may have important potential for promoting vascular and metabolic health. *Open Heart.* 2(1):e000262.

Meghvansi MK, Siddiqui S, Khan MH, Gupta VK, Vairale MG, Gogoi HK, *et al.* (2010).Naga chilli: a potential source of capsaicinoids with broad-spectrum ethnopharmacological applications. *J Ethnopharmacol*. 132(1):1-14.

Meydani M, Hasan ST. (2010). Dietary polyphenols and obesity. Nutrients. 2(7):737-51.

Mittelstadt SW, Nelson RA, Daanen JF, King AJ, Kort ME, Kym PR, *et al.* (2012).Capsaicin-induced inhibition of platelet aggregation is not mediated by transient receptor potential vanilloid type 1. *Blood Coagul Fibrinolysis*.23(1):94-7.

Moon, D.O., Kang, C.H., Kang, S.H., Choi, Y.H., Hyun, J.W., Chang, W.Y., Kang, H.K., Koh, Y.S., Maeng, Y.H., Kim, Y.R., Kim, G.Y.(2012). Capsaicin sensitizes TRAIL- induced apoptosis through Sp1-mediated DR5 up-regulation: involvement of Ca^{+2} influx. *Toxicol. Appl. Pharmacol*. 259, 87–95.

Moreno-Aliaga MJ, Martínez JA, Stanhope KL, Fernández-Otero MP, Havel PJ. (2002).Effects of Trecadrine, a beta3-adrenergic agonist, on

leptin secretion, glucose and lipid metabolism in isolated rat adipocytes. Int J Obes Relat Metab Disord. 26(7):912-9.

Mori A, Lehmann S, O'Kelly J, Kumagai T, Desmond JC, Pervan M, *et al.* (2006). Capsaicin, a component of red peppers, inhibits the growth of androgen-independent, p53 mutant prostate cancer cells. *Cancer Res*.66(6):3222-9.

Mueller E, Drori S, Aiyer A, Yie J, Sarraf P, Chen H, *et al.* (2002). Genetic analysis of adipogenesis through peroxisome proliferator-activated receptor gamma isoforms. J Biol Chem.277(44):41925-30.

Nguyen P, Leray V, Diez M, Serisier S, Le Bloc´h J, Sillart B, *et al*. (2008). Liver lipid metabolism. *J Anim Physiol Anim Nutr*. 92(3):272-83.

Nordestgaard, B. G. *et al.*(2012). The effect of elevated body mass index on ischemic heart disease risk: causal estimates from a Mendelian randomisation approach. *PLoS Med.* 9, e1001212.

Palou A, Picó C, Bonet ML, Oliver P. (1998).The uncoupling protein, thermogenin. *Int J Biochem Cell Biol.* 30(1):7-11.

Park, M. H., Sovio, U., Viner, R. M., Hardy, R. J. & Kinra, S. (2013). Overweight in childhood, adolescence and adulthood and cardiovascular risk in later life: pooled analysis of three British birth cohorts. *PLoS ONE* 8, e70684.

Perry L, Dickau R, Zarrillo S, Holst I, Pearsall DM, Piperno DR, *et al.* (2007).Starch fossils and the domestication and dispersal of chili peppers (Capsicum spp. L.) in the Americas. *Science.* 315(5814):986-8.

Petrovic, N., T. B. Walden, I. G. Shabalina, J. A. Timmons, B. Cannon and J. Nedergaard (2010). Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes.*The Journal of Biological Chemistry.* 285(10): 7153-7164.

Popkin, B.M., L.S. Adair and S.W. Ng (2012) “Global Nutrition Transition and Thepandemic of Obesity in Developing Countries” Nutr Rev 70(1): 3-21.

Prakash UN, Srinivasan K. (2010). Gastrointestinal protective effect of dietary spices during ethanol-induced oxidant stress in experimental rats. *Appl Physiol Nutr Metab.* 35(2):134-41.

Prieto-Hontoria , P.L., Pérez-Matute, M. Fernández-Galilea, *et al*.(2011). Role of obesity-associated dysfunctional adipose tissue in cancer: a molecular nutrition approach. Biochim Biophys Acta 1807(6):664-678.

Raghavendra RH, Naidu KA. (2009). Spice active principles as the inhibitors of human platelet aggregation and thromboxane biosynthesis. *Prostaglandins Leukot Essent Fatty Acids.* 81(1):73-8.

Reilly CA, Yost GS. (2006). Metabolism of capsaicinoids by P450 enzymes: a review of recent findings on reaction mechanisms, bio-activation, and detoxification processes. *Drug Metab Rev.* 38(4):685-706.

Renehan, A. G. *et al.* (2010). Incident cancer burden attributable to excess body mass index in 30 European countries. *Int. J. Cancer* 126, 692–702.

Rosen ED, Spiegelman BM (2000). Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol. 16, 145-71.

Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM.(2000). Transcriptional regulation of adipogenesis. Genes Dev.14(11):1293-307.

Rosenwald M, Perdikari A, Weber E, Wolfrum C. (2013).Phenotypic Analysis of BAT versus WAT Differentiation. Curr Protoc Mouse Biol. 3(4):205-16.

Ross, J. A., Kasum, C. M. (2002). Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu. Re*V*. Nutr*. *22*, 19–34.

Rothwell NJ, Stock MJ. (1997). A role for brown adipose tissue in diet-induced thermogenesis. Obes Res. 5(6):650-6.

Rothwell NJ, Stock MJ.(1979). A role for brown adipose tissue in diet-induced thermogenesis. Nature. 281(5726):31-5.

Rutkowski, J.M., J.H. Stern and P.E. Scherer (2015). The cell biology of fat expansion. J Cell Biol 208(5): 501-512.

Saltiel, A.R. and J.M. Olefsky (2017). “Inflammatory mechanisms linking obesity and metabolic disease” J Clin Invest 127(1): 1-4

Sam,S. and T. Mazzone (2014). “Adipose tissue changes in obesity and the impact on metabolic function” Transl Res 164(4): 284-292

Scalbert A, Williamson G. (2000).Dietary intake and bioavailability of polyphenols. *J Nutr*. 130(8S Suppl):2073S-85S.

Schweiger M, Schreiber R, Haemmerle G, Lass A, Fledelius C, Jacobsen P, *et al.* (2006).Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. J Biol Chem. 281(52):40236-41.

Scully T. (2014).Public health: society at large. Nature.508:S50-1.

Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. (2008). PRDM16 controls a brown fat/skeletal muscle switch. Nature. 454(7207):961-7.

Seidell, J.C. and J. Halberstadt (2015). “The Global Burden of Obesity and the Challenges of Prevention.”Annu Rev Immunol 25:101-137.

Sekine, R., Satoh, T., Takaoka, A., Saeki, K., Yokozeki, H. (2012). Antipruritic effects of topical crotamiton, capsaicin, and a corticosteroid on pruritogen-induced scratching behavior. *Exp. Dermatol.* 21, 201–204.

Sethi JK, Vidal-Puig AJ. (2007). Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation. J Lipid Res.48(6):1253-62.

Sexton, A., McDonald, M., Cayla, C., Thiemermann, C., Ahluwalia, A. (2007). 12-Lipoxygenase-derived eicosanoids protect against myocardial ischemia/ reperfusion injury via activation of neuronal TRPV1. FASEB J.: Off. Publ. Fed. *Am. Soc. Exp. Biol*. 21, 2695–2703.

Shan T, Liang X, Bi P, Zhang P, Liu W, Kuang S. (2013). Distinct populations of adipogenic and myogenic Myf5-lineage progenitors in white adipose tissues. *J Lipid Res.* 54(8):2214-24.

Sharma, S. K., Vij, A. S., Sharma, M. (2013). Mechanisms and clinical uses of capsaicin. *Eur J Pharmacol*. *720*, 55-62.

Smith CJ, Wejksnora PJ, Warner JR, Rubin CS, Rosen OM. (1979). Insulin-stimulated protein phosphorylation in 3T3-L1 preadipocytes. Proc Natl Acad Sci U S A.76(6):2725-9.

Strissel, K.J., Stancheva, Z., Miyoshi, H., Perfield 2nd, J.W., DeFuria, J., Jick, Z., Greenberg, A.S. and Obin, M.S. (2007) Adipocyte death, adipose tissue remodeling, and obesity complications. Diabetes 56, 2910–2918.

Sylvester DM, LaHann TR. (1989). Effects of capsaicinoids on platelet aggregation. *Proc West Pharmacol Soc.* 32:95-100.
Tanaka, Y., Hosokawa, M., Otsu, K., Watanabe, T., Yazawa, S.(2009). Assessment of capsiconinoid composition, nonpungent capsaicinoid analogues, in capsicum cultivars. *J. Agric. Food Chem*. 57, 5407–5412

Tansey JT, Sztalryd C, Gruia-Gray J, Roush DL, Zee JV, et al. (2001) Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. Proc Natl Acad Sci U S A 98: 6494–6499.

Thiele, R., Mueller-Seitz, E., Petz, M. (2008). Chili pepper fruits: presumed precursors of fatty acids characteristic for capsaicinoids. *J Agric Food Chem.56*, 4219-4224.

Thompson BR, Lobo S, Bernlohr DA. (2010) Fatty acid flux in adipocytes: the in's and out's of fat cell lipid trafficking. Mol Cell Endocrinol. 318 (1-2):24-33

Toh CC, Lee TS, Kiang AK. (1955).The pharmacological actions of capsaicin and analogues. *Br J Pharmacol Chemother.* 10(2):175-82.

Townsend, K. and Y. H. Tseng (2012). "Brown adipose tissue: Recent

insights into development, metabolic function and therapeutic potential." Adipocyte 1(1): 13-24.

Trigueros, L.,Peña, S., Ugidos, A.,Sayas-Barberá, E., Pérez-Álvarez, J.,Sendra, E. (2013).Food ingredients as anti-obesity agents: A review. *Crit. Rev. Food Sci. Nutr. 53*, 929–942.

Tsou MF, Lu HF, Chen SC, Wu LT, Chen YS, Kuo HM, *et al.* (2006).Involvement of Bax, Bcl-2, Ca2+ and caspase-3 in capsaicin-induced apoptosis of human leukemia HL-60 cells. *Anticancer Res*. 26(3A):1965-71.

Umar S, Kumar A, Sajad M, Zargan J, Ansari M, Ahmad S, *et al.* (2013). Hesperidin inhibits collagen-induced arthritis possibly through suppression of free radical load and reduction in neutrophil activation and infiltration. *Rheumatol Int*. 33(3):657-63.

Virtue, S and A.Vidal-Puig (2010). Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome-an allostatic perspective. Biochim Biophys Acta 1801(3):338-349.

Waldén TB, Hansen IR, Timmons JA, Cannon B, Nedergaard J. (2012).Recruited vs. nonrecruited molecular signatures of brown, "brite," and white adipose tissues. Am J Physiol Endocrinol Metab. 302(1):E19-31.

Walgren, R. A.; Walle, U. K.; Walle, T. (1998).Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochem. Pharmacol. 55* (10), 1721–7.

Walle, U. K.; French, K. L.; Walgren, R. A.; Walle, T. (1999).Transport of genistein-7-glucoside by human intestinal CACO-2 cells: Potential role for MRP2. *Res. Commun. Mol. Pathol. Pharmacol*,.*103* (1), 45–56.

Wang *et al*. (2011). Health and economic burden of the projected obesity trends in the USA and the UK Lancet 378,(9805):1778.

Wang HM, Chuang SM, Su YC, Li YH, Chueh PJ. (2011).Down-regulation of tumor-associated NADH oxidase, tNOX (ENOX2), enhances capsaicin-induced inhibition of gastric cancer cell growth. *Cell Biochem Biophys.* 61(2):355-66.

Wang JP, Tseng CS, Sun SP, Chen YS, Tsai CR, Hsu MF. (2005).Capsaicin stimulates the non-store-operated Ca2+ entry but inhibits the store-operated Ca2+ entry in neutrophils. *Toxicol Appl Pharmacol*. 209(2):134-44.

Wang QA, Tao C, Gupta RK, Scherer PE (2013). Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nat Med 19(10): 1338-1344.

Watt MJ, Steinberg GR. (2008).Regulation and function of triacylglycerol lipases in cellular metabolism. *Biochem J.* 414(3):313-25.

Westerterp-Plantenga MS, Smeets A, Lejeune MP. (2005). Sensory and gastrointestinal satiety effects of capsaicin on food intake. Int J Obes (Lond). 29(6):682-8.

WHO. Obesity and overweight. Fact sheet N°311. Updated oct 2017.

World Health Organization. *Obesity: preventing and managing the global epidemic. Report of a WHO Consultation (WHO Technical Report Series 894)* (WHO, 2000).

Wu, J., P. Boström, L. M. Sparks, L. Ye, J. H. Choi, A. H. Giang, M. Khandekar, K. A. Virtanen, P. Nuutila, G. Schaart, K. Huang, H. Tu, W. D. van Marken Lichtenbelt, J. Hoeks, S. Enerbäck, P. Schrauwen and B. M. Spiegelman (2012). "Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human." Cell 150(2): 366-376.

Xu X, So JS, Park JG, Lee AH (2013). Transcriptional control of hepatic lipid metabolism by SREBP and ChREBP. Semin Liver Dis. 33(4):301-11.

Yamada, M.; Tanabe, F.; Arai, N.; Mitsuzumi, H.; Miwa, Y.; Kubota, M.; Chaen, H.; Kibata, M. (2006). Bioavailability of glucosyl hesperidin in rats. *Biosci., Biotechnol., Biochem. 70* (6), 1386–94.

Yamamoto, M., Suzuki, A., Hase, T.(2008). Short-term effects of glucosyl hesperidin and hesperetin on blood pressure and vascular endothelial function in spontaneously hypertensive rats. *J Nutr Sci Vitaminol (Tokyo).54*, 95-98.

Yoshioka M, Lim K, Kikuzato S, Kiyonaga A, Tanaka H, Shindo M, *et al.(*1995). Effects of red-pepper diet on the energy metabolism in men. *J Nutr Sci Vitaminol* (Tokyo). 41(6):647-56.

Yoshioka M, St-Pierre S, Suzuki M, Tremblay A. (1998). Effects of red pepper added to high-fat and high-carbohydrate meals on energy metabolism and substrate utilization in Japanese women. *Br J Nutr.* 80(6):503-10

Yoshioka, M., St-Pierre, S., Drapeau, V., Dionne, I.*, et al.*(1999). Effects of red pepper on appetite and energy intake. *Br J Nutr.82*, 115-123.

Yu, C.S., 2011. Study on HIF-1alpha gene translation in psoriatic epidermis with the topical treatment of capsaicin ointment. *ISRN Pharm.* 821874.

Zamora-Ros R, Knaze V, Rothwell JA, Hémon B, Moskal A, Overvad K, *et al.* (2016).Dietary polyphenol intake in Europe: the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Eur J Nutr.55(4):1359-75.

Zdunczyk, Z., S. Frejnagel, M. Wroś blewska, J. Juś kiewicz, J. Oszmianski, and I. Estrella. (2002). Biological activity of polyphenol extracts from different plant sources. Food Res. Int. Poult. Sci. 35:183–186.

Zechner R, Madeo F. Cell biology: Another way to get rid of fat. (2009). Nature. 458(7242):1118-9.

Zechner R, Zimmermann R, Eichmann TO, Kohlwein SD, Haemmerle G, Lass A, *et al*. (2012).FAT SIGNALS--lipases and lipolysis in lipid metabolism and signaling. Cell Metab. 15(3):279-91.

Zhang, J.H., Lai, F.J., Chen, H., Luo, J., Zhang, R.Y., Bu, H.Q., Wang, Z.H.,

Lin, H.H., Lin, S.Z. (2013). Involvement of the phosphoinositide 3-kinase/Akt pathway in apoptosis induced by capsaicin in the human pancreatic cancer cell line PANC-1. *Oncol. Lett.* 5, 43–48.

Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, et al. (2004). Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 306(5700): 1383-1386.

J Physiol Biochem
DOI 10.1007/s13105-015-0464-2

ORIGINAL PAPER

Limited beneficial effects of piceatannol supplementation on obesity complications in the obese Zucker rat: gut microbiota, metabolic, endocrine, and cardiac aspects

E. Hijona · L. Aguirre · P. Pérez-Matute · M. J. Villanueva-Millán · A. Mosqueda-Solis · M. Hasnaoui · F. Nepveu · J. M. Senard · L. Bujanda · L. Aldámiz-Echevarría · M. Llarena · F. Andrade · P. Perio · F. Leboulanger · L. Hijona · J. M. Arbones-Mainar · M.P. Portillo · C. Carpéné

Received: 14 September 2015 / Accepted: 28 December 2015

Abstract Resveratrol is beneficial in obese and diabetic rodents. However, its low bioavailability raises questions about its therapeutic relevance for treating or preventing obesity complications. In this context, many related natural polyphenols are being tested for their putative antidiabetic and anti-obesity effects. This prompted us to study the influence of piceatannol, a polyhydroxylated stilbene, on the prevention of obesity complications in Zucker obese rats. A 6-week supplementation was followed by the determination of various

E. Hijona and L. Aguirre contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s13105-015-0464-2) contains supplementary material, which is available to authorized users.

E. Hijona · L. Bujanda · L. Hijona
Department of Gastroenterology, Biodonostia Research Institute, University of Basque Country (UPV/EHU), San Sebastián, Spain

E. Hijona · L. Bujanda · L. Hijona
Centro de Investigación Biomédica en Enfermedades Hepáticas (CIBERehd), Instituto de Salud Carlos III, Madrid, Spain

L. Aguirre (✉) · A. Mosqueda-Solis · M. Portillo
Nutrition and Obesity Group, Department of Nutrition and Food Science, University of Basque Country (UPV/EHU), Vitoria, Spain
e-mail: leixuri.aguirre@ehu.eus

L. Aguirre · J. M. Arbones-Mainar · M. Portillo
CIBER Fisiopatología Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III, Madrid, Spain

P. Pérez-Matute · M. J. Villanueva-Millán
HIV and Associated Metabolic Alterations Unit, Infectious Diseases Department, Center for Biomedical Research of La Rioja (CIBIR), Logroño, Spain

M. Hasnaoui · J. M. Senard · F. Leboulanger · C. Carpéné
INSERM U1048, Institute of Metabolic and Cardiovascular Diseases (I2MC), Toulouse, France

F. Nepveu · P. Perio
University of Toulouse, UPS and IRD, UMR 152 (PHARMA-DEV), F-31062 Toulouse cedex 9, France

L. Aldámiz-Echevarría · M. Llarena · F. Andrade
Division of Metabolism, Cruces University Hospital and BioCruces Health Research Institute, Plaza de Cruces s/n, 48903 Barakaldo, Spain

L. Aldámiz-Echevarría · M. Llarena · F. Andrade
GCV-CIBER de Enfermedades Raras (CIBERer), Instituto de Salud Carlos III, Madrid, Spain

J. M. Arbones-Mainar
Adipocyte and Fat Biology Laboratory (AdipoFat), Unidad de Investigación Traslacional, Instituto Aragonés de Ciencias de la Salud (IACS), Zaragoza, Spain

Published online: 20 January 2016

markers in plasma, liver, adipose tissue and heart, together with a large-scale analysis of gut microbiota composition. When given in doses of 15 or 45 mg/kg body weight/day, piceatannol did not reduce either hyperphagia or fat accumulation. It did not modify the profusion of the most abundant phyla in gut, though slight changes were observed in the abundance of several *Lactobacillus*, *Clostridium*, and *Bacteroides* species belonging to *Firmicutes* and *Bacteroidetes*. This was accompanied by a tendency to reduce plasma lipopolysaccharides by 30 %, and by a decrease of circulating non-esterified fatty acids, LDL-cholesterol, and lactate. While piceatannol tended to improve lipid handling, it did not mitigate hyperinsulinemia and cardiac hypertrophy. However, it increased cardiac expression of ephrin-B1, a membrane protein that contributes to maintaining cardiomyocyte architecture. Lastly, ascorbyl radical plasma levels and hydrogen peroxide release by adipose tissue were similar in control and treated groups. Thus, piceatannol did not exhibit strong slimming capacities but did limit several obesity complications.

Keywords Piceatannol · Zucker rat · Adipose tissue · Liver · Gut microbiota · Adipokines · Cardiomyocytes

Introduction

Currently, the number of drugs approved for obesity treatment remains limited. Therefore, the scientific community is actively searching for biomolecules which are naturally present in foodstuffs and plants, and which may be useful in the prevention or treatment of this disorder. In this context, considerable attention has been paid to polyphenols [20, 21] of which resveratrol is undoubtedly one of the most widely studied. Numerous studies, the majority essentially performed in mice and rats, have reported that resveratrol exerts beneficial effects on adipose tissue excess [2, 55], and on metabolic, hepatic, cardiac, and kidney complications associated with excessive body weight [1, 3, 56]. Although several studies have also shown similar effects in primates [19, 30], it has not yet been possible to draw a clear conclusion in humans, at least to the best of our knowledge [11, 14, 17, 32, 45, 62].

Despite resveratrol's promising effects, its poor bioavailability and rapid metabolism [59, 60] can limit its benefits for human dietary interventions. Therefore, there is a great interest in other resveratrol-related molecules. Piceatannol (*trans*-3,4,3′,5′-tetrahydroxystilbene) is a polyphenol belonging to the group of stilbenes, naturally present in plants [6, 48]. In addition, it is a phase I metabolite of resveratrol, which is produced by cytochrome P450 enzyme CYP1B1 [44]. The chemical structure of piceatannol shows an extra hydroxyl group at the 3′ position (Fig. 1). Although little information concerning piceatannol bioavailability has been reported to date, Setoguchi and coworkers have demonstrated that piceatannol displayed higher metabolic stability than resveratrol since, under their experimental conditions, the plasma concentration curve for piceatannol was 2.1 times greater than that for resveratrol [51].

Considering that piceatannol is present in edible plants, fruits as well as in red wines (up to 5 mg/L), at approximately the same levels as resveratrol [7], we considered that piceatannol could be an alternative to resveratrol when preventing or treating obesity complications. Although piceatannol has already been reported to induce several beneficial effects [43, 57], its influence on adipose tissue development, obesity and its comorbidities, has not been definitely addressed. A study performed in cultured adipocytes reported a delipidating effect for this resveratrol derivative [34], while another study demonstrated a mitigation of impaired glucose tolerance in diabetic and obese mice [40]. However, obesity complications such as liver steatosis, cardiac hypertrophy, or oxidative stress have not been addressed in these studies.

The aim of this study was therefore to analyze the effect of treatment with piceatannol on obesity and several related complications in a model already tested to demonstrate the beneficial effects of resveratrol: the genetically obese Zucker rat (*fa/fa*). Piceatannol was orally administered at 15 and 45 mg/kg/day during 6 weeks, i.e., in the same conditions in which resveratrol was reported to improve hepatic triacylglycerol metabolism [28], adipose tissue functions, and disturbed lipid handling [27, 49].

Material and methods

Chemicals

Piceatannol used for the 6-week oral supplementation was provided by Great Forest Biomedical Ltd. (Hangzhou, P.R. China) while piceatannol used for in vitro

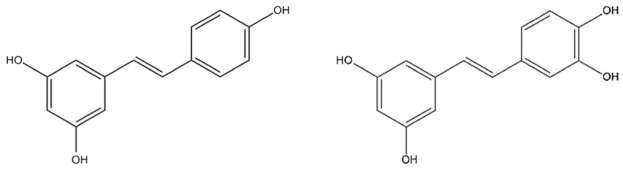


Fig. 1 Chemical structures of resveratrol and piceatannol. A hydroxyl group at the 3′ position of the stilbene structure is present in piceatannol, not in resveratrol (its parent compound)

studies was from Selleck Chemicals (Munich, Germany). Both compounds were of purity ≥99 % and exhibited chemical identity when analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS) with an ACE C18 column (1150 × 4.6 mm–5 μm) and a gradient of formic acid/acetonitrile for elution. Both commercial sources generated two peaks when using UV-visible detection as shown in Fig. S1. They corresponded to the same ion (*m/z* 245): protonated piceatannol under its *cis* and *trans* forms, as already reported [7]. The proportion of these isomers was the same in the two used batches. Moreover, comparison of the chemical purity at the start and the end of treatment showed that piceatannol was not altered under our storage conditions.

Animals, diet, and experimental design

The experiment was conducted on 30 male Zucker rats (Crl:ZUC(Orl)-Lepr^{fa}, hereafter named *fa/fa*, at the age of 6 weeks (233 ± 2 g). They were purchased from Charles River Laboratories France (L'Arbresle Cedex, France) and took place in accordance with Biodonostia Institute's guide for the care and use of laboratory animals (ANIM21/13). The rats were housed in individually ventilated cages and placed in an air-conditioned room (22 ± 2 °C) with a 12-h light–dark cycle. After a 6-day adaptation period, the rats were randomly distributed into three experimental groups of 10 animals each and fed on a standard laboratory diet (Harlan Laboratories, Barcelona, Spain). Rats in the piceatannol groups were orally given this polyphenol (15 mg/kg body weight/day in PICEAT-15 group and 45 mg/kg body weight/day in PICEAT-45 group) through an orogastric catheter for 6 weeks. Piceatannol was diluted in 1 mL of ethanolic solution (20 %). Rats from control group (CONTROL) group received only the vehicle. All animals had free access to food and water. Food intake and body weight were measured daily.

At the end of the experimental period, and after a fasting period of 6–8 h, rats were sacrificed by cardiac exsanguination under isoflurane anesthesia. White adipose depots from different anatomical locations (perirenal, epididymal, mesenteric, and subcutaneous), the heart and liver were dissected, weighed, and immediately frozen or fixed in 10 % formalin. Blood was collected. For serum obtention, blood was allowed to clot at 4 °C before centrifugation (1000×*g* for 10 min, at 4 °C). Serum aliquots were prepared and stored at −80 °C until further analysis.

Metabolite and adipokine circulating levels

Serum glucose, lactate, and circulating lipids were spectrophotometrically determined with an ABX Pentra 400 device and recommended reagents (Horiba, ABX, Montpellier, France) as previously reported in mice [35]. Insulin and adipokine circulating levels were simultaneously determined on 80 μL serum volume with a Luminex 100/200 System, a flexible analyzer based on the principles of flow cytometry, enabling a measurement of various analytes in a single microplate well, (Luminex, Austin, TX, USA). A rat metabolic hormone panel from Millipore (St Quentin, France) was used according to the manufacturer's recommendations with this compact analyzer using xMAP technology to perform bioassays on the surface of the provided color-coded microspheres. Adiponectin was determined with an ELISA kit supplied by R&D Systems (Lille, France). LPS levels in plasma were measured with the Limulus

Amebocyte Lysate kit (LAL, QCL-1000, Lonza) using pyrogen-free-water and -microtubes (Lonza, Basel, Switzerland), and following the manufacturer's instructions.

Feces collection and DNA extraction

Fresh fecal contents were removed from each animal, weighted, and kept at −80 °C for subsequent analysis. DNA from fecal microbiota was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands). Its purity and concentration were subsequently determined by a Nanodrop spectrophotometer 1000 (ND-1000; Thermo Scientific, USA).

Bacterial 16S rDNA pyrosequencing and sequence postprocessing

Samples for MiSeq pyrosequencing were amplified for the 16S rDNA hypervariable sequence V4 using the primers previously described (515F-806R) [9] and using the Illumina MiSeq Instrument (reads, 2 × 150 bp). Bioinformatic analysis was developed by Era7 Bioinformatics (Granada, Spain) and nucleotide filiations were assigned using the Ribosomal Database Project (RDP) [16]. Two different taxonomic assignment approaches were used: BBH (*Best Blast Hit*: each read was assigned to the taxon corresponding to the Best Blast Hit over a threshold of similarity) and LCA (*Lowest Common Ancestor*: adopted by advanced tools of metagenomics analysis as the last version of MEGAN) [29]. The direct assignments (calculated as counting reads specifically assigned to a node, not including the reads assigned to the descendant nodes in the taxonomy tree) and the cumulative assignment frequencies (calculated by including the direct frequencies and also the frequencies of the descendant nodes) for each taxonomy node (with some read assigned) were analyzed. Finally, a β diversity analysis was carried out in order to analyze in depth the distinctness between communities [42].

Hepatic steatosis assessment

Total lipids were extracted from liver following the method described by Folch [25]. The lipid extract was dissolved in isopropanol. TG and cholesterol contents were measured by using commercial kits (Spinreact, Barcelona, Spain).

Cardiac tissue histomorphology

For histological analysis, hearts which had been immediately fixed in 10 % formalin at sacrifice were embedded in paraffin and sectioned at 6 μm. Hematoxylin/eosin and Masson's trichrome staining were performed according to standard procedures. Apical cardiac fibrosis was quantified on Masson trichrome-stained paraffin-embedded heart sections using NIS Element Basic Research version 2.31 (Nikon imaging software, Tokyo, Japan).

For the quantification of cardiomyocyte size, slides were incubated for 30 min at room temperature with Texas red®-conjugated wheat germ agglutinin (WGA: 1/500, Life Technologies), rinsed in phosphate-buffered saline (PBS) and mounted in fluorescent mounting medium (DAKO). CM area was measured in cross-sectional tissue orientation by manually tracing the cell contour on image scan acquired on digital slide scanner NanoZoomer (Hamamastu, Tokyo, Japan) and using Zen 2011 software (Carl Zeiss, Jena, Germany).

For protein analysis, cardiac tissue samples were directly lysed in RIPA buffer (25 mM Tris–HCl, 150 mM NaCl, 1 % NP-40, 1 % NaDoc, 0.1 % SDS, pH 7.6) in the presence of protease inhibitor cocktail (Roche, Basel, Switzerland). Immunoprecipitation studies were conducted as previously described from cardiomyocyte lysates [26]. Protein concentration of extracts was determined by the Bradford method (Bio-Rad) and equal amounts of proteins were either immunoprecipitated (2 mg) with monoclonal antibody to ephrin-B1 (R&D) or subjected directly (50 μg) to SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Proteins were detected with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Little Chalfont, UK) using enhanced chemoluminescence detection reagent (GE Healthcare, Little Chalfont, UK). Protein quantification was obtained by densitometric analysis of the Western blots using ImageQuant 5.2 software and was normalized to that of GAPDH expression and expressed in arbitrary units (AU).

Assessment of ascorbyl radicals in plasma

Electron paramagnetic resonance (EPR) spectra were obtained at X-band and at room temperature on a Bruker EMX-8/2.7 (9.86 GHz) equipped with a high-sensitivity cavity (4119/HS 0205) and a gaussmeter (Bruker,

Wissembourg, France). A flat quartz cell FZKI160-5 X 0.3 mm (Magnettech, Berlin, Germany) was used for plasma, and quartz tubes of 4 mm in diameter were used for tissues analysis. EPR data processing and spectrum computer simulation were performed using WINEPR and SIMFONIA software (Bruker, Wissembourg, France). Typical scanning parameters were as follows: scan rate, 1.2 G/s; scan number, 5; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 20 mW; sweep width, 100 G; sweep time, 83.88 s; time constant, 40.96 ms; magnetic field, 3460–3560 G. Five hundred microliters of plasma samples thawed immediately before measurement was introduced in the flat quartz cell. Twenty to 30 mg of tissue samples was cryoground before being introduced into the quartz capillary. It was verified that none of the cells, capillaries, and compounds used contained traces of free radicals. While ascorbyl radical is directly detectable, spin trapping techniques were performed to detect others radicals, using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and α-phenyl-*tert*-butyl-nitrone (PBN) for oxygen or carbon-centered radicals and iron(II)-*bis*-diethyldithiocarbamate [$Fe(DETC_2$] for the NO radical as previously reported (Reybier, 2006; Loibl S, 2006).

Measurement of hydrogen peroxide in white adipose tissue

Hydrogen peroxide release was measured using Amplex Red (10-acetyl-3,7-dihydrophenoxazine) as a fluorescent probe, as previously described [10]. Briefly, quantification was performed by means of a chromogenic mixture containing 40 μM Amplex Red, 4 U/mL horseradish peroxidase, and the parallel use of a standard hydrogen peroxide solution ranging from 0.05 to 5 μM. Thawed subcutaneous white adipose tissue samples were homogenized in 200 mM phosphate buffer (pH 7.4) just prior to the determination of basal and amine oxidase-dependent hydrogen peroxide release on 30 min at 37 °C in the dark. Fifty microliters of homogenates was distributed in 96-well dark microplates and preincubated for 15 min without (control) or with 1 mM of the reference inhibitors pargyline and semicarbazide to inhibit MAO and SSAO activities, or with increasing doses of piceatannol. Then, buffer or tested amine and chromogenic mixture was added to reach a final volume of 200 μL. Benzylamine is a preferred substrate of SSAO, and can be oxidized by MAO-B, while tyramine is a substrate of MAO-A and MAO-B, and of SSAO; both exhibit K_m for amine oxidases in the 10–500 micromolar range. Fluorescence readouts (ex/em, 540/590 nm) were collected in a Fluoroskan Ascent plate reader (ThermoLabsystems, Finland). DMSO vehicle used to solubilize the higher piceatannol dose had no without influence in the tested conditions (final concentrations were 100 μM for piceatannol and 1/100 *v*/*v* for DMSO).

In vitro assessment of piceatannol delipidating effect

3T3-L1 pre-adipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10 % fetal calf serum (FCS). Two days after confluence (day 0), the cells were stimulated to differentiate with DMEM containing 10 % FCS, 10 μg/mL insulin, 0.5 mM isobutylmethylxanthine, and 1 μM dexamethasone for 2 days. On day 2, the differentiation medium was replaced by FBS/DMEM medium (10 %) containing 0.2 μg/mL insulin. This medium was changed every 2 days until mature adipocytes were harvested (day 12). All media contained 1 % penicillin/streptomycin (10,000 U/mL), and the media for differentiation and maturation contained 1 % (*v*/*v*) of biotin and pantothenic acid. Cells were maintained at 37 °C in a humidified 5 % CO_2 atmosphere.

Mature adipocytes grown in six-well plates were incubated with either 0.1 % ethanol (95 %) (control group) or with piceatannol, at 1, 10, and 25 μM (diluted in 95 % ethanol) on day 12 after differentiation. After 24 h, supernatant was removed and cells were used for triacylglycerol determination. To this aim, cells were washed with PBS and lipids extracted three times with 800 μL of hexane/isopropanol (2:1). After evaporation under nitrogen, the pellet was resuspended in 200 μL 1 % Triton X-100 by sonication. TG content was measured using Infinity Triglycerides reagent (Thermo Scientific, Rockford, IL, USA). For protein determination, cells were lysed in 0.3 N NaOH, 0.1 % SDS and measurements were performed using BCA reagent (Thermo Scientific, Rockford, IL, USA).

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 17·0 (SPSS® Inc. Chicago, IL, USA) and Graph Pad Prism 5. All the parameters were normally

distributed according to the Shapiro-Wilks test. Subsequently, in the in vivo study, the data were analyzed by ANOVA I test and Newman-Keuls as a post-hoc test, unless otherwise stated. In the in vitro study, comparisons between each treatment and the controls were analyzed by Student's *t* test. In both studies, statistical significance was set at the $P < 0.05$ level.

Results

Body weight, food consumption, adipose tissue weights, and circulating parameters

At the end of the 6-week supplementation period, no significant difference was observed between CONTROL and PICEAT-15 or PICEAT-45 groups for body weight, food intake, and adipose tissue weights (Table 1). In all the groups, the mass of all dissected fat depots represented 14 % of the body weight, indicating that oral piceatannol at 15 or 45 mg/kg body weight/day had no apparent anti-obesity effect in the obese Zucker rats, which exhibited severe hyperphagia.

In fact, during the treatment, each rat approximately doubled its body mass (+200 g) and consumed more than 1.000 g of pelleted diet, regardless of its experimental group. With regard to serum metabolic parameters, no significant differences were found in glucose, insulin, urea, triacylglycerols, and HDL-cholesterol. By contrast, non-esterified fatty acids (NEFA) and LDL-cholesterol were reduced in PICEAT-45 group and lactate in PICEAT-15 group (Table 2). This indicated that the insulin resistance was not modified by piceatannol, while lipid handling was slightly improved. At the same time, the percent of glycated hemoglobin in the whole blood remained at similar levels in the three groups (data not shown). Serum adiponectin was not modified while the hyperleptinemia of these genetically obese rats, which lack efficient leptin receptors, was decreased in PICEAT-45 group. Finally, a decrease of 33 %, not reaching statistical significance, was observed for lipopolysaccharide plasma levels in both groups of rats treated with piceatannol (Table 2).

Feces bacterial composition

Bacterial composition of feces from the groups of rats treated with piceatannol was not significantly affected at the phylum level (Table 3), and only slight changes were observed in piceatannol-treated rats in comparison with the control group when less abundant phyla were studied, such as the reduction observed in *Deferribacteres* ($P = 0.07$, PICEAT-45 group vs. CONTROL), *Planctonmycetes* ($P = 0.08$, PICEAT-45 group vs. CONTROL), and *Verrucomicrobia* ($P = 0.08$, PICEAT-15 group vs. CONTROL; $P < 0.05$, PICEAT-45 group vs. CONTROL) (Fig. 2a–c). A tendency to decrease the *Prevotella* genus, a group of bacteria associated with inflammation, was also observed ($P = 0.06$, PICEAT-15 group vs. CONTROL, data not shown). Although no significant effects were observed at the class, family, or genus levels, piceatannol supplementation significantly modified the abundance of several species belonging to the *Firmicutes* and *Bacteroidetes* phyla, as reported in Fig. 2d–f.

Liver weight, hepatic triglyceride content, and histological analysis

Neither liver weight nor hepatic triacylglycerol content were modified by piceatannol supplementation (Table

Table 1 Final body weight, daily food intake, and adipose tissue weights in rats from control and piceatannol-treated groups

	CONTROL	PICEAT-15	PICEAT-45	ANOVA
Final body weight (g)	403 ± 12	401 ± 11	402 ± 10	NS
Food intake (g/day)	25.4 ± 0.5	24.5 ± 0.4	24.5 ± 0.5	NS
Adipose tissue weights (g)				
Epididymal (g)	6.9 ± 0.3	7.7 ± 0.4	7.7 ± 0.5	NS
Perirenal (g)	7.6 ± 0.3	7.0 ± 0.2	7.4 ± 0.5	NS
Mesenteric (g)	4.6 ± 0.4	4.2 ± 0.3	4.2 ± 0.3	NS
Subcutaneous (g)	38.3 ± 1.4	37.7 ± 1.4	37.6 ± 1.7	NS

Values are means ± SEM ($n = 10$)

NS not significant

Table 2 Serum biochemical parameters in rats from control and piceatannol-treated groups

	CONTROL	PICEAT-15	PICEAT-45	ANOVA
Glucose (mmol/L)	8.3 ± 1.1	9.1 ± 0.5	11.2 ± 1.2	NS
Insulin (ng/mL)	2.1 ± 0.4	1.4 ± 0.1	2.0 ± 0.5	NS
Lactate (mmol/L)	5.0 ± 0.7 a	3.0 ± 0.4 b	3.3 ± 0.5 ab	$P < 0.05$
Urea (mmol/L)	6.5 ± 0.2	6.1 ± 0.3	6.7 ± 0.5	NS
Triacylglycerols (mmol/L)	11.8 ± 1.5	8.2 ± 1.2	8.8 ± 1.2	NS
NEFA (mmol/L)	1.19 ± 0.10 a	1.04 ± 0.05 a	0.91 ± 0.08 b	$P < 0.05$
Cholesterol (mmol/L)	4.8 ± 0.2	5.2 ± 0.2	4.7 ± 0.2	NS
HDL-cholesterol (mmol/L)	1.30 ± 0.03	1.30 ± 0.04	1.30 ± 0.03	NS
LDL-cholesterol (mmol/L)	0.74 ± 0.05 a	0.78 ± 0.05 a	0.58 ± 0.05 b	$P < 0.05$
Lipopolysaccharides (EU/mL)	3.23 ± 0.57	2.29 ± 0.34	2.32 ± 0.33	NS
Adiponectin (μg/mL)	2.65 ± 0.13	2.49 ± 0.06	2.58 ± 0.09	NS
Leptin (ng/mL)	26.0 ± 6.2 a	16.5 ± 8.6 a	8.28 ± 1.6 b	$P < 0.05$

Values are means ± SEM ($n = 10$)

NS non-significant, *NEFA* non-esterified fatty acids

4). Steatosis degree assigned after liver histology was unchanged (not shown). The concentration of glutamate-pyruvate (GPT/ALT) and glutamic oxaloacetic transaminases (GOT/AST) remained unchanged (Table 4).

Cardiac morphology

The heart weight/body weight ratio, usually used to assess cardiac hypertrophy, tended to decrease only slightly after piceatannol supplementation, without reaching statistical significance (CONTROL, 2.74 ± 0.20; PICEAT-15, 2.55 ± 0.20; PICEAT-45, 2.58 ± 0.22, NS). This indicated that the cardiac hypertrophy occurring in obese Zucker rats was not abolished by the treatment (Fig. 3a). Microscopical observations showed that the cardiomyocyte transversal area was unaffected by piceatannol, regardless of the dose (Fig. 3b). In the same way, no difference in the percentage of fibrosis was observed among the three groups (Fig. 3c). These data strongly suggested that piceatannol neither prevents cardiac remodeling associated with obesity development nor has a toxic effect on the obese Zucker rat heart, known to develop cardiac hypertrophy and fibrosis.

To further assess the effects of piceatannol on the heart, the protein levels of ephrin-B1, a structural protein essential for cardiac tissue architecture, were quantified in whole heart tissue. ANOVA indicated a significant increase in ephrin-B1 expression in the PICEAT-45 group ($P < 0.01$ vs. CONTROL) but not in the PICEAT-15 group (Fig. 3d). Since a marked decrease in ephrin-B1 in heart failure has been described in the literature [26], the increase observed in this protein can be considered as a marker of cardiac compensation.

Table 3 Phylum abundance in gut microbiota from control and piceatannol-treated rats

Phyla	CONTROL	PICEAT-15	PICEAT-45	ANOVA
Firmicutes	37.98 ± 3.43	40.37 ± 2.19	35.10 ± 2.83	NS
Bacteroidetes	36.47 ± 1.98	33.61 ± 1.60	36.07 ± 1.03	NS
Proteobacteria	5.36 ± 0.48	5.64 ± 0.62	5.70 ± 0.64	NS
Actinobacteria	2.08 ± 0.40	2.58 ± 0.47	2.65 ± 0.55	NS
Bacteroidetes/Firmicutes	0.89 ± 0.09	0.87 ± 0.09	1.18 ± 0.16	NS
Proteobacteria/Firmicutes	0.13 ± 0.01	0.14 ± 0.02	0.22 ± 0.05	NS
Actinobacteria/Firmicutes	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	NS

Data are expressed as LCA cumulative (%)

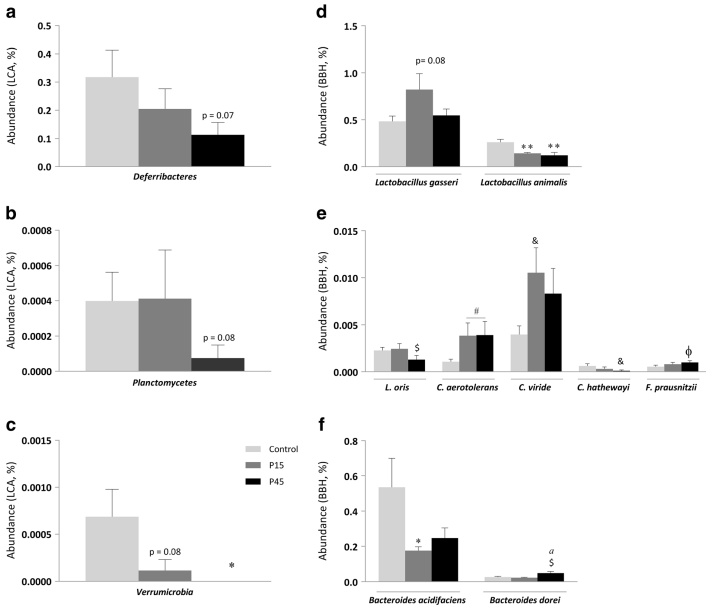


Fig. 2 Influence of piceatannol on gut microbiota composition: effects on three low abundant phyla in gut (**a**–**c**) and actions on several bacterial species belonging to *Firmicutes* and *Bacteroidetes* phyla (**d**–**f**). Values are means ± SEM (10 rats/group). $^{*}P < 0.05$ PICEAT-45 vs. CONTROL, $^{\&}P = 0.05$ PICEAT-45 vs. CONTROL, $^{\$}P = 0.06$ PICEAT-45 vs. CONTROL, $^{\#}P = 0.07$ PICEAT-15 and PICEAT-45 vs. CONTROL, $^{\phi}P = 0.08$ PICEAT-45 vs. CONTROL and $^{a}P = 0.07$ PICEAT-45 vs. PICEAT-15

Assessment of ascorbyl radical in serum and hydrogen peroxide in white adipose tissue

An estimation of ROS production was applied using EPR spectroscopy for the detection of radicals in plasma and tissues. Under our experimental conditions, only the ascorbyl radical endogenously present in plasma was detected (data not shown), giving EPR signal intensities of 35051 ± 350, 35438 ± 3544, and 38755 ± 387 arbitrary units in CONTROL, PICEAT-15, and PICEAT-45 rats, respectively. Thus, there was no difference between control and piceatannol-treated rats regarding the concentration of ascorbyl radical in plasma. While it is not a member of the ROS family, it is considered an oxidative stress marker since it mainly results from the interaction between ascorbate and potentially harmful radicals such as those of the ROS family. No free radicals were detected in adipose tissue samples, irrespective of the group (data not shown).

Hydrogen peroxide, a member of the ROS family, was determined in adipose tissue. Spontaneous hydrogen peroxide production by white adipose tissue preparations was not significantly modified by piceatannol supplementation (Fig. 4) but was readily increased by

Table 4 Liver weight, hepatic triacylglycerol content, and transaminases from control and piceatannol-treated groups

	CONTROL	PICEAT-15	PICEAT-45	ANOVA
Liver (g)	20.7 ± 0.6	21.8 ± 0.7	20.6 ± 0.9	NS
Triacylglycerols (mg/g)	29.1 ± 7.4	32.3 ± 8.5	31.8 ± 6.5	NS
GOT/AST (U/L)	167 ± 17	229 ± 29	116 ± 12	NS
GPT/ALT (U/L)	86 ± 9	116 ± 12	94 ± 7	NS

Values are means ± SEM ($n = 10$)

NS not significant

the addition of 1 mM tyramine or 0.1 mM benzylamine. These are doses previously reported to induce maximal activation of their metabolizing enzymes: amine oxidases [46]. The combination of the inhibitors semicarbazide and pargyline at 1 mM completely abolished the amine-induced hydrogen peroxide release by white adipose tissue preparations, confirming that the amine oxidases present in adipocytes were implicated in the response to amines (Fig. 4). In fact, hydrogen peroxide is a common end-product of amine oxidation, irrespective of the nature of the enzyme substrate and of the stimulated amine oxidase. The repeated ingestion of piceatannol at the dose of 15 mg/kg body weight/day only tended to lower the activation of hydrogen peroxide production by amines. However, this tendency never reached statistical significance (Fig. 4).

Nevertheless, when directly added in vitro to white adipose tissue preparations, piceatannol dose-dependently impaired the detection of hydrogen peroxide resulting from amine oxidase activation. The increased levels of hydrogen peroxide detected in response to the substrates benzylamine and tyramine were lowered by 100 μM of piceatannol with a maximal inhibition similar to that obtained with the combination

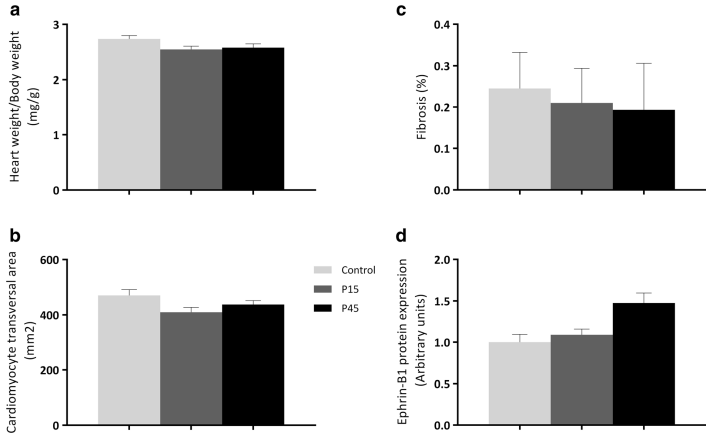


Fig. 3 Influence of piceatannol on heart cardiac morphology: weight/body weight ratio (**a**), cardiomyocyte transversal area (**b**), fibrosis (%) (**c**), ephrin-B1 protein expression (**d**). Values are means ± SEM (10 rats/group). $^{**}P < 0.01$ PICEAT-45 vs. other groups

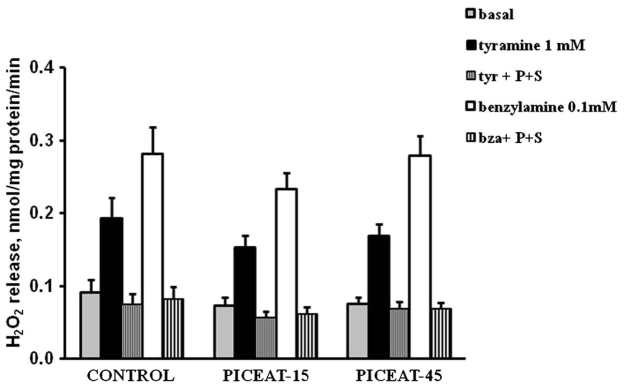


Fig. 4 Hydrogen peroxide release in white adipose tissue preparations. Release was measured over a 30-min period without any added agent, except the chromogenic mixture for H_2O_2 detection (basal, *gray columns*), or in the presence of amine substrates, tyramine 1 mM (*black columns*) or benzylamine 0.1 mM (*open columns*), without or with the inhibitors pargyline plus semicarbazide at 1 mM (P + S, *shaded columns*). Values are means ± SEM (10 rats/group)

of semicarbazide plus pargyline (Fig. 5a, b). The acute inhibitory properties of piceatannol on amine oxidase activities were not altered after chronic treatment since the inhibition curves of piceatannol on amine-induced hydrogen peroxide release were superimposed in the three experimental groups (Fig. 5).

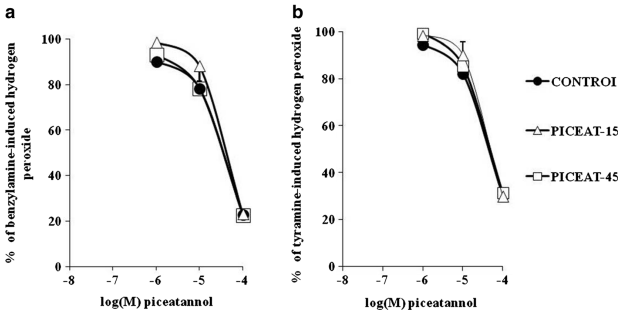


Fig. 5 Inhibition by piceatannol of the amine-induced release of hydrogen peroxide by white adipose tissue. Increasing concentrations of piceatannol were added 15 min before and during the 30-min incubation of WAT preparations from the indicated groups: control (*closed circles*), PICEAT-15 (*open triangles*), or PICEAT-45 (*open squares*). Oxidation of 1 mM tyramine (**a**). Oxidation of 0.1 mM benzylamine (**b**). Data are expressed as percentage of amine-induced H_2O_2 signal without any inhibitor. Values are means ± SEM of 10 rats/group

In vitro effects of piceatannol on lipid accumulation in cultured adipocytes

Piceatannol significantly reduced triacylglycerol accumulation in mature 3T3-L1 adipocytes at a dose of 25 μM ($P < 0.01$), but was without effect at lower doses (10 and 1 μM) (Fig. 6).

Discussion

The present work aimed to study the potential anti-obesity action of piceatannol. For this purpose, we tested its oral administration in *fa/fa* Zucker rat, a model of genetic obesity that already revealed the beneficial effects of resveratrol supplementation [27, 49]. The *fa/fa* Zucker rat exhibits an early overweight increase shortly after weaning, accompanied by many of the human metabolic syndrome features, such as insulin resistance and dyslipidemia. Moreover, in this model, the liver synthesizes an excess of triacylglycerols and oxidizes a small amount of fatty acids, which also lead to a rapid onset of hepatic steatosis [5].

Under our experimental conditions, piceatannol did not show a clear-cut anti-obesity effect since it did not limit the adipose tissue expansion in either subcutaneous or internal (epididymal, perirenal, and mesenteric) anatomical locations. Along the same lines, no changes were observed in liver triacylglycerol content after piceatannol treatment, which indicated that this polyphenol was not able to prevent liver steatosis, a common obesity comorbidity. These results were surprising because a previous study from our group, performed by using exactly the same model and experimental conditions, showed that resveratrol, the parent compound of piceatannol, clearly exerts both a body fat-lowering effect [27], at least in internal fat depots, as well as a liver delipidating effect [28]. It is worth mentioning that resveratrol has also been demonstrated by other studies to exert clear anti-obesity action in these obese rats [49], as well as in other rodent models of dietary-induced obesity [13, 31, 52].

The discrepancy between the present study and these previous reports could be due to a difference between piceatannol and resveratrol, in terms of antioxidant properties, bioavailability, or intrinsic activity on target cells. It is well known that a small change, such as a hydroxyl group position in the chemical structure of a molecule, influences not only its antioxidant or scavenger capacities. The extended conjugation stabilizes the phenoxyl radical, after H-atom transfer to the scavenged radical, as it was predicted by calculations [61]. It also influences its bioavailability, as mentioned in the "Introduction" section, and its activity. Thus, it could be hypothesized that the additional hydroxyl group of piceatannol in 3′ position might also reduce its ability to act on cells managing lipid storage and mobilization, especially hepatocytes and adipocytes.

The only study aiming to assess the delipidating effect of piceatannol reported so far showed a reduction in triacylglycerol accumulation in 3T3-L1 pre-adipocytes treated with 50 μM of this polyphenol, indicating a substantial anti-adipogenic effect [33]. These results indicated that piceatannol is active on adipose cells, but it should be pointed out that such a 50 μM concentration tested in vitro is likely to be higher than plasma

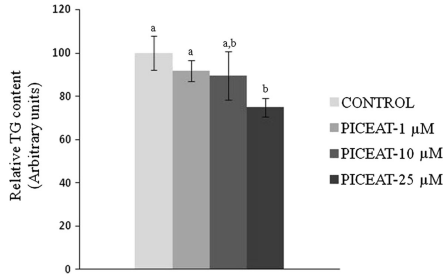


Fig. 6 Inhibition of triacylglycerol accumulation in 3T3-L1 mature adipocytes. Values are means ± SEM of three experiments. Comparisons were made between each treatment and the control cells (non-treated cells). $^{**}P < 0.01$

concentrations reached in animals orally treated with piceatannol, approximately 10 μM [34]. Since the literature lacks data concerning the in vitro delipidating effect of this polyphenol at low doses, we studied in the present work the effect of piceatannol at 1, 10, and 25 μM on cultured 3T3-L1 mature adipocytes. The dose of 25 μM induced a significant reduction in adipocyte triacylglycerol content, but not the lower doses, 10 and 1 μM (close to that found in serum and tissues from animals treated with piceatannol [51]. In a previous study carried out in our laboratory devoted to analyzing the delipidating effect of resveratrol in this cell line, we observed that the reference polyphenol reduced adipocyte triacylglycerol content at a dose of 1 μM [36]. All these results suggest that the presence of the hydroxyl group of piceatannol in 3′ position reduces its delipidating ability at low doses, and therefore partly explain why nutritional supplementation with this polyphenol did not limit fat accumulation in obese rats at 15 or 45 mg/kg body weight/day.

In spite of no reduction in the excessive food intake and fat accumulation, the lowered serum NEFA, LDL-cholesterol concentrations, and the tendency to lower triacylglycerol levels represented a modest improvement in lipid handling in the obese rats on piceatannol supplementation. In other words, piceatannol may limit the lipotoxicity of elevated circulating lipids accompanying excessive fat accumulation. The decreased fasting levels of lactate were somehow indicative of an improved liver metabolic function since hepatocytes normally break down lactic acid to perform gluconeogenesis during fasting. Although fasting serum glucose and insulin remained unchanged at the end of the treatment, and in spite of no reduction in glycated hemoglobin, it could be suggested that piceatannol supplementation may help delay the age-associated development of metabolic disturbances occurring in obese Zucker rats.

Although Zucker rats lack an efficient leptin system and so are clearly hyperphagic, the surprising decrease observed in this adipokine in serum after piceatannol treatment is in agreement with the direct reduction of leptin expression that resveratrol, its parent compound, exerts on pre-adipocytes [22] or mature adipocytes [54], as well as in the same animal model as that used in the present study [49]. As a result of its defective mutated leptin receptor, the Zucker rat is peculiarly resistant to leptin, and the observed changes of leptinemia were not accompanied by a change in food intake. However, the leptin-reducing effect of piceatannol described here might be of interest in the prevention of diet-induced leptin resistance.

Obesity is also associated with changes in gut microbiota composition [58]. Gut microbiota is even considered a potential contributory factor in obesity-associated disorders, since, when altered, it induces increased intestinal permeability and thus increased plasma lipopolysaccharide (LPS), leading to endotoxemia. This, in turn, triggers inflammation and metabolic disorders [8]. For this reason, our study of piceatannol effects on body fat accumulation, serum and liver lipids, and cardiac remodelling, also included a gut microbiota analysis using a metagenomic approach. Indeed, it is considered both that polyphenols may be possible therapeutic tools to restore microbial imbalance, as well as that gut microbiota is also involved in polyphenol metabolization, thus likely to influence their beneficial actions [24, 38, 50].

Some studies have also suggested that the beneficial effects of resveratrol on obesity and related disorders could be mediated by mechanisms of regulating gut microbes [47]. However, the effects of resveratrol on gut microbiota are still not clear since the study by Etxeberria et al. [23] shows that despite resveratrol treatment (15 mg/kg body weight/day) being able to decrease some features of insulin resistance in high-sucrose fed rats, it hardly modifies the profile of gut bacteria, but rather the host/bacteria reciprocal interactions. Concerning piceatannol, our study demonstrated that the administration of this polyphenol barely modified gut microbiota composition in this model of genetic obesity, in agreement with the lack of significant actions observed in body weight gain, fat pads, liver, and biochemical markers of glucose and lipid metabolism. However, in both the study from Etxeberria et al. (2015) and the present one, a significant decrease in the abundance of *Clostridium hathewayi*, which belongs to the *Clostridium cluster XIVa*, was observed. These bacteria are considered major butyrate producers and, therefore, the less abundance of these bacteria could suggest a decrease in the production of this short chain fatty acid (SCFA), which provides energy for cellular metabolism and also induces the expression of leptin in adipose tissue [58]. However, since a non-significant increase was also observed in the abundance of *Faecabacterium prausnitzii*, an intestinal anaerobe that mainly produces butyrate, this hypothesis needs to be further investigated.

Concerning the biochemical parameters that have been slightly improved by piceatannol treatment

(NEFA; LDL-cholesterol and lactate), a positive association was observed between *Lactobacillus animalis* and lactate plasma levels and LDL-cholesterol ($r=0.451$, $P=0.012$ and $r=0.322$, $P=0.08$, respectively). As a significant decrease in the abundance of *L. animalis* was observed after piceatannol treatment, it is tempting to suggest that the improvements observed in lactate and LDL-cholesterol could be explained, at least in part, by the less abundance of *L. animalis*. However, more studies are needed in this regard as no studies have demonstrated a role of *L. animalis* in obesity and associated metabolic disorders. Finally, and although *Lactobacillus* and *Bacteroides* are the predominant genera of murine intestinal microbiota, only slightly changes were observed in some of their species and none of them (with the exception of the aforementioned *L. animalis*) was associated with any of the metabolic improvements caused by the polyphenol. Thus, and although the physiological relevance of the bacterial species modified by piceatannol needs to be further determined, our results suggest that these discrete changes in microbiota do not extrapolate into significant impacts on physiology.

Piceatannol (15 and 45 mg/kg body weight/day) failed to reduce cardiomyocyte hypertrophy or heart fibrosis deposition associated with obesity and insulin resistance in the obese Zucker rat. In fact, cardiomyocyte cross sectional areas were approximately twice as large in the endocardium of left ventricle from obese rats irrespective of the studied group than from age-matched lean rats (personal communication from C. Guilbeau-Frugier, Univ. Toulouse). All these observations confirmed the presence of a left ventricle hypertrophy in the obese rats. Moreover, an increased expression of ephrin-B1, a protein expressed at the lateral membrane of the cardiomyocyte and essential for maintenance of their rod-shape and for cardiac tissue cohesion [26], was evidenced after treatment with 45 mg/kg piceatannol. Such increased expression suggested that this polyphenol could have some protective properties against cardiomyocyte lateral membrane remodeling during the development of obesity-associated cardiac hypertrophy and fibrosis. Indeed, ephrin-B1 and claudin are associated at the intercellular surfaces and contribute to the maintenance of good cardiomyocyte architecture [26, 53]. This finding would justify future studies to investigate the effect of piceatannol on lateral membrane structure using electron microscopy or atomic force microscopy [18] in order to extend previous functional explorations at molecular level which reported antiarrhythmic activity of piceatannol (1–10 μM) in isolated rat hearts subjected to ischemia-reperfusion injury [15].

Since polyphenols are also known as anti-oxidants, we expected a recovery of the exaggerated oxidative stress that occurs with obesity in serum and organs after piceatannol supplementation. It must be noted that piceatannol displays radical scavenging activities in vitro, and it has been demonstrated to be over 1250-fold and 3300-fold more active than resveratrol on superoxide and DPPH radicals, respectively [41].

Taking this into account, the ascorbyl free radical was determined since it is considered as a reliable marker of oxidative stress, located upstream from more classical markers of excessive oxidation of carbohydrates, lipids, proteins, or nucleic acids by the reactive oxygen species (ROS) [43]. We also determined hydrogen peroxide levels, another member of the ROS family, in white adipose tissue for the following reasons: (i) several polyphenols have been demonstrated to generate hydrogen peroxide when they spontaneously undergo rapid oxidation in incubation media [37]; (ii) polyphenols also promote amine oxidation [4], a reaction that releases hydrogen peroxide, but which is physiologically supported by the family of amine oxidases, encompassing monoamine oxidases (MAO) and semicarbazide-sensitive amine oxidases (SSAO); (iii) both MAO and SSAO are highly expressed in white adipose tissue [10]; and (iv) MAO and SSAO inhibitors have been reported to limit fattening in obese Zucker rats [12, 46] or in other rodents [39].

Piceatannol seemed to behave in vitro as an inhibitor of MAO and SSAO, somewhat resembling to resveratrol, already reported to inhibit human MAO [63]. When added in vitro to white adipose tissue preparations, piceatannol dose-dependently impaired the amine-stimulated hydrogen peroxide release, regardless the rats have been previously treated or not with piceatannol. However, in vivo piceatannol treatment did not change basal or amine-induced hydrogen peroxide production in white adipose tissue of obese Zucker rats. This difference suggests that the effective concentration tested in vitro (100 μM) was clearly supranutritional. Accordingly, when tested in vitro at 1 μM, piceatannol was without detectable acute effect on adipose tissue preparations, as it was also the case with the in vitro approach on cultured mature adipocytes. Taken together, our findings definitely indicate that piceatannol can directly and substantially impact fat cells only at doses equivalent or higher than 25 μM.

In summary, our study has compiled and integrated observations of multiple weak effects of piceatannol on various complications triggered by severe hyperphagia, including gut microbiota, metabolic and cardiovascular alterations. Under our experimental conditions, while piceatannol tends to improve lipid handling, it does not mitigate hyperinsulinemia obesity and cardiac hypertrophy in genetically obese rats.

Acknowledgments This study was supported by grants from Refbio Pyrenees Biomedical Network (POLYFrEsNOL project), Instituto de Salud Carlos III (CIBERobn, CIBERehd, CIBERer), Government of the Basque Country (IT-572-13), University of the Basque Country (UPV/EHU) (ELDUNANOTEK UFI11/32), Instituto Biodonostia, and Fundación Rioja Salud. We acknowledge with thanks M.A. Fernandez (Univ. La Rioja) and J. Marimon (Univ. Basque Country) for analyzing chemical purity, anti/prooxidant, and antibacterial properties of piceatannol sources. We thank J. Daydé and colleagues (Pôle Aliment Sécurité Sanitaire et Santé, Toulouse) for their expertise in Food & Nutrition. We acknowledge C. Sarasqueta for statistical support, M. Larzabal, M. Zabala, J.I. Martinez and E. Recio-Fernández for their technical support. We also thank A. Bouloumié (INSERM U1048, I2MC, Toulouse) and Dr. J.A. Oteo (Hospital San Pedro-CIBIR, Logroño) for their scientific advices in the discussion of the results.

Compliance with ethical standards

Conflict of interest None declared.

References

1. Agouni A, Lagrue-Lak-Hal AH, Mostefai HA, Tesse A, Mulder P, Rouet P, Desmoulin F, Heymes C, Martínez MC, Andriantsitohaina R (2009) Red wine polyphenols prevent metabolic and cardiovascular alterations associated with obesity in Zucker fatty rats (Fa/Fa). PLoS One 4:e5557. doi:10.1371/journal.pone.0005557
2. Aguirre L, Fernández-Quintela A, Arias N, Portillo MP (2014) Resveratrol: anti-obesity mechanisms of action. Molecules 19:18632–18655. doi:10.3390/molecules191118632
3. Aguirre L, Portillo MP, Hijona E, Bujanda L (2014) Effects of resveratrol and other polyphenols in hepatic steatosis. World J Gastroenterol 20:7366–7380. doi:10.3748/wjg.v20.i23.7366
4. Akagawa M, Shigemitsu T, Suyama K (2005) Oxidative deamination of benzylamine and lysine residue in bovine serum albumin by green tea, black tea, and coffee. J Agric Food Chem 53:8019–8024. doi:10.1021/jf050843f
5. Argilés JM (1989) The obese Zucker rat: a choice for fat metabolism 1968–1988: twenty years of research on the insights of the Zucker mutation. Prog Lipid Res 28:53–66
6. Bavaresco L (2003) Role of viticultural factors on stilbene concentrations of grapes and wine. Drugs Exp Clin Res 29:181–187
7. Buiarelli F, Coccioli F, Jasionowska R, Merolle M, Terracciano A (2007) Analysis of some stilbenes in Italian wines by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 21:2955–2964. doi:10.1002/rcm.3174
8. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R (2008) Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 57:1470–1481. doi:10.2337/db07-1403
9. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6:1621–1624. doi:10.1038/ismej.2012.8
10. Carpéné C, Bizou M, Tréguer K, Hasnaoui M, Grès S (2015) Glitazones inhibit human monoamine oxidase but their anti-inflammatory actions are not mediated by VAP-1/semicarbazide-sensitive amine oxidase inhibition. J Physiol Biochem. doi:10.1007/s13105-014-0379-3
11. Carpéné C, Gomez-Zorita S, Gupta R, Grès S, Rancoule C, Cadoudal T, Mercader J, Gomez A, Bertrand C, Iffiu-Soltész Z (2014) Combination of low dose of the anti-adipogenic agents resveratrol and phenelzine in drinking water is not sufficient to prevent obesity in very-high-fat diet-fed mice. Eur J Nutr 53:1625–1635. doi:10.1007/s00394-014-0668-1
12. Carpéné C, Iffiú-Soltesz Z, Bour S, Prévot D, Valet P (2007) Reduction of fat deposition by combined inhibition of monoamine oxidases and semicarbazide-sensitive amine oxidases in obese Zucker rats. Pharmacol Res 56:522–530. doi:10.1016/j.phrs.2007.09.016
13. Carreras A, Zhang SX, Almendros I, Wang Y, Peris E, Qiao Z, Gozal D (2015) Resveratrol attenuates intermittent hypoxia-induced macrophage migration to visceral white adipose tissue and insulin resistance in male mice. Endocrinology 156:437–443. doi:10.1210/en.2014-1706
14. Chachay VS, Macdonald GA, Martin JH, Whitehead JP, O'Moore-Sullivan TM, Lee P, Franklin M, Klein K, Taylor PJ, Ferguson M, Coombes JS, Thomas GP, Cowin GJ, Kirkpatrick CM, Prins JB, Hickman IJ (2014) Resveratrol does not benefit patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol 12:2092–2103.e2096. doi:10.1016/j.cgh.2014.02.024
15. Chen WP, Hung LM, Hsueh CH, Lai LP, Su MJ (2009) Piceatannol, a derivative of resveratrol, moderately slows I(Na) inactivation and exerts antiarrhythmic action in ischaemia-reperfused rat hearts. Br J Pharmacol 157:381–391. doi:10.1111/j.1476-5381.2008.00106.x
16. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM (2014) Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acids Res 42:D633–642. doi:10.1093/nar/gkt1244
17. Crandall JP, Oram V, Trandafirescu G, Reid M, Kishore P, Hawkins M, Cohen HW, Barzilai N (2012) Pilot study of resveratrol in older adults with impaired glucose tolerance. J

Gerontol A Biol Sci Med Sci 67:1307–1312. doi:10.1093/gerona/glr235
18. Dague E, Genet G, Lachaize V, Guilbeau-Frugier C, Fauconnier J, Mias C, Payré B, Chopinet L, Alsteens D, Kasas S, Severac C, Thireau J, Heymes C, Honton B, Lacampagne A, Pathak A, Sénard JM, Galés C (2014) Atomic force and electron microscopic-based study of sarcolemmal surface of living cardiomyocytes unveils unexpected mitochondrial shift in heart failure. J Mol Cell Cardiol 74:162–172. doi:10.1016/j.yjmcc.2014.05.006
19. Dal-Pan A, Blanc S, Aujard F (2010) Resveratrol suppresses body mass gain in a seasonal non-human primate model of obesity. BMC Physiol 10:11. doi:10.1186/1472-6793-10-11
20. Del Rio D, Costa LG, Lean ME, Crozier A (2010) Polyphenols and health: what compounds are involved? Nutr Metab Cardiovasc Dis 20:1–6. doi:10.1016/j.numecd.2009.05.015
21. Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A (2013) Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal 18:1818–1892. doi:10.1089/ars.2012.4581
22. Eseberri I, Lasa A, Churruca I, Portillo MP (2013) Resveratrol metabolites modify adipokine expression and secretion in 3T3-L1 pre-adipocytes and mature adipocytes. PLoS One 8:e63918. doi:10.1371/journal.pone.0063918
23. Etxeberria U, Arias N, Boqué N, Macarulla MT, Portillo MP, Martínez JA, Milagro FI (2015) Reshaping faecal gut microbiota composition by the intake of trans-resveratrol and quercetin in high-fat sucrose diet-fed rats. J Nutr Biochem 26:651–660. doi:10.1016/j.jnutbio.2015.01.002
24. Etxeberria U, Fernández-Quintela A, Milagro FI, Aguirre L, Martínez JA, Portillo MP (2013) Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. J Agric Food Chem 61:9517–9533. doi:10.1021/jf402506c
25. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
26. Genet G, Guilbeau-Frugier C, Honton B, Dague E, Schneider MD, Coatrieux C, Calise D, Cardin C, Nieto C, Payré B, Dubroca C, Marck P, Heymes C, Dubrac A, Arvanitis D, Despas F, Altié MF, Seguelas MH, Delisle MB, Davy A, Sénard JM, Pathak A, Galés C (2012) Ephrin-B1 is a novel specific component of the lateral membrane of the cardiomyocyte and is essential for the stability of cardiac tissue architecture cohesion. Circ Res 110:688–700. doi:10.1161/CIRCRESAHA.111.262451
27. Gómez-Zorita S, Fernández-Quintela A, Lasa A, Hijona E, Bujanda L, Portillo MP (2013) Effects of resveratrol on obesity-related inflammation markers in adipose tissue of genetically obese rats. Nutrition 29:1374–1380. doi:10.1016/j.nut.2013.04.014
28. Gómez-Zorita S, Fernández-Quintela A, Macarulla MT, Aguirre L, Hijona E, Bujanda L, Milagro F, Martínez JA, Portillo MP (2012) Resveratrol attenuates steatosis in obese Zucker rats by decreasing fatty acid availability and reducing oxidative stress. Br J Nutr 107:202–210. doi:10.1017/S0007114511002753
29. Huson DH, Weber N (2013) Microbial community analysis using MEGAN. Methods Enzymol 531:465–485. doi:10.1016/B978-0-12-407863-5.00021-6
30. Jimenez-Gomez Y, Mattison JA, Pearson KJ, Martin-Montalvo A, Palacios HH, Sossong AM, Ward TM, Younts CM, Lewis K, Allard JS, Longo DL, Belman JP, Malagon MM, Navas P, Sanghvi M, Moaddel R, Tilmont EM, Herbert RL, Morrell CH, Egan JM, Baur JA, Ferrucci L, Bogan JS, Bernier M, de Cabo R (2013) Resveratrol improves adipose insulin signaling and reduces the inflammatory response in adipose tissue of rhesus monkeys on high-fat, high-sugar diet. Cell Metab 18:533–545. doi:10.1016/j.cmet.2013.09.004
31. Kim S, Jin Y, Choi Y, Park T (2011) Resveratrol exerts anti-obesity effects via mechanisms involving down-regulation of adipogenic and inflammatory processes in mice. Biochem Pharmacol 81:1343–1351. doi:10.1016/j.bcp.2011.03.012
32. Konings E, Timmers S, Boekschoten MV, Goossens GH, Jocken JW, Afman LA, Müller M, Schrauwen P, Mariman EC, Blaak EE (2014) The effects of 30 days resveratrol supplementation on adipose tissue morphology and gene expression patterns in obese men. Int J Obes 38:470–473. doi:10.1038/ijo.2013.155
33. Kwon JY, Seo SG, Heo YS, Yue S, Cheng JX, Lee KW, Kim KH (2012) Piceatannol, natural polyphenolic stilbene, inhibits adipogenesis via modulation of mitotic clonal expansion and insulin receptor-dependent insulin signaling in early phase of differentiation. J Biol Chem 287:11566–11578. doi:10.1074/jbc.M111.259721
34. Kwon JY, Seo SG, Yue S, Cheng JX, Lee KW, Kim KH (2012) An inhibitory effect of resveratrol in the mitotic clonal expansion and insulin signaling pathway in the early phase of adipogenesis. Nutr Res 32:607–616. doi:10.1016/j.nutres.2012.06.014
35. Labialle S, Marty V, Bortolin-Cavaillé ML, Hoareau-Osman M, Pradère JP, Valet P, Martin PG, Cavaillé J (2014) The miR-379/miR-410 cluster at the imprinted Dlk1-Dio3 domain controls neonatal metabolic adaptation. EMBO J 33:2216–2230. Doi: 10.15252/embj.201387038.
36. Lasa A, Churruca I, Eseberri I, Andrés-Lacueva C, Portillo MP (2012) Delipidating effect of resveratrol metabolites in 3T3-L1 adipocytes. Mol Nutr Food Res 56:1559–1568. doi:10.1002/mnfr.201100772
37. Long LH, Hoi A, Halliwell B (2010) Instability of, and generation of hydrogen peroxide by, phenolic compounds in cell culture media. Arch Biochem Biophys 501:162–169. doi:10.1016/j.abb.2010.06.012
38. Manach C, Donovan JL (2004) Pharmacokinetics and metabolism of dietary flavonoids in humans. Free Radic Res 38:771–785
39. Mercader J, Iffiú-Soltész Z, Bour S, Carpéné C (2011) Oral administration of semicarbazide limits weight gain together with inhibition of fat deposition and of primary amine oxidase activity in adipose tissue. J Obes 2011:475786. doi:10.1155/2011/475786
40. Minakawa M, Miura Y, Yagasaki K (2012) Piceatannol, a resveratrol derivative, promotes glucose uptake through glucose transporter 4 translocation to plasma membrane in L6 myocytes and suppresses blood glucose levels in type 2 diabetic model db/db mice. Biochem Biophys Res Commun 422:469–475. doi:10.1016/j.bbrc.2012.05.017
41. Murias M, Jäger W, Handler N, Erker T, Horvath Z, Szekeres T, Nohl H, Gille L (2005) Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-

activity relationship. Biochem Pharmacol 69:903–912. doi:10.1016/j.bcp.2004.12.001
42. Pasari JR, Levi T, Zavaleta ES, Tilman D (2013) Several scales of biodiversity affect ecosystem multifunctionality. Proc Natl Acad Sci U S A 110:10219–10222. doi:10.1073/pnas.1220333110
43. Piotrowska H, Kucinska M, Murias M (2012) Biological activity of piceatannol: leaving the shadow of resveratrol. Mutat Res 750:60–82. doi:10.1016/j.mrrev.2011.11.001
44. Potter GA, Patterson LH, Wanogho E, Perry PJ, Butler PC, Ijaz T, Ruparelia KC, Lamb JH, Farmer PB, Stanley LA, Burke MD (2002) The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. Br J Cancer 86:774–778. doi:10.1038/sj.bjc.6600197
45. Poulsen MM, Vestergaard PF, Clasen BF, Radko Y, Christensen LP, Stødkilde-Jørgensen H, Møller N, Jessen N, Pedersen SB, Jørgensen JO (2013) High-dose resveratrol supplementation in obese men: an investigator-initiated, randomized, placebo-controlled clinical trial of substrate metabolism, insulin sensitivity, and body composition. Diabetes 62:1186–1195. doi:10.2337/db12-0975
46. Prévot D, Soltesz Z, Abello V, Wanecq E, Valet P, Unzeta M, Carpéné C (2007) Prolonged treatment with aminoguanidine strongly inhibits adipocyte semicarbazide-sensitive amine oxidase and slightly reduces fat deposition in obese Zucker rats. Pharmacol Res 56:70–79. doi:10.1016/j.phrs.2007.04.002
47. Qiao Y, Sun J, Xia S, Tang X, Shi Y, Le G (2014) Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity. Food Funct 5:1241–1249. doi:10.1039/c3fo60630a
48. Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR (2004) Resveratrol, pterostilbene, and piceatannol in vaccinium berries. J Agric Food Chem 52:4713–4719. doi:10.1021/jf040095e
49. Rivera L, Morón R, Zarzuelo A, Galisteo M (2009) Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats. Biochem Pharmacol 77:1053–1063. doi:10.1016/j.bcp.2008.11.027
50. Scalbert A, Williamson G (2000) Dietary intake and bioavailability of polyphenols. J Nutr 130:2073S–2085S
51. Setoguchi Y, Oritani Y, Ito R, Inagaki H, Maruki-Uchida H, Ichiyanagi T, Ito T (2014) Absorption and metabolism of piceatannol in rats. J Agric Food Chem 62:2541–2548. doi:10.1021/jf404694y
52. Sun L, Wang Y, Song Y, Cheng XR, Xia S, Rahman MR, Shi Y, Le G (2015) Resveratrol restores the circadian rhythmic disorder of lipid metabolism induced by high-fat diet in mice. Biochem Biophys Res Commun 458:86–91. doi:10.1016/j.bbrc.2015.01.072
53. Swager SA, Delfín DA, Rastogi N, Wang H, Canan BD, Fedorov VV, Mohler PJ, Kilic A, Higgins RS, Ziolo MT, Janssen PM, Rafael-Fortney JA (2015) Claudin-5 levels are reduced from multiple cell types in human failing hearts and are associated with mislocalization of ephrin-B1. Cardiovasc Pathol 24:160–167. doi:10.1016/j.carpath.2014.10.006
54. Szkudelska K, Nogowski L, Szkudelski T (2009) Resveratrol, a naturally occurring diphenolic compound, affects lipogenesis, lipolysis and the antilipolytic action of insulin in isolated rat adipocytes. J Steroid Biochem Mol Biol 113:17–24. doi:10.1016/j.jsbmb.2008.11.001
55. Szkudelska K, Szkudelski T (2010) Resveratrol, obesity and diabetes. Eur J Pharmacol 635:1–8. doi:10.1016/j.ejphar.2010.02.054
56. Szkudelski T, Szkudelska K (2011) Anti-diabetic effects of resveratrol. Ann N Y Acad Sci 1215:34–39. doi:10.1111/j.1749-6632.2010.05844.x
57. Tang YL, Chan SW (2014) A review of the pharmacological effects of piceatannol on cardiovascular diseases. Phytother Res 28:1581–1588. doi:10.1002/ptr.5185
58. Villanueva-Millán MJ, Pérez-Matute P, Oteo JA (2015) Gut microbiota: a key player in health and disease. A review focused on obesity. J Physiol Biochem. doi:10.1007/s13105-015-0390-3
59. Walle T (2011) Bioavailability of resveratrol. Ann N Y Acad Sci 1215:9–15. doi:10.1111/j.1749-6632.2010.05842.x
60. Wenzel E, Somoza V (2005) Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res 49:472–481. doi:10.1002/mnfr.200500010
61. Wright JS, Johnson ER, DiLabio GA (2001) Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. J Am Chem Soc 123:1173–1183
62. Yoshino J, Conte C, Fontana L, Mittendorfer B, Imai S, Schechtman KB, Gu C, Kunz I, Rossi Fanelli F, Patterson BW, Klein S (2012) Resveratrol supplementation does not improve metabolic function in nonobese women with normal glucose tolerance. Cell Metab 16:658–664. doi:10.1016/j.cmet.2012.09.015
63. Yáñez M, Fraiz N, Cano E, Orallo F (2006) Inhibitory effects of cis- and trans-resveratrol on noradrenaline and 5-hydroxytryptamine uptake and on monoamine oxidase activity. Biochem Biophys Res Commun 344:688–695. doi:10.1016/j.bbrc.2006.03.190

Gómez-Zorita *et al. J Transl Med (2017) 15:237*
DOI 10.1186/s12967-017-1343-0

Journal of Translational Medicine

RESEARCH Open Access

Phenolic compounds apigenin, hesperidin and kaempferol reduce in vitro lipid accumulation in human adipocytes

Saioa Gómez-Zorita[1,3], Arrate Lasa[1,3*], Naiara Abendaño[1], Alfredo Fernández-Quintela[1,3], Andrea Mosqueda-Solís[1], Maria Pilar Garcia-Sobreviela[2], Jose M. Arbonés-Mainar[2,3] and Maria P. Portillo[1,3]

Abstract

Background: Adipocytes derived from human mesenchymal stem cells (MSCs) are widely used to investigate adipogenesis. Taking into account both the novelty of these MSCs and the scarcity of studies focused on the effects of phenolic compounds, the aim of the present study was to analyze the effect of apigenin, hesperidin and kaempferol on pre-adipocyte and mature adipocytes derived from this type of cells. In addition, the expression of genes involved in TG accumulation was also measured.

Methods: Pre-adipocytes were cultured from day 0 to day 8 and mature adipocytes for 48 h with the polyphenols at doses of 1, 10 and 25 μM.

Results: Apigenin did not show an anti-adipogenic action. Pre-adipocytes treated with hesperidin and kaempferol showed reduced TG content at the three experimental doses. Apigenin did not modify the expression of the main adipogenic genes (*c/ebpβ, c/ebpa, ppary* and *srebp1c*), hesperidin inhibited genes involved in the three phases of adipogenesis (*c/ebpβ, srebp1c* and *perilipin*) and kaempferol reduced *c/ebpβ*. In mature adipocytes, the three polyphenols reduced TG accumulation at the dose of 25 μM, but not at lower doses. All compounds increased mRNA levels of *atgl*. Apigenin and hesperidin decreased *fasn* expression. The present study shows the anti-adipogenic effect and delipidating effects of apigenin, hesperidin and kaempferol in human adipocytes derived from hMSCs. While hesperidin blocks all the stages of adipogenesis, kaempferol only inhibits the early stage. Regarding mature adipocytes, the three compounds reduce TG accumulation by activating, at least in part, lipolysis, and in the case of hesperidin and apigenin, also by reducing lipogenesis.

Conclusions: The present study shows for the first time the anti-adipogenic effect and delipidating effect of apigenin, hesperidin and kaempferol in human adipocytes derived from MSCs for the first time.

Keywords: Adipocytes, Apigenin, Hesperidin, Kaempferol, Obesity, Human mesenchymal stem cells

Background

Overweight and obesity are considered a serious threat to public health, due to their high prevalence in our society and their association with co-morbidities such as type 2 diabetes, hypertension and cardiovascular diseases [1, 2].

In vitro studies performed using adipocytes derived from human mesenchymal stem cells (MSCs) represent a good method to analyze the two main processes that lead to adipose tissue increase and thus, to obesity development: hypertrophy (increased adipocyte size) and hyperplasia (increased adipocyte number) [1, 2]. MSCs are fibroblastoid multipotent adult stem cells with a high capacity for self-renewal. These cells have been isolated from several human tissues, such as bone marrow, adipose tissue, umbilical cord matrix, tendon, lung and periosteum, among others [3]. MSCs become adipoblasts,

*Correspondence: arrate.lasa@ehu.eus
[1] Nutrition and Obesity Group, Department of Nutrition and Food Science and Lucio Lascaray Research Center, University of the Basque Country (UPV/EHU), Vitoria, Spain
Full list of author information is available at the end of the article

which subsequently commit to pre-adipocytes. Thereafter, upon adipogenic stimuli, pre-adipocytes undergo terminal differentiation into mature adipocytes [4]. Recently, it has been suggested that MSCs are a main source of adipocyte generation. Therefore, the biology of MSCs is studied in this work, as is the possible roles of MSCs in managing different components of metabolic syndrome [5–7].

In recent years, a great deal of attention has been paid to new active biomolecules that could be effective in preventing or treating obesity and its co-morbidities. Numerous studies have been carried out with phenolic compounds, which are members of a very large family of plant-derived compounds in the form of a wide variety of chemical structures [8]. Of these, resveratrol, quercetin and epigallocatechin have been shown to prevent fat accumulation in adipocytes through different mechanisms: by adipogenesis and lipogenesis inhibition as well as by lipolysis and fatty acid oxidation stimulation [9–11]. It must be pointed out that the majority of these studies have been performed in 3T3-L1 or mouse and rat primary adipocytes. However, studies demonstrating the effect of these phenolic compounds on human adipocytes are scarce to date [12–19]. Furthermore, important differences in adipocyte function exist among species, which complicates the extrapolation of results from murine adipocytes to human adipocytes [20]. Consequently, human MSCs provide an important alternative model system which represents a valuable instrument for experimental human fat cell investigation [21, 22].

Taking into account the novelty of these MSCs as an in vitro model for the study of obesity, and the few studies performed on human cells with phenolic compounds, the aim of the present study was to analyze the effect of three polyphenols (two flavonoids: apigenin and hesperidin; and one non-flavonoid: kaempferol). These polyphenols were previously studied in 3T3-L1 cells in our laboratory [23], in pre-adipocyte and mature adipocytes derived from this type of cells. In addition, the expression of genes involved in TG accumulation was also measured.

Methods

Cell samples

Human mesenchymal fat cells (hMSCs) were obtained from subcutaneous abdominal fat from a 59 year-old man with overweight and without type 2 diabetes mellitus, hypertension or dyslipidemia or obesity, as previously described [24], and approved by Clinical Investigation Ethics Committee of Aragon (Acta 11/2013).

Experimental design

Cells were cultured until confluence in DMEM 1 g/L glucose (Lonza, Verviers, Belgium) containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 4 mM glutamine, 1% penicillin/streptomycin. Differentiation was induced 2 days post-confluence (designated as day 0) with DMEM 4.5 g/L glucose (Lonza, Verviers, Belgium) supplemented with 10% FBS, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, 1 µM rosiglitazone and 1.67 µM human insulin. This medium was changed every 72 h. At day 6, cells were incubated in a DMEM 4.5 g/L glucose medium (Lonza, Verviers, Belgium) containing 1 mM sodium pyruvate, 4 mM glutamine, 10% FBS, 1% penicillin/streptomycin and this medium was changed every 72 h until cells were treated. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Each experiment was performed three times.

Differentiating cells were grown in 6-well plates and incubated together with either 0.1% ethanol (95%) (control group) or with apigenin (Extrasynthese, Lyon, France), hesperidin (Extrasynthese, Lyon, France) or with kaempferol (Genay, Lyon, France) at 1, 10 or 25 µM (diluted in 95% ethanol). In a first experiment, on day 3 cells treated with phenolic compounds were used at 25 µM for TG content determination and RNA extraction. In a second experiment, on day 8 cells treated with phenolic compounds were used at 1, 10 or 25 µM for TG content determination and cells treated with 25 µM were used for RNA extraction and cytotoxicity determination.

Mature adipocytes grown in 6-well plates were also incubated with either 0.1% ethanol (95%) (control group) or with apigenin, hesperidin or kaempferol at 1, 10 or 25 µM (diluted in 95% ethanol) on day 12 after differentiation. After 48 h of treatment, supernatant was removed and cells were used for triacylglycerol (TG) determination and RNA extraction.

Triacylglycerol content

For TG determination, cells were washed with phosphate buffer saline (PBS) and incubated 3 times with 800 µL of hexane/isopropanol (2:1). The total volume was evaporated under nitrogen, and the pellet was resuspended in 100 µL of Triton X-100 in 1% distilled water. Afterward, TG were solubilized by a sonicator, and the content was measured using infinity triglycerides reagent (Spinreact, Girona, Spain). For protein determination, cells were lysed in 0.3 N NaOH and 0.1% SDS. Protein measurements were performed using the BCA reagent (Thermo Fischer Scientific, Rockford, USA).

Extraction and analysis of RNA and quantification by real-time PCR

RNA samples were extracted from cells treated with 25 µM of each phenolic compound by using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using a

RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with DNase I kit (Applied Biosystems Inc., Foster City, CA, USA) to remove any contamination with genomic DNA.

One microgram of total RNA in a total reaction volume of 20 µL was reverse transcribed using the iScript cDNA Kit (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocols. Reactions were incubated initially at 25 °C for 10 min and subsequently at 37 °C for 120 min and at 85 °C for 5 min.

Relative mRNA levels were quantified using real-time PCR with an iCycler MyiQ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). 18S mRNA levels were similarly measured and served as the reference gene. The PCR reagent mixture consisted of 4.75 µL of each diluted cDNA. SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and of the upstream and downstream primers (300 nM). Specific primers for c/*ebpβ* (CCAAT/enhancer-binding protein beta), *c/ebpα* (CCAAT/enhancer-binding protein alpha), *ppary* (peroxisome proliferator factor gamma), *srebp1c* (sterol regulatory element-binding protein 1c), *acc* (acetyl-CoA carboxylase), *perilipin*, *scd1* (stearoyl-CoA desaturase 1), *atgl* (adipose triglyceride lipase), *hsl* (hormone sensitive lipase), *fasn* (fatty acid synthase) and *dgat* (diglyceride acyltransferase) were synthesized commercially and the sequences are shown in Table 1.

PCR parameters were as follows: initial 2 min at 50 °C, denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C, except 64.5 °C for *scd1* and 66.3 °C for *dgat*, for 30 s, and extension at 60 °C for 30 s. The results are expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [25].

Table 1 Primer sequences for PCR amplification of each gene studied

	Sense primer	Antisense primer
c/ebpβ	5′-GGCAGCACCACGACTTCCT-3′	5′-CGCCCCAGGCTCACGTAG-3′
c/ebpa	5′-AGGGTCTCTAGTTCCACGCC-3′	5′-CAAGGGGAAGCCCAGCCTATA-3′
ppary	5′-TAGATGACAGCGACTTGGCAA TAT-3′	5′-GAATGTCTTCAATGGGCTTCA CA-3′
srebp1c	5′-ACGCCCCACTTCATCAAGG-3′	5′-ACTGTTGCCAAGATGGTTCCG-3′
acc	5′-CATCAGCAGAGACTACGTCCT CAA-3′	5′-CATGGCAACCTCTGGATTGG-3′
perilipin	5′-TGGAGACTGAGGAGAAC AAG-3′	5′-ATGTCACAGCCGAGATGG-3′
scd1	5′-GCAGGACGATATCTCTAGCT-3′	5′-GTCTCCAACTTATCTCCTCCA TTC-3′
atgl	5′-GTGTCAGACGGCGAGAATG -3′	5′-TGGA GGGAGGGAGGGATG-3′
hsl	5′-TCAGTGTCTAGGTCAGACTGG-3′	5′-AGGCTTCTGTTGGGTATTGGA-3′
fasn	5′-TATGCTTCTTCGT-GCAGCAGTT-3′	5′-GCTGCCACACGCTCCTCTAG-3′
dgat	5′-ATTGCTGGCTCATCGCTGT-3′	5′-GGGAAAGTAGTCTCGAAAGTA GC-3′
18S	5′-TTCGAACGTCTGCCCTATCAA-3′	5′-ATGGTAGGCACGGCGACTA-3′

c/ebpβ, CCAAT/enhancer-binding protein beta; *c/ebpα*, CCAAT/enhancer-binding protein alpha; *ppary*, peroxisome proliferator fator gamma; *srebp1c*, sterol regulatory element-binding protein 1c; *acc*, acetyl-CoA carboxylase; *scd1*, stearoyl-CoA desaturase 1; *atgl*, adipose triglyceride lipase; *hsl*, hormone sensitive lipase; *fasn*, fatty acid synthase; *dgat*, diglyceride acyltransferase

Cytotoxicity assay

Cell viability was assessed using the neutral red assay (TOX4 kit, Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's recommendations.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using SPSS 24.0 (SPSS, Chicago IL, USA). Comparisons between each treatment and the controls were carried out by Student's *t* test. Statistical significance was set at the $P < 0.05$ level.

Results

Effect of apigenin, hesperidin and kaempferol on triacylglycerol content and cell viability in hMSC-derived adipocytes during differentiation

While apigenin did not decrease TG content, hesperidin induced a decrease at both 10 and 25 µM and kaempferol at the three doses used (Fig. 1a–c). Apigenin, hesperidin or kaempferol did not produce a loss of viability of hMSCs even when exposed to the highest concentration of each compound (25 µM) (Fig. 2).

Effect of apigenin, hesperidin and kaempferol in hMSC-derived adipocytes during differentiation

On day 3, the expression of *c/ebpβ* and *ppary* was measured in cells treated with the three phenolic compounds at 25 µM. Apigenin did not modify the expression of these genes. By contrast, hesperidin-treated cells showed lower mRNA levels of both genes. In the case of kaempferol, cells showed a significantly reduction in *ppary* gene expression and a trend towards lower values in the case of *c/ebpβ* ($P = 0.06$) (Fig. 3).

On day 8, apigenin did not modify *c/ebpβ* gene expression, while hesperidin and kaempferol-treated cells showed lower mRNA levels than the control cells (Fig. 4). Furthermore, gene expression of transcription factors involved in the intermediate stage of adipogenesis, *c/ebpα*, *ppary and srebp1c*, is presented in Fig. 5. Only hesperidin significantly reduced *srebp1c* mRNA levels. Regarding the late stage of adipogenesis, *acc*, *perilipin* and *scd1* gene expression was determined. Apigenin reduced *acc* and *perilipin* gene expression. In turn, hesperidin induced a reduction in *perilipin* mRNA levels (Fig. 6).

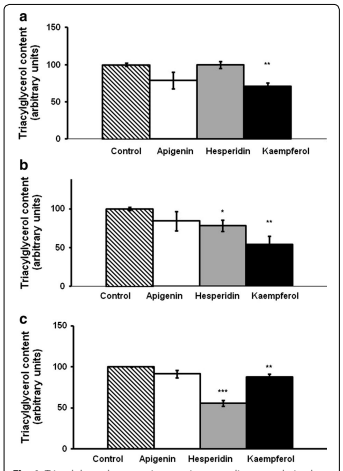


Fig. 1 Triacylglycerol content in maturing pre-adipocytes derived from human mesenchymal stem cells treated with apigenin, hesperidin or kaempferol 1 μM (**a**), 10 μM (**b**) or 25 μM (**c**) or not (control cells) for 8 days. Values are mean ± SEM. Comparisons between each treatment and the control were analyzed by Student's *t* test. Asterisks represent differences between phenolic compounds-treated cells and control cells. **P* < 0.05; ***P* < 0.01; ****P* < 0.001

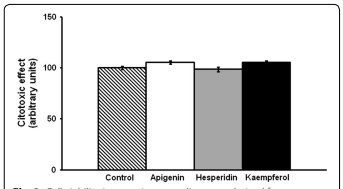


Fig. 2 Cell viability in maturing pre-adipocytes derived from human mesenchymal stem cells treated with apigenin, hesperidin or kaempferol at 25 μM, or not (control cells) for 8 days. Values are mean ± SEM. Comparisons between each treatment and the control were analyzed by Student's *t* test

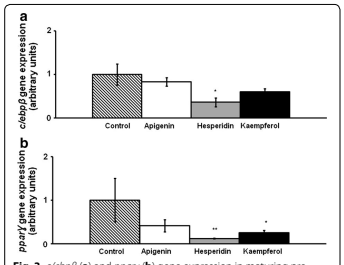


Fig. 3 *c/ebpβ* (**a**) and *ppary* (**b**) gene expression in maturing pre-adipocytes derived from human mesenchymal stem cells treated with apigenin, hesperidin or kaempferol at 25 μM or not (control cells) for 3 days. Values are mean ± SEM. Comparisons between each treatment and the control were analyzed by Student's *t* test. **P* < 0.05; ***P* < 0.01

Effect of apigenin, hesperidin and kaempferol on triacylglycerol content in mature adipocytes derived from hMSCs

The lowest doses, 1 and 10 μM of apigenin, hesperidin or kaempferol did not reduce TG content in mature adipocytes (Fig. 7a and b). However, 25 μM of the three phenolic compounds led to a significant diminution of TG (Fig. 7c).

Effect of apigenin, hesperidin and kaempferol on gene expression of mature adipocytes derived from hMSCs

Gene expression in mature adipocytes was measured at 25 μM. The three compounds increased *atgl* mRNA levels (Fig. 8a). In addition, apigenin and hesperidin decreased gene expression of *fasn* (Fig. 8e). No changes were observed in the expression of *hsl*, *acc*, *dgat2* and *scd1* (Fig. 8b–d, f).

Discussion

In recent years, a large number of scientific studies have focused on phenolic compounds as potential new tools for obesity management. In this context, our group previously analyzed the effect of fifteen phenolic compounds, belonging to different chemical groups, on 3T3-L1 pre-adipocytes, in order to know the potential relationship between the efficacy on adipogenesis inhibition and the chemical structure [23].

The present work aimed to study the responses of human pre-adipocytes and adipocytes obtained from

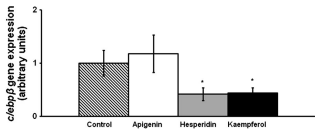


Fig. 4 *c/ebpβ* gene expression in maturing pre-adipocytes derived from human mesenchymal stem cells treated with apigenin, hesperidin or kaempferol at 25 μM or not (control cells) for 8 days. Values are mean ± SEM. Comparisons between each treatment and the control were analyzed by Student's *t* test. **P* < 0.05

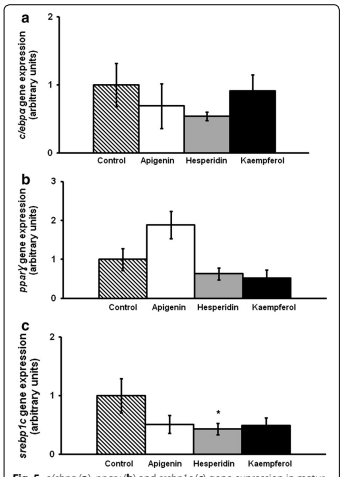


Fig. 5 *c/ebpα* (**a**), *ppary* (**b**) and *srebp1c* (**c**) gene expression in maturing pre-adipocytes derived from human mesenchymal stem cells treated with hesperidin or kaempferol at 25 μM or not (control cells) for 8 days. Values are mean ± SEM. Comparisons between each treatment and the control were analyzed by Student's *t* test. **P* < 0.05

stem cells to three polyphenols: (a) apigenin, a natural flavonoid widely distributed in plant foods such as chamomile tea, grapefruit, onions, oranges and some spices like parsley, (b) hesperidin, a flavanone glycoside abundant in citrus fruits and (c) kaempferol, a natural flavonol originally isolated from tea, broccoli and other plant sources, and to compare them with those obtained previously in murine adipocytes under the same experimental conditions. We selected these three compounds because they showed greater effectiveness in our previous study carried out in 3T3-L1 [18] and because their mechanisms of action have been little studied. We carried out the experiment in a range from a physiological dose of 1 μM [26–32] to a high dose of 25 μM.

Apigenin did not show an anti-adipogenic action. By contrast, pre-adipocytes treated with hesperidin and kaempferol showed reduced TG content at the end of the maturation process, suggesting that these compounds induced a reduction in adipogenesis. These results are different from those obtained in our laboratory in 3T3-L1 pre-adipocytes [23]. In these cells, apigenin and kaempferol were anti-adipogenic at 10 and 25 μM, and hesperidin was effective at the three experimental doses. These results suggest that kaempferol, and mainly hesperidin, could be useful to prevent obesity in those stages of life where adipogenesis, significantly contributes to obesity development, such as childhood, adolescence and adults with severe levels of obesity. By comparing these results with those previously observed in 3T3-L1 pre-adipocytes, we can state that the latter seem to be more responsive to apigenin and hesperidin than pre-adipocyte derived from human stem cells, but not to kaempferol. This confirms the existence of important interspecies differences in adipocyte function and thus the difficulty in extrapolating results from murine adipocytes to human adipocytes.

In order to discard a potential involvement of cytotoxic effects in adipogenesis reduction, we measured cell viability when cells were incubated with the highest dose (25 μM). The study showed that the molecules did not decrease cell viability. These results are in good accordance with Morikawa et al. [33] who reported no cytotoxic effect for hesperidin at 500 μM in adipocytes derived from human bone marrow stromal cells. Other authors have performed experiments treating hMSCs at different doses, not using isolated phenolic compounds but plant extracts containing kaempferol, hesperidin and apigenin among other phenolic compounds [34, 35]. The only toxic effect was observed at the highest doses used (above 0.5 mg/mL). The authors concluded that this effect might be due to synergism between compounds present in the plant extract.

One of the objectives of the present study was to elucidate the mechanism by which apigenin, hesperidin and kaempferol reduced adipogenesis, and for that purpose the expression of genes involved in this pathway was analyzed. Three phases can be distinguished in adipogenesis: early, intermediate and late [36]. During clonal expansion, there is an induction of the early phase

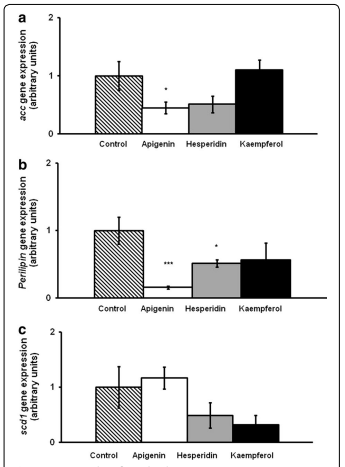


Fig. 6 *acc* (**a**), *perilipin* (**b**) and *scd1* (**c**) gene expression in maturing pre-adipocytes derived from human mesenchymal stem cells treated with apigenin, hesperidin or kaempferol at 25 μM or not (control cells) for 8 days. Values are mean ± SEM. Comparisons between each treatment and the control were analyzed by Student's *t* test. **P* < 0.05; ***P* < 0.01

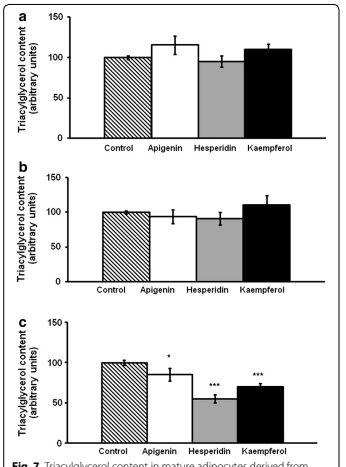


Fig. 7 Triacylglycerol content in mature adipocytes derived from human mesenchymal stem cells treated with apigenin, hesperidin or kaempferol 1 μM (**a**), 10 μM (**b**) or 25 μM (**c**) or not (control cells) for 48 h. Values are mean ± SEM. Comparisons between each treatment and the control were analyzed by Student's t test. Asterisks represent differences between phenolic compounds-treated cells and control cells. *P < 0.05; **P < 0.01; ***P < 0.001

transcription factor, *c/ebpβ*, which leads to transactivation of the transcription factors of the intermediate stage *ppary*, *c/ebpα* and *srebp1c* [36]. During the late phase of differentiation, adipocytes markedly increase de novo lipogenesis and acquire sensitivity to insulin. The mRNA levels of enzymes involved in TG metabolism, including *acc* and *scd1* among others, increase 10–100 fold. The transcription factors of the intermediate stage are implicated in the activation of these last genes [37].

Under our experimental conditions, apigenin did not modify the expression of the main adipogenic genes (*c/ebpβ*, *c/ebpα*, *ppary* and *srebp1c*) [38]. This is in good accordance with the observed lack of reduction in TG content. As far as we know, there are no studies in the literature on human adipocytes derived from stem cells treated with this phenolic compound, and thus comparisons cannot be made. By contrast, in 3T3-L1 pre-adipocytes many authors analyzed the anti-adipogenic effect of this phenolic compound. Kim et al. [39], observed that 70 μM apigenin reduced cell TG content by modulating the expression of the adipogenic transcriptional factors C/EBPβ, PPARγ and C/EBPα. These results are in good accordance with that of Ono et al. [40], who observed an anti-adipogenic effect of apigenin at 10 and 50 μM, but not at 1 mM. In our laboratory we analyzed the effect of this compound on 3T3-L1 cells in the same experimental conditions, and we observed reductions in TG content when incubations were carried out with 10 and 25 μM [23]. Other authors did not observe a reduction in TG content in the same cells at a dose of 10 μM [41].

Hesperidin inhibited genes involved in the three phases of adipogenesis, *c/ebpβ srebp1c*, *ppary* and *perilipin*. These results justify the reduction in TG content induced by this phenolic compound. To the best of our knowledge, there are no in vitro studies analyzing the anti-adipogenic effect of pure hesperidin in pre-adipocytes derived from hMSCs. There is a study performed with an extract of *Citrus bergamia*, which contains 13%

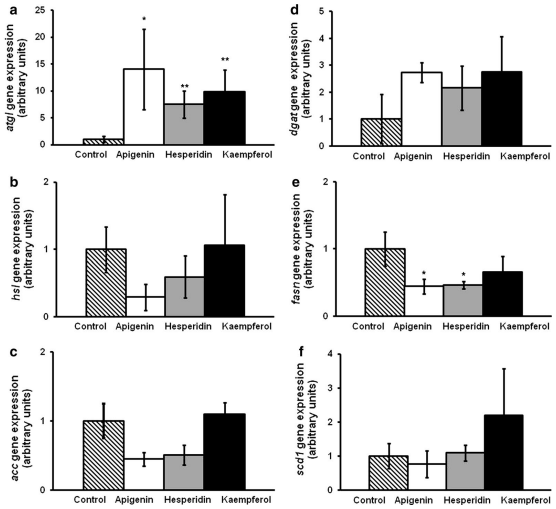


Fig. 8 *Atgl* (**a**), *hsl* (**b**), *acc* (**c**), *fasn* (**d**), *dgat* (**e**) and *scd1* (**f**) gene expression in mature adipocytes derived from hMSCs17 stem cells treated with hesperidin or kaempferol at 25 μM or not. Values are mean ± SEM. Comparisons between each treatment and the control were analyzed by Student's *t* test. **P* < 0.05; ***P* < 0.01

hesperidin, where hMSCs treated during differentiation showed a significant reduction in TG accumulation and *ppary* expression [34]. Nevertheless, the comparison with our results is difficult because the amount of hesperidin provided to the cells in that study is far lower than that used in our experiment. In addition, it contains other phenolic compounds that may also have an effect on pre-adipocytes and interact among themselves. In 3T3-L1 pre-adipocytes, Jeon et al. [42], in good accordance with our results, observed a delipidating effect in pre-adipocytes treated with 10 and 20 μM hesperidin. Moreover, in our laboratory we observed a decrease in TG content in this type of cells when they were incubated for 8 days with 1, 10 or 25 μM. The effect was mediated by a decrease in *srebp1c* [23].

As far as kaempferol-treated cells are concerned, despite only *c/ebpβ* and *ppary* being significantly reduced, an anti-adipogenic effect was observed, suggesting that the inhibition of genes involved in the early and intermediate stages of adipogenesis was enough to reduce this process. In fact, a similar situation took place in a previous study from our laboratory when 3T3-L1 pre-adipocytes were incubated with resveratrol or some of its metabolites [15]. As far as we are aware, there are no studies in the literature that demonstrate the anti-adipogenic effect of this compound in adipocytes derived from hMSCs. In 3T3-L1 pre-adipocytes, doses higher than 5 μM of kaempferol exerted a significant anti-adipogenic effect [43]. Moreover, it was observed that also at 40 μM, this compound decreased TG accumulation by the down-regulation of *ppary* and *srebp1c* [44]. In our studies performed in this type of cells, kaempferol reduced TG content at 10 and 25 μM, but not at 1 μM. The effect was mediated by a decrease in *c/ebpβ* and *srebp1c* [23].

The present study also aimed to determine the effect of the three phenolic compounds in mature adipocytes. All of them reduced TG accumulation at the dose of 25 μM

after 48 h of treatment, but not at lower doses. Taking into account that 25 μM is far higher than the serum concentrations and tissue amounts found in animals treated with polyphenols, we may say that in all likelihood these phenolic compound are not useful to reduce body fat accumulation in adult humans. Unfortunately, there are no studies in the literature showing the effects of pure apigenin, hesperidin or kaempferol on mature adipocytes derived from hMSCs. As far as plant extracts containing these phenolic compounds are concerned, Lo Furno et al. [34] showed that treating hMSCs cells with 10 or 100 μg/mL of *C. bergamia* extract during 7 or 14 days reduced TG accumulation. Colitti et al. [45] demonstrated that a plant extract from *Citrus aurantium*, containing hesperidin, reduced lipid accumulation in primary human mature adipocytes.

When the expression of genes involved in TG metabolism of mature adipocytes was analyzed, all compounds increased mRNA levels of a very well known lipase, *atgl*. By contrast, *hsl* remained unchanged. This could be explained by the fact that *hsl* mediates lipolysis stimulated by catecholamines and by natriuretic peptide, whereas *atgl* mediates the hydrolysis of TG during basal lipolysis [46]. Moreover, apigenin and hesperidin decreased gene expression of *fasn*. These results as a whole suggest that while the three compounds reduced TG content in mature human adipocytes by affecting, at least in part, the lipolytic process, in the case of apigenin the decrease in the lipogenic pathway also contributed to the delipidating effect.

This is the first time that the effect of pure apigenin, hesperidin and kaempferol has been tested in mature adipocytes derived from hMSCs. However, in the study of Lo Furno et al. [34], where *C. bergamia* extracts were used to treat hMSCs for 14 days, the authors observed that 10 and 100 μg/mL of the plant extract increased lipase protein levels, results that are in line with those of the present study [27]. As explained previously in this manuscript, a comparison with our results is difficult because of the presence of other active phenolic compounds and the potential synergism.

Conclusions

In summary, the present study shows the anti-adipogenic and delipidating effects of apigenin, hesperidin and kaempferol in human adipocytes derived from hMSCs for the first time. While hesperidin and kaempferol reduce adipogenesis, apigenin was ineffective. Regarding mature adipocytes, the three compounds (hesperidin, kaempferol and apigenin) reduce TG accumulation by activating, at least in part, lipolysis, and in the case of hesperidin and apigenin, also by reducing lipogenesis. Nevertheless, doses higher than those found in serum and plasma of animals treated with polyphenols are needed to show the delipidating effect in mature adipocytes.

Abbreviations

acc: acetyl-CoA carboxylase; *atgl*: adipose triglyceride lipase; Ct: threshold cycle; *dgat*: diglyceride acyltransferase; *c/ebpα*: CCAAT/enhancer-binding protein alpha; *c/ebpβ*: CCAAT/enhancer-binding protein beta; *fasn*: fatty acid synthase; FBS: fetal bovine serum; *hsl*: hormone sensitive lipase; MSC: mesenchymal stem cells; PBS: phosphate buffer saline; *ppary*: peroxisome proliferator factor gamma; *scd1*: stearoyl-CoA desaturase 1; *srebp1c*: sterol regulatory element-binding protein 1c; TG: triacylglycerol.

Authors' contributions

SGZ, AL, NA, AMS carried out experiments; AFQ performed experimental design and supervised results; MPGS obtained and isolated stem cells; MPO, JMAM wrote the manuscript and coordinated experiments. All authors read and approved the final manuscript.

Author details

[1] Nutrition and Obesity Group, Department of Nutrition and Food Science and Lucio Lascaray Research Center, University of the Basque Country (UPV/EHU), Vitoria, Spain. [2] Adipocyte and Fat Biology Laboratory (AdipoFat), Unidad de Investigación Traslacional, Instituto Aragonés de Ciencias de la Salud (IACS), Instituto de Investigación Sanitaria (IIS) Aragón, Zaragoza, Spain. [3] CIBER Fisiopatología Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III, Madrid, Spain.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Declarations

Not applicable.

Ethics approval and consent to participate

The study was approved by Clinical Investigation Ethics Committee of Aragon (Acta 11/2013).

Funding

This study was supported by grants from the Instituto de Salud Carlos III (CIBERObn), Government of the Basque Country (IT-572-13) and University of the Basque Country (UPV/EHU) (ELDUNANOTEK UFI11/32). Saioa Gómez-Zorita is a recipient of a post-doctoral fellowship from the University of the Basque Country (UPV/EHU). Jose M. Arbones-Mainar is partially funded by the Project PI14/00508 (Instituto de Salud Carlos III) and by the Fondo Europeo de Desarrollo Regional (FEDER) funds: "Una manera de hacer Europa".

Publisher's Note

Received: 7 April 2017 Accepted: 12 November 2017
Published online: 21 November 2017

References

1. de Ferranti S, Mozaffarian D. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. Clin Chem. 2008;54:945–55.
2. Tang QQ, Lane MD. Adipogenesis: from stem cell to adipocyte. Annu Rev Biochem. 2012;81:715–36.
3. Vaz-da-Silva M, Loureiro A, Falcao A, Nunes T, Rocha J, Fernandes-Lopes C, Soares E, Wright L, Almeida L, Soares-da-Silva P. Effect of food on the pharmacokinetic profile of trans-resveratrol. Int J Clin Pharmacol Ther. 2008;46:564–70.
4. Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol. 2006;7:885–96.
5. El-Badri N, Ghoneim MA. Mesenchymal stem cell therapy in diabetes mellitus: progress and challenges. J Nucleic Acids. 2013;2013:194858.
6. Marketou ME, Parthenakis FI, Kalyva A, Pontikoglou C, Maragkoudakis S, Kontaraki JE, Zacharis EA, Chlouverakis G, Patrianakos A, Papadaki HA, Vardas PE. Increased mobilization of mesenchymal stem cells in patients with essential hypertension: the effect of left ventricular hypertrophy. J Clin Hypertens (Greenwich). 2014;16:883–8.
7. Shen H, Wang Y, Zhang Z, Yang J, Hu S, Shen Z. Mesenchymal stem cells for cardiac regenerative therapy: optimization of cell differentiation strategy. Stem Cells Int. 2015;2015:524756.
8. Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. Dietary (poly) phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal. 2013;18:1818–92.
9. Eseberri I, Miranda J, Lasa A, Churruca I, Portillo MP. Doses of quercetin in the range of serum concentrations exert delipidating effects in 3T3-L1 preadipocytes by acting on different stages of adipogenesis, but not in mature adipocytes. Oxid Med Cell Longev. 2015;2015:480943.
10. Rayalam S, Yang J, Ambati S, Della-Fera M, Baile C. Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. Phytother Res. 2008;22:1367–71.
11. Wu M, Liu D, Zeng R, Xian T, Lu Y, Zeng G, Sun Z, Huang B, Huang Q. Epigallocatechin-3-gallate inhibits adipogenesis through down-regulation of PPARγ and FAS expression mediated by PI3K-AKT signaling in 3T3-L1 cells. Eur J Pharmacol. 2017;795:134–42.
12. Casado-Díaz A, Anter J, Dorado G, Quesada-Gómez JM. Effects of quercetin, a natural phenolic compound, in the differentiation of human mesenchymal stem cells (MSC) into adipocytes and osteoblasts. J Nutr Biochem. 2016;32:151–62.
13. Fischer-Posovszky P, Kukulus V, Tews D, Unterkircher T, Debatin K, Fulda S, Wabitsch M. Resveratrol regulates human adipocyte number and function in a Sirt1-dependent manner. Am J Clin Nutr. 2010;92:5–15.
14. Gomez-Zorita S, Tréguer K, Mercader J, Carpéné C. Resveratrol directly affects in vitro lipolysis and glucose transport in human fat cells. J Physiol Biochem. 2013;69:585–93.
15. Lasa A, Schweiger M, Kotzbeck P, Churruca I, Simón E, Zechner R, Portillo MP. Resveratrol regulates lipolysis via adipose triglyceride lipase. J Nutr Biochem. 2012;23:379–84.
16. Leiherer A, Stoemmer K, Muendlein A, Saely CH, Kinz E, Brandtner EM, Fraunberger P, Drexel H. Quercetin impacts expression of metabolism- and obesity-associated genes in SGBS adipocytes. Nutrients. 2016;8:282.
17. Les F, Deleruyelle S, Cassagnes LE, Boutin JA, Balogh B, Arbones-Mainar JM, Biron S, Marceau P, Richard D, Nepveu F, et al. Piceatannol and resveratrol share inhibitory effects on hydrogen peroxide release, monoamine oxidase and lipogenic activities in adipose tissue, but differ in their antilipolytic properties. Chem Biol Interact. 2016;258:115–25.
18. Park H, Yang J, Ambati S, Della-Fera M, Hausman D, Rayalam S, Baile C. Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes. J Med Food. 2008;11:773–83.
19. Warnke I, Jocken JW, Schoop R, Toepfer C, Goralczyk R, Schwager J. Combinations of bio-active dietary constituents affect human white adipocyte function in-vitro. Nutr Metab (Lond). 2016;13:84.
20. Entenmann G, Hauner H. Relationship between replication and differentiation in cultured human adipocyte precursor cells. Am J Physiol. 1996;270:C1011–6.
21. Janderová L, McNeil M, Murrell AN, Mynatt RL, Smith SR. Human mesenchymal stem cells as an in vitro model for human adipogenesis. Obes Res. 2003;11:65–74.
22. Nakamura M, Toyama Y. Transplantation of neural stem cells into spinal cord after injury. Nihon Rinsho. 2003;61:463–8.
23. Mosqueda-Solís A, Lasa A, Gómez-Zorita S, Eseberri I, Picó C, Portillo MP. Screening of potential anti-adipogenic effects of phenolic compounds showing different chemical structure in 3T3-L1 preadipocytes. Food Funct. 2017;8:3576–86.
24. Perez-Diaz S, Garcia-Rodriguez B, Gonzalez-Irazabal Y, Valero M, Lagos-Lizan J, Arbones-Mainar JM. Knockdown of PTRF ameliorates adipocyte differentiation and functionality of human mesenchymal stem cells. Am J Physiol Cell Physiol. 2017;312:C83–91.
25. Livak K, Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the 2(—Delta Delta C(T)) method. Methods. 2001;25:402–8.
26. Kanaze FI, Bounartzi MI, Georgarakis M, Niopas I. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. Eur J Clin Nutr. 2007;61:472–7.
27. Jin MJ, Kim U, Kim IS, Kim Y, Kim DH, Han SB, Kim DH, Kwon OS, Yoo HH. Effects of gut microflora on pharmacokinetics of hesperidin: a study on non-antibiotic and pseudo-germ-free rats. J Toxicol Environ Health Part A. 2010;73:1441–50.
28. Chang L, Ren Y, Cao L, Sun Y, Sun Q, Sheng N, Yuan L, Zhi X, Zhang L. Simultaneous determination and pharmacokinetic study of six flavonoids from Fructus Sophorae extract in rat plasma by LC-MS/MS. J Chromatogr B. 2012;904:59–64.
29. Sun H, Dong T, Zhang A, Yang J, Yan G, Sakurai T, Wu S, Han Y, Wang X. Pharmacokinetics of hesperetin and naringenin in the Zhi Zhu Wan, a traditional chinese medicinal formulae, and its pharmacodynamics study. Phytother Res. 2013;27:1345–51.
30. Zhou Z, Wang M, Guo Z, Zhang X. Pharmacokinetic evaluation of the interaction between oral kaempferol and ethanol in rats. Acta Pharm. 2016;66:563–8.
31. Dong X, Lan W, Yin X, Yang C, Wang W, Ni J. Simultaneous determination and pharmacokinetic study of quercetin, luteolin, and apigenin in rat plasma after oral administration of *Matricaria chamomilla* L. extract by HPLC-UV. Evid Based Complement Altern Med. 2017;2017:8370584.
32. Elhennawy MG, Lin HS. Quantification of apigenin trimethyl ether in rat plasma by liquid chromatography-tandem mass spectrometry: application to a pre-clinical pharmacokinetic study. J Pharm Biomed Anal. 2017;142:35–41.
33. Morikawa K, Nonaka M, Mochizuki H, Handa K, Hanada H, Hirota K. Naringenin and hesperetin induce growth arrest, apoptosis, and cytoplasmic fat deposit in human preadipocytes. J Agric Food Chem. 2008;56:11030–7.
34. Lo Furno D, Graziano AC, Avola R, Giuffrida R, Perciavalle V, Bonina F, Mannino G, Cardile V. A *Citrus bergamia* extract decreases adipogenesis and increases lipolysis by modulating PPAR levels in mesenchymal stem cells from human adipose tissue. PPAR Res. 2016;2016:4563815.
35. Yunita O, Mochammad Yuwono M, Rantam FA. In vitro cytotoxicity assay of *Sauropus androgynus* on human mesenchymal stem cells. J Toxicol Environ Chem. 2013;95:679–86.
36. Ntambi J, Young-Cheul K. Adipocyte differentiation and gene expression. J Nutr. 2000;130:3122S–6S.
37. Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. Physiol Rev. 1998;78:783–809.
38. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. Genes Dev. 2000;14:1293–307.
39. Kim MA, Kang K, Lee HJ, Kim M, Kim CY, Nho CW. Apigenin isolated from *Daphne genkwa* Siebold et Zucc. inhibits 3T3-L1 preadipocyte differentiation through a modulation of mitotic clonal expansion. Life Sci. 2014;101:64–72.
40. Ono M, Fujimori K. Antiadipogenic effect of dietary apigenin through activation of AMPK in 3T3-L1 cells. J Agric Food Chem. 2011;59:13346–52.
41. Nishina A, Ukiya M, Fukatsu M, Koketsu M, Ninomiya M, Sato D, Yamamoto J, Kobayashi-Hattori K, Okubo T, Tokuoka H, Kimura H. Effects of various 5,7-dihydroxyflavone analogs on adipogenesis in 3T3-L1 cells. Biol Pharm Bull. 2015;38:1794–800.
42. Jeon HJ, Seo MJ, Choi HS, Lee OH, Lee BY. *Gelidium elegans*, an edible red seaweed, and hesperidin inhibit lipid accumulation and production of reactive oxygen species and reactive nitrogen species in 3T3-L1 and RAW264.7 cells. Phytother Res. 2014;28:1701–9.
43. Fang XK, Gao J, Zhu DN. Kaempferol and quercetin isolated from *Euonymus alatus* improve glucose uptake of 3T3-L1 cells without adipogenesis activity. Life Sci. 2008;82:615–22.

Annex 2

44. Park UH, Jeong JC, Jang JS, Sung MR, Youn H, Lee SJ, Kim EJ, Um J. Negative regulation of adipogenesis by kaempferol, a component of Rhizoma Polygonati falcatum in 3T3-L1 cells. Biol Pharm Bull. 2012;35:1525–33.
45. Colitti M, Stefanon B. Different anti-adipogenic effects of bio-compounds on primary visceral pre-adipocytes and adipocytes. EXCLI J. 2016;15:362–77.
46. Langin D, Dicker A, Tavernier G, Hoffstedt J, Mairal A, Rydén M, Arner E, Sicard A, Jenkins CM, Viguerie N, et al. Adipocyte lipases and defect of lipolysis in human obesity. Diabetes. 2005;54:3190–7.

Resumen

Este trabajo de Tesis Doctoral interuniversitaria se ha llevado a cabo en dos universidades, la primera parte en la Universidad del País Vasco dentro del grupo de investigación "Nutrición y Salud" dirigido por la Catedrática María del Puy Portillo. Este grupo tiene una amplia experiencia en el estudio de los efectos de los ingredientes funcionales sobre el metabolismo de los lípidos en el campo de la obesidad y co-morbilidades. En los últimos años se han estudiado ácidos grasos conjugados y distintos compuestos fenólicos, como la quercetina, resveratrol y pterostilbeno. Para esta Tesis, se ha decidido implementar una nueva búsqueda en compuestos fenólicos que puedan tener efectos en la regulación del metabolismo de los triglicéridos. Después, en la Universidad de las Islas Baleares, en el Laboratorio de Biología Molecular, Nutrición y Biotecnología el cuál se centra en la investigación y el desarrollo en el campo de bioquímica y nutrición, este grupo es liderado por el Prof. Andreu Palou y por la Catedrática Catalina Picó, la cuál es co-directora de este trabajo. Además, dentro del marco de Tesis Internacional, se realizó una estancia internacional entre los meses Marzo y Junio del 2017 teniendo lugar en el Departamento de Biología Celular y Molecular del Instituto Karolinska dirigido por la Doctora Kirsty Spalding, donde principalmente se investiga el origen y la renovación de los adipocitos, sus células progenitoras y las reservas de lípidos en individuos delgados y obesos. Bajo la supervisión de la Dra. Firoozeh Salehzadeh se trabajo durante la estancia en proyectos enfocados en la renovación celular en distintos depósitos adiposos (subcutáneo y visceral) con el objetivo de comprender la regulación del tejido adiposo humano, y así, la dinámica de recambio celular para poder descubrir

tratamientos potenciales para la obesidad. Una de las técnicas utilizadas para la heterogeneidad de los adipocitos utilizada en este laboratorio es single cell sequencing, técnica basada en la utilización de células individuales para comprender las complejidades celulares del tejido adiposo.

En esta Tesis Doctoral, se llevaron a cabo dos enfoques. Primero, estudios *in vivo* que consistieron en una exploración de 15 compuestos fenólicos para estudiar y comparar sus efectos a diferentes dosis en la adipogénesis. Segundo, la aplicación *in vivo* donde evaluamos los efectos potenciales de hesperidina (seleccionada del estudio in vivo debido a sus efectos antiadipogénicos) y capsaicina (seleccionada tras una amplia búsqueda en la literatura científica debido a sus potenciales efectos termogénicos), por separado y en combinación, para prevenir el desarrollo de obesidad y alteraciones metabólicas inducidas por la ingesta de una dieta obesogénica en ratas.

A)Detección de posibles efectos antiadipogénicos de compuestos fenólicos con diferentes estructuras químicas en preadipocitos 3T3-L1

Los compuestos fenólicos son miembros de una gran familia de moléculas derivadas de plantas que exhiben una extensa variedad de estructuras químicas. La estructura química de los compuestos fenólicos tiene influencia no solo en su biodisponibilidad, sino también en sus acciones biológicas. Teniendo todo esto en cuenta y considerando el gran problema de salud que representa la obesidad hoy en día, nuestro interés se centró en los efectos de los compuestos fenólicos en la obesidad y más específicamente en la adipogénesis, proceso que

permite la expansión del tejido adiposo mediante la transformación de precursores de adipocitos (pre-adipocitos) en adipocitos maduros.

Se analizó el efecto antiadipogénico de 15 compuestos fenólicos de diversos grupos químicos en preadipocitos 3T3-L1. Con este fin, los modelos *in vitro* han sido seleccionados para determinar los mecanismos implicados en la proliferación, diferenciación y expresión génica de los adipocitos.

Los compuestos fenólicos utilizados fueron flavonas (apigenina y luteolina), flavan-3-oles (catequina, epicatequina y epigalocatequina), isoflavonas (genisteína y daizeína), flavanonas (naringenina y hesperidina), flavonoles (quercetina y kaempferol), estilbenos (resveratrol, piceatannol y pterostilbeno) y ácido hidroxibenzoico (ácido vanílico). Se trabajó con un intervalo de dosis de 25 µM a una dosis fisiológica de 1 µM. La cuantificación de triglicéridos mostró que a 25 µM todos los compuestos, con la excepción de catequina, epicatequina y epigalocatequina, redujeron la acumulación de triglicéridos. A una dosis de 10 µM de apigenina, luteolina, naringenina, hesperidina, quercetina y kaempferol indujeron reducciones significativas, y a 1 µM solo la naringenina, la hesperidina y la quercetina fueron efectivas. Estos resultados mostraron que la estructura química de las flavanonas fue la más potente en la inhibición de la adipogénesis, al menos en nuestras condiciones experimentales, porque ambos compuestos que muestran esta estructura fueron capaces de reducir la acumulación de triglicéridos en los preadipocitos en las tres dosis estudiadas, incluyendo 1 µM.

Se sabe que la estructura química de los compuestos fenólicos tiene influencia, no solo en su biodisponibilidad, sino también en sus

acciones biológicas. Cuando se compararon los efectos de compuestos fenólicos pertenecientes al mismo grupo químico, se observó que ambas flavonas estaban activas a 25 y 10 µM, ambas isoflavonas estaban activas a 25 µM, ambas flavanonas estaban activas en las tres dosis y los tres estilbenos estaban activos a 25 µM. Estos resultados sugieren que pequeñas diferencias, basadas principalmente en grupos sustituyentes, entre compuestos que muestran una estructura química general similar no son cruciales para la actividad antiadipogénica de estas moléculas. En el caso de los flavonoles, el patrón de respuesta fue diferente porque mientras que la quercetina fue efectiva en las tres dosis, el kaempferol solo mostró un efecto antiadipógenico a 25 µM y 10 µM. Esta diferencia podría deberse a la presencia de un grupo hidroxilo adicional en la estructura de quercetina.

Con el fin de obtener más información sobre los efectos de los compuestos fenólicos en la adipogénesis, analizamos los posibles cambios en los genes que desempeñan un papel clave en este proceso. La adipogénesis está regulada por una elaborada red de factores de transcripción. En términos generales, se pueden distinguir dos etapas, la etapa pre mitótica (etapa temprana de diferenciación, 60 horas después de la confluencia), regulada por *c/ebpβ* y *srebp1c*, y la etapa post mitótica (etapa tardía) regulada por *pparγ* y *c/ebpα* (Farmer *et al.,* 2007; Fajas *et al.,* 1998; Tang *et al.,* 2003). Sin embargo, la influencia de *pparγ* en estas dos etapas es difícil de establecer. Ntambi *et al*., informaron que este factor de transcripción comienza su expresión alrededor de las 48 horas y alcanza su expresión máxima alrededor de 3-4 días de diferenciación (Ntambi *et al.,* 2000).

Después de analizar los efectos de los compuestos fenólicos en la expresión de estos genes, se observó que todos actuaron inhibiendo la etapa inicial de la adipogénesis porque redujeron significativamente la expresión génica de *srebp1c*, o ambas *c/ebpβ* y *srebp1c* (genisteína y kaempferol). Esta propuesta se confirma por la reducción de triglicéridos observada cuando las células se trataron de 0 a 60 horas con todos los compuestos fenólicos analizados en el segundo experimento (luteolina, genisteína, hesperidina, kaempferol, pterostilbeno, ácido vanílico). Ninguno de los compuestos probados modificó la expresión del gen *c/ebpa*. En el caso de la expresión del gen *pparγ*, tanto la genisteína como el pterostilbeno indujeron reducciones significativas, pero este efecto no se acompañó de la reducción esperada en el contenido de triglicéridos cuando las células se incubaron de 60 horas al día 8. Para explicar este hecho, la expresión proteica de PPARγ2 se midió y no se encontraron diferencias significativas cuando las células tratadas con estos compuestos fenólicos se compararon con las células de control. Estos resultados están de acuerdo con la falta de efecto delipidante mostrado en la última etapa de diferenciación.

Con respecto a los genes expresados en la última fase, los compuestos luteolina, genisteína, kaempferol y pterostilbeno disminuyeron la expresión del gen *acc* y kaempferol la de *lpl*, lo que sugiere una capacidad de reducir la acumulación de triglicéridos en adipocitos maduros previamente tratados con estos compuestos. La reducción observada en la expresión de genes desde la etapa temprana de la adipogénesis debería haber conducido a una reducción en la expresión de genes reguladores de la etapa tardía. Sin embargo, en el

presente estudio, no todos los compuestos demostraron este efecto. No tenemos una explicación para este hecho, pero no es inusual porque esta situación también se ha encontrado en otros estudios de nuestro grupo (Eseberri *et al.,* 2015) y por otros autores (Ntambi *et al.,* 2000; Fajas *et al.,* 1998; Tang *et al.,* 2003; Lane *et al*., 1999; Erickson *et al.,* 2000; Dowell *et al.,* 2000; Allender y Rayner, 2007). Sin embargo, deberíamos indicar como observación final que, en general, los compuestos fenólicos experimentados actúan en la etapa temprana de la adipogénesis.

B) Hesperidina y capsaicina, pero no la combinación, previenen la esteatosis hepática y otras alteraciones relacionadas con el síndrome metabólico en ratas alimentadas con dieta occidental.

Entre todos los compuestos fenólicos analizados en la selección previamente descrita en el Manuscrito 1, elegimos la hesperidina para el estudio *in vivo* debido a los interesantes resultados obtenidos *in vitro* con esta molécula. Como estudios previos mostraron que diferentes combinaciones de compuestos fenólicos podían reducir la comorbilidad de la grasa corporal y la obesidad incluso más que la administración individual de estos compuestos, surge el interés en combinar la hesperidina con otro compuesto (De Santi et al., 2000; Ohara et al., 2015; Arias et al., 2015, Arias et al., 2016; Arias et al., 2017). En este sentido, consideramos la capsaicina, debido a los posibles efectos antiobesidad descritos asociados con la capacidad de inducir termogénesis (Saito, 2015) y marronización del tejido adiposo blanco (Baskaran et al., 2016).

En este contexto, el objetivo fue evaluar los efectos potenciales de la hesperidina y la capsaicina en la dieta, de forma independiente, y la combinación de ambos compuestos para prevenir el desarrollo de la obesidad y alteraciones metabólicas relacionadas. El experimento *in vivo* se realizó con ratas Wistar divididas aleatoriamente en 5 grupos: Grupo de control: alimentado con una dieta estándar de chow, grupo WD: animales alimentados con una dieta alta en grasas y alta en sacarosa (dieta occidental), HESP: animales alimentados con una dieta occidental y tratados con hesperidina (100 mg / kg / día), CAP: animales alimentados con una dieta occidental y tratados con capsaicina (4 mg / kg / día) y HESP + CAP: animales alimentados con una dieta occidental y tratados con la combinación de hesperidina (100 mg / kg / día) + capsaicina (4 mg / kg / día).

Después de 8 semanas de tratamiento, no se encontraron diferencias significativas entre los 5 grupos de animales con respecto al peso corporal. Sin embargo, el grupo WD, pero no el grupo CAP, mostró una mayor ganancia de peso corporal que los controles. Los animales de los grupos HESP y HESP + CAP mostraron valores intermedios. El tratamiento con capsaicina también se muestra al final del período de intervención, con menor porcentaje de grasa corporal que los animales del grupo WD, pero mayor que el grupo control. El contenido de grasa corporal en los grupos HESP y HESP + CAP fue ligeramente más alto que el del grupo CAP y no significativamente diferente del grupo WD. Estos resultados concuerdan con otros autores que tampoco han encontrado efectos significativos de la hesperidina en el peso corporal en ratones obesos con una dieta baja en grasas. Sin embargo, cuando glucosyl-

hesperidin se combinó con cafeína, se observaron efectos significativos (Ohara et al., 2014). La acción antiobesidad de la capsaicina ha sido descrita previamente por varios autores (Watanabe et al., 1986; Sherriffs et al., 2010; Li et al., 2010 y Mattes, 2011), lo que sugiere que la capsaicina disminuye la grasa corporal. En particular, la combinación de hesperidina y capsaicina mostró menor efecto sobre la ganancia de grasa corporal que los observados cuando se administraron por separado.

La expresión de diferentes genes se estudió con el fin de encontrar mecanismos potenciales subyacentes a los efectos de la capsaicina que previene la acumulación de grasa por los efectos sobre el metabolismo de los lípidos en el tejido adiposo. Los animales tratados con capsaicina en el tejido adiposo blanco retroperitoneal disminuyeron los niveles de expresión de genes relacionados con la lipogénesis (*Pparg* y *Srebf1*), con la absorción de ácidos grasos (*Lpl*, *Cd36*) y oxidación (*Cpt1b*), lipólisis (*Pnpla2*, *Lipe*) , y captación y metabolismo de la glucosa (*Slc2a4*, *Hk2*). Los animales tratados con hesperidina también mostraron niveles de expresión disminuidos de algunos de los genes mencionados anteriormente (*Pparg*, *Lpl*, *Pnpla2*, *Cpt1b*, *Hk2* e *Insr*) con respecto a animales del grupo WD, pero los efectos fueron generalmente menos marcados que los observados. mediante tratamiento con capsaicina, de acuerdo con el efecto más modesto de la hesperidina sobre la ganancia de grasa corporal. La combinación de CAP y HESP tampoco provocó efectos adicionales a los observados administrando únicamente capsaicina.

Con respecto al sistema de glucosa-insulina, los resultados obtenidos para la evaluación del modelo de homeostasis de la

resistencia a la insulina (HOMA-IR) indican que el tratamiento con capsaicina evitó el aumento de HOMA-IR en ratas alimentadas con dieta occidental, pero notablemente los efectos se redujeron cuando la capsaicina se combinó con hesperidina. La capacidad de la capsaicina para mejorar la resistencia a la insulina se ha descrito previamente en roedores (Kang et al., 2010). Varios estudios en humanos también han revelado que la capsaicina tiene un efecto modesto en la diabetes tipo 2. A diferencia de la capsaicina, no se observaron efectos significativos para la hesperidina en la resistencia a la insulina en ratas alimentadas con dieta occidental.

El hígado juega un papel importante en la acumulación y oxidación de los lípidos (Lazarow, 1978; Vernon, 1980). Bajo nuestras condiciones experimentales, los animales en el grupo WD, pero no los animales en el grupo CAP, mostraron un mayor peso del hígado que los controles, mientras que los animales tratados con hesperidina o con la combinación de ambos compuestos mostraron valores intermedios. Además, medimos el contenido de lípidos hepáticos para saber si este órgano acumuló menos lípidos por los efectos de estos bioactivos. Los animales del grupo WD mostraron un aumento significativo en el contenido de lípidos con respecto a los controles. Notablemente, los animales alimentados con WD tratados con hesperidina o capsaicina por separado y, en menor medida, con la combinación mostraron un contenido de lípidos hepáticos significativamente menor con respecto al grupo de WD. El análisis histológico reveló que tanto la hesperidina como la capsaicina, cuando se administraban solos, conferían un efecto protector sobre el desarrollo de enfermedad de hígado graso no

alcohólica (NAFLD) y evitaban las características de esteatohepatitis no alcohólica (EHNA), hepatocitos en balón, los hepatocitos necróticos y los linfocitos infiltrados, encontrados en animales alimentados con dieta occidental.

Con respecto a los triglicéridos circulantes, el tratamiento con hesperidina o con capsaicina sola, pero no con la combinación, atenuó el aumento de los niveles circulantes de triglicéridos en el estado alimentado que ocurre en ratas alimentadas con dieta occidental. Mitsuzumi *et al*., Describieron previamente que la hesperidina reduce los triglicéridos séricos en modelos animales y en sujetos hipertrigliceridémicos (Mitsuzumi *et al*., 2011). Específicamente, se informó que los mecanismos implicados en los efectos reductores de los triglicéridos de la hesperidina son la reducción del contenido de triglicéridos hepáticos mediante la inhibición de la lipogénesis y la inducción de la oxidación de ácidos grasos (Mitsuzumi *et al.,* 2011) y la baja regulación de la síntesis y secreción de lipoproteínas de muy baja densidad (VLDL) (Miwa *et al.,* 2006). De acuerdo con esta noción, después de analizar la expresión hepática de genes seleccionados, observamos que los animales tratados con hesperidina mostraron niveles de expresión disminuidos de tres genes clave relacionados con la lipogénesis, *Srebf1*, *Fasn* y *Scd1*, así como la normalización de la expresión del gen *Pklr*. Además, estos animales exhibieron niveles de expresión hepática disminuidos de los genes *Insr* y *Lepr*, en comparación con los niveles de expresión en ratas alimentadas con dieta occidental, lo que sugiere una disminución de la acción lipogénica de la insulina, junto con una menor actividad de oxidación grasa. Aunque no existe un

consenso claro en la literatura sobre los efectos de la leptina en el hígado (Ceddia et al., 2002), se ha propuesto que la leptina puede tener efectos sensibilizadores a la insulina, controlando el grado de acción de la insulina en este tejido (Huynh *et al* ., 2010). En particular, se ha demostrado que la interrupción de la señalización de la leptina hepática protege a los ratones de la intolerancia a la glucosa relacionada con la dieta (Huynh *et al,* 2010). Por lo tanto, se sugiere que la disminución de la lipogénesis hepática en los animales tratados con hesperidina, asociada a una disminución de la acción de la insulina sobre este tejido, puede ser uno de los mecanismos por los cuales este bioactivo protege contra las patologías hepáticas inducidas por la dieta.

A su vez, el tratamiento con capsaicina provocó pocos efectos sobre la expresión hepática de los genes relacionados con la lipogénesis en comparación con los cambios observados con la hesperidina. Investigadores han revelado que la capsaicina regula el metabolismo de los lípidos hepáticos y evita la deposición de lípidos en el hígado a través de la activación de TRPV1 (Li *et al.,* 2012). La acción de la capsaicina en TRPV1 implica la regulación al alza de UCP2, un transportador de membrana mitocondrial que puede proporcionar la translocación de ácidos grasos, evitando su acumulación en la matriz mitocondrial (Li *et al*., 2012). En este trabajo, la presencia de mayores niveles de expresión de *Ucp2* en animales tratados con capsaicina en comparación con los otros grupos experimentales está de acuerdo con la participación de UCP2 en los efectos protectores de la capsaicina sobre la deposición de lípidos en el hígado, proporcionando efectos protectores de la lipotoxicidad hepatocelular (Baffy, 2005).

Por lo tanto, la capsaicina como la hesperidina, por separado, tienen efectos en el metabolismo de los lípidos. Inesperadamente, los efectos fueron atenuados o incluso insignificantes cuando los animales fueron tratados con ambos compuestos simultáneamente. Se desconocen los mecanismos que subyacen a esta interacción, pero se ha observado que el tratamiento con capsaicina bloqueó la disminución de la expresión hepática de *Lepr* inducida por hesperidina, que puede asociarse tentativamente con un aumento de la actividad de la lipogénesis inducida por la insulina en este tejido. De hecho, la combinación de ambos compuestos regulaba al alza la expresión del gen lipogénico *Gadph*, alcanzando niveles superiores a los de los animales tratados con hesperidina sola. Por otro lado, la hesperidina parece anular los efectos de la capsaicina a través de su receptor TRPV1. De hecho, la inducción de la expresión de *Trpv1* por la capsaicina se atenuó con el tratamiento simultáneo con hesperidina, lo que sugiere que la respuesta a la acción de la capsaicina se redujo en animales tratados con la combinación de compuestos bioactivos. Por consiguiente, los animales tratados con la combinación de compuestos bioactivos no mostraron el aumento en los niveles de expresión de Ucp2 característicos de los animales tratados con capsaicina. Por lo tanto, se sugiere que ambos bioactivos pueden perjudicar mutuamente sus formas de acción sobre la mejora de la salud hepática en animales alimentados con dieta occidental.

Con respecto a otros componentes del síndrome metabólico, los resultados del presente estudio también aportan evidencia que respalda los efectos hipotensivos de la hesperidina y, en menor medida, de la

capsaicina. De hecho, la presión arterial sistólica y diastólica disminuyó en los animales tratados con hesperidina, en comparación con las ratas alimentadas con dieta occidental no tratada, alcanzando niveles más bajos que los controles. Los animales tratados con capsaicina o la combinación de hesperidina y capsaicina también mostraron una presión arterial diastólica más baja que los animales alimentados con dieta occidental, y similar a los animales de control. Esto sugiere interesantes efectos antihipertensivos de la hesperidina, más allá de sus posibles efectos reductores de la grasa.

Los resultados del presente estudio muestran que la capsaicina y hesperidina, por separado, exhiben diferentes efectos beneficiosos para la salud que mejoran la acumulación de grasa y las alteraciones relacionadas con el síndrome metabólico en ratas alimentadas con dieta occidental. Sin embargo, la falta o los efectos menores encontrados con la combinación de ambos compuestos merecen tenerse en cuenta al considerar posibles mezclas de compuestos bioactivos como estrategias para la prevención de la obesidad.

C) Efectos de capsaicina y hesperidina y su combinación en la morfología del tejido adiposo y la inducción de marronización en ratas alimentadas con dieta occidental

Teniendo en cuenta la capacidad de capsaicina y hesperidina, por separado, para exhibir los efectos beneficiosos para la salud mencionados anteriormente, exploramos las propiedades potenciales de estos compuestos bioactivos, por separado y en combinación, para investigar la morfología del tejido adiposo blanco e inducción de marronización, como una ayuda en el manejo del peso corporal en las

ratas alimentan con una dieta obesogénica. La marronización puede aparecer en el tejido adiposo blanco en respuesta a estímulos específicos, como la exposición al frío, pero también en respuesta a componentes dietéticos específicos (Bonet, Oliver y Palou, 2013) o dietas particulares, como las dietas ricas en grasas (García-Ruiz et al., 2015). Por lo tanto, las moléculas bioactivas capaces de aumentar el tejido adiposo marrón y / o convertir el blanco en adipocitos "brite" pueden ayudar a corregir el desequilibrio energético que subraya la obesidad. Además, considerando que los factores nutricionales generalmente ejercen efectos limitados, la combinación con otros agentes bioactivos que se dirigen a otros procesos relacionados con el metabolismo de los adipocitos, como adipogénesis y / o lipólisis, puede facilitar aún más el metabolismo energético y el control del peso (Stohs y Badmaev, 2016) . Por ejemplo, se conoce que la combinación de resveratrol y quercetina ejerce mayores efectos sobre la marronización en ratas alimentadas con una dieta obesogénica, mientras que ambos compuestos ejercen individualmente pocos efectos (Arias *et al*., 2017).

Los capsaicinoides (también conocidos como capsinoides), son un grupo de moléculas presentes de forma natural en los chiles. El más abundante y estudiado es la capsaicina, que tiene propiedades antiobesidad, antidiabéticas y antiinflamatorias (Whiting, Derbyshire y Tiwari, 2012). Sus efectos antiobesidad están relacionados en parte con su capacidad para activar el sistema simpático e inducir la termogénesis BAT (Saito, 2015) y desencadenar la marronización de WAT activando el miembro V de la subfamilia V del canal de cationes potencialmente transitorio (TRPV1) (Baskaran et al. 2016). Notablemente, la capsaicina y

sus análogos no pungentes tienen un interés potencial como agentes de control de peso porque actúan como agentes termogénicos, sin causar estimulantes y efectos cardiovasculares adversos (Stohs y Badmaev, 2016). Por otro lado, los flavonoides cítricos, como la hesperidina, han surgido como agentes terapéuticos prometedores para el tratamiento de la desregulación metabólica. Varios estudios han señalado los efectos bioactivos de la hesperidina contra las alteraciones relacionadas con la obesidad, principalmente debido a sus efectos reductores de la grasa, antiinflamatorios, antioxidantes y sensibilizadores a la insulina (Li y Schluesener, 2017). Cabe destacar que, en un estudio en humanos, se ha demostrado que la hesperidina potencia el efecto termogénico de la p-sinefrina, el protoalcaloide primario en la naranja amarga (Stohs *et al*., 2011). Por lo tanto, se sugiere que la combinación apropiada de agentes termogénicos con flavonoides u otros bioactivos reductores de grasa podría potencialmente aumentar los efectos de compuestos individuales y ser altamente valioso en el campo nutricional para ayudar al control de peso.

En el presente estudio, los animales tratados con capsaicina mostraron menor ganancia de peso corporal y contenido de grasa en comparación con el grupo de WD, pero no se detectaron diferencias en cuanto al gasto de energía. Esto también se asoció con una disminución del tamaño de los adipocitos, particularmente en la almohadilla de grasa retroperitoneal. El efecto reductor de la grasa puede deberse, en parte, a la inducción de marronización en el tejido adiposo blanco. Este efecto fue morfológicamente evidente en el depósito retroperitoneal, donde el tratamiento con capsaicina se asoció con la aparición de adipocitos

multiloculares similares a UCP1 y CIDE-A-marrones. En consecuencia, también se observó una tendencia a una mayor expresión de la proteína UCP1 en este depósito, aunque los niveles no fueron diferentes de los del grupo WD, probablemente debido a la alta variabilidad entre este grupo. Teniendo en cuenta que UCP1 es la proteína de sello responsable de la termogénesis, la inducción de la capsaicina en la dieta de las células marrones que expresan esta proteína sugiere que el tejido adiposo de estos animales puede tener una capacidad termogénica aumentada. Sin embargo, en el depósito inguinal, aunque el proceso de marronización no fue evidente a nivel morfológico, el tratamiento con capsaicina produjo un aumento significativo en los niveles de expresión génica de *Prdm16* y *Cidea*, y aumentó los niveles de proteína de CIDE-A con respecto al control y / o el grupo WD. El regulador transcripcional PRDM-16 es un determinante del linaje de adipocitos marrones.

PRDM-16 estimula la adipogénesis marrón mediante la unión a PPAR-γ (receptor-γ activado por peroxisoma-proliferador) y activando su función transcripcional (Seale et al., 2008). Por lo tanto, la capsaicina en la dieta podría inducir la conversión molecular de adipocitos blancos a células brite al aumentar la expresión de *Prdm-16*. Esto se afirma con el trabajo previo que muestra una mayor expresión de Prdm-16 y Pgc-1α en ratones debido a la ingesta dietética de capsaicina junto con una dieta alta en grasas (Baskaran et al., 2016). El mecanismo por el cual la capsaicina induce la marronización implica la inducción de la expresión y actividad de la sirtuina-1 a través de la activación del canal TRPV1. A su vez, la sirtuina-1 desencadena la desacetilación e interacción de PRDM-16 y PPARγ para mediar el oscurecimiento del tejido adiposo blanco

(Baskaran et al., 2016). TRPV1 ha demostrado ser esencial para los efectos de la capsaicina, ya que no tiene efectos sobre los depósitos de grasa en ratones TRPV1 - / - (que carecen genéticamente de los canales TRPV1) (Baskaran et al., 2016).

El mecanismo del factor A de fragmentación de ADN del factor alfa efector (CIDE-A) no se conoce con profundidad, pero se ha demostrado que está implicado en la formación de gotas lipídicas y el almacenamiento de lípidos en el tejido adiposo (Wu et al. 2014). Particularmente, los ratones transgénicos que expresan CIDE-A humana muestran un aumento robusto de la capacidad de almacenamiento de lípidos del tejido adiposo con una dieta alta en grasas, lo que sugiere que está involucrado en la protección contra las complicaciones metabólicas de la obesidad (Abreu-Vieira et al., 2015). En humanos, *Cidea* se expresa en el tejido adiposo blanco y los niveles de expresión se correlacionan positivamente con la sensibilidad a la insulina y obesidad; de hecho, los bajos niveles de expresión están asociados con varias características del síndrome metabólico (Nordström et al., 2005). Por lo tanto, la expresión incrementada de *Cidea* en animales tratados con capsaicina puede ser indicativa de una mejora en la salud metabólica.

Los resultados actuales que muestran la inducción de marronización por capsaicina están de acuerdo con estudios previos (Baboota et al., 2014; Baskaran et al., 2016), aunque los efectos dependen del modelo animal y las condiciones de tratamiento. Por ejemplo, la combinación de capsinoides con exposición leve al frío (17 ° C) en ratones promovió sinérgicamente la biogénesis de adipocitos brite en el tejido adiposo blanco inguinal y mejoró la obesidad inducida por la

dieta (Ohyama et al., 2016).Debe mencionarse el hallazgo de una respuesta específica de depósito a los tratamientos, ya que la capsaicina conlleva una mayor expresión de marcadores marrones en el depósito inguinal, mientras que la evidencia histológica de marronización solo se encontró en la grasa retroperitoneal. Esto es consistente con el trabajo previo que muestra que la inducción de marronización por la ingesta de una dieta de cafetería solo fue evidente a nivel histológico en el depósito retroperitoneal, a pesar de que el depósito inguinal mostró niveles de expresión aumentados de marcadores marrón / brite (García-Ruiz et al. 2015).

Además de la capsaicina, la hesperidina tiene propiedades interesantes para el tratamiento de las alteraciones relacionadas con la obesidad, principalmente debido a su efecto reductor de la grasa. Sin embargo, a diferencia de la capsaicina, no se han descrito efectos claros de la hesperidina en el control del peso corporal, ni se ha informado que promuevan la marronización del tejido adiposo blanco o la termogénesis (Li y Schluesener, 2017), aunque se ha demostrado que potencia el efecto termogénico de p- sinefrina (Stohs et al., 2011). Aquí, describimos que el tratamiento con hesperidina indujo cambios morfológicos en el tejido adiposo blanco, concretamente esto se asoció con una disminución del tamaño medio de los adipocitos en los depósitos retroperitoneal e inguinal, al igual que la capsaicina. En particular, el tratamiento con hesperidina también indujo la aparición de adipocitos multiloculares, positivos para UCP1 y CIDE-A, en el depósito retroperitoneal. Los niveles de proteína de CIDE-A también se incrementaron en el depósito inguinal, de forma similar a lo observado

por el tratamiento con capsaicina, pero no se observó una inducción significativa al nivel de expresión génica en ninguna de las dos almohadillas de grasa analizadas. Por lo tanto, la hesperidina junto con una dieta occidental puede tener el potencial de inducir un cierta marronización del tejido blanco, aunque en menor medida en comparación con la capsaicina. Sin embargo, además de esto, no se han observado efectos significativos sobre el aumento de peso corporal o el porcentaje de grasa corporal aquí en ratas tratadas con hesperidina junto con una dieta occidental, más allá de los efectos que disminuyen el tamaño de los adipocitos. Los estudios en humanos también han descrito que la suplementación con jugo a base de cítricos, rico en vitamina C y hesperidina, mejora los factores de riesgo en pacientes con síndrome metabólico, pero no tiene ningún efecto sobre los parámetros antropométricos (Mulero et al., 2012).

Además de la inducción de marronización, es importante mencionar que ambos tratamientos de capsaicina y hesperidina condujeron a un aumento significativo en los niveles totales de UCP1 en el tejido adiposo marrón interescapular, en comparación con animales de control, lo que sugiere que estos animales pueden tener una capacidad termogénica aumentada frente al grupo de control. a los animales alimentados con dieta occidental. Sin embargo, a pesar de los efectos de la capsaicina y la hesperidina, no observamos ningún aumento significativo en el gasto energético diario de capsaicina o hesperidina. Otros autores como Baskaran *et al*., han demostrado una mejora en el gasto de energía en ratones, pero después de 26 semanas de tratamiento con capsaicina en la dieta (Baskaran *et al.,* 2016). Podría

ser posible que el potencial aumento en la capacidad termogénica presentado por los animales tratados con capsaicina o hesperidina solo resultara en un mayor gasto energético efectivo bajo estímulos apropiados. De hecho, Ohyama *et al.,* han descrito que la suplementación dietética de capsinoides promueve la termogénesis inducida por frío in vivo, pero el efecto antiobesidad de los capsinoides se atenuó por completo en condiciones termoneutrales de 30 ° C (Ohyama *et al*., 2016). Con respecto a la hesperidina, no hay estudios previos que demuestren sus efectos termogénicos potenciales in vivo.

A pesar del potencial de la capsaicina y hesperidina, individualmente, para inducir marronización, fue sorprendente que la combinación de ambos bioactivos no ejerció efectos, ni a nivel histológico, ni en el nivel de la expresión génica, en cualquiera de los depósitos de grasa estudiados. Esto está de acuerdo con los efectos menores ejercidos por la combinación de ambos compuestos frente a la capsaicina sola que contrarresta el aumento inducido por la dieta occidental en el peso corporal y la adiposidad. Los mecanismos que subyacen a la interacción negativa entre ambos compuestos son desconocidos. Teniendo en cuenta la participación del canal TRPV1 en el programa de marronización de tejido adiposo blanco inducida por la capsaicina, se podría decir como hipótesis de que la hesperidina podría anular los efectos de la capsaicina a través de su receptor TRPV1. De hecho, las ratas tratadas con hesperidina mostraron menores niveles de expresión de TRPV1 en el tejido adiposo blanco en comparación con las ratas de control, lo que sugiere que la capacidad de respuesta a la acción de la capsaicina se vio afectada en los animales tratados con la

combinación de bioactivos. Esto está de acuerdo con los resultados descritos en el hígado (manuscrito 2), donde la inducción de la expresión de TRPV1 por la capsaicina fue atenuada con el tratamiento simultáneo con hesperidina, y por lo tanto los efectos beneficiosos de la capsaicina en la salud del hígado no fueron evidentes con la combinación de ambos bioactivos . Sin embargo, no podemos descartar la participación de otros mecanismos, pero no han sido explorados aquí. El conocimiento de estas interacciones puede ser de interés para diseñar y descartar combinaciones apropiadas de bioactivos en el tratamiento de la obesidad y sus complicaciones metabólicas relacionadas.